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ORIGINAL ARTICLE

Bacillus licheniformis, a potential probiotic, inhibits obesity by modulating colonic microflora in C57BL/6J mice model

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Keywords
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Abstract

Aims: This study evaluated the effects of a potential probiotic, *Bacillus* sp., on the growth, serum and hepatic triglyceride, histological features of liver tissues and colonic microflora in high-fat diet-induced obese mice.

Methods and Results: Sixty male C57BL/6J mice were randomly divided into five groups: mice fed a low-fat diet (Cont), mice fed a high-fat diet (Hf), Hf and orally challenged with *Bacillus subtilis* (Bs), *B. licheniformis* (Bl) and a mixture of *B. subtilis* and *B. licheniformis* (Bls). Gavage feeding was provided at week 9 and the experiment was continued for 8 weeks. Treatment with *B. licheniformis* and a mixture of *Bacillus* sp. attenuated body weight gain at the end of study and enhanced glucose tolerance by sensitizing insulin action in the Hf-fed mice. Lower serum and hepatic triglyceride and epididymal fat weight were observed in Bl and Bls groups than that of Hf group. Lesser hepatic fat deposition was observed in the Bl and Bls groups than in the Hf group. High-throughput sequencing showed that *Bacillus* sp. supplementation dramatically changed the colonic bacterial community in obese mice.

Conclusions: *Bacillus licheniformis* reduced body weight and improved glucose tolerance, obesity and insulin resistance in Hf-fed mice by changing colonic microbiota composition.

Significance and Impact of the Study: Orally administration of *Bacillus licheniformis* may reduce body weight and decrease fat deposition by modulating colonic bacterial community in Hf model.

Introduction

With economic growth, overnutrition leads to excessive ectopic lipid accumulation and development of obesity (Ruderman *et al.*, 2013). Obesity is an excessive or abnormal accumulation of fat in the body resulting in several metabolic disorders, such as type 2 diabetes, fatty liver and dyslipidaemia (He *et al.*, 2018). Normally, glucose intolerance and fat deposition are treated as indicators of diabetes and obesity respectively (Balakumar *et al.*, 2018). Dihydrocapsiate was reported to reduce high-fat diet-induced weight gain and significantly prevented hyperglyceridaemia and hyperinsulinaemia (Baboota *et al.*, 2018). Actually, specific substances have been found to inhibit obesity, such as plant and seed extracts, probiotics and prebiotics.

16S rRNA gene sequencing of samples showed major modulation of the gut microbial community in obese C57BL/6J male mice (Jaja-Chimedza *et al.*, 2018). Several studies have confirmed that imbalanced gut microbiota is closely related to metabolic dysregulation leading to obesity (Johnson and Olefsky 2013; An *et al.*, 2018). A recent study revealed that specific probiotics could be used as potential therapeutic agents in diabetes or obesity by beneficially modulating the gut microbiota (Balakumar *et al.*, 2018). Different species of *Bacillus*, such as *B. subtilis*, *B. cereus*, *B. licheniformis*, *B. pumilus*, *B. clausii*, *B. coagulans* and *B. sonorensis* have been shown to ameliorate dysbiosis

of gut microbiota (Elshaghabee *et al.*, 2017). Fang *et al.* (2019) confirmed that *Lactobacillus plantarum* ameliorates high-fat diet-induced inflammation and insulin resistance, and promotes thermogenesis in adipose tissues of gnotobiotic mice. This study was conducted to investigate the effects of *B. subtilis* and *B. licheniformis* on the growth performance, fat deposition and colonic bacterial community in high-fat diet-induced obese mice.

Materials and methods

Animal care and experimental design

This study was conducted after obtaining approval from the animal welfare committee of the Institute of Zhejiang A&F University, and all procedures were carried out according to the rules established by the committee. Sixty male C57BL/6J mice (8 weeks old) were obtained from the SLAC Laboratory Animal Central (Changsha, China). All mice were housed under standard conditions and were provided with food and water *ad libitum*. After an adaptation period of 1 week, the mice were fed a control diet. The mice were randomly divided into five treatment groups (n = 12 in each group) as follows: control (Cont) (10% fat, 70% carbohydrate, and 20% protein by gavage feeding on every alternate day), Hf (45% fat, 35% carbohydrate, and 20% protein by gavage feeding on every alternate day), Hf (gavage feeding) along with 1·0 ml 10^8 CFU of *B. subtilis* per ml (Bs) on every alternate day, Hf (gavage feeding) along with 1·0 ml 10^8 CFU of *B. licheniformis* per ml (Bl) on every alternate day and Hf (gavage feeding) along with 1·0 ml 10^8 CFU of each *B. subtilis* and *B. licheniformis* per ml (1 : 1) (Bl) on every alternate day. Blood samples were collected from the abdominal aorta at 17 weeks of age, and then the mice were anesthetized with diethyl ether and sacrificed by cervical dislocation. Samples from the liver and colonic content were collected and stored at −80°C for further analysis. The probiotics were provided by Zhejiang Huijia Biotechnology Co. Ltd. (Anji, China) including *B. subtilis* (CGMCC 9383) and *B. licheniformis* (CGMCC 9385).

Estimation of growth performance

Body weight of each mice and total food intake were estimated for calculating the final body weight and average daily intake at the end of the experiment.

Estimation of serum and hepatic triglyceride levels and epididymal fat weight

Serum was separated after 15 min of centrifugation at 3000 ***g*** and was stored at −80°C for triglyceride estimation. The liver tissues were treated following the method described by Zhou (2017). Serum and hepatic triglyceride levels were measured by a kit following the manufacturer's instructions (Sigma-Aldrich, Shanghai, China). The epididymal fat weight was measured with a digital balance.

Intraperitoneal glucose and insulin tolerance test

Intraperitoneal glucose test (IPGTT) and intraperitoneal insulin tolerance test (IPITT) were performed 9 days and 1 week before the end of experiment respectively. A hand-held glucometer (OneTouch Ultra Easy, LifeScan) was used to measure the blood glucose level according to the method described by Zhou et al. (2018). Briefly, the mice were intraperitoneally injected with a dose of 1·0 g glucose or 0·65 U insulin (Novolin R; Novo Nordisk, Copenhagen, Denmark) per kg body weight after 6 h of fasting. Blood samples were collected from the tail veins and the glucose levels were measured at 0, 30, 60 and 120 min.

Hepatic morphology

Haematoxylin–eosin (H&E) staining was used to examine the hepatic lipid deposition. The liver samples were fixed with 4% paraformaldehyde, immediately embedded in paraffin and sliced. Then, 8-μm-thick tissue sections were stained with H&E.

High-throughput sequencing

A total of 30 mice (n = 6 in each group) were selected to analyse the colonic microflora as per our previous study (Cao *et al.*, 2019). DNA from the colonic contents was isolated using the MoBio Power Soil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer's manual. DNA testing and purity check were conducted by 1% agarose gel electrophoresis. DNA was diluted with sterile water up to a concentration of 1 ng μl^{-1} and stored at −80°C until further sequencing (Yin *et al.*, 2017, 2018). The V3 + V4 region of the bacterial 16S rRNA gene was amplified using the universal primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACVSGGGTATCTAAT-3′) and using the Illumina MiSeq platform (Novogene, Beijing, China).

Microbial bioinformatics analysis

The low-quality genes were filtered in raw reads, and the chimeras were deleted using the Cutadapt software (ver. 1.9.1). The Ion Plus Fragment Library Kit 48 rxns (Thermo Fisher Scientific, Waltham, MA, USA) was used

for building the database, and Ion S5 TM XL platform was used for further sequencing. The reads were clustered as operational taxonomic units (OTUs) by the UPARSE software (ver. 7.0) with a 97% similarity threshold. After the species annotation of all the samples using MOTHUR software and SILVA database (SSUrRNA), the top 10 phyla and genera were selected for the following analysis. The α-diversity (Shannon and Simpson index) analysis was then performed by the QIIME software (ver. 1.7.0). The β-diversity was estimated from the weighted UniFrac distances and visualised with the coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) using the R software (ver. 2.15.3). Finally, the differential bacterial taxa were identified among the groups by a linear discrimination analysis coupled with an effect size (LEfSe) analysis (Segata *et al.*, 2011).

Statistical analyses

All statistical analyses were performed using SPSS software ver. 16.0. One-way ANOVA was performed to analyse the growth performance, glucose intolerance and a *P*-value of <0·05 was considered statistically significant. For investigating the effect of probiotics on the colonic bacterial communities in the Cont/Hf, Hf/Bl and Hf/Bls groups, Student's *t*-test was performed.

Results

Effects of *Bacillus* sp. probiotic administration on the growth, serum and hepatic triglyceride levels, and epididymal fat weight

The effects of *Bacillus* sp. probiotic administration on the growth, serum and hepatic triglyceride levels, and epididymal fat weight in high-fat diet-induced obese mice are shown in Fig. 1. Supplementation with *B. licheniformis* or mixture of *Bacillus* significantly reduced the final body weight as compared to the mice in the Hf group, although there was no significant difference between the Bs and Hf groups (Fig. 1a). No obvious difference was found among the control, high fat diet and probiotic plus high-fat diet-fed mice (Fig. 1b). In addition, supplementation of mice in the Bl or Bls groups significantly reduced serum and hepatic triglyceride levels and epididymal fat weight as compared to those in the

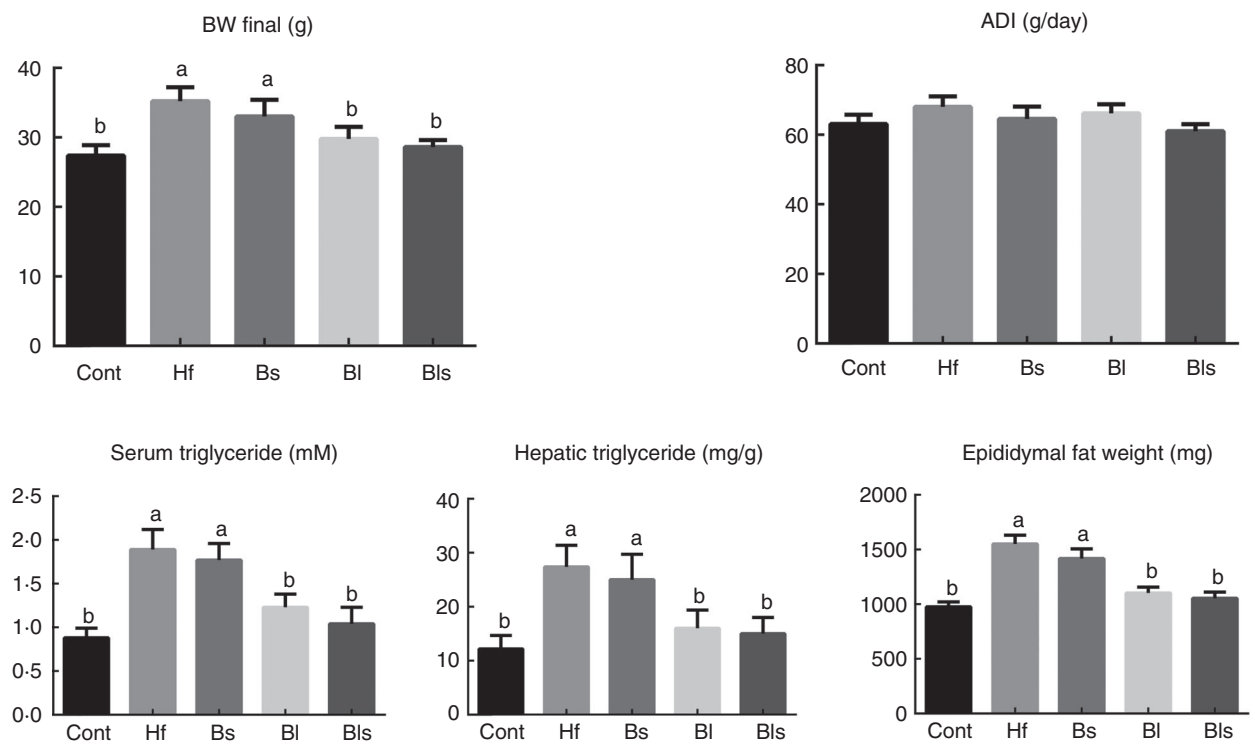

Figure 1 Effects of *Bacillus* sp. probiotic on the growth performance, serum and hepatic triglyceride and epididymal fat weight in high-fat diet-induced mice. a, b represent significant difference. Cont, mice were fed a low-fat diet; Hf, mice were fed a high-fat diet; Bs, mice were fed a high-fat diet and orally administrated with *B. subtilis*; Bl, mice were fed a high-fat diet and orally administrated with *B. licheniformis*; Bls, mice were fed a high-fat diet and orally administrated with *B. subtilis* and *B. licheniformis*, *N* = 6 ((■) Cont; (■) Hf; (■) Bs; (■) Bl; (■) Bls).

Hf and Bs groups. Moreover, there was no significant difference in serum and hepatic triglyceride level and epididymal fat weight among the Cont, Bl and Bls groups.

Glucose homeostasis

The effects of *Bacillus* sp. probiotics on glucose and insulin tolerance in high-fat diet-induced mice are shown in Fig. 2. In IPGTT, either Hf or Bs groups of mice had higher glucose level than the Cont, Bl and Bls groups of mice from 30 to 120 min, while the Cont, Bl and Bls groups of mice had similar glucose tolerances (Fig. 2a). For IPITT, Cont, Bl and Bls groups of mice had lower glucose levels as compared to that in the Hf and Bs groups of mice (Fig. 2b). These results indicate that *B. licheniformis* alleviate insulin resistance caused by a high-fat diet in mice.

H&E staining of the hepatocytes

The effects of *Bacillus* sp. administration on the lipid droplet accumulation in the hepatocytes of high-fat diet-induced mice are presented in Fig. 3. In H&E staining, lipid droplet accumulations were highest in Hf group among the five groups, and that in the Con, Bl and Bls groups were not remarkably different from each other. Meanwhile, extremely less lipid droplet accumulations were found in the Bl and Bls groups than in Hf group. Interestingly, there was still a certain amount of lipid droplet accumulation in the Bs group as compared to that in the Bl and Bls groups. Based on these findings, we inferred that supplementation with *B. licheniformis* had positive effects against lipid accumulation in the liver in high-fat diet-induced obese mice.

The modulation of the colonic microflora by the probiotics in high-fat diet-induced mice is shown in Fig. 4. There were 209 common OTUs shared within the five treatment groups; and 141, 17, 29, 28 and 54 unique OTUs were presented in the Cont, Hf, Bl, Bs and Bls groups of mice respectively (Fig. 4a). Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia were the predominant phyla within all the groups (Fig. 4b) with a relative abundance of >1%. Meanwhile, Lactobacillus, Bifidobacterium, Romboutsia, Alistipes, Faecalibaculum, Clostridium_sensu_stricto_1, Ruminococcaceae_UCG-014, Akkermansia and Lachnospiraceae_NK4A136_ were present in abundance in all the groups (Fig. 4c). Compared to the Cont group, the Bl and BIs groups had remarkable higher ($P < 0{\cdot}001$ each) Shannon and Simpson (α-diversity) index (Fig. 4d,e). The Bl group had a significantly higher ($P < 0{\cdot}01$) Shannon index than the Hf and Bs groups, and the Hf group had a significantly higher ($P < 0{\cdot}01$) Shannon and Simpson index than the Cont group. From PCoA analysis, we found that the samples of Cont mice had more distance than the others, while the samples of the Hf group were separated from those of the Bs, Bl and Bls groups (Fig. 4f). In addition, the NMDS plots also indicated that there was a further distance between the samples of Cont and Hf groups. Moreover, as compared to the Hf group, the samples of the Bs or Bls groups had more distance than that of the Bl group.

LEfSe analysis indicated that genus *Lactobacillus*, species *Bifidobacterium pseudocatenulatum* and family Streptococcaceae were present in abundance in the Cont group; class Clostridia, family Ruminococcaceae and Peptococcaceae were present in abundance in the Hf group; genus *Clostridium_sensu_stricto*_1, *Romboutsia* and *Turicibacter* were present in abundance in the Bs group; genus *Faecalibaculum* and *Acinetobacter* were present in abundance in the Bls group; genus *Ruminiclostridium*, *Akkermansia*, *Bilophila* and *Anaerotruncus*, and species *Parabacteroides_goldsteinii* were present in abundance in the Bl group (Fig. 5a). Moreover, *t*-test was used to

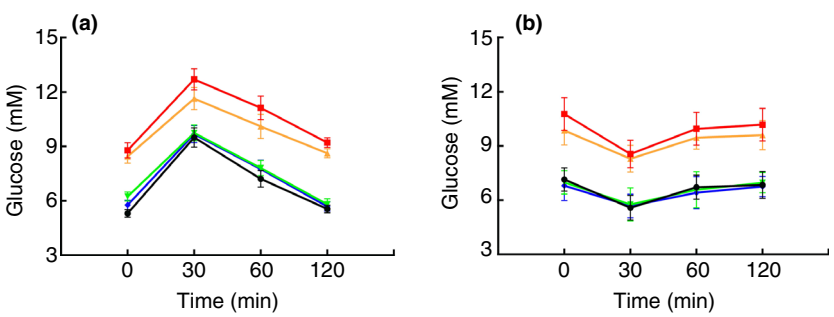

Figure 2 Effects of *Bacillus* sp. probiotics supplementation on the glucose tolerance and insulin tolerance in high-fat diet-induced mice. Cont, mice were fed a low-fat diet; Hf, mice were fed a high-fat diet; Bs, mice were fed a high-fat diet and orally administrated with *B. subtilis*; Bl, mice were fed a high-fat diet and orally administrated with *B. licheniformis*; Bls, mice were fed a high-fat diet and orally administrated with *B. subtilis* and *B. licheniformis*, $N = 6$ ((●) Cont; (■) Hf; (▲) Bs; (▼) Bl; (◆) Bls).

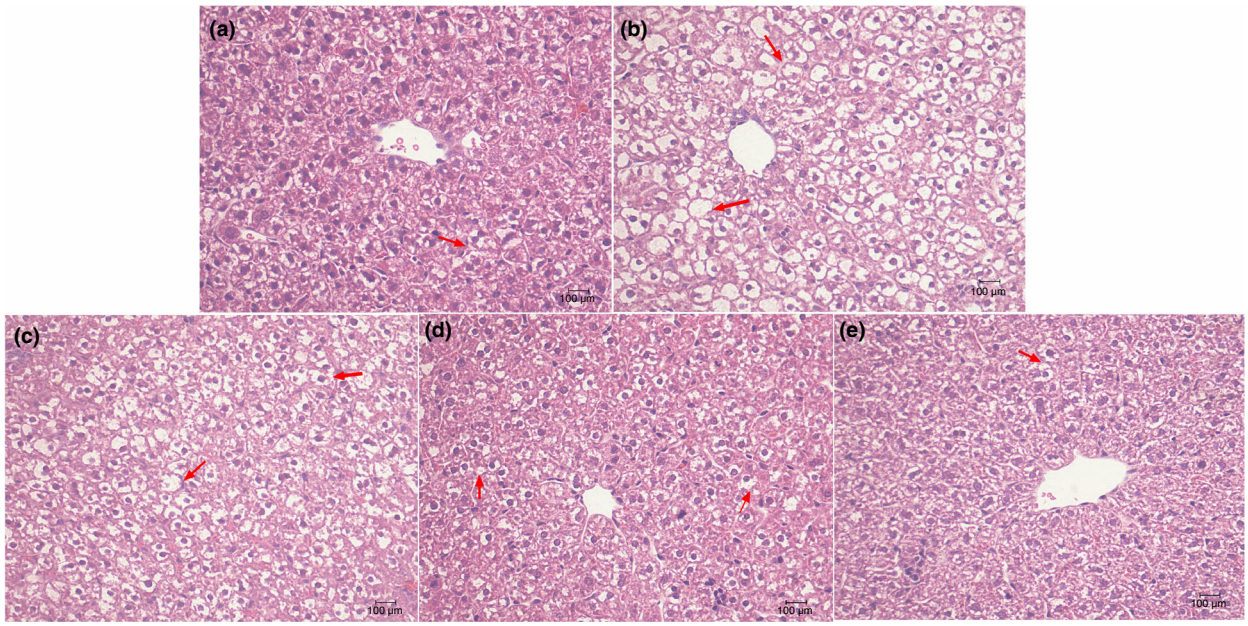

Figure 3 Effects of *Bacillus* sp. probiotic supplementation on hepatic morphology of high-fat diet-induced mice. H&E staining (200×). (a) Represents Cont mice fed a low-fat diet; (b) represents Hf mice fed a high-fat diet; (c) represents Bs mice fed a high-fat diet and orally administrated with *B. subtilis*; (d) represents Bl mice fed a high-fat diet and orally administrated with *B. licheniformis*; (e) represents Bls mice fed a high-fat diet and orally administrated with *B. subtilis* and *B. licheniformis*.

analyse the distinguished genera within the differential treatments in the present study (Fig. 5b–d). Many genera changed dramatically ($P < 0{\cdot}05$) between the Cont and Hf groups; while the Cont group had more abundance of *Lactobacillus* and *Odoribacter*, the Hf group had more abundance of *Romboutsia*, *Parabacteroides*, *Ruminiclostridium*, *Intestinimonas*, *Anaerotruncus*, *Lachnoclostridium* and *Oscillibacter*. Meanwhile, the Bl group had more abundance of *Bilophila*, unidentified_*Ruminococcaceae* and *Marvinbryantia* than the Hf group; the Bls group had more abundance of *Bacillus* and *Enterorhabdus* than the Hf group, and the Hf group had more *Ruminococcaceae*_UGG-009 than the Bls group.

Discussion

The global burden of overweight and obesity results from the intake of diets containing high proportions of fat and carbohydrates (Elena *et al.*, 2018). As a potential approach, probiotics have been reported to prevent and treat metabolic diseases by modulation of gut microbiota (Paolella *et al.*, 2014). Treatment with *B. subtilis* caused a reduction in weight gain, serum glucose activity and hepatic triglyceride in high-fat diet-induced obese mice (Lei *et al.*, 2015). In the present study, supplementation with *B. licheniformis* and *Bacillus* mixture reduced the final body weight of mice in the Hf group, while no significant change was found in the daily food intake. High serum cholesterol and triglyceride levels were induced by a high-fat diet in a mouse model of obesity (Kim *et al.*, 2013). A study confirmed that fermented Cheonggukjang powder led to lower cholesterol and triglyceride levels in mice fed a high-fat diet (Kim *et al.*, 2013). *Bacillus licheniformis*-fermented pepper powder inhibited the deposition of fat and normalised lipid metabolism in mice fed a high-fat diet (Hf) (Yeon *et al.*, 2013). Our findings revealed that *B. licheniformis*, but not *B. subtilis*, significantly lowered the hepatic and epididymal triglyceride levels. In addition, supplementation with *B. licheniformis* and *Bacillus* mixture also lowered the levels of serum and hepatic triglyceride and epididymal fat weight. Based on these results, we hypothesised that the different probiotic strains have differential effects on high-fat diet-induced obesity in mice.

High-fat diet consumption-induced insulin resistance has been proven by high fasting plasma glucose and insulin levels in C57BL/6J mice (Elena *et al.*, 2018). Balakumar *et al.* (2018) indicated that insulin resistance, glucose intolerance, hyperglycaemia and dyslipidaemia were observed in Hf. Probiotic administration (*L. plantarum* and *Lactobacillus fermentum*) prevented the development of insulin resistance and diabetes in Hf (Balakumar *et al.*, 2018). A probiotic mixture combined with different *Bacillus* sp. was also reported to protect the mice from

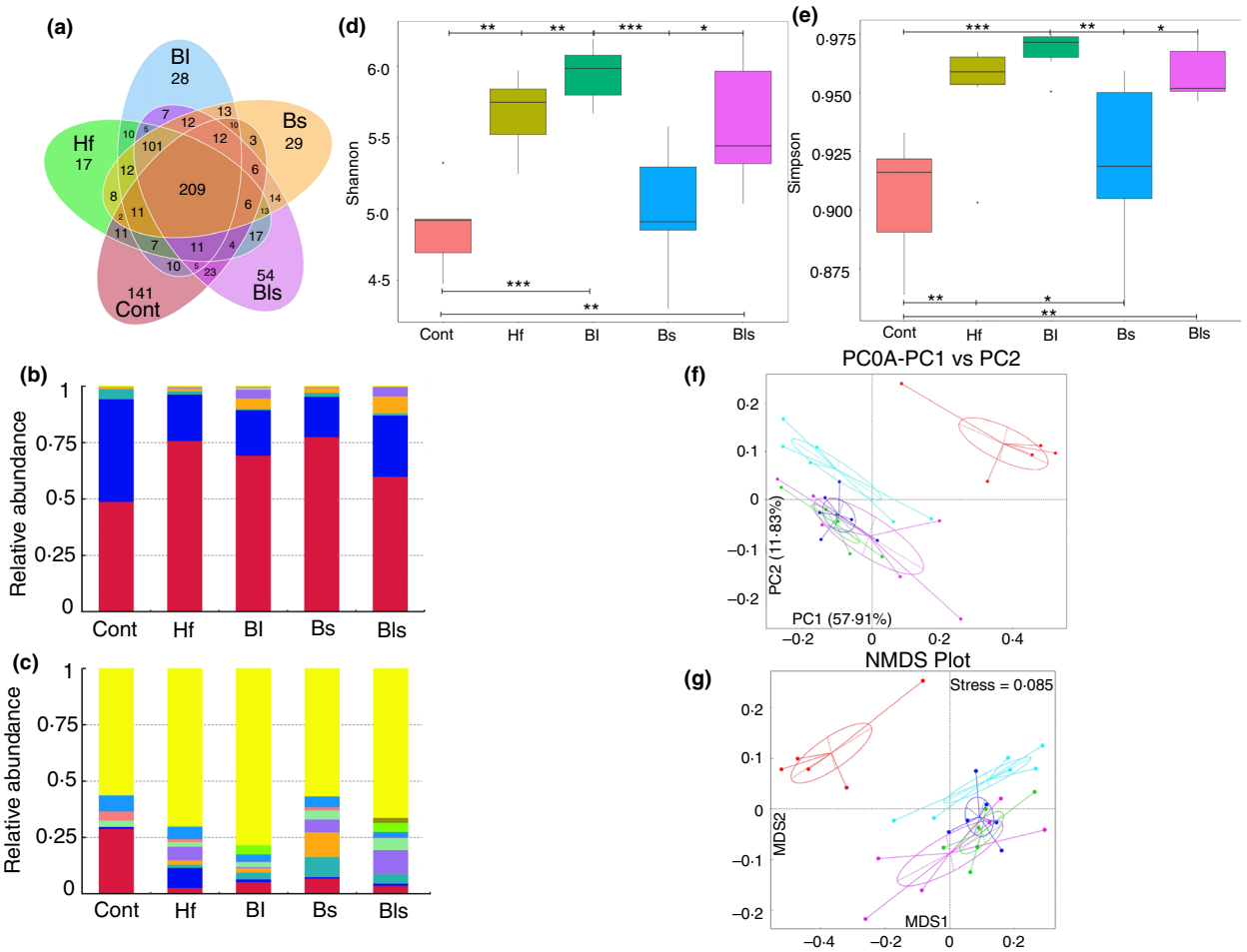

Figure 4 Summary of *Bacillus* sp. probiotics modulated the colonic microflora community in high-fat diet-induced mice. (a) OTUs in all mice samples, (b) representative phylum in all mice ((■) others; (■) Acidobacteria; (■) Saccharibacteria; (■) Cyanobacteria; (■) Deferribacteres; (■) Tenericutes; (■) Verrucomicrobia; (■) Proteobacteria; (■) Actinobacteria; (■) Bacteroidetes; (■) Firmicutes), (c) representative genus in all mice ((■) others; (■) Acinetobacter; (■) Akkermansia; (■) Lachnospiraceae_NK4A136_group; (■) Bifidobacterium; (■) Alistipes; (■) Faecalibaculum; (■) Clostridium_sensu_stricto_1; (■) Romboutsia; (■) Ruminococcaceae_UCG-014; (■) Lactobacillus), (d) Shannon index of all mice samples, (e) Simpson index of all mice samples, (f) PCoA analysis of all mice samples, (g) NMDS analysis of all mice samples ((●) Cont; (●) Hf; (●) Bl; (●) Bs; (●) Bls). *N* (Cont) = 5, *N* (Hf, Bl, Bs and Bls) = 6. * represents '$P < 0{\cdot}05$', ** represents '$P < 0{\cdot}01$', *** represents '$P < 0{\cdot}001$'.

high-fat diet-induced obesity and insulin resistance (Kim *et al.*, 2018). Administration of *Bacillus* strains mixed with long-term fermented soybean pastes protected the mice against high-fat diet-induced adiposity and glucose intolerance (Kim *et al.*, 2018). Our findings suggest that intake of probiotics (*B. licheniformis* and *Bacillus* sp.) could improve glucose tolerance and offer protection against insulin resistance, which was similar to the results of Choi *et al.* (2016).

The liver is one of the main organs involved in the maintenance of glucose and lipid homeostasis. High-fat diet consumption disrupts the homeostasis and in parallel causes deposition of fat metabolism in the liver (König *et al.*, 2012). A study confirmed that treatment with probiotics (*Bacillus*) significantly reversed hepatic steatosis in Hf (Kim *et al.*, 2018, 2018a). In the present study, H&E staining showed that supplementation with *B. licheniformis* or *Bacillus* mixture prevented liver fat deposition. These results also support the hypothesis that administration of *B. licheniformis* reduces lipid accumulation in high-fat diet-induced obese mice.

The host intestine harbours millions of microbes, and bacteria are considered to be the predominant organism. The colon is the last part of the gastrointestinal tract and it harbours more than 1000 bacterial strains. It is confirmed that intestinal microflora plays a key role in the development of obesity. Machado and Cortez-Pinto (2016) demonstrated that obese mice had a differential

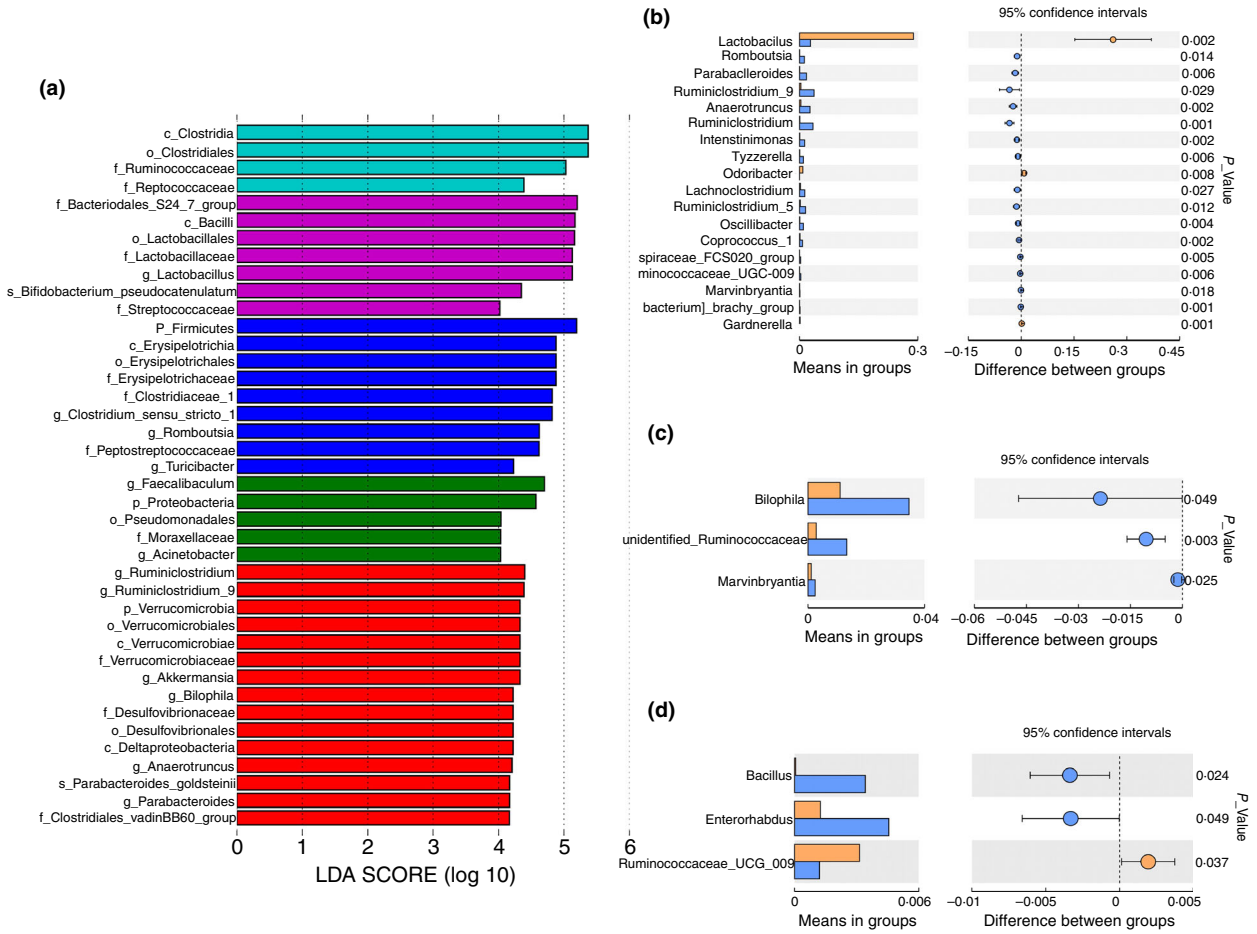

Figure 5 LEfSe analysis and Students' *t*-test about the relative abundance of predominant microflora in high-fat diet-induced mice. (a) LEfSe analysis ((■) Bl; (■) Bls; (■) Bs; (■) Cont; (■) Hf), (b) Students' *t*-test about 'Cont *vs* Hf' ((■) Cont; (■) Hf), (c) Students' *t*-test about 'Hf *vs* Bl' ((■) Hf; (■) Bl), (d) Students' *t*-test about 'Hf *vs* Bls'. *N* (Cont) = 5, N (Hf, Bl, Bs and Bls) = 6 ((■) Hf; (■) Bls).

gut microbiota composition as compared to normal mice. There were fewer Bacteroidetes and more Firmicutes in the high-fat diet-induced obese mice. Similarly, our study found that the relative abundance of phylum Firmicutes in the Cont group was less than that in the Hf group, and Bacteroidetes in the Cont group was higher than that in the Hf group. Moreover, Bl and Bls supplementation induced the reduction of the relative abundance of Firmicutes, and Bls increased the colonization of Bacteroidetes in the colonic microflora. More Firmicutes increased the enzymes that disintegrate polysaccharides from diet and release short-chain fatty acids (Machado and Cortez-Pinto 2016). Turnbaugh *et al.* (2006) supported our data showing that a low-energy diet increased the proportion of Bacteroidetes in the digestive tract. Moreover, *Lactobacillus* species are known to contribute to the maintenance of normal body weight. In our study, the mice in the Cont, Bl and Bls groups had a similar abundance of *Lactobacillus*. Interestingly, the Hf group had a significantly higher Shannon and Simpson index than the Cont group, while Bl group had a significantly higher Shannon index than the Hf and Bs groups. Both the PCoA and NMDS plot indicated that the colonic microbiota of the Cont and Hf groups were identical; whereas that in the Hf, Bl and Bls groups were different. These results reflected the changes in the colonic microbiota after oral administration of probiotics (*Bacillus* sp.).

Henning *et al.* (2017) found that the relative abundance of *Blautia*, *Bryantella*, *Collinsella*, *Lactobacillus*, *Marvinbryantia*, *Turicibacter*, *Barnesiella* and *Parabacteroides* in mice were significantly associated with weight loss caused by tea extracts. In addition, Rikenellaceae was more abundant in the faecal microflora of high-fat diet-induced males, while *Bacteroides* species,

Bilophila, *Sutterella* sp., *Parabacteroides*, *Bifidobacterium longum*, *Akkermansia muciniphila* and *Desulfovibrio* sp. were higher in number in the control male mice (Javurek *et al.*, 2016). Zhou *et al.* (2016) reported that using *B. licheniformis* as a direct-fed microbial agent significantly upregulated the catabolism-related genes in the liver and modulated the expression of lipid-anabolism genes in *Clostridium perfringens*-induced necrotic enteritis in broiler chickens. Jumpertz *et al.* (2011) confirmed that the gut microbiota is related to energy metabolism. Our results implied that the colonic microflora community in high-fat diet-induced obese mice was dramatically changed by supplementation with probiotics (*Bacillus* sp.) compared to that in the Hf and Cont groups.

Thus, to conclude, in the present study, high-fat diet-induced obese mice were treated with probiotic supplements (*B. subtilis*, *B. licheniformis* and their mixture) to examine their protective effects against fat deposition. Our results showed that *B. licheniformis* or a mixture of *Bacillus* stains significantly reduced final body weight, enhanced glucose intolerance and decreased hepatic fat deposition in mice without any change in food intake. Moreover, there was a dramatic alteration in the colonic microflora between the *Bacillus*-supplemented and high-fat diet-fed mice. These results suggest that *B. licheniformis*-based probiotics have a potential role in the treatment of different metabolic disorders.

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Conflict of Interest

None of authors declare a conflict of interest.

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