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Enterococcus faecium WEFA23 from infants lessens high-fat-diet-induced hyperlipidemia via cholesterol 7-alpha-hydroxylase gene by altering the composition of gut microbiota in rats

Fuqing Huang,* Fen Zhang,* Di Xu,* Zhihong Zhang,* Feng Xu,* Xueying Tao,* Liang Qiu,†[1] and Hua Wei*[1]
*State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang, Jiangxi, 330047, P. R. China
†Jiangxi University of Traditional Chinese Medicine, Nanchang, Jiangxi, 330004, P. R. China

ABSTRACT

Enterococcus faecium WEFA23 is a potential probiotic strain from Chinese infants with the ability to decrease cholesterol levels. Aiming to explore the mechanism of *E. faecium* WEFA23 in lowering cholesterol in vivo, we examined the gene transcriptions related to cholesterol metabolism, the composition of bile acids in feces, the synthesis of trimethylamine *N*-oxide (TMAO) in liver, and the composition of the gut microbiota of rats. We found that *E. faecium* WEFA23 enhanced the synthesis of bile acids by promoting cholesterol excretion, upregulating the genes transcript level relevant to cholesterol decomposition and transportation, and downregulating the genes involved in cholesterol synthesis. In addition, *E. faecium* WEFA23 not only downregulated the transcript levels of farnesoid X receptor and fibroblast growth factor 15 as well as flavin-containing monooxygenase 3, but also decreased the TMAO production followed by increasing the *CYP7A1* transcript level. Furthermore, when orally administered to rats for 35 d, *E. faecium* WEFA23 improved the gut microbiota diversity of rats fed a high-fat diet. Therein, the ratio of *Bacteroidetes* to *Firmicutes* and the abundance of *Rikenellaceae* increased, whereas the number of *Veillonellaceae* decreased. These results suggest that reduction of cholesterol level by *E. faecium* WEFA23 might be related to the changes in the gut microbiota. Our finding provides important information on lowering cholesterol by *E. faecium* and reveals that *Enterococcus* spp. might have the potential to decrease the TMAO level.

Key words: cholesterol 7-alpha-hydroxylase, trimethylamine N-oxide, intestinal microbiota, bile acid

INTRODUCTION

High cholesterol level is the leading cause of cardiovascular diseases (Prospective Studies Collaboration et al., 2007), which increase the number of human deaths worldwide (Mendis et al., 2011). Hypercholesterolemia leads to 45% of heart diseases in Western Europe and 35% of those in Central and Eastern Europe (Yusuf et al., 2004). The metabolism process of cholesterol includes synthesis, decomposition, and transportation in the host; among them, decomposition (i.e., bile acid synthesis) plays a crucial role in maintaining cholesterol homeostasis (Gupta et al., 2001). Cholesterol 7-α-hydroxylase (**CYP7A1**) is a critical enzyme that catalyzes the conversion of cholesterol into primary bile acids, which are then hydrolyzed into secondary bile acids. Parts of bile acids are reabsorbed in the ileum by active transport and return to the liver for secretion into the biliary system and gallbladder of the host (Hofmann, 1999). The other bile acids are eliminated by feces. Inhibiting the level of *CYP7A1* can reduce the biosynthesis of bile acids (Pullinger et al., 2002). *CYP7A1* is repressed by farnesoid X receptor (*FXR*), which functions as bile acid receptor (Chiang, 2009) and fibroblast growth factor 15 (*FGF15*; Inagaki et al., 2005). The *CYP7A1* is also inhibited by trimethylamine *N*-oxide (**TMAO**), which is converted from trimethylamine (**TMA**) by flavin-containing monooxygenase 3 (*FMO3*) in the liver (Tang et al., 2013).

Many publications have demonstrated that the synthesis of TMAO (Wang et al., 2011; Miller et al., 2014) and bile acids (Pavlović et al., 2012) is significantly affected by gut microbiota; however, few reports have stated that the regulation of *CYP7A1* affects the TMAO level by remodeling the microbiota. Moreover, no studies have reported that oral administration with probiotics (e.g., *Lactobacillus plantarum*, *Bifidobacterium* spp., and *Enterococcus* spp.) reshapes the gut microbiota structure and subsequently affects *CYP7A1* via TMAO.

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[1] Corresponding authors: weihua@ncu.edu.cn and qiuliang2016@126.com

Cholesterol levels in patient with hypercholesterolemia are mainly reduced in humans by administering drugs. For instance, statins, specific inhibitors of hydroxymethylglutaryl-CoA reductase (*HMG-CoAR*), are the rate-limiting enzymes in cholesterol biosynthesis (Farmer, 1998). However, most drugs are expensive and may have side effects, such as muscle pain and increased risk of diabetes mellitus and abnormalities in liver enzyme tests (Naci et al., 2013). Development of novel alternative methods for lowering cholesterol is necessary.

Probiotics are defined as live microorganisms that can confer health benefits on the host when administered in adequate amounts (Hotel and Cordoba, 2001). Accumulating evidence suggests that oral administration of probiotics (e.g., *Bifidobacterium* spp. and *Lactobacillus* spp.) effectively alleviated the level of serum cholesterol and should be a promising option for treatment of cardiovascular diseases. A previous study reported that *Bifidobacterium bifidum* PRL2010 can decrease cholesterol levels by modulating the gut microbiota (Zanotti et al., 2015). Meanwhile, *L. plantarum* can reduce the absorption of cholesterol and accelerate the elimination of biliary cholesterol by downregulating Niemann–Pick C1-like 1 (*NPC1L1*) via upregulating the liver X receptor (*LXR*; Ishimwe et al., 2015).

Enterococcus spp. are common lactic acid bacterial species in humans, animals, and fermented foods (Franz et al., 2011), and exhibit cholesterol-lowering effect in vitro (De Rodas et al., 1996; Agerholm-Larsen et al., 2000). However, few studies have demonstrated the cholesterol-lowering effect of *Enterococcus* spp. in vivo (Hlivak et al., 2005; Zhang et al., 2017). Moreover, the mechanism underlying the cholesterol lowering property of the strain remains unclear.

In our previous study, *E. faecium* WEFA23 isolated from infants (Zhang et al., 2016) presented high activity for synthesis of bile salt hydrolase (**BSH**; Zhang et al., 2017), which can catalyze conjugated bile acids into nonconjugated bile acids (Pavlović et al., 2012). Another study showed the effect of lowering serum cholesterol in rats fed a high-fat diet by *E. faecium* WEFA23 (Zhang et al., 2017). However, the molecular mechanisms of lowering serum cholesterol in vivo are unknown yet.

In this study, critical factors (e.g., relevant genes and gut microbiota) and their relevance in the metabolism of cholesterol in vivo were systematically analyzed. The decrease in cholesterol level from the aspects of synthesis, decomposition, and transportation in rats fed a high-fat diet treated with *E. faecium* WEFA23 was also investigated. The whole study was performed as follows: establishment of a model of hyperlipidemia, analyzing the TMAO level by ultra HPLC/MS/MS and cholesterol metabolism-related gene transcript level by real-time quantitative PCR as well as the bioinformation of the gut microbiota composition by 16S rRNA gene amplicon sequencing.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Enterococcus faecium WEFA23 was grown on brain heart infusion agar (Oxoid, Basingstoke, UK) and incubated anaerobically at 37°C for 16 h.

Animals and Experimental Design

The protocol for the animal experiment was approved by Nanchang University animal ethical committee, all the ethical requirements for conducting the experiment were met (approval number 0064257). Fifteen 5-wk-old male Sprague Dawley rats were acclimatized for 1 wk and randomly divided into 3 groups: (1) **ND** group, fed with a standard chow diet and PBS (1.0 mL/d); (2) **HFD** group, which received a high-fat diet and PBS (1.0 mL/d); and (3) **WEFA23** group, which received a high-fat diet and *E. faecium* WEFA23 (5.0×10^9 cfu/mL in PBS, 1 mL/d). 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%). The weight, food intake, and water consumption of the rats were measured once a week. After intervention for 35 d, the rats were fasted overnight and anesthetized using diethyl ether. Blood was collected from the orbit. Serum was separated from blood samples by centrifugation at 4°C and $4,000 \times g$ for 20 min then stored at −80°C for use. All the rats were killed by cervical dislocation and autopsied in a sterile environment. The liver and feces in the colon and cecum were collected and stored at −80°C for analysis.

Cholesterol Content of Serum and Feces

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 corresponding kits (Jiancheng, Nanjing, China) according to the manufacturer's instructions.

Total RNA Isolation and Quantitative Reverse-Transcription PCR Analyses

Primers (Supplemental Table S1; https://doi.org/10.3168/jds.2017-13713) for quantitative reverse-transcription PCR were designed then synthesized by

Sangon (Shanghai, China). The Takara MiniBEST Universal RNA Extraction Kit (Takara, Shiga, Japan) was used to extract total RNA. Takara Prime-Script RT reagent Kit (catalog #RR047A, lot #AK2802) with gDNA Erase was used for reverse transcription of total RNA into cDNA. The SYBR Premix Ex Taq II (Takara Code: DRR820A) was used. Quantitative PCR analysis performed on 7900HT Fast Real-Time System (Applied Biosystems, Foster City, CA) in 96-well optical reaction plates by a reaction mixture (20 μL) containing 0.8 μL (10 μmoL/L) of forward and reverse primers, 0.4 μL of ROX Reference Dye I (50×; lot #AA4001A), 10 μL of SYBR Premix Ex Taq II (2×), 2 μL of cDNA samples, and 6 μL of nuclease-free water. The PCR program included the following: pre-denaturation at 95°C for 10 s and then 40 cycles at 95°C for 30 s and 60°C for 1 min. All mRNA quantitative data were normalized to *GAPDH*. Relative quantification was calculated using $2^{-\Delta\Delta Ct}$ method. All the results were expressed as the ratio of each mRNA to the mRNA transcript level of *GAPDH*.

Determination of Bile Acid Content of Feces

Feces were prepared as previously described (Chen et al., 2016). In brief, 40 mg of feces was mixed with 400 μL of 80% acetonitrile; then, 10 μg/mL D5-TCA (Toronto Research Chemicals Inc., Toronto, ON, Canada) or D4-CA (CDN Isotopes Inc., Pointe-Claire, QC, Canada) was added as internal standard. The final concentrations of D5-TCA and D4-CA were both 0.2 μg/mL. The supernatant was collected and subjected to LC/MS analysis. Bile acids were analyzed by an AB SCIEX QTRAP 4500 LC/MS/MS system with an Acquity HSS T3 column (Waters, Milford, MA), and gradient elution with 10 m*M* formic acid in water and 10 m*M* formic acid in acetonitrile:methanol (35:65) as mobile phases. The cone voltage and collision energy were set as 70 V and 2 eV for nonconjugated bile acid, and 90 V and 65 eV for taurine conjugates, respectively. All standard solutions were stored at −20°C. Supplemental Table S2 (https://doi.org/10.3168/jds.2017-13713) shows the retention times of the analytes.

Quantification of Total Bile Acids and Total Cholesterol

The TC in the liver, the TC, and the total bile acid in feces of rats were determined by the following process. Feces was pretreated by air drying and ground into powder. The powdered feces and thawed liver were prepared using a previously reported method with minor modification (Kim and Shin, 1998). In brief, the dry feces powder and liver were mixed with the same solution (methanol:chloroform = 2:1). The mixture of feces or liver samples was placed in a water bath at 45°C for 1 h. The supernatant was separated by centrifugation at 4°C and 2,500 × *g* for 10 min. Total cholesterol and total bile acid in the samples were determined by TC assay kit (Jiancheng) and total bile acid (**TBA**) kits (Jiancheng), respectively.

Evaluation of Serum TMAO and Cecal TMA

Serum samples were spiked with 2.5 μ*M* deuterium-labeled TMAO (Cambridge Isotope Laboratories, Andover, MA) as the internal standard. Cecal contents were filtered using a 0.2-μm filter (Millipore, Billerica, MA), and 10-μL aliquots were subjected to uHPLC/MS/MS analysis. The samples were treated with 7 volumes of ice-cold methanol to precipitate proteins, incubated at 4°C for 2 h, and then centrifuged at 4°C and 12,000 × *g* for 15 min. The supernatant was analyzed through uHPLC-MS/MS in positive-ion electrospray mode (Applied Biosystems, Waltham, MA). Separation was performed as previously reported with minor modifications (Bennett et al., 2013). In brief, liquid chromatography separation was performed on a Waters Acquity BEH Amide (2.1 mm × 50 mm, 1.7 μm; Waters) analytical column in gradient mode with 100% water: 0.1% formic acid as mobile phase A and acetonitrile as mobile phase B. The ion transitions (m/z 60→44 for TMA, m/z 69→44 for d9-TMA, m/z 76.2→58.3 for TMAO, m/z 85.1→66.2 for d9-TMAO) were used to quantify TMA, d9-TMA, TMAO, and d9-TMAO. Different concentrations of TMAO and TMA standards and fixed amounts of internal standards were spiked into the control serum to prepare calibration curves for quantification of serum TMAO and cecal TMA.

Total DNA Extraction and 16S rRNA Gene Amplicon Sequencing

Total DNA was extracted and purified from feces samples by using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The DNA quality was assessed by agarose gel electrophoresis and spectrophotometry using NanoDrop (Thermo Scientific, Wilmington, DE). The purified DNA samples were used for 16S rRNA gene amplicon sequencing on the Illumina MiSeq platform (BGI TechSolutions Co., Ltd., Shenzhen, China) according to previously discussed protocols (Caporaso et al., 2012). The primers targeting the hypervariable V4 regions of 16S RNA genes were designed using the method reported by Pan et al. (2016).

Table 1. The effect of *Enterococcus faecium* WEFA23 on the lipid levels of serum [total cholesterol (TC), triacylglycerols (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C)] of rats[1]

Group	n	TG (mmoL/L)	TC (mmoL/L)	HDL-C (mmoL/L)	LDL-C (mmoL/L)
ND	5	0.6102 ± 0.1593*	1.4812 ± 0.1117***	0.9874 ± 0.2886*	1.0442 ± 0.1769***
HFD	5	0.9274 ± 0.1826	2.9022 ± 0.2034	0.601 ± 0.0689	2.07 ± 0.1918
WEFA23	5	0.6012 ± 0.0688**	1.9184 ± 0.3319***	0.7984 ± 0.1397*	1.4486 ± 0.2730**

[1]Values shown as means ± standard errors. ND = normal diet; HFD = high-fat diet; WEFA23 = high-fat diet with *E. faecium* WEFA23; differences between HFD group and ND group or WEFA23 group were determined by 1-way ANOVA.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Bioinformatics Analysis

Quantitative Insights into Microbial Ecology (http://qiime.sourceforge.net/) pipeline was used for bio-information analysis of sequencing data. Raw data from all samples were filtered to eliminate adapter pollution. Clean reads were filtered from the raw reads by eliminating low-quality reads. High-quality paired-end reads were combined to tags using Flash (v 1.2.11; Fast Length Adjustment of Short Reads; http://www.cbcb.umd.edu/software/flash). The tags were clustered to operational taxonomic units at 97% sequence similarity by using UPARSE (http://www.drive5.com/uparse/). Ribosomal Database Project Classifier v 2.2 (Wang et al., 2007) was used to analyze the phylogenetic affiliation of each 16S rRNA gene sequence. Finally, differences among the 3 groups (e.g., ND, HFD, and WEFA23) of rats were analyzed using α diversity (within a sample), β diversity (among samples), and different species screening. Principal component analysis of operational taxonomic units was performed with the relative abundance value by package "ade4" (https://pbil.univ-lyon1.fr/CRAN/web/packages/ade4/index.html) of software R (v3.1.1; https://cran.rstudio.com/). Different-species screening among the 3 groups was conducted using linear discriminant analysis effect size method.

Statistical Analysis

All data are expressed as mean ± standard deviation unless otherwise noted. Significant differences among the 3 groups were determined by 1-way ANOVA and evaluated by 2-tailed unpaired Student's *t*-test. A *P*-value < 0.05 was considered statistically significant. All statistical analyses were conducted using GraphPad Prism v5.01 (GraphPad Software Inc., La Jolla, CA).

RESULTS

Effect of E. faecium WEFA23 on Serum Lipid Level in Rats Fed a High-Fat Diet

A model of rats fed a high-fat diet was successfully established to investigate the effect of *E. faecium* WEFA23 on lowering serum cholesterol; meanwhile, the rats in WEFA23 group were orally administered with 5×10^9 cfu/mL of *E. faecium* WEFA23 per day for 5 wk. As shown in Table 1, the indexes of serum lipids significantly differed between HFD and ND groups. Compared with the HFD group, the WEFA23 group showed a significant decrease in the levels of serum TG ($P < 0.01$), TC ($P < 0.001$), and LDL-C ($P < 0.01$) and an increase in the level of HDL-C ($P < 0.05$). This result indicates the potential capacity of *E. faecium* WEFA23 in decreasing the serum lipid level in high-fat diet rats.

Effect of E. faecium WEFA23 on the Transcript Levels of Genes Related to Cholesterol Metabolism

Serum lipid level was affected partially by the expression of genes related to cholesterol metabolism in the host. Upon the lowering of the serum lipid level by *E. faecium* WEFA23, we further examined the expression of hepatic genes, including *CYP7A1*, *HMGCoAS*, *SCD1*, *FAS*, *SCARB1*, *LDLR*, and *FMO3*, as well as intestinal genes, including *FGF15* and *FXR*. To determine their involvement in regulating cholesterol levels initiated by *E. faecium* WEFA23, all of the gene transcript level was standardized by the basic value of the HFD group (Figure 1). The transcript levels of *CYP7A1* ($P < 0.05$) and *LDLR* ($P > 0.05$) increased (Figure 1A), whereas those of *HMGCoAS*, *SCD1*, *FAS*, *SCARB1*, and *FMO3* in the liver decreased significantly ($P < 0.05$; Figure 1A). Similarly, the transcript levels of *FXR* ($P < 0.05$) and *FGF15* ($P > 0.05$) in the intestine were decreased (Figure 1B). All of these results show that *E. faecium* WEFA23 up- or downregulated gene transcript level related to cholesterol metabolism either in the liver or intestinal tract.

Effect of E. faecium WEFA23 on Bile Acid Metabolism

Following the upregulation of *CYP7A1*, *E. faecium* WEFA23 might influence the TC in the liver and feces, the level of TBA, and the ratio of conjugated to

nonconjugated bile acid in feces. As shown in Figure 2, the TC level in the liver significantly differed between HFD and ND groups. *Enterococcus faecium* WEFA23 significantly decreased TC level in the liver of rats fed with high-fat diet ($P < 0.05$; Figure 2A) and slightly increased the TC level in feces ($P > 0.05$; Figure 2B). Comparing with that in the HFD group, the concentration of TBA in feces increased (Figure 2C) and the ratio of conjugated to nonconjugated bile acids decreased (Figure 2D). Supplemental Figure S1 (https://doi.org/10.3168/jds.2017-13713) shows the content of conjugated and nonconjugated bile acids.

Effect of E. faecium WEFA23 on TMAO Synthesis

The TMAO affected the metabolism of cholesterol and bile acid by inhibiting *CYP7A1* expression. Therefore, we examined the level of serum TMAO and cecal TMA of rats. When compared with that in the HFD group, the serum TMAO levels obviously decreased ($P < 0.05$; Figure 3A), whereas the level of TMA in cecum slightly deceased (Figure 3B).

Effect of E. faecium WEFA23 on the Intestinal Microbiota

The gut microbiota plays a crucial role in bile acid metabolism and synthesis of cecal TMA, which is subsequently oxidized into TMAO in the liver (Tang et al., 2013). In this regard, changes in the gut microbiota pattern in feces of rats in the ND, HFD, and WEFA23 groups were determined. The HFD reduced the α-diversity (decreased the Shannon's index and increased the Simpson index) compared with those in the ND group (Figure 4A and 4B). Moreover, *E. faecium* WEFA23 retarded the deduced tendency of the α diversity in the intestinal microbiota. The relative abundance showed that the intestinal microbiota significantly differed at family and genus levels; that is, some taxa (*RF16*, *Helicobacteraceae*) were significantly ($P < 0.05$) increased in the WEFA23 group, and one taxon (*Veillonelaceae*) was overgrown ($P < 0.05$) in the HFD group. Moreover, significant differences were found in the microbiota with low abundance, including *Rikenellaceae*, *Peptococcaceae*, *RFP12*, *Odoribacteraceae*, and *Pseudomonadaceae* at the family level, *Butyricimonas*,

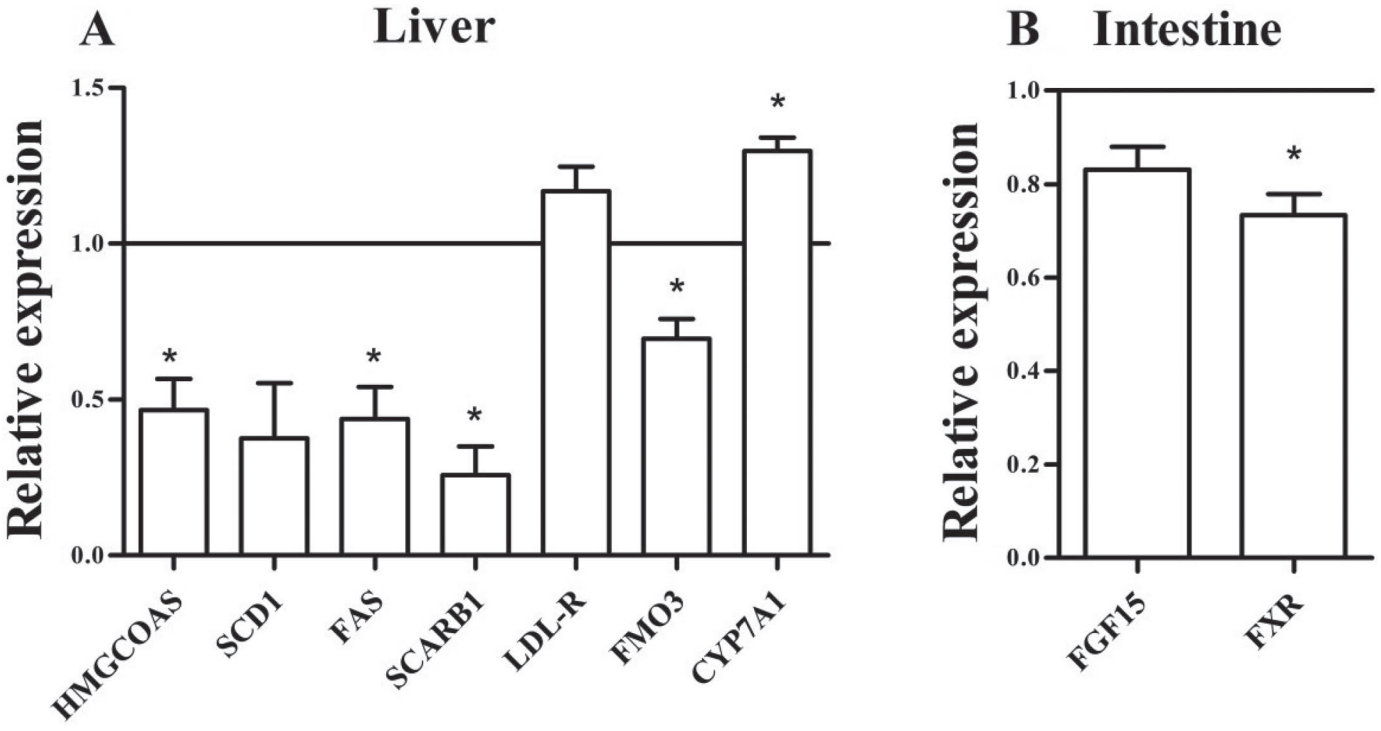

Figure 1. Relative transcript level of genes related to cholesterol metabolism in the liver (A) and intestinal tract (B) of rats by quantitative reverse-transcription PCR analyses with *GAPDH* as the internal reference. Relative expression indicates the *Enterococcus faecium* WEFA23 group compared with the high-fat diet (HFD) group. Values are expressed as mean ± SD (n = 5 per group). *$P < 0.05$.

Succinivibrio, *YRC22*, *rc4–4*, *CF231*, and *Pseudomonas* at the genus level. At the phylum level, the abundance of *Firmicutes* in the WEFA23 group decreased by 12.2% (from 32.3 to 20.1%) and *Bacteroidetes* increased by 11.4% (from 43 to 54.4%) when compared with those in the HFD group. However, The *Proteobacteria* increased from 15.7 to 18.9% and *Fusobacteria* decreased from 5.6 to 4.3% (Supplemental Figure S2; https://doi.org/10.3168/jds.2017-13713). Principal component analysis indicated that the similarity in the WEFA23 group is higher than that in the HFD group when compared with the ND group (Supplemental Figure S3; https://doi.org/10.3168/jds.2017-13713). Therefore, we predicted that the reduction of the serum cholesterol level by administering *E. faecium* WEFA23 may be due to the changes in the intestinal microbiota.

DISCUSSION

Scholars have reported an increasing number of probiotic strains (e.g., *Lactobacillus* spp. and *Bifidobacterium* spp.) with cholesterol-lowering potential (Sumarno et al., 2011; Fuentes et al., 2013; Kuda et al., 2013), among them, *Enterococcus* spp. have been rarely investigated (Hlivak et al., 2005). Nevertheless, few reports interpreted the mechanism of this species in lowering cholesterol. In this study, based on the data of cholesterol level in serum, gene regulation, and the changes in bile acids and microbiota constitution in the gut, we inferred that the mechanisms cholesterol reduction by *E. faecium* WEFA23 might be via decreasing cholesterol synthesis, increasing cholesterol excretion, and promoting cholesterol transportation from serum

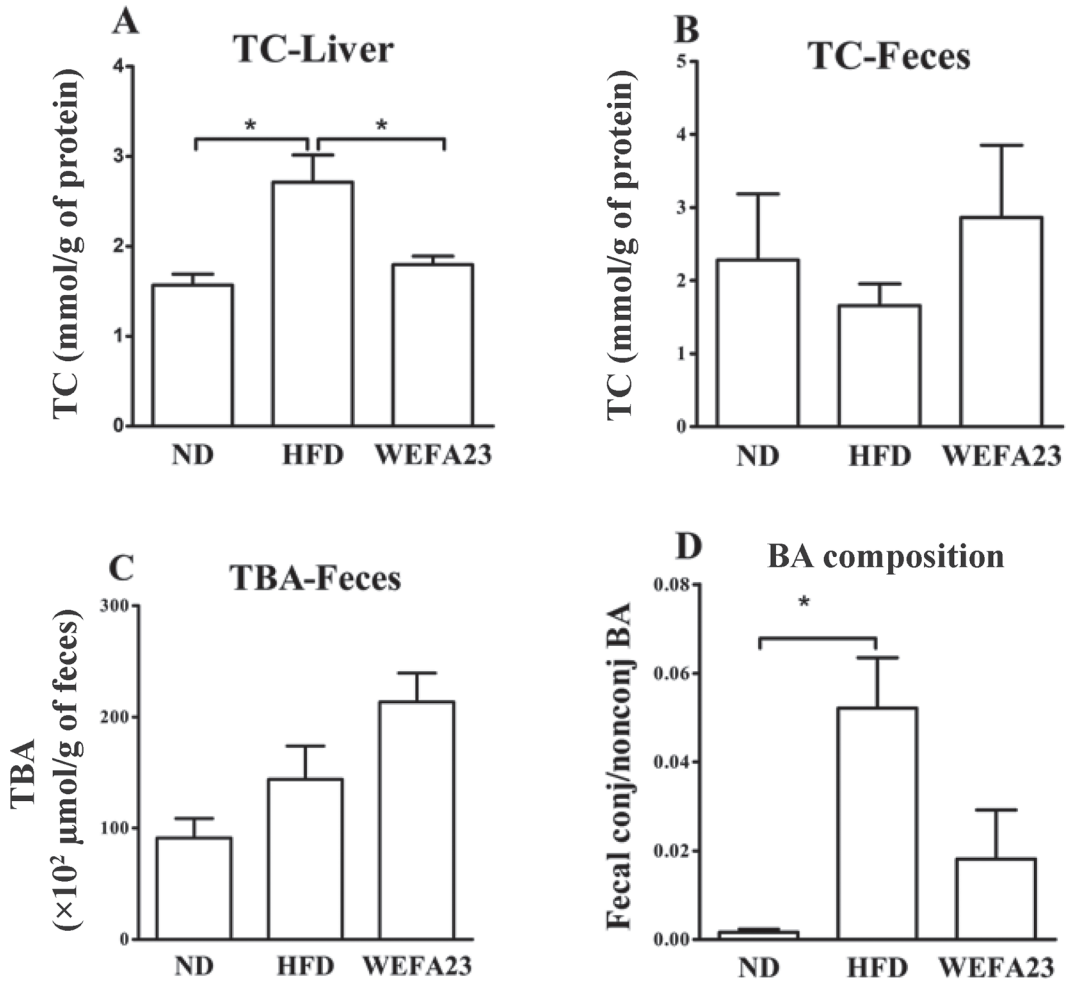

Figure 2. Influence of *Enterococcus faecium* WEFA23 on bile acid synthesis, total cholesterol (TC) in the liver (A) and feces (B), total bile acid (TBA) in feces (C), and the ratio of conjugated (conj.) to nonconjugated (nonconj.) bile acid (BA) in feces (D). ND = normal diet; HFD = high-fat diet; WEFA23 = high-fat diet with *E. faecium* WEFA23. Values are expressed as mean ± SD (n = 5 per group). *$P < 0.05$ versus HFD.

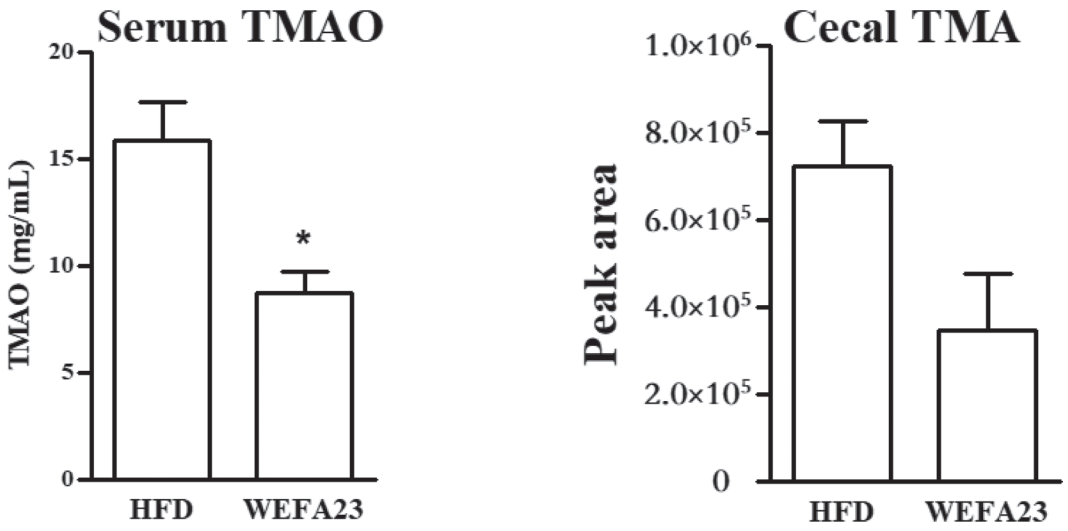

Figure 3. Effect of *Enterococcus faecium* WEFA23 on the level of trimethylamine N-oxide (TMAO) in serum (left) and the peak area of trimethylamine (TMA) in cecum (right). $^*P < 0.05$, versus high-fat diet (HFD). WEFA23 = high-fat diet with *E. faecium* WEFA23.

to liver in the model of high-fat diet rats with hyperlipidemia.

Bordoni et al. (2013) reported that the mixture of probiotics (*B. bifidum* MB 109, *B. breve* MB 113, and *B. animalis* ssp. *lactis* MB 2409) significantly decreased the TC and LDL-C levels. Similarly, *E. faecium* WEFA23 increased the serum HDL-C level and decreased the TC, TG, and LDL-C levels. Cholesterol is converted into a variety of bile acids in the liver (Javitt, 1994). CYP7A1 is the key rate-limiting enzyme in the classic bile acid biosynthesis pathway (Chiang, 2009). Moreover, *E. faecium* WEFA23 increased the levels of *CYP7A1* in the liver and might contribute to the transformation of cholesterol to conjugated bile acids. After transportation from the liver to the intestinal tract and subsequent hydrolysis from BSH in the gut, the conjugated bile acids were converted into nonconjugated bile acids, which are key metabolic targets for hypercholesteremia (Joyce et al., 2014). In our previous study, *E. faecium* WEFA23 exhibited high activity of BSH (Zhang et al., 2017). In the present study, oral administration of *E. faecium* WEFA23 decreased the ratio of conjugated to nonconjugated bile acids and increased the total amount of bile acids in feces. Therefore, we concluded that *E. faecium* WEFA23 altered the composition of bile acids and promoted the transformation of cholesterol to bile acids.

Except for *CYP7A1*, some genes in the liver (e.g., *HMGCoAS*, *SCD1*, *LDLR*, and *SCARB1*) or those (e.g., *FXR* and *FGF15*) in the intestinal tract participated in cholesterol metabolism. Oral administration of *E. faecium* WEFA23 decreased the levels of both *FXR* ($P < 0.05$) and *FGF15* ($P > 0.05$), which might be relevant to the upregulation of *CYP7A1*. Lidbury et al. (2014) proposed that *FGF15* and *FXR* suppressed the expression of *CYP7A1*, whereas the expression of *FGF15* was induced by *FXR* (Cashman and Zhang, 2006); this result is similar to the present results. Expression of *CYP7A1* was also affected by other factors (e.g., TMAO). An increase in TMAO promoted the reduction of *CYP7A1* expression and might decrease the reverse cholesterol transport, leading to increased levels of serum cholesterol (Koeth et al., 2013). Thus, we inferred that TMAO level should be negatively related to the expression level of *CYP7A1*. Furthermore, oral administration of *E. faecium* WEFA23 significantly decreased the level of TMAO and the gene expression of *FMO3*. FMO3 is the limiting enzyme in the conversion of TMA into TMAO (Hisamuddin and Yang, 2007), so we can deduce that the dramatic decrease ($P < 0.05$) in *FMO3* transcript level might result in the significant reduction ($P < 0.05$) of TMAO. This phenomenon led to subsequent increase in the *CYP7A1* transcript level, following the complex decomposition process of cholesterol. The *HMGCoAS* gene is a critical factor in cholesterol synthesis. Overexpression of *SCD1* in humans may be involved in the development of hypertriglyceridemia (Mar-Heyming et al., 2008). Our results suggested that *E. faecium* WEFA23 might reduce the synthesis of cholesterol by downregulating *SCD1* and *HMGCoAS*, leading to the reduction of serum cholesterol levels. For cholesterol transportation, the *LDLR* gene mediates the endocytosis of LDL-C (Leren, 2014) and the scavenger receptor class B member 1

(*SCARB1*) functions as a receptor for HDL (Valacchi et al., 2011). Therefore, upregulation of *LDLR* might account for the reduction of LDL-C and the increase of HDL-C by downregulation of *SCARB1*.

Intestinal microbiota plays a crucial role in decreasing cholesterol levels. On the one hand, conjugated bile acids are hydrolyzed and converted into unconjugated bile acids by BSH, which were produced by intestinal microbiota [e.g., *E. faecium* AK61 (Knarreborg et al., 2002) and *E. faecium* WEFA23 (Zhang et al., 2017)]. On the other hand, intestinal microbiota affects dietary choline metabolism, transforming it into TMA (Tang et al., 2015) and subsequently from TMA to TMAO (Koeth et al., 2014), which is metabolized by FMO3. The inhibition of intestinal microbiota by broad-spectrum antibiotics resulted in the dramatic decrease of TMAO level (Wang et al., 2011). Interestingly, instead of using antibiotics, oral administration of *E. faecium* WEFA23 led to a significant decrease of both TMA and TMAO, accompanying the changes of intestinal microbiota constitution (e.g., *Rikenellaceae*, *Bacteroidetes*, and *Firmicutes*), which might be relevant to the development of obesity. High-fat diet significant reduced the abundance of *Rikenellaceae* and the proportion of *Bacteroidetes* to *Firmicutes* (den Besten et al., 2013; Daniel et al., 2014). In our study, *E. faecium* WEFA23 significantly increased the ratio of *Bacteroidetes* to *Firmicutes* and the abundance of *Rikenellaceae* as well as decreased the abundance of *Veillonellaceae*. *Veillonellaceae* was demonstrated to be positively correlated with obesity (Marchandin and Jumas-Bilak, 2014). Meanwhile, *E. faecium* WEFA23 significantly increased the diversity

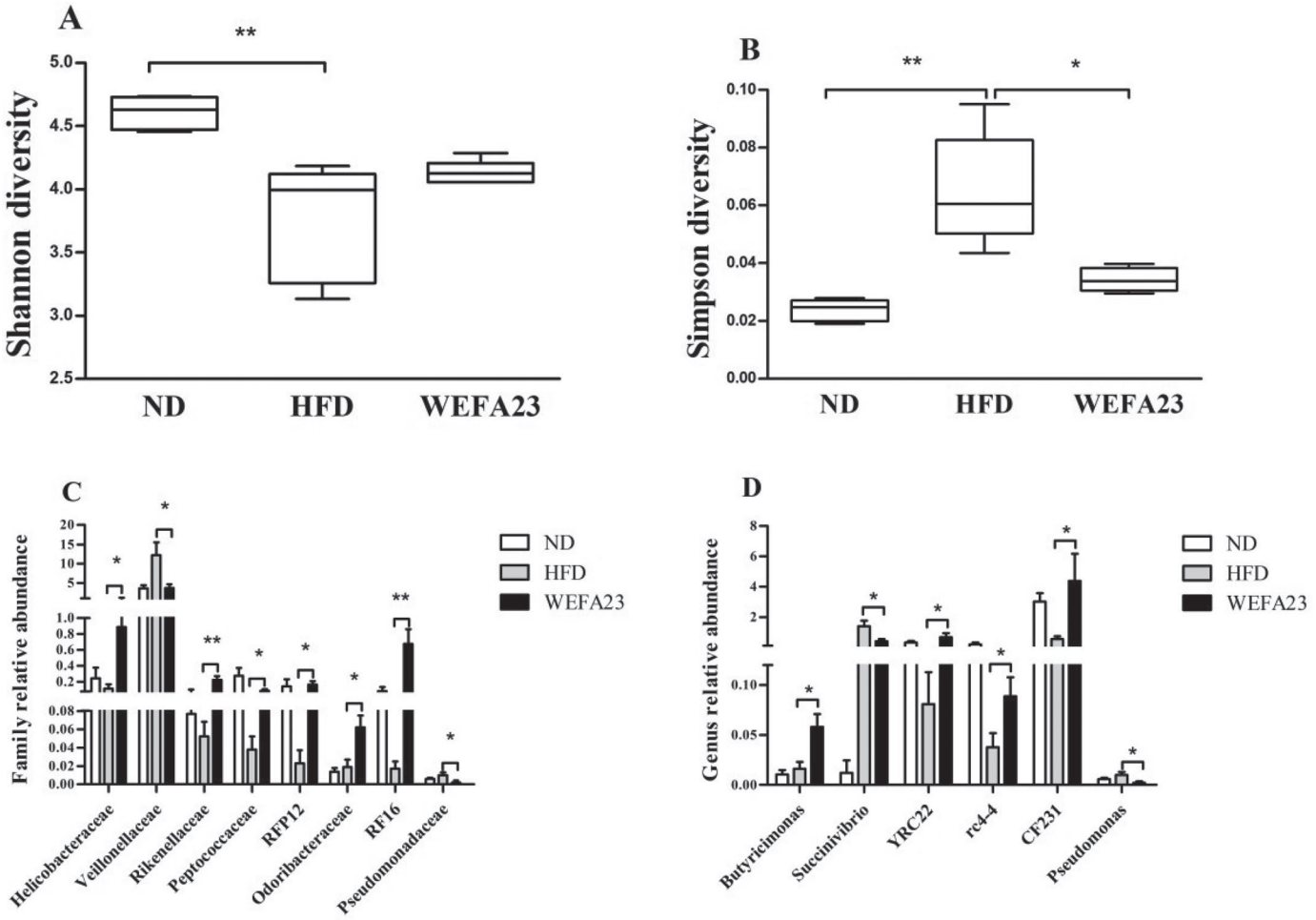

Figure 4. Effect of *Enterococcus faecium* WEFA23 on the intestinal microbiota. Shannon diversity (A) and Simpson diversity (B) were used to assess the α diversity of intestinal microbiota; the relative abundance at family (C) and genus (D) level were calculated and compared among 3 groups. ND = normal diet; HFD = high-fat diet; WEFA23 = high-fat diet with *E. faecium* WEFA23. $^{*}P < 0.05$; $^{**}P < 0.01$. Boxplot was used to visually display the differences of the alpha diversity among groups. The 5 lines from bottom to top are the minimum value, the first quartile, median, the third quartile, and the maximum value, respectively.

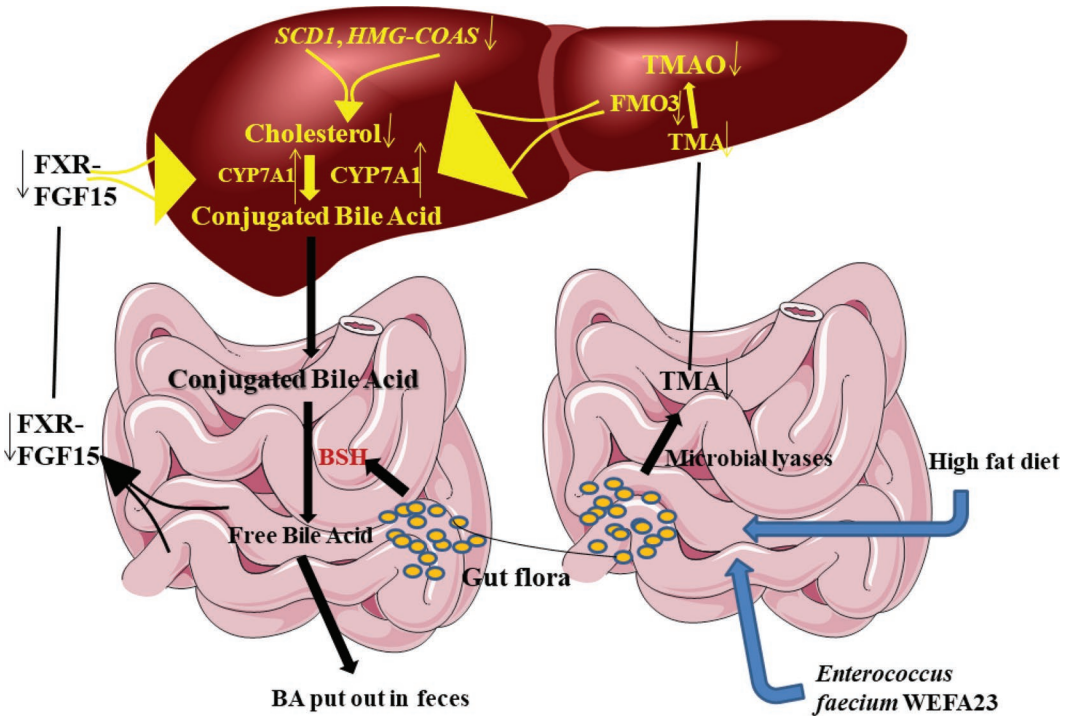

Figure 5. Mechanisms of reducing serum cholesterol level in rats by *Enterococcus faecium* WEFA23. *Enterococcus faecium* WEFA23 lessens high-fat-diet-induced hyperlipidemia via increase of the cholesterol 7-α-hydroxylase (CYP7A1) gene transcript by altering the composition of gut microbiota in rats and decrease of the level of trimethylamine-*N*-oxide (TMAO). BA = bile acid; TMA = trimethylamine; BSH = bile salt hydrolase. FXR = farnesoid X receptor; FGF15 = fibroblast growth factor 15; FXR-FGF15 = FXR-controlled fibroblast growth factor 15 signaling pathway, which controls the expression levels of hepatic CYP7A1; FMO3 = flavin-containing monooxygenase, which can oxidize TMA into TMAO. Color version available online.

of intestinal microbiota in rats fed a high-fat diet and changed the microbiota structure similar to rats fed a normal diet.

Based on our data and the above discussion, the mechanisms of reducing serum cholesterol level in rats by *E. faecium* WEFA23 are proposed in Figure 5. Cholesterol is transformed into conjugated bile acid by CYP7A1 and converted into nonconjugated bile acid (i.e., free bile acid) by BSH. Reduction of the ratio of conjugated to nonconjugated bile acids increased the expression of *CYP7A1* via inhibition of *FXR-FGF15* axis. Furthermore, decreasing the level of genes (e.g., *SCD1* and *HMGCoAS*) reduced the synthesis of cholesterol. High-fat diet generated TMA by the catalysis of microbial lyases, remodeling the intestinal microbiota by *E. faecium* WEFA23 decreased the level of TMA, resulting in reduction of TMAO and upregulation of *CYP7A1*.

CONCLUSIONS

Enterococcus faecium WEFA23 can improve hyperlipidemia in rats fed with high-fat diet via a complex mechanism that includes upregulating or downregulating genes relevant to the decomposition (*CYP7A1*), synthesis (*HMGCoAS*, *SCD1*), and transportation (*LDLR*, *SCARB1*) of cholesterol, and remodeling the intestinal microbiota similar to that in rats fed with normal diet. *Enterococcus faecium* WEFA23 altered the bile acid composition, thereby inhibiting the *FXR-FGF15* axis to upregulate the *CYP7A1* transcript level, and subsequently promoting the metabolism of cholesterol into bile acids. Reshaping of the intestinal microbiota reduced cecal TMA, leading to decrease of serum TMAO level and subsequent increase of the transcript level of *CYP7A1*, and promotion of the metabolism of cholesterol into bile acids. The mechanism of improving hyperlipidemia by *E. faecium* WEFA23 is mainly due to the upregulated expression of key genes (i.e., *CYP7A1*) via reshaping the composition of the gut microbiota in rats fed a high-fat diet.

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