Beneficial effects of probiotic cholesterol-lowering strain of *Enterococcus faecium* WEFA23 from infants on diet-induced metabolic syndrome in rats

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ABSTRACT

The aim of this study was to select probiotic *Enterococcus* strains that have the potential to improve metabolic syndrome (MS). Ten *Enterococcus* strains isolated from healthy infants were evaluated for their probiotic properties in vitro, and *Enterococcus faecium* WEFA23 was selected due to its cholesterol removal ability (1.89 ± 0.07 mg/10¹⁰ cfu), highest glycodeoxycholic acid-hydrolase activity (1.86 ± 0.01 U/mg), and strong adhesion capacity to Caco-2 cells ($17.90 \pm 0.19\%$). The safety of *E. faecium* WEFA23 was verified by acute oral administration in mice, and it was found to have no adverse effects on general health status, bacterial translocation, and gut mucosal histology. Moreover, the beneficial effects of *E. faecium* WEFA23 on high-fat diet-induced MS in rats were investigated, and we found WEFA23 significantly decreased body weight, serum lipid levels (total cholesterol, triacylglycerols, and low-density lipoprotein cholesterol), blood glucose level, and insulin resistance in rats fed with a high-fat diet. This indicated that administration of *E. faecium* WEFA23 improved almost all key markers of MS, including obesity, hyperlipidemia, hyperglycemia, and insulin resistance. Our results supported *E. faecium* WEFA23 as a candidate for cholesterol-lowering dairy products and improvement of MS. Our research provided novel insights on *Enterococcus* as a strategy to combat MS.

Key words: enterococci, cholesterol, probiotic, metabolic syndrome

INTRODUCTION

The metabolic syndrome (MS), which was defined by a series of obesity-related metabolic alterations,

became a major public health concern and clinical challenge due to an increase risk of cardiovascular disease and type 2 diabetes (Kaur, 2014). The International Diabetes Federation suggested that the main features of MS include central obesity, hyperlipidemia, and raised blood pressure and fasting glucose (Festi et al., 2014). Worldwide incidence of MS ranged from 10 to 84%, and prevalence among the adult population is 25% (Kaur, 2014). Lifestyle modifications and pharmacologic agents, which are main treatment methods for MS at present, remain inefficient. Most patients have difficulties achieving goals for lipid, cholesterol, and glucose levels (Lebovitz, 2011).

Probiotics are defined as live microorganisms that provide beneficial effects to the host when administered in adequate amounts (WHO, 2001). Lactic acid bacteria with active bile salt hydrolase (BSH) have been suggested to attenuate the unbalanced homeostasis and cholesterol levels through interaction with host bile salt metabolism (Kumar et al., 2012), thereby inhibiting fat accumulation, which can result in satisfactory effect on the improvement of MS, including obesity, insulin resistance, and other metabolic diseases (Mei et al., 2015). A variety of lactic acid bacteria were found to have the potential to improve MS, such as *Bifidobacterium* spp. (Amar et al., 2011; Chen et al., 2012), *Lactobacillus* spp. (Kim et al., 2013; Park et al., 2013; Yoo et al., 2013), *Streptococcus thermophilus* (Ma et al., 2008), and *Pediococcus pentosaceus* (Zhao et al., 2012).

However, the beneficial effects of enterococci on MS have been seldom reported. To best of our knowledge, limited data involving cholesterol-lowering activity by *Enterococcus* strains are available (Agerholm-Larsen et al., 2000; Hlivak et al., 2005; Cavallini et al., 2009; Guo et al., 2016). Furthermore, the beneficial effects of enterococci on insulin resistance remain barely reported.

In our previous study (Zhang et al., 2016), 10 strains of *Enterococcus* from healthy Chinese infants were found to possess good ability of bile tolerance and exhibit BSH activity. However, whether those strains

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of *Enterococcus* can effectively reduce cholesterol levels and exert beneficial effects on MS is unknown. Therefore, in the present study, we selected candidates from those *Enterococcus* based on their cholesterol-lowering capacity, BSH activities, and adhesion to human intestinal epithelial Caco-2 cells. A strain of *Enterococcus* presenting desirable properties was further clarified for safety by acute oral administration. Thereafter, the selected strain was assayed for metabolic changes to elucidate use for treatment and prevention of MS, including obesity, dyslipidemia, hypercholesterolemia, hyperglycemia, and hyperinsulinemia and insulin resistance.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Ten *Enterococcus* strains (*Enterococcus faecium* strains WEFA23, WEFA24, WEFA26, WEFA28, WEFA30, and WEFA32; *Enterococcus hirae* strains WEHI01 and WEHI02; *Enterococcus durans* WEDU02; *Enterococcus casseliflavus* WECA01) were previously isolated from healthy infants (Zhang et al., 2016), grown on brain heart infusion (BHI) agar (Oxoid, Basingstoke, UK), and incubated anaerobically at 37°C.

Animals

Female 7-wk-old BALB/c mice and male 5-wk-old Sprague-Dawley rats (weighing 150 g) were obtained from Nanchang University Animal Science Department (Jiangxi, China). All animals were housed in a controlled atmosphere (temperature of $23 \pm 1^\circ\text{C}$, humidity $54 \pm 2\%$) and fed a standard diet and fresh water freely. All experimental procedures were approved by the Nanchang University Medical College Animal Care Review Committee (approval number 0064257).

In Vitro Cholesterol-Lowering Ability

Assimilation of cholesterol was tested as described before (Tomaro-Duchesneau et al., 2014) with slight modification. Briefly, 1% (vol/vol) inoculums of overnight-cultured enterococci were inoculated into BHI broth supplemented with 100 $\mu\text{g/mL}$ of water-soluble cholesterol (Sigma-Aldrich, St. Louis, MO) in the presence or absence of 0.3% (wt/vol) bile salts. Both BHI and cholesterol-BHI were used as control. After 24 h of incubation at 37°C, the supernatants were harvested by centrifugation ($12,000 \times g$, 10 min, 4°C) and the viable counts of enterococci were measured by using colony-counting method.

The residual cholesterol concentration in the supernatants was measured by using the *o*-phthalaldehyde method (Tomaro-Duchesneau et al., 2014). Briefly, 500 μL of the supernatants were added into the tube containing 1 mL of absolute ethanol and 500 μL of 33% (wt/vol) KOH. Then, the solution was mixed by vortexing for 1 min and incubated at 60°C for 10 min. After being cooled, 1.5 mL of hexane and 1 mL of deionized water were added and vortexed for 2 min. For phase separation, the tube was allowed to sit at room temperature for 10 min, and 1 mL of hexane layer was carefully transferred to clean tube; then, the hexane layer was evaporated to dryness by blowing nitrogen gas. Subsequently, 500 μL of 5 mg/mL fresh *o*-phthalaldehyde reagent in acetic acid was added to each tube to redissolve the samples, and 250 μL of concentrated H_2SO_4 was added and vortexed for 2 min. After incubation at room temperature for 20 min, the resulting absorbance was read at 570 nm. A standard curve was determined for deriving cholesterol concentrations. The cholesterol assimilation was calculated as (Tomaro-Duchesneau et al., 2014):

$$\begin{aligned} \text{Cholesterol removal (\%)} = \\ 100\% \times [\text{cholesterol } (\mu\text{g/mL})_{\text{oh}} \\ - \text{cholesterol } (\mu\text{g/mL})_{24\text{h}}] / \text{cholesterol } (\mu\text{g/mL})_{\text{oh}}, \text{ and} \end{aligned}$$

$$\begin{aligned} \text{Cholesterol removal (mg/10}^{10} \text{ cfu)} = \\ [\text{cholesterol } (\mu\text{g/mL})_{\text{oh}} - \text{cholesterol } (\mu\text{g/mL})] / \\ [\text{probiotic cell viability (cfu/mL)} \times 10^{10}]. \end{aligned}$$

Quantitative BSH Activity

Bile salt hydrolase activity was measured by using ninhydrin assay (Dong et al., 2012). Briefly, 30 mL of overnight cultures were harvested by centrifugation ($5,000 \times g$, 10 min, 4°C), washed twice with phosphate buffer (pH 7.2), and resuspended with 3 mL of the same buffer. Ten microliters of 10 mM dithiothreitol were added to avoid oxidation of BSH. Then the bacterial suspensions (3 mL) were sonicated for 20 min (each time for 3 s and separated by 9 s) and cooled in ice. Afterward, the supernatant was obtained by centrifugation ($5,000 \times g$, 10 min, at 4°C) and stored as cell-free extracts at -20°C .

To 90 μL of phosphate buffer (pH 5.1), 10 μL of 10 mM glycodeoxycholic acid (GDCA) or taurodeoxycholic acid (TDCA) and 10 μL of cell-free extracts were mixed together, and the reaction mixture was handled at 37°C for 30 min, then 200 μL of 15% (wt/vol) trichloroacetic acid were added to terminate the reaction.

Then the mixture was centrifuged ($12,000 \times g$, 10 min, 4°C) to obtain the supernatant. To measure the AA contents in the supernatant, 100 μL of the supernatant were mixed with 1.9 mL of ninhydrin reagent [0.5 mL of 1% (wt/vol) ninhydrin in 0.5 M sodium citrate buffer (pH 5.5), 0.2 mL of the same sodium citrate buffer and 1.2 mL of glycerol] and boiled for 14 min; after cooling, absorbance at 570 nm was read. A standard curve of AA (glycine or taurine) contents versus absorbance was measured. The BSH activity (U/mg) was defined as the amount of enzyme that liberated 1 μM of AA per min per milligram of protein. Protein concentrations were determined using the Bradford Protein Assay kit (Beijing Solarbio Science and Technology Co. Ltd., Beijing, China), and a standard curve was measured using BSA.

Adhesion Assay

The adhesion of the enterococci to Caco-2 cells was performed as described previously (Jiang et al., 2016). Briefly, overnight cultures of the enterococci were harvested by centrifugation ($8,000 \times g$, 5 min, 4°C), and resuspended in antibiotic-free Dulbecco's modified Eagle's medium (ExCell Bio Co., Ltd., Shanghai, China) with 10% heat-inactivated fetal bovine serum at a final concentration of about 2.5×10^7 cfu/mL. Then, 2 mL of the prepared bacterial suspensions were added to Caco-2 cell monolayer with a ratio of bacteria to cells of 1:50. After incubation for 2 h at 37°C under 5% CO_2 atmosphere, wells were gently washed 3 times with PBS solution to remove any unbound bacteria. Then EDTA-trypsin solutions (Sigma-Aldrich) were added to release adherent Caco-2 cells and the attached bacteria were counted by plating on BHI agar. The results were expressed as the percentage of the number of bacteria adhered compared with bacteria added initially.

Acute Oral Toxicity Test and Bacterial Translocation

To investigate the safety of consumption of *E. faecium* WEFA23, acute oral test and bacteria translocation assay were carried out. Briefly, 16 female BALB/c mice were acclimatized for 1 wk and randomly divided into 2 groups: one group receiving 100 μL of cell suspension containing 10^{11} cfu of *E. faecium* WEFA23 (treatment), another group receiving equal volume of sterile PBS (pH 7.4) as control. All mice were gavaged twice a day for 1 wk. Throughout the experiment, live weight, food intake, and water consumption of the mice were measured daily. The activity, behavior, and general health status of mice were observed twice daily.

After 7 d of intervention, the mice were anesthetized and blood samples were collected by cardiac puncture,

then the mice were humanely euthanatized. Before collecting tissue samples in sterile conditions, the surface of viscera was swiped with a sterile swab, which was then plated in Luria-Bertani (LB), de Man, Rogosa, and Sharpe (MRS), and BHI media to examine the contamination of viscera. The spleen and liver tissues were excised and homogenized in PBS solution (1 g/mL). Then, the tissue suspensions (100 μL) and blood samples were inoculated separately on MRS, LB, or BHI agar plates and incubated at 37°C for 72 h under aerobic (LB and BHI plates) or anaerobic (MRS plates) conditions. The cecum was excised for histological examination.

Probiotic Effect on MS in High-Fat Diet-Fed Rats

Twenty male 5-wk-old Sprague-Dawley rats were acclimatized for 1 wk and randomly divided into 4 groups: (1) rats received the normal diet and PBS (pH 7.4, 1 mL/d by gavage); (2) rats received a high-fat diet and PBS (HFD; pH 7.4, 1 mL/d by gavage); (3) rats received a high-fat diet and low rate of *E. faecium* WEFA23 (WEFA23-L; 5×10^8 cfu/mL in PBS, 1 mL/d by gavage); (4) rats received a high-fat diet and high rate of *E. faecium* WEFA23 (WEFA23-H; 5×10^9 cfu/mL in PBS, 1 mL/d by gavage). The composition of the high-fat diet was a normal diet (66.5%, wt/wt), lard (10.0%), sucrose (20.0%), cholesterol (2.5%), and sodium cholate (1.0%; Xing et al., 2009). Throughout the experiment, the rats' live weight, food intake, and water consumption were measured once a week. After the 35-d intervention, the overnight-fasted rats were anesthetized and blood samples were collected by cardiac puncture. Serum was collected from blood samples by centrifugation ($4,000 \times g$, 20 min, 4°C) and stored at -80°C for further analysis.

Biochemical Analysis

The concentrations of serum lipids including total cholesterol (TC), triacylglycerols (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured by assay kits (Jiancheng, Nanjing, China) according to the manufacturer's instructions. The level of blood glucose and blood insulin was determined by using assay kits (Rongsheng, Shanghai, China) and Rat INS ELISA kit (Jiancheng, Nanjing, China), respectively. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as (Matthews et al., 1985)

$$\text{HOMA-IR} = \frac{\text{fasting insulin (mIU/L)}}{\text{fasting blood sugar (mmol/L)/22.5}}.$$

Oral Glucose Tolerance Test

After the 30-d intervention, the oral glucose tolerance test was performed. The rats were fasted for 12 h and received glucose (2 g/kg of BW) by oral administration. Blood samples were collected from caudal vein at times 0, 30, 60, and 120 min following glucose administration. The concentration of glucose was determined as described above.

Statistical Analysis

Results were expressed as the mean $\pm$ standard error of the mean, and the data were analyzed using the SPSS 17.0 software (SPSS Inc., Chicago, IL). One-way ANOVA followed by Duncan's multiple range tests was used to compare the differences among 2 groups. Differences with P -value <0.05 were considered statistically significant.

RESULTS

Cholesterol-Lowering Ability of *Enterococcus* Isolates

Ten *Enterococcus* strains were selected and evaluated for their cholesterol assimilation abilities. The result is shown in Figure 1A. All strains were able to assimilate cholesterol in the range of 21.38 to 67.88%. Generally, the percentage of cholesterol assimilation was significantly higher for *E. hirae* WEHI01 ($61.06 \pm 1.29\%$), WEHI02 ($67.88 \pm 2.50\%$), and *E. faecium* WEFA24 ($61.22 \pm 1.28\%$) than that of other *Enterococcus* strains. As cholesterol-removal ability could be associated with BSH activity (Taranto et al., 1997), the removal of cholesterol was tested in BHI broth supplemented with 0.3% bile salt (Figure 1A). The percentage of cholesterol removal increased tremendously, up to 60.85 to 86.09%, with the addition of bile salts. Four *Enterococcus* strains were significantly better ($P < 0.05$) than other strains at the removal of cholesterol: *E. faecium* WEFA23 ($86.09 \pm 1.36\%$), *E. hirae* WEHI01 ($85.85 \pm 3.90\%$), WEHI02 ($85.50 \pm 1.12\%$), and *E. casseliflavus* WECA01 ($81.49 \pm 2.25\%$).

Taking into account of the effect of biomass on the cholesterol removal ability, we used milligrams per 10^{10} cfu instead of percentage to make an evaluation. Similarly, as seen by the cholesterol-removal tendency in Figure 1A, *E. hirae* WEHI02 assimilated more cholesterol than other strains, with 1.89 ± 0.07 mg/ 10^{10} cfu. All of the strains presented higher removal ability when bile salt was added, except for *E. hirae* WEHI02 and *E. durans* WEDU02. *Enterococcus faecium* WEFA23 (2.84

mg/ 10^{10} cfu) exhibited the best cholesterol-removal ability (Figure 1B).

BSH Activities of *Enterococcus* Isolates

The BSH activities of 10 strains toward GDCA or TDCA were measured. As shown in Figure 2, the BSH

Figure 1. Cholesterol removal of 10 *Enterococcus* isolates (*Enterococcus faecium* WEFA23, WEFA24, WEFA26, WEFA28, WEFA30, and WEFA32; *Enterococcus hirae* WEHI01, WEHI02; *Enterococcus durans* WEDU02; *Enterococcus casseliflavus* WECA01) after incubation at 37°C for 24 h in brain heart infusion broth containing 100 μ g/mL of water-soluble cholesterol with or without 0.3% (wt/vol) bile salts. (A) The percentage of cholesterol removal; (B) amount of cholesterol removal per 10^{10} cells in brain heart infusion broth. Data are expressed as the mean $\pm$ SD of triplicate tests.

activities varied with strains, ranging from 0.109 to 1.860 U/mg for GDCA hydrolase activities (Figure 2A) and 0.004–0.131 U/mg for TDCA hydrolase activities (Figure 2B). The highest GDCA hydrolase activity was observed for *E. faecium* WEFA23 (1.86 ± 0.005 U/mg) and *E. hirae* WEHI01 (1.339 ± 0.009 U/mg), whereas the highest TDCA hydrolase activity also were recorded for *E. hirae* WEHI01 (0.131 ± 0.002 U/mg). All of strains exhibited their preference toward GDCA as compared with TDCA, and their GDCA hydrolase activities were above 10 fold that of TDCA hydrolase activities. Therefore, GDCA hydrolase activity was a better criterion selection for potential probiotic strains.

Adhesion Capacity of *Enterococcus* Isolates

As shown in Figure 3, adhesion percentage of *Enterococcus* strains to Caco-2 cells varied from 7.50 to 37.35%. *Enterococcus faecium* WEFA24 had the highest adhesion, whereas *E. durans* WEDU02 had the lowest.

Safety Evaluation of *E. faecium* WEFA23

Based on the results of in vitro evaluation of 10 strains, *E. faecium* WEFA23 was selected mainly due to having the highest GDCA hydrolase activity and best cholesterol-removal ability in the environment containing 3% bile salt. *Enterococcus faecium* WEFA23 was further evaluated for its safety through acute oral toxicity, intestinal mucosal histology, and bacterial translocation assay. Throughout the experimental period, no records of treatment-related illness and death of mice took place. We observed no changes in activity, behavior, and fur luster. The BW of mice in treatment and control group showed no significant difference (data not shown). Histopathology of cecum showed no noticeable difference in the size and arrangement of epithelial cells between treatment and control groups, and no signs of inflammation and damage were observed (Figure 4). No bacterium was observed in spleen and liver of the experimental animals. Translocation of bacteria from the gut to spleen and liver was not detected in all animals (data not shown).

Effect of *E. faecium* WEFA23 on HFD-Fed Rats

All the groups did not significantly differ in feed intake (data not shown). By the end of the feeding period, WEFA23-L and WEFA23-H significantly ($P < 0.05$) gained less BW than the HFD group (218.80 ± 17.8 and 198.60 ± 15.65 vs. 264.60 ± 15.29 g, respectively; Figure 5B). The WEFA23-H group demonstrated a significant ($P < 0.05$) decrease of BW beginning in wk 4 (Figure 5A). In addition, the treatment with *E. fae-*

Figure 2. The bile salt hydrolase (BSH) activities of cell-free extracts toward different substrates from *Enterococcus* isolates (*Enterococcus faecium* WEFA23, WEFA24, WEFA26, WEFA28, WEFA30, and WEFA32; *Enterococcus hirae* WEHI01, WEHI02; *Enterococcus durans* WEDU02; *Enterococcus casseliflavus* WECA01). (A) Glycodeoxycholic acid (GDCA) as the substrate; (B) taurodeoxycholic acid (TDCA) as the substrate. Data are expressed as the mean $\pm$ SD of triplicate tests. Different letters (a–i) indicate significant differences ($P < 0.05$) between the groups.

cium WEFA23 led to a significant decrease ($P < 0.01$) of epididymis adipose tissue index in rats fed the HFD diet (Figure 5C).

Effect of *E. faecium* WEFA23 on Serum Lipid Levels in HFD-Fed Rats

The effect on serum lipid levels varied among 10 strains of *E. faecium* in HFD-fed rats (data not shown).

Figure 3. Adhesion percentage of *Enterococcus* strains (*Enterococcus faecium* WEFA23, WEFA24, WEFA26, WEFA28, WEFA30, and WEFA32; *Enterococcus hirae* WEHI01, WEHI02; *Enterococcus durans* WEDU02; *Enterococcus casseliflavus* WECA01) to Caco-2 cells. Each adhesion assay was conducted in triplicate. Data are expressed as the mean $\pm$ SD of triplicate tests. Different letters (a–d) indicate significant differences ($P < 0.05$) between the groups.

For the efficiency of *E. faecium* WEFA23, we noted a significant ($P < 0.05$) decrease of the serum concentrations of TC (Figure 6A), TG (Figure 6B), and LDL-C

(Figure 6D), and a nonsignificant ($P > 0.05$) increase of HDL-C (Figure 6C) in HFD-fed rats. Moreover, the effects were more obvious when administrated with a higher dose of 5×10^9 cfu of *E. faecium*.

Effect of *E. faecium* WEFA23 on Glucose Level and Insulin in HFD-Fed Rats

As shown in Figure 7, when compared with the HFD group, the oral glucose tolerance (Figure 7A and 7B), fasting blood glucose (Figure 7C), fasting insulin (Figure 7D), and HOMA-IR score (Figure 7E) of the WEFA23-H group were significantly decreased ($P < 0.05$). Among these parameters, the change of HOMA-IR was the most remarkable ($P < 0.001$), reaching 55.6%; however, the WEFA23-L group did not significantly differ in these parameters except for HOMA-IR.

DISCUSSION

Oral administration of probiotics (e.g., *Lactobacillus* and *Bifidobacterium*) as a strategy to combat MS has been studied very little in humans and animals. Therefore, in our research 10 strains of *Enterococcus*, selected from our previous study (Zhang et al., 2016), were used in a series of experiments in vitro and in vivo to obtain candidates as biotherapeutics to improve host MS.

Figure 4. Safety assessment of *Enterococcus faecium* WEFA23 with acute oral toxicity method. Histological examination of mice cecum ($\times 400$ magnification). Control = mice fed PBS; treatment = mice fed suspension of *E. faecium* WEFA23 (10^{11} cfu) in PBS. Color version available online.

Figure 5. Probiotic effect of *Enterococcus faecium* WEFA23 on high-fat diet-induced mice by monitoring the BW (A), BW gain after 5 wk (B), and epididymis adipose tissue index (C). Data are expressed as the mean $\pm$ SD of 5 replicates. Significant differences between the high-fat diet (HFD) group versus normal diet (ND) group are indicated as ### P < 0.001. Significant differences for low dose of *E. faecium* WEFA23 (WEFA23-L) or high dose of *E. faecium* WEFA23 (WEFA23-H) group versus HFD group are indicated as * P < 0.05, ** P < 0.01, and *** P < 0.001.

Cholesterol-lowering ability and BSH activity in vitro were critical parameters for screening probiotics possessing potential activity against MS in vivo (Tsai et al., 2014; Song et al., 2015). Evidence suggested a link between cholesterol-lowering activity and BSH in probiotics (Ishimwe et al., 2015). Through bacterial BSH activity, glycine- and taurine-conjugated bile salts were hydrolyzed into free bile acids and AA residues. Free bile acids were co-precipitated with cholesterol, resulting in a decrease of intestinal absorption and increase of fecal elimination (Ooi and Liong, 2010). Moreover, this process increased demand for cholesterol of de novo synthesis to replace the loss of bile acids (Ishimwe et al., 2015). In our study, *E. faecium* WEFA23 and *E. hirae* WEHI01 and WEHI02 all showed good ability to remove cholesterol, with *E. faecium* WEFA23 showing the best ability considering the factors of biomass and bile salts. Moreover, *E. faecium* WEFA23 was proved to an ideal strain for yielding BSH due to its highest GDCA hydrolase activity. In fact, Brashears et al. (1998) suggested that a BSH-active strain with more efficiency in hydrolyzing sodium glycocholate may have far more potential to reduce serum cholesterol levels, as glycocholate is considered to be the most predominating bile salt. We found also that TDCA hydrolase activities of 10 strains were only one-tenth of GDCA hydrolase activities. Therefore, although *E. hirae* WEHI01 showed both good TDCA hydrolase and GDCA hydrolase activities, *E. faecium* WEFA23 might be still better candidate for further evaluation. Furthermore, *E. faecium* WEFA23 adhered well to Caco-2 cells, indicating that the strain had potential to colonize in human intestine. These results provided evidence for the potential improvement of MS by *E. faecium* WEFA23 in vivo.

To test the safety of *E. faecium* WEFA23 as a probiotic, we tested acute oral toxicity via gavage of the strain to mice for 1 wk; that test was fundamental and classical for in vivo safety assessment of bacteria and had been applied in many similar studies (Zhou et al., 2000; Tan et al., 2013; Fernández-Murga and Sanz, 2016). The ability to damage the integrity of intestinal mucosa and of translocation was also important for probiotic safety evaluation, as it is a prerequisite for infectivity and pathogenicity for many opportunistic pathogens (Ford et al., 1996; Marteau and Salminen, 1997; Martín-Platero et al., 2009; Muñoz et al., 2011). After feeding mice a high dose of *E. faecium* WEFA23, no adverse effects were detected regarding live weight, feed intake, general health status, gut mucosal histology, and bacterial translocation, indicating that *E. faecium* WEFA23 was indeed a nonpathogenic strain.

Based on the in vivo anti-MS potential and safety evaluation of *E. faecium* WEFA23, we used the model of HFD-fed rats to test the effect of *E. faecium* WEFA23

on MS. Clinical treatment of MS was quite difficult due to the fact that available methods did not effectively prevent or treat the whole syndrome. Namely, most drugs can only treat one aspect of MS and cannot achieve the goal of lower weight, blood glucose, and triglycerides (Kaur, 2014). Therefore, *E. faecium* WEFA23 was evaluated not only for beneficial effects

on obesity and hyperlipidemia, but also for hyperglycemia and insulin resistance.

In the model, HFD-fed rats developed obesity, hyperlipidemia, hyperglycemia, and insulin resistance, which mimics almost all of the clinical aspects of MS (Eckel et al., 2005). We observed that *E. faecium* WEFA23 effectively improved diet-induced obesity by greatly

Figure 6. Probiotic effect of *Enterococcus faecium* WEFA23 on serum lipid levels of (A) total cholesterol (TC), (B) triacylglycerols (TG), (C) high-density lipoprotein cholesterol (HDL-C), and (D) low-density lipoprotein cholesterol (LDL-C). Data are expressed as the mean $\pm$ SD of 5 replicates. Significant differences between the high-fat diet (HFD) group versus normal diet (ND) group are indicated # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$. Significant differences for low dose of *E. faecium* WEFA23 (WEFA23-L) or high dose of *E. faecium* WEFA23 (WEFA23-H) group versus HFD group are indicated as * $P < 0.05$ and *** $P < 0.001$.

Figure 7. Probiotic effect of *Enterococcus faecium* WEFA23 on blood glucose levels and insulin resistance. (A) Curve of the oral glucose tolerance tests; (B) areas under the curve (AUC); (C) fasting blood glucose; (D) fasting insulin; and (E) homeostasis model assessment of insulin resistance (HOMA-IR), calculated by fasting blood glucose (mmol/L) $\times$ fasting insulin (mU/L)/22.5. Data were expressed as the mean $\pm$ SD of 5 replicate tests. Significant differences between the high-fat diet (HFD) group versus normal diet (ND) group are indicated # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$. Significant differences for low dose of *E. faecium* WEFA23 (WEFA23-L) or high dose of *E. faecium* WEFA23 (WEFA23-H) group versus HFD group are indicated as * $P < 0.05$ and *** $P < 0.001$.

reducing BW gain and index of epididymis adipose tissue whose hyperplasia and hypertrophy propagates an overall systemic inflammation associated with the development of MS (Kaur, 2014). We also found that administration of *E. faecium* WEFA23 dramatically decreased the serum level of TC, TG, and LDL-C to combat hyperlipidemia, which usually came with elevation of serum TC, TG, and LDL-C levels and a reduction of HDL-C levels and is also a major risk factor for cardiovascular disease (Kobayashi et al., 2012). It's worth noting that *E. faecium* WEFA23 significantly decreased the concentrations of fasting blood glucose, fasting insulin levels, ameliorated glucose tolerance, and insulin resistance in HFD rats. The results suggested that *E. faecium* WEFA23 might enhance the sensitivity of the peripheral tissues to insulin and improve the key components of MS and marks of type 2 diabetes mellitus, including hyperglycemia, hyperinsulinemia, and insulin resistance.

Isolates from various resources (e.g., infants and adults, fermented food, and environment) might have different efficiency on MS. With the development of microbiota analysis, previous studies disclosed that gut microbiota played a key role in the development of MS (Lecomte et al., 2015; Wang et al., 2015). Alard et al. (2015) found that the number of enterococci significantly increased in HFD-fed mice. BaiYang et al. (2009) demonstrated that *Bifidobacterium adolescentis* reduced the level of serum lipid in type 2 diabetes mellitus rats, and the beneficial effect was associated with a significant decrease in enterococci. In contrast, the strain *E. faecium* WEFA23 was found to markedly improve MS. We suspected that the mechanism may be different from other probiotics, such as *Lactobacillus* and *Bifidobacterium*, especially for the change of gut microbiota composition.

In conclusion, strain *E. faecium* WEFA23 showed desirable probiotic properties of cholesterol removal, BSH activity, and adhesion ability. Acute oral administration of *E. faecium* WEFA23 to mice showed that this strain was safe to use. Furthermore, *E. faecium* WEFA23 was found to have potential for improvement of MS symptoms, including obesity and hyperlipidemia in HFD rats. Interestingly, the strain was able to notably attenuate hyperglycemia and insulin resistance, which was seldom observed in the literature. The present study provides evidence that probiotic *E. faecium* WEFA23 effectively attenuates MS induced in a high-fat diet.

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