Ligustrum robustum Intake, Weight Loss, and Gut Microbiota: An Intervention Trial

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Ligustrum robustum (LR) shows antiobesity effects in animal studies. However, little is known about the effect on human. The present study aimed to investigate the effect of LR intake on weight change in obese women and the role of gut microbiota. Thirty overweight and obese female participants (BMI \( \geq 24 \) kg/m\(^2\)) were recruited in the current study. The participants drank LR 10 g/d for 12 wks. Their body composition and related biomarkers were assessed. Alterations of the gut microbiota were analyzed using 16S rRNA sequencing. The primary outcome was the change in body weight. LR intake resulted in 2.5% weight loss over 12 wks (\( P<0.01 \)). Change in body fat at 12 wk was -1.77 ± 1.19 kg (\( P<0.01 \)). In addition, increased Firmicutes-to-Bacteroidetes ratio (\( P=0.03 \)), increased richness (the ACE estimator, \( P<0.01 \); the Chao1 estimator, \( P<0.01 \)), and altered representative taxa of the gut microbiota were observed. Bacteroidaceae, Bacteroides, Bacilli, and Lactobacillales were higher while Ruminococcaceae, Enterobacteriaceae, Enterobacteriales, Lachnospiraceae, Clostridia, and Clostridiales were lower at 12 wk. Moreover, LR intervention decreased fasting glucose (\( P<0.01 \)), serum leptin (\( P<0.01 \)), and IL8 (\( P=0.02 \)) and increased HOMA-\( \beta \) (\( P<0.01 \)). LR intervention moderately decreased the body weight in overweight and obese women and such effect might be due to modulation of gut microbiota.

1. Introduction

Obesity has become an epidemic worldwide [1, 2]. Gut microbiota has recently emerged as a novel, metabolically active organ, tightly linked to obesity [3, 4], through various mechanisms such as the production of bioactive compounds influencing fat storage and metabolism [5]. Thus, dietary modulation of gut bacteria is considered to be a promising method for preventing and treating obesity.

Ligustrum robustum (LR), a traditional Chinese beverage plant, contains abundant bioactive compounds with health benefits, such as flavonoids, total phenolic acid, polysaccharide, and triterpenoid [6, 7]. Animal studies have shown the antiobesity, antioxidative, anti-inflammatory, antitumor, and hepatoprotective effects of LR [8–10]. Moreover, the LR-induced changes in the composition of gut microbiota were also observed accompanied with weight loss in an animal study [11].

Although accumulating data highlight the potential effects of LR on weight loss, the direct evidence from human studies is lacking; and little is known about whether LR affects gut microbiota in humans. Because women have a higher
2. Materials and Methods

2.1. Components Analysis of LR. To preparation of LR extract for component analysis, 50 g LR was ground to a fine powder and suspended in 1500 mL distilled water and incubated with shaking at 80°C for 3 h. Then the extract was filtered and further evaporated on a rotary evaporator to a concentration to 1 g/mL and stored at −20°C until further use [10]. The analytical solution was prepared by filtering 220 μL supernatant using a 0.22 μm filter.

An ACQUITY UPLC system (Waters Corporation, Milford, USA) coupled with an AB SCIEX Triple TOF 5600 System (AB SCIEX, Framingham, MA) was used to analyze the metabolic profiling in both ESI positive and ESI negative ion modes. An ACQUITY UPLC BEH C18 column (1.7 μm, 2.1 × 100 mm) was used in both positive and negative modes. The analysis was performed in binary gradient mode, with water (containing 0.1% formic acid, v/v) as mobile phases A and acetonitrile (containing 0.1% formic acid, v/v) as mobile phases B. The separation was achieved using the following gradient: 0 min, 5% B; 2 min, 20% B; 4 min, 25% B; 9 min, 60% B; 14 min, 100% B; 18 min, 100% B; 18.1 min, 5% B; and 19.5 min, 5% B, with a flow rate of 0.4 mL/min and column temperature of 45°C. All the samples were kept at 4°C during the analysis. Full scan mode (m/z ranges from 70 to 1000) combined with IDA mode was used in data acquisition. The ion source temperature was 550°C for positive and negative ion mode. The ion spray voltage was 5500 V for positive ion mode and 4500 V for negative ion mode. For IDA analysis, the range of m/z was from 25 to 1000 and the collision energy was 30 eV. The injection volume was 2 μL. The measurement was performed by Shanghai Lu-Ming Biotech Co., Ltd., Shanghai, China. Raw data was analyzed by Progenesis QI software (Nonlinear Dynamics, Newcastle, UK).

2.2. Subjects. Community female volunteers aged 30 to 60 years and whose body mass index (BMI) over 24 kg/m² were invited to join the study [13]. Subjects who were using medications that affect body weight, having a history of chronic illness, taking antibiotic within 3 months, changing their diet recently, or showing insufficient motivation to complete the study, were excluded. Thirty-six volunteers attended the first interview. One volunteer was excluded for basic disease, four for BMI <24 kg/m² and one volunteer dropped out because of work. Demographic characteristics of participants including age and medical history were obtained by questionnaire.

The Medical Ethics Committee of Sichuan University approved the study (Approval No. K2016025). Written informed consent was obtained from all participants. This trial was registered at http://www.chictr.org.cn (ChiCTR-OOh-17011832).

2.3. LR Intervention. Processed leaves of LR were obtained from China’s largest LR provider, Green Hills and Blue Waters Co., Ltd. (Junliang, Sichuan, China). One tea bag was packed with 5g grounded LR leaves. No additional chemical was introduced during preparation of the tea powder. One tea bag was brewed with 420 mL boiled water for at least 10 minutes for one cup of tea. Additional hot water was permitted for the same tea bag when needed.

Participants were instructed to drink LR twice per day after meals for 12 wks. Participants were told to consume their habitual diets throughout the study. Other tea and antibiotic were prohibited in the study. At the end of the intervention, the number of tea bags left was counted to evaluate the compliance.

2.4. Anthropometric Measures and Biochemical Analysis. Body composition measurement was introduced by multifrequency BIA with eight tactile electrodes (InBody 720; Biospace, Seoul, Korea). Body weight, abdomen circumference, hip circumference, and body composition (i.e., body fat, visceral fat area, body protein, and body muscle) were measured in the morning with light clothing before breakfast with an empty bladder at baseline, 4 wk, and 12 wk. Height was measured without shoes. Reduced weight (%) was defined as [(initial body weight - body weight at follow-up)/initial body weight] × 100 [14]. Triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), insulin, C-Peptide, glucose, and biomarkers for safety assessment were assessed by an automated biochemical analyzer in No. 4 West China Teaching Hospital at baseline and 12 wk. We further calculated homeostasis model assessment of insulin resistance (HOMA-IR) by fasting insulin (mU/L) × fasting glucose (mmol/L)/22.5 and HOMA of β-cell function (HOMA-β) by 20 × fasting insulin (mU/L)/[fasting glucose (mmol/L) × 3.5] × 100%. Fasting blood samples were stood for 30 minutes to clot before centrifugation for 10 minutes at 1000×g. Then the serum samples were frozen at −80°C until analysis. Moreover, we measured leptin, interleukin-6 (IL6) and interleukin-8 (IL8) using the LUMINEX technology (Millipore, Billerica, MA, USA).

2.5. Dietary Assessment and Physical Activity Monitor. To evaluate the dietary adherence, we collected food frequency questionnaires (FFQs) which measured the frequency and quantity of major food and beverages intake from all participants at baseline and 12 wk [15]. Physical activity was evaluated at baseline and 12 wk by International Physical Activity Questionnaire (IPAQ) [16]. The frequency and duration of various types of physical activity were obtained from participants. The metabolic equivalent (MET) value was defined as the ratio of the work metabolic rate to a standard resting metabolic rate (RMR). One MET is defined as 1.0 kcal/kg/hour [17]. We multiplied the corresponding MET value by the number of hours per week involved in the activity to get an average energy expenditure for each activity.

2.6. 16S rRNA Sequencing. The microbial communities of 29 participants were analyzed by high-throughput sequencing.
Fecal samples collected at baseline and 12 wk were immediately stored at -80°C for gut microbiota analysis. DNA of each fecal sample was extracted by using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). The DNA concentration was determined by Nanodrop spectrophotometer (NanoDrop 2000, Wilmington, USA). V4 region of 16S rRNA was amplified using primer 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAAT-3') with the barcode. Sequencing was performed on an Illumina Miseq platform (Novogene, Beijing, China) based on a standard protocol from the manufacturer. Raw sequences were processed by using the QIME software package. Sequences with ≥97% similarity were assigned to the same OTUs. The representative sequence for each OTU was screened for further annotation.

2.7. Adverse Event Monitoring. Adverse events associated with LR intake were self-reported by the participants. Additionally, biomarkers such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum were measured to evaluate liver function.

2.8. Statistics. All statistical comparisons are 2-sided. The level of significance for all tests was set to P < 0.05. Baseline data are reported as means ± standard deviation or median and interquartile range when appropriate. The concentration of biomarkers only available at baseline and 12 wk was analyzed using paired T-test for normal distribution variables and Wilcoxon signed-rank test for nonparametric tests. Above statistical analyses were performed with SAS version 9.4 (SAS Institute Inc., Cary, NC). Linear discriminant analysis coupled with effect size (LEfSe) was used to identify the taxa differentially represented between baseline and 12 wk. Alpha diversity, Principal Coordinate Analysis (PCoA) and clustering in our samples were calculated and displayed with R software (Version 3.3.3). The study was powered to detect a 0.5 kg weight loss as an effect of LR intake over the intervention. Power calculations were performed with G*Power 3.1.

3. Results

3.1. Characterization of LR Composition. A total of 7477 components were identified in LR. The most abundant top 25 components of both positive ion mode and negative ion mode were shown in Table 1.

3.2. Characteristics of Study Participants. Characteristics of participants are shown in Table 2. At baseline, the average age of the participants was 47.9±5.4 years, the mean BMI was 29.85±3.70 kg/m², and the mean percentage of body fat was 41.33±5.27%. Participants had higher physical activities at baseline than at 4 wk and 12 wk (P=0.01). No significant change in dietary energy intake was found across the 12-wk study (P=0.62).

3.3. Effects of LR on Body Composition. Participants showed significant weight loss in response to LR intervention (P < 0.01). The average reduced weight (%) was 0.6% at 4 wk and 50% of the participants achieved a weight loss of 2.5% at 12 wk. In addition, the changes in fat from baseline to 12 wk were -1.77 ± 1.19 kg (P < 0.01). Corresponding change in the visceral fat area was -5.25 cm² (P < 0.01). Change in abdomen circumference was significant at 12 wk (P=0.01). No significant changes were observed in hip circumference, body protein, and muscle (Table 2).

3.4. Metabolic Biomarkers. The change of biochemical markers between baseline and 12 wk was analyzed (Table 3). There were significant decreases in serum leptin, fasting glucose, and IL8 at 12 wk compared with baseline (all P<0.05). We also observed a significant increase in HDL. No changes in TC, TG, LDL, insulin, and C-Peptide were observed. In addition, HOMA-β increased at 12 wk (P<0.05). Moreover, except decreases in ALT and AST, other parameters did not differ between the baseline and 12 wk (Table 3).

3.5. Effects of LR on Gut Microbiota. The gut microbiota was dominated by Firmicutes, Bacteroidetes, and Proteobacteria at the phylum level across the study. A greater community richness was shown at 12 wk compared with baseline (the ACE estimator, P<0.01; the Chao1 estimator, P<0.01). We also detected a significant difference at the observed species level (P<0.01) and increased phylogenetic diversity (P<0.01; the Chao1 estimator, P<0.01), while there is no significant change in Shannon (P=0.99) and Simpson (P=0.85) indices (Figure 1).

We found a significantly decreased Firmicutes-to-Bacteroidetes ratio before and after LR intervention (0.53 versus 0.42, P=0.03) (Figures 2(a) and 2(b)). We further analyzed the beta diversity of gut microbiota. PCoA showed distinct bacterial community structures between baseline and 12 wk (Figure 2(c)). We also identified microbial taxa which accounted for the differences between baseline and 12 wk. In comparison with those at baseline, Bacteroidaceae, Bacteroides, Bacilli, and Lactobacillales were higher while Ruminococcaceae, Enterobacteriaceae, Enterobacteriales, Lachnospiraceae, Clostridia, and Clostridiales were lower at 12 wk (Figure 2(d)).

We further examined whether the gut microbiota differs between those achieving a 2.5% weight loss and those not; however, no significant difference was found on the composition of gut microbiota.

3.6. Safety Assessment. No participants withdrew from the study for discomfort or adverse effects in response to the invention. According to the follow-up, though 2 participants reported mild abdominal pain at the first 2 days, the discomfort was soon relieved. After the study, 6 participants (including 1 participant who had reported mild abdominal discomfort) reported a beneficial effect of LR on promoting defecation. In addition, AST and ALT decreased after the intervention. No difference was found for other measurement of safety assessment.
Table 1: Characterization of chemical compounds of *Ligustrum robustum*.

| ID  | m/z  | Rt(min) | Metabolites                             | Formula     | Me (ppm)   | Rc  |
|-----|------|---------|-----------------------------------------|-------------|------------|-----|
| **Positive mode** |       |         |                                         |             |            |     |
| 1   | 256  | 11.79   | 3-Methylcyclopentadecanone              | C16H30O     | -2.91      | 392814266.4 |
| 2   | 280  | 11.56   | 2-(5,8-Tetradecadienyl)cyclobutanone    | C18H30O     | -0.69      | 189789298.9 |
| 3   | 152  | 1.20    | L-Malic acid                            | C4H6O5      | 3.48       | 888675126.2 |
| 4   | 338  | 12.59   | 13Z-Docosanamide                        | C22H43NO    | -3.19      | 88330756.9  |
| 5   | 338  | 12.05   | 13E-Docosanamide                        | C22H43NO    | -4.16      | 83549663.1  |
| 6   | 136  | 0.69    | 4-Hydroxy-L-threonine                   | C3H3ClO     | -3.71      | 55202432.5  |
| 7   | 129  | 13.31   | cis-3-Chloroallyl aldehyde              | C3H3ClO     | -3.71      | 55202432.5  |
| 8   | 579  | 6.84    | ROHOFOLIN                               | C7H30O14    | -1.14      | 48595635.5  |
| 9   | 247  | 11.85   | 5,7-Diethyl-9-methyl-3E,5E,7E,9E-tridecatraene | C18H34O      | -0.81      | 38479884.8  |
| 10  | 310  | 12.19   | 3,4-Epoxy-6Z,9Z-eicosadiene             | C20H36O     | 2.35       | 26746448.6  |
| 11  | 163  | 5.23    | 4-Hydroxycoumarin                       | C9H6O3      | -4.18      | 2346225.0   |
| 12  | 303  | 6.49    | Delphinidin                             | C15H10O7    | -4.26      | 21141278.3  |
| 13  | 455  | 7.24    | Ampdopsin D                             | C28H22O6    | 0.65       | 20516556.0  |
| 14  | 312  | 10.97   | 8E-Heneicosene                          | C21H42      | -1.90      | 18469775.4  |
| 15  | 284  | 1.19    | Guanosine                               | C10H13N5O5  | -3.09      | 17980999.0  |
| 16  | 309  | 5.74    | p-Coumaroyl-D-glucose                   | C15H18O8    | -2.65      | 16642683.2  |
| 17  | 611  | 6.48    | Rutin                                   | C27H30O16   | -0.83      | 16302462.1  |
| 18  | 151  | 5.71    | 4-Hydroxyphthalalde                      | C8H6O3      | -4.75      | 16973233.7  |
| 19  | 147  | 6.50    | 3(S)-hydroxy-l-3-cis-docosenoyl-CoA      | C4H4CIN3    | -1.02      | 15818105.7  |
| 20  | 243  | 5.86    | 3-Furanmethanold glucoside              | C11H16O7    | -3.69      | 15268333.3  |
| 21  | 309  | 9.04    | 6-O-p-Coumaroyl-D-glucose               | C15H18O8    | -2.11      | 15264538.9  |
| 22  | 163  | 4.25    | UMBELLIFERONE                           | C9H6O3      | -0.74      | 15248352.7  |
| 23  | 455  | 7.90    | Benzyl gentiobioside                    | C19H28O11   | 3.86       | 11520413.1  |
| 24  | 284  | 11.68   | Octadecanamide                          | C18H37NO    | -2.35      | 10503591.6  |
| 25  | 309  | 6.91    | 3-Hydroxy-4,6-heptadiyne-1-yl-1-glucoside | C13H18O7   | 3.03       | 10093831.0  |
| **Negative mode** |       |         |                                         |             |            |     |
| 1   | 737  | 7.16    | N-Deisopropyl-fluvastatin               | C2H20FNO4   | -3.49      | 161866859.3 |
| 2   | 785  | 6.28    | Echinacoside                            | C3H5H6O20   | -0.09      | 93138299.4  |
| 3   | 625  | 8.07    | PI(22.6(4Z,7Z,10Z,13Z,16Z,19Z)/0.0)     | C3H14H9O2P  | 3.38       | 64107509.6  |
| 4   | 769  | 6.87    | Nilvadipine                             | C19H9N3O6   | 2.80       | 63743793.5  |
| 5   | 753  | 7.09    | Kinetin-7-N-glucoside                   | C16H9N5O6   | -4.83      | 51797213.7  |
| 6   | 389  | 7.01    | Monotropein                             | C16H22O11   | -3.80      | 46844488.5  |
| 7   | 693  | 5.81    | Methyl 3,4,5-trimethoxycinnamate         | C2H142O19   | 0.18       | 43832212.1  |
| 8   | 621  | 8.36    | Tolazamide                              | C4H11N3O3S  | 2.36       | 31866436.9  |
| 9   | 641  | 7.43    | Famiclovir                              | C4H19N5O4   | 0.87       | 30737133.3  |
| 10  | 553  | 8.09    | Gibberellin A37 glucosyl ester          | C26H36O10   | -1.60      | 30651726.6  |
| 11  | 639  | 6.42    | Didymin                                 | C28H34O14   | -0.45      | 19767532.3  |
| 12  | 623  | 6.47    | Acteoside                               | C2H26O15    | 0.09       | 19487425.6  |
| 13  | 571  | 6.11    | Demethylxyleuropepe                     | C24H30O13   | 1.29       | 12702192.5  |
| 14  | 282  | 1.19    | 8-[(Aminomethyl)sulfanyl]-6-sulfanyloctanoic acid | C9H9NO2S2   | -4.38      | 12218110.0  |
| 15  | 477  | 7.61    | Ala Leu Phe Glu                         | C23H34N4O7  | -4.65      | 11066566.9  |
| 16  | 725  | 9.24    | Licorice glycoside A                    | C36H38O16   | 4.95       | 10528525.7  |
| 17  | 461  | 7.87    | Neryl rhamnosyl-glucoside              | C22H38O10   | -1.66      | 10026632.6  |
| 18  | 533  | 0.63    | 3-Fucosylactose                         | C18H32O15   | 1.30       | 10007452.3  |
| 19  | 745  | 7.51    | Pentacarboxyl porphyrinogen III         | C37H40N4O10 | 3.66       | 914090.1    |
| ID | m/z | Rt(min) | Metabolites                                      | Formula         | Me (ppm) | Rc       |
|----|-----|---------|-------------------------------------------------|-----------------|----------|----------|
| 20 | 493 | 7.98    | Neryl arabinofuranosyl-glucoside                | C21H36O10       | -1.92    | 8954593.3|
| 21 | 393 | 4.78    | Foeniculoside VIII                              | C16H28O8        | -2.70    | 8892977.2|
| 22 | 461 | 8.12    | 3-O-(alpha-L-rhamnopyranosyl-(1-2)-alpha-L-rhamnopyranosyl)-3-hydroxydecanoic acid | C22H40O11       | -1.01    | 8361152.7|
| 23 | 739 | 6.42    | Rubrofusarin 6-[glucosyl-(1>3)-glucosyl-(1>6)-glucoside] | C33H42O20       | 1.62     | 7957872.9|
| 24 | 327 | 8.30    | Corchorifatty acid F                            | C18H32O5        | -4.39    | 7845309.6|
| 25 | 551 | 7.06    | Caryatin glucoside                              | C24H26O12       | 0.79     | 7582397.4|

Rt, retention time; Me, mass error; Rc, relative content.
4. Discussion

In the current study, we found that LR intervention for 12-wk showed favorable impact on weight, body fat, visceral fat area, HDL, leptin, and abdominal circumference among overweight and obese female participants. In addition, we found that LR intervention decreased the Firmicutes-to-Bacteroidetes ratio, increased richness, and altered representative taxa of the gut microbiota. Our observation in overweight and obesity middle-aged females is in line with our previous animal study, in which we found that LR extractive intake decreased weight and changed gut bacteria in rat [11]. A recent mice study also showed the antiobesity effects of phenylpropanoid glycosides extracted from LR [9].

Gut microbiota has been demonstrated to be a cause of obesity [3]. Efficacy of energy harvest from food can be affected by the composition of gut microbiota [18]. Modifying the intestinal microbial community by diet has become a new approach to prevent and treat obesity [4, 19, 20]. Obesity has been associated with a low bacterial richness [21, 22]. Dietary and several natural beverage plants have shown modification on the composition of gut microbiota [18, 23, 24]. In the present study, we found that richness of the gut microbiota was higher at 12 wk than baseline, and this change was similar to the effect caused by energy-restricted diet intervention and Roux-en-Y gastric bypass surgery [18, 25]. Though mechanisms remain unknown, our data suggest that bioactive compounds such as plant polysaccharides contained in LR [7] may partly account for the effects, and such postulation is supported by the study conducted by De Filippo et al., in which diet nutrients were associated with bacterial richness and diversity [26].

We also found that drinking LR for 12 wks resulted in a significant decrease in the ratio of Firmicutes to Bacteroidetes (F/B ratio). A study showed that, in obese individuals, the F/B ratio was higher than that of lean individuals. The increase of the abundance of Bacteroides was associated with the percentage of weight loss caused by fat restricted or carbohydrate restricted diet [20]. Bariatric surgery was also found to diminish the F/B ratio during weight loss [27]. Thus, the decreased body weight observed in our study might be related to the regulation of F/B ratio [20, 28]. In addition, bacterial communities clustered according to prior and after LR intervention, highlighting the fact that the enrichments and diversity of gut microbiota were affected by LR [29]. Furthermore, human twins study revealed that obesity was associated with the altered representation of bacterial genes [22]. We showed a significantly different abundance of specific taxa between baseline and 12 wk. We noted an increase in Lactobacillales group (which was in line with our previous rat study) and a decrease in Clostridiales and Lachnospiraceae groups with the same trend as other weight loss related studies described [30]. A previous study has shown that excessive fat accumulation was associated with a low-grade chronic inflammatory [31]. The endotoxin activity of LPS from members of the families Enterobacteriaceae is 1000-fold higher than that from Bacteroidaceae [3]. As an endotoxin, LPS is the major component of Gram-negative bacteria that causes inflammation related to obesity. We observed an increase in Bacteroidaceae and reduction of Enterobacteriaceae. Therefore, the anti-inflammatory effect of

### Table 2: Characters of the study participants at baseline and 12 wk.

| Characters                        | Baseline             | 4 wk                 | 12 wk                | P       |
|---|---|---|---|---|
| Weight, kg                      | 70.27±8.75          | 69.82±8.69          | 68.50±8.44          | <.01*   |
| BMI, kg/m²                      | 29.85±3.70          | 29.67±3.73          | 29.09±3.54          | <.01*   |
| Fat, kg                         | 29.35±6.84          | 28.64±7.00          | 27.24±7.12          | <.01*   |
| Fat, %                          | 41.33±5.27          | 40.53±5.74          | 39.25±6.37          | <.01*   |
| Protein, kg                     | 8.04±0.70           | 8.12±0.69           | 8.08±0.73           | 0.46    |
| Muscle, kg                      | 22.23±2.11          | 22.49±2.11          | 22.40±2.19          | 0.41    |
| Viscera fat area, cm²           | 107.65, 32.50       | 105.20, 37.10       | 102.40, 28.20       | <.01*   |
| Fitness score                   | 67.00, 8.00         | 67.00, 7.00         | 68.00, 8.00         | 0.03*   |
| Abdomen Circumference, cm       | 95.43±8.19          | 95.56±8.67          | 92.26±8.78          | <.01*   |
| Hip Circumference, cm           | 100.88±5.51         | 100.76±5.47         | 100.28±5.53         | 0.45    |
| Metabolic rate, kcal/d          | 1253.74±76.90       | 1259.46±76.80       | 1261.0±82.03        | 0.59    |
| Physical activity, MET-h/wk     | 140.88, 162.75      | 65.13, 70.75        | 67.90, 0.67         | 0.01*   |

**Dietary intake per day**

| Energy, kcal               | 1998.23±529.47      | NA                   | 1920.45±611.10      | 0.62    |
| Protein, g                 | 68.88±20.03         | NA                   | 65.67±17.18         | 0.55    |
| Fat, g                     | 79.56±38.06         | NA                   | 69.65±24.57         | 0.85    |
| Carbohydrate, g            | 257.36±70.12        | NA                   | 261.81±108.78       | 0.27    |

Data with normal distribution are presented as mean ± SD and data with nonnormal distribution are presented as median, interquartile range. P values were calculated by paired T test for normal distribution variables and Wilcoxon signed-rank test for nonparametric tests. We used linear models and Friedman's test to analyze repeated measurement at baseline and 4 and 12 weeks when needed.

* P<0.05.
Table 3: Metabolic variables at baseline and 12 wk after *Ligustrum robustum* intervention.

| Biomarkers               | Baseline       | 12 wk          | P   |
|--------------------------|----------------|----------------|-----|
| **Lipid profile**        |                |                |     |
| TG, mmol/L               | 1.57±0.96      | 1.48±0.87      | 0.33|
| CHOL, mmol/L             | 4.75±0.74      | 4.72±0.95      | 0.81|
| LDL cholesterol, mmol/L  | 3.00±0.77      | 3.06±0.80      | 0.67|
| HDL cholesterol, mmol/L  | 1.33±0.27      | 1.42±0.33      | 0.01*|
| **Glucose metabolism**   |                |                |     |
| Glucose, mmol/L          | 5.45, 0.85     | 5.27, 0.71     | <.01*|
| Insulin, mU/L            | 9.50, 6.14     | 9.84, 4.75     | 0.48|
| C-Peptide, pmol/mL       | 0.77, 0.41     | 0.77, 0.39     | 0.09|
| HOMA-IR                  | 2.42, 2.56     | 2.24, 1.41     | 0.87|
| HOMA-β, %                | 87.53, 50.88   | 103.99, 62.66  | <.01*|
| **Cytokines and hormones**|               |                |     |
| IL6, pg/mL               | 0.86, 0.47     | 0.68, 0.79     | 0.39|
| IL8, pg/mL               | 6.04, 4.21     | 4.79, 2.45     | 0.02|
| Leptin, pg/mL            | 6856.5, 5203.25| 5628, 3290.25  | <.01*|
| **Safety assessment**    |                |                |     |
| Total protein, g/L       | 73.67±3.94     | 73.99±4.36     | 0.48|
| Globulin, g/L            | 28.36±3.27     | 28.39±3.42     | 0.91|
| Albumin/Globulin (A/G)   | 1.62±0.19      | 1.63±0.18      | 0.58|
| ALB, g/L                 | 45.31±1.79     | 45.60±1.86     | 0.30|
| ALT, U/L                 | 30.90±20.49    | 22.43±9.76     | 0.02*|
| AST, U/L                 | 27.57±11.80    | 22.03±7.35     | 0.01*|
| GGT, U/L                 | 31.37±27.48    | 25.37±20.77    | 0.18|
| DBIL, umol/L             | 3.70±1.24      | 3.63±1.09      | 0.75|
| IDBIL, umol/L            | 6.17±2.57      | 6.50±2.54      | 0.55|
| TBIL, umol/L             | 9.87±3.71      | 10.13±3.55     | 0.73|
| UREA, mmol/L             | 5.30±1.33      | 5.44±1.37      | 0.59|

Data with normal distribution are presented as mean ± SD and data with nonnormal distribution are presented as median, interquartile range. P values were calculated by paired T test for normal distribution variables and Wilcoxon signed-rank test for nonparametric tests.

ALB, albumin; ALT, alanine aminotransferase; AST, aspartic transaminase; CHOL, total cholesterol; DBIL, direct bilirubin; GGT, gamma-glutamyl transferase; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; TBIL, total bilirubin; TG, triglycerides; IDBIL, Indirect bilirubin.

* P < 0.05.

*LR*, which was further confirmed by the downregulation of IL 8 [32], might also account for weight loss induced by *LR* intervention. Another potential mechanism of the weight loss effect induced by *LR* may be through the improvement in metabolic biomarkers. A previous in vitro study demonstrated that *LR* extract could downregulate the activities of α-amylase and α-glucosidase in Caco-2 cell monolayer [33]. α-amylase and α-glucosidase are associated with starch and glycogen digestion and glucose absorption, respectively [34, 35]. Evidence suggests that weight loss is associated with improvement of insulin resistance, β cell function, and a reduced risk of developing type 2 diabetes [36–38]. The increase in HOMA-β, together with the decreases in fasting glucose after the trial, suggest a potential role of *LR* in control of postprandial hyperglycemia. As the product of the adipose-specific *ob* gene, leptin is secreted by adipose tissue and plays a role in regulating food intake and energy expenditure. Compared with lean people, the concentration of leptin in the peripheral circulation is approximately fourfold higher in obese people and this may lead to leptin resistance [39]. We observed a decrease of leptin along with weight loss, which was in accordance with the study performed by May Faraj [40]. With a reduction in leptin level in our study, *LR* intervention presented a potential role in downregulating energy store [41]. However, our result was different with a previous mice study in which the antiobesity effect caused by *LR* was associated with increased leptin [9]. This discrepancy may be due to the different study subjects.

To our knowledge, this is the first intervention trial on the effect of *LR* on the changes in weight, body composition measures, and other obesity-related biomarkers among overweight and obese middle-aged Chinese women. A major strength is that we analyzed the role of *LR* intervention on the modulation of gut microbiota. However, several limitations should be considered. First, because the flavor of *LR* is quite bitter, it was difficult to find an ideal placebo to conduct a blinded controlled study. Lack of participants as a control...
group and the small sample size might lead to uncertainty in our estimates. Second, we only performed analysis in women; further studies in men are needed. Third, data of contraception method which might influence the related measurement was not obtained in the current study. Fourth, we measured body composition by BIA, which is less reliable than dual-energy X-ray absorptiometry. Also, weight loss effects of LR in different doses warrant further studies.

Taken together, this study suggests that daily drinking of LR 10g per day for 12 weeks may improve body weight. This favorable outcome is associated with the alterations of gut microbiota and metabolic status. Our results support consumption of the LR, a traditional beverage, in the treatment of obesity.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors’ Contributions

Tao Zhou, Jingyuan Xiong, and Xiaofang Pei designed research; Tao Zhou, Jiayi Chen, Jiayi Xu, and Sijing Liu conducted research; Tao Zhou and Tianli Zheng collected data. Tao Zhou and Dianjianyi Sun analyzed data; Tao Zhou and Jingyuan Xiong wrote the paper; Lu Qi critical revised the manuscript; Xiaofang Pei had primary responsibility for final content. All authors read and approved the final manuscript.

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Figure 2: Modifications in gut microbiota after *Ligustrum robustum* (baseline and 12 wk). (a)-(b) 16S rRNA sequencing analysis and taxonomy classification of the gut microbiota at the phylum levels. (c) Principal coordinate analysis (PCoA) of the microbial community structure before and after *Ligustrum robustum* drinking (N=28). The analysis was based on the abundances of all class of bacteria acquired by high-throughput sequencing by unweighted UniFrac analysis. The bacterial community of baseline was represented in red circle and 12 wk in blue triangle. (d) Linear discriminant analysis (LDA) effect size (LEfSe) identified taxa most characteristic (increased and decreased abundance) at 12 wk. c, class; o, order; f, family; g, genus; WK0, baseline; WK12, 12 wk.

the United States of America as a joint Ph.D. Student (File no. 201606240145).

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