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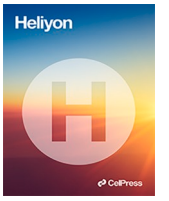

Research article

Postbiotic potential of *Bacillus velezensis* KMU01 cell-free supernatant for the alleviation of obesity in mice

Hee Hyun Shin [a], Jong-Hoon Kim [b], Ye-Jin Jung [b], Mi-Sun Kwak [b], Moon-Hee Sung [b], Jee-Young Imm [a,*]

[a] *Department of Foods and Nutrition, Kookmin University, Seoul, 02707, South Korea*
[b] *KookminBio Corporation, Seoul, 02826, South Korea*

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ABSTRACT

Attention toward the preventive effects of postbiotics on metabolic diseases has increased because of greater stability and safety over probiotics. However, studies regarding the bioactive effects of postbiotics, especially from probiotic *Bacillus* strains, are relatively limited. The anti-obesity effects of the cell-free culture supernatant of *Bacillus velezensis* KMU01 (CFS–*B.vele*) were evaluated using high-fat-diet (HFD)-induced mice. HFD-induced mice (n = 8 per group) received equal volumes of (1) CFS-*B.vele* (114 mg/kg) in PBS, (2) Xenical in PBS, or (3) PBS alone by oral gavage daily for 13 weeks. The results demonstrated that CFS-*B.vele* changed the gut microbiota and showed anti-obesity effects in HFD-induced obese mice. The elevated *Firmicutes/Bacteroidota* ratio induced by HFD was decreased in the CFS-*B.vele* group compared to the other groups ($p < 0.05$). The CFS-*B.vele* intervention led to the enrichment of SCFA-producers, such as *Roseburia* and *Eubacterium*, in the cecum, suggesting their potential involvement in the amelioration of obesity. Due to these changes, the various obesity-related biomarkers (body weight, fat in tissue, white adipose tissue weight and size, serum LDL-cholesterol level, hepatic lipid accumulation, and adipogenesis/lipogenesis-related gene/protein expression) were improved. Our findings suggest that CFS-*B.vele* has potential as a novel anti-obesity agent through modulation of the gut microbiota.

1. Introduction

Obesity is a global public health problem that has continued to increase over the years. It is closely linked to cardiovascular disease, diabetes, and osteoporosis [1]. Given the impact of obesity on global health there is a pressing need for innovative approaches to manage this complex and multifactorial condition. Recent evidence has suggested that the gut microbiota plays a critical role in food digestion and metabolic regulation [2]. The gut microbiota can alleviate obesity by modulating energy harvesting, fat deposition, pro-inflammatory cytokines, and damaged intestinal barrier function [3,4].

Probiotics are a potential strategy to modify the gut microbiota, but they struggle to reach the gut due to inherent instability [5]. In addition, an excess dose of probiotics is often administered to ensure efficacy. Postbiotics are non-viable microbial products generated during fermentation and include cell wall components, extracellular polysaccharides, and microbial metabolites of

* Corresponding author.
E-mail addresses: shh6355@kookmin.ac.kr (H.H. Shin), kbjh0701@kmbio.co.kr (J.-H. Kim), jeongyj@kmbio.co.kr (Y.-J. Jung), mskwak@kmbio.co.kr (M.-S. Kwak), smoonhee@kmbio.co.kr (M.-H. Sung), jyimm@kookmin.ac.kr (J.-Y. Imm).

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carbohydrates/proteins, such as short-chain fatty acids (**SCFA**) and branched-chain fatty acids [6].

Postbiotics have excellent stability during production and subsequent storage, making them more promising than probiotics for application as novel anti-obesity agents. Culture supernatants of *Bifidobacterium bifidum* DS0908 and *Bifidobacterium longum* DS0950 suppressed obesity by promoting thermogenesis in obese mice [7]. In addition, SCFA prevented obesity by regulating gut hormones [8]. According to these findings postbiotics may represent a novel, safe, and effective strategy for the alleviation of obesity, which warrants further investigation.

Bacillus species are commonly found in fermented foods, and a *Bacillus velezensis* strain has been isolated from Korean fermented squid products containing high salt [9]. Although *B. velezensis* has a limited probiotic potential, several *B. velezensis* strains displayed strong inhibitory activity against pathogens and improved intestinal gut damage and inflammation [10,11]. According to a recent study, postbiotics produced by *B. velezensis* Kh2-2 exerted immune-enhancing effects in a cyclophosphamide-induced mouse model and improved gut dysbiosis in mice [12]. Another recent study found that *B. velezensis* A2 decreased zearalenone, a mycotoxin that induces cecal inflammation, by modulating the intestinal flora and SCFA [13]. These results suggest that physiologically active metabolites produced by *B. velezensis* strains might modulate systemic inflammation or suppress the entry of lipopolysaccharide (LPS) by improving gut barrier function.

The correlation between obesity and the gut microbiota has been well supported in animal and human studies, and modulation of the gut microbiota by postbiotics is a promising strategy for the management of obesity [14,15]. In the present study, the anti-obesity effect of the cell-free supernatant of *B. velezensis* (**CFS–*B.vele***) was evaluated in a high fat diet(HFD)-induced obesity mouse model.

2. Materials and methods

2.1. Materials

Triglyceride (TG) and cholesterol assay kits (LabAssay™) were purchased from Abcam (Cambridge, MA, USA) and Wako (Osaka, Japan), respectively. TRIzol reagent for total RNA extraction and sterol regulatory element-binding protein-1c (**SREBP-1c**), and diacylglycerol acyltransferase (**DGAT**) antibodies were obtained from Invitrogen Corp. (Carlsbad, CA, USA). Other antibodies including acetyl-CoA carboxylase (**ACC**), phosphor-acetyl-CoA carboxylase (**p-ACC**), fatty acid synthase (**FAS**), CCAAT/enhancer-binding protein alpha (**C/EBPα**), peroxisome proliferator-activated receptor gamma (**PPARγ**), stearoyl-CoA desaturase 1 (**SCD-1**), β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). Taqman® Universal Master Mix, Taqman® probes, and high-capacity RNA-to-cDNA kit were purchased from Applied Biosystems (Foster City, CA, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of CFS-B. vele

B. velezensis KMU01 inoculated (2 %, v/v) into medium designed for protease production (0.1 % casein peptone, 0.5 % yeast extract, 0.1 % soy peptone, 0.1 % K_2HPO_4, 0.5 % KH_2PO_4, 0.25 % maltodextrin, 0.1 % $MgSO_4$ and 1.8 % glucose) and incubated at 37 °C for 12 h. CFS-B. vele was collected by centrifugation (13,000 rpm, 15 min) followed by filtration through a 0.22-μm membrane (Sartorius Stedim Biotech, Göettingen, Germany) and used as a sample.

2.3. Animal experiments

Male C57BL/6 J mice (5 weeks old; RaonBio, Yongin, Korea) were maintained in ventilated cages at 22 ± 1 °C and 40−60 % relative humidity with a 12-h light−dark cycle. The animal experiment protocol was approved by the Institutional Animal Care and Use Committee of Kookmin University (KMU-2022-01). After a week adaptation period, the mice were randomly assigned (n = 8/group, 4 mice/cage) as follows: 1) normal diet (NOR; 6 % kcal from fat; Envigo, UK), 2) high-fat diet (HFD; 45 % kcal from fat; Research Diets, Inc., USA), 3) *B. velezensis* (*B.vele*; HFD + 114 mg CFS/kg), 4) Xenical (Xen; HFD + 50 mg Xenical/kg; positive control). Mice had ad libitum access to feed and water. CFS-*B.vele* suspended in PBS was administered to animals once a day by gavage. Xenical dissolved in PBS or PBS alone was given to animals of the Xen and NOR (HFD) groups, respectively. Body weight and feed intake were measured every week. The feed efficiency ratio (FER) was calculated as weight gain (g/week)/feed intake (g/week) × 100. After 13 weeks of sample administration, the mice were sacrificed, and blood and tissues were collected for further analysis.

2.4. Body composition, organs and white adipose tissue (WAT) weight

Body composition were measured before sacrifice (at 13 weeks) using dual-energy X-ray absorptiometry (DEXA; Medikors, Seongnam, Korea). The weights of organs such as the heart, liver, kidney, and spleen were measured. WAT including epididymal WAT (**eWAT**), inguinal WAT (**iWAT**), mesenteric WAT (**mWAT**), and retroperitoneal WAT (**rWAT**) were collected, and their weights were separately recorded.

2.5. Serum biochemical analysis

Whole blood was collected from the heart after an 18-h fasting period and the serum was immediately. Blood glucose, aspartate aminotransferase (**AST**), alanine aminotransferase (**ALT**), and blood urea nitrogen (**BUN**) were analyzed using a chemical analyzer

(Fuji Dri Chem 3500i, Fujiflim, Ltd., Tokyo, Japan). TG, total cholesterol (**TCHO**), and high density lipoprotein cholesterol (**HDL-C**) in the serum were measured using TG assay kit and LabAssay™ Cholesterol kit, respectively. Low density lipoprotein cholesterol (LDL-C) in the serum was calculated as follows:

$$\text{LDL-C} = \text{TCOH-} \{(\text{HDL-C}) + (\text{TG} / 5)\}$$

2.6. Histological analysis of eWAT and hepatic TG content

The histological analysis of eWAT was performed using hematoxylin and eosin (**H&E**) staining assay and the average size of adipocytes was analyzed from 15 adipocytes located in center of stained section using KFBIO Slide Manager (KFBIO, Ningbo, China). The hepatic TG content was measured using TG assay kit and TG content was normalized by cellular protein content.

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated from WAT and the RNA was reverse-transcribed to cDNA using a high capacity RNA-to-cDNA kits (Applied Biosystems) by the method of Jung et al. [16]. The gene expression of C/EBPα (Mm00514283_s1) and PPARγ (Mm00440940_m1) was normalized by β-actin (Mm00607939_s1).

2.8. Immunoblotting

The liver was homogenized in radioimmunoprecipitation assay **(RIPA)** buffer containing 1 % protease inhibitor and 1 % phosphatase inhibitor using a bullet blender (Next Advance, Troy, NY, USA). After obtaining tissue lysates, equal amounts of total cellular proteins were separated on 10 % SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA).

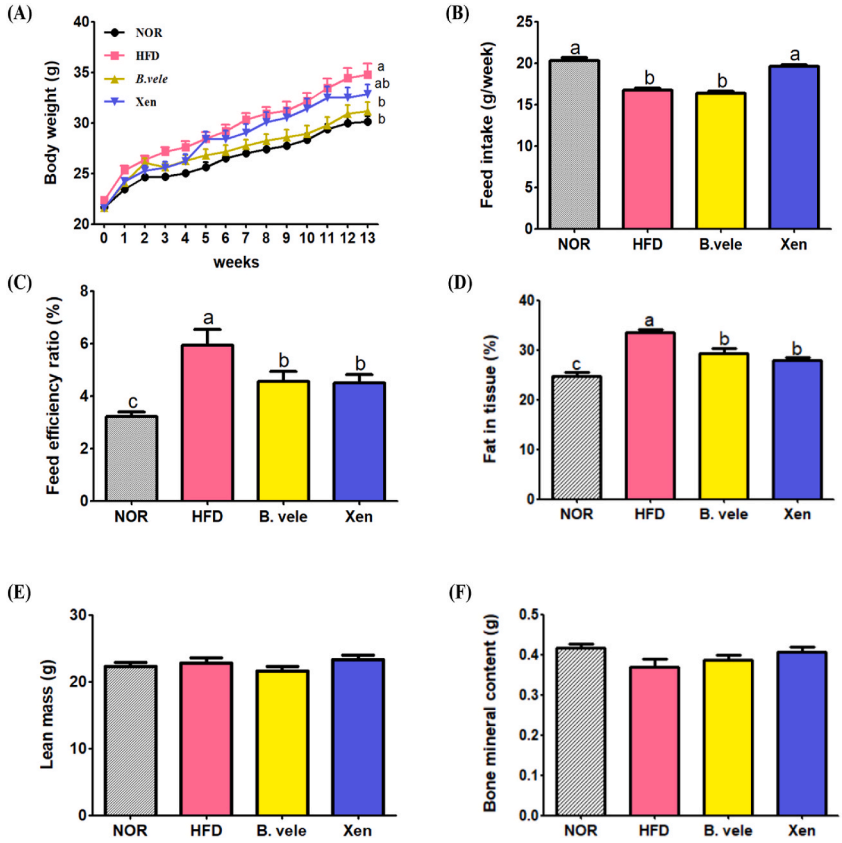

Fig. 1. Effect of CFS-*B.vele* administration on growth performance and body composition: (**A) body weight; (B) feed intake; (C) feed efficiency ratio; (D) fat in tissue; (E) lean mass; (F) bone mineral content.** CFS, cell-free culture supernatant; NOR, normal diet (6 % kcal from fat); HFD, high fat diet (45 % kcal from fat); B. vele, HFD+114 mg/kg CFS of *Bacillus velezensis*; Xen, HFD+50 mg/kg Xenical. Each value represents mean ± standard error (n = 8). Body composition [(D), (E), and (F)] was measured using dual-energy X-ray absorptiometry. a,b: Different letters indicate significant differences at $p < 0.05$.

The immunoblotting procedures were performed [17].

2.9. Cecal microbiota analysis

Gut microbiome DNA was extracted from cecum of mice using a Qiagen DNeasy PowerSoil Pro extraction kits (Qiagen, CA, USA). DNA concentration was quantified by 260/280 nm and 260/230 nm absorption ratio. The V3–V4 variable region of the 16S rRNA gene was amplified and sequenced on an Illumina MiSeq sequencing system (Illumina, San Diego, CA, USA) to generate 300 bp PE reads with a target sequencing depth of >50,000 reads per sample. Low-quality reads were trimmed, noisy reads were error corrected, and chimeric sequences were removed. Taxonomic classification analysis was performed for the ASVs using Qiime2 Naïve bayes classifier against SILVA database v138. The linear discriminant analysis (LDA) effect size (LEfSe) was used to find differences in relative abundances in the microbiota between groups. Taxa with LDA score >2.0 and significance of $p < 0.05$ were considered as significant difference.

2.10. Statistical analysis

All data are presented as the mean ± standard error (SE). One-way ANOVA was performed to test the significant differences ($p < 0.05$) in groups means, followed by Duncan tests with SPSS Statistics V. 26 (SPSS Inc., IL, USA).

3. Results and discussion

3.1. The effects of CFS-B.vele on growth performance and body composition

After 13 weeks of the feeding trial, the body weight of the HFD group increased significantly compared to the NOR group. It has been reported that diet containing 60 % calorie from fat was more effective inducing obesity than the 45 % fat diet [18]. However, the 45 % fat diet was chosen because it represents the typical fat intake of US adults (30–40 %) [19]. The average body weight gain after 13 weeks was about 12 and 5 g for the HFD and the NOR group, respectively, demonstrating that HFD-induced obesity. The body weight of the B. vele group was significantly decreased compared to the HFD group ($p < 0.05$; Fig. 1A). There was no difference in feed intake between the HFD and *B.vele* groups (Fig. 1B). The FER of the HFD group increased significantly compared to the NOR group. The FER of the B. vele and Xen groups decreased significantly compared to the HFD group ($p < 0.05$; Fig. 1C). These results suggest that the administration of CFS-*B.vele* effectively suppressed weight gain induced by the HFD. Similar to the result of this study, some probiotics, including *Lactobacillus paracasei* CNCM I-4270, and *L. rhamnosus* I-3690, lowered weight gain without affecting feed intake in an HFD-fed mice model, but the effects on body composition and WAT remain to be fully investigated [20].

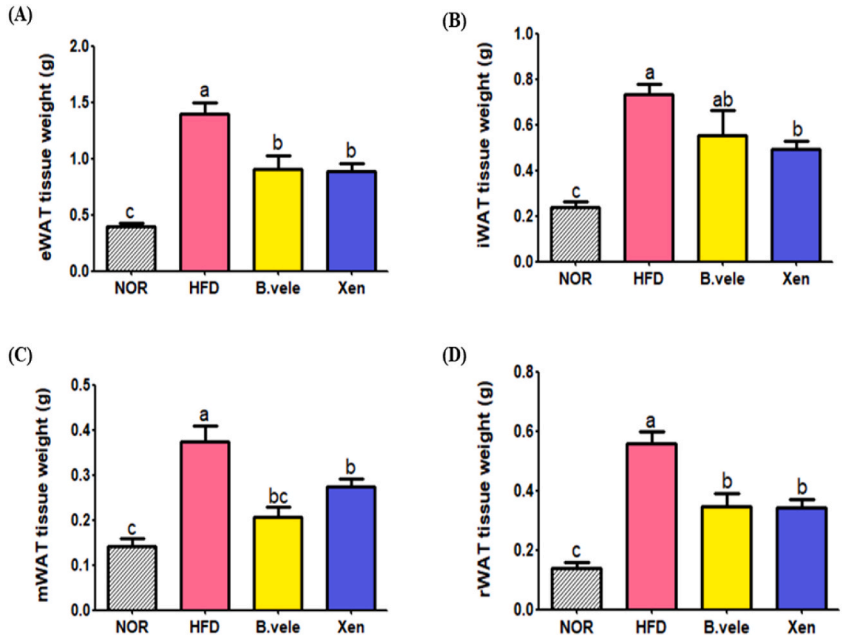

Fig. 2. Effect of CFS-*B.vele* administration on weight of white adipose tissue (WAT): (A) epididymal WAT (eWAT); (B) inguinal WAT (iWAT); (C) mesenteric WAT (mWAT); (D) retroperitoneal WAT (rWAT). CFS, cell-free culture supernatant; NOR, normal diet (6 % kcal from fat); HFD, high-fat diet (45 % kcal from fat); B. vele, HFD+114 mg/kg CFS of *Bacillus velezensis*; Xen, HFD+50 mg/kg Xenical. Each value represents mean ± standard error ($n = 8$). a,b: Different letters indicate significant differences at $p < 0.05$.

The body composition of mice was determined by DEXA. As shown in Fig. 1D, fat in tissue (%) of the B. vele and Xen groups was significantly lower than in the HFD group, whereas no significant differences were observed in both lean mass and bone mineral content among groups (Fig. 1E and F). This indicates that decreased weight gain was the major contributor to the body weight change between the HFD and B.vele groups.

3.2. The effects of CFS-B.vele on organ and WAT weights

There was no difference in the weights of organs, such as the heart, liver, kidney, and spleen among the groups (Supplement Table 1). This indicates that the administration of CFS-*B.vele* did not cause any adverse effects on these organs. However, consistent with the result of body composition analysis using DEXA, the HFD group showed significantly greater eWAT, mWAT, and rWAT, weights compared to those of the *B.vele* group ($p < 0.05$; Fig. 2A–D). In addition, a strong correlation was proposed between the chemically quantified body fat content/serum leptin concentration in mice and the amount of body fat estimated by the DEXA image procedure [21].

WAT is classified into subcutaneous (below the skin) and visceral (close to internal organs) fat based on its location, with visceral adipocytes (eWAT, mWAT, and rWAT) being more closely associated with insulin resistance and obesity-related pathogenesis than subcutaneous fat mass [22]. In previous studies, the administration of *L. johnsonii* 3121, *L. rhamnosus* 86, and *Pediococcus pentosaceus* KID7 reduced body eWAT and iWAT mass in an HFD-induced obese mice model [23]. *L. reuteri* 263 has suggested to play a role in remodeling energy metabolism by increasing oxygen consumption in WAT [24]. Interestingly, the administration of heat-killed *L. reuteri* 263 had similar effects to the administration of viable *L. reuteri* 263 on improving metabolic functions in HFD-induced obese rats, suggesting that the viability of probiotics may not be essential for attenuating obesity [25]. Moreover, heat-killed *L. pentosus* S-PT84 was found to elicit a stronger release of anti-inflammatory cytokines, such as interleukin (**IL**)-12 and interferon (**IFN**)-γ, than its live counterpart [26]. Overall, the administration of CFS-*B.vele* effectively suppressed HFD-induced fat accumulation in the body.

3.3. Effect of CFS-B.vele on serum biochemical parameters

The serum concentrations of TG and TCHO exhibited a decreasing trend in the *B. vele* group, although no significant difference was found between the HFD and *B. vele* groups (Table 1). However, the level of serum LDL-C was significantly decreased in the *B. vele* group compared to the HFD group ($p < 0.05$). There is a growing body of evidences that the ingestion of postbiotics can provide beneficial effects on body weight gain, blood cholesterol and adipose tissue weights [27,28]. LDL-C is a major risk factor for atherosclerotic cardiovascular disease and is considered as a primary therapeutic target [29]. The results suggest that the continuous administration of CFS-*B.vele* provides a protective effect on metabolic disease risk by lowering HFD-mediated elevated serum LDL-C. AST, ALT, and BUN levels were not significantly different among the treatment groups. These results confirm that the administration of CFS-*B.vele* did not cause any damage to the liver and kidney.

3.4. Effects of CFS-B.vele on adipocyte size and hepatic TG content

The accumulation of fat in the adipocytes is a key feature of obesity and can occur through the expansion of existing adipocytes (hypertrophy), the number of adipocytes (hyperplasia), or the combination of these two phenomena [30]. Adipocyte hypertrophy is associated with increased cellular stress and various metabolic dysfunctions such as cellular hypoxia, oxidative stress and chronic inflammation [31]. The mean size of adipocytes was compared after H&E staining of eWAT. The administration of CFS-*B.vele* significantly suppressed the HFD-induced adipocyte hypertrophy, resulting in a smaller mean adipocyte size compared to the HFD group ($p < 0.05$; Fig. 3A and B).

The liver plays a central role in lipid metabolism and hepatic fat accumulation is often increased in patients with obesity [32]. In terms of hepatic fat accumulation, a much smaller lipid droplets deposit was observed in the *B.vele* group than in the HFD group, and

Table 1
Effect of CFS-*B.vele* administration on serum biochemical parameters.

Parameters	NOR	HFD	B. vele	Xen
TG (mg/dL)	58.41 ± 16.43a	59.61 ± 11.76a	50.92 ± 26.35 ab	30.56 ± 8.97b
TCHO (mg/dL)	150.33 ± 21.91b	178.80 ± 9.04a	156.40 ± 10.11 ab	173.60 ± 19.24a
HDL-C (mg/dL)	113.74 ± 6.09b	131.07 ± 6.70a	133.96 ± 2.71a	135.94 ± 2.71a
LDL-C (mg/dL)	24.91 ± 18.01 ab	35.81 ± 6.36a	12.26 ± 9.28b	31.55 ± 17.44a
Glucose (mg/dL)	209.29 ± 17.40	217.60 ± 66.05	191.60 ± 29.91	173.56 ± 21.07
AST (U/L)	66.43 ± 16.69	69.40 ± 11.72	59.00 ± 15.84	60.22 ± 17.38
ALT (U/L)	27.71 ± 3.99a	26.00 ± 7.18 ab	20.00 ± 9.19b	20.00 ± 2.78b
BUN (mg/dL)	31.81 ± 5.30	26.96 ± 4.90	27.96 ± 6.90	26.78 ± 4.00

CFS, cell free culture supernatant, NOR, normal diet (6 % kcal from fat); HFD, high fat diet (45 % kcal from fat); B. vele, HFD+114 mg/kg CFS of *Bacillus velezensis*; Xen, HFD+50 mg/kg Xenical. TG, triglyceride; TCHO, total cholesterol; HDL-C, high density lipoprotein cholesterol, LDL-C, low density lipoprotein cholesterol, AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen. Each value represents mean ± standard error ($n = 8$). [a,b]: Different superscripts indicate significant differences within the same row ($p < 0.05$).

the increased hepatic TG content by HFD was significantly reduced by the administration of CFS-*B.vele* ($p < 0.05$; Fig. 3C). Both the average adipocyte size and hepatic TG content showed no significant differences between the CFS-*B.vele* and Xen groups.

The beneficial effects of probiotics and postbiotics on adipocyte size and hepatic steatosis. *L. plantarum* NCU116 has been shown to decrease fat accumulation in an HFD-induced NAFLD rat model by inhibiting lipopolysaccharide (LPS) and pro-inflammatory cytokines [33]. A mixture of five *Bacillus* strains (*B. sonorensis* JJY12-3, *B. paralicheniformis* JJY12-8, *B. sonorensis* JJY13-1, *B. sonorensis* JJY 13-3, and *B. sonorensis* JJY 13-8) significantly reduced adipocyte size and restored hepatic steatosis in HFD-fed mice [34]. Zhang et al. [35] reported that *B. velezensis* T23 significantly increased antioxidant activity and suppressed hepatic TG accumulation and inflammation mediated by HFD in a zebrafish model. Our previous study with *B. velezensis* postbiotics showed inhibitory effects on tumor necrosis factor alpha (**TNF-α**) production in LPS-stimulated RAW 264.7 cells [36]. Treatment with another *B. velezensis* strain isolated from yaks also effectively lowered IL-6 and IL-8 levels while increasing anti-inflammatory IL-10 levels in a mice model [37]. Overall, our findings provide another piece of evidence supporting the potential use of *B. velezensis* as an anti-obesity agent.

3.5. Effects of CFS-B.vele on adipogenesis in WAT and lipogenesis in the liver

The effect of CFS-*B.vele* administration on adipogenic gene expressions in WAT was analyzed using qRT-PCR. As shown in Fig. 4, HFD stimulated gene expression of C/EBPα and PPARγ but the elevated gene expressions were down-regulated by CFS-*B.vele* ingestion to levels similar to those in the NOR group. Adipogenesis is the differentiation of preadipocytes into mature adipocytes, and it involves the coordinated binding of two master transcriptional factors, C/EBPα and PPARγ to adipogenesis associated genes to regulate mature fat cell development [38].

De novo lipogenesis (DNL) is a metabolic process that produces TG, followed by fatty acids synthesis. Liver and adipose tissue are major tissues for DNL, but the liver plays a more substantial role than adipose tissue in terms of quantitative efficiency [39]. It has been demonstrated that hepatic DNL contributed to increased fat mass in individuals with obesity [40].

Consistent with the effect of CFS-*B.vele* on adipogenic gene expressions in WAT, the upregulated protein expression of C/EBPα and PPARγ by HFD was significantly suppressed in the liver by CFS-*B.vele* (Fig. 4). In the development of NAFLD, PPARγ stimulates ACC and FAS, through the regulation of SREBP-1. The phosphorylation of ACC inhibits the conversion of acetyl-CoA to malonyl-CoA, which is necessary for fatty acid synthesis [41]. SCD-1 catalyzes the synthesis of palmitic acid and oleic acid, the main substrates of TG synthesis and DGAT catalyzes the acylation of diacylglycerol into TG, which is the last step of TG synthesis [42].

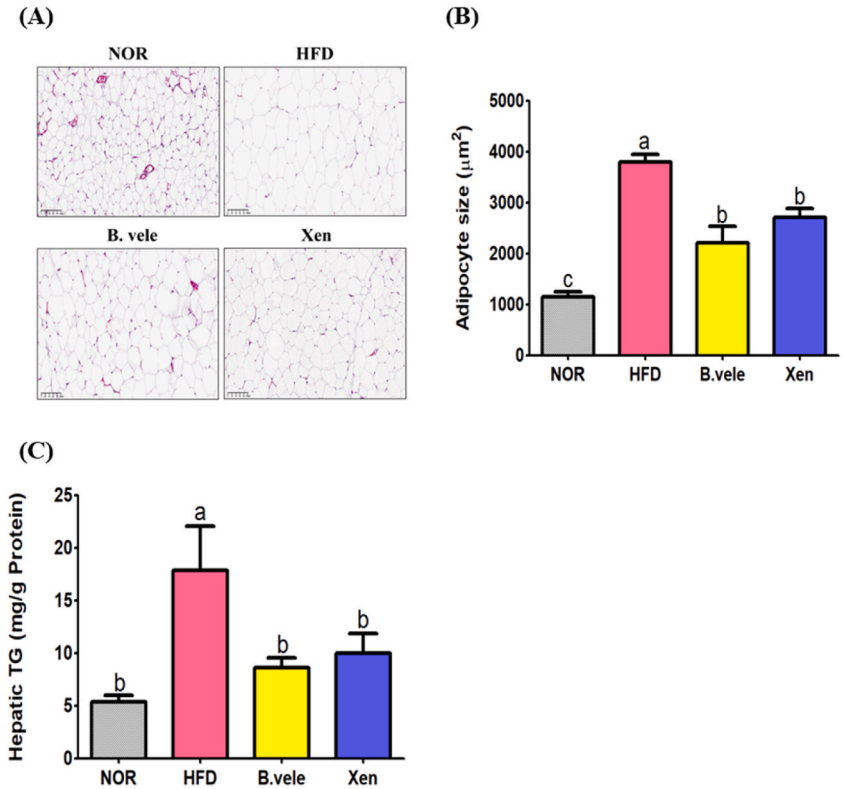

Fig. 3. Effect of CFS-*B.vele* administration on mean adipocyte size and hepatic TG accumulation: (A) representative H&E staining image of epididymal white adipose tissue; (B) mean adipocyte size; (C) hepatic TG accumulation. CFS, cell-free culture supernatant; NOR, normal diet (6 % kcal from fat); HFD, high fat diet (45 % kcal from fat); B. vele, HFD+114 mg/kg CFS of *Bacillus velezensis*; Xen, HFD+50 mg/kg Xenical. TG, triglyceride. Each value represents mean ± standard error ($n = 8$). a,b,c: Different letters indicate significant differences at $p < 0.05$.

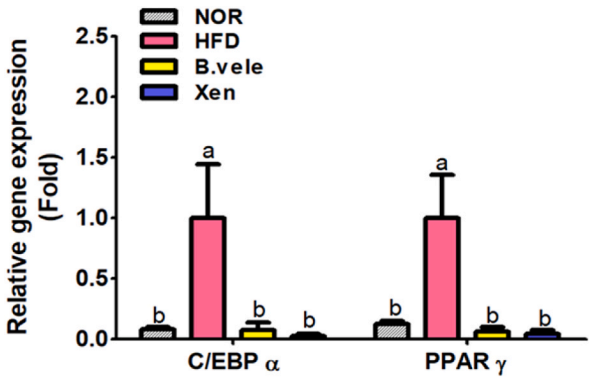

Fig. 4. Effect of CFS-*B.vele* administration on adipogenesis-related gene expression in white adipose tissue. CFS, cell-free culture supernatant; NOR, normal diet (6 % kcal from fat); HFD, high-fat diet (45 % kcal from fat); B. vele, HFD+114 mg/kg CFS of *Bacillus velezensis*; Xen, HFD+50 mg/kg Xenical. C/EBPα, CCAAT/enhancer-binding protein alpha; PPARγ, peroxisome proliferator-activated receptor gamma. Each value represents mean ± standard error (n = 8). a,b: Different letters indicate significant differences at $p < 0.05$.

As shown in Fig. 5, the level of ACC phosphorylation was significantly increased after CFS-*B.vele* administration. Consequently, the expression levels of FAS, SREBP-1, SCD-1, and DGAT were downregulated by CFS-*B.vele.* This result clearly indicates that CFS-*B.vele* exerted an anti-obesity effect by regulating adipogenesis and lipogenesis. The oral intake of postbiotics prepared from *L. plantarum* L-14 alleviated adipogenesis in HFD-fed mice through Toll-like receptor 2 and AMP-activated protein kinase pathway [43]. The authors reported that the exopolysaccharide of postbiotics might be responsible for the beneficial effect. Pan et al. [44] reported that postbiotics derived from *L. paracasei* CCFM1224 prevented NAFLD by modulating the gut microbiota and liver metabolism. Collectively, decreased adipogenesis and lipogenesis in the liver by CFS-*B.vele* led to a reduction in weight gain, serum LDL-C and hepatic TG accumulation and ultimately enhanced anti-obesity effects.

3.6. *Effects of CFS-B.vele on modulation of gut microbiome*

The gut microbiota play an important role in metabolic homeostasis, and HFD-induced obesity is closely associated with the alteration of the intestinal microbiota [45]. Changes in the community structure of cecal microbiota were analyzed following the administration of CFS-*B.vele*. The relative abundance of *Deferribacterota* significantly increased in the HFD group, and it was decreased by the administration of either CFS-*B.vele* or Xenical (Fig. 6A). Serino et al. [46] reported that the gut microbiota reflects the host metabolic phenotype and that the relative proportion of the phylum *Deferribacterota* was increased only in HFD-diabetic mice but not in HFD-diabetic resistant mice. Structural microbial community analysis at phylum level indicated that the relative abundance of *Firmicutes* increased, while *Bacteroidota* decreased by HFD at the phylum level. The elevated Firmicutes/Bacteroidota (F/B) ratio was reversed by CFS-*B.vele* intervention consistent with previous report [47] (Fig. 6B).

At the family level, HFD increased the relative abundance of *Lachnospiraceae,* but the abundance of *Muribaculaceae* was

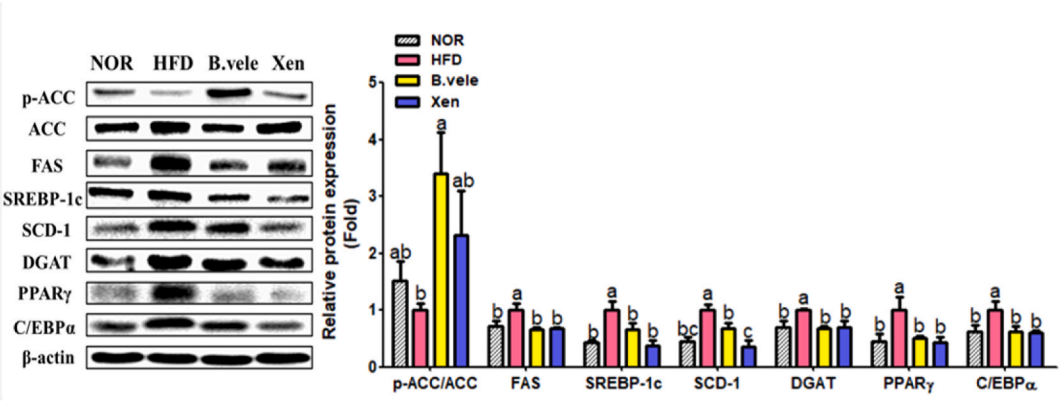

Fig. 5. Effect of CFS-*B.vele* administration on adipogenesis and lipogenesis-related protein expression in the liver. CFS, cell-free culture supernatant; NOR, normal diet (6 % kcal from fat); HFD, high-fat diet (45 % kcal from fat); B. vele, HFD+114 mg/kg CFS of *Bacillus velezensis*; Xen, HFD+50 mg/kg Xenical. p-ACC, phosphor-acetyl-CoA carboxylase; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase, SREBP-1c, sterol regulatory element-binding protein-1c; DGAT, diacylglycerol acyltransferase; PPARγ, peroxisome proliferator-activated receptor gamma; C/EBPα, CCAAT/enhancer binding protein alpha. Each value represents mean ± standard error (n = 8). a,b,c: Different letters indicate significant differences at $p < 0.05$.

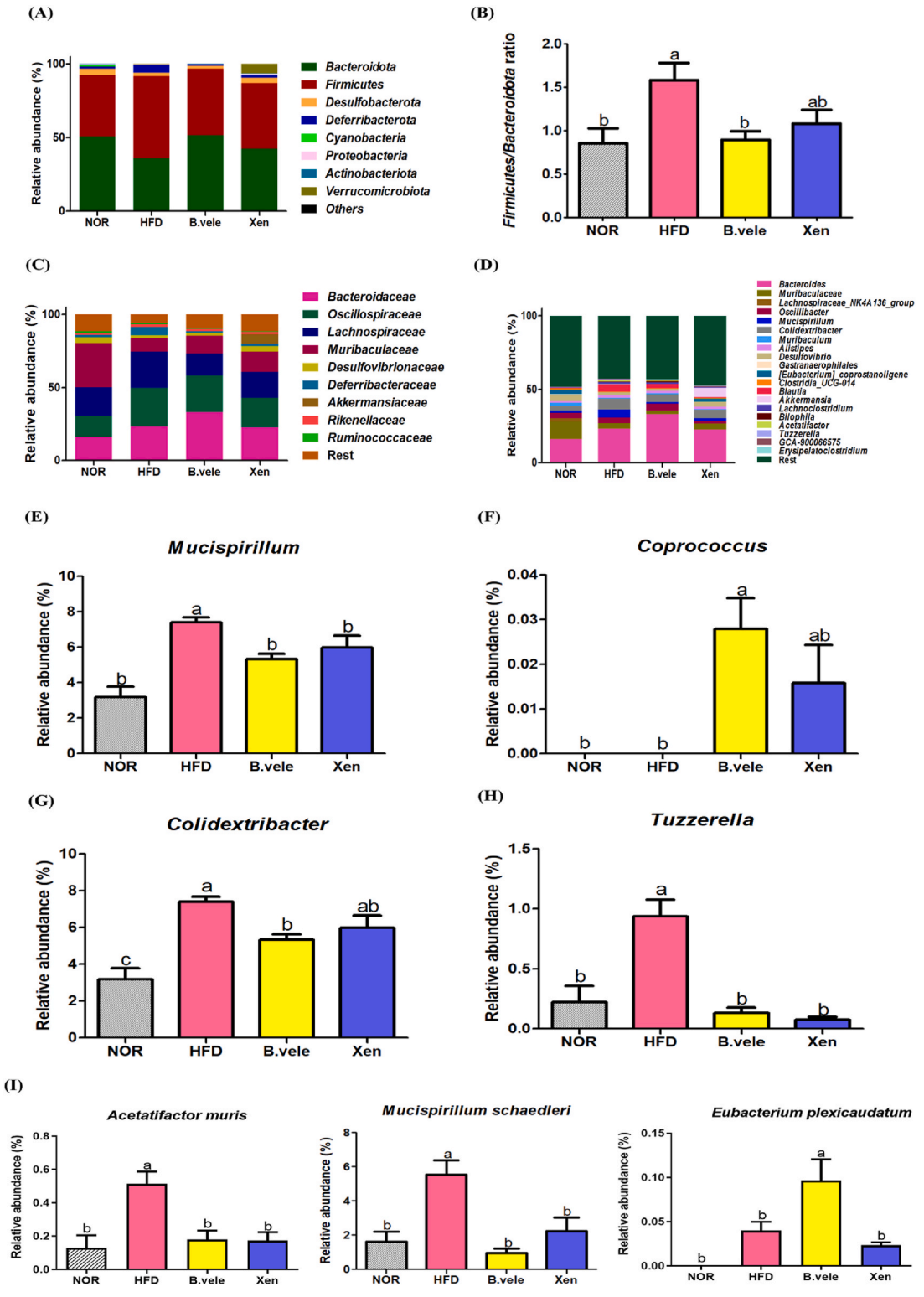

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Fig. 6. Effect of CFS-*B.vele* administration on cecal microbiota: (A) taxonomy analysis at phylum level; (B) *Firmicutes/Bacteroidota* ratio; (C) taxonomy analysis at family level; (D) taxonomy analysis at genus level; (E) relative abundance of *Mucispirillum*; (F) relative abundance of *Coprococcus*; (G) relative abundance of *Colidextribacter*; (H) relative abundance of *Tuzzerella*; (I) relative abundance of *Acetatifactor muris*, relative abundance of *Mucispirllum schaedleri*, relative abundance of *Eubacterium plexicaudatum*. CFS, cell-free culture supernatant; NOR, normal diet (6 % kcal from fat); HFD, high-fat diet (45 % kcal from fat); B. vele, HFD+114 mg/kg CFS of *Bacillus velezensis*; Xen, HFD+50 mg/kg Xenical. a,b: Different letters indicate significant differences at $p < 0.05$.

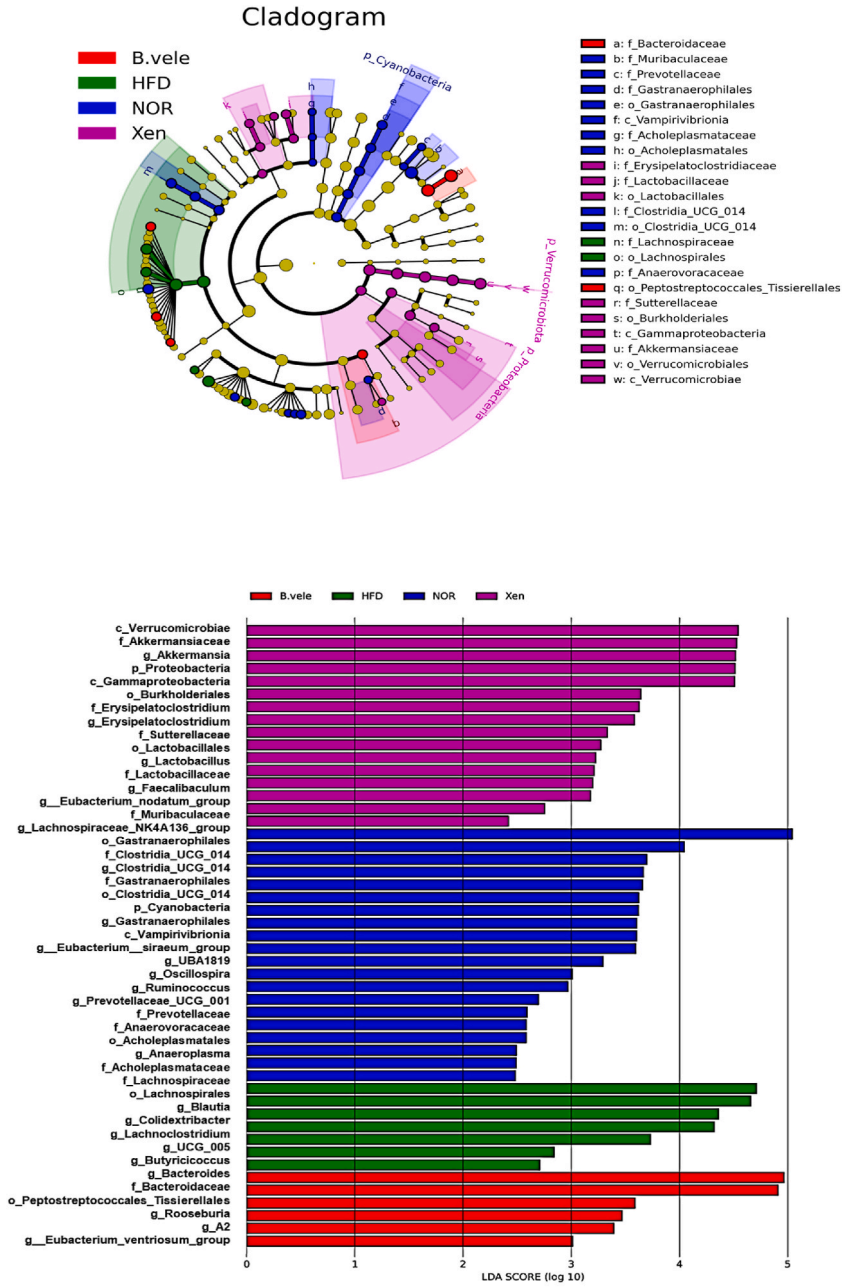

Fig. 7. LEfSe analysis of microbial abundance among different cecal samples. Taxonomic cladogram and LDA scores for LEfSe analysis of cecal microbiota. (A) The cladogram diagram shows the microbial species with significant differences in the four groups. (B) LEfSe analysis results comparing all four groups. LEfSe, the linear discriminant analysis effect size; NOR, normal diet (6 % kcal from fat); HFD, high-fat diet (45 % kcal from fat); B. vele, HFD+114 mg/kg CFS of *Bacillus velezensis*; Xen, HFD+50 mg/kg Xenical. Significance obtained by LDA effect size at $P < 0.05$, (Kruskal-Walis test) and LDA score >2.0.

significantly decreased compared to the NOR group (Fig. 6C). Following the administration of CFS-*B.vele*, the relative proportion of *Lachnospiraceae* was decreased, whereas *Muribaculaceae* increased. Previous studies demonstrated that *Lachnospiraceae* is positively correlated with obesity and NAFLD-related disease [48,49]. Cao et al. [50] reported that the proportion of *Muribaculaceae* was significantly increased in obesity-resistant mice. They concluded that the gut microbiota improved obesity by regulating the utilization efficiency of HFD.

At the genus level, the abundance of *Mucispirillum*, *Colidextribacter*, and *Tuzzerella* is significantly increased in the HFD group, but it decreased in mice who received CFS- *B.vele* (Fig. 6D–E, 6G-H). Zhang et al. [51] reported that the level of *Mucispirillum* was closely associated with systemic inflammation, which contributes to metabolic dysfunction in HFD-fed mice. The abundance of *Mucispirillum schaedleri* has been found to be significantly increased in mice with nonalcoholic steatohepatitis. Moreover, its abundance showed a strong correlation with the level of free fatty acids in HFD-fed mice [52]. *Colidextribacter* has a positive correlation with obesity [53]. HFD increased the abundance of *Colidextribacter*, while the administration of flavonoids from whole-grain oats decreased the abundance of *Colidextribacter* [54]. Qiao et al. [55] reported that *Tuzzerella* showed a positive correlation with obesity biomarkers, including body weight and serum lipids. As shown in Fig. 6F, the administration of CFS-*B.vele* increased the abundance of *Coprococcus*, which showed a negative correlation with BMI and body fat (%) [56].

At the species level, the abundance of *Acetatifactor muris* increased in the HFD group, but it was significantly decreased by the administration of either CFS-*B.vele* or Xenical ($p < 0.05$, Fig. 6I). *Acetatifactor muris* is a secondary bile acid producer that has been isolated originally from obese mice intestine [57]. The decreased abundance of *Acetatifactor muris* by CFS-*B.vele* suggests a possible link between bile acid metabolism and anti-obesity effect mediated by CFS-*B.vele*. The abundance of *Mucispirillum schaedleri* was significantly increased in response to HFD, while the administration of either CFS-*B.vele* or Xenical reversed the increased abundance of *Mucispirillum schaedleri* ($p < 0.05$). Zhang et al. [58] reported that the level of *Mucispirillum* was closely associated with systemic inflammation, which contributes to metabolic dysfunction in HFD fed mice. Moreover, the abundance of *Mucispirillum schaedleri* has been found to be significantly increased in livers with nonalcoholic steatohepatitis in mice and its abundance showed strong correlation with level of free fatty acid in HFD-fed mice [52]. The abundance of *Eubacterium plexicaudatum* was significantly increased by *B. vele* compared to the rest of groups ($p < 0.05$). *Eubacterium plexicaudatum* is known as a butyrate producer, and an increase in the proportion of butyrate producing bacteria in the gut has been shown to contribute to the prevention of western style diet-induced obesity in mice [59].

Taxonomic biomarker identification using LEfSe analysis indicated that the administration of CFS-*B.vele* led to significant enrichment in the abundances of *Bacteroides, Peptostreptococcales/Tissierellales, Roseburia,* and *Eubacterium ventrisum*, (Fig. 7A and B). These significant differences in relative abundance of cecal microbes among the experimental groups likely reflects alterations in bacterial composition resulting from CFS-*B.vele* intervention. Modulation of gut microbiome can help metabolic disturbances caused by HFD.

Although the active anti-obesity components in CFS-*B.vele* are still uncertain, it is speculated that beneficial bacterial metabolites, such as SCFA enhance lipid metabolism and glucose homeostasis through the activation of G protein-coupled receptors [60]. In line with this speculation, the administration of CFS-*B.vele* increased the abundance of SCFA producers, such as *Roseburia* and *Eubacterium*. The significant reduction of commensal *Roseburia* spp. Has been observed in obese mice and human population [61]. Furthermore, a negative correlation has been demonstrated between arterial lipid accumulation and the depletion of *Roseburia* and *Eubacterium* [62].

Diet is the most important regulator of the gut microbiota and host metabolism. Postbiotics alleviate HFD-induced obesity through multiple mechanisms, such as regulation of energy expenditure, adipogenesis/inflammation, food/feed intake, and gut permeability [63,64]. The administration of *B. velezensis* led to changes in microbial community structure by increasing *Lactobacillus* and *Ruminococcus* while decreasing *Acinetobacter* and Helicobacter [65]. This study suggested that CFS of *B. velezensis* exerts anti-obesity effects through modulation of the gut microbiota. Chen et al. [66] reported that *B. velezensis* FJAT-52631 produced lipopeptides (fengycin, iturin, and surfactin) with potent lipase inhibitory activity ($IC_{50} = 0.01-0.05$ mg/mL). This suggests that lipase inhibition cannot be ruled out as an anti-obesity mechanism of CFS-*B.vele*, and clarification of the details of the mode of action will require further investigation.

4. Conclusion

This study demonstrated that intake of CFS-*B.vele* significantly improved obesity biomarkers such as body weight, fat in tissue, weight of WAT, adipocyte size, and hepatic lipid accumulation in HFD-fed mice. Additionally, the expression of key proteins involved in adipogenesis (PPAR γ, and C/EBPα) and lipogenesis (ACC, FAS, SREBP-1, SCD-1, and DGAT) were significantly downregulated by administration of CFS-*B.vele*. The elevated F/B ratio induced by HFD was decreased after administration of CFS-*B.vele*. The CFS-*B.vele* intervention led to the enrichment of SCFA producers, such as Roseburia and Eubacterium, suggesting their potential involvement in the amelioration of obesity. Overall, these findings strongly suggest that CFS-*B.vele* has a potential as a novel anti-obesity postbiotic ingredient.

Declarations

This study was reviewed and approved by [Institutional Animal Care and Use Committee of Kookmin University], with the approval number: [KMU-2022-01].

Data availability statement

The raw data of 16s RNA sequencing and taxonomy are available at the following link: DOI 10.17605/OSF.IO/8S36F.

CRediT authorship contribution statement

Hee Hyun Shin: Writing – original draft, Methodology, Investigation. **Jong-Hoon Kim:** Resources, Formal analysis, Data curation. **Ye-Jin Jung:** Methodology, Formal analysis. **Mi-Sun Kwak:** Visualization, Validation. **Moon-Hee Sung:** Supervision, Conceptualization. **Jee-Young Imm:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Moon-Hee Sung reports financial support was provided by Korea Ministry of Small and Medium Enterprises and Startups. Moon Hee Sung reports financial support was provided by Korea Ministry of Agriculture, Food and Rural Affairs.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e25263.

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