

Basic Study

Effects of *Ligustrum robustum* on gut microbes and obesity in rats

Zhi-Mei Xie, Tao Zhou, Hong-Yu Liao, Qian Ye, Shan Liu, Lu Qi, Jing Huang, Hao-Jiang Zuo, Xiao-Fang Pei

Zhi-Mei Xie, Tao Zhou, Hong-Yu Liao, Qian Ye, Shan Liu, Hao-Jiang Zuo, Xiao-Fang Pei, Department of Laboratory Science of Public Health, West China School of Public Health, Sichuan University, Chengdu 610041, Sichuan Province, China

Hong-Yu Liao, Sichuan Provincial Disease Prevention and Control Center, Chengdu 610041, Sichuan Province, China

Shan Liu, Department of Clinical Laboratory, Sichuan Provincial People's Hospital, Chengdu 610041, Sichuan Province, China

Lu Qi, Department of Nutrition, Harvard School of Public Health, Boston, MA 02115, United States

Lu Qi, Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Boston, MA 02115, United States

Lu Qi, Harvard Medical School, Boston, MA 02115, United States

Jing Huang, Department of Chemistry of Medicinal Natural Products, West China School of Pharmacy, Sichuan University, Chengdu 610041, Sichuan Province, China

Author contributions: Pei XF and Zuo HJ designed the research; Zuo HJ, Xie ZM, Zhou T, Liao HY, Ye Q, Huang J and Pei XF performed the research; Zuo HJ and Liao HY analyzed the data; Zuo HJ, Xie ZM and Pei XF wrote the paper; Qi L made critical revisions of this manuscript.

Supported by National Natural Science Foundation of China, No. 81273055; Sichuan Provincial Department of Science and Technology, No. 2014JY0001; and The Youth Foundation of Sichuan University, No. 2012SCU11099.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of the State Key Laboratory of Oral Diseases, Sichuan University, China.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Ethics Committee of the State Key Laboratory of Oral Diseases (protocol number: SKLODLL2013A067).

Conflict-of-interest statement: The authors declare that there is

no conflict of interest in this study.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Hao-Jiang Zuo, PhD, Laboratory Science of Public Health, West China School of Public Health, Sichuan University, No. 3-13 Renminnanlu, Chengdu 610041, Sichuan Province, China. zuohaojiang@scu.edu.cn

Received: March 17, 2015

Peer-review started: March 18, 2015

First decision: June 2, 2015

Revised: June 19, 2015

Accepted: September 28, 2015

Article in press: September 30, 2015

Published online: December 14, 2015

Abstract

AIM: To investigate the anti-obesity and antibacterial effects of *Ligustrum robustum* (*L. robustum*) *in vivo* and *in vitro* and its possible mechanisms.

METHODS: The effects of *L. robustum* aqueous extract (LR) on various gut bacteria *in vitro* were evaluated. The effects of LR on high-fat diet-fed (HFD) rats *in vivo* were also assessed. Culture methods, quantitative polymerase chain reaction, and terminal-restriction fragment length polymorphism were used to analyze the effects of LR on gut bacteria. Biochemical tests were also performed to detect the changes in

obesity-related indicators after LR treatment.

RESULTS: LR treatment lowered adipose weight and decreased Lee's index, blood glucose, total cholesterol, and lipid in the tested groups relative to control ($P < 0.05$). To determine the reasons for these changes, we assessed the potential bacteriostatic and bactericidal effects of LR on specific bacterial species *in vitro*. LR affected the richness, diversity, and evenness of gut bacteria, increased fecal *Lactobacillus*, and decreased *Enterococci* in HFD rats ($P < 0.05$).

CONCLUSION: *L. robustum* may be a safe and effective food for weight loss and obesity control, and the effects of *L. robustum* might be mediated by the regulation of gut bacteria.

Key words: Gut bacteria; *Ligustrum robustum*; Culture methods; Quantitative polymerase chain reaction; Terminal-restriction fragment length polymorphism; Obesity

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Gut microbes play important roles in fat storage and metabolism. The control of gut microbes is considered promising in the prevention of obesity. In this study, the regulatory effect of *Ligustrum robustum* aqueous extract (LR) on gut bacteria *in vivo* and *in vitro* and body weight was determined. Certain doses of LR prevented obesity without sacrificing daily food or energy intake. LR may affect the richness, diversity, and evenness of gut bacteria by increasing *Lactobacillus* and decreasing *Enterococci* in the host gut.

Xie ZM, Zhou T, Liao HY, Ye Q, Liu S, Qi L, Huang J, Zuo HJ, Pei XF. Effects of *Ligustrum robustum* on gut microbes and obesity in rats. *World J Gastroenterol* 2015; 21(46): 13042-13054 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i46/13042.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i46.13042>

INTRODUCTION

Recently, emerging data have implicated gut microbes in the development of obesity, and the regulation of gut microbes as a potential strategy in the prevention and treatment of obesity has attracted significant attention. Dietary factors, which possess anti-obesity effects, may also play a key role in regulating gut microbes. However, few foods have been investigated for their effects on gut microbes and the development of obesity.

Ligustrum robustum (*L. robustum*) was classified as food by the Chinese Ministry of Health in 2011^[1]. In southwest China, the leaves of *L. robustum* are processed as Ku-Ding tea. Previous studies have

shown that this tea might have anti-oxidative, anti-inflammatory, and anti-obesity effects^[2-5]. In addition, it was found that *L. robustum* may affect a variety of gut microorganisms^[6,7]. Thus, we hypothesized that *L. robustum* might exert anti-obesity effects by regulating gut microbes.

In this study, we examined the effects of *L. robustum* on different types of gut bacteria. We also assessed the effects of *L. robustum* on the changes in host body weight and gut flora in high-fat diet-fed rats.

MATERIALS AND METHODS

Preparation of *L. robustum* aqueous extract

L. robustum was previously identified by Professor Guomin Liu of the Ku-Ding Tea Research Institute, Hainan University, China and was obtained from China's largest *L. robustum* provider, Green Hills and Blue Waters Co., Ltd. (Junlian, Sichuan, China) (<http://jlqingshanlvshui.1688.com/>). The extraction was guided by Professor Jing Huang of the West China School of Pharmacy, Sichuan University and prepared by Chengdu Push Bio-Technology Co., Ltd. (Chengdu, China). In brief, dried *L. robustum* was ground and extracted with 10 times its volume of water for 60 min at 80 °C. The residue was re-extracted twice, the extracted solution was filtered, and the combined supernatants were concentrated to 1 g/mL in a vacuum evaporator at 60 °C. *L. robustum* aqueous extract (LR) was sterilized at 115 °C for 15 min and stored in bottles at -20 °C in the dark.

In vitro effects of LR on microorganisms

The inhibitory effects of LR on intestinal symbiotic bacteria, conditional pathogenic bacteria, and enteropathogenic bacteria were determined. The symbiotic bacteria included: *Escherichia coli* (*E. coli*) ATCC 8099, *Bifidobacterium bifidum* CICC 6071, *Bifidobacterium infantis* CICC 6069, *Lactobacterium bulagrium* (separated from yogurt), *Enterococcus faecalis* ATCC 29212, and *Bacteroides thetaiotaomicron* ATCC 29741. The conditional pathogenic bacteria included: *Bacteroides fragilis* ATCC 25285 and *Clostridium perfringens* ATCC 13124. The enteropathogenic bacteria included: *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 15442, *Salmonella enteritidis* CMCC 50335, *Shigella flexneri* CMCC 51061, *E. coli* O157:H7 ATCC 882364, *Enterobacter Aggregative E. coli* (clinical isolates), *Enterobacter Hemorrhagic E. coli* (clinical isolates), *Enterobacter Toxigenic E. coli* (clinical isolates), *Enterobacter Pathogenic E. coli* (clinical isolates), *Enterobacter Invasive E. coli* (clinical isolates), *Bacillus cereus* (Military Medical Science Academy of the PLA reference strain 4001), and *Vibrio parahemolyticus* ATCC 17802. All clinical isolates in our laboratory were identified by conventional biochemical and serological tests.

The micro-dilution susceptibility assay was

Table 1 Culture media and incubation conditions

Bacterial group	Bacteria preparation		MIC	
	Medium	Incubation conditions	Medium	Incubation conditions
<i>Bacteroides</i>	GAM ¹ agar	37 °C anaerobic, 48 h	BHI ² (10% HS ⁴)	37 °C Anaerobic, 48 h
<i>Bifidobacteria</i>	BBL ² agar	37 °C anaerobic, 48 h	RC ²	37 °C Anaerobic, 48 h
<i>Lactobacilli</i>	LBS ² agar	37 °C aerobic with 5% CO ₂ 48 h	RC ²	37 °C Anaerobic, 48 h
<i>C. perfringens</i>	SPS ² agar	37 °C anaerobic, 48 h	RC ²	37 °C Anaerobic, 48 h
Aerobic <i>E. coli</i>	EMB ² agar	37 °C aerobic, 18 h	MH ²	37 °C Aerobic, 18 h
<i>Enterococci</i>	BEA ² agar	37 °C aerobic, 18 h	MH ²	37 °C Aerobic, 18 h
<i>Candida albicans</i>	Salouraud ² agar	37 °C aerobic, 18 h	Salouraud ²	37 °C Aerobic, 18 h
<i>Staphylococcus aureus</i>	blood plate ³	37 °C aerobic, 18 h	MH ²	37 °C Aerobic, 18 h
Other bacteria	LB ¹ agar	37 °C aerobic, 18 h	MH ²	37 °C Aerobic, 18 h

¹Prepared in the laboratory according to methods defined by the Chinese Ministry of Health^[8]; ²Purchased from Beijing Land Bridge Technology Co., Ltd., China; ³Purchased from Chengdu RICH Technology Co., Ltd., China; ⁴Purchased from Hyclone. MIC: Minimum inhibitory concentration; GAM: Gifu anaerobic medium; BBL: Bifidobacterium culture; LBS: Lactobacillus selection; SPS: Sulfite polymyxin-sulfadiazine; EMB: Eosin methylene blue; BEA: Bile esculin azide; LB: Luria-Bertani; BHI (10% HS): Brain heart infusion with 10% horse serum; RC: Reinforced Clostridium medium; MH: Mueller-Hinton medium.

performed to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Dilutions were prepared in 96-well plates to obtain final concentrations ranging from 200 mg/mL to 0.1 mg/mL of LR in the medium. Fresh target bacteria were prepared (Table 1), and an inoculum of 10 μ L (approximately 10⁶ CFU/mL–10⁷ CFU/mL) was inoculated into the microplates, and the tests were performed in a volume of 100 μ L. The homogenated 96-well plates were incubated aerobically or anaerobically at 37 °C in duplicate for 24–48 h (Table 1), and then the culture solution containing bacteria was re-suspended. The lowest concentration of the tested samples, which did not show any visual growth after macroscopic valuation, was determined as the MIC. Using the results of the MIC assay, the concentrations showing complete absence of visual bacterial growth were identified, and 10 μ L of each culture broth was transferred onto agar plates and incubated for the specified time and temperature, as mentioned above. The complete absence of growth on the agar surface at the lowest concentration of sample was defined as the MBC.

In vivo experiments

Animals and treatments: Male Sprague Dawley rats [220 \pm 20 g, specific pathogen free (SPF) grade, Certified No. SCXK (Jing) 2009-0004], normal chow diets (No. D12450B), and high-fat diets (HFDs, No. D12492) were obtained from the Beijing HFK Bioscience Co., Ltd. (Beijing, China). The animals were kept in an environmentally controlled breeding room (temperature: 23 °C \pm 2 °C, humidity: 50%–60%, 12 h dark/light cycle). The protocol was approved by the Ethics Committee of the State Key Laboratory of Oral Diseases, Sichuan University, China. After 3 d of adaptive feeding, the rats were randomly assigned to one of five groups according to their body weight, with each group comprising 10 rats. The treatment was as follows: the control group (Normal) was fed

normal chow diet and infused with distilled water; the obese model group (HFD) was fed HFD and infused with distilled water; and low-, medium- and high-dose groups (LR-L, LR-M, LR-H) were fed HFD and infused with a solution of LR at 2.5 mL/kg body weight, 5 mL/kg body weight, and 10 mL/kg body weight per day, respectively.

Experimental protocol

Body weight and food intake were monitored once a week. Total calorie intake was calculated according to dietary calorie intake and expressed as kcal/rat per day. Freshly collected feces were used for bacteria number assays. After 6 wk of normal chow diet or HFD feeding, the animals were weighed and fasted for 24 h before the experiments. Fasted blood was collected from the femoral artery. Serum was isolated by centrifugation at 3000 *g* and 4 °C for 10 min and stored at -70 °C until it was used for blood biochemical assays. Following blood collection, the rats were sacrificed by cervical dislocation. Visceral adipose tissue, liver, and spleen were immediately weighed after removal.

Biochemical analysis

Blood glucose (GLU), total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured using an automatic biochemical analyzer (Olympus AU400; Olympus, Tokyo, Japan).

Fecal sample preparation, bacterial cultivation, and counting

Fecal samples were obtained before the animals were killed and stored at -70 °C for culture independent analysis. For bacterial cultivation and counting, fresh fecal samples were cultured quantitatively for aerobic, facultative, and anaerobic bacteria using methods defined by the Chinese Ministry of Health^[8]. In brief, freshly voided feces (less than 10 min after defecation)

Table 2 Primers for target gut bacteria

Target bacteria (amplicon size, bp)	Sequence (5' to 3')
<i>Bifidobacteria</i> sp. (243)	F: TCGCGTC(C/T)GGTGTGAAAG R: CCACATCCAGC(A/G)TCCAC
<i>Lactobacilli</i> sp. (341)	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG
<i>Clostridium perfringens</i> groups (120)	F: ATGCAAGTCGAGCGA(G/T)G R: TATGCGGTATTAATCT(C/T)CCITT
<i>Enterobacteriaceae</i> (195)	F: CATTGACGTTACCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC
<i>Enterococci</i> sp. (144)	F: CCCTTATGTGTTAGTTGCCATCATT R: ACTCGTTGTACTTCCCATTTGT
<i>Bacteroidetes</i> (126)	F: GGARCATGTGGTTTAATTCGATGAT R: AGCTGACGACAACCATGCAG
<i>Firmicutes</i> (126)	F: GGAGYATGTGGTTTAATTCGAAGCA R: AGCTGACGACAACCATGCAC

F: Leading strand; R: Lagging strand.

were collected in a sterile box. Fecal samples (approximately 1000 mg) were homogenized and serially diluted in sterile anaerobic solution. Appropriate dilutions were incubated aerobically or anaerobically at 37 °C in duplicate using selective media within 2 h of collection. The culture conditions are shown in Table 1. The target bacterial colonies in each medium at the corresponding dilution were counted, and the bacteria were subsequently characterized by Gram staining and polymerase chain reaction (PCR) identification. Colony counts are expressed as the log of colony forming units per gram of wet feces (log colony forming units/g, log CFU/g).

Nucleic acid extraction

Genomic DNA was extracted from 200 mg (wet weight) of frozen rat feces using a TIANamp Stool DNA Kit (Tiangen, Beijing, China).

Quantitative polymerase chain reaction

In our laboratory, 16S rRNA analysis of the main gut bacteria (Table 2) in feces was performed in accordance with a previously published method^[9-13] with slight modifications, which demonstrated good repeatability and specificity^[14].

Terminal-restriction fragment length polymorphism

A double fluorescence labeled terminal-restriction fragment length polymorphism (T-RFLP) analysis was performed based on the technique described by Kato *et al.*^[15]. Bacterial 16S rRNA genes were amplified with the universal bacterial primers Bac8F (5'-FAM-AGAGTTTGATCCTGGCTCAG) and Univ1492R (5'-HEX-GGTTACCTTGTTACGACTT) (Sangon, Shanghai, China). For T-RFLP, the forward primer was fluorescently 5'-labeled with FAM and the reverse primer was 5'-labeled with HEX, respectively, to enable subsequent detection of terminal restriction fragments (T-RFs).

The PCR mixture contained approximately 50 ng DNA template, 12.5 µL Hot Start Taq Master Mix

(Dongsheng Biotech, Guangdong, China), 0.2 µmol/L labeled primer, 0.5 µL Taq Enzyme, and water in a final volume of 25 µL. The PCR reaction was performed in a PCR Thermo cycler (S1000, Bio-Rad, Hercules, CA, United States) using the following program: an initial activation of Taq polymerase at 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 53 °C (56 °C and 60 °C were also tested) for 30 s, and 72 °C for 90 s, with a final extension at 72 °C for 7 min. Negative controls containing all reagents, but without DNA template, were included throughout the analysis to exclude DNA contamination.

Approximately 100 ng purified PCR products (TIANgel Midi Purification Kit DP209, Tiangen, Beijing, China) were digested with 1 µL of the restriction enzyme *Hae* III or *Hha* I at 37 °C for 1 h in a final volume of 20 µL following the manufacturer's instructions (Takara, Dalian, China). DNA fragments were separated by capillary electrophoresis on an ABI 3730xl DNA analyzer (Invitrogen, Shanghai, China). The lengths of fluorescently labeled T-RFs were determined by comparison with an internal standard (GeneScan 1200 LIZ[®]; Applied Biosystems, Foster City, CA, United States), and data were analyzed using GeneMapper 4.1 software (Applied Biosystems, Foster City, CA, United States). T-RFs > 15 bp with more than 1% abundance of bacterial 16S rRNA gene fragments were identified using the MiCA online software^[16,17]. In order to quantify the differences in community structures of the studied groups, the fragment richness, Shannon-Weiner diversity index, and Evenness value were calculated by double fluorescence labeled T-RFLP^[16]. The Jaccard similarity index^[18] of T-RFs between the HFD group and the other groups were then investigated using the equation, $J = C/(A + B - C)$, in which A is the total size of T-RFs present in group A, B is the total size of T-RFs present in group B, and C is the total size of T-RFs present in both groups.

Statistical analysis

Continuous variables were expressed as mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) or nonparametric tests, where appropriate. Spearman's correlation was used to determine the relationship between body weight and Jaccard similarity index derived from T-RFLP. *P* values less than 0.05 were considered statistically significant. SPSS 13.0 software (SPSS Inc., Chicago, IL, United States) was used for all statistical analyses.

The statistical methods of this study were reviewed by assistant professor Xing Zhao from the Department of Biostatistics, West China School of Public Health, Sichuan University, China.

RESULTS

In vitro effect of LR on microorganisms

Following culture *in vitro*, the MIC and MBC of 20 types of bacteria were obtained. The tested strains

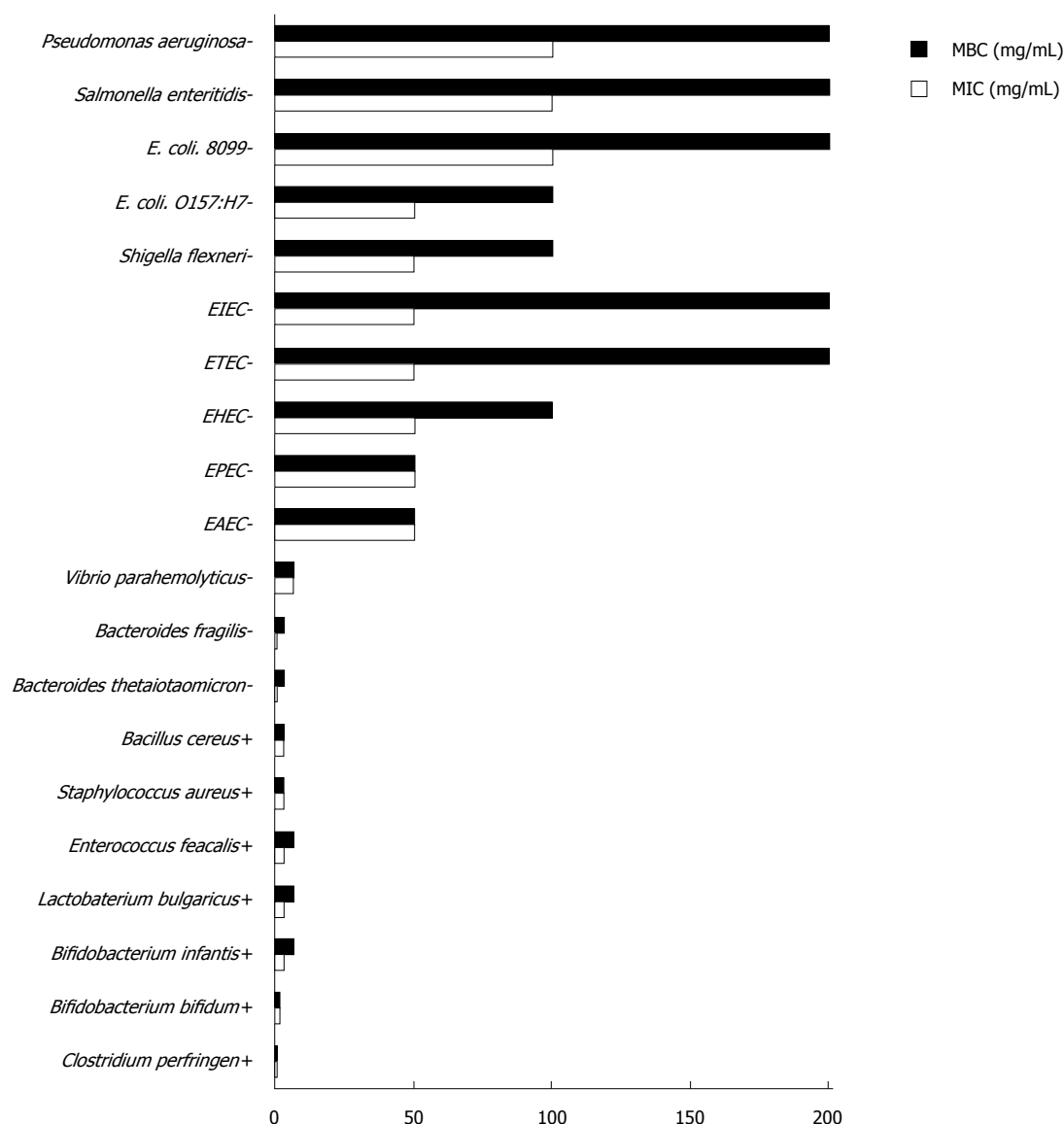


Figure 1 Antimicrobial activity of *Ligustrum robustum* on gut bacteria. MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; -: Gram negative bacteria; +: Gram positive bacteria; EAEC: Enteroto-aggregative *Escherichia coli*; EHEC: Enteroto-hemorrhagic *Escherichia coli*; ETEC: Enteroto-toxigenic *Escherichia coli*; EPEC: Enteroto-pathogenic *Escherichia coli*; EIEC: Enteroto-invasive *Escherichia coli*.

had different MIC and MBC to LR: 0.20 mg/mL and 0.20 mg/mL for *Clostridium perfringens*; 0.39 mg/mL and 0.39 mg/mL for *Bacteroides thetaiotaomicron* and *Bacteroides fragilis*; 1.56 mg/mL and 1.56 mg/mL for *Bifidobacterium bifidum*; 3.13 mg/mL and 6.25 mg/mL for *Bifidobacterium infantis*; 3.13 mg/mL and 6.25 mg/mL for *Lactobacterium bulagricum* and *Enterococcus faecalis*; 3.13 mg/mL and 3.13 mg/mL for *Staphylococcus aureus* and *Bacillus cereus*; 6.25 mg/mL and 6.25 mg/mL for *Vibrio parahemolyticus*; 1.56 mg/mL and no bactericidal effect for *Candida albicans*. The MICs for other strains were all greater than 25 mg/mL. Taken together, nonparametric test results indicated that LR exhibited higher inhibitory effects on gram positive bacteria than gram negative bacteria in the *in vitro* experiment ($P < 0.05$, Figure 1).

Animal experimental results

Food and energy intake: As indicated in Figure 2A, the average daily food intake in the Normal group was higher than that in the HFD model group (23.47 ± 1.69 g vs 19.16 ± 1.33 g, $P < 0.05$). In contrast, the average daily energy intake in the Normal group was markedly lower than that in the HFD model group (98.52 ± 6.67 kcal vs 88.49 ± 6.72 kcal, $P < 0.05$, Figure 2B), which could lead to greater body weight gain in the HFD group. In addition, LR did not significantly affect average daily food intake (19.95 ± 0.98 g, 19.99 ± 1.78 g, 18.37 ± 1.13 g, respectively) and energy intake (101.74 ± 5.02 g, 101.94 ± 9.07 g, 93.67 ± 5.75 g, respectively) compared with the HFD model group, suggesting that *L. robustum* did not affect food intake and energy intake.

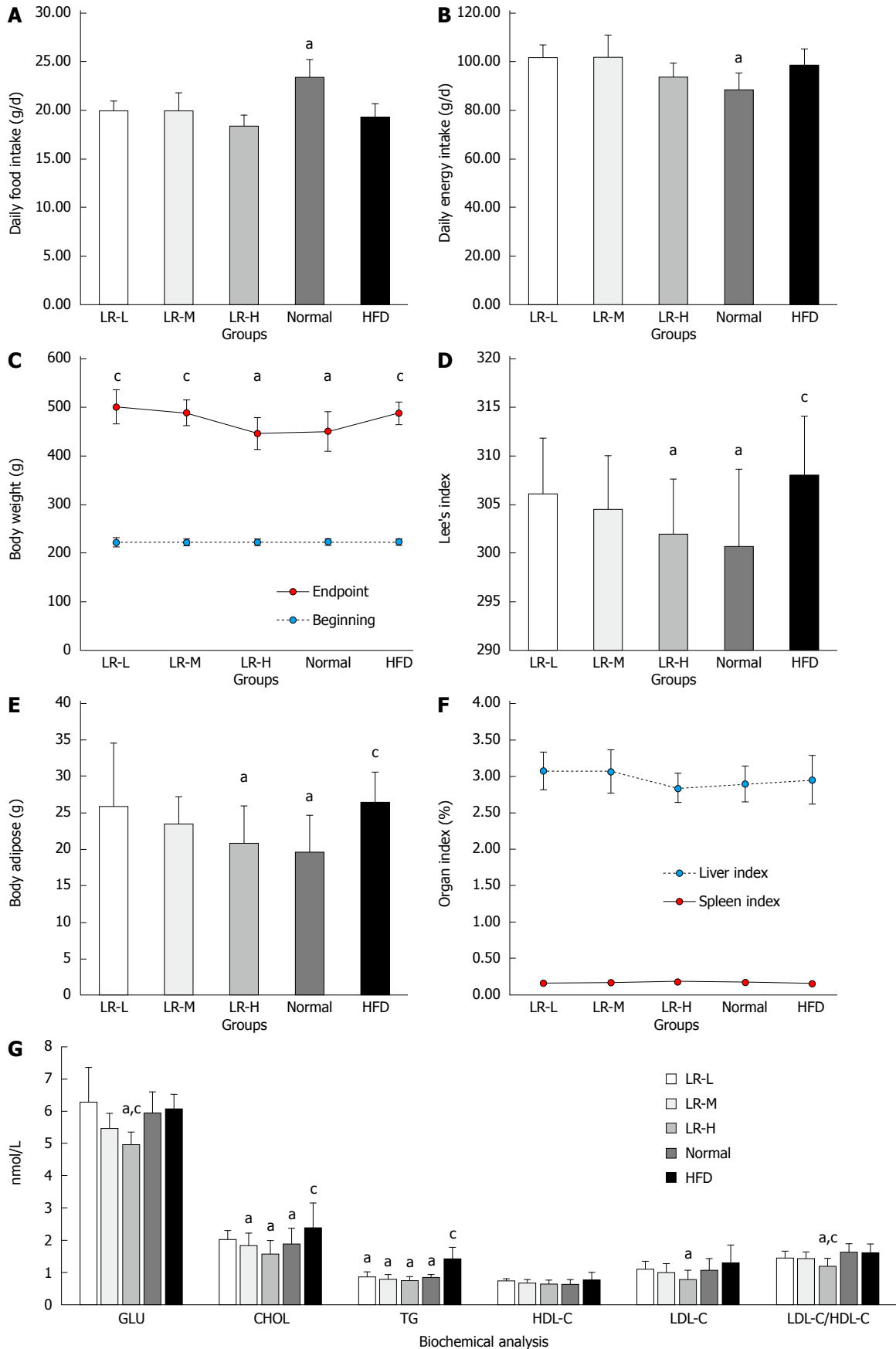


Figure 2 Effects of *Ligustrum robustum* on macroscopic obesity related parameters. A: Daily food intake; B: Daily energy intake; C: Body weight; D: Lee's index; E: Body adipose; F: Liver index and Spleen index; G: Blood glucose and lipid. Normal: Normal chow diet-fed mice; HFD: High-fat diet-fed control mice; LR-L/M/H: Low-, medium- and high-dose of *Ligustrum robustum* aqueous extract-treated HFD rats; $n = 10$ for LR-L, LR-M, LR-H; $n = 9$ for Normal and HFD; ^a $P < 0.05$ vs HFD group; ^c $P < 0.05$ vs Normal group.

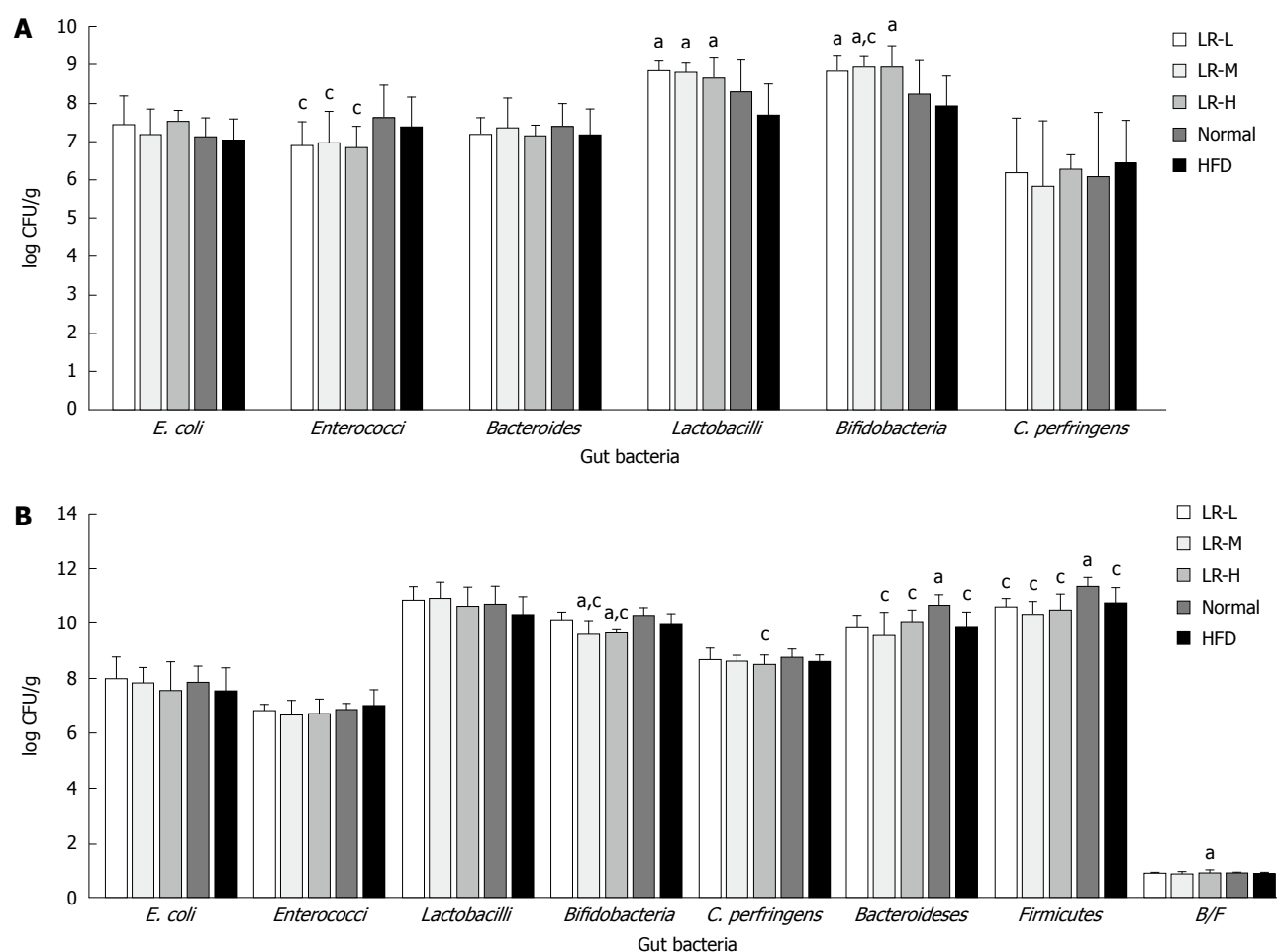


Figure 3 Effect of *Ligustrum robustum* on gut bacteria. A: Viable counts of six gut bacteria; B: 16S rRNA relative gene expression of gut bacteria. Normal: Normal chow diet-fed mice; HFD: High-fat diet-fed control mice; LR-L/M/H: Low-, medium- and high-dose of *Ligustrum robustum* aqueous extract-treated HFD rats; B/F: *Bacteroidetes*/*Firmicutes*; $n = 10$ for LR-L, LR-M, LR-H; $n = 9$ for Normal and HFD; $^aP < 0.05$ vs HFD group; $^cP < 0.05$ vs Normal group.

Body and organ weight: Compared with the control group, the HFD group showed increases in body weight, Lee's index, and body adipose tissue ($P < 0.05$, Figure 2C-E). However, no significant increase in Lee's index was found in LR-L, LR-M, and LR-H compared with the Normal group, and high dose LR suppressed the increases in body weight and fat in HFD rats ($P < 0.05$, Figure 2F).

Blood glucose and lipid parameters: The high-fat diet increased plasma CHOL and TG relative to the Normal group ($P < 0.05$, Figure 2G). LR-H reduced the increases in GLU, TG, CHOL, LDL-C, and the ratio of LDL-C to HDL-C relative to the HFD group ($P < 0.05$, Figure 2G).

Viable counts of six gut bacteria: After 24-48 h of culture *ex vivo*, no significant differences in *E. coli*, *Bacteroides*, and *Clostridium perfringens* growth were observed between the experimental groups (Figure 3A). However, compared with the HFD group, the LR groups had a greater increase in the growth of *Lactobacilli* and *Bifidobacteria* ($P < 0.05$), and compared with the Normal group, the LR groups had a

lower number of *Enterococci* ($P < 0.05$).

Quantitative PCR: As indicated in Figure 3B, there were no significant differences in *E. coli*, *Enterococci*, and *Lactobacilli*, although LR showed a tendency to decrease *Enterococci* and increase *Lactobacilli* in the feces of HFD rats, which is in accordance with the results of the culture method. In addition, the HFD group had the lowest ratio of *Bacteroidetes* to *Firmicutes* in all the groups, and had a decreased number of *Firmicutes* compared with the Normal group ($P < 0.05$). In contrast, certain doses of LR increased the ratio of *Bacteroidetes* to *Firmicutes* ($P < 0.05$) and decreased *Clostridium perfringens* and *Bifidobacteria* in the feces of HFD rats ($P < 0.05$). These data indicated that the effects of LR on certain types of gut bacteria, using quantitative PCR, were partly in accordance with those derived from the culture method (*Enterococci* and *Lactobacilli*), although *Bifidobacteria* was different.

T-RFLP community analysis: In this study, as previously mentioned, the forward primer was labeled with FAM fluorescein and the reverse primer was labeled with HEX fluorescein. Two incision enzymes,

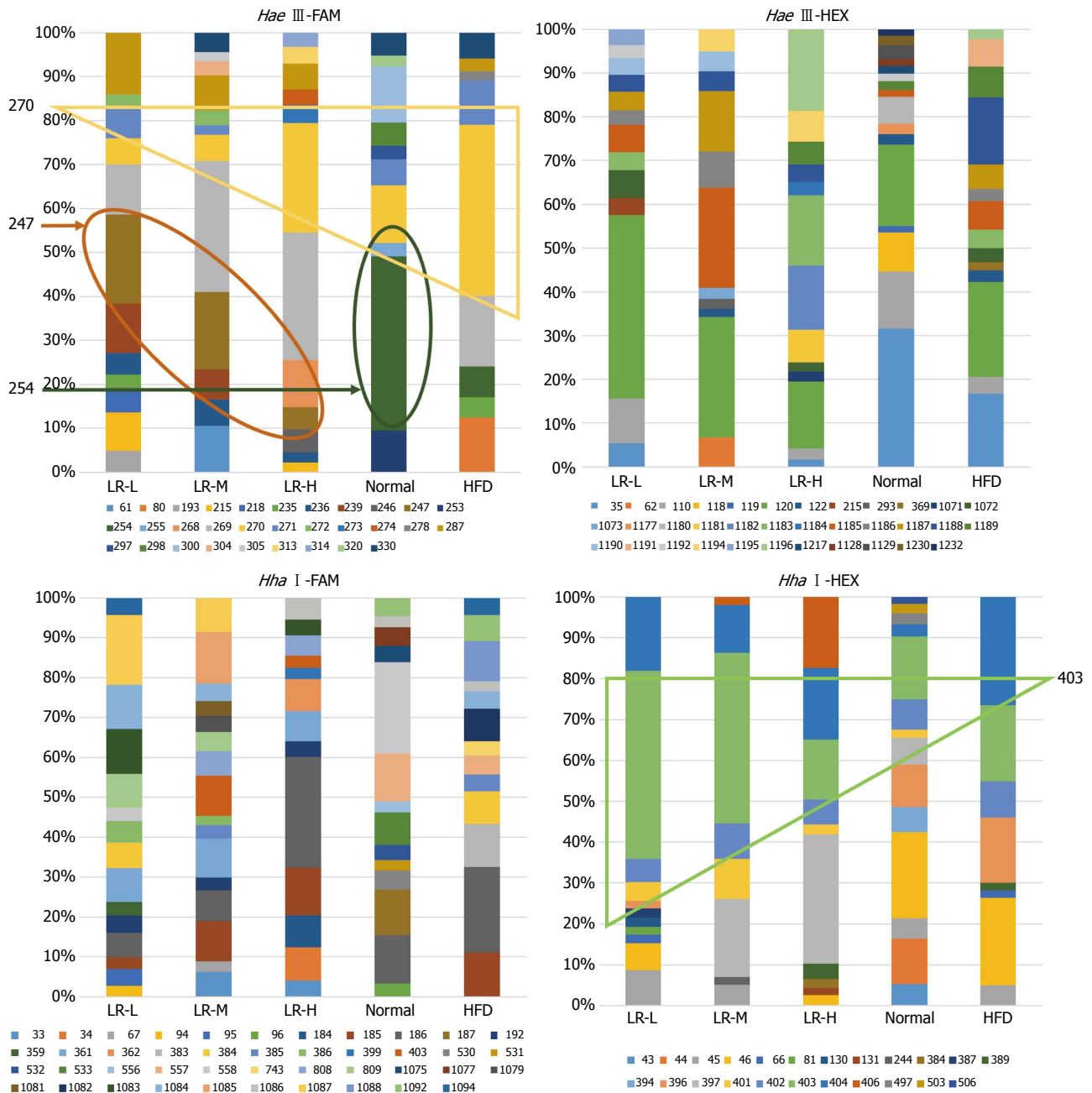


Figure 4 Results of terminal-restriction fragment length polymorphism analyses. Normal: Normal chow diet-fed mice; HFD: High-fat diet-fed control mice; LR-L/ M/H: Low-, medium- and high-dose of *Ligustrum robustum* aqueous extract-treated HFD rats; Peaks > 15 bp with more than 1% abundance of bacterial 16S rRNA gene fragments retrieved from four different treatments. *Hae* III (*Hha* I)-FAM (HEX): Purified PCR products digested by the restriction enzyme *Hae* III (*Hha* I) with FAM (HEX)-labeled DNA fragments separated by capillary electrophoresis; the length of T-RFs in base pairs (bp) is indicated; $n = 10$ for LR-L, LR-M, LR-H; $n = 9$ for Normal and HFD.

Hha I and *Hae* III, were used separately to obtain T-RFs. Finally, four types of treatment, namely *Hha* I-HEX, *Hha* I-FAM, *Hae* III-HEX, and *Hae* III-FAM, were analyzed separately. The relative abundance of T-RFs in each sample was determined by calculating the ratio between the size of each peak and the total peak size of all peaks detected in one sample. Distinct T-RF was defined as an abundance of more than 1% (Figure 4). According to the Mica III database, in the *Hae* III-FAM group, the probable bacteria in T-RF 247 were *Lactobacillus gallinarum*, uncultured *Lactobacillus*

sp., uncultured Chloroflexi bacteria, and uncultured bacteria. T-RF 247 was found in LR-L, LR-M, and LR-H but not in the Normal or HFD groups, indicating that LR treatment might increase *Lactobacillus*. T-RF 254 was identified as *Clostridium subterminale*, uncultured *Clostridiales* bacteria, uncultured rumen bacteria, and uncultured bacteria. T-RF 254 was detected in the Normal group (19.9%) and HFD group (3.4%). However, T-RF 254 was not detected in the treatment groups, indicating that LR may decrease bacteria such as *Clostridium*. T-RF 270 was identified as

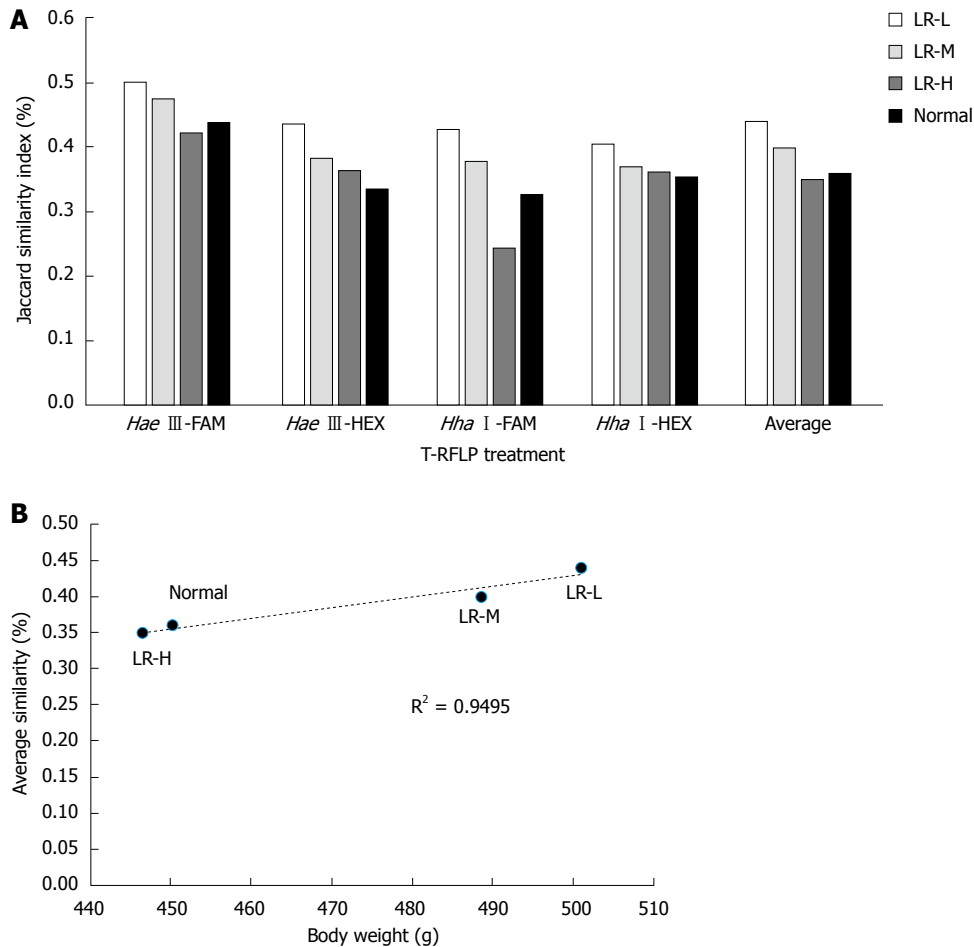


Figure 5 Similarity of gut microbiota compared with the obese group. A: The Jaccard similarity index of T-RFs in the HFD group and the other groups; B: Correlation of body weight with average similarity. Normal: Normal chow diet-fed mice; HFD: High-fat diet-fed control mice; LR-L/M/H: Low-, medium- and high-dose of *Ligustrum robustum* aqueous extract-treated HFD rats; Hae III (Hha I)-FAM (HEX): Purified PCR products digested by the restriction enzyme Hae III (Hha I) with FAM (HEX)-labeled DNA fragments separated by capillary electrophoresis; $n = 10$ for LR-L, LR-M, LR-H; $n = 9$ for Normal and HFD.

Streptococcus equi subsp., uncultured rumen bacteria, *Helicobacter* sp., uncultured *Acidobacteria* bacteria, and uncultured bacteria. T-RF 271, using Hae III-FAM, was identified as *Helicobacter pylori*, uncultured rumen bacteria, uncultured *Fusobacterium* sp., *Fusobacterium nucleatum* subsp. and uncultured bacteria. The HFD group had a higher percentage of T-RF 270 and T-RF 271 than the other groups (T-RF 270: 2.8%, 2.9%, 12.1%, 6.6%, 19.1%; T-RF 271: 3.4%, 1.0%, 0%, 3.0%, 5.0% in the LR-L, LR-M, LR-H, Normal, and HFD groups, respectively), which indicated that the HFD group may contain more *Streptococcus equi* subsp. and *Helicobacter* sp. compared with the other groups. In the Hae III-HEX group, the bacteria of T-RF 35 were suggested to be uncultured bacteria. The abundance of T-RF 35 in the LR-L, LR-M, LR-H, Normal and HFD groups was 2.2%, 0%, 1.03%, 20.69%, and 10.25% respectively. In the Hha I-FAM group, bacteria of T-RF 361 were mainly uncultured rumen bacteria. The abundance of T-RF 361 in the LR-L, LR-M, LR-H, Normal, and HFD groups was 3.4%, 4.3%, 3.1%, 0%, and 0%, respectively, indicating that LR treatment may increase the abundance of uncultured rumen bacteria.

In the Hha I-HEX group, bacteria of T-RF 403 were mainly *Lactobacillus* sp. and uncultured bacteria when compared with the database. This also indicated that LR treatment may increase the abundance of *Lactobacillus* sp. The findings from T-RF 403 (Hha I-HEX) and T-RF 247 (Hae III-FAM) indicated that LR effectively increased some types of *Lactobacillus* sp. (Figure 4). This finding was partly in accordance with the results obtained from the culture method and quantitative PCR.

The Jaccard similarity index in the different treatments (*i.e.*, Hae III-FAM, Hae III-HEX, Hha I-FAM, and Hha I-HEX) between the HFD group and the other groups is shown in Figure 5A. The average similarity compared with the HFD group was 0.44%, 0.40%, 0.35%, and 0.36%, respectively. There was a correlation between body weight and average similarity compared with the obese group ($P < 0.05$, Figure 5B), which indicated that gut microbiota in the heavier body weight group had higher similarity with the HFD group. These results demonstrated that the anti-obesity effect of LR might be derived from its effect on gut bacteria.

In this study, we found that bacterial richness was

Table 3 Richness, Shannon-Weiner diversity indices, and evenness

Group	n	Richness				Shannon-Weiner				Evenness			
		Hae III-FAM	Hae III-HEX	Hha I -FAM	Hha I -HEX	Hae III-FAM	Hae III-HEX	Hha I -FAM	Hha I -HEX	Hae III-FAM	Hae III-HEX	Hha I -FAM	Hha I -HEX
LR-L	10	44.80 ± 8.64 ^{bc}	32.50 ± 5.20 ^{bc}	44.90 ± 14.90	24.00 ± 7.77	3.16 ± 0.31 ^{ac}	2.76 ± 0.22 ^{ac}	3.23 ± 0.20 ^a	2.36 ± 0.16	0.84 ± 0.07	0.80 ± 0.05	0.86 ± 0.03 ^a	0.76 ± 0.05
LR-M	10	31.70 ± 8.08	26.00 ± 5.68	39.40 ± 5.32	25.00 ± 4.99	2.93 ± 0.25	2.54 ± 0.18 ^c	3.19 ± 0.11 ^a	2.34 ± 0.15	0.85 ± 0.04	0.79 ± 0.05	0.87 ± 0.03 ^a	0.73 ± 0.05
LR-H	10	31.80 ± 11.79	24.50 ± 4.58	37.00 ± 7.56	26.40 ± 6.11	2.88 ± 0.38	2.50 ± 0.25 ^c	3.04 ± 0.18	2.51 ± 0.22	0.85 ± 0.04	0.79 ± 0.06	0.85 ± 0.04	0.77 ± 0.07
Normal	9	29.11 ± 10.40	21.56 ± 8.17	35.89 ± 7.03	21.44 ± 4.50	2.66 ± 0.25	2.28 ± 0.23 ^a	3.09 ± 0.22	2.35 ± 0.16	0.81 ± 0.08	0.77 ± 0.09	0.87 ± 0.04 ^a	0.77 ± 0.06
HFD	9	33.56 ± 11.20	27.11 ± 5.80	38.67 ± 12.75	24.78 ± 6.36	2.80 ± 0.47	2.50 ± 0.20 ^c	2.95 ± 0.39	2.38 ± 0.23	0.81 ± 0.06	0.76 ± 0.06	0.82 ± 0.04 ^c	0.75 ± 0.08

S (Richness) = $\sum p_i$; H (Shannon-Weiner diversity index) = $-\sum p_i \ln p_i$; D (Simpson index) = $1 / \sum p_i^2$; E (Evenness) = H/H_{max} ($H_{max} = \ln S$). ^a $P < 0.05$ vs HFD group; ^c $P < 0.05$ vs Normal group. Normal: Normal chow diet-fed mice; HFD: High-fat diet-fed control mice; LR-L/M/H: Low-, medium- and high-dose of *Ligustrum robustum* aqueous extract-treated HFD rats.

higher in the LR-L group than in the HFD group when Hae III-FAM and Hae III-HEX were applied ($P < 0.05$, Table 3). When Hae III-FAM, Hae III-HEX, and Hha I-FAM were used, a higher diversity index was found in the LR-L group compared with the HFD group ($P < 0.05$). When Hha I -FAM was used, a higher diversity index was also found in the LR-M group compared with the HFD group ($P < 0.05$). In addition, both the LR-L group and the LR-M group had higher E values compared with the HFD group when using Hha I -FAM ($P < 0.05$), while no difference between the LR-H and obese group was observed.

DISCUSSION

Various studies have demonstrated that gut bacteria play an important role in obesity development. Here, we analyzed the regulatory effect of LR on gut bacteria *in vivo* and *in vitro* and body weight control. We found that a high dose of LR resulted in a significant decrease in body weight and Lee's index compared with rats fed the HFD not treated with LR, and certain doses of LR decreased CHOL, TG, LDL-C, and LDL-C/HDL-C in rats fed the HFD. These results suggest that LR may have anti-obesity and lipid-reducing effects. To determine whether the effect of weight loss was related to food and energy intake, we evaluated food and energy intake in all LR treated rats and in rats fed the HFD and found no significant differences, indicating that LR may prevent obesity without sacrificing daily food intake or daily energy intake. This led us to further investigate the mechanism of LR on body weight control. It is widely accepted that gut microbes play an important role in host energy metabolism and obesity development. Combined with the already existing evidence that LR has a regulatory effect on microorganisms^[6,7], we speculated that the anti-obesity effects of LR may be mediated by the regulation of host gut microbes. To verify our hypothesis, we tested the regulatory effects of LR on gut bacteria *in vivo* and *in vitro* using different methods.

We first tested the effect of LR on the changes in rat gut bacteria. The culture results demonstrated that all three groups treated with LR had a lower level of *Enterococci* ($P < 0.05$). The quantitative PCR results also indicated a decreasing tendency in the *Enterococci* population compared with the HFD model group. In our previous study, we found that the number of *Enterococci* in the obese group was higher than that in the normal weight group^[19]. The studies by Mozes *et al.*^[20] and Zhou *et al.*^[21] showed that an increase in *Enterococci* might help promote the development of obesity. These findings demonstrated that the anti-obesity effect of LR may be partly due to its ability to decrease the number of *Enterococci*.

In addition to *Enterococci*, we also found that the numbers of *Bifidobacteria* in the LR-L, LR-M, and LR-H groups were higher than that in the HFD group ($P < 0.05$). However, the results from quantitative PCR demonstrated that the numbers of *Bifidobacteria* in the LR-M and LR-H groups were lower than those in the HFD model group and control group ($P < 0.05$). What are the probable reasons contributing to the controversial results obtained from the different detection methods? In Matsuki *et al.*^[22], it was reported that not all *Bifidobacteria* could be identified by the culture method, and various types of *Bifidobacteria* required different culture conditions to grow on the plates. Thus, BBL, the only medium used, may not satisfy the requirements of all types of *Bifidobacteria*, especially those requiring strict culture conditions. Moreover, it is worth noting that a previous study indicated that many cultivable *Bifidobacteria* strains were important for host health and energy intake^[22]. Therefore,

in our study, the decreased amount of *Bifidobacteria* detected by quantitative PCR might be vulnerable to LR treatment. According to our other results, this kind of *Bifidobacteria* might contribute less to the control of host's body weight than those that could be grown on BBL plates. Besides, there are only 2000 types of bacteria that can be used for 16S rRNA sequencing, and a large proportion of the 16S rRNA sequence is still unknown. Therefore, we speculate that the reason we obtained controversial results for *Bifidobacteria* may be partly due to the different principles of the detection methods. However, further studies are needed to confirm our speculation.

Lactobacillus consists of more than 90 types of bacteria, and approximately 30% can be cultured from stool samples^[23]. The beneficial effect of *Lactobacillus* has been widely known for a long time. In this study, the culture results showed that the populations of *Lactobacillus* at different doses of LR were higher than those in the HFD model group ($P < 0.05$). The quantitative PCR results also demonstrated a similar tendency without significant variance. Furthermore, T-RF 247 (*Hae* III-FAM) and T-RF 403 (*Hha* I-HEX) analyses showed that LR treatment increased the abundance of *Lactobacillus* sp. Several previous studies reported that *Lactobacillus* regulates host metabolism and energy intake^[24,25]. These results may indicate a potential role for cultivable *Lactobacillus* in LR-mediated weight loss.

Clostridium perfringens is a harmful gut bacterium, which can cause many diseases^[26,27]. In this study, *C. perfringens* in LR-H treated group was lower than that in the normal group ($P < 0.05$), therefore, LR might inhibit the growth of *C. perfringens* and result in a healthier gut environment. However, this is a preliminary conclusion, and more studies are needed to confirm these findings.

Bacteroides, the most predominant bacterial group in the gut, is involved in the regulation of host metabolism and energy intake by regulating the metabolism of polysaccharide in the gut^[28]. Previous studies have shown that normal rats contained more *Bacteroides* compared with the obese group^[19,20]. In the present study, no significant difference was found using the culture method. However, the number of *Bacteroidetes* in the control group was higher than that in the group fed a HFD ($P < 0.05$). This was partly consistent with previous findings.

The relative proportions of gut bacteria can also affect body weight. *Bacteroidetes* and *Firmicutes* are the main gut bacteria that affect energy homeostasis^[29]. It was reported that obese rats had less *Bacteroidetes* and more *Firmicutes* than the lean group^[30,31]. In the present study, the HFD model group had the highest Lee's index and the lowest ratio of *Bacteroidetes* to *Firmicutes* compared with the other groups. On the other hand, the RL-H group had the lowest Lee's index and the highest ratio of *Bacteroidetes* to *Firmicutes* ($P < 0.05$). In addition, our results showed that the

numbers of *Bacteroidetes* and *Firmicutes* in the normal control group were higher than those in the other four HFD groups, indicating that a HFD may lower the total number of gut bacteria.

In addition to the animal experiment, the different bacteriostatic and bactericidal effects of LR on gut bacteria were studied. Strongly selective bacteriostasis of LR was found by the MIC and MBC tests, and LR showed a higher inhibitory effect on the growth of gram positive bacteria than on gram negative bacteria. Interestingly, *Enterococci*, *Bifidobacteria*, *Lactobacillus*, and *C. perfringens* are all gram positive bacteria, and it was speculated that the growth of these gram positive bacteria would be inhibited and not enhanced according to the *in vitro* experiment. However, in the *in vivo* experiment, the number of some gram positive bacteria, such as *Enterococci* and *C. perfringens* decreased, while the number of *Lactobacillus* increased. This may be attributed to the fact that LR did not reach the effective concentrations required *ex vivo* when in the intestinal tract. The *in vitro* experiment targeted specific strains that partly represented the whole gut flora, whereas the results from the animal experiment focused on the genus level of different gut bacteria. Furthermore, LR is a mixture of different components, and the digestive tract is a complicated channel with various enzymes and microorganisms. LR would gradually be degraded in the gastrointestinal tract, thus the effective components in LR would probably be metabolized. As a result, in the *ex vivo* and *in vivo* experiments, the active ingredients of LR on gut bacteria would be different. However, we still observed the same trend in some bacteria after LR treatment both *in vivo* and *in vitro*.

In the present study, double fluorescence labeled T-RFLP was used to analyze the richness, diversity, and evenness of gut microbiota. Analysis of dominant flora showed that LR changed the advantageous bacterial species and their percentage in the total flora. By analyzing the percentage of advantageous flora in the total flora, we found that a certain amount of LR increased the percentage of *Lactobacillus* sp. in the total flora. We also found that LR treatment, especially LR-L, increased the richness and species diversity of the gut. Similar analysis of gut microbiota in the LR treated groups and the normal group compared with the HFD group indicated that gut microbiota in the heavier body weight group had higher similarity with the HFD group, which revealed a possible role for LR in obesity prevention by affecting the gut microbiota.

In conclusion, our study demonstrated significant bacteriostatic and bactericidal effects of LR on specific gut bacteria *in vitro*, and certain doses of LR altered the richness and diversity of gut bacteria in HFD rats. This study also found increased number of fecal *Lactobacilli* and decreased number of *Enterococci*, together with a decrease in Lee's index and blood concentration of fat storage-related indicators in HFD

rats. These findings indicated that LR may have a potential anti-obesity effect by regulating gut bacteria.

COMMENTS

Background

Gut microbes play important roles in fat storage and metabolism. The control of gut microbes is considered to be a promising method for preventing and treating obesity. The identification of safe and efficient modulatory agents to regulate body weight is a promising strategy for obese individuals and is of great interest to researchers.

Research frontiers

To date, few foods have been investigated for their effects on gut microbes and the prevention of obesity. Recent studies indicated that *Ligustrum robustum* (*L. robustum*), a safe food-grade drink, can influence body weight and may have different bacteriostatic activities on different bacteria. However, the exact effect and mechanism of *L. robustum* require further investigation. The relationship between gut bacteria and obesity following treatment with *L. robustum* remains poorly defined.

Innovations and breakthroughs

In this study, the authors analyzed the regulatory effect of *L. robustum* aqueous extract (LR) on gut bacteria *in vivo* and *in vitro* and its effect on body weight control. The data showed that LR had significant bacteriostatic and bactericidal effects on specific gut bacteria *in vitro* and *in vivo*, and certain doses of LR altered the richness and diversity of gut bacteria. This study also found increased numbers of fecal *Lactobacilli* and decreased numbers of *Enterococci* together with a decrease in Lee's index and in the blood concentration of fat storage-related indicators in high-fat diet-fed rats. These findings indicated that LR may have a potential anti-obesity effect by regulating gut bacteria.

Applications

The data suggested that *L. robustum* may be regarded as a safe and effective food for weight loss and obesity control, and these functions might be mediated by the regulation of gut bacteria. These findings may help to boost people's recognition of *L. robustum*, promote the consumption of *L. robustum*, and facilitate the expansion and development of *L. robustum*.

Peer-review

The authors are provided interesting and original data about the effects of LR on host body weight and gut flora in high-fat diet-fed rats and its possible mechanisms. This study is well carried out, and the applied methods are sound. Overall, the study showed that *L. robustum* may have a potential anti-obesity effect by regulating gut bacteria.

REFERENCES

- 1 Ministry of Health PR, China. The official approval for *Ligustrum robustum* as a food. *Zhongguo Shipin Weisheng Zazhi* 2012; **2**: 165
- 2 Yu ZL, Zeng WC. Antioxidant, antibrowning, and cytoprotective activities of *Ligustrum robustum* (Roxb.) Blume extract. *J Food Sci* 2013; **78**: C1354-C1362 [PMID: 23924383 DOI: 10.1111/1750-3841.12224]
- 3 Lau KM, He ZD, Dong H, Fung KP, But PP. Anti-oxidative, anti-inflammatory and hepato-protective effects of *Ligustrum robustum*. *J Ethnopharmacol* 2002; **83**: 63-71 [PMID: 12413708 DOI: 10.1016/S0378-8741(02)00192-7]
- 4 He ZD, Lau KM, But PP, Jiang RW, Dong H, Ma SC, Fung KP, Ye WC, Sun HD. Antioxidative glycosides from the leaves of *Ligustrum robustum*. *J Nat Prod* 2003; **66**: 851-854 [PMID: 12828473 DOI: 10.1021/np020568g]
- 5 Huang LF, Wan DG. Study on the weight loss effect of *Ligustrum robustum* derived from Sichuan. *Chengdu Zhongyiyao Daxue Xuebao* 2004; **9**: 49
- 6 Ye Q, Zuo HJ, Liao HY, Cao Y, Cao X, Pei XF. Study on in vitro antimicrobial activity of *L. purpurascens* Y.C. Yang. *Xiandai Yufang Yixue* 2013; **40**: 1320-1322
- 7 Du XX, Ye Q, Chen F, Zuo HJ, Huang MJ, Dong ZF, Pei XF. Study on the antibacterial activity of *Ligustrum robustum* combined with antibiotics to antibiotic resistant *Staphylococcus aureus* isolates. *Xiandai Yufang Yixue* 2014; **41**: 894-897
- 8 Method for the Assessment of Regulating Gastrointestinal Tract Flora Function. Ministry of Health PR, China. Technical Standards for Testing & Assessment of Health Food. Beijing: People's Medical Publishing House, 2003: 148-153
- 9 Rinttilä T, Kassinen A, Malinen E, Krogus L, Palva A. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J Appl Microbiol* 2004; **97**: 1166-1177 [PMID: 15546407 DOI: 10.1111/j.1365-2672.2004.02409.x]
- 10 Calame W, Weseler AR, Viebke C, Flynn C, Siemensma AD. Gum arabic establishes prebiotic functionality in healthy human volunteers in a dose-dependent manner. *Br J Nutr* 2008; **100**: 1269-1275 [PMID: 18466655 DOI: 10.1017/S0007114508981447]
- 11 Metzler-Zebeli BU, Hooda S, Pieper R, Zijlstra RT, van Kessel AG, Mosenthin R, Gänzle MG. Nonstarch polysaccharides modulate bacterial microbiota, pathways for butyrate production, and abundance of pathogenic *Escherichia coli* in the pig gastrointestinal tract. *Appl Environ Microbiol* 2010; **76**: 3692-3701 [PMID: 20382813 DOI: 10.1128/AEM.00257-10]
- 12 Guo X, Xia X, Tang R, Wang K. Real-time PCR quantification of the predominant bacterial divisions in the distal gut of Meishan and Landrace pigs. *Anaerobe* 2008; **14**: 224-228 [PMID: 18524640 DOI: 10.1016/j.anaerobe.2008.04.001]
- 13 Bahl MI, Bergström A, Licht TR. Freezing fecal samples prior to DNA extraction affects the Firmicutes to Bacteroidetes ratio determined by downstream quantitative PCR analysis. *FEMS Microbiol Lett* 2012; **329**: 193-197 [PMID: 22325006 DOI: 10.1111/j.1574-6968.2012.02523.x]
- 14 Cheng Y, Zuo HJ, Liao HY, Chen JY, Zheng TL, Pei XF, Xu X. Establishment of real-time PCR method for detection of intestinal bacteria. *Xiandai Yufang Yixue* 2014; **41**: 4338-4341
- 15 Kato M, Ishige A, Anjiki N, Yamamoto M, Irie Y, Taniyama M, Kibe R, Oka J, Benno Y, Watanabe K. Effect of herbal medicine Juzentaihoto on hepatic and intestinal heat shock gene expression requires intestinal microflora in mouse. *World J Gastroenterol* 2007; **13**: 2289-2297 [PMID: 17511026 DOI: 10.3748/wjg.v13.i16.2289]
- 16 Shyu C, Soule T, Bent SJ, Foster JA, Forney LJ. MiCA: a web-based tool for the analysis of microbial communities based on terminal-restriction fragment length polymorphisms of 16S and 18S rRNA genes. *Microb Ecol* 2007; **53**: 562-570 [PMID: 17406775 DOI: 10.1007/s00248-006-9106-0]
- 17 Blasco L, Kahala M, Tampio E, Ervasti S, Paavola T, Rintala J, Joutsjoki V. Dynamics of microbial communities in untreated and autoclaved food waste anaerobic digesters. *Anaerobe* 2014; **29**: 3-9 [PMID: 24791674 DOI: 10.1016/j.anaerobe.2014.04.011]
- 18 Bellou N, Papathanassiou E, Dobretsov S, Lykousis V, Colijn F. The effect of substratum type, orientation and depth on the development of bacterial deep-sea biofilm communities grown on artificial substrata deployed in the Eastern Mediterranean. *Biofouling* 2012; **28**: 199-213 [PMID: 22352335 DOI: 10.1080/08927014.2012.662675]
- 19 Zuo HJ, Xie ZM, Zhang WW, Li YR, Wang W, Ding XB, Pei XF. Gut bacteria alteration in obese people and its relationship with gene polymorphism. *World J Gastroenterol* 2011; **17**: 1076-1081 [PMID: 21448362 DOI: 10.3748/wjg.v17.i8.1076]
- 20 Mozes S, Bujnáková D, Sefčíková Z, Kmet V. Intestinal microflora and obesity in rats. *Folia Microbiol (Praha)* 2008; **53**: 225-228 [PMID: 18661297 DOI: 10.1007/s12223-008-0031-0]
- 21 Zhou LP, Hu LX, Deng XM, Li WZ, Yang HY, Ding WJ. Study on the gut bacteria alternation of patient undergo acupuncture weight control therapy. *Sichuan Zhongyiyao Daxue Xuebao* 2011; **29**: 124-126

- 22 **Matsuki T**, Watanabe K, Fujimoto J, Kado Y, Takada T, Matsumoto K, Tanaka R. Quantitative PCR with 16S rRNA-gene-targeted species-specific primers for analysis of human intestinal bifidobacteria. *Appl Environ Microbiol* 2004; **70**: 167-173 [PMID: 14711639 DOI: 10.1128/AEM.70.1.167-173.2004]
- 23 **Hase K**, Ohno H. [Epithelial cells as sentinels in mucosal immune barrier]. *Nihon Rinsho Meneki Gakkai Kaishi* 2006; **29**: 16-26 [PMID: 16505599 DOI: 10.2177/jsci.29.16]
- 24 **Kang JH**, Yun SI, Park MH, Park JH, Jeong SY, Park HO. Anti-obesity effect of *Lactobacillus gasseri* BNR17 in high-sucrose diet-induced obese mice. *PLoS One* 2013; **8**: e54617 [PMID: 23382926 DOI: 10.1371/journal.pone.0054617]
- 25 **Miyoshi M**, Ogawa A, Higurashi S, Kadooka Y. Anti-obesity effect of *Lactobacillus gasseri* SBT2055 accompanied by inhibition of pro-inflammatory gene expression in the visceral adipose tissue in diet-induced obese mice. *Eur J Nutr* 2014; **53**: 599-606 [PMID: 23917447 DOI: 10.1007/s00394-013-0568-9]
- 26 **Nicholson JK**, Holmes E, Wilson ID. Gut microorganisms, mammalian metabolism and personalized health care. *Nat Rev Microbiol* 2005; **3**: 431-438 [PMID: 15821725 DOI: 10.1038/nrmicro1152]
- 27 **Van Immerseel F**, De Buck J, Pasmans F, Huyghebaert G, Haesebrouck F, Ducatelle R. *Clostridium perfringens* in poultry: an emerging threat for animal and public health. *Avian Pathol* 2004; **33**: 537-549 [PMID: 15763720 DOI: 10.1080/03079450400013162]
- 28 **Hooper LV**, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI. Molecular analysis of commensal host-microbial relationships in the intestine. *Science* 2001; **291**: 881-884 [PMID: 11157169 DOI: 10.1126/science.291.5505.881]
- 29 **Nadal I**, Santacruz A, Marcos A, Warnberg J, Garagorri JM, Moreno LA, Martin-Matillas M, Campoy C, Martí A, Moleres A, Delgado M, Veiga OL, García-Fuentes M, Redondo CG, Sanz Y. Shifts in clostridia, bacteroides and immunoglobulin-coating fecal bacteria associated with weight loss in obese adolescents. *Int J Obes (Lond)* 2009; **33**: 758-767 [PMID: 19050675 DOI: 10.1038/ijo.2008.260]
- 30 **Ley RE**, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. *Proc Natl Acad Sci USA* 2005; **102**: 11070-11075 [PMID: 16033867 DOI: 10.1073/pnas.0504978102]
- 31 **Ley RE**, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. *Nature* 2006; **444**: 1022-1023 [PMID: 17183309 DOI: 10.1038/4441022a]

P- Reviewer: Mallard BL **S- Editor:** Kong JX **L- Editor:** Filipodia
E- Editor: Ma S





Published by **Baishideng Publishing Group Inc**

8226 Regency Drive, Pleasanton, CA 94588, USA

Telephone: +1-925-223-8242

Fax: +1-925-223-8243

E-mail: bpgoffice@wjgnet.com

Help Desk: <http://www.wjgnet.com/esps/helpdesk.aspx>

<http://www.wjgnet.com>



ISSN 1007-9327



9 771007 932045