Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Heat-killed *Lactobacillus brevis* KB290 attenuates visceral fat accumulation induced by high-fat diet in mice

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Keywords
anti-adiposity effect, high-fat diet, intestinal microbiota, *Lactobacillus brevis*, metabolic syndrome.

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2020/2194: received 11 October 2020, revised 9 February 2021 and accepted 11 March 2021

doi:10.1111/jam.15079

Abstract

Aims: This study aimed to evaluate the anti-adiposity effect of heat-killed *Lactobacillus brevis* KB290 originating from traditional Japanese fermented pickles in mice fed a high-fat diet (HFD).

Methods and Results: C57BL/6J mice were fed a normal-fat diet, HFD or HFD supplemented with heat-killed KB290 for 8 weeks. Epididymal and renal adipose tissue weights, as well as areas of epididymal adipocytes, were significantly lower in the mice fed a HFD supplemented with KB290 than in those fed an unsupplemented HFD. Mice whose diets were supplemented with KB290 had elevated adiponectin and β3-adrenergic receptor expression in epididymal adipose tissue and an accompanying higher serum free fatty acid level. Furthermore, the HFD-induced elevations in serum glucose, insulin and HOMA-IR were significantly suppressed by dietary supplementation with KB290. Amplicon sequencing of 16S rRNA genes revealed that KB290 ingestion altered the composition of the intestinal microbiota.

Conclusions: Heat-killed *L. brevis* KB290 suppressed diet-induced visceral fat accumulation and ameliorated diet-induced metabolic symptoms and intestinal gut microbiota modifications, suggesting possibility of novel paraprobiotic.

Significance and Impact of the Study: Heat-killed *L. brevis* KB290 is useable as a material to develop functional foods that attenuate visceral fat accumulation.

Introduction

Abnormal accumulation of lipids in adipose tissue is a typical feature of obesity, which is leaded by ingestion of a high-fat diet (HFD) (Flanagan *et al.* 2008). Obesity is a major health concern in both developing and developed countries, and the worldwide prevalence has increased more than two-fold in the last 30 years. A combination of environmental and genetic factors contributes to obesity (Cox *et al.* 2015). Obesity is associated with multiple clinical complications and diseases, including insulin resistance, hypertension, inflammation, oxidative stress and dyslipidaemia (Hutcheson and Rocic 2012). Consequently, obesity increases the risk of various metabolic diseases, such as cardiovascular diseases, hypertension and type 2 diabetes (Emanuela *et al.* 2012; Bastien *et al.* 2014). Obesity is also associated with low-grade systemic inflammation, which is considered a major mechanism driving insulin resistance in obese individuals (Wolowczuk *et al.* 2008; Sanz *et al.* 2010).

Live lactic acid bacteria (LAB), which are Gram positive and acid tolerant, are commonly consumed as probiotics (Behnsen *et al.* 2013). Some strains of probiotic *Lactobacillus* were reported to be effective against obesity and obesity-related metabolic syndromes and against gastrointestinal disorders, such as irritable bowel syndrome and immune disorders (Parvez *et al.* 2006; Masood *et al.* 2011). Although probiotics should be alive in order to provide health benefits to the host according to the current definition of probiotic, administration of some inactivated probiotic strains was found to suppress diet-induced obesity in animals (Shin *et al.* 2010) as well as in

obese individuals (Higashikawa *et al.* 2016; Pedret *et al.* 2019). Those inactivated probiotic microorganisms are frequently called as paraprobiotics (de Almada *et al.* 2016; Cuevas-González *et al.* 2020). The addition of living LAB to foods or vegetables has the potential to impair the product quality owing to the production of unfavourable fermentation end products (Garofalo *et al.* 2015; Tomita *et al.* 2018). In addition, food packages could become inflated as a result of LAB fermentation, especially by facultatively hetero-fermentative species like *Lactobacillus brevis*. To prevent quality impairment, foods containing live LAB are usually chilled for distribution. Thus, from the viewpoint of quality maintenance and ease of distribution, the supplementation of processed foods or vegetables with paraprobiotics is considered advantageous.

We have focused on *L. brevis* KB290 isolated from 'Suguki', a traditional pickle in Japan. *L. brevis* KB290 has been maintained at KAGOME CO., LTD., and has also been deposited as strain *L. brevis* JCM 17312 in the Japan Collection of Microorganisms, Riken BioResource Research Center (Ibaraki, Japan). *Lactobacillus brevis* KB290 is recognized as a probiotic because the strain has been verified to improve bowel habits and to be safe for human consumption (Nobuta *et al.* 2009). However, paraprobiotic functions including anti-adiposity effect of *L. brevis* KB290 were unclear.

In this study, we evaluated the anti-adiposity effect of heat-killed *L. brevis* KB290 in mice fed an HFD aiming to find a possibility as a paraprobiotic. We compared the intestinal microbiota and gene expression in epididymal adipose tissue between mice fed an HFD and those fed an HFD supplemented with heat-killed *L. brevis* KB290 to clarify the mechanisms involved.

Materials and methods

Animals and diets

Five-week-old male C57BL/6J mice in specific-pathogen free were purchased from Japan Charles River (Yokohama, Japan) and housed in standard plastic cages (four mice par cage) in a temperature-controlled room (23 ± 2°C) with a dark period from 20:00 to 08:00 hours. Sphered paper (Paper Clean; Japan SLC, Hamamatsu, Japan) was used as bedding materials for mice and was changed every other day. Prior to their use in our experiment, they were acclimatized for 1 week during which they were fed a normal-fat diet (NFD, 10% energy from fat, D19071803; Research Diet, New Brunswick, NJ). Mice weighing 20·9 ± 0·1 g were divided into three groups of 12 animals each. Each group of mice was fed an NFD, HFD (60% energy from fat, D19032801; Research Diet) or HFD supplemented with 2% (w/w) of heat-killed *L. brevis* KB290 powder (KB). In order to obtain heat-killed *L. brevis* KB290 powder, *L. brevis* KB290 cultured in a food grade medium was heated at 95°C for 2 min and then washed and lyophilized. Mice were fed each diet ad libitum for 8 weeks. At the end of the experimental period, mice were fasted for 12 h, anaesthetized via isoflurane inhalation and exsanguinated by cardiac puncture. The liver and epididymal, mesenteric and renal adipose tissues were excised and weighed. Part of the collected epididymal adipose tissue was soaked in RNAlater (Qiagen, Valencia, CA) for use in RNA extraction, and part was soaked in Mildform 10N (Wako Pure Chemicals, Osaka, Japan) for use in histological observations. The caecal contents were snap frozen in liquid nitrogen for use in a microbiota analysis.

This animal experiment was approved by the Animal Use Committee of the Food Research Institute, National Agriculture and Food Research Organization, and all mice were maintained in accordance with the guidelines for the care and use of laboratory animals of the research organization (approval no. H31-026).

Serum biochemical analyses

Sera were prepared from whole blood by centrifugation (3500 ***g***, 5 min). Serum triacylglycerol (TG), total cholesterol (TC), free fatty acids (FFA), glucose and insulin levels were measured using commercial enzyme kits (Wako Pure Chemicals). The homeostatic model assessment of insulin resistance (HOMA-IR) value was calculated from the serum glucose and insulin levels by using the homeostasis model assessment method (Matthews *et al.* 1985).

Histology of epididymal adipose tissue

Histological evaluation of epididymal adipose tissue was performed according to our previous method (Yin *et al.* 2020). Briefly, fixed epididymal adipose tissue was embedded in paraffin. The sections (3 μm) were stained with haematoxylin and eosin. Images were taken under a microscope (BZ-8000; Keyence, Osaka, Japan) at a magnification of 200×, and the adipocyte area of at least 30 randomly selected cells was analysed using Image J software (National Institutes of Health, Bethesda, MD).

Reverse transcription-PCR

Reverse transcription-PCR was conducted according to our previous method with slight modifications (Yin *et al.* 2020). Briefly, total RNA from epididymal adipose tissue was reverse-transcribed using ReverTra Ace (Toyobo,

Osaka, Japan) and an oligo $(dT)_{15}$ primer (TakaraBio, Otsu, Japan). The resulting cDNA was used for quantitative PCR with a KAPA SYBR Fast qPCR Master Mix (KAPA Biosystems, Wilmington, MA). The relative quantification of expression levels was calculated by applying the $^{\Delta\Delta}$Ct method with normalization to β-actin. The primer sequences used are shown in Table S1.

Microbiota analysis

DNA was extracted from the caecal content via bead-beating using a Multi-Beads Shocker (Yasui Kikai Co., Osaka, Japan) and then purified using a QIAamp DNA Stool Mini Kit (Qiagen). DNA samples were quantified, after which the V1 and V2 regions of 16S rRNA genes were amplified by PCR with 27Fmod (5′-AGRGTTTGATYMTGGCTCAG-3′) and 338R (5′-TGCTGCCTCCCGTAGGAG-3′) joined to the Illumina overhang adapter sequences (Kim *et al.* 2013). A second PCR was performed to add barcodes to each sample. Amplicons were pooled in equal amounts, and pair-end 2 × 300 bp sequencing was performed using a MiSeq System (Illumina Inc., San Diego, CA) and MiSeq Reagent Kit v3 (Illumina Inc.).

Sequences in demultiplexed format were analysed using QIIME2 2020.8 (https://qiime2.org). Merged paired-end reads were denoised using DADA2 (Callahan *et al.* 2016). Sequence variants assigned as originating from chloroplasts or mitochondria were eliminated from further analyses. For each representative sequence, the GreenGene database (McDonald *et al.* 2012) was used to annotate the taxonomic information (DeSantis *et al.* 2006). Alpha- and beta-diversities were analysed by rarefying the feature table at a consistent sample depth of 10 000. Because one mouse in the HFD group produced fewer than 1000 valid sequences, data from this mouse were removed from the analyses. To identify the representative genera of each group, the linear discriminant analysis effective size (LEfSe) algorithm was then performed (Segata *et al.* 2011).

Statistical analyses

One mouse in the HFD group continuously showed a lower bodyweight than the other mice in that group, and the final bodyweight of this animal was judged as a statistical outlier using the Smirnov–Grubbs test. Additionally, an apparent abnormality was observed in the kidneys of a mouse in the NFD group. Thus, data from these mice were excluded from our analysis. All data are presented as the mean ± SEM. To compare the mean values between groups, a one-way ANOVA followed by the Tukey–Kramer or the Dunn–Bonferroni post hoc tests was applied. Bartlett's test for homogeneity of variances was performed to determine whether the variances were equal. All statistical analyses were conducted with a significance level of $P < 0{\cdot}05$ using R ver. 4.0.2.

Results

Bodyweights and organ weights

Increases in bodyweight over time and the weights of the liver and epididymal, mesenteric, and renal adipose tissues of mice at 8 weeks are shown in Fig. 1. Mice in the HFD and KB groups showed significantly higher bodyweights from 4 days after starting the experimental feeding than those in the NFD group (Fig. S1). In addition, the epididymal, renal and mesenteric adipose tissue weights for the HFD-fed groups were all significantly higher than those for the NFD group (Fig. 1b–d). These results suggest that obesity was successfully induced by HFD consumption. The bodyweight gain was not significantly different between the HFD and KB groups (16·03 ± 0·9 vs. 14·9 ± 0·9 g, Fig. 1a). Liver weight relative to bodyweight was lower in the HFD-fed groups than in the NFD group, and that of the KB group was higher than that of the HFD group (3·2 ± 0·1, 2·7 ± 0·0 and 2·9 ± 0·0 g 100 gBW^{-1} for NFD, HFD and KB groups, respectively, Fig. 1e). Conversely, the weights of epididymal and renal adipose tissue for the HFD-fed groups were significantly higher than those for the NFD-fed group, and these weights were significantly lower for the KB group than for the HFD group (epididymal adipose tissue weight; 5·5 ± 0·2 vs. 4·6 ± 0·2, and renal adipose tissue weight; 2·1 ± 0·1 vs. 1·6 ± 0·1 g $100gBW^{-1}$, Fig. 1b,c). Although the mesenteric adipose tissue weight was significantly higher in the HFD-fed groups than in the NFD group, this weight did not differ significantly between the HFD and KB groups (Fig. 1d). The average diet consumption was not significantly different among the HFD-fed groups (3·0 ± 0·4 and 3·0 ± 0·6 g d^{-1} for HFD and KB groups, respectively).

Serum parameters

The serum glucose and TC levels of the HFD and KB groups were significantly higher than those of the NFD group (Fig. 2a,b). The serum TG level was significantly higher in the KB group than in the HFD group (111 ± 6·0 vs. 85·1 ± 4·5 mg dl^{-1}, Fig. 2c). Additionally, the serum FFA level was significantly lower in mice fed an HFD than in those fed an NFD (0·23 ± 0·01, 0·17 ± 0·01 and 0·19 ± 0·01 mEq dl^{-1} for NFD, HFD and KB groups, respectively, Fig. 2d). Epididymal, renal and mesenteric adipose tissue weights were negatively

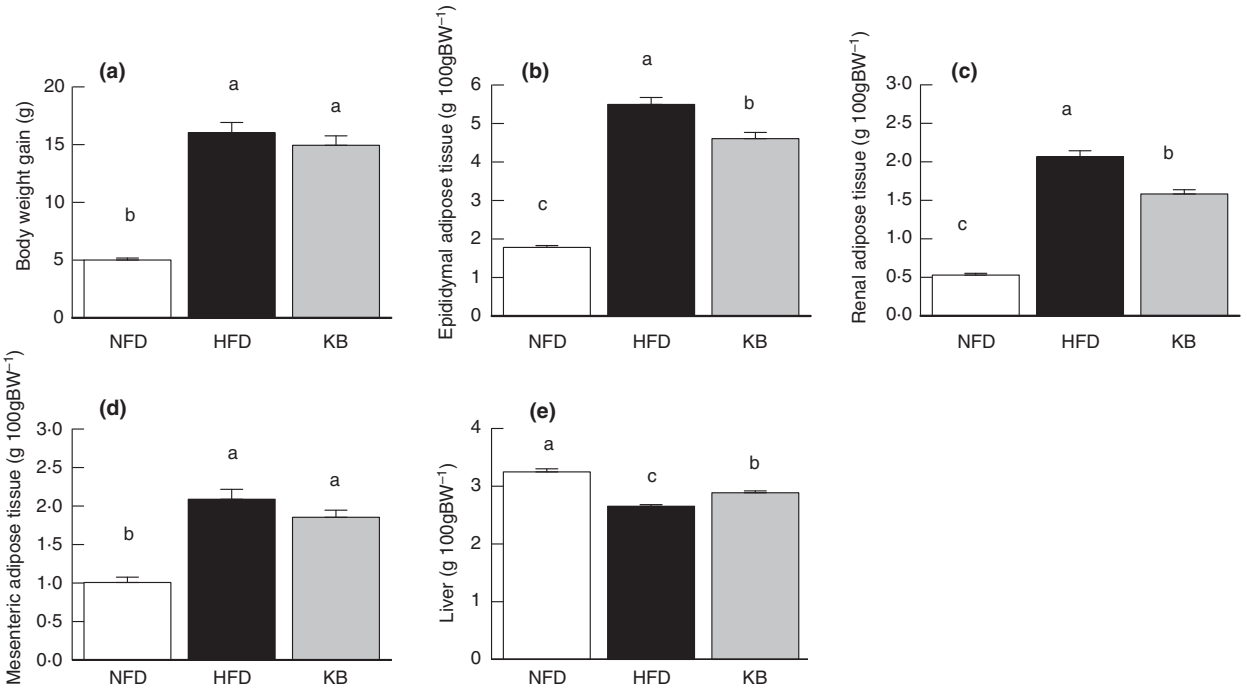

FIGURE 1 Bodyweight gain (a), weights of epididymal (b), renal (c) and mesenteric (d) adipose tissue and of liver (e) at 8 weeks of mice fed (□) NFD, a normal-fat diet; (■) HFD, high-fat diet or (▨) KB, HFD supplemented with *Lactobacillus brevis* KB290. Values are expressed as the means ± SEM (n = 11–12). Values without the same letters are significantly different (P < 0·05, Tukey–Kramer post hoc test).

correlated with serum FFA levels (Fig. S2). The serum insulin level was significantly higher in mice fed an HFD than in those fed an NFD or KB (98·3 ± 29·1, 785·2 ± 200·4 and 303·6 ± 65·4 U dl^{-1} for NFD, HFD and KB groups, respectively, Fig. 2e). Similarly, the HOMA-IR value was significantly higher for the HFD group than for the NFD group; supplementation with KB290 recovered the HFD-induced elevation of HOMA-IR (0·21 ± 0·05, 3·30 ± 0·91 and 0·97 ± 0·20 for NFD, HFD and KB groups, respectively, Fig. 2f).

Histological observation of epididymal adipose tissue

The cross-sectional area of adipocytes in epididymal adipose tissue was significantly higher in the HFD and KB groups than in the NFD group (Fig. 3). Mice in the KB group had a significantly lower cross-sectional area of adipocytes than those in the HFD group (4644 ± 319 vs. 5699 ± 373 μm^2, Fig. 3d).

Gene expression in epididymal adipose tissue

To explore the mechanisms by which KB290 affects lipid metabolism in HFD-fed mice, we used RT-PCR to assess the expression of genes encoding proteins related to lipogenesis (*Fas*) and lipid β-oxidation (*Acox1*, *Cpt1*) as well as the expression of genes for adiponectin and β3-adrenergic receptor in epididymal adipose tissue (Fig. 4). *Acox1* expression was significantly lower in the HFD group than in the NFD group, and *Acox1* expression in the KB group did not differ significantly from those in the other two groups (Fig. 4a). The levels of *Cpt1* and *Fas* expression were not significantly different among the three groups (Fig. 4b,c). The levels of adiponectin and β3-adrenergic receptor gene expression were significantly lower in the HFD group than in the NFD group; however, compared with the HFD group, the expression level of adiponectin was significantly higher and that of β3-adrenergic receptor trended higher in the KB group (adiponectin; 0·56 ± 0·04 vs. 0·82 ± 0·10, β3-adrenergic receptor; 0·30 ± 0·04 vs. 0·52 ± 0·07, Fig. 4d,e).

Intestinal microbiota

High-quality 16S rRNA gene sequences (n = 2 812 924) from the caecal content of mice in the NFD, HF and KB groups were analysed (average of 85 240 ± 6077 sequences per sample). After quality filtration, denoising and the elimination of chimeric sequences with DADA2 (Callahan *et al.* 2016), 1 107 780 sequences (average of 33 569 ± 2659 sequences per sample) were deemed valid. After sequence variants assigned as originating from

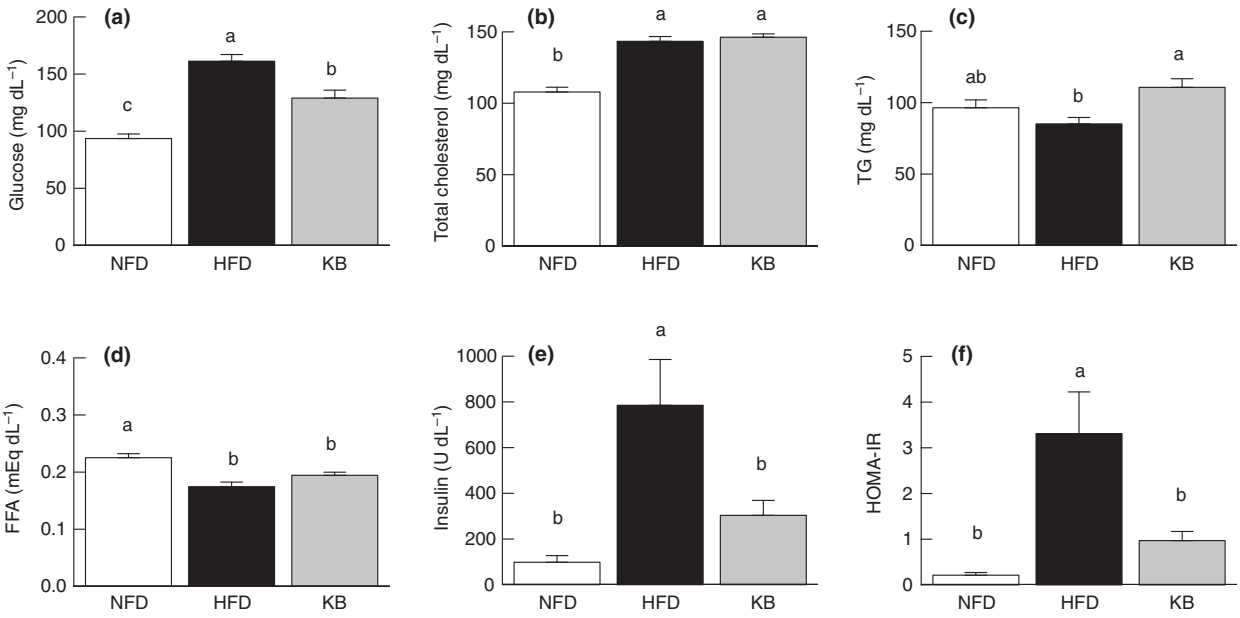

FIGURE 2 Levels of serum glucose (a), total cholesterol (b), triglyceride (TG; c), free fatty acids (FFA; d), insulin (d) and HOMA-IR (e) of mice fed (□) NFD, a normal-fat diet; (■) HFD, high-fat diet or (▨) KB, HFD supplemented with *Lactobacillus brevis* KB290. Values are expressed as the means ± SEM (n = 11–12). Values without the same letters are significantly different (P < 0·05, Tukey–Kramer post hoc test).

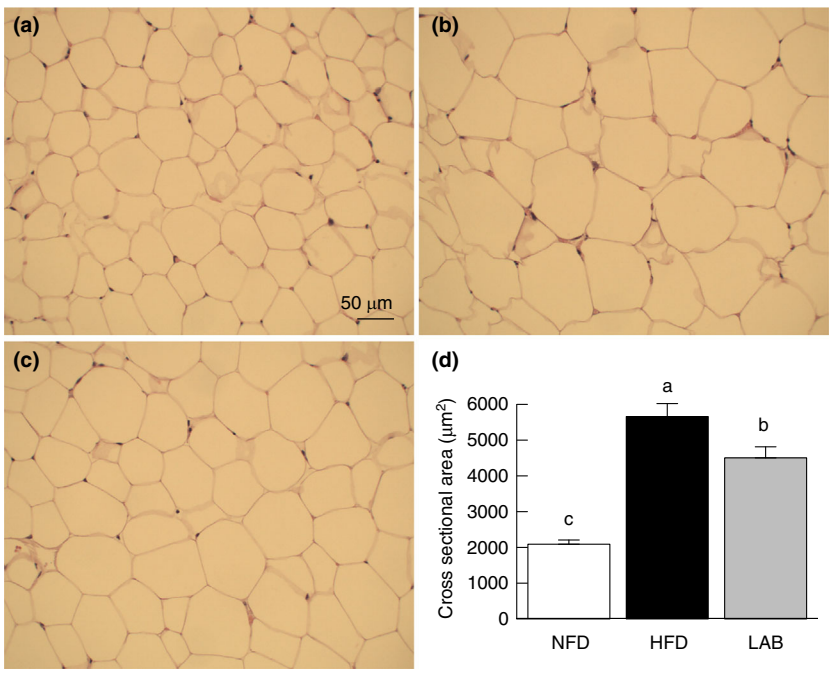

FIGURE 3 Histological evaluation of epididymal adipose tissue in mice fed a normal-fat diet (NFD; a), high-fat diet (HFD; b) or HFD supplemented with *Lactobacillus brevis* KB290 (KB; c). (d) Cross-sectional area of adipocytes in the epididymal adipose tissue of these mice (□ NFD; (■) HFD; (▨) KB). Sections of fixed epididymal adipose tissue were stained with haematoxylin and eosin. The adipocyte area of at least 30 randomly selected cells was analysed. Bar in the figure indicates 50 μm. Values are expressed as the means ± SEM (n = 11–12). Values without the same letters are significantly different (P < 0·05, Tukey–Kramer post hoc test). [Colour figure can be viewed at wileyonlinelibrary.com]

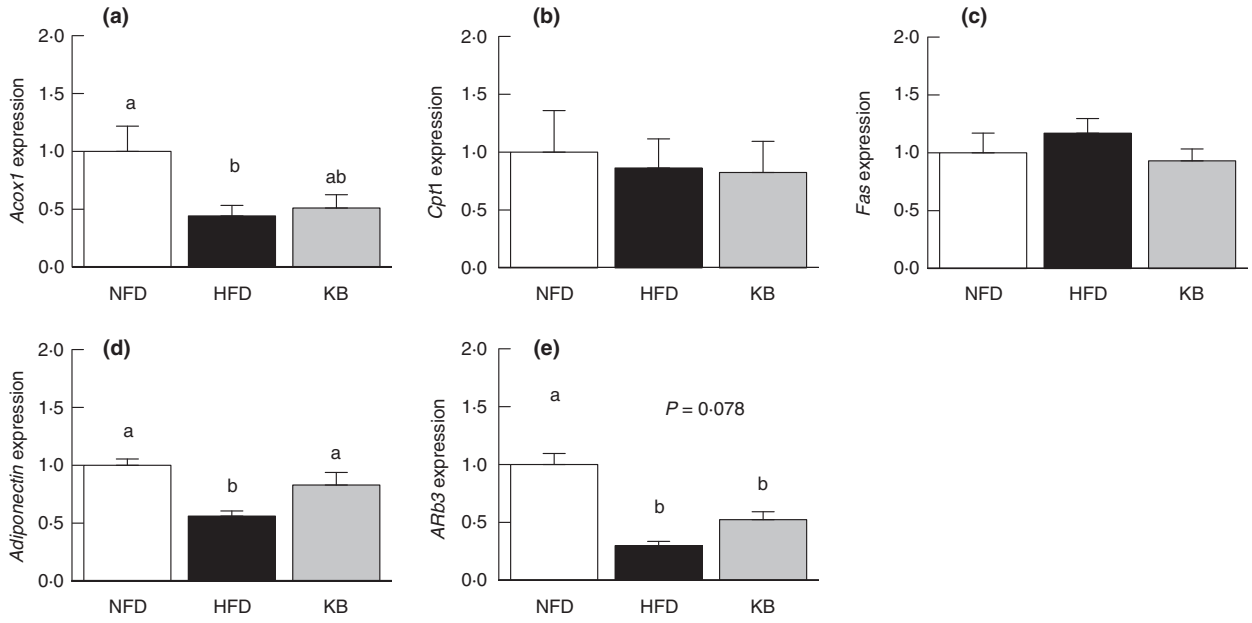

FIGURE 4 Expression levels of genes relating to β-oxidation (*Acox1*; acyl-CoA oxidase 1 (a), *Cpt1*; carnitine palmitoyltransferase 1 (b)) or lipogenesis (*Fas*; fatty acid synthase (c)), *Adiponectin* (d), or *ARb3* (adrenaline receptor β3 (e)) in the epididymal adipose tissue of mice fed (□) NFD, a normal-fat diet; (■) HFD, high-fat diet or (▨) KB, HFD supplemented with *Lactobacillus brevis* KB290. Values are expressed as the means ± SEM (n = 11–12). Values without the same letters are significantly different ($P < 0{\cdot}05$, Tukey–Kramer post hoc test).

chloroplasts and mitochondria were eliminated, 630 sequence variants were obtained.

Comparing the intestinal microbiota alpha diversity among the groups revealed that mice in the KB group had a higher Shannon index than those in the HFD group (5·22 ± 0·09, 5·09 ± 0·09 and 5·37 ± 0·03 for NFD, HFD and KB groups, respectively, Fig. 5a), although the observed features, Faith's phylogenetic diversity and Pielou's evenness were not significantly different among any groups (Fig. 5b and Fig. S3). Beta-diversity (weighted UniFrac) distances revealed that the microbiotas of mice in KB group were more similar to those in NFD group than were those in HFD group (Fig. 5c).

To identify the representative genera of each group of mice, we applied LEfSe analysis (Fig. S4). The bacterial genera with a LDA score was >3 and with a relative abundance was >1% in at least one mouse (except for that of *Lactobacillus*) were selected, and the relative abundances were compared among the groups of mice (Table 1). The relative abundance of *Lactobacillus* was almost negligible in the NFD and HFD groups; conversely, it was significantly higher in the KB group. Representative sequences of sequence variants that were assigned as *Lactobacillus* showed the highest similarity with *L. brevis* (data not shown). Mice in the KB group had a significantly higher abundance of *Bacteroides* compared with the other groups. The abundances of f_*S24-7*: g_ in *Bacteroidetes* and *Oscillospira* in *Firmicutes* were significantly lower in mice fed an HFD than in those fed an NFD; dietary supplementation with *L. brevis* KB290 partly recovered the HFD-induced deficits in abundance observed for these bacterial groups. Additionally, *Clostridium* and *Akkermansia* were significantly more abundant in the mice fed an HFD than in those fed an NFD; dietary supplementation with *L. brevis* KB290 also partly recovered the HFD-induced elevated abundances observed for these bacterial groups.

Discussion

We investigated the anti-adiposity activity of heat-killed *L. brevis* KB290 isolated from traditional Japanese fermented pickles in an HFD-induced obesity murine model with regards to the amelioration of metabolic symptoms and modification of the intestinal gut microbiota. The rodent HFD model has been widely used to study visceral obesity and metabolic disorders because the pathogenesis of obesity in this model is similar to that in humans (Liao *et al.* 2013). We demonstrated here that mice fed an HFD showed remarkable increases in bodyweight (Fig. S1 and Fig. 1a), adiposity (Fig. 1b–d) and enlargement of adipocytes in epididymal adipose tissue (Fig. 3) compared with those fed an NFD, and that the supplementation of an HFD with *L. brevis* KB290 significantly suppressed the HFD-induced increase in epididymal and

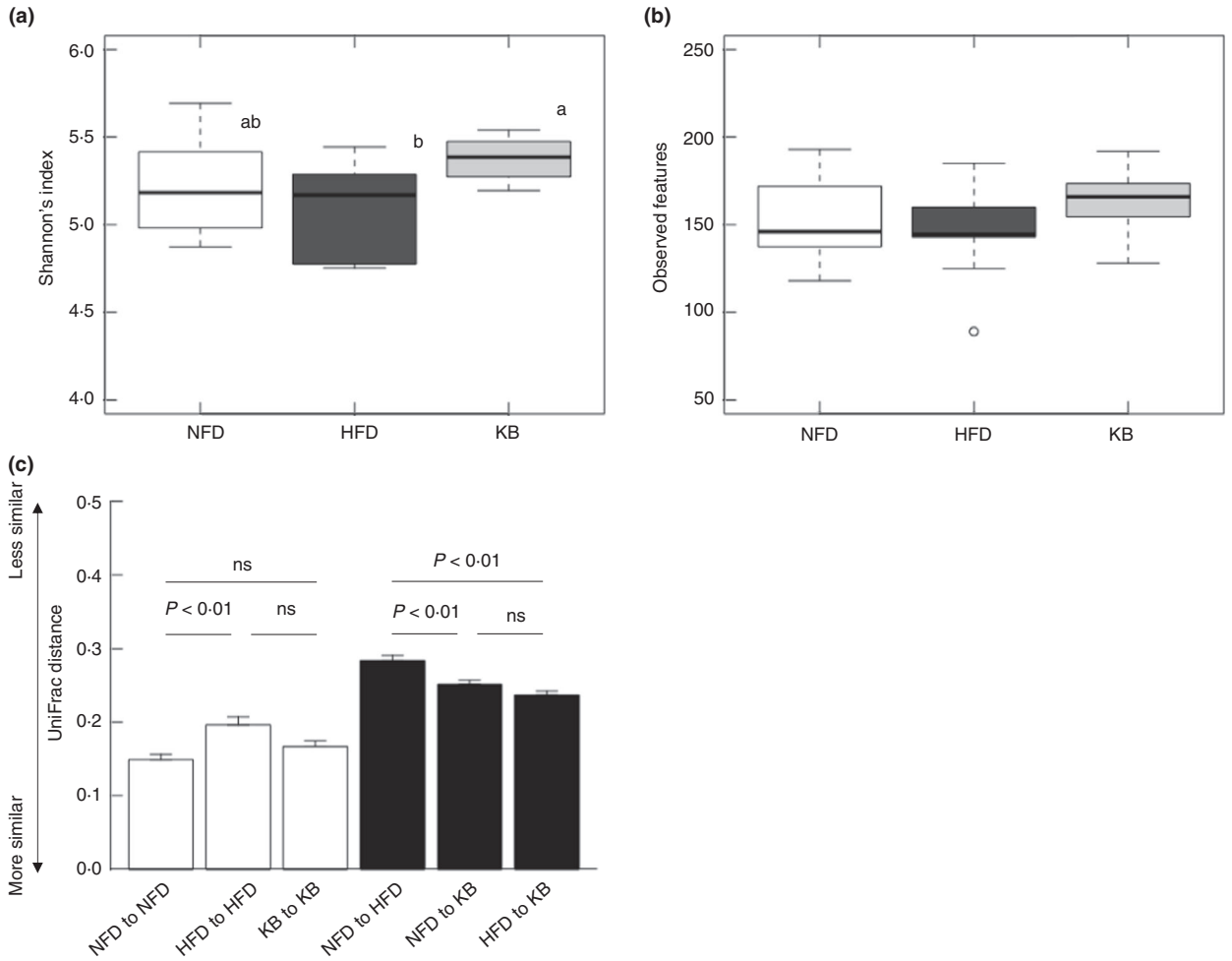

FIGURE 5 Alpha diversity parameters Shannon index (a) and observed features (b) of the intestinal microbiota from mice fed (□) NFD, a normal-fat diet; (■) HFD, high-fat diet; or (▨) KB, HFD supplemented with *Lactobacillus brevis* KB290. (c) Beta-diversity measures shown as weighted UniFrac distances ((□) within the group; (■) between groups). Values are expressed as the means ± SEM (n = 11–12). Values without the same letters are significantly different. The Dunn–Bonferroni post hoc test was applied, and values with significant differences are shows as different letters ($P < 0·05$) or P values.

renal adipose tissue weights (Fig. 1b,c) and the cross-sectional area of adipocytes (Fig. 3d). Thus, we confirmed that the model used here induced obesity via the consumption of an HFD and that dietary supplementation with *L. brevis* KB290 reduced markers of obesity. Supplementation of the HFD with KB290 did not affect the average diet consumption; thus, appetite control probably does not contribute to the anti-adiposity effect of KB290.

Adipose tissue is an energy-storing tissue; it also serves as an integrator of various physiological pathways, including those related to energy balance and glucose homeostasis (Rosen and Spiegelman 2006). Obesity-induced insulin resistance is a major contributor to the worldwide prevalence of type 2 diabetes (Meigs *et al.* 2007). Higher fasting serum glucose and insulin levels were observed in the mice fed an HFD than in those fed an NFD, and these elevations were significantly suppressed in mice fed an HFD supplemented with KB290 (Fig. 2a,e). In addition, HOMA-IR, which is commonly measured parameter of insulin resistance, was significantly higher for mice in the HFD group compared with those in the NFD group, whereas the HOMA-IR of mice in the KB group was significantly lower than that of mice in the HFD group (Fig. 2f). These observations suggest that the ingestion of an HFD-induced insulin resistance and that dietary supplementation with KB290 ameliorated HFD-induced insulin resistance. Furthermore, increased adipocyte size is reportedly correlated with the development of insulin resistance (Fang *et al.* 2015) as well as a marked impairment in adipokine secretion (Skurk *et al.* 2007; Dong *et al.* 2020). The higher expression of adiponectin in the epididymal adipose tissue (Fig. 4d) may

TABLE 1 Relative abundances of bacterial genera in the intestinal microbiotas of mice

Taxonomy	Relative abundance (%)		
	NFD	HFD	KB
Bacteroidetes			
o__*Bacteroidales*;f__*Bacteroidaceae*;g__*Bacteroides*	7·69 ± 0·67^{b}	5·03 ± 0·53^{c}	11·69 ± 0·67^{a}
o__*Bacteroidales*;f__*Porphyromonadaceae*;g__*Parabacteroides*	25·27 ± 1·21^{a}	25·62 ± 1·80^{a}	18·96 ± 0·90^{b}
o__*Bacteroidales*;f__*Rikenellaceae*;g__*Alistipes*	2·66 ± 0·37^{b}	3·37 ± 0·40^{ab}	4·26 ± 0·36^{a}
o__*Bacteroidales*;f__*S24-7*;g__	10·95 ± 0·63^{a}	1·84 ± 0·32^{c}	3·65 ± 0·27^{b}
Deferribacteres			
o__*Deferribacterales*;f__*Deferribacteraceae*;g__*Mucispirillum*	2·35 ± 0·23^{b}	3·54 ± 0·45^{ab}	4·90 ± 0·89^{a}
Firmicutes			
o__*Lactobacillales*;f__*Lactobacillaceae*;g__*Lactobacillus*	0·01 ± 0·00^{b}	0·02 ± 0·01^{b}	0·16 ± 0·02^{a}
o__*Clostridiales*;f__;g__	3·29 ± 0·53^{b}	11·46 ± 1·57^{a}	12·47 ± 1·43^{a}
o__*Clostridiales*;f__*Lachnospiraceae*;__	1·09 ± 0·09^{b}	2·66 ± 0·25^{a}	2·82 ± 0·33^{a}
o__*Clostridiales*;f__*Lachnospiraceae*;g__	0·52 ± 0·05^{a}	0·73 ± 0·11^{a}	0·77 ± 0·07^{a}
o__*Clostridiales*;f__*Lachnospiraceae*;g__*Dorea*	0·27 ± 0·06^{b}	1·08 ± 0·18^{ab}	1·48 ± 0·32^{a}
o__*Clostridiales*;f__*Lachnospiraceae*;g__*Roseburia*	0·01 ± 0·01^{b}	0·17 ± 0·05^{ab}	0·61 ± 0·22^{a}
o__*Clostridiales*;f__*Peptostreptococcaceae*;g__	0·17 ± 0·02^{b}	0·78 ± 0·12^{a}	0·63 ± 0·11^{a}
o__*Clostridiales*;f__*Ruminococcaceae*;__	6·54 ± 0·46^{a}	3·14 ± 0·49^{b}	2·39 ± 0·13^{b}
o__*Clostridiales*;f__*Ruminococcaceae*;g__	1·47 ± 0·14^{b}	3·05 ± 0·28^{a}	3·19 ± 0·35^{a}
o__*Clostridiales*;f__*Ruminococcaceae*;g__*Oscillospira*	22·48 ± 1·58^{a}	13·96 ± 0·89^{c}	18·46 ± 0·78^{b}
o__*Clostridiales*;f__*Ruminococcaceae*;g__*Ruminococcus*	1·63 ± 0·23^{a}	0·74 ± 0·15^{b}	1·06 ± 0·12^{ab}
o__*Erysipelotrichales*;f__*Erysipelotrichaceae*;g__*Clostridium*	0·82 ± 0·11^{b}	1·71 ± 0·17^{a}	1·06 ± 0·18^{b}
Verrucomicrobia			
o__*Verrucomicrobiales*;f__*Verrucomicrobiaceae*;g__*Akkermansia*	9·64 ± 0·94^{b}	16·29 ± 1·01^{a}	8·58 ± 0·58^{b}

Values are the relative abundance (%) of each genus in the caecal microbiota of mice in the NFD, HFD and KB groups and are shown as means ± SE (n = 11–12). Bacterial genera with a LDA score of LEfSe analysis was >3 and a relative abundance was >1% in at least one mouse (except for that of *Lactobacillus*) are shown. Values with different letters significantly differ from one another ($P < 0·05$, Tukey–Kramer post hoc test).

HFD, high-fat diet; NFD, normal-fat diet; KB, HFD supplemented with Lactobacillus brevis KB290.

contribute to the improvement of insulin sensitivity caused by KB290 consumption because adiponectin acts as an anti-inflammatory molecule that in turn improves insulin sensitivity (Farias *et al*. 2012). Serum TC levels were significantly higher for mice in the HFD and KB groups than for those in the NFD group (Fig. 2b), which suggests that the lard in the HFD elevated the serum TC level and that KB290 consumption failed to alter the HFD-induced TC elevation.

Ingestion of an HFD significantly reduced the serum FFA level in mice, suggesting a decreased level of lipolysis in the adipose tissues (Fig. 2d). Although a Tukey–Kramer test did not detect significant differences in serum FFA levels between the HFD and KB groups, the result of an unpaired Student's t test indicates that the HFD-induced reduction in serum FFA level tended to be recovered by HFD supplementation with KB290 ($P < 0·05$ by unpaired Student's t test; Fig. 2d). Moreover, the epididymal, renal and mesenteric adipose tissue weights were negatively correlated with serum FFA levels (Fig. S2). In addition, the HFD-induced downregulation of β3 adrenergic receptor expression in epididymal adipose tissue was partly recovered by dietary supplementation with KB290 (Fig. 4e). Because adrenergic stimulation promotes lipolysis in the adipose tissues, serum FFA levels may reflect β3 adrenergic receptor expression (Matthews *et al*. 1985). However, the level of *Fas* expression was not significantly different among groups (Fig. 4c), and the serum TG level in the KB group was not significantly lower than that in the HFD group (Fig. 2c). Because the TG level in adipose tissues is determined by the FFA secretion into blood, lipogenesis from glucose and uptake of serum TG, these observations suggest that reduced visceral fat accumulation in mice fed a KB290-supplemented HFD is more likely the result of elevated lipolysis, rather than decreased synthesis and uptake of TG. It has been reported that fermented dairy products with mixtures of the probiotic strains *Lactobacillus rhamnosus* GG, *Lactobacillus paracasei* TMC0409 and *Streptococcus thermophilus* TMC1543 enhance lipolysis in the adipose tissue (Yoda *et al*. 2015). Additionally, Jocken *et al*. (2018) found that β adrenergic receptor-mediated lipolysis was enhanced by butyrate treatment of human multipotent adipose tissue-derived stem cells. Thus, dietary supplementation with *L. brevis* KB290 may enhance lipolysis by upregulating the β3

adrenergic receptor and boosting the signal. We have not yet evaluated changes in the mass or functions of brown adipose tissues following KB290 consumption, so further examinations on brown adipose tissues are necessary to investigate this possibility.

The gut microbiota are reportedly associated with induction of obesity and obesity-associated disorders (Lee *et al.* 2019). Manipulation of the intestinal microbiota via the administration of probiotics (Kim *et al.* 2017; Park *et al.* 2017) has been shown to have a beneficial effect on adiposity, insulin sensitivity and the development of metabolic syndrome. We performed amplicon sequencing on fragments of the 16S rRNA gene to clarify the effects of ingesting heat-killed KB290 on the intestinal microbiota composition of mice. The relative abundance of *Lactobacillus* was almost negligible in the NFD and HFD groups, whereas it was significantly higher in the KB group (Table 1). Representative sequences of sequence variants that were assigned as *Lactobacillus* showed the highest similarity with *L. brevis*. In this experiment, heating at 95°C for 2 min was chosen for the preparation of heat-killed *L. brevis* KB290. These results suggest that heat-killed KB290 cells are enough stable to reach a distal part of the murine gastrointestinal tract and possibly altered the intestinal microbiota. Regarding alpha-diversity parameters, the Shannon index was significantly increased in mice fed a KB290-supplemented HFD (Fig. 5a), although observed features, Faith's phylogenetic diversity and Pielou's evenness were not significantly different among the groups (Figs 5b and Fig. S3) which suggest slight effects of ingested KB290 on the diversity of intestinal microbiota.

Beta-diversity analyses based on weighted UniFrac distance revealed that the microbiotas of mice in KB group were more similar to those in NFD group than were those in HFD group (Fig. 5c). The relative abundances of some bacterial genera within *Bacteroidetes*, *Defferibacteres*, *Firmicutes* or *Verrucomicrobia* were significantly different among the groups of mice (Table 1). The abundances of f_*S24-7*:g_ in *Bacteroidetes* and *Oscillospira* in *Firmicutes* were significantly lower in mice fed an HFD compared with those fed an NFD, and dietary supplementation with KB290 partly recovered the HFD-induced decreases in abundance observed for these bacterial groups (Table 1). Notably, family *S24-7* is associated with improved gut function and metabolic health (Ormerod *et al.* 2016). For example, an increased abundance of *S24-7* resulting from supplementation with dietary fibre was strongly correlated with a suppression of inflammatory markers in obese mice (Serino *et al.* 2012), and co-treatment of quercetin and resveratrol suppressed HFD-induced obesity with an accompanying increase in the intestinal population of *S24-7* (Zhao *et al.* 2017). Additionally, a lower abundance of *Oscillospira* in the intestine was observed for mice fed an HFD supplemented with flaxseed (Yang *et al.* 2020) or isoxanthohumol (Fukizawa *et al.* 2020), both of which have anti-obesity effects. *Akkermansia* is a mucin-degrading bacterium, and the anti-obesity effect of apple procyanidins was found to accompany an increased population of *Akkermansia* in the intestines of mice (Masumoto *et al.* 2016). Here, the relative abundance of *Oscillospira* was significantly lower in mice fed an HFD than in those fed an NFD, and the HFD-induced decrease in the abundance of *Oscillospira* was partly recovered by dietary supplementation with KB290. In contrast, the abundance of *Akkermansia* was significantly higher in mice fed an HFD than in those fed an NFD, and dietary supplementation with KB290 partly recovered the HFD-induced increase in abundance of this bacterial group (Table 1). Although the reason for these contradicting observations is currently unclear, changes in the abundances of *Oscillospira* and *Akkermansia* may not have contributed to the anti-adiposity effect of heat-killed KB290. Gut epithelia protect against the invasion of intact bacterial cells, including LAB, which suggests that KB290, even when inactivated by heat, is unlikely to directly affect the metabolism of mice. Thus, the anti-adiposity effects of dietary supplementation with KB290 may be at least partly induced by changes in the composition of gut microbiota.

In this study, we evaluated the anti-adiposity effect of heat-killed *L. brevis* KB290 using an HFD-induced obesity model. Supplementation of heat-killed KB290 suppressed HFD-induced elevations in epididymal and renal adipose tissue weight. Enhanced lipolysis via upregulation of the β3 adrenergic receptor and boosting the signal might have contributed to the anti-adiposity effect of KB290. Elevated serum glucose, insulin and HOMA-IR levels by ingestion of HFD were also suppressed by supplementation of heat-killed KB290, suggesting ameliorated metabolic symptoms. Although an altered composition of the intestinal microbiota was observed following ingestion of heal-killed KB290, the contribution of the intestinal microbiota to the anti-adiposity effect of KB290 remains unclear. Overall, our results indicate that heat-killed KB290 is useable as a paraprobiotic to develop functional foods that attenuate visceral fat accumulation.

Acknowledgements

We would like to express our appreciation to Ms. Y. Toyama for technical support. We thank Katie Oakley, PhD, from Edanz Group (https://en-author-services.edanzgroup.com/ac) for editing a draft of this manuscript.

Conflict of Interest

This study was funded by KAGOME CO., Ltd. CA, TI and SS are employees of KAGOME CO., Ltd.

Author contributions

All of the authors have made substantive intellectual contributions to this study. JW, NH and CA designed the experiments. JW, NH, TY, BS and CA performed most of the experiments and generated figures and tables. TI and SS provided reagents and facilities and participated in discussion. All authors contributed to interpreting the data and writing and editing the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Sequences of the primers used for reverse transcription-PCR.

Figure S1. Bodyweight from weeks 0 to 8 of mice fed (—●—) NFD, a normal-fat diet; (—▲—) HFD, high-fat diet; or (—■—) KB, HFD supplemented with *Lactobacillus brevis* KB290.

Figure S2. Correlations between serum free fatty acids levels, and epididymal (A), renal (B), and mesenteric (C) adipose tissue weights relative to bodyweight. The correlations were assessed by Spearman's correlation method.

Figure S3. Alpha diversity parameters Faith's phylogenetic diversity (A) and Pielou's evenness (B) of the intestinal microbiota from mice fed (□) NFD, a normal-fat diet; (■) HFD, high-fat diet; or (▨) KB, HFD supplemented with *Lactobacillus brevis* KB290.

Figure S4. Logarithmic linear discriminant analysis of intestinal microbiota from mice fed (□) NFD, a normal-fat diet; (■) HFD, high-fat diet; or (▨) KB, HFD supplemented with *Lactobacillus brevis* KB290.