Evaluation of the efficacy and safety of *Ganoderma lucidum* mycelium-fermented liquid on gut microbiota and its impact on cardiovascular risk factors in human†

Qizheng Wu, a Houcheng Zhang, a Peng Gorge Wang*ab and Min Chen a,*ab

*Ganoderma lucidum* is a Chinese traditional medicine with various bioactivities. However, the impacts of a *Ganoderma lucidum* mycelium-fermented liquid (GLFL), as a promising alternative product, on the gut microbes and cardiovascular risk factors have not been explored in humans to date. In this study, the composition of polysaccharides in GLFL was analyzed, and eight volunteers were fed with GLFL to investigate its influence on gut microbes and cardiovascular risk factors. High-throughput 16S rRNA gene sequencing technique was utilized to investigate the effects of GLFL on the diversity of the bacterial communities and the composition of gut microbiota. The results demonstrated that GLFL significantly altered β diversity and several phyla of gut microbiota in volunteers. Moreover, GLFL provided protection to humans by promoting the growth of probiotics, incorporating genus *Lactobacillus* (p < 0.05), and reducing pathogens containing genus *Campylobacter* and *Agregatibacter* (p < 0.05); however, GLFL also had detrimental effects as it increased the population of *Firmicutes/Bacteroidetes* and opportunistic pathogens including genus *Acinetobacter*, *Pseudomonas*, *Serratia*, *Stenotrophomonas*, *Peptococcus* and *S24-7* (p < 0.05) and reduced that of probiotics genus *Lactococcus*. Finally, we found that GLFL reduced plasma low-density lipoprotein cholesterol (LDL-c) in volunteers. This is helpful for further understanding the effect of *Ganoderma lucidum* or its related products on human body.

Introduction

*Ganoderma lucidum*, which is a traditional medical mushroom, has been used to promote health and longevity. Since *G. lucidum* is rare in nature, as an alternative method, submerged fermentation of *Ganoderma lucidum* mycelium is carried out as it requires shorter time for cultivation. Prior assays indicated that the *G. lucidum* mycelium-fermented liquid (GLFL) included ganoderic acid and extracellular and intracellular polysaccharides as effective constituents.

The *Ganoderma lucidum* mycelium-fermented liquid was verified to strengthen health in vivo. Previous report suggested that components of GLFL had antitumor activity, and recent assays also found that GLFL induced human peripheral blood mononuclear cells (PBMC) to synthesize the tumor necrosis factor α (TNF-α) as a supporting therapy in cancer patients. Moreover, another assay manifested that GLFL had hepatoprotective properties in rat. However, the biological activities and functions of GLFL have not been extensively studied especially in the fields of regulation of gut microbiota and cardiovascular diseases.

The gut microbiota and cardiovascular risk factors play a crucial role in host health. Several reports have found that *Ganoderma lucidum*-related products could regulate the gut microbiota and cardiovascular risk factors. Previous reports suggested that a water extract of *Ganoderma lucidum* mycelium reversed the composition of gut microbiota in obese mice. GLFL was proven to reduce plasma low-density lipoprotein cholesterol (LDL-c) and triglycerides, total cholesterol, and increased high-density lipoprotein cholesterol (HDL-c) in rat. However, it is unclear whether the *Ganoderma lucidum* mycelium-fermented liquid produces any effect on gut microbiota and cardiovascular risk factors in human.

In this study, to evaluate the efficacy and safety of *Ganoderma lucidum* mycelium-fermented liquid, which contained extracellular and intracellular biopolymers, we examined the composition of polysaccharides of GLFL purchased from market, performed a systematic analysis of gut microbiota following oral administration of GLFL by the 16S rRNA sequencing technique, as well as analyzed the plasma cardiovascular risk factors. Our study will provide the foundation of how GLFL influences the human body.
Materials and methods

Volunteers and administration

A cohort of eight healthy volunteers, consisting of equal number of females and males, participated in this study. Volunteers drank 50 mL GLFL every day for one month from April 1 to 30, 2016. The administration was random in a day. Volunteers were needed to conform to all the following conditions: (1) females or males should be ≥20 years of age; (2) they should have normal body mass index (BMI = 18.5–24.9 kg m⁻²); (3) they should have no smoking history, (4) no severe drinking history, and (5) no medical history; (6) they should not be vegetarians; (7) they should not be taking probiotics, prebiotics or antibiotics one month before commencement of the study; and (8) they should not be taking probiotics, prebiotics or antibiotics within one month of enrollment. All procedures of this study involving human participants were performed in compliance with the guidelines of Biomedical research ethics review method involving people (China). The study was approved by the Ethics Committee of Shandong University, and written informed consent was obtained from each volunteer before commencement of the study.

Experimental design

The fresh stool and serum samples were obtained before GLFL administration (named pre-feeding group), and the fresh stool and serum samples were also obtained at the end of the experiment (named post-feeding group). The gut microbiota was extracted from stool and then analyzed by the high-throughput 16S rRNA gene sequencing technique; the plasma lipids and lipoproteins were analyzed by an automatic biochemical analyzer. Finally, the data of the pre-feeding group was compared to the data of the post-feeding group. Moreover, monosaccharides of GLFL were analyzed by gas chromatography/mass spectrometry.

The components of GLFL

In our study, the *Ganoderma lucidum* mycelium-fermented liquid was purchased from a commercial producer (Hongshun Biological Technology Co., Ltd, Weihai, China), which comprised extracellular and intracellular biopolymers, containing 2.9% (w/w) carbohydrate, 0.19% (w/w) protein, <0.1% (w/w) lipids, 96.5% (w/w) water, 0.2% (w/w) dietary fiber, and 0.25% (w/w) other components. The dry biomass of GLFL was ≈3.5% (w/v), whereas the percentage of polysaccharides in GLFL was ≈0.7% (w/v).

Extraction of the GLFL crude polysaccharides

GLFL was mixed with four-fold volume of 100% EtOH at 4 °C for 12 hours to precipitate the GLFL crude polysaccharides. The precipitate was pelleted (15 000 × g, 4 °C, 10 minutes), and then, the supernatant was drained and lyophilized.

Gas chromatography/mass spectrometry analysis for monosaccharides

Monosaccharide acetate derivatization. We took 2 mg of the purified GLFL crude polysaccharide sample and added 1 mL 2 M trifluoroacetic acid to it; the mixture was sealed and then hydrolyzed for 9 h at 105 °C. After cooling, TFA was removed by vacuum evaporation. For this, 2 mL methanol was added, the mixture was dried by vacuum evaporation, and this step was repeated several times to remove TFA. Finally, hydrolyzate was dissolved in 2 mL double distilled water. Hydrolyzate and standard monosaccharides were reduced by 60 mg sodium borohydride for 8 hours, and then, acetic acid was added to decompose excess sodium borohydride. After vacuum evaporation, we added 3 mL methanol and dried it to powder by rotary evaporator. The reaction mixture was dried at 110 °C, and 1 mL acetic anhydride was added to the reaction mixture for 1 h at 100 °C; the reaction mixture was then cooled and 3 mL toluene was added; it was dried four times by vacuum evaporation to remove the excess acetic anhydride. The acetylated product was dissolved in 3 mL chloroform, and then, it was transferred into a separatory funnel. A little amount of distilled water was added to it, and then, the solution was sufficiently shaken. The upper aqueous solution was removed, and above-mentioned process was repeated 4 times. The layer of chloroform was dried with an adequate amount of anhydrous sodium sulfate, and the layer of chloroform was set to 10 mL by adding chloroform for GC-MS analysis.

GC-MS conditions. Hp-5 (Agilent 19091J-413) column (30 m × 0.25 mm × 320 μm); program temperature conditions: starting temperature was 120 °C, temperature increased to 250 °C at 3 °C min⁻¹ and kept at 250 °C for 5 minutes. The temperature of inlet was 250 °C, the temperature of detector was 250 °C, the hydrogen flow rate was 30 mL min⁻¹, the air flow rate was 400 mL min⁻¹; the carrier gas was N₂; and its flow rate was 1 mL min⁻¹.

Collection of stool specimens and gut microbiota

The fresh stool specimens were obtained at the start and end of the experiment. Fresh stool specimens were processed immediately after defecation as follows: in brief, a stool specimen weighing 500 mg was suspended in 10 mL 0.01 M pH 7.4 PBS, and then was centrifuged at 12000 g for 3 min, 25 cycles at 95 °C for 10 minutes to obtain the precipitates containing gut microbiota. The precipitate was resuspended in 10 mL 0.01 M pH = 7.2–7.4 sterile phosphate buffered solution (PBS). Then, the mixture was stirred for 10 minutes. The supernatant was obtained by centrifugation at 100g for 3 minutes. The supernatant was centrifuged at 12 000g for 10 minutes to obtain the supernatants containing gut microbiota. The precipitate was resuspended in 10 mL 0.01 M pH = 7.2–7.4 PBS, and then was centrifuged at 12 000g for 10 minutes to obtain the supernatants. The abovementioned process was repeated 3 times. The bacterial pellets were resuspended in 1 mL sterile PBS and saved at 4 °C for DNA extraction.

DNA extraction from gut microbiota and sequencing

Gut bacterial DNA was extracted using the TIANGEN® TIANamp Bacteria DNA Kit (TIANGEN Biotech Co., Ltd, Beijing, China) according to the manufacturer’s instructions.

The gut bacterial V4 hypervariable region of 16S rDNA was amplified with the universal primer pair B341F (5'-CCTACGGGNGGCWGCAG-3') and B785R (5'-GACTACHVGGGTATCTAATCC-3') using the following conditions: initial denaturation at 95 °C for 3 min, 25 cycles at 95 °C for 30 s, 55 °C for
Bioinformatic analysis

After sequence assembly and sequence filtration, high-quality sequences were clustered into OTUs based on 97% sequence similarity according to USEARCH. OTUs were used for analysis of α diversity (nsqs, sobs, chao, ace, coverage, Shannon, and Simpson) by the mothur method. Rarefaction curves were calculated for all the samples. For β diversity, the OTU sequences were aligned with the SILVA database using PyNAST, and the phylogenetic tree was built with the FastTree software. The unweighed and weighed distance matrix between communities was generated with the UniFrac software, which was the basis of principal coordinates analysis (PCoA).

Analysis of the major cardiovascular risk factors

Herein, 3 mL blood was obtained from 8 volunteers before feeding and after feeding, and then, it was put into the Automatic Biochemical Analyzer, Hitachi 7100 (Hitachi, Ltd, Japan), to analyze the major cardiovascular risk factors.

Statistical analysis

The data of the pre-feeding group as a baseline (control) was compared with the data of the post-feeding group. The MetaStats statistical software was used to test the differences of species abundance between two groups. Student’s t-test was performed to test the differences. Differences were considered significant at \( P < 0.05 \). Data are denoted as mean ± standard deviation (SD).

Results and discussion

Composition of the polysaccharides of GLFL

Ganoderma polysaccharide is considered as one of the beneficial ingredients. However, there is a wide diversity in the chemical composition among different Ganoderma polysaccharides; this results in various bioactivities. Thus, characterization of the composition of polysaccharide is crucial. We analyzed the composition of the polysaccharides by gas chromatography/mass spectrometry. The carbohydrate portion was found to comprise glucose (52.01%), galactose (19.36%), arabinose (6.41%), fucose (4.67%), xylose (7.45%), and mannose (10.11%). Our results supported the data obtained by other researchers indicating that the polysaccharides isolated from Ganoderma consisted of mannose, glucose, arabinose, fucose, xylose, and galactose with different combinations. GLFL changed the diversity of gut bacteria

To address the changes in the gut bacterial structure, we characterized the gut bacterial community diversity in the pre-feeding and post-feeding GLFL group by pyrosequencing of 16S rDNA covering the V4 hypervariable regions. To exclude the influence of host adiposity, age, and geographical location on the gut microbiome, the eight volunteers selected for this study were healthy adults, living in northern China, and had a normal body mass index (BMI = 18.5–24.9 kg m\(^{-2}\)) (ESI Table 1†).

After selecting the high-quality sequences, we obtained 120 259 sequences (mean ± S.D., 15 032 ± 3829) from the pre-feeding group and 89 760 sequences (mean ± S.D., 11 220 ± 2571) from the post-feeding group (Table 1). Moreover, rarefaction curve analysis indicated that sufficient sequences were obtained to conduct the pyrosequencing analysis (Fig. 1A). More OTUs (524 OTUs) were detected in the post-feeding GLFL group as compared to those in the pre-feeding GLFL group (502 OTUs), but both groups shared 410 OTUs (Fig. 1B). Therefore, GLFL assisted in the generation of 22 OTUs.

Although no significant differences in the α diversity were detected between two groups (ESI Table 2†), the β diversity between two groups had evident difference, as observed by calculation of unweighed unifrac. Principal coordinates analysis (PCoA) suggested that the second principal component PC2, which accounted for 20.3% of variance in the data, could completely differentiate the post-feeding group from the pre-feeding groups (Fig. 2). This indicated that the composition of gut microbiota changed greatly after the administration of GLFL.

The composition of fecal microbiota was changed after feeding the GLFL

To address how GLFL altered the gut microbiome, we compared the microbiota composition in the pre-feeding and post-feeding GLFL groups. Taxonomic profiling demonstrated that Firmicutes and Bacteroidetes were the main gut bacteria in two groups.

Table 1 16S rDNA sequences statistics

<table>
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<tr>
<th>Sample</th>
<th>Clean_seqs</th>
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<tbody>
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<td>7859</td>
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<tr>
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<tr>
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<td>18 063</td>
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<td>79.85%</td>
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<tr>
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<td>17 741</td>
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<td>56.91%</td>
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<tr>
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<td>15 492</td>
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<tr>
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<tr>
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<tr>
<td>B8</td>
<td>23 152</td>
<td>14 777</td>
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</tr>
</tbody>
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\( a \) A and B correspond to pre-feeding group and post-feeding group, respectively.
At the phylum level, in the pre-feeding group, the percentage of Firmicutes (F) was 62.98% and that of Bacteroidetes (B) was 23.91%, and F/B = 2.63. However, in the post-feeding group, the percentage of Firmicutes was 68.54% and that of Bacteroidetes was 19.23%, and F/B = 3.56. It was noted that F/B increased 1.35 times, which was consistent with the previous result obtained after feeding Ganoderma lucidum polysaccharides in mice and contrary to the result obtained after feeding water extracts of cultured Ganoderma lucidum mycelium in mice. A previous report suggested that the gut microbiota of obese human and obese mice had a significantly greater F/B ratio. Moreover, GLFL administration increased the population of Acidobacteria (0 vs. 0.0520%, p < 0.05), Lentisphaerae (0 vs. 0.0057%, p < 0.05), Planctomycetes (0.0034% vs. 0.0424%, p < 0.05), and Tenericutes (0 vs. 0.0049%, p < 0.05) at the phylum level. Another feature was that the population of phylum Proteobacteria reduced from 8.822% to 6.758% (p > 0.05) (Fig. 4). This result was contrary to previous result, which suggested that mushroom polysaccharide induced elevation of Proteobacteria. In murine models, recent result suggested that less Firmicutes and more Bacteroidetes and Proteobacteria were associated with low grade intestinal inflammation. Thus, GLFL might reduce the probability of low-grade intestinal inflammation.

Focusing on the genus level allowed us to further understand how GLFL altered the gut microbiome. In this study, there were 39 genera, which were significantly altered (P < 0.05) (ESI Table 3†). Herein, we mainly analyzed numerous genera, which had relation with the health.

There were 12 genera that significantly decreased (Fig. 5A). The population of the Campylobacter genus decreased significantly (p = 0.045) after administration of GLFL. Campylobacter genus is associated with diarrheal disease, and it has been viewed as a crucial risk factor for the development of inflammatory bowel disease. The population of Aggregatibacter also decreased significantly (p = 0.000999) from 0.0105% to 0. Aggregatibacter is a genus of the phylum Proteobacteria, which contains three species, namely A. actinomycetemcomitans, A. actinomyces, and A. nodatum.
**A. aphrophilus**, and **A. segnis**. **A. actinomycetemcomitans** is a causative agent of periodontal disease, and a recent study suggested that **A. aphrophilus** is associated with cerebral abscess. The population of **Succinivibrio** genus decreased from 0.0274% to 0. Previous study indicated that the decreased abundance of **Succinivibrio** was related to parasite infection in pigs. However, the population of two beneficial gut bacteria also decreased in this study. The population of **Lactococcus** genus decreased from 0.2556 to 0.0041. **Lactococcus** genus decreased from 0.0057% to 0 and that of the **Turicibacter** genus increased mostly from 0.0042% to 0.7015%. **Acinetobacter** genus increased from 0.0022% to 0.0304%. Previous study indicated that infectious species, such as **Pseudomonas aeruginosa**, of this genus was a key conditioned pathogen in immunocompromised patients and caused life-threatening lung infections in individuals with cystic fibrosis. The population of the **Serratia** genus increased from 0 to 0.0155%, and this bacterium was an opportunistic human pathogen, causative agent of severe nosocomial infections. The population of the **Stenotrophomonas** genus increased from 0 to 0.086%. The numerous recent literatures reported to date have shown that **Stenotrophomonas maltophilia**, belonging to **Stenotrophomonas** genus, is an opportunistic pathogen that causes nosocomial infections. The population of the **Peptococcus** genus also increased from 0 to 0.0053%. Species in this genus are a normal part of the human gut microbiome, but **Peptococcus magnus** is a human pathogen. Interestingly, the population of the **S24-7**, belonging to the **Bacteroidales** family, increased from 0 to 0.1428%. The recent study indicated that this **Bacteroidales** family was related to the degradation of particular carbohydrates such as α-glucan, host glycan, and plant glycan. In our study, GLFL could contain analogous carbohydrates; thus, the abovementioned family consumed them and then proliferated acutely. Another study suggested that mice fed with a high-fat carbohydrate-free diet had more **S24-7**; thus, **S24-7** might be bad gut microbiota.

Water extracts of the cultured **Ganoderma lucidum** mycelium (WEGL) have been proven to alter the gut microbiota significantly ($P < 0.05$). Chang et al. proved that a water extract of **Ganoderma lucidum** mycelium decreased Firmicutes-to-Bacteroidetes ratios and **Lactobacillus** genus level in mice. However, our results suggested that GLFL elevated the Firmicutes-to-Bacteroidetes ratios and **Lactobacillus** genus level in humans. However, both results suggested that WEGL and GLFL decreased **Proteobacteria** phylum level and **Lactococcus** genus lowering activity, and antioxidant activity. The population of **Acinetobacter** genus increased mostly from 0.0042% to 0.7015%. This genus is the main reason for infection in fragile patients in the hospital, particularly the species **Acinetobacter baumannii**, causing skin infections. As is known, **Acinetobacter** species are found frequently in natural environment such as water and soil, as well as in hospital environment, human skin, and foods. Interestingly, it was also found in gut microbiota in this study. The population of **Pseudomonas** genus increased from 0.0022% to 0.0304%. Previous study indicated that infectious species, such as **Pseudomonas aeruginosa**, of this genus was a key conditioned pathogen in immunocompromised patients and caused life-threatening lung infections in individuals with cystic fibrosis. The population of the **Serratia** genus increased from 0 to 0.0155%, and this bacterium was an opportunistic human pathogen, causative agent of severe nosocomial infections. The population of the **Stenotrophomonas** genus increased from 0 to 0.086%. The numerous recent literatures reported to date have shown that **Stenotrophomonas maltophilia**, belonging to **Stenotrophomonas** genus, is an opportunistic pathogen that causes nosocomial infections. The population of the **Peptococcus** genus also increased from 0 to 0.0053%. Species in this genus are a normal part of the human gut microbiome, but **Peptococcus magnus** is a human pathogen. Interestingly, the population of the **S24-7**, belonging to the **Bacteroidales** family, increased from 0 to 0.1428%. The recent study indicated that this **Bacteroidales** family was related to the degradation of particular carbohydrates such as α-glucan, host glycan, and plant glycan. In our study, GLFL could contain analogous carbohydrates; thus, the abovementioned family consumed them and then proliferated acutely. Another study suggested that mice fed with a high-fat carbohydrate-free diet had more **S24-7**; thus, **S24-7** might be bad gut microbiota.

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level. As GLFL is a complex containing WEGL, extracellular biopolymers, and other compounds, the discrepancy and similarity of the effect of GLFL and WEGL on gut microbiota can be explained by their components. Moreover, the difference of animal model was another reason for discrepancy.

GLFL decreased plasma low-density lipoprotein in humans

Major cardiovascular risk factors, including triglycerides (TG), total cholesterol (TC), glucose, low-density lipoprotein cholesterol (LDL-c), and high-density lipoprotein cholesterol (HDL-c),
were analyzed between pre and post-feeding groups. There was no significant difference between two groups in terms of these factors, but every volunteer after being administered with GLFL exhibited a lower plasma LDL-c (Table 2). This effect may be attributed to the elevation of cholesterol-lowering Lactobacillus genus, as abovementioned. Yang et al. found that the extracts of submerged mycelium culture of Ganoderma lucidum not only reduced low-density lipoprotein cholesterol, but also decreased total cholesterol and triglyceride, and increased high-density lipoprotein cholesterol in rats. Moreover, Tong et al. revealed that Ganoderma lucidum without fermentation decreased the LDL-c, TG, and TC level significantly \((P < 0.05)\), whereas further increased the plasma concentration of HDL-c in rats. However, we found that GLFL only decreased low-density lipoprotein cholesterol in humans. There were two reasons for the discrepancy. First, their animal model was diet-induced hyperlipidemic rat, whereas our model was a healthy human. Second, they utilized the extracts of submerged mycelium culture of Ganoderma lucidum or G. lucidum fruiting body, whereas we used the submerged mycelium culture of Ganoderma lucidum containing all extracts and other contents. Therefore, difference of model and aliment may result in different consequences.

**Conclusion**

When Ganoderma lucidum mycelium-fermented liquid was fed to humans, it profoundly altered the gut microbiota. In the post-feeding group, there was evident difference in \(\beta\) diversity as compared to the case of the pre-feeding group; this suggested that GLFL had altered the composition of gut microbiota. At the phylum level, GLFL increased the population of Firmicutes, whereas it decreased the population of Bacteroidetes and Proteobacteria. At the genus level, there were 39 genera, which were significantly altered \((P < 0.05)\). As we expected, GLFL promoted the growth of probiotics such as Lactobacillus genus, whereas it suppressed the growth of pathogens such as Campylobacter genus. However, we also found that GLFL was unsafe as it increased the population of opportunistic pathogens, such as Acinetobacter genus, and reduced the population of probiotics such as Lactococcus genus. In addition, there was no doubt that GLFL could reduce plasma LDL-c. These results were not identical with previous consequences that were produced from Ganoderma lucidum without fermentation, WEGL or GLFL, which were fed to murine. Based on our results, we appeal to re-examine the effect of Ganoderma lucidum or its derived products on humans and the consequences may be different from previous successful results in murine.

**Conflicts of interest**

There are no conflicts of interest to declare.

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