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Citrus depressa peel extract act as a prebiotic to reduce lipid accumulation and modulate gut microbiota in obese mice

ORIGINAL ARTICLE

Citrus depressa peel extract acts as a prebiotic to reduce lipid accumulation and modulate gut microbiota in obese mice

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Abstract

Citrus peels contain abundant polyphenols, particularly flavonoids, and have been shown to exert lipid accumulation decreasing ability. In this study, *Citrus depressa* peel applied to oven drying and extracted with ethanol extract as CDEE to analyze its flavonoids compositions and investigated its effects on a high-fat diet (HFD)-induced obese mice model. CDEE contained several flavonoids such as hesperidin, sinesentin, nobiletin, tangeretin, 5-demethylnobiletin, and 5-demethyltangeretin. The mice fed an HFD, and administration of 2% CDEE to could decrease weight gain, abdominal fat weight, inguinal fat weight, and the adipocyte size, and CDEE also reduced serum total cholesterol (TCHO), triacylglycerol (TG) compared with mice fed only on HFD. CDEE hindered lipid accumulation through a decreased fatty acid synthase (FAS) protein expression via upregulation of the protein expression of AMP-activated protein kinase α (AMPKα). Moreover, CDEE modulated gut microbiota that altered by HFD through an increased abundance of *Lactobacillus reuteri* compared with the HFD group. The results demonstrated that CDEE helps decrease lipid accumulation through the AMPK pathway, which also indicates a prebiotic-like effect on gut microbiota.

Keywords: *Citrus depressa*, Citrus peels, Gut microbiota, Lipid accumulation

1. Introduction

According to the information from World Health Organization (WHO) in 2021, 39% of men and 40% of women is overweight, and 11% of men and 15% of women is obese in 2016 [1]. The relative low price of calorie-dense process foods is correlation to the obesity epidemic in upper-income and higher-income countries [2]. Energy intake is more than their energy expenditure, which is the major cause of obesity [1]. In the first phase, fat is abnormally accumulated in the adipose tissues, especially in the abdominal region, which is associated with obesity-related comorbidities [3,4]. The excessive energy is stored as triacylglycerol (TG) in the adipose tissue via the fatty acid pathway. Acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase-1 are lipogenic enzymes [5]. AMP-activated protein kinase (AMPK) plays an important role in regulating energy homeostasis and metabolic pathways [6]. Since the 1970s, when AMPK was discovered, it has been known that this enzyme negatively regulates the rate-limiting acetyl-CoA carboxylase for fatty acid synthesis and the rate-limiting HMG-CoA reductase for cholesterol synthesis, also activation of AMPK was found to inhibit lipogenesis by down-regulating the activity of fatty acid synthase in the liver and adipose tissue [7]. Increasing evidence shows that the gut microbiota plays a key role in the host's health. The gut microbiota is related to many conditions such as obesity, type 2 diabetes, fatty liver disease, intestinal bowel disease, and cancer, and may involve immunity, energy, lipid, and glucose metabolic pathways [8]. An unstable microbiome and the dysbiotic status of the gut

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microbiota may be related to metabolic pathology [9]. Recently, researchers have shown that polyphenols modulate the gut microbiota; in turn, microorganisms can modulate the activity of polyphenols. This bidirectional interaction would regulate the metabolism, and the bioavailability of polyphenols or their conversion into metabolites might have different health effects on the host [10].

Citrus fruits are the most consumed fruits and the crops with the highest yields worldwide; thus, citrus production has grown in the last decades [11]. However, the production of juice generates massive amounts of peels as waste [11]. Nevertheless, this waste contains various bioactive components such as flavonoids, limonoids, coumarins, and other polyphenols [11]. The dried citrus peel is kind of traditional Chinese medicine known as chenpi is a traditional medicine used for alleviating the symptoms of digestive disorders. Chenpi extract contains flavonoids such as narirutin, hesperidin, nobiletin, tangeretin, and 5-demethylnobiletin, which show antioxidant, anti-inflammatory, and anti-obesity effects [12,13]. *Citrus depressa* Hayata is a small citrus known as hirami lemon, flat lemon, thin-skinned flat lemon, and is distributed in Japan and Taiwan [14]. Research has shown that the ethanol or methanol extract of the peel of *C. depressa* contains polymethoxylated flavonoids such as nobiletin, tangeretin, sinensetin, and 5-demethylnobiletin, which showed inhibitory effects on lipid accumulation in the liver and adipose tissue [15,16]. Therefore, the objective of this study was to evaluate the effects of the peel of *C. depressa* Hayata treated by hot drying and extracted with ethanol (*C. depressa* ethanol extract, CDEE) on lipid accumulation and the alteration of the intestinal flora in high-fat diet-induced obese mice.

2. Materials and methods

2.1. Chemicals

Antibodies against fatty acid synthase (FAS) were purchased from Cell Signaling Technology (Beverly, MA, USA). The antibody of AMP-activated protein kinase (AMPK) was purchased from ABclonal (Woburn, MA, USA). The GADPH control antibody was purchased from Proteintech (Rosemont, IL, USA). The rabbit and mouse secondary IgG antibodies were procured from Croyez Bioscience Co. (Taipei, Taiwan). Ethyl alcohol and acetonitrile were procured from Sigma Chemical Co. (St. Louis, MO, USA). Hesperidin was purchased from Tokyo Chemical Industry (Tokyo, Japan). *Citrus depressa* Hayata fruit were purchased from Yung-Hsin Cooperative (Pingtung, Taiwan). Nobiletin, tangeretin, 5-demethylnobiletin, 5-demethyltangeretin, and sinensetin were kindly provided by Dr. Guor-Jien Wei.

2.2. Sample preparation and flavonoid content analysis

C. depressa Hayata were bought from Yung-Hsin Cooperative at Pingtung, Taiwan during March, 2021, and peel was collected manually, then dried in an oven at 50 °C for 80 h, ground into powder, and extracted with 95% ethyl alcohol for 48 h. The extract was evaporated in a rotary vacuum evaporator and freeze-dried to obtain the *C. depressa* ethanolic extract (CDEE), which was stored at −20 °C for further experiments. The high-performance liquid chromatography (HPLC) method was based previous studies with modifications [12,17]. Briefly, the CDEE extract solution was applied to a Shimadzu LC-10AT HPLC system with a C18 column (250 mm × 4.6 mm, 5 μm) and a photodiode array (PDA) at a 280 nm wavelength (Shimadzu, Kyoto, Japan). The injection volume was 20 μL and the flow rate was maintained at 1.0 mL/min. Mobile phase A consisted of deionized water, whereas mobile phase B was acetonitrile.

2.3. Animal experiments

Four-week-old male C57BL/6 mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and housed under a controlled atmosphere (25 ± 1 °C at 50% relative humidity) with a 12 h light/12 h dark cycle. Mice had free access to food and water during the whole experiment. After 1 week of acclimation, animals were randomly distributed into four groups (n = 7): normal diet (ND, 15% energy from fat), high-fat diet (HFD, 50% energy from fat), ND supplemented with 2% CDEE (NDCDEE), and HFD supplemented with 2% CDEE (HFDCDEE); the trial lasted 12 weeks. LabDiet (laboratory rodent diet) 5001 was used as the ND and was purchased from Young Li Trading Company (Taipei, Taiwan). HFD was a modified normal diet according to our previous study [18] (Supplementary Table 1). Food consumption was recorded daily and the body weight was recorded weekly. At 12 weeks, all animals were sacrificed by CO_2 asphyxiation. Blood samples were collected by cardiac puncture. The epididymal, retroperitoneal, mesenteric, and inguinal fat, and feces were immediately removed and stored at −80 °C until further analysis. All animal experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee of Chung

Yuan Christian University (IACUC, Approval No: 11005).

2.4. Serum analysis

The analysis was performed according to our previous study [18]. Blood samples were centrifuged at 4000×*g* for 10 min at 4 °C and stored at −20 °C until analysis. The serum levels of alanine transaminase (ALT), total cholesterol (TCHO), triacylglycerol (TG), high-density lipoprotein (HDL-C), and low-density lipoprotein (LDL-c) were analyzed at the National Laboratory Animal Center (NLAC; Taipei, Taiwan), using a Hitachi 7080 biochemical analyzer (Tokyo, Japan) according to the manufacturer's instructions.

2.5. Histopathological examinations and adipocyte size

The histopathological examination was performed according to our previous study [18]. Epididymal and inguinal adipose tissue were dissected at a 4 μm thickness and fixed in 10% buffered formalin, dehydrated with a sequence of ethanol solutions, and processed for embedding in paraffin. Then, stained with hematoxylin and eosin (H&E) and subjected to microscopic observation. The adipocyte size was measured at a 100 × magnification and determined by the Image J software (Bethesda, MD, USA).

2.6. Western blotting

Epididymal tissue was homogenized with RIPA buffer to extract total protein as described in our previous study [12] with slight modifications. The cell lysate was centrifuged at 17,000×*g* for 1 h at 4 °C. The total protein content was measured using the bicinchoninic acid assay. Cell lysate containing 35 μg of protein was heated at 95 °C for 5 min and subjected to SDS-PAGE. After 3–4 h, the SDS-PAGE was transferred to a PVDF membrane with a transfer buffer. The primary antibodies FAS, AMPK, and pAMPK were applied against the target proteins and GADPH. The blots were visualized by a luminescent image analyzer (Fujifilm, Tokyo, Japan) and quantified using the Image J software (Bethesda, MD, USA).

2.7. Gut microbiota classification by next-generation sequencing (NGS)

This procedure was conducted according to our previous study [18]. Fecal DNA was extracted using the innuPREP Stool DNA Kit (Jena, Germany) according to the manufacturer's instructions. All PCR reactions were carried out using a Phusion® High-Fidelity PCR Kit (MA, USA). Then, the mixed PCR products were purified using a Qiagen gel extraction kit (Hilden, Germany). Finally, the library was sequenced using an Illumina HiSeq 2500 platform; 250 bp paired-end reads were generated and the classification of operational taxonomic units was based on the Greengenes database (https://greengenes.lbl.gov/).

2.8. Statistical analysis

Data are represented as the means ± SD and significant differences between groups were identified by one-way analysis of variance (ANOVA) and Duncan's multiple range test, using SAS (version 9.4, SAS Institute Inc., Cary, NC, USA). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Flavonoid content of CDEE

The HPLC analysis showed that the ethanol extract of the peel of *C. depressa* contained various flavonoids such as hesperidin (2844.6 mg/100 g dry extract), nobiletin (10283.4 mg/100 g dry extract), tangeretin (5622.5 mg/100 g dry extract), and sinensetin (627.9 mg/100 g dry extract), which are generally found in citrus peels. In this study, we also found 5-demethylnobiletin (667.3 mg/100 g dry extracts), and, especially, 5-demethyl-tangeretin (26 mg/100 g dry extract), which are reported for the first time in *C. depressa* (Bi˘an shí níngméng) peel extract from Taiwan (Table 1 & Supplementary Figure 1).

3.2. Effects of CDEE on body weight and food intake

At the beginning of the experiment, the initial body weight of the four groups did not show a significant difference. After 12 weeks, the final body

Table 1. The flavonoids content of CDEE.

Flavonoids	(mg/100 g dry extracts)
Hesperidin	2844.6
Sinesentin	627.9
Nobiletin	10283.4
Tangeretin	5622.5
[a]5-OH Nobiletin	667.3
[b]5-OH Tangeretin	26.0

[a] 5-demethylnobiletin
[b] 5-demethyltangeretin

weight of the HFD group was 30.2 ± 0.9 g, which was significantly higher than that of the ND group (23.1 ± 1.1 g). Mice fed a normal diet supplemented with CDDE (NDCDEE) did not show a final body weight difference with the ND group. On the other hand, mice fed a high-fat diet supplemented with CDDE (HFDCDEE), had a final body weight of 26.2 ± 0.9 g, which was significantly decreased compared with that of the HFD group. During the whole experiment, the HFD group had a significantly higher weight gain (2.5%) compared with the ND group. The HFDCDEE group had a significantly lower (0.8%) weight gain compared with the HFD group. The effect of food intake in the ND and NDCDEE groups was significantly greater than in the HFD and HFDCDEE groups. However, ND compared with NDCDEE or HFD compared with HFDCDEE did not show significant differences. Although the ND and NDCDEE groups had a significantly higher food intake than the HFD and HFDCDEE groups, the food efficiency in the HFD and HFDCDEE mice was significantly higher than in the ND and NDCDEE mice (Table 2).

3.3. Effects of CDEE on blood biochemistry

The TCHO, HDL-C, and LDL-C levels in the HFD group were significantly higher than in the ND and NDCDEE groups (Table 3). In this study, the HFDCDEE groups had significantly lower serum TCHO levels compared with the HFD groups, which suggests that CDEE might alleviate the high serum cholesterol caused by an HFD. The NDCDEE and HFDCDEE groups did not show significant differences in serum AST levels compared with the ND or HFD groups. AST is a crucial liver toxicity index; thus, the result obtained proved that mice administered with CDEE did not experience liver damage [19]. Nevertheless, the NDCDEE group had significantly increased serum TG levels compared to other groups.

3.4. Effects of CDEE on the weight of adipose tissue and lipogenesis-related protein expression

The HFD group had significantly higher epididymal, retroperitoneal, mesenteric, and inguinal fat, and body fat ratios compared with the ND group. The HFDCDEE group had significantly decreased weights of the epididymal, retroperitoneal, mesenteric, and inguinal fat, and body fat ratio compared with the HFD group. The NDCDEE groups had significantly lower mesenteric fat weights (Fig. 1). Furthermore, the epididymal fat of the HFD group had a substantially larger adipocyte size compared with that of the ND group (Fig. 2A). By contrast, the HFDCDEE group had epididymal fat with a significantly decreased adipocyte size, which was caused by the HFD. In this study, we found a significantly upregulated pAMPK protein expression and downregulated FAS protein expression (Fig. 2B). The results showed that CDEE decreased lipid accumulation by regulating pAMPK and FAS protein expression.

3.5. Effects of CDEE on gut microbiota

After the mice were fed with the HFD for 12 weeks, the composition of the gut microbiota was different from that of the ND group. Firmicutes (F) predominated in the HFD group (33.0%) in contrast with the 22.5% observed in the ND group. The NDCDEE group had 31.8% Firmicutes, and the HFDCDEE group had 41.2%. Bacteroidetes (B), the other major bacterial phylum in the gut, was less abundant (55.77%) in the HFD group than in the ND

Table 2. The physiological profiles.

Groups	ND	NDCDEE	HFD	HFDCDEE
Initial Weight (g)	19.1 ± 1.1^{a}	19.0 ± 0.6^{a}	19.0 ± 1.0^{a}	18.3 ± 0.7^{a}
Final Weight (g)	23.1 ± 1.1^{c}	23.0 ± 0.7^{c}	30.2 ± 0.9^{a}	26.2 ± 0.9^{b}
Weight gain (g)	4.2 ± 0.5^{c}	4.2 ± 0.3^{c}	10.7 ± 1.0^{a}	8.8 ± 0.7^{b}
Food intake (g)	3.4 ± 0.02^{a}	3.3 ± 0.06^{a}	2.7 ± 0.15^{b}	2.8 ± 0.20^{b}
Food efficiency (g/day/mice)#	0.1 ± 0.2^{b}	0.1 ± 0.2^{b}	0.3 ± 0.3^{a}	0.2 ± 0.2^{ab}

Table 3. The blood biochemical analysis.

Groups	ND	NDCDEE	HFD	HFDCDEE
AST(U/L)	254.8 ± 56.1^{a}	324.7 ± 127.0^{a}	226.2 ± 57.8^{a}	313.1 ± 37.4^{a}
TCHO(mg/L)	104.8 ± 15.0^{c}	108.6 ± 8.0^{c}	192.2 ± 26.6^{a}	170.1 ± 6.8^{b}
TG(mg/L)	46.4 ± 16.1^{b}	86.6 ± 17.3^{a}	43.8 ± 17.5^{b}	39.2 ± 4.8^{b}
HDL-C(mg/L)	80.5 ± 13.9^{b}	89.7 ± 12.7^{b}	134.3 ± 21.5^{a}	127.9 ± 5.4^{a}
LDL-C(mg/L)	14.6 ± 2.1^{b}	9.9 ± 1.2^{b}	40.0 ± 9.2^{a}	40.2 ± 4.8^{a}

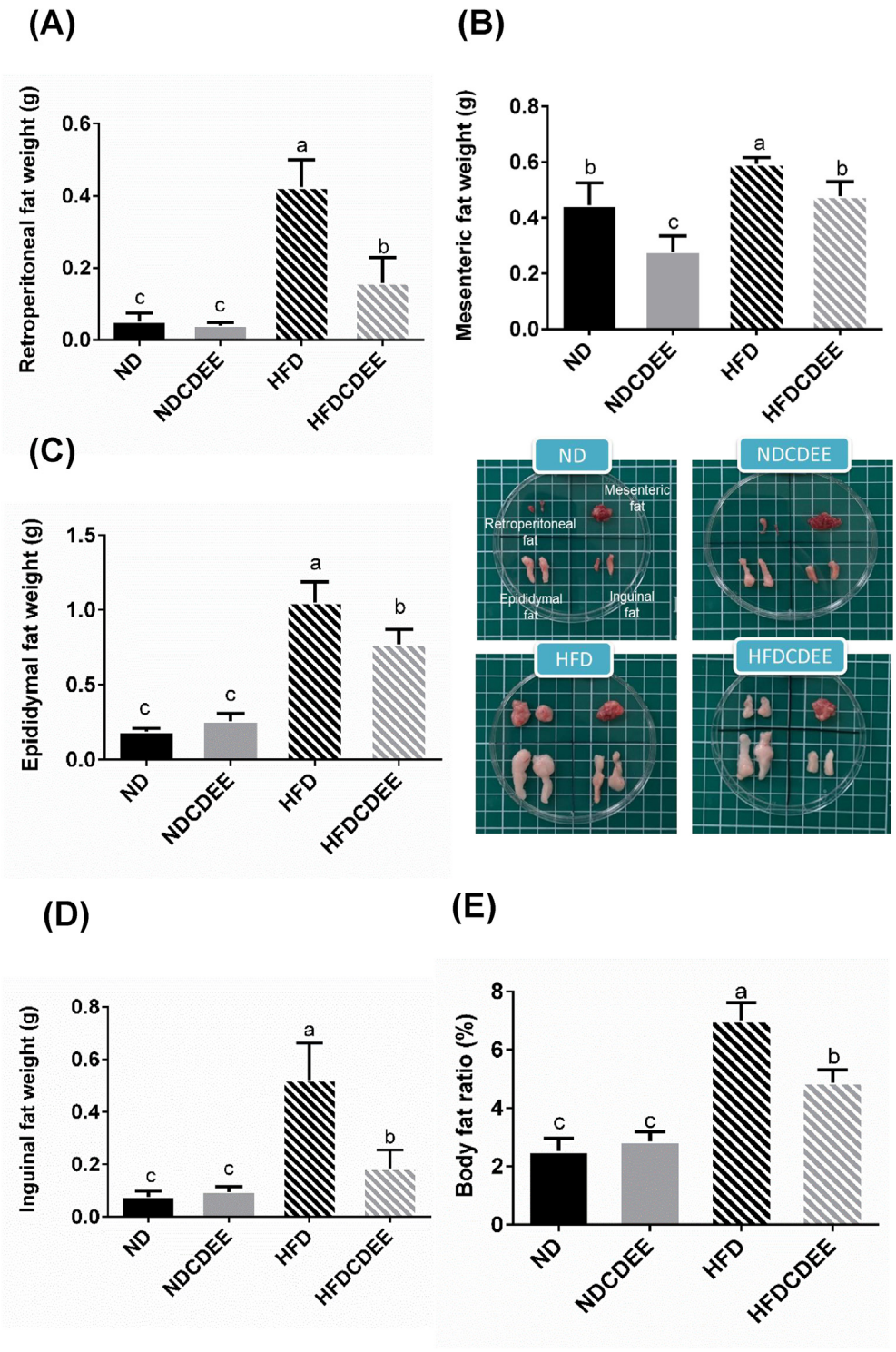

*Fig. 1. **Effect of CDEE on the weight of adipose tissue.** Weights of the (A) retroperitoneal, (B) mesenteric, (C) epididymal, and (D) inguinal adipose tissues. (E) Body fat ratio (perigonadal weight + retroperitoneal + mesenteric/final body weight). Statistical differences were detected by one-way ANOVA with Duncan's tests. Different letters (a–c) indicate significant differences ($p < 0.05$) among the groups.*

group (74.8%). The NDCDEE group had 58.5% Bacteriodetes, and the HFDCDEE group had 52.8% (Fig. 3A). The HFD and HFDCDEE groups had significantly higher Firmicutes/Bacteriodetes ratios than the ND group (Fig. 3B). The HFD group had a higher abundance of *Akkermansia*, *Lachnospiraceae NK4A13*, and *Desulfovibrio*, and a lower abundance of *Muribaculum* and *Blautia*. The HFDCDEE group

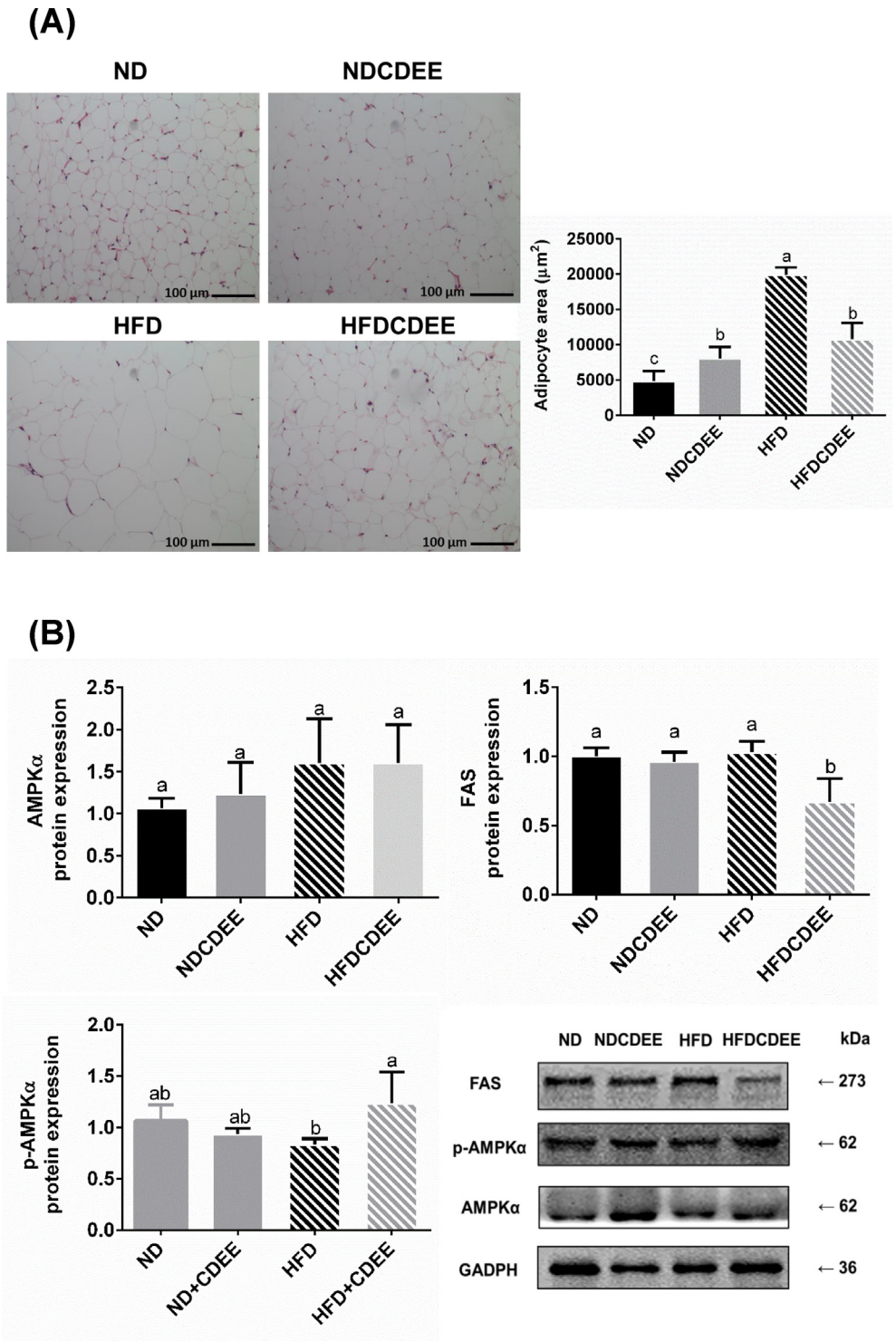

Fig. 2. ***Effect of CDEE on the size of the adipose tissue and AMPK pathway-related protein expression.*** *(A) The size of the epididymal adipocyte was evaluated by H&E staining, and representative images were taken at a 200 × magnification; (B) Protein expression of AMPK, p-AMPK, and FAS. Statistical differences were detected by one-way ANOVA with Duncan's tests. Different letters (a–c) indicate significant differences ($p < 0.05$) among the groups.*

had a higher abundance of *Oscillibacter* and *Ruminiclostridium 9* (Fig. 3C). The Venn diagram showed that the ND group had four operational taxonomic units (OTUs), the NDCDEE group had 17 OTUs, the HFD group had 10 OTUs, and the HFD had eight OTUs, which were different for each group (Fig. 4A and B). The Chao1 and ACE indices are used to estimate community richness; the higher the value,

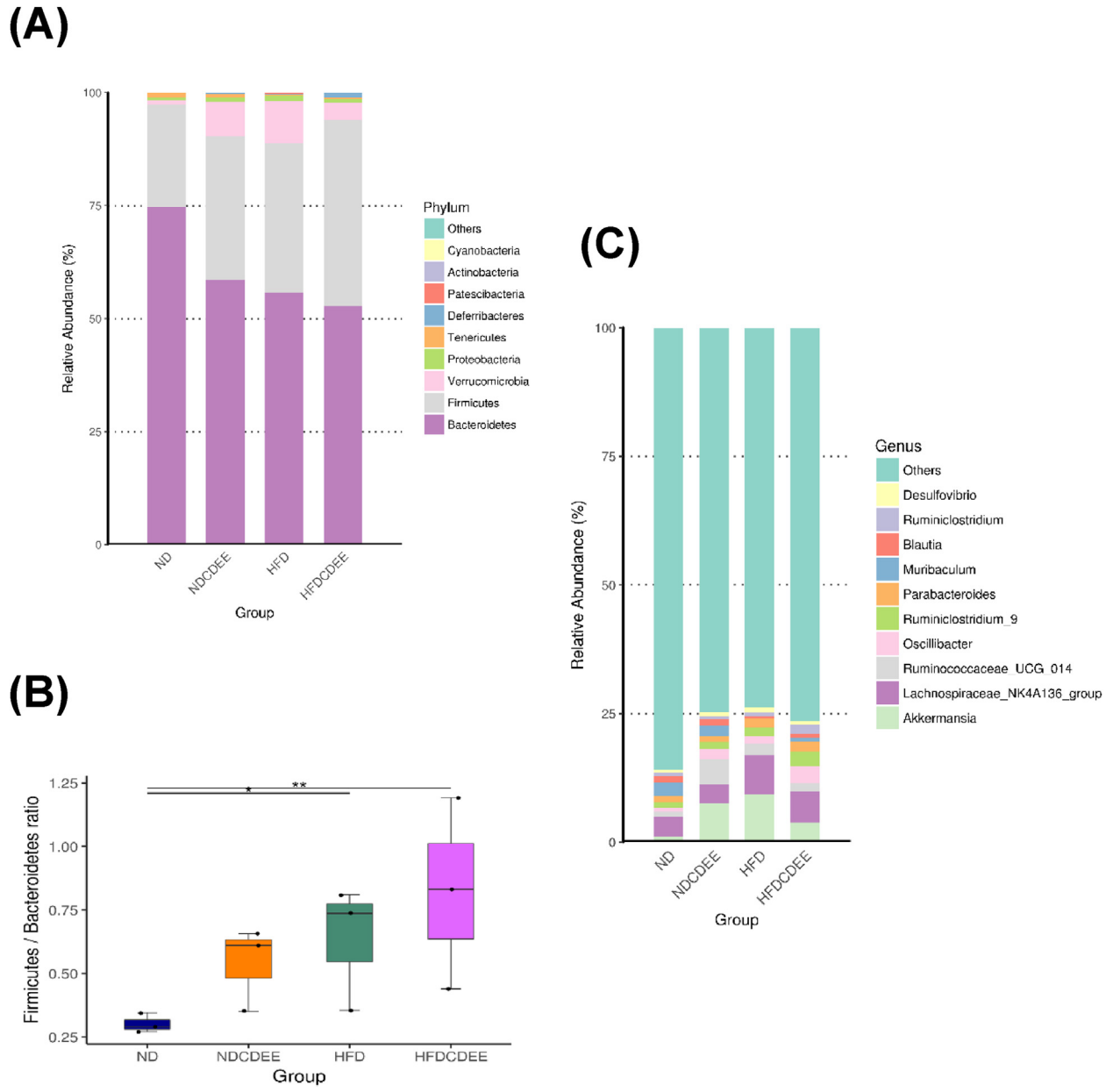

Fig. 3. ***Effects of CDEE on gut microbiota.*** *(A) Top 10 bacteria at the phylum level; (B) Firmicutes/Bacteroidetes ratio; (C) Top 10 bacterial taxa at the genus level.*

the more abundant the gut microbiota [20]. Although the HFDCDEE group had lower Chao1 and ACE indices, there were no significant differences among other groups (Fig. 4C and D). The Shannon and Simpson indices are used to reflect the diversity of gut microbiota; the higher the Shannon index, the higher the diversity of the gut microbiota [20]. By contrast, the higher the Simpson index, the lower the diversity of the gut microbiota. Here, there were no significant differences among the four groups (Fig. 4E and F). These results demonstrate that different diets affect the bacterial richness and diversity in the gut. We further analyzed the whole bacterial composition in the gut using Welch's *t*-test and metagenomeSeq statistic methods. At the genus level, the HFD group had significantly more abundance of *Turicibacter, GCA 900066575, Faecalibaclum,* and *Bifidobacterium,* and less abundance of *Ruminococcaceae UCG013* than the ND group. The NDCDEE group had significantly less abundance of *ASF356* and Ruminococcaceae *UCG013* than the ND group. The HFDCDEE group had a significantly greater abundance of *Acetatifactor* and a lower abundance of *Negativibacillus* than the HFD group (Fig. 5A). The

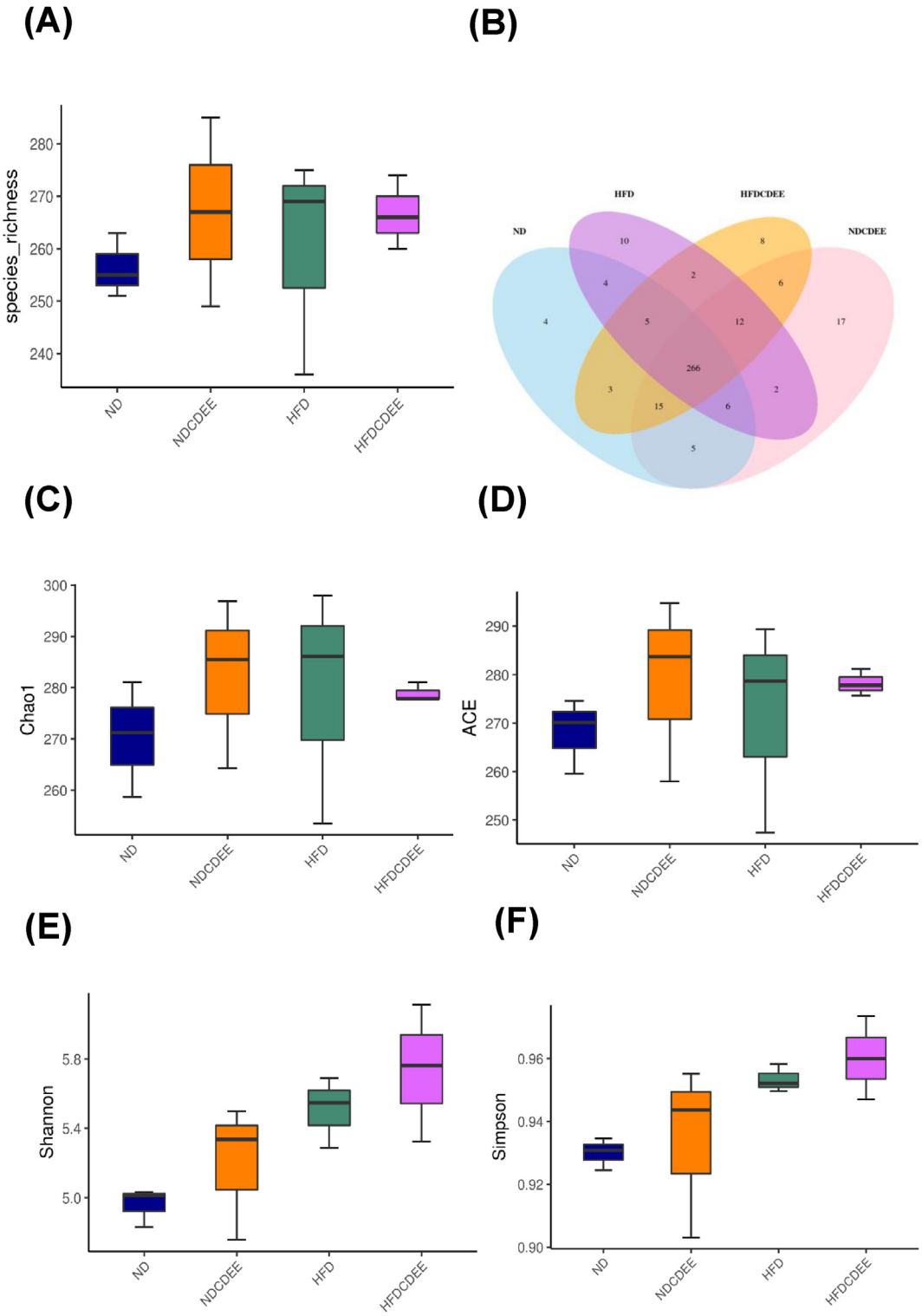

Fig. 4. ***Effects of CDEE on the alpha diversity of gut microbes.*** *(A) Species richness; (B)Venn diagram; (C) Chao1; (D) ACE; (E) Shannon; and (F) Simpson indices.*

metagenomeSeq results showed that the ND group had a significantly greater abundance of *Ruminococcaceae UCG013* than the HFDCDEE groups. HFDCDEE showed a higher abundance of *Ruminococcaceae UCG013* than the HFD group, although the difference was not significant. The ND and NDCDEE groups had a significantly lower abundance of *Turicibacter* compared with the HFD

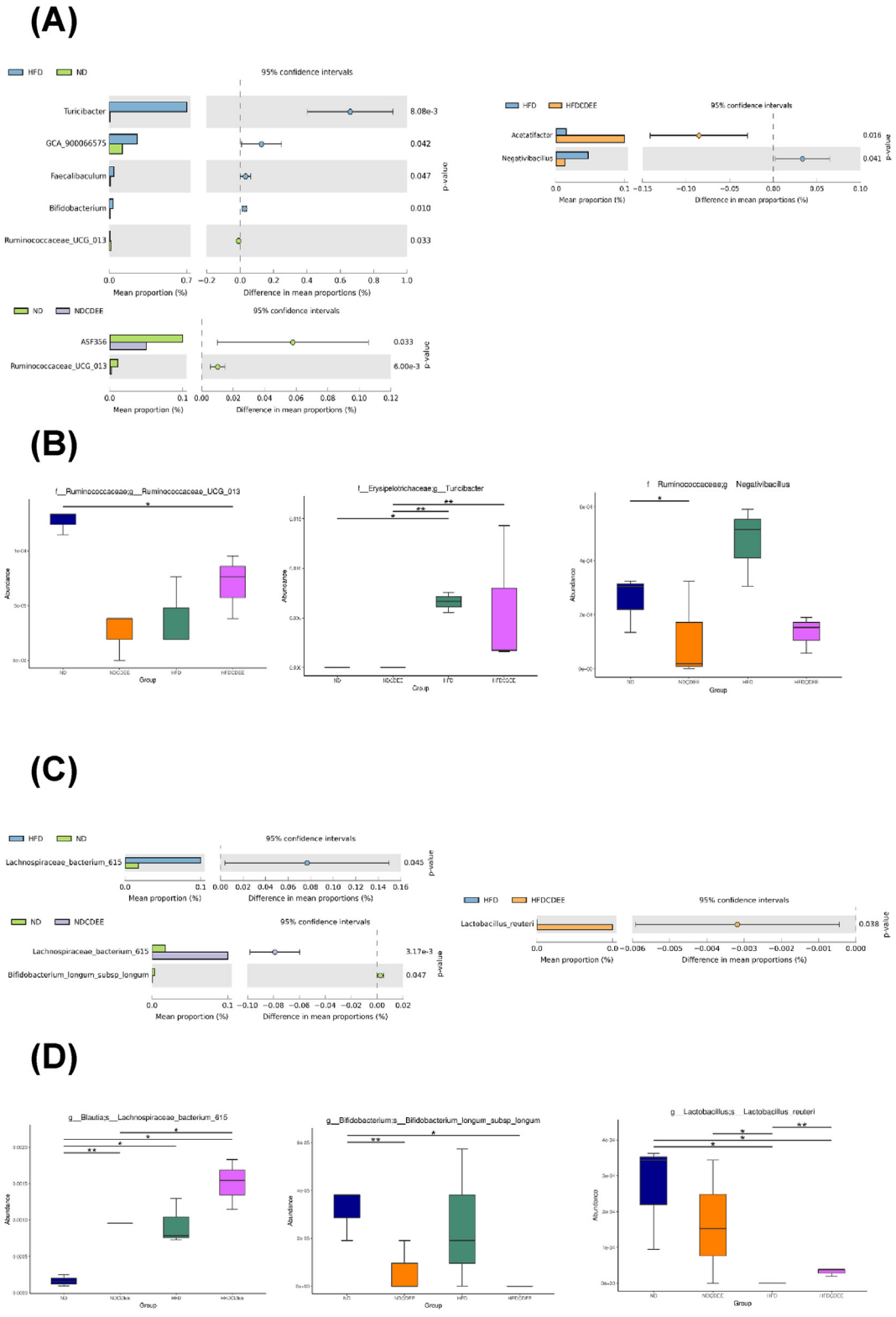

Fig. 5. ***Effects of CDEE on specific gut bacteria.*** *(A) Analysis of the bacterial abundance at the phylum level by Welch's t-test; (B)Analysis of the bacterial abundance at the phylum level by metagenomeSeq; (C) Analysis of the bacterial abundance at the genus level by Welch's t-test; (D) Analysis of the bacterial abundance at the genus level by metagenomeSeq.*

group. HFDCDEE also had a lower abundance of *Turicibacter* than the HFD group, although the difference was non-significant. HFDCEE and NDCDEE had a lower abundance of *Negativibacillus* than ND and HFD (Fig. 5B). At the species level, the HFD and NDCDEE groups had a significantly greater abundance of *Lachnospiraceae bacterium 615* than the ND group. The ND group had a significantly greater abundance of *Bifidobacterium longum* than the HFD group. The HFDCDEE group had a significantly greater abundance of *Lactobacillus reuteri* than the HFD group (Fig. 5C). The metagenomeSeq results showed that mice administered CDEE with the ND or HFD diet had a higher abundance of *Lachnospiraceae bacterium 615* and a lower abundance of *B. longum* compared with the ND and HFD groups. The ND group had a significantly higher abundance of *L. reuteri* than the HFD and HFDCDEE groups. The HFDCDEE group also had a significantly higher abundance of *L. reuteri* than the HFD group (Fig. 5D). In this study, CDEE demonstrated prebiotic potential by reacting with *L. reuteri* to ameliorate the adverse effects caused by an HFD. We input the intestinal bacterial composition to the PICRUSt functional predictions and the clusters of orthologous groups (COG) results annotated that the bacterial composition of the HFDCDEE groups had a higher abundance related to carbohydrate transport and metabolism and a lower abundance related to lipid transport and metabolism compared with the HFD group. Furthermore, the results of the Kyoto Encyclopedia of Genes and Genomes (KEGG) annotated that the bacterial composition of the HFDCDEE group had a lower abundance related to amino acid metabolism and lipid metabolism (Fig. 6).

4. Discussion

Citrus peel contained lot of flavonoids, and Li et al. obtained *C. depressa* peel powder after drying the peels and extracting them with methanol and dimethyl sulfoxide and reported the presence of hesperidin, nobiletin, and tangeretin [21]. The ethanol extract of *C. depressa* from Japan (shiikuwasa) contained nobiletin, tangeretin, sinensetin, and 5-demethylnobiletin [16]. In this study, CDEE contain hesperidin, nobiletin, tangereti, and sinensetin, the 5-demethylnobiletin and firstly detected 5-demethyl-tangeretin in CDEE means that *C. depressa* peel extracted with ethanol contain variety of citrus flavonoids (Table 1). Citrus showed its ability on body weight. In a 2012 study, the administration of 1.5% methanol extract of *C. depressa* to ICR mice fed an HFD reduced body weight and prevented weight gain [16]. In other study, mice administered the chenpi extract gained less body weight [22]. In this study, CDEE prevented body weight gain when mice were fed a high-energy diet (Table 2). Obesity is related to the dyslipidemia, a research showed that ICR mice fed an HFD with 1.5% methanol extract of *C. depressa* peel also showed decreased serum TCHO and TG levels. Moreover, the extract did not increase the serum HDL-C level compared with the HFD group [16]. Li (2022) administered 4% *C. depressa* peel extract to Wistar rats. As a result, the serum TCHO and TG levels did not decrease [21]. However, chenpi extract decreased the plasma TCHO but not the plasma TG levels [22]. Although, in this study, NDCDEE had highest TG level (86 mg/dL), but a research showed that 20-week-old male C57BL/6J mice, the serum TG level was 1.0 mM (89 mg/dL) [23,24]. The mice fed with normal diet or HFD and CDEE did not affect the TG level, but HFDCDEE group could significantly decease TCHO level compared with HFD group. CDEE had potential to reverser the TCHO level in obese mice (Table 3). Excessive energy would store as triacyglycerol in the abdominal region. The administration of 1.5% methanol extract of *C. depressa* peel decreased the epididymal and perirenal fat weight, reduced the adipocyte size compared with the HFD group, and downregulated the FAS mRNA level [15]. CDEE could decrease body fat weight, based on the abdominal fat weight (Fig. 1). Chenpi extract (0.5%) decreased the weight of the epididymal fat and its adipocyte size and upregulated pAMPK protein expression [22]. CDEE also decreased the lipid accumulation by increased pAMPK protein expression, and lipogenesis related protein FAS expression (Fig. 2). Diet is one of the exogenous factors behind the differences in the gut microbiota of different individuals. Approximately 57% of the intestinal flora composition is related to the diet alone [9,25]. Some studies have shown that lean individuals have a higher Bacteriodetes/Firmicutes ratio, whereas the opposite is true for obese individuals [9]. However, HFDCDEE showed higher Firmicutes/Bacteriodetes ratio same as HFD group (Fig. 3A), but Sze et al. found that the Bacteriodetes/Firmicutes ratio was only loosely associated with the obesity status. Furthermore, some human studies and meta-analyses have not found a relationship between the Bacteriodetes/Firmicutes ratio and obesity [9,26]. Nevertheless, more evidence is needed to determine what kind of intestinal flora composition is needed for a healthy gut. The diversity of the intestinal flora is, however, an essential element for the host's health [9,27]. Mice fed different diets would have different bacterial

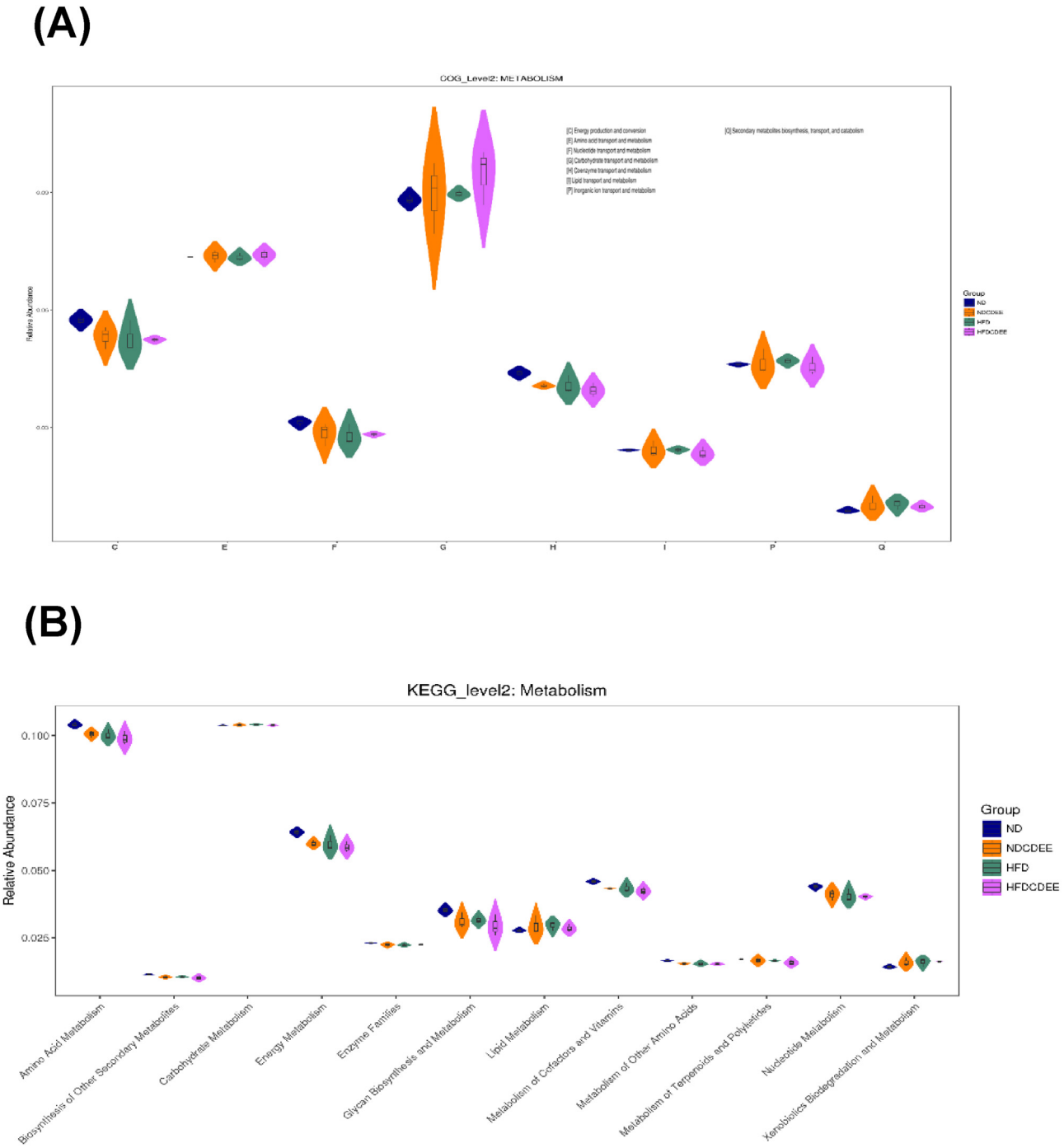

*Fig. 6. **Effects of CDEE on gut bacteria based on the PICRUSt functional predictions.** (A) Clusters of orthologous groups (COG) annotated at Level 2; (B) Kyoto Encyclopedia of Genes and Genomes (KEGG) annotated at Level 2.*

compositions. HFDCDEE showed eight different OTUs compared other group, although, the richness and abundance did not showed significant different (Fig. 3), but we further investigate CDEE effect on gut composition in genus and species level. A 0.25% concentration of chenpi extract promoted the growth of *Lactobacillus*, whereas 0.5% chenpi extract promoted the growth of *Bifidobacterium* [28]. The consumption of ultra-processed food, which has a high energy density, is positively associated with the abundance of *Negativibacillus* in overweight elderly as well as elderly suffering from obesity and metabolic syndrome [29]. Yiqi-Bushen-Tiaozhi is a traditional medicine that restored the abundance of *Acetatifactor* when mice were fed high-fat and high-fructose diets [20]. HFDCDEE group had higher

abundance of *Acetatifactor* and a lower abundance of *Negativibacillus* compared with the HFD group (Fig. 5A), CDEE could be a positive supplement for modulated the composition of gut microbiota. The obesity-resistant mice treated with HFD had a higher abundance of *Ruminococcaceae UCG013* than obesity-prone mice fed an HFD, and a negative correlation with serum TCHO, TG, LDL-C, and body weight was observed [30]. *Ruminococcaceae UCG013* is a butyrate-producing bacterium that can degrade cellulose and hemicellulose; thus, the population that consumes various fruits and vegetables has a greater abundance of this bacterium [30]. Lynch et al. indicated that the relative abundance of *Turicibacter* is inversely related to dietary fat and host adiposity, although some researchers have obtained the opposite result in rodent and human studies [31]. These differences may be due to the different *Turicibacter* strains in the host, host genetics, and sex, leading to a different lipid response [31]. Although, HFDCDEE group did not had significant higher abundance of *Ruminococcaceae UCG013* than the HFD group (Fig. 5B), but it restore the abudnace of *Ruminococcaceae UCG013,* might it is a factor that CDEE could decrease the TCHO level. HFDCDEE decrease the abundance of *Turicibacter* compared with the HFD group, maybe the abundance of *Turicibacter* affect the lipid accumulation in HFDCDEE group (Fig. 5B). ApoE −/− mice fed a Western diet supplemented with *L. reuteri* for 11 weeks exhibited a decreased body weight and epididymal fat weight [32]. *Lactobacillus* spp., such as *L. acidophilus, L. rhamnosus, L. bulgaricus, L. casei, and L. reuteri*, are the most used probiotics in the food industry and are commonly used in food fermentation [33]. These microorganisms are also found in the human and animal GI system. *L. reuteri* is a well-studied probiotic bacterium that has shown beneficial effects on the health of the host [33]. Multi-strain *L. reuteri* (JBD301, GMNL-263, and 6475) exhibited a negative correlation with obesity in mice. Gibson et al. defined the term prebiotic as a digestible food ingredient fermented by intestinal flora that is selectively used by host microorganisms, leading to health effects [9,34]. HFDCDEE might increase the abundance probiotic microorganism-*L. reuteri* to restore the adiposity cause by HFD, therefore, CDEE might had potential act like a prebiotic (Fig. 5D).

5. Conclusion

In this study, we found that *Citrus depressa* peel ethanol extract (CDEE) contained various flavonoids including hesperidin, nobiletin, tangeretin, sinensentin, 5-demethyl-nobiletin, and 5-demethyltangeretin. Mice fed a high-fat diet (HFD) supplemented with CDEE had a lower body weight as well as an epididymal adipose tissue with a lower weight and cell size as a result of the high energy intake. The HFDCDEE group also had lower blood cholesterol compared with the HFD group. The results of the gut bacterial composition showed that the CDEE supplement increased the abundance of *L. reuteri* in mice fed an HFD. *L. reuteri* is a well-studied probiotic bacterium that showed that CDEE might have a prebiotic-like effect to prevent lipid accumulation because of its flavonoid content.

Conflict of interest

The authors declare that there are no known conflicts of interest or personal relationships that could have affected the work presented in this paper.

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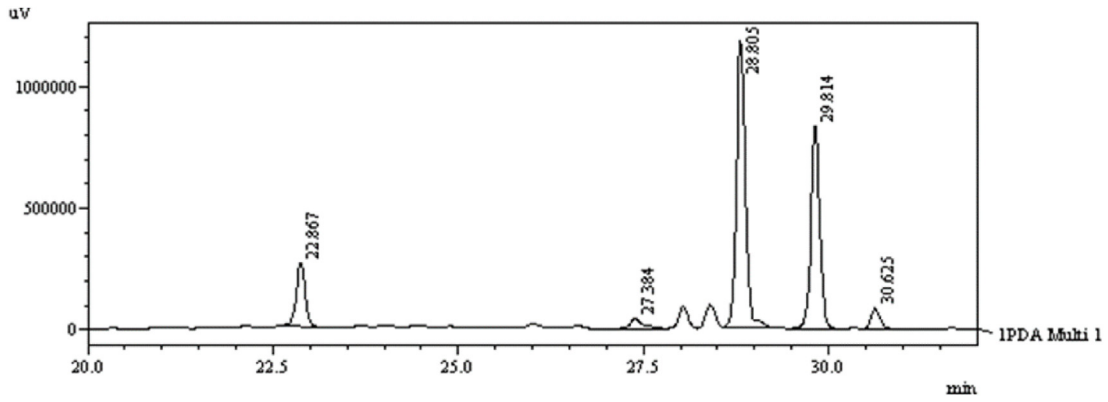
Appendix

Supplementary Table 1. Nutritional composition of the normal diet (ND) and high-fat diet (HFD).

Compositions (Calories; %)	ND	HFD
Protein	28.5	16.5
Fat	13.5	50
Carbohydrate	58.0	33.5
Total	100	100



Supplementary figure 1. The HPLC profile of CDEE.

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