Effects of dihydroartemisinin on the gut microbiome of mice

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Abstract. Dihydroartemisinin (DHA) is a semisynthetic derivative of artemisinin, which has been found to exhibit a broad range of biological activities, excluding antimalarial effects; however its effects on the gut microbiota remain poorly understood. The present study aimed to investigate the effects of DHA on the gut microbiome in mice and to determine its potential biological and pharmaceutical activities through its alteration of the gut microbiota. Serum glucose, triglyceride (TG), total cholesterol, lipopolysaccharide, high density lipoprotein-cholesterol, low density lipoprotein-cholesterol, alanine aminotransferase and aspartate aminotransferase levels in mice treated with DHA were analyzed using the corresponding detection kits. In addition, hematoxylin and eosin staining was performed to determine the pathological effects of DHA on the liver, kidney and intestinal tissues of mice, and the effects of DHA on the gut microbiome were analyzed using 16S ribosomal (r)DNA gene analysis. The results demonstrated that the TG serum levels of mice treated with DHA were significantly decreased compared with the control group. Furthermore, 16S rDNA gene analysis demonstrated that the bacterial diversity of mice treated with DHA was enriched compared with the control group. The DHA group exhibited increased numbers of *Firmicutes* and *Saccharibacteria*, and decreased *Deferribacteres* and *Actinobacteria* compared with the control group at the phylum level. Kyoto Encyclopedia of Genes and Genomes signaling pathway enrichment analysis also revealed that the signaling pathways associated with 'Energy metabolism' and 'Nucleotide metabolism' were upregulated, whereas the signaling pathways associated with 'Infectious diseases and 'Neurodegenerative diseases' were downregulated in the DHA group compared with the control group. In conclusion, the findings of the present study indicated that DHA may significantly decrease the serum TG levels and alter the gut microbiota, which suggested its potential to be used for the treatment of hyperlipidemia, inflammatory and neurodegenerative disorders.

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Introduction

Artemisinin is a sesquiterpene lactone that is isolated from the Chinese plant Artemisia annua (Qinghao) (1). Artemisinin has been reported to possess antimalarial properties and it has also been discovered to serve roles in several types of disease, including different types of cancer and inflammatory disorders (1-3). Dihydroartemisinin (DHA) is a semisynthetic derivative of artemisinin that has exhibited a broad range of biological activities, excluding antimalarial effects (2). For example, Chen *et al* (4) demonstrated that DHA ameliorated liver damage, steatosis and hepatocyte lipoapoptosis of alcoholic fatty liver model mice by regulating lipin-1 signaling. Li *et al* (5) revealed that DHA also inhibited the proliferation and migration of breast cancer cell lines. DHA was also reported to serve important roles in downregulating the prostaglandin E2 synthesis signaling cascade and the inflammation of endothelial cells in the cardiovascular system (6). Furthermore, Zhu and Ji (7) suggested that DHA may have the potential to be used as a treatment option for asthma by

regulating the microRNA-183C and interleukin-6/STAT3 signaling pathway.

In more recent years, gut microbiota has become a research hotspot due to its involvement in several types of disease, including obesity, diabetes, stroke, allergic asthma, nonalcoholic fatty liver disease/nonalcoholic steatohepatitis, cancer, inflammation, and behavioral and physiological abnormalities (8-10). DHA has demonstrated the potential to be used as a treatment option for fatty liver disease, inflammatory disorders and different types of cancer (4-6), except for malaria; however, its underlying molecular mechanism of action remains unknown. Chen *et al* (11), reported that artesunate (AS) exhibited the potential to regulate the gut microbiota, ameliorate inflammation and improve the conditions of the liver and intestine following the damage caused by carbon tetrachloride, alcohol and a high fat diet in Sprague Dawley rats, suggesting that DHA may have similar effects on the gut microbiota. Thus, the present study aimed to investigate the effects of DHA in the liver, kidney, intestine and gut microbiota to identify the underlying molecular mechanisms of DHA and its pharmacological activities.

Materials and methods

Animal studies and tissue collection. The experimental procedures in the present study were approved by the Committee on Laboratory Animal Care and Use of Guangdong Pharmaceutical University (Guangzhou, China). A total of 18 male C57BL/6 mice (weight, 22.53±1.23 g; age, 7 weeks old; provided by Hunan Lex Jingda Laboratory Animal Co., Ltd.) were housed in a temperature-controlled environment at 25°C and 50±10% humidity, with a 12-h light/dark cycle and free access to a standard diet and water. The mice were randomly divided into two groups (n=9/group): Control and DHA group. According to the results obtained in a pre-experiment and previous studies (12-14), the experimental group was treated with DHA (150 mg/kg; Shanghai Aladdin Biochemical Technology Co., Ltd.) diluted in 0.5% carboxymethyl cellulose-Na (CMC-Na; Tianjin Zhiyuan Chemical Reagent Co., Ltd.), once daily for 7 days by oral gavage. The control group was treated with 0.5% CMC-Na once daily for 7 days by oral gavage. After 7 days, the whole blood was collected from the orbit; the mice were adequately anesthetized with ether for blood collection and the pinch reflex was monitored to ensure full anesthesia. After 1 ml blood collection, the mice were sacrificed by cervical dislocation. The liver, kidney and intestine tissues were collected, and fixed in 4% paraformaldehyde overnight at 4°C for hematoxylin and eosin (H&E) staining. The experimental procedures were performed as previously described (15).

Serum lipid profile assays. Serum was obtained from the blood by centrifugation (1,160 x g) at 4°C for 15 min. The concentrations of serum triglyceride (TG), total cholesterol (TC), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed using their respective kits (Nanjing Jiancheng Bioengineering Institute; cat. no. A110-1-1 for TG; cat. no. A111-1-1 for TC; cat. no. A112-1-1 for HDL-C; cat. no. A113-1-1 for LDL-C; cat.

no. C009-2-1 for ALT; cat. no. C010-2-1 for AST), according to the manufacturer's protocols. The serum glucose levels were detected using a detection kit purchased from Shanghai Rongsheng Biopharmaceutical Co., Ltd., according to the manufacturer's protocols, and the serum liposaccharide (LPS) concentration was determined using a detection kit purchased from Jiangsu Enzyme Immunity Co., Ltd., according to the manufacturer's protocols.

16S ribosomal (r)DNA gene analysis. Fecal bacterial DNA extraction, 16S rDNA gene amplification and subsequent 16S rDNA gene analysis were performed by Gene Denovo Biotechnology Company (Guangzhou, China). The experimental procedures were performed as previously described (15,16). All α diversity indexes were calculated in QIIME (17) (version 1.9.1; http://qiime.org). Shannon rarefaction curves, Operational taxonomic units (OTU) rarefaction curve and rank abundance curves were plotted in R project ggplot2 package (18) (v2.2.1).

The abundance of bacteria was calculated using the method of Ace and Chao (19), and the diversity of bacteria was estimated using the method of Shannon and Simpson (20). Principal coordinates analysis (Vegan; v2.5-4; https://cran.r-project.org/web/packages/vegan) was performed in order to present the differences between the gut microbial communities of different groups. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed at http://www.kegg.jp/kegg/kegg1.html. Canonical Correspondence Analysis (CCA) was performed using Vegan (21), and the correlation network analysis was performed using Psych (v1.8.4; https://cran.r-project.org/web/packages/psych/index.html).

H&E staining. Mouse tissues were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated with ascending series of alcohol (70% alcohol for 45 min, 75% alcohol for 45 min, 80% alcohol for 45 min, 85% alcohol for 45 min, 95% alcohol for 45 min, pure alcohol twice for 1 h each) and embedded in paraffin. Paraffin-embedded tissues were sliced into 4-μm sections and stained with hematoxylin for 3 min and eosin for 20 sec, both at room temperature (Sigma-Aldrich; Merck KGaA). The stained sections were visualized using an automated quantitative pathology system (Vutomated Quantitative Pathology Imaging System; PerkinElmer, Inc.; the magnification of kidney and liver was x40, and the magnification of intestinal tissue was x20).

Statistical analysis. Statistical analysis was performed using SPSS software (v23.0; National Institutes of Health) and the data are presented as the mean ± standard deviation (SD). Statistical differences among the two groups were determined using an unpaired Student's t-test for equal variances or a U-Mann Whitney test for unequal variances. Each experiment was repeated ≥3 times. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of DHA on the serum lipid profile and important organs. DHA was demonstrated to significantly decrease the
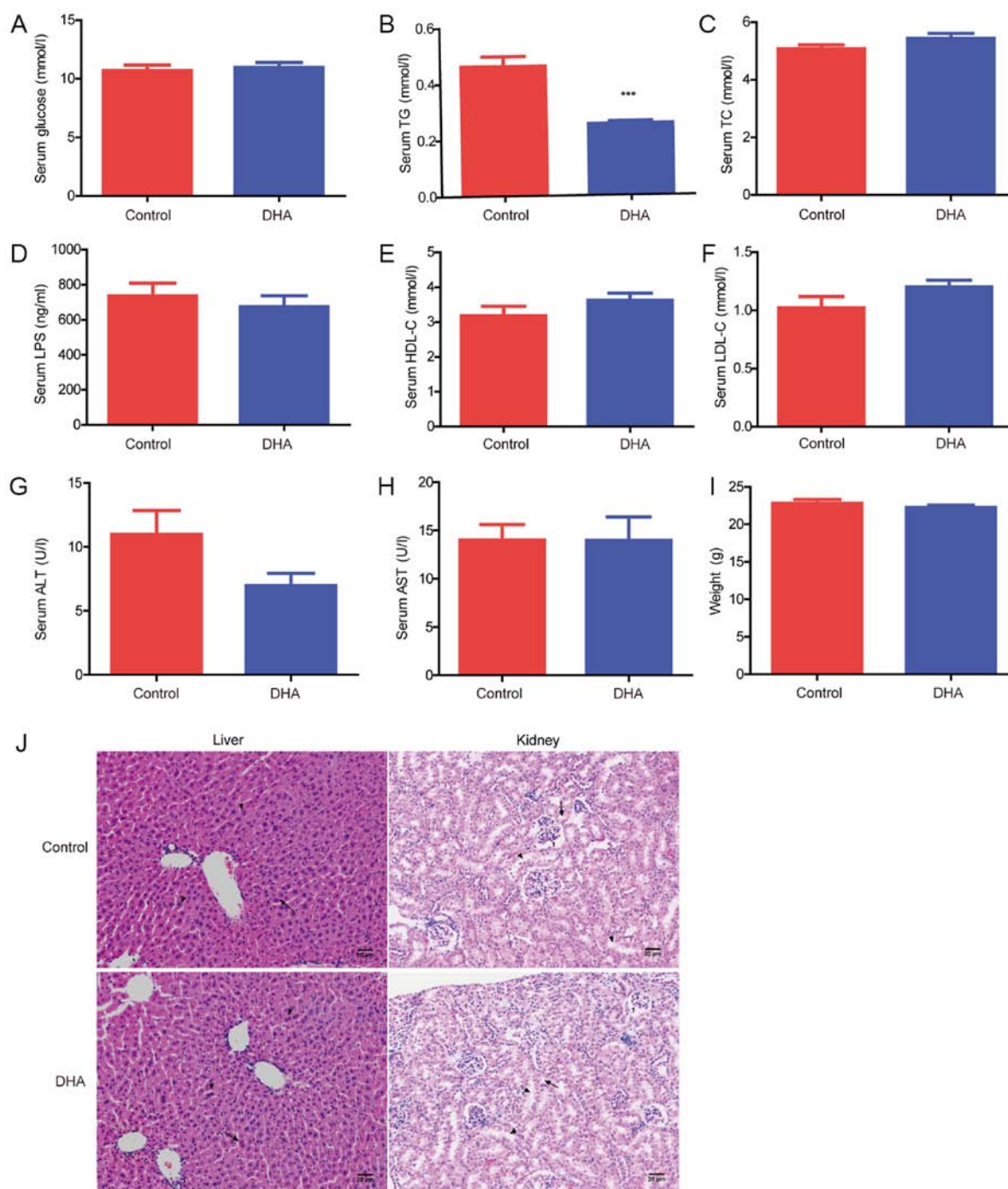
Figure 1. Effects of DHA on the serum lipid profile and important organs. Serum (A) glucose, (B) TG, (C) TC, (D) LPS, (E) HDL-C, (F) LDL-C, (G) ALT and (H) AST levels were analyzed in the DHA and control groups. (I) Body weight of the mice in the DHA and control groups. (J) Hematoxylin and eosin staining of the liver and kidney tissues obtained from the DHA and control groups. Scale bar, 20 μm. In the liver: Arrows, hepatocytes; triangles, hepatic cords. In the kidney: 1, spherical renal corpuscles; arrows, proximal convoluted tubule; triangles, distal convoluted tubule. ***P<0.001 vs. control. TG, triglyceride; TC, total cholesterol; LPS, lipopolysaccharide; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DHA, dihydroartemisinin.

serum TG levels compared with the control group (Fig. 1B); however, no significant differences were observed in the serum glucose, TC, LPS, HDL-C, LDL-C, ALT and AST levels, or the body weight, between the DHA and control groups (Fig. 1A, C-I). H&E staining revealed that the liver and kidney tissues of the DHA group were similar in pathology compared with the control group (Fig. 1J). Furthermore, no significant differences were observed in the pathology of the

intestine, including the duodenum, jejunum, ileum and colon, between the two groups (Fig. S1).

Overview of the 16S rDNA gene sequencing. In order to determine the effect of DHA on the gut microbiota, mice fecal samples were collected and the 16S rDNA genes were sequenced. The indices of Shannon and Simpson (calculated for the diversity of bacteria), Chao and Ace (calculated for

Table I. Diversity estimation of the 16S ribosomal RNA gene library of the DHA-treated mice and the control group.

Group	Total tags	Operational taxonomic units	Shannon	Simpson	Chao	Ace
Control	119009.11±17477.13	953.22±84.17	6.09±0.12	0.97±0.01	1661.94±104.26	1717.02±110.90
DHA	132965.38±10623.35	1010.13±51.92	6.32±0.27	0.97±0.01	1763.40±149.16	1728.54±120.93

DHA, dihydroartemisinin.

Table II. Taxonomic composition of the gut bacteria on the phylum level of the DHA and control groups.

Phylum	Control (%)	DHA (%)	DHA/control	P-value
Bacteroidetes	63.61675556	59.1229500	0.92936129	0.262040377
Firmicutes	19.26385556	27.8502625	1.44572629	0.046519675
Verrucomicrobia	8.32234444	7.2688500	0.87341374	0.723052299
Proteobacteria	7.55047778	5.1879000	0.68709559	0.119181387
Deferribacteres	0.67160000	0.0777500	0.11576831	0.002711787

the abundance of bacteria), together with the total tags and OTUs of each group are presented in Table I. The Shannon rarefaction curves for each group were observed to reach the saturation plateau, suggesting the sufficient sequence coverage of the samples to describe the composition of the gut microbiota (Fig. 2A). The Venn diagram demonstrated that there were 597 common OTUs identified between the DHA and control groups, and there were 195 and 110 OTUs specific to the DHA-treated mice and the control group, respectively (Fig. 2B).

Effect of DHA on the relative abundance and composition of the gut microbiota of mice. The α diversity analysis of the two groups demonstrated that the bacterial diversity of the DHA group was richer compared with the control group (Fig. 2C). Furthermore, principal coordinates analysis revealed that the DHA and the control groups could be distinguished from each other (Fig. 2D), suggesting that DHA may alter the gut bacteria of the mice.

The taxonomic compositions of the bacterial phylum of the two groups are presented in Fig. 3A and B. The majority of the samples exhibited high percentages of *Bacteroidetes*, *Firmicutes*, *Verrucomicrobia* and *Proteobacteria* on the phylum level, and the DHA group had increased *Firmicutes* and *Saccharibacteria* species, and decreased *Deferribacteres* and *Actinobacteria* species compared with the control group (Fig. 3A and B; Table II). The taxonomic compositions of the two groups on the class and family level are presented in Fig. S2, Table SI and Table SII.

The heatmap of KEGG signaling pathway enrichment analysis demonstrated that the following signaling pathways were upregulated in the DHA group: 'ABC transporters', 'Two-component system', 'Purine metabolism', 'Pyrimidine metabolism', 'Amino sugar and nucleotide sugar metabolism', 'Starch and sucrose metabolism', 'Arginine and proline metabolism', 'Peptidoglycan biosynthesis', 'Fructose and

mannose metabolism', 'Homologous recombination', 'Nitrogen metabolism', 'Cell cycle-Caulobacter', 'Methane metabolism' and 'Alanine, aspartate and glutamate metabolism' (Fig. 4A). Conversely, the following signaling pathways were downregulated in the DHA group compared with the control group: 'Aminoacyl-tRNA biosynthesis', 'Ribosome', 'Oxidative phosphorylation', 'Bacterial secretion system', 'RNA degradation' and 'Porphyrin and chlorophyll metabolism' (Fig. 4A). The signaling pathways associated with 'Energy Metabolism' and 'Nucleotide Metabolism' were demonstrated to be significantly upregulated in the DHA group compared with the control group (Fig. 4B), while the signaling pathways associated with 'Infectious Diseases' and 'Neurodegenerative Diseases' were significantly downregulated in the DHA group compared with the control group (Fig. 4B). The associations between HDL-C, LDL-C, TG, TC and LPS with the bacterial taxa are presented in Fig. S3. The result of CCA demonstrated a correlation of the bacteria with different environmental factors, and that LDL-C, LPS and TC influenced the structure and composition of gut microbiota most (Fig. S3A). The result of the correlation network analysis indicated that LDL-C had a positive correlation with *Verrucomicrobia*, but had a negative correlation with *Bacteroidetes*. HDL had a positive correlation with *Saccharibacteria*, and TC had a positive correlation with Fimicutes, but had a negative correlation with *Proteobacteria*. Furthermore, TG had a positive correlation with *Deferribacteres*, but had a negative correlation with *Saccharibacteria* (Fig. S3B).

Discussion

Artemisinin is well-known for its significant role in the treatment of malaria (22) and it was previously demonstrated that artemisinin had the potential to manage 'artemisinin-resistance' (23). In addition to its antimalarial properties, artemisinin and its derivatives were discovered to exhibit
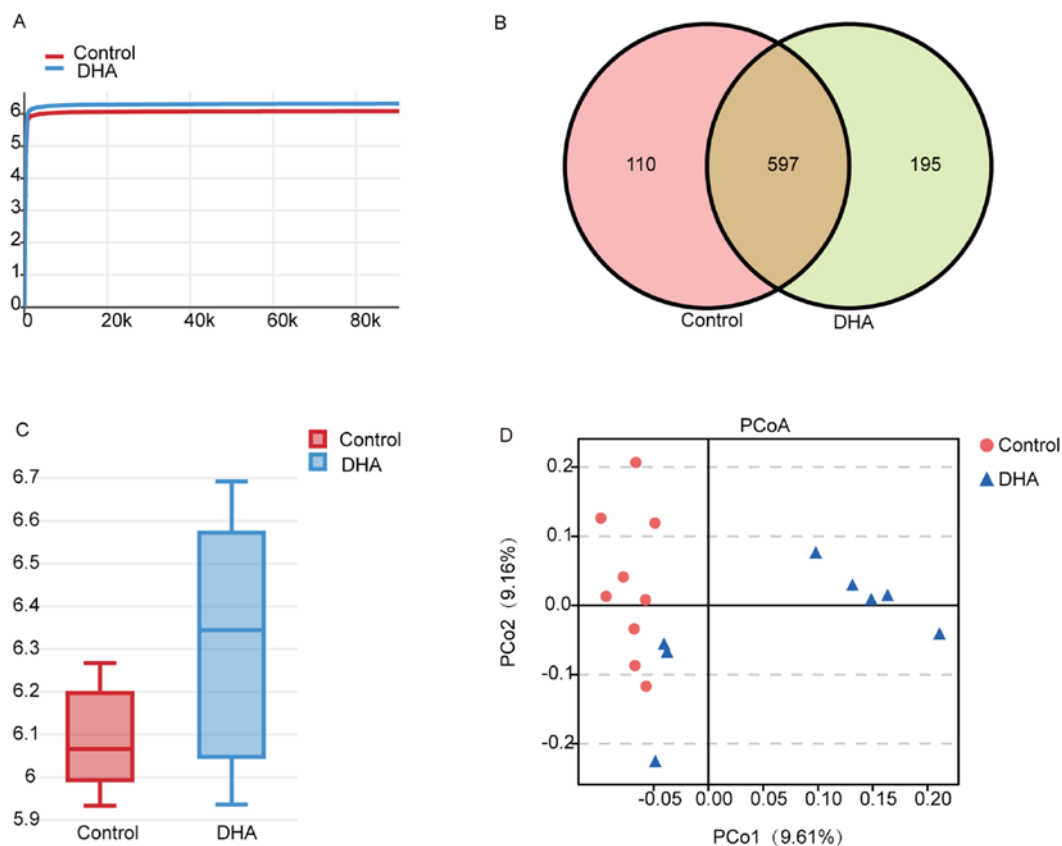
Figure 2. Overview of the 16S ribosomal DNA gene analysis. (A) Shannon rarefaction curves for the control and DHA groups. (B) Venn diagram of the DHA and control groups demonstrated the number of OTUs of the control group and the DHA group. The control group had 110 specific OTUs, the DHA group had 195 OTUs and the two groups had 597 OTUs in common. (C) α diversity of the DHA and control groups. (D) PCo analysis of the gut microbiota in the DHA and control groups. DHA, dihydroartemisinin; PCo, Principle coordinates; OTU, Operational taxonomic units.
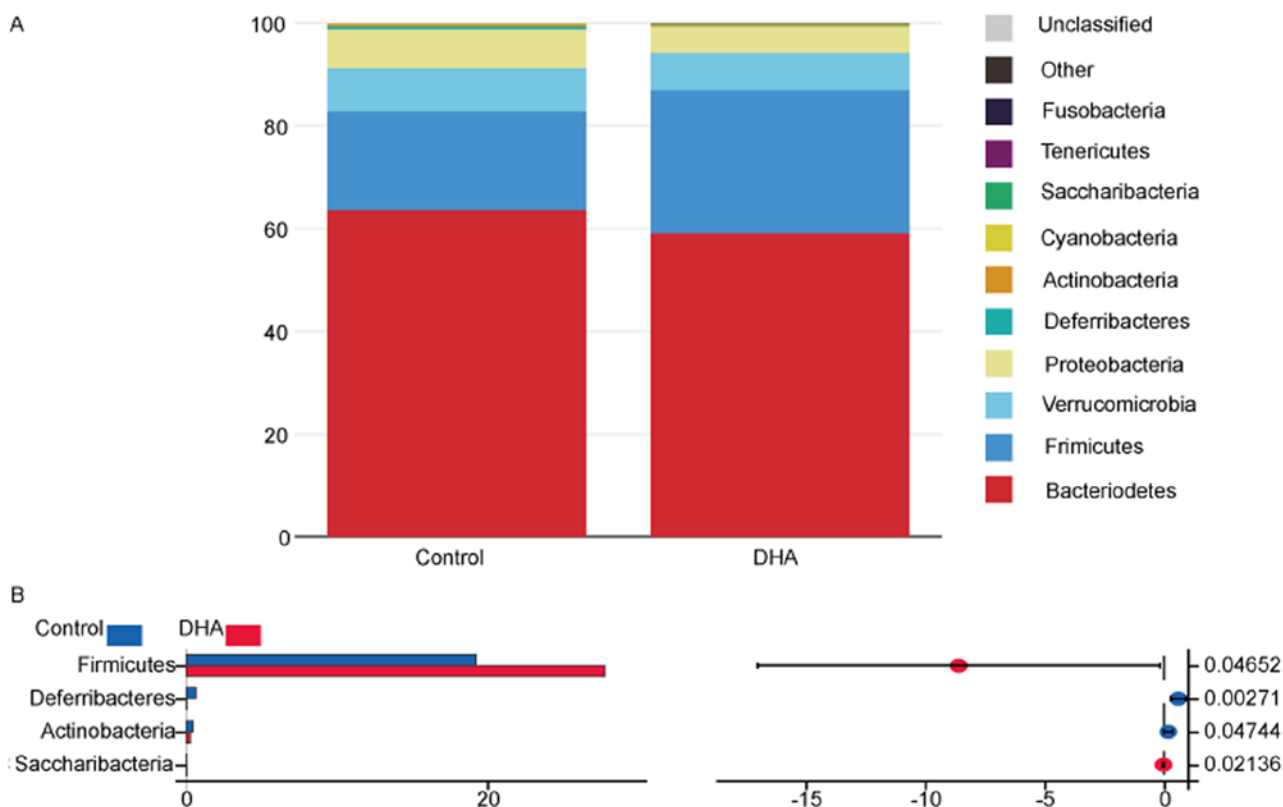

Figure 3. Relative abundance of the dominant gut microbiota found in the DHA and control groups. (A and B) Relative abundance of the gut microbiota at the bacterial phylum level in the two groups. DHA, dihydroartemisinin.
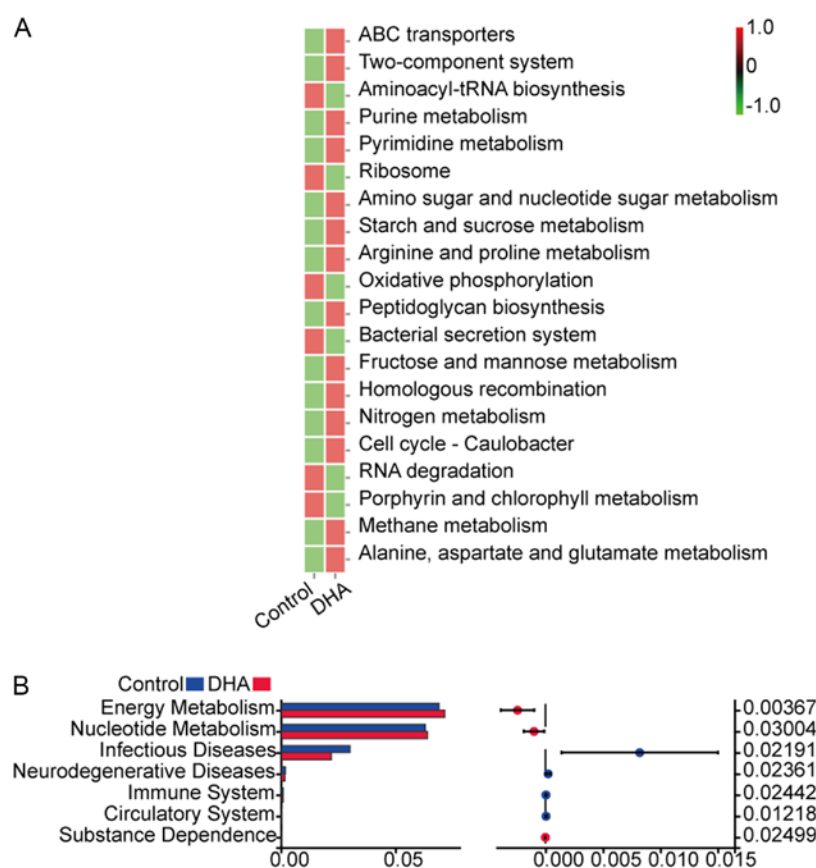
Figure 4. Kyoto Encyclopedia of Genes and Genomes signaling pathway enrichment analysis of the altered signaling pathways in the DHA and control groups. (A) Top 20 signaling pathways in the DHA and control groups. (B) Significantly altered signaling pathways in the DHA and control groups. DHA, dihydroartemisinin.

a range of pharmaceutical activities, such as anti-viruses, anti-inflammation and anti-tumors (1). DHA, an important derivative of artemisinin, was revealed to be associated with several biological activities, including improving liver damage, inhibiting cancer and inflammation (4-7); however, its effect on the gut microbiota remains poorly understood. In the present study, C57BL/6 mice were treated with DHA to determine its effects on the gut microbiota, lipid metabolism and important organs, including the liver, kidney and intestine. The H&E staining results of the liver, kidney and intestinal tissues demonstrated that there were no significant differences in the pathology between the DHA group and the control group, indicating that DHA did not exert toxic effects on these organs. TG serum levels were significantly decreased in the DHA group compared with the control group; however, there was no significant differences observed in the serum levels of glucose, TC, LPS, HDL-C, LDL-C, ALT and AST between the two groups. It was previously reported that AS significantly decreased the TG plasma levels, thus these findings suggested the potential of DHA to be used as a treatment for hyperlipidemia when combined with ursolic acid (24,25). The results of the present study indicated that DHA may exert similar biological functions as AS.

The effects of DHA on the gut microbiota were further analyzed via 16S rDNA gene analysis; the α diversity accounts for the abundance of species residing in the host, whereby a high α diversity indicates a healthy host pattern. For example, Wolff *et al* (26) reported that the healthy oral microbiome had

a significantly higher diversity compared with the advanced caries microbiome, whereas Clarke *et al* (27) reported that the gut microbiota of athletes was more diverse compared with the controls. The results of the present study demonstrated that the bacterial diversity of the DHA group was richer compared with the control group, suggesting that DHA may have the potential to increase the diversity of the gut bacteria.

Obese individuals have exhibited a tendency to have an increased ratio of *Firmicutes* to *Bacteroidetes* in the gut microbiota compared with lean humans and mice (28-30). In the present study, DHA was demonstrated to increase the relative abundance of the *Firmicutes* phylum from 19.26 to 27.85%, while the *Bacteroidetes* phylum decreased from 63.62 to 59.12% compared with the control group, respectively. Although DHA increased the ratio of *Firmicutes* to *Bacteroidetes*, the mice treated with DHA failed to exhibit an obese phenotype as there was no difference in body weight between the control and DHA groups. Furthermore, there were no significant differences observed in the body weights of the mice between the DHA and control groups. According to Mazloom *et al* (31), the importance of the *Firmicutes* to *Bacteroidetes* ratio in obesity remains controversial as several studies have reported no significant differences between the two phyla in obesity. The results of the present study suggested that the effect of DHA on the *Firmicutes* to *Bacteroidetes* ratio was not associated with obesity. The present study also revealed that DHA significantly decreased TG serum levels, whilst KEGG signaling pathway enrichment analysis indicated that the signaling pathways asso-

ciated with 'Energy Metabolism' and 'Nucleotide Metabolism', such as 'Purine metabolism', 'Pyrimidine metabolism', 'Amino sugar and nucleotide sugar metabolism', 'Starch and sucrose metabolism', 'Arginine and proline metabolism', 'Peptidoglycan biosynthesis', 'Fructose and mannose metabolism', 'Nitrogen metabolism', 'Methane metabolism' and 'Alanine, aspartate and glutamate metabolism' were upregulated in the DHA group compared with the control group. Overall, these results suggest that DHA may affect lipid metabolism by regulating the gut microbiota. The effects of DHA on the gut microbiota were evaluated in the present study. A high fat diet was previously discovered to affect the relative abundance and composition of the gut microbiota (15). To ensure that the alterations of the gut microbiota were due to the effects of DHA, mice were fed a normal diet in the present study. Since the serum TG levels were observed to be significantly decreased in the DHA group, the effect of DHA on the lipid metabolism will be further evaluated using mice fed with a high fat diet in our future study.

In the present study, KEGG signaling pathway enrichment analysis revealed that the signaling pathways involved in 'Infectious Diseases' and 'Neurodegenerative Diseases' were significantly downregulated in the DHA group compared with the control group. Notably, Liu *et al* (2) reported that DHA had the potential to suppress the NF-κB signaling pathway and inhibit the inflammatory response in lipopolysaccharide-induced septic acute kidney injury within a mouse model. In addition, Kim *et al* (32) reported that artemisinin inhibited the interaction between nod-like receptor protein 3 (NLRP3) and never in mitosis geneA-related kinase 7 (Nek7) in NLRP3 inflammasome activation. Furthermore, it was indicated that artemisinin may inhibit AMP kinase/NF-κB/NLRP3 inflammasome signaling in macrophages in order to protect the aorta from atherosclerotic lesions (33). Neurodegenerative diseases are characterized by the deterioration of neuronal structures and functions (34), and it has been suggested that artemisinin may be used as a treatment for neurodegenerative diseases by inhibiting oxidation, inflammation and decreasing the level of amyloid β protein (34). The results of the present study indicated that DHA may suppress the signaling pathways involved in 'Infectious Diseases' and 'Neurodegenerative Diseases' by regulating the gut microbiota.

In conclusion, the findings of the present study revealed that DHA decreased the TG serum levels in mice and 16S rDNA gene analysis suggested that DHA may have the potential to increase the diversity of the gut bacteria. Mice treated with DHA exhibited increased numbers of *Firmicutes* and *Saccharibacteria*, and decreased *Deferribacteres* and *Actinobacteria* on the phylum level compared with the control group. KEGG signaling pathway enrichment analysis subsequently demonstrated that DHA upregulated the signaling pathways involved in 'Energy Metabolism' and 'Nucleotide Metabolism', while downregulating the signaling pathways involved in 'Infectious Diseases' and 'Neurodegenerative Diseases'. Altogether, these results suggested that DHA may exert its functions, including decreasing the serum TG levels and inducing anti-inflammatory and anti-neurodegenerative responses through regulating the composition of the gut microbiota.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZL and JY designed and conceived the study; YYL established the model mice and performed the serum analysis; YY designed the study, analyzed and interpreted the results, wrote the first draft of the manuscript and critically revised the manuscript; and YTL, LY and XZ performed the H&E staining. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experimental procedures in the present study were approved by the Committee on Laboratory Animal Care and Use of Guangdong Pharmaceutical University (Guangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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