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Optimized Sugar-Free Citrus Lemon Juice Fermentation Efficiency and the Lipid-Lowering Effects of the Fermented Juice

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Abstract: Aging and obesity make humans more prone to cardiovascular and metabolic syndrome diseases, leading to several serious health conditions, including dyslipidemia, high blood pressure, and sleep disturbance. Hyperlipidemia means elevated blood lipid levels, including cholesterol and triglycerides. This study aimed to explore the hypolipidemic effect of fermented citrus lemon juice using a hyperlipidemic hamster model. Sugar-free lemon juice's fermentation was optimized, and the characteristics of fresh and fermented lemon juice (FLJ) were evaluated and compared. In addition, tests were conducted to determine the appropriate hamster feed for the functional animal tests. Then, experiments were conducted to evaluate the hypolipidemic effect of the FLJ, which contained polyphenols and superoxide dismutase-like activity, on a hyperlipidemic hamster model. This study's pre-fermentation efficiency evaluation found that 21–30 days of bacterial DMS32004 and DMS32005 fermentation of fresh lemon juice provided the best fermentation benefits. In addition, the fermentation evaluation showed that a sugar-free fermentation method provided FLJ with the best benefits. Results showed that the absorption and utilization efficiency of FLJ was higher compared with the unfermented lemon juice. After six weeks of feeding, the total cholesterol and triglyceride values in the blood and liver of the FLJ treatment groups were decreased compared with the high-fat diet (HFD) group. In addition, the blood low-density lipoprotein cholesterol levels were significantly reduced in the FLJ treatment groups compared with the HFD group. In contrast, the blood high-density lipoprotein to low-density lipoprotein cholesterol ratio increased considerably in the FLJ treatment groups, and the total to high-density lipoprotein ratio was significantly lower than in the HFD group. Compared with the HFD group, the total cholesterol content in the FLJ treatment groups' feces increased significantly. This study demonstrated that the sugar-free fermentation method and fermentation cycle management provided FLJ with the potential to regulate blood lipids. Further research and verification will be carried out to isolate specific substances from the FLJ and identify their mechanisms of action.

Keywords: efficiency; hyperlipidemia; fermented lemon juice; fermentation evaluation; lipid-lowering

1. Introduction

Lemon is a popular citrus fruit that contains bioflavonoids and other bioactive compounds, such as phenolic compounds, organic acids, essential oils, vitamins, carotenoids, pectins, and minerals. Lemons are recognized to prevent diseases and have anticancer, antimicrobial, and lipid-lowering

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effects. Furthermore, lemons have been demonstrated to have a protective effect against cardiovascular diseases [1].

According to the World Health Organization (2021)[2], about 40% of the world's population is obese, and the epidemic of childhood and adult obesity is growing. Obesity can cause serious health problems and increase the risk of heart and circulatory disease, including dyslipidemia, hypertension, and sleep disorders. Hyperlipidemia means that blood has too many lipids, which is characterized by elevated plasma levels of total cholesterol (TC), triglycerides (TGs), and low-density lipoprotein cholesterol (LDL-C), which is 'bad cholesterol' that clogs arteries. Cholesterol, particularly low-density lipoprotein cholesterol, triglycerides, and other fats can build up in the arteries, narrowing the blood vessels and making it harder for blood to pass. In contrast, high-density lipoprotein cholesterol (HDL-C) is protective against heart and blood vessel diseases because it absorbs cholesterol in the blood and takes it to the liver, which eliminates it from the body [3–5].

Studies suggest that the intake of citrus fruits and their juices can prevent cardiovascular disease, which may be related to citrus bioflavonoids. Citrus bioflavonoids are a class of antioxidant compounds, such as naringenin, hesperidin, nobiletin, and tangerine [6,7]. For example, hesperidin has been demonstrated to improve blood lipid regulation in animals with casein-induced hyperlipidemia [8,9]. In addition, hesperidin metabolites improve blood lipid levels, which are speculated to be related to the decreased activity of cholesterol synthase and esterase [10–12].

Previous animal studies have demonstrated that fermentation improves the bioavailability of substances, such as flavanols, in orange juice [9]. In studies on the effect of fermentation on the absorption rate of polyphenols in humans, the results showed that the polyphenols in fermented orange juice were absorbed more rapidly after ingestion [13,14].

Unlike rats and mice, which are often used in experiments, hamsters have lipid metabolism pathways similar to humans [15,16]. For example, hampster plasma contains cholesteryl ester transfer protein, absent in rats and mice, which can transfer HDL-C to LDL-C when cholesterol levels are elevated. In addition, hamsters have similar enzymatic pathways in lipoprotein and bile metabolism. Therefore, this study evaluated the preventive effect of fermented lemon juice (FLJ) supplementation on blood lipid regulation by lipid regulation using hyperlipidemic hamsters.

The purpose of this study is to explore the impact of fermentation engineering and its cycle management on the benefits of FLJ. In addition, the study investigated if FLJ had a hypolipidemic effect and identified its effects on hamsters fed a high-fat diet (HFD) to establish if it may effectively prevent obesity.

2. Materials and Methods

2.1. Preparation of Lemon Juice and Fermentation

Contracted farmers supplied organic green lemons from the southern part of Taiwan. After washing, lemon juice is extracted by squeezing whole lemons, including the peel and seed. To prepare the FLJ, the extracted lemon juice was inoculated with cultivated DMS32004 and DMS32005 isolated from organic lemons (Jian Mao Biotechnology Co., Ltd., Kaohsiung City, Taiwan). The yeast concentration was $5 \times 10^6 \sim 5 \times 10^7$ CFU/mL and fermentation was conducted at 28°C at pH 2.3. After 21 days of fermentation, the FLJ was sterilized at 90°C for 15 min and stored in a sealed container at room temperature until used.

2.2. Animal Care

The Institutional Animal Care and Use Committee (IACUC) approved this study (IACUC approval number MG-109328). In addition, all animal experiments were performed following the IACUC protocol. Fifty male Syrian hamsters were purchased from the National Laboratory Animal Center (Taipei, Taiwan). Animals were housed in the MedGaea Life Sciences Institute Animal Room (Medgaea Life Science Ltd., Taipei, Taiwan) in hamster cages under a 12-hour light/dark cycle (6 a.m. light on and at 6 p.m. light off) at an air-conditioned temperature of 22°C ± 3°C. After one week, healthy hamsters were selected for the experiments. Food (LabDiet® 5001; Purina Mills, Inc., Richmond, IN, USA) and purified water were provided ad libitum. The experimental hamsters are randomly divided into five equl groups (10 hamsters per group).

2.3. High-Fat Diet Composition

Hamsters, except the control group, were fed a HFD to establish an animal model of hyperlipidemia. The standard chow (LabDiet® 5001) contained 3.36 kcal/g with 28.5% protein, 13.5% fat, and 58.0% carbohydrates. The HFD was standard chow, 91.7% (wt/wt), supplemented with cholic acid, 0.1% (wt/wt), cholesterol, 0.2% (wt/wt), lard oil, 3% (wt/wt), and soybean oil, 5% (wt/wt). The control group was fed standard feed, whereas the hyperlipidemia model hamsters were fed a HFD daily for six weeks to induce hyperlipidemia.

2.4. Remedy Designs

Animals were randomly assigned to five groups (n = 10 each) of similar average body weight as follows: control (standard chow), HFD without FLJ (HFD), HFD with low-dose FLJ (3.1 mL/kg/day), HFD with medium-dose FLJ (6.2 mL/kg/day), and HFD with high-dose FLJ (9.3 mL/kg/day) groups. The hamsters were housed, one per cage, in a controlled environment (22°C ± 3°C, 12-hour light/dark cycle) with free access to food and water during the acclimatization and study periods.

The hamster dose of FLJ was based on the US Food and Drug Administration's human-equivalent dose to estimate the maximum safe starting dose in initial clinical trials for therapeutics in healthy adult volunteers. The recommended use of FLJ (includes polyphenols and superoxide dismutase-like activity) for humans is about 25 mL daily for a normal diet. Therefore, assuming a human weight of 60 kg, the human equivalent dose would be (25 mL/day/60 kg); this equates to a hamster dose of 3.1 mL/kg/day using the conversion coefficient 7.4 to account for differences in body surface area between hamsters and humans. The FLJ administered dose in this test is once (low-dose), double (medium-dose) and triple (high-dose) of the recommended human dose.

2.5. Data Collection

Collection of blood: At the end of 6 weeks, all animals were fasted for 12 hours. The blood samples were obtained by cardiac puncture under isoflurane inhalation anesthesia. The fresh blood was collected, settled for 2 hours, and centrifuged at 1,200 × g for 15 min to obtain the serum samples.

Collection of feces: Body weight was measured every week, and feces were collected during the final 3 days of the experiment for analysis.

Collection of tissue samples: The hamsters were sacrificed simultaneously at the end of the experimental period under isoflurane inhalation anesthesia, and the heart, liver, spleen, and kidney were removed immediately. The organs were weighed after washing with normal cold saline and sucked updry. The largest lobe of the liver is stored at −70°C for further use.

2.6. Determination of Serum Total Cholesterol and Triglycerides

The hamster serum collected as described previously was assayed for levels of TC, TG, using a BioTek Eon microplate spectrophotometer (Agilent Technologies Inc., Santa Clara, CA, USA).

Total triglycerides: Serum (10 µL) was combined with 1 mL Triglyceride FS 5′ multi-purpose kit (Cat No. 1 5760 99 10 023; DiaSys Diagnostic Systems GmbH, Holzeim, Germany) at 37°C for 10 min. The resulting sample's absorbance was determined at the optical density at 500 nm (OD500) and compared with the calibrator TruCal U (Cat No. 5 9100 99 10 064; DiaSys Diagnostic Systems GmbH, Holzeim, Germany) using the following formula:

Triglyceride (mg/dL) = (ODSample − ODblank) / (ODcalibrator − ODblank) × 135

Where 135 mg/dL is the calibrator concentration and OD is the optical density at 500 nm.

Total cholesterol: Serum (10 µL) serum was combined with 1mL Cholesterol FS 10′ multi-purpose kit (Cat No. 1 1300 99 10 024; DiaSys Diagnostic Systems GmbH, Holzeim, Germany) at 37°C for 10 min. The sample's absorbance was then determined at an OD500 and compare with the calibrator

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TruCal U (Cat No. 5 9100 99 10 064; DiaSys Diagnostic Systems GmbH, Holzeim, Germany) using the following formula:

Cholesterol (mg/dL) = (ODSample − ODblank) / (ODcalibrator − ODblank) × 155

Where 155 mg/dL is the calibrator concentration and OD is the optical density at 500 nm.

2.7. Determination of Serum Lipoprotein-Cholesterol Concentrations

The centrifugation method specified by the Taiwan Food and Drug Administration (Evaluation method for regulating blood lipid function of healthy food, No. 0960403114) was used to determine the serum samples' very-low-density lipoprotein cholesterol (VLDL-C), LDL-C, and HDL-C. First, 0.5 mL serum was added to 2.5 mL sodium bromide (NaBr) (Density [D] = 1.006 g/mL), and the samples were centrifuged at 453,000 × g for 3.5 hours at 4°C; 0.5 mL of the supernatant's top fraction, VLDL-C (D ≤ 1.006 g/mL), was collected. Then 0.5 mL NaBr (D = 1.230 g/mL) was added, and the sample was centrifuged at 453,000 × g for 3.5 hours at 4°C. Then, 1 mL of the supernatant's top fraction, LDL-C (1.006 g/mL < D ≤ 1.063 g/mL), was collected. Finally, 1.5 mL NaBr (D = 1.406 g/mL) was added before centrifuging at 453,000 × g for 3.5 hours at 4°C; the top fraction was HDL-C (1.063 g/mL < D ≤ 1.210 g/mL). The various lipoproteins were measured using a Cholesterol FS 10′ multi-purpose kit.

2.8. Determination of Total Cholesterol and Triglycerides in the Liver

The protocol of Folch et al. [17] was used to determine the TC and TG in the hampsters' livers. First, chloroform:methanol (2:1,v/v) (TEDIA Chloroform, Cat No. CS1332-001; Tedia Company Inc., Fairfield, OH, USA; Macron Fine Chemicals™ Methanol, Lot No. 000209176; Avantor Inc., Radnor, PA, USA) was used to homogenize the liver tissue. The homogenzed sample was then centrifuged at 3,000 × g for 10 min, and the supernatant was collected. Following the protocol of Carlson and Goldfarb [18], 0.9% saline was added to the homogeneous lipid liquid, and it was mixed well before being centrifuged at 1,200 × g for 5 min. The upper supernatant layer was removed, keeping the lipid phase layer, and it was placed in an oven at 95°C until the organic solvent was volatilized. The resulting material was dissolved and mixed with lipid liquid (tert-butyl alcohol:Triton X-100:methanol, 2:1:1) (Sigma-Aldrich® tert-butyl alcohol, Lot No. SHBJ9404; Sigma-Aldrich® Triton™ X-100, Lot No. SLBN2536V, both Merck KGaA, Darmstadt, Germany). The livers' TC and TG ere measured using a Cholesterol FS 10′ multi-purpose kit.

2.9. Determination of Total Cholesterol and Triglycerides in the Feces

The protocol of Folch et al. [17] was used to extract the fecal lipids, which were extracted using chloroform:methanol (2:1, v/v) to homogenize the tissue. The homogenized samples were centrifuged at 3,000 × g for 10 min, and the supernatant was collected. Then, following the protocol of Carlson and Goldfarb [18], 0.9% saline was added to the homogeneous lipid liquid. The solution was mixed well and then centrifuged at 1,200 × g for 5 min. The upper layer of the supernatant was removed, and the lipid phase layer was placed in an oven at 95°C until the organic solvent was completely volatilized. The remaining residue was dissolved and mixed with lipid liquid (tert-butyl alcohol:Triton X-100:methanol, 2:1:1). The TC and TG in the feces were measured using a Cholesterol FS 10′ multi-purpose kit.

2.10. Statistical Analysis

All data was expressed as mean ± standard deviation (SD). Significant differences were established with one-way analysis of variance and Duncan's multiple range test. Statistical significance was considered at $p < 0.05$.

3. Results

3.1. Lemon Juice Fermentation Evaluation

First, the total phenol content of the control unfermented lemon juice and the FLJ were compared to identify the optimal fermentation time. The results showed that the total phenol content on the 21st and 28th days of fermentation were significantly higher than that of the unfermented lemon juice (Figure 1).

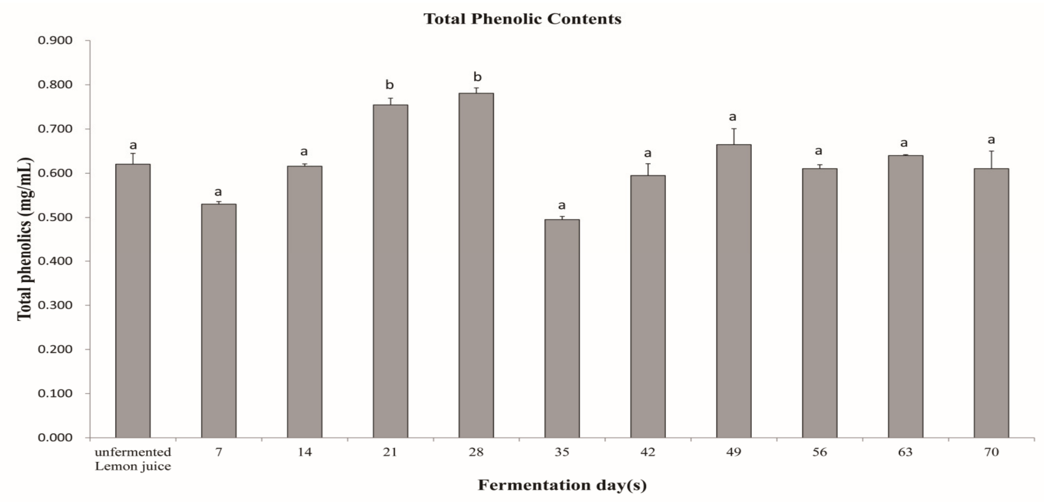

Figure 1. Total phenolic content of the unfermented and fermented lemon juice over time (days). Data are mean ± SD. Values with different superscript letters in the same row are significantly different, $p < 0.05$, by one-way analysis of variance and Duncan's multiple range test post hoc tests.

3.2. Changes in the Experimental Hamster's Body and Organ Weights

During the test period, the test animals' activity, coat color, and reactions were normal, and there were no cases of hair loss, abnormal clinical symptoms, or death. There was no significant difference in the body weight of the experimental animals in each group (Tables 1 and 2). In terms of the relative weight of the liver (g/100g body weight), the HFD group's liver weight was significantly higher compared with the control group ($p < 0.05$) (Tables 1 and 2).

Table 1. Weeks 0–4 average body weights of the treatment groups.

Groups	FLJ dose (mL/kg)	Body weight (g)				
		Week 0	Week 1	Week 2	Week 3	Week 4
Control	-	114.90 ± 15.69[a]	113.77 ± 15.29[a]	121.1 3± 13.38[a]	125.91 ± 12.48[a]	130.52 ± 12.81[a]
HFD	-	114.80 ± 14.90[a]	112.20 ± 16.21[a]	121.11 ± 14.14[a]	127.75 ± 14.43[a]	133.79 ± 14.24[a]
Low-dose	3.1	112.74 ± 3.30[a]	111.59 ± 12.31[a]	119.54 ± 12.55[a]	125.73 ± 10.91[a]	130.53 ± 12.47[a]
Medium-dose	6.2	114.59 ± 12.99[a]	111.96 ± 13.23[a]	121.60 ± 12.55[a]	128.25 ± 11.59[a]	133.64 ± 12.30[a]
High-dose	9.3	116.46 ± 11.77[a]	113.48 ± 13.23[a]	122.56 ± 10.18[a]	129.86 ± 11.05[a]	133.99 ± 11.42[a]

#Week 0: Body weight on Day 0 at the start of the trial; Data are mean ± SD, n = 10 hamsters per group. Values with different superscript letters in the same row differ significantly ($p < 0.05$). FLJ, fermented lemon juice; HFD, high-fat diet.

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Table 2. Weeks 5 and 6 average and the initial and final body weights of the treatment groups.

Groups	FLJ dose (mL/kg)	Body weight (g)				Liver relative percentage (g/100g BW)
		Week 5	Week 6	Before fasting	Fasted	
Control	-	134.24 ± 13.48[a]	137.89 ± 13.41[a]	142.64 ± 14.16[a]	132.49 ± 13.27[a]	3.564 ± 0.297[a]
HFD	-	137.83 ± 13.57[a]	142.59 ± 14.15[a]	147.82 ± 14.01[a]	137.24 ± 13.83[a]	5.852 ± 0.498[a]
Low-dose	3.1	135.11 ± 12.85[a]	138.97 ± 11.12[a]	144.14 ± 10.98[a]	134.22 ± 10.77[a]	4.656 ± 0.206[a]
Medium-dose	6.2	138.39 ± 13.11[a]	142.67 ± 12.79[a]	147.38 ± 12.61[a]	137.78 ± 11.82[a]	4.556 ± 0.198[a]
High-dose	9.3	138.16 ± 12.01[a]	142.61 ± 12.53[a]	145.78 ± 13.23[a]	136.61 ± 12.31[a]	4.546 ± 0.228[a]

Data are mean ± SD, n = 10 hamsters per group. Values with different superscript letters in the same row differ significantly ($p < 0.05$). BW, body weight; FLJ, fermented lemon juice; HFD, high-fat diet.

Furthermore, the results showed that the relative weight of the liver in each FLJ dose group significantly differed from that of the HFD group ($p < 0.05$). These results suggested that FLJ could reduce fat accumulation in the liver. During the test period, except for the control group, there was no significant difference in the average daily food intake between the low-dose, medium-dose, and high-dose FLJ groups and the HFD group (Tables 3 and 4).

Table 3. Weeks 0–4 average body weights of the treatment groups.

Groups	FLJ dose (mL/kg)	Feed intake (g/day)		
		Week 1	Week 2	Week 3
Control	-	8.23 ± 0.91[a]	9.03 ± 0.67[b]	9.13 ± 0.90[b]
HFD	-	7.43 ± 1.24[a]	8.50 ± 0.94[a]	8.18 ± 0.59[a]
Low-dose	3.1	7.31 ± 1.06[a]	7.96 ± 0.72[a]	7.86 ± 0.69[a]
Medium-dose	6.2	7.53 ± 0.81[a]	7.90 ± 0.62[a]	7.80 ± 0.86[a]
High-dose	9.3	7.66 ± 0.93[a]	8.06 ± 0.64[a]	7.56 ± 0.65[a]

Data are mean ± SD, n = 10 hamsters per group. Values with different superscript letters in the same row differ significantly ($p < 0.05$). BW, body weight; FLJ, fermented lemon juice; HFD, high-fat diet.

Table 4. Average daily feed intake of the treatment group: Weeks 4–6.

Groups	FLJ dose (mL/kg)	Feed intake (g/day)		
		Week 4	Week 5	Week 6
Control	-	9.22 ± 0.83[b]	9.00 ± 0.65[b]	9.16 ± 0.96[b]

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HFD	-	8.39 ± 0.79[a]	8.24 ± 0.92[a]	7.81 ± 0.86[a]
Low-dose	3.1	7.81 ± 1.19[a]	8.03 ± 0.45[a]	7.81 ± 0.60[a]
Medium-dose	6.2	7.91 ± 0.65[a]	7.94 ± 0.64[a]	7.84 ± 0.38[a]
High-dose	9.3	7.70 ± 0.82[a]	7.80 ± 0.62[a]	7.60 ± 1.00[a]

Data are mean ± SD, n = 10 hamsters per group. Values with different superscript letters in the same row differ significantly ($p < 0.05$). FLJ, fermented lemon juice; HFD, high-fat diet.

3.3. Total Cholesterol, Triglycerides, and Lipoprotein Levels in the Serum

After the experimental period of 6 weeks, the serum TG and TC values of each FLJ dose group were significantly lower compared with the HFD group ($p < 0.05$), as shown in Table 5.

Table 5. Serum total cholesterol (TC) and triglyceride (TG) values in the treatment groups.

Groups	FLJ dose (mL/kg)	TC (mg/dL)	TG (mg/dL)
		Week 6	Week 6
Control	-	104.15 ± 12.34[a]	61.62 ± 10.42[a]
HFD	-	268.37 ± 22.17[c]	209.04 ± 24.44[d]
Low-dose	3.1	218.48 ± 28.4[b]	131.08 ± 52.38[c]
Medium-dose	6.2	213.7 ± 12.81[b]	104.76 ± 34.85[b]
High-dose	9.3	210.89 ± 22.96[b]	97.3 ± 37.2[b]

Data are mean ± SD, n = 10 hamsters per group. Values with different superscript letters (a–d) in the same row differ significantly ($p < 0.05$). FLJ, fermented lemon juice; HFD, high-fat diet.

The HDL-C and LDL-C values in the serum are shown in Table 6. The HDL-C of each experimental group was not significantly increased compared with the HFD group ($p > 0.05$). However, the LDL-C of each FLJ dose group was significantly lower compared with that of the HFD group ($p < 0.05$). These results suggest that FLJ can effectively reduce the blood's TC, TG, and LDL-C concentrations.

Table 6. Serum lipoprotein cholesterol values of the treatment groups.

Groups	FLJ dose (mL/kg)	VLDL-C (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)
		Week 6		
Control	-	14.02 ± 3.73[a]	20.25 ± 3.39[a]	69.79 ± 11.05[a]
HFD	-	64.26 ± 6.80[c]	107.67 ± 10.37[c]	94.14 ± 11.54[b]
Low-dose	3.1	54.47 ± 11.51[b]	70.52 ± 13.85[b]	93.33 ± 8.24[b]
Medium-dose	6.2	53.56 ± 7.87[b]	66.43 ± 10.81[b]	93.47 ± 8.17[b]
High-dose	9.3	51.90 ± 11.55[b]	63.31 ± 12.95[b]	90.06 ± 12.31[b]

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Data are mean ± SD, n = 10 hamsters per group. Values with different superscript letters (a–c) in the same row differ significantly ($p < 0.05$). FLJ, fermented lemon juice; HFD, high-fat diet; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; VLDL-C, very-low-density lipoprotein cholesterol.

The elevated ratio of HDL-C/LDL-C is a negatively correlated risk factor for atherosclerosis disease [19,20]. Compared with the HFD group, the HDL-C/LDL-C ratio of each FLJ dose group was significantly increased ($p < 0.05$). However, the TC/HDL-C ratios of all the FLJ dose groups were significantly lower compared with the HFD group ($p < 0.05$) (Table 7).

Table 7. Serum lipoprotein cholesterol values of the treatment groups.

Groups	FLJ dose (mL/kg)	HDL-C/LDL-C	TC/HDL-C
		Week 6	Week 6
Control	-	3.555 ± 0.971^{c}	1.505 ± 0.123^{a}
HFD	-	0.880 ± 0.119^{a}	2.868 ± 0.234^{c}
Low-dose	3.1	1.366 ± 0.273^{b}	2.339 ± 0.222^{b}
Medium-dose	6.2	1.416 ± 0.322^{b}	2.310 ± 0.260^{b}
High-dose	9.3	1.510 ± 0.524^{b}	2.402 ± 0.555^{b}

Data are mean ± SD, n = 10 hamsters per group. Values with different superscript letters (a-c) in the same row differed significantly ($p < 0.05$). FLJ, fermented lemon juice; HFD, high-fat diet; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol; VLDL-C, very-low-density lipoprotein cholesterol.

3.4. Total Cholesterol and Triglyceride Levels in the Liver

The test results showed that the TC and TG contents in the HFD group's livers were significantly higher compared with those of the control group ($p < 0.05$). Compared with the HFD group, the livers' TC and TG concentration were significantly decreased compared with each FLJ dose group ($p < 0.05$) (Table 8).

Table 8. Total cholesterol (TC) and triglyceride (TG) levels in the livers of the treatment groups.

Groups	FLJ dose (mL/kg)	Liver	
		TC (mg/g)	TG (mg/g)
Control	-	1.651 ± 0.506^{a}	1.763 ± 0.975^{a}
HFD	-	41.598 ± 4.148^{d}	30.866 ± 7.467^{c}
Low-dose	3.1	26.989 ± 4.664^{c}	15.187 ± 4.324^{b}
Medium-dose	6.2	24.821 ± 6.206^{c}	17.890 ± 2.458^{b}
High-dose	9.3	19.312 ± 2.491^{b}	15.484 ± 3.706^{b}

Data are mean ± SD, n = 10 hamsters per group. Values with different superscript letters (a–d) in the same row differed significantly ($p < 0.05$). FLJ, fermented lemon juice; HFD, high-fat diet.

3.5. Total Cholesterol and Triglyceride Levels in the Feces

At the end of the sixth week, the TC and TG levels in the feces of each FLJ dose group were significantly higher than those of the HFD group ($p < 0.05$) (Table 9).

Table 9. Fecal triglycerides (TG) and total cholesterol (TC) levels in the feces.

Groups	FLJ dose (mL/kg)	Feces	
		TC (mg/g)	TG (mg/g)
Control	-	1.038 ± 0.150^{a}	1.272 ± 0.116^{a}
HFD	-	6.281 ± 0.748^{b}	1.611 ± 0.201^{b}
Low-dose	3.1	8.359 ± 1.105^{c}	2.136 ± 0.190^{c}
Medium-dose	6.2	9.095 ± 0.998^{d}	2.358 ± 0.297^{cd}
High-dose	9.3	9.830 ± 1.463^{c}	2.584 ± 0.299^{d}

Data are mean ± SD, n = 10 hamsters per group. Values with different superscript letters (a–d) in the same row differ significantly ($p < 0.05$). FLJ, fermented lemon juice; HFD, high-fat diet.

4. Discussion

Chronic diseases are major health problems faced by most countries. It is generally believed that excessive total calories, alcohol, and fat intakes, lack of exercise, and such as disorders of metabolism are the leading causes of hyperlipidemia [21]. Common lipid-lowering drugs can be divided into four types: hydroxymethylglutaryl-CoA reductase inhibitors (statins), fibric acid derivatives (fibrates), niacin (nicotinic acid), and bile acid sequestrants; however, these drugs have many side effects [22]. Previous studies have shown that fermented orange juice attenuates dietary glycemic and triglyceride responses [23]. Therefore, diet and preventing lipid deposition in blood vessels and the liver may provide a solution for improving this chronic syndrome [24–26].

The literature shows that bioflavonoids in citrus may prevent neovascular diseases. For example, bergamot (Citrus bergamia) juice significantly reduces serum cholesterol, triglycerides, and LDL and increases serum HDL levels [27]. The flavonoids in lemons, including hesperidin, eriocitrin, naringin, and narirutin, are often present in glycosylated forms. Among them, naringenin has been shown to have an anti-inflammatory effect and hypolipidemic activity, while naringenin has a hypocholesterolemic effect [28–30]. Flavonoid substances are often degraded by heat treatment. In contrast, the fermentation process can enhance the production of bioactive extractable phytochemicals, ensuring that heat-treated products retain bioactive components and improving bioavailability [31].

This experiment evaluated the blood lipid regulation effect of FLJ in hamsters with HFD-induced hyperlipidemia. Unlike the typical fermentation with sugar and a long fermentation time, this study used organic lemon raw materials and selected specific strains DMS32004 and DMS32005 of bacteria to shorten the fermentation time without added sugar, improving the fermentation efficiency through fermentation management effects. The subjects who consumed sugar-free FLJ had similar indices and doses as the unfermented lemon juice group; the data showed that the polyphenol concentrations in the subjects' blood of the FLJ group at 1.5 and 3 hours were higher than those in the unfermented lemon juice group, indicating higher absorption and utilization of polyphenols. The fermentation cycle evaluation demonstrated that the total phenolic content of the FLJ was 0.754mg/mL on the 21st day of fermentation and 0.781mg/mL on the 28th day, and the total phenolic content of unfermented lemon juice was 0.620mg/mL; furthermore, the total phenolic content was higher than unfermented lemon juice. 21-day fermentation was chosen for this study based on the

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fermentation evaluation results. Therefore, fermentation process has been reported to produce higher levels of bioactive extractable phytochemicals [8,9].

Elevated cholesterol or triglyceride levels can easily cause vascular endothelial cell dysfunction. In addition, lipoproteins can freely enter and exit the blood vessel walls when their concentration in the blood is too high. Moreover, lipoproteins can accumulate in the inner layer of the arterial vessel walls, causing local inflammation and attracting monocytes to adhere to the blood vessels' inner layer, differentiating them into macrophages, which devour oxidized LDL-C to form foam cells. The death and accumulation of foam cells formed by macrophages, coupled with the proliferation and repair of connective tissue, may create early fatty streaks. If arteriosclerosis continues, then atherosclerotic plaque will be formed. Macrophages also secrete some cytokines, stimulate the proliferation of smooth muscle cells on the vessel wall, cause plaque fibrosis, accelerate arteriosclerosis, make the vessel lumen smaller, and make blood flow difficult [32,33].

In addition to causing heart disease, hyperlipidemia is closely related to chronic conditions, such as strokes, high blood pressure, diabetes, and kidney disease. The LDL-C/HDL-C ratio is reported to be a more valuable biomarker than LDL-C or HDL-C levels alone, especially for predicting the risk of multiple diseases. If the ratio is low, atherosclerosis risk factors are reduced [34–36]. Based on the experimental results obtained from the sugar-free fermentation method and fermentation cycle, the hypolipidemic effect of FLJ on the hyperlipidemic hamster model was investigated. This study's data after six weeks showed that FLJ could effectively reduce the concentration of TC, TG, and LDL-C in blood. Compared with the negative control group, the ratio of HDL-C/LDL-C in each FLJ group was significantly increased, while the TC/HDL-C ratio was significantly reduced ($p < 0.05$).

A normal liver contains 4%–5% of the liver weight as fat. If the fat content exceeds 10%–15% of the liver weight, it is called fatty liver disease, typically an increase of oil in the whole liver [37]. More than half of patients with hyperlipidemia also suffer from fatty liver. In addition, patients with high triglycerides are more likely to suffer from fatty liver compared with those with high cholesterol. Fatty liver is generally divided into two categories according to different causes, namely alcoholic and non-alcoholic [38,39]. Hyperlipidemia and obesity are also common risk factors for non-alcoholic fatty liver disease[40]. Some animal studies have shown that a high-cholesterol diet with hesperidin can reduce triglycerides in blood lipids and increase cholesterol content in feces [41]. The HFD group was fed a high-fat and high-cholesterol feed, which resulted in excessive fatty liver accumulation. Therefore, the relative liver weight (g/100g body weight) of the HFD group was significantly increased compared with the control group. In this study, the liver's TC and triglyceride levels showed that FLJ effectively reduced the blood's TC, TG and LDL-C concentrations.

After six weeks of FLJ supplementation, the feces from all hamster groups were collected and analyzed for TC and TG. The test results showed that the contents of TC and TG in the feces of each FLJ dose group were significantly higher than those in the HFD group. Therefore, supplementing FLJ can promote the excretion of fecal TC and TG.

This study demonstrated the sugar-free fermentation method and fermentation cycle management of lemon juice. The potential use of FLJ without added sugar in regulating blood lipids was investigated. Futurer research and verification will be carried out to isolate specific substances from the sugar-free FLJ and examine their mechanism of action.

5. Conclusions

This study showed that FLJ could reduce the content of TC, TG, LDL-C, and TC/HDL-C in blood, reduce the content of TC and TG in the liver, improve the HDL-C/LDL-C ratio, and promote the excretion of TC and TG in the feces. Therefore, based on this study's results, the effect of fermentation engineering and its cycle management on fermentation benefits can optimize fermentability. The results of the study confirmed that FLJ produced by the sugar-free fermentation method and fermentation evaluation method had the effect of regulating blood lipids in hyperlipidemic hamsters.

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Data Availability Statement: We encourage all authors of articles published in MDPI journals to share their research data. In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Where no new data were created, or where data is unavailable due to privacy or ethical restrictions, a statement is still required. Suggested Data Availability Statements are available in section "MDPI Research Data Policies" at https://www.mdpi.com/ethics.

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