

Astaxanthin, *Haematococcus pluvialis* and *Haematococcus pluvialis* Residue Alleviate Liver Injury in D-Galactose-induced Aging Mice through Gut-liver Axis

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Abstract: Astaxanthin is a keto-based carotenoid mainly obtained from marine organisms, like *Haematococcus pluvialis* (*H. pluvialis*). Previous studies indicated the protective effects of Astaxanthin and *H. pluvialis* on aging related oxidative injury in liver, while the potential mechanisms are largely unknown. In addition, *H. pluvialis* residue is a by-product after astaxanthin extraction, which is rarely studied and utilized. The present study aimed to compare the effects of astaxanthin, *H. pluvialis* and *H. pluvialis* residue on the oxidant injury of liver in D-galactose-induced aging mice and explore the potential mechanisms through gut-liver axis. The results showed that all the three supplements prevented D-galactose-induced tissue injury, oxidative stress and chronic inflammation in liver and improved liver function. Gut microbiota analysis indicated that astaxanthin notably increased fecal levels of *Bacteroidetes*, *unclassified_f_Lachnospiraceae*, *norank_f_Lachnospiraceae*, *norank_o_Clostridia_UCG-014*, *Prevotellaceae_UCG-001*, *unclassified_f_Prevotellaceae* in D-galactose-fed mice ($p < 0.05$). Compared to aging mice, *H. pluvialis* group had higher fecal levels of *norank_f_Lachnospiraceae* and *Lachnospiraceae_UCG-006* ($p < 0.05$). *H. pluvialis* residue group displayed higher relative levels of *Bacteroidetes*, *Streptococcus*, and *Rikenellaceae_RC9_gut_group* ($p < 0.05$). Moreover, the production of fecal microbial metabolites, like SCFAs and LPS was also differently restored by the three supplements. Overall, our results suggest astaxanthin, *H. pluvialis* and *H. pluvialis* residue could prevent aging related hepatic injury through gut-liver axis and provide evidence for exploiting of *H. pluvialis* residue as a functional ingredient for the treatment of liver diseases. Future studies are needed to further clarify the effect and mechanism of dominant components of *H. pluvialis* residue on liver injury, which is expected to provide a reference for the high-value utilization of *H. pluvialis* resources.

Key words: astaxanthin, *H. pluvialis*, *H. pluvialis* residue, intestinal microbiota, liver injury

1 Introduction

Aging is an complex, inevitable and spontaneous degenerative process, that is associated with damage in all vital organs and frequently accompanied with many diseases, such as cardiovascular diseases, diabetes, neurodegenera-

tive diseases, and liver diseases¹⁾. Liver is an important organ that is essential for maintaining whole-body metabolic homeostasis. The process of aging leads to gradual damage to the structure and function of liver²⁾. One of the most accepted mechanisms underlying aging is oxidative

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stress caused by excessive accumulation of reactive oxygen species (ROS)³. As playing central roles in oxidative metabolism, the liver is particularly vulnerable to attacked by ROS causing oxidative stress and subsequent swelling and necrosis of hepatocytes, and ultimately resulting in hepatic injury⁴. Therefore, antioxidative agents are considered to be potential therapeutic drugs against aging induced liver injury.

Astaxanthin is a keto-based carotenoid with super antioxidant activity that is mainly obtained from marine organisms, like *Haematococcus pluvialis* (*H. pluvialis*) and shrimps⁵. The microalga *H. pluvialis* is a single-cell chlorophyte organism with strong capacity to accumulate astaxanthin and have very wide applications in food industry⁶. Previous studies have revealed that both astaxanthin⁷ and *H. pluvialis*⁸ had a protective effects on aging related oxidative injury in liver. For instance, Chen *et al.*⁹ reported that 5–15 mg/kg astaxanthin attenuated hepatic oxidative injury in D-galactose (D-gal) induced aging rats. Another study also indicated 450 mg/kg *H. pluvialis* treatment ameliorated D-gal-induced hepatic injury and oxidative stress in aging rats⁸. It's worth mentioning that the D-gal-induced aging model is commonly used due to it induced a physiological state similar to natural aging, with increased levels of free radicals and oxidative stress in all organ system. While, the potential mechanisms underlying the protective effects of astaxanthin or *H. pluvialis* on aging related liver injury are still largely unknown.

Recent evidence revealed that the communication between intestinal microbiota and liver, named gut-liver axis, plays a vital role in aging related liver injury¹⁰. Plenty of microbial communities are colonized in gut that maintain health of host and are interactive with various factors, like diets, diseases and drugs. A series of reports in aging patients or rodents have observed gut microbial dysbiosis¹¹. Fecal microbiota transplantation from health donors reversed aging related oxidative injuries in rodents¹⁰. Compelling evidences have proved that intestinal bacteria produce numerous substances that interact with host liver via portal vein and initiate hepatic oxidative stress and chronic inflammation¹². Lipopolysaccharide (LPS), derives from the outer membrane of gram-negative bacteria, triggers oxidative stress and inflammation in liver by targeting Toll-like receptor 4 (TLR4)¹³. Short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate etc., are fermentation products from dietary fibers by gut microbiota that play beneficial roles in maintaining gut barrier, inhibition hepatic oxidative stress and chronic inflammation via the combination of cell surface G-protein-coupled receptor 41 (GPCR 41) and G-protein-coupled receptor 43 (GPR43)¹⁴. The modulation effects of astaxanthin on gut microbiota was reported in high-fat diet¹⁵ or alcohol¹⁶ induced fatty liver mice. While, no study has explored the roles of gut microbiota and microbial metabo-

lites in the protective effect of astaxanthin or *H. pluvialis* on aging related hepatic injury. In addition, *H. pluvialis* residue is a by-product after astaxanthin extraction, which is rich in protein, fibers, and lipids, etc¹⁷. Currently, limited studies have investigated the effective utilization approach of *H. pluvialis* residues. Investigating the biological effect of *H. pluvialis* residues *in vivo* might provide guiding significance for its exploitation.

In this study, we aimed to compare the effects of astaxanthin, *H. pluvialis* and *H. pluvialis* residue on the liver injury in D-gal-induced aging mice. Potential mechanisms regarding the intestinal microbiota and microbial metabolites were evaluated. The findings will provide further evidence for improvement mechanisms of astaxanthin and *H. pluvialis* on aging related oxidative injury in liver and for the development and utilization of *H. pluvialis* residue.

2 Materials and Methods

2.1 Materials

Astaxanthin, astaxanthin-rich *H. pluvialis* (1% astaxanthin), and *H. pluvialis* residues of cell disruption after astaxanthin extraction were purchased from Yunnan Alphy Biotech Co., Ltd. (Yunnan, China). The nutritional composition of the *H. pluvialis* was 1% astaxanthin, 7.3% moisture, 23.8% protein, 15.9% fat and 13.5% ash, while the *H. pluvialis* residue was 7.8% moisture, 36.1% protein, 1.7% fat and 8.4% ash. D-gal was provided by Macklin Biochemical Co., Ltd. (Shanghai, China).

2.2 Protocol of animal study

The laboratory animal welfare ethics committee of Qingdao University approved this study (QDU-AEC-2023345). Forty eight-week-old Kunming (KM) mice, which is a frequently been utilized strain in D-gal-induced aging models^{18, 19}, were purchased from SPF Biotechnology Co., Ltd. (Beijing, China) and housed under a standard laboratory condition. After one week of adaptation, they were randomly divided into 5 groups (n = 8): (1) Control group (C); (2) Model group (M); (3) Astaxanthin group (M + A); (4) *H. pluvialis* group (M + H); (5) *H. pluvialis* residue group (M + HR). All the mice were fed with a normal chow diet. The C group was intraperitoneally injected with normal saline and the rest mice were intraperitoneally injected with 200 mg/kg/day D-gal daily. Meanwhile, the M + A, M + H and M + HR groups were orally gavaged with 10 mg/kg/day astaxanthin⁹, 1 g/kg/day *H. pluvialis* (equality to 10 mg/kg/day astaxanthin), and 1 g/kg/day *H. pluvialis* residue, respectively. The three supplements were dissolved in sunflower oil, which was purchased from Yihai Kerry Arawana Holdings Co., Ltd. (Shanghai, China) and the mice in C and M groups were gavaged sunflower oil. The gavage volume of sunflower oil for each group was 10

mL/kg. After 10 weeks, feces were acquired and the mice were fasted 12 hours to collect blood and tissues.

2.3 Evaluation of biochemical parameters

Serum levels of total cholesterol (TC), triglyceride (TG), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured on an automatic analyser (Rayto Life and Analytical Sciences Co., Ltd., Shenzhen, China). Chemical kits (Jiancheng, Nanjing, China) were utilized to evaluate levels of superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), total antioxidant capacity (T-AOC), and malondialdehyde (MDA) in serum and liver. TG and TC in liver were detected by commercial kits (Jiancheng, Nanjing, China). Serum and hepatic concentrations of tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6) and interleukin-1 β (IL-1 β) were evaluated by ELISA kits (Jiangsu Jingmei Biological Technology Co., Ltd., Jiangsu, China). Serum and fecal levels of LPS were also detected through ELISA kits.

2.4 Histological analysis of liver

The fresh livers were fixed in a 4% paraformaldehyde for 24 hours. H&E staining was referenced to our previous publication²⁰⁾.

2.5 Measurement of short chain fatty acids in feces

Fecal levels of SCFAs were detected through GC/MS (Agilent 7890-7250 GC/MSD, Santa Clara, CA, USA) according to our previous method²¹⁾.

according to our previous method²¹⁾.

2.6 Microbial diversity analysis

Fecal bacterial DNA was extracted and sequenced by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China)²⁰⁾. The variable region 3–4 (V3–V4) was employed to conduct PCR amplification. All data were analyzed online (<https://www.majorbio.com>).

2.7 Statistical analysis

Data are shown as mean $\pm$ standard deviation (SD). Mean differences were evaluated via ANOVA followed by LSD post hoc test. Spearman correlation analysis was utilized to explore the relationships among indices. Statistically significant was recognized as $p < 0.05$. Data analysis was accomplished by SPSS 21.0 software.

3 Results

3.1 Effect of astaxanthin, *H. pluvialis* and *H. pluvialis* residue on the growth parameters

As shown in Table 1, no change on body weight gain, food intake, liver weight, spleen weight, spleen index, kidney weight, kidney index, heart weight, and heart index was found among the five groups. M + H group displayed higher liver index than M + A group ($p < 0.05$). Thymus weight and thymus index were lower in M + H group compared to other groups.

Table 1 Growth parameters of experimental groups.

	C	M	M+A	M+H	M+HR
Growth parameters					
Initial BW (g)	43.56 $\pm$ 0.43 ^a	43.66 $\pm$ 1.13 ^a	44.18 $\pm$ 2.28 ^a	43.35 $\pm$ 0.95 ^a	43.72 $\pm$ 0.79 ^a
Final BW (g)	52.85 $\pm$ 1.35 ^a	53.48 $\pm$ 1.77 ^a	54.85 $\pm$ 1.69 ^a	52.76 $\pm$ 3.34 ^a	55.48 $\pm$ 2.91 ^a
BW gain (g)	9.29 $\pm$ 1.27 ^a	9.82 $\pm$ 2.10 ^a	10.67 $\pm$ 2.27 ^a	9.42 $\pm$ 3.14 ^a	11.76 $\pm$ 3.28 ^a
Food intake (g/day)	6.07 $\pm$ 0.50 ^a	5.84 $\pm$ 0.47 ^a	5.92 $\pm$ 0.66 ^a	5.72 $\pm$ 0.66 ^a	6.82 $\pm$ 0.56 ^a
Liver weight (g)	1.94 $\pm$ 0.15 ^a	2.03 $\pm$ 0.07 ^a	1.93 $\pm$ 0.06 ^a	2.10 $\pm$ 0.24 ^a	2.08 $\pm$ 0.18 ^a
Liver index (mg/g)	36.79 $\pm$ 2.65 ^{ab}	37.91 $\pm$ 1.44 ^{ab}	35.23 $\pm$ 2.05 ^a	39.80 $\pm$ 3.65 ^b	37.42 $\pm$ 1.70 ^{ab}
Spleen weight (g)	0.14 $\pm$ 0.03 ^a	0.12 $\pm$ 0.02 ^a	0.13 $\pm$ 0.02 ^a	0.16 $\pm$ 0.05 ^a	0.12 $\pm$ 0.03 ^a
Spleen index (mg/g)	2.74 $\pm$ 0.56 ^a	2.32 $\pm$ 0.42 ^a	2.30 $\pm$ 0.42 ^a	2.93 $\pm$ 0.96 ^a	2.21 $\pm$ 0.38 ^a
Thymus weight (g)	0.06 $\pm$ 0.01 ^{ab}	0.07 $\pm$ 0.02 ^a	0.06 $\pm$ 0.02 ^{ab}	0.05 $\pm$ 0.01 ^b	0.06 $\pm$ 0.02 ^{ab}
Thymus index (mg/g)	1.18 $\pm$ 0.27 ^{ab}	1.37 $\pm$ 0.32 ^a	1.17 $\pm$ 0.28 ^{ab}	0.95 $\pm$ 0.23 ^b	1.09 $\pm$ 0.30 ^{ab}
Kidney weight (g)	0.66 $\pm$ 0.06 ^a	0.64 $\pm$ 0.08 ^a	0.68 $\pm$ 0.08 ^a	0.64 $\pm$ 0.08 ^a	0.69 $\pm$ 0.07 ^a
Kidney index (mg/g)	12.43 $\pm$ 0.90 ^a	12.01 $\pm$ 1.34 ^a	12.31 $\pm$ 1.46 ^a	12.16 $\pm$ 1.15 ^a	12.53 $\pm$ 1.25 ^a
Heart weight (g)	0.27 $\pm$ 0.03 ^a	0.24 $\pm$ 0.04 ^a	0.26 $\pm$ 0.01 ^a	0.24 $\pm$ 0.02 ^a	0.25 $\pm$ 0.03 ^a
Heart index (mg/g)	5.08 $\pm$ 0.57 ^a	4.48 $\pm$ 0.61 ^a	4.67 $\pm$ 0.25 ^a	4.58 $\pm$ 0.69 ^a	4.43 $\pm$ 0.60 ^a
Perirenal adipose tissue (g)	0.20 $\pm$ 0.11 ^b	0.72 $\pm$ 0.25 ^a	0.76 $\pm$ 0.12 ^a	0.78 $\pm$ 0.29 ^a	0.82 $\pm$ 0.23 ^a
Epididymal adipose tissue (g)	0.80 $\pm$ 0.16 ^b	1.43 $\pm$ 0.62 ^a	1.38 $\pm$ 0.22 ^a	1.31 $\pm$ 0.44 ^{ab}	1.67 $\pm$ 0.47 ^a

^{a)} a-b, different letters indicate statistical different between groups ($p < 0.05$)

pared to M group ($p < 0.05$). The weights of both perirenal and epididymal adipose tissue were notably higher in the M group than C group ($p < 0.05$). Astaxanthin, *H. pluvialis* or residue treatment had no significant influence on the weights of adipose tissue.

3.2 Astaxanthin, *H. pluvialis* and *H. pluvialis* residue ameliorated D-gal induced hepatic injury in mice

The results of H&E staining (Fig. 1A) showed that the C group had a well-organized liver structure with radial arrangement of hepatic cords and cells with clear boundaries. While in the liver of M group, destroyed cell morphology, disordered arrangement of liver cords and blurred cell boundaries were observed. Astaxanthin, *H. pluvialis* or residue supplementation notably attenuated the D-gal induced injury of liver. As shown in Figs. 1B and 1C, D-gal treatment dramatically increased serum levels of ALT, AST. All the three dietary supplementation decreased serum levels of ALT ($p < 0.05$). M + A and M + HR group displayed lower serum levels of AST ($p < 0.05$). No difference on hepatic concentrations of TG, TC (Figs. 1D and 1E) and serum levels of TC (Fig. 1F) was noticed among the experimental groups. Astaxanthin treatment markedly decreased the elevated serum TG levels (Fig. 1G) in the M group ($p < 0.05$).

3.3 Astaxanthin, *H. pluvialis* and *H. pluvialis* residue ameliorated hepatic oxidation stress and inflammation in mice

D-gal induced hepatic injury is accompanied by oxidation stress and chronic inflammation. As shown in Figs. 2A–2G, D-gal treatment dramatically decreased levels of SOD, GSH-PX, T-AOC and enhanced that of MDA, TNF- α , IL-6, IL-1 β in liver, indicating the elevated hepatic oxidation stress and chronic inflammation. M + A and M + H groups displayed significantly higher hepatic levels of SOD, GSH-PX ($p < 0.05$) and lower that of MDA ($p < 0.05$) compared to M group. Astaxanthin supplementation also notably increased levels of T-AOC in liver ($p < 0.05$). *H. pluvialis* residue administration significantly decreased hepatic concentrations of MDA compared to M group ($p < 0.05$). M + A, M + H and M + HR groups showed markedly declined levels of TNF- α , IL-6 in the liver of D-gal-fed mice ($p < 0.05$). M + HR group had higher hepatic levels of IL-1 β than M group ($p < 0.05$).

Serum parameters of oxidation stress and chronic inflammation were also measured (Figs. 2H–2N). D-gal treatment dramatically increased serum levels of MDA, TNF- α , and IL-6 ($p < 0.05$). Compared with M group, M + A and M + H groups displayed higher levels of SOD ($p < 0.05$).

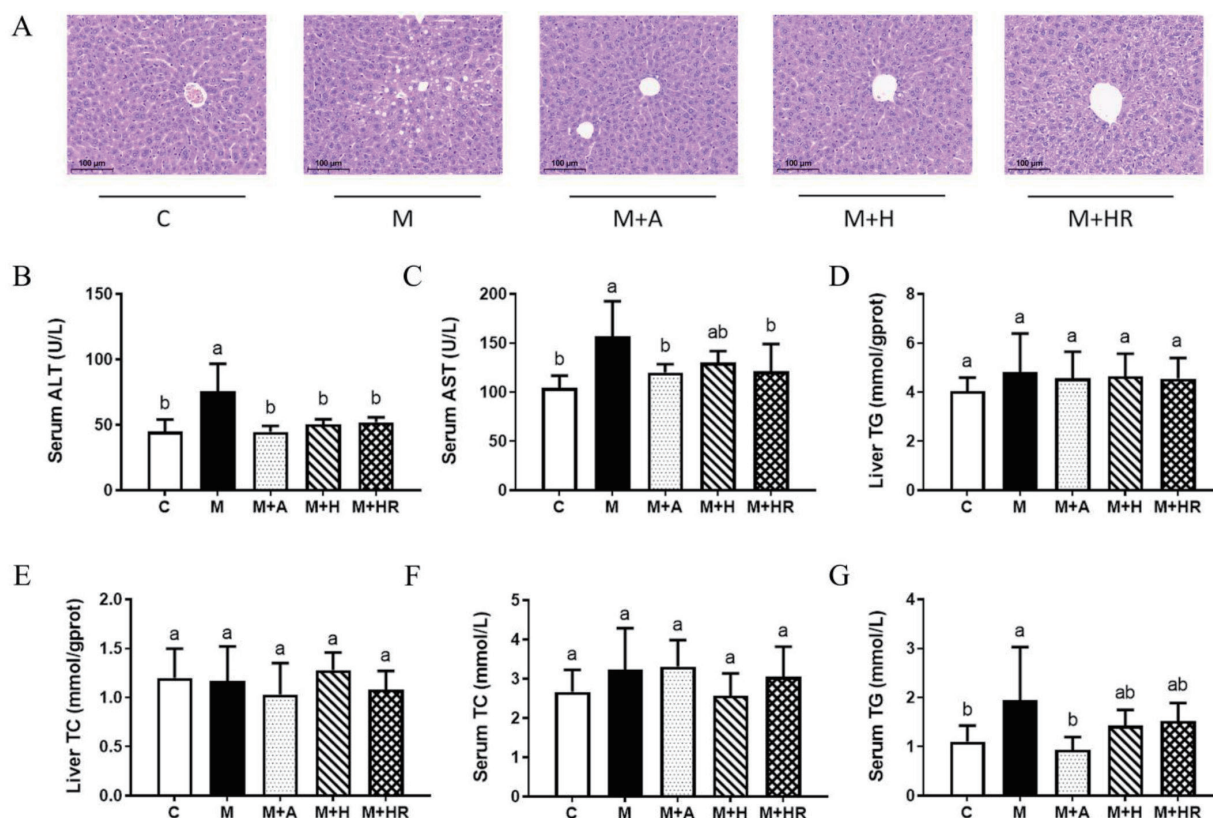


Fig. 1 Effects of astaxanthin, *H. pluvialis* and *H. pluvialis* residue on hepatic lipids and function. (A) H&E staining of liver tissues (400 $\times$); (B-C) Serum levels of ALT and AST; (D-E) Liver TG and TC levels; (F-G) Serum levels of TC and TG; a-b, different letters indicate statistical different between groups ($p < 0.05$).

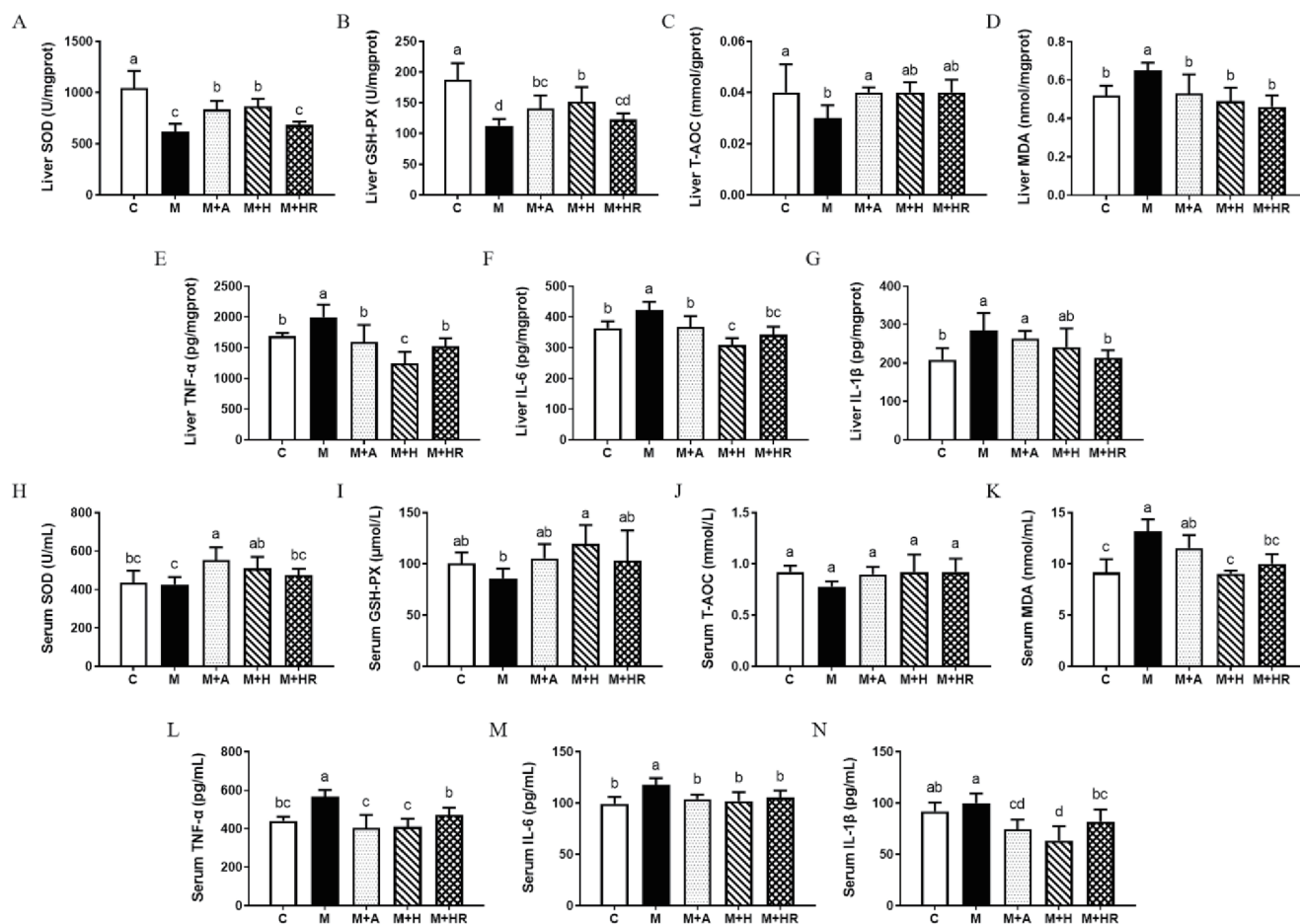


Fig. 2 Effects of astaxanthin, *H. pluvialis* and *H. pluvialis* residue on oxidative stress and chronic inflammation related indices in liver and serum. (A-D) Liver SOD, GSH-PX, T-AOC, MDA; (E-G) Liver TNF- α , IL-6, IL-1 β ; (H-K) Serum SOD, GSH-PX, T-AOC, MDA; (L-N) Serum TNF- α , IL-6, IL-1 β ; a-d, different letters indicate statistical different between groups ($p < 0.05$).

M + H and M + HR groups showed lower MDA ($p < 0.05$). M + H group had higher GSH-PX ($p < 0.05$). M + A, M + H and M + HR groups showed markedly declined TNF- α , IL-6 and IL-1 β ($p < 0.05$).

3.4 Overall fecal microbial community structure

Alpha and beta diversity were conducted to evaluate the overall community structure of fecal microbiota. The rarefaction curves became flat with increased optimized sequences, indicating sufficiency of sequencing depth (Fig. 3A). Shannon and Simpson indices reflected the community diversity, while Ace and Chao indices determined the richness of the microbial communities (Figs. 3B–3E). The Shannon index was lower and Simpson index was higher in M group than in C group ($p < 0.05$), indicating decreased community diversity in the D-gal treated mice. M + A, M + H and M + HR groups displayed markedly higher Shannon index and lower Simpson index compared to M group ($p < 0.05$). No change on Chao and Ace indices was noticed

between C and M groups. Dietary astaxanthin significantly increased Chao and Ace indices compared to M group, indicating enhanced richness of the microbial community in M + A group. The Venn of the operational taxonomic units (OTUs) (Fig. 3F) displayed 645 OTUs were acquired and the five groups shared 274 OTUs.

β -Diversity analysis determined via PCoA showed the diversity of fecal microbial structure (Fig. 3G). The five groups were divided from each other, indicating their distinct microbial compositions.

3.5 Astaxanthin, *H. pluvialis* and *H. pluvialis* residue restored intestinal microbiota dysbiosis in D-gal-fed mice

Figure 4A shows the primary phyla of bacterial communities. *Firmicutes* and *Bacteroidetes* took approximately 90% of the total microbiota. As shown in Figs. 4B and 4C, D-gal administration led to increased relative abundance of *Firmicutes* and decline of *Bacteroidetes* ($p < 0.05$). The

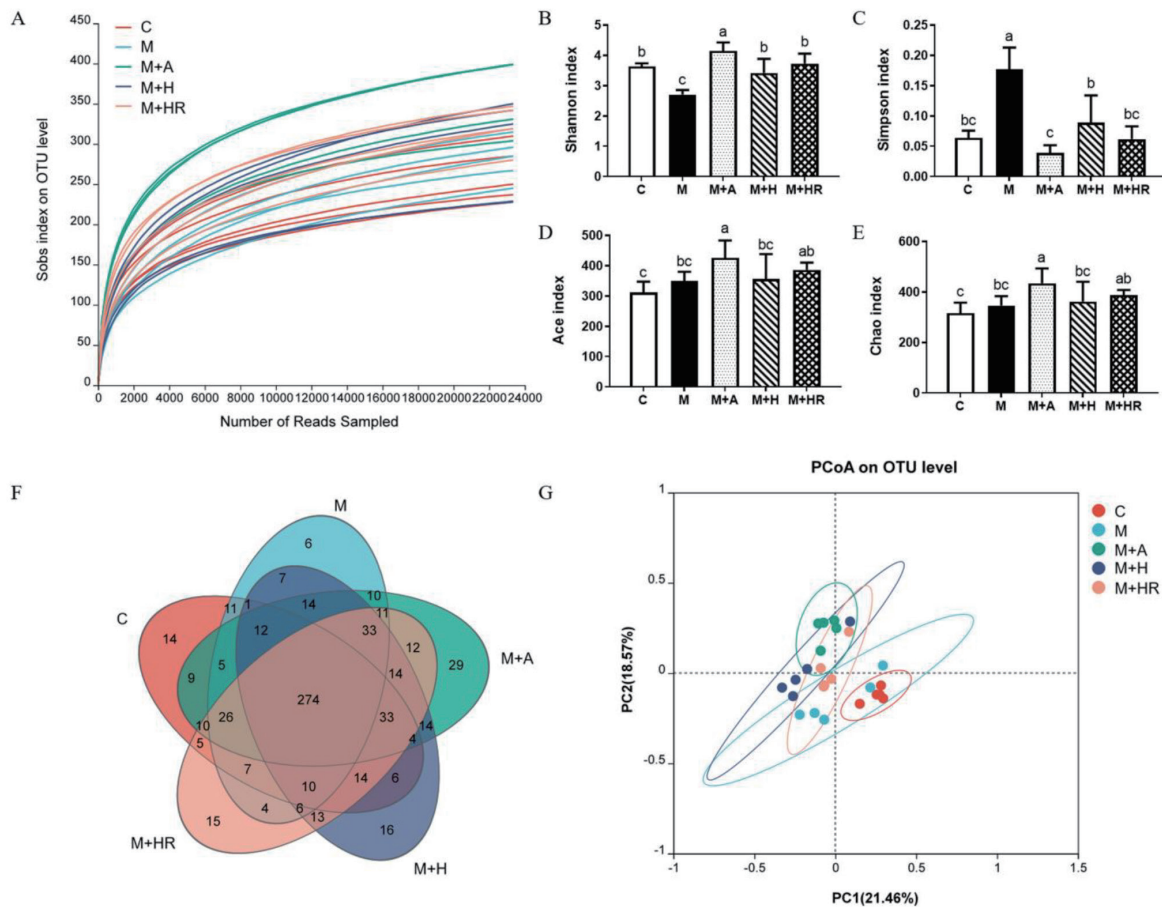


Fig. 3 Overall community structure of fecal microbiota. (A) Rarefaction curves; (B) Shannon index; (C) Simpson index; (D) Ace index; (E) Chao index; (F) Venn of the OTUs; Simpson index; (G) Principal coordinates analysis. a-c, different letters indicate statistical different between groups ($p < 0.05$).

ratio of F/B (Fig. 4D) was also calculated and M group showed higher ratio of F/B than C group ($p < 0.05$). M + A and M + HR groups had lower levels of *Firmicutes*, ratio of F/B and higher levels of *Bacteroidetes* than M group ($p < 0.05$).

The top 30 most abundant fecal genera are displayed in Figs. 4E-4Q. The relative levels of *Lactobacillus* were markedly higher, while that of *norank_f__Muribaculaceae*, *Lachnospiraceae_UCG-006*, *Streptococcus*, *Alistipes*, *Staphylococcus* were lower in M group than C group ($p < 0.05$). The bacterial genera of *Lactobacillus* were declined in M + A, M + H and M + HR groups than in M group ($p < 0.05$). M + A and M + H groups had higher levels of *norank_f__Lachnospiraceae* than M group ($p < 0.05$). M + A group showed elevated levels of *unclassified_f__Lachnospiraceae*, *norank_f__norank_o__Clostridia_UCG-014*, *Prevotellaceae_UCG-001*, *unclassified_f__Prevotellaceae* than M group ($p < 0.05$). Fecal levels of *Lachnospiraceae_UCG-006* in M + H group was higher than that in M group ($p < 0.05$). M + HR group displayed higher relative levels of *Streptococcus*, *Rikenellaceae*

RC9_gut_group than M group ($p < 0.05$).

3.6 Effects of astaxanthin, *H. pluvialis* and *H. pluvialis* residue on the gut microbiota-derived metabolites

Figure 5 displayed the determined gut microbiota metabolites (SCFAs and LPS). No notably difference was observed on fecal levels of SCFAs and LPS between C and M group. Administration of astaxanthin and *H. pluvialis* residue dramatically increased fecal concentrations of total SCFAs, acetic acid, isobutyric acid, and valeric acid compared to M group ($p < 0.05$). M + A group displayed higher fecal levels of propionic acid than M group ($p < 0.05$). The feces of M + H group had higher concentrations isobutyric acid compared to M group ($p < 0.05$). M + H and M + HR groups showed significantly lower fecal levels of LPS than M group ($p < 0.05$). Astaxanthin and *H. pluvialis* markedly decreased D-gal induced elevated serum levels of LPS compared to M group ($p < 0.05$). Moreover, the effects of astaxanthin was superior to *H. pluvialis* ($p < 0.05$).

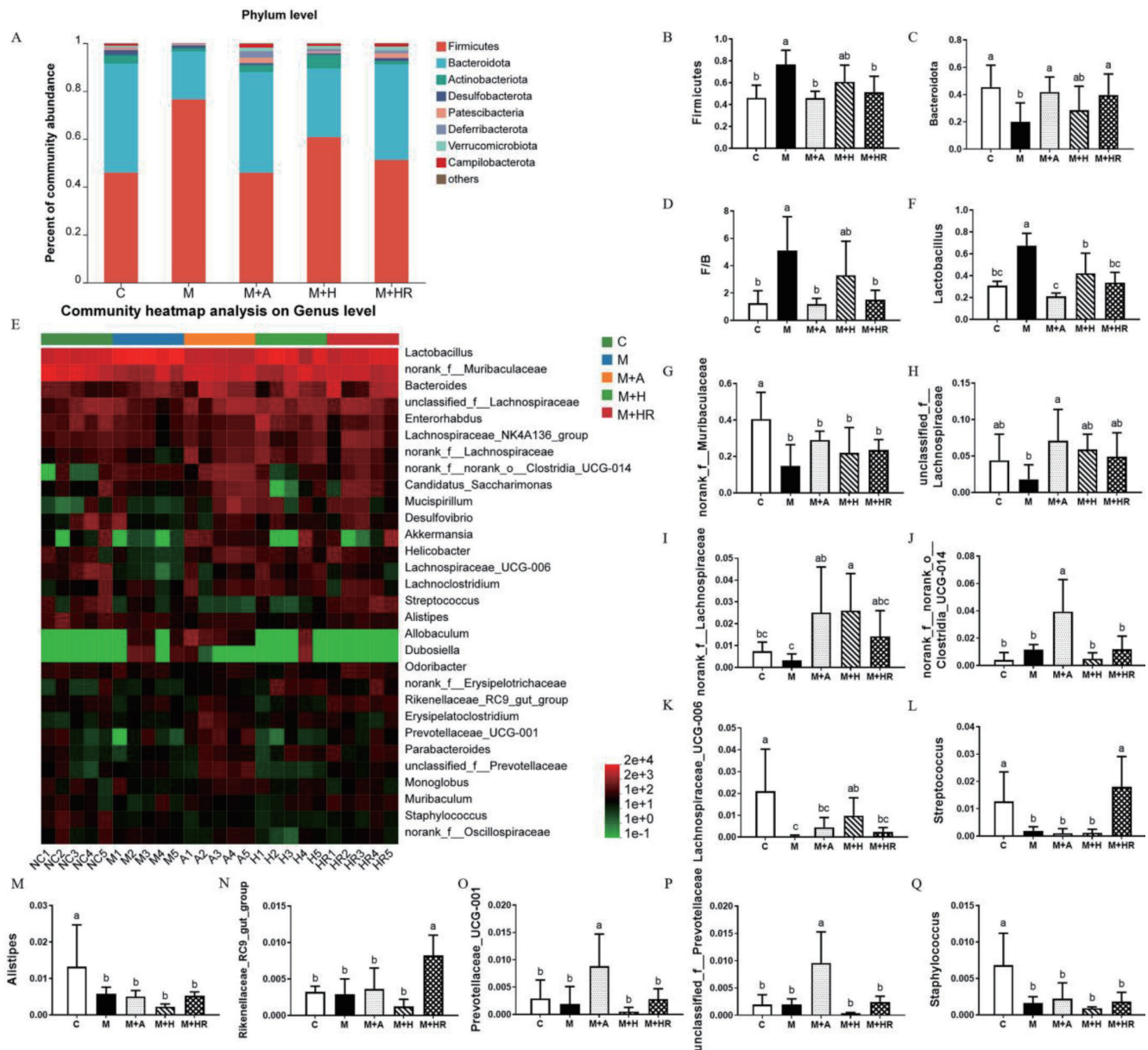


Fig. 4 Gut microbial composition among groups. (A) Distributions of microbial phyla; (B–C) Relative abundance of microbial phyla; (D) Ratios of F/B; (E) Distributions of microbial genera; (F–Q) Relative abundance of microbial genera. a–c, different letters indicate statistical different between groups ($p < 0.05$).

3.7 Associations of astaxanthin, *H. pluvialis* and *H. pluvialis* residue modified gut microbiota and microbial metabolites with liver injury related indices

To reveal the roles of gut microbiota in modulation of hepatic injury, relationships among the supplements regulated bacteria with oxidant injury parameters of liver were also analyzed (Fig. 6A). Fecal levels of *Firmicutes* was positively associated with serum AST, liver MDA, liver IL-6, liver IL-1 β , serum TNF- α , serum IL-6 and negatively correlated with liver GSH-PX, liver T-AOC ($p < 0.05$). *Bacteroidetes* was negatively associated with liver IL-1 β , serum IL-6 and positively associated with liver T-AOC ($p < 0.05$).

F/B was positively related with serum AST, liver MDA, liver IL-1 β , serum TNF- α , serum IL-6 and negatively correlated with liver T-AOC ($p < 0.05$). *Lactobacillus* was positively associated with serum AST, liver MDA, liver IL-6, liver IL-1 β , serum MDA, serum TNF- α , serum IL-6 and negatively correlated with liver SOD ($p < 0.05$). Fecal levels of *unclassified_f_Lachnospiraceae* was negatively related with serum ALT, liver MDA, liver TNF- α ($p < 0.05$). *Norank_f_Lachnospiraceae* was positively associated with serum T-AOC and negatively related with liver IL-1 β ($r: -0.443, p < 0.05$). Fecal levels of *norank_f_norank_o_Clostridia_UCG-014* was positively associated with

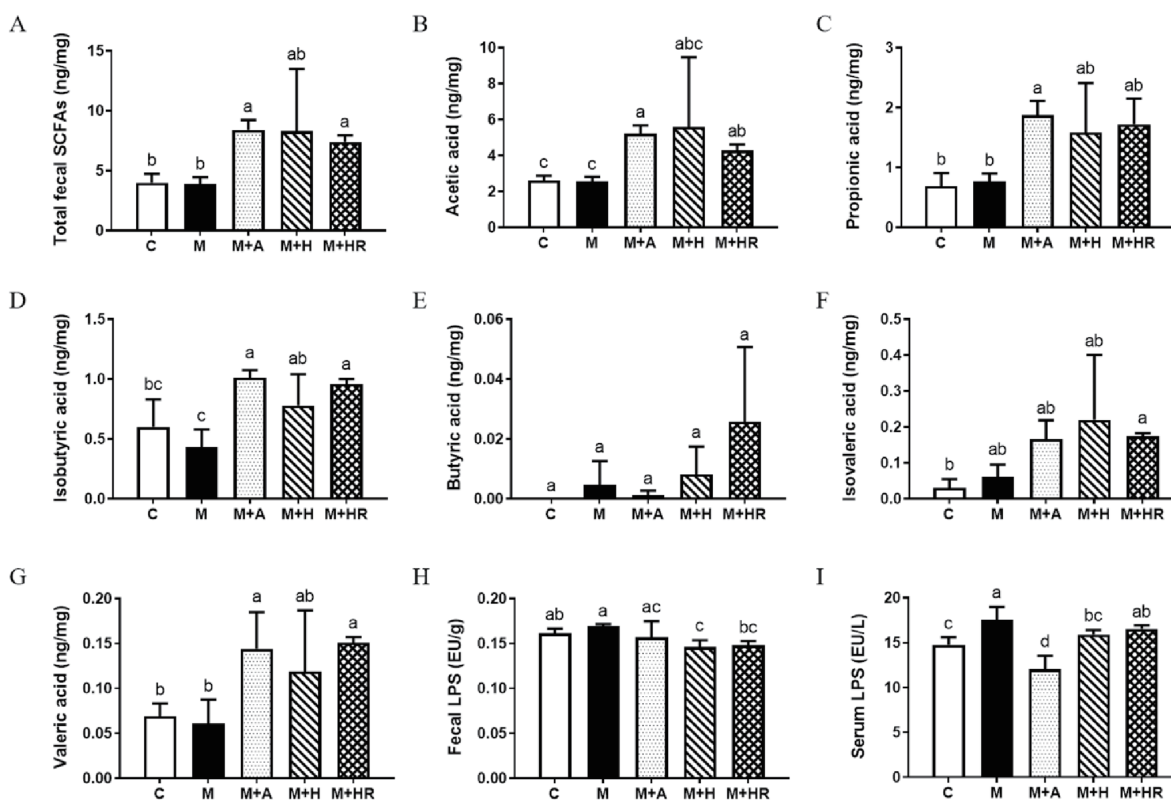


Fig. 5 Effects of astaxanthin, *H. pluvialis* and *H. pluvialis* residue on gut microbial-derived metabolites. (A) Fecal levels of total SCFAs; $n=8$ each group; (B-G) Fecal levels of various SCFAs; (H) Fecal levels of LPS; (I) Serum levels of LPS. a-c, different letters indicate statistical different between groups ($p < 0.05$).

liver T-AOC ($p < 0.05$). *Lachnospiraceae_UCG-006* was negatively associated with serum AST, serum MDA and positively related with liver SOD, liver GSH-PX, serum T-AOC ($p < 0.05$). *Streptococcus* was negatively associated with serum AST, serum ALT, liver MDA, liver TNF- α , serum TNF- α , serum IL-1 β and positively correlated with serum SOD ($p < 0.05$). *Rikenellaceae_RC9_gut_group* was negatively associated with liver MDA and liver TNF- α ($p < 0.05$). *Prevotellaceae_UCG-001* was negatively associated with serum ALT, liver MDA, liver TNF- α , serum TNF- α , serum IL-1 β and positively correlated with serum SOD, serum T-AOC ($p < 0.05$). *Unclassified_f__Prevotellaceae* was negatively associated with serum ALT, liver MDA, liver TNF- α , serum TNF- α and positively correlated with liver SOD ($p < 0.05$).

We also explored the associations of microbial-derived metabolites with the hepatic injury parameters (Fig. 6B). Fecal levels of total SCFAs, acetic acid, propionic acid, isovaleric acid was negatively correlated with liver TNF- α and positively correlated with serum SOD ($p < 0.05$). Acetic acid was also negatively related with serum IL-1 β ($p < 0.05$). Isobutyric acid was negatively correlated with liver TNF- α ($p < 0.05$). Valeric acid was negatively associated with liver MDA, liver TNF- α and positively related with liver SOD ($p < 0.05$). Fecal LPS was positively related with

liver MDA, liver IL-6, serum TNF- α , serum IL-1 β and negatively related with serum T-AOC ($p < 0.05$). Serum LPS was positively associated with serum ALT, serum AST, serum TNF- α , serum IL-6 and negatively related with liver SOD, liver GSH-PX, liver T-AOC and serum SOD ($p < 0.05$).

4 Discussion

In this work, we evaluated the distinct effects of astaxanthin, *H. pluvialis* and *H. pluvialis* residue on the hepatic injury, gut microbiota and microbial metabolites in D-gal-induced aging mice for the first time. The three supplements all markedly prevented D-gal-induced tissue injury, oxidative stress and chronic inflammation in liver. Amelioration of intestinal microbiota dysbiosis and production of fecal microbial metabolites might partially explain their protective effects.

D-gal-induced aging process mimics the senescence progress *in vivo* and is broadly applied for anti-aging research²². Numerous studies have shown that overload of D-gal lead to excessive ROS production in the body, causing oxidative stress and chronic inflammation that attack liver and ultimately result in hepatic injury². Herein, we successfully built a D-gal (200 mg/kg/day I.P) induced

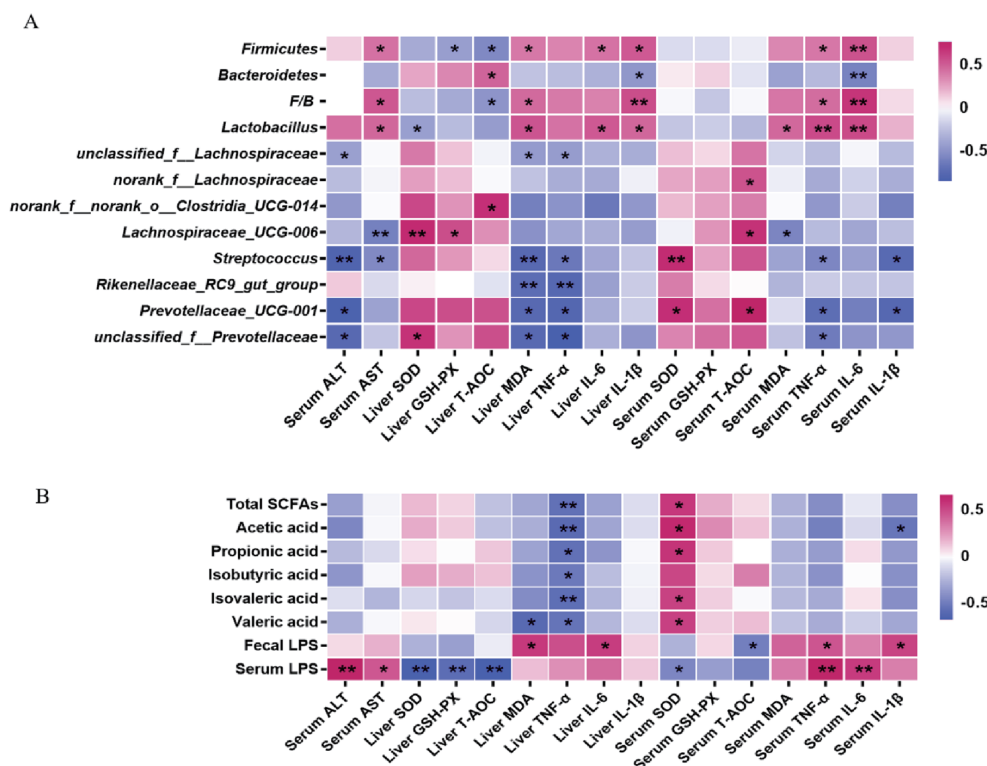


Fig. 6 Spearman correlation between the modified gut microbiota and microbial metabolites with hepatic injury-related parameters. (A)Heat map shows the Spearman correlation coefficient between the gut bacteria and liver injury related indices; (B)Heat map shows the Spearman correlation coefficient between the microbial metabolites and liver injury related indices; * $p < 0.05$, ** $p < 0.01$.

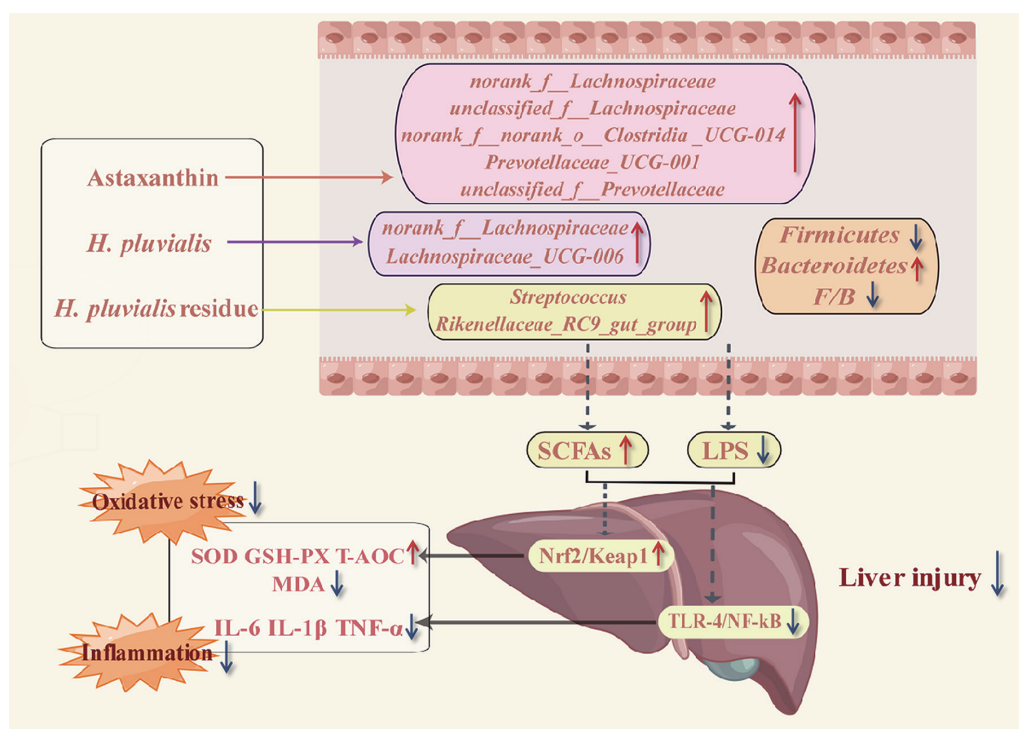


Fig. 7 Potential mechanisms underlying the preventive effect of astaxanthin, *H. pluvialis* and *H. pluvialis* residue on aging related liver injury. This Figure is made by FigDraw (ID:YAIWP71688).

hepatic injury model characterized by damaged liver structure, elevated serum liver function parameters ALT and AST, exacerbated hepatic oxidative stress (decreased SOD, GSH-PX, T-AOC and increased MDA) and enhanced chronic inflammation related cytokines in liver, like TNF- α , IL-6 and IL-1 β . Astaxanthin is a well-known antioxidant natural ingredient and *H. pluvialis* is an one of the major sources of astaxanthin with broad potentials of exploitation and application. We found both astaxanthin (10 mg/kg/day, equivalent to approximately 60 mg/day for human), *H. pluvialis* (1% astaxanthin, 1000 mg/kg/day, equivalent to approximately 6 g/day for human) and *H. pluvialis* residue (1000 mg/kg/day) supplementation remarkably ameliorated liver morphology, decreased serum levels of ALT, attenuated hepatic oxidative stress and chronic inflammation in the aging mice, suggesting the protective effect of them on D-gal induced liver injury. Numerous reports revealed the protective effect of astaxanthin (2-100 mg/kg/day) on liver in high calorie or chemicals induced liver disease rodent models through anti-oxidant and anti-inflammation⁷⁾. Few studies have reported the liver protective effects of astaxanthin in aging models. Chen *et al.*⁹⁾ found 5-15 mg/kg astaxanthin administration reversed injures in liver through regulating the balance of oxidative and antioxidant systems, activating transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) and cytoplasmic protein kelch-like ECH-associated protein 1 (Keap1) mediated antioxidant pathway, and inhibiting nuclear factor kappa-B (NF- κ B) mediated inflammation pathway in D-gal-induced aging rats. El-Baz *et al.*⁸⁾ reported that 450 mg/kg astaxanthin-rich *H. pluvialis* supplementation for 2 weeks significantly attenuated D-gal induced liver injury in rats through activating the Nrf2/Keap1 antioxidant pathway. These reports are consistent with our findings. While, *H. pluvialis* is also abundant in other ingredients, like approximately 50% fibers, 25% proteins, few carbohydrates, lipids, and ash²³⁾. These constituents have potential bioactivities²⁴⁾. This might also explain the protective effects of *H. pluvialis* residue on liver injuries in this study. Fibers and proteins are the major components in the residue. Dietary fibers are well recognized for their regulative roles on intestinal microbiome composition and affect the physiology of liver through gut-liver communication²⁵⁾. Enzymatic hydrolysates of *H. pluvialis* residue protein was also reported to play antioxidant properties *in vitro*²⁶⁾. A recent study reported peptides derived from *H. pluvialis* residue displayed anti-aging activity in *Caenorhabditis elegans*²⁷⁾. Our study was the first to explore the hepatic protective effects of *H. pluvialis* residue *in vivo*. In addition, the ameliorative effects of the three supplements on D-gal induced liver injury were comparable with slightly different. For instance, the results showed that M + A group had a lower liver index than to M + H group. Astaxanthin, but not *H. pluvialis* or residue notably reduced serum TG

compared to model mice. Astaxanthin and *H. pluvialis* are more effective in attenuating liver oxidative stress induced by D-gal than *H. pluvialis* residue, with markedly enhanced liver SOD and GSH-PX levels. While, *H. pluvialis* is more effective in reducing liver inflammation than astaxanthin and residue. We suppose these differences might be attribute to the different components of the three supplements and different absorption rate of astaxanthin between pure astaxanthin and that in *H. pluvialis*. It's worth noting that the dose of astaxanthin we used was higher than the recommended dose for human. The recommended or approved doses of astaxanthin in different countries ranged from 2 to 24 mg²⁸⁾, and no serious adverse events were observed in clinical indices with the high dose treatment (45 mg/kg)²⁹⁾. In China, there is no recommended nutrient intake (RNI) or tolerable upper intake levels (UL) for astaxanthin. While, it is suggested by National Health Commission of China that the daily intake of *H. pluvialis* should be less than 800 mg/day. Further studies are needed to compare lower doses of the three supplements on D-gal induced liver injury. Moreover, also we have measured the general nutritional components (moisture, protein, fat, ash) of *H. pluvialis* and residue, there are other trace elements that we failed to measure. As reported, dry *H. pluvialis* contains approximately 0.01% β -carotene, 0.03% canthaxanthin, 0.01% lutein, 0.01% adonixanthin 0.01% adonirubin, 0.003% echinenone, etc^{30, 31)}. While, due to the low contents of these trace elements, we suppose the ameliorative effect of *H. pluvialis* or *H. pluvialis* residue might be mainly attributed the major general components in them. Further studies are warranted to clarify the distinct roles of specific substrate from *H. pluvialis* residue on liver protection.

Growing evidence proves that the communication between gut microbiota and liver is important in the pathogenesis of aging related liver injury¹¹⁾. Dietary habits play a primary role in shaping intestinal environment³²⁾. In this study, alpha diversity analysis revealed that D-gal induced decreased community diversity were reversed by astaxanthin, *H. pluvialis* and *H. pluvialis* residue. Astaxanthin treatment also enhanced the microbial community richness. Numerous specific intestinal bacteria were identified to exhibit critical roles in liver diseases. *Firmicutes* and *Bacteroidetes* comprises the top two major phyla in the feces of primates and rodents, and F/B is widely accepted as a marker for gut microbiota dysbiosis³³⁾. Enriched abundance of *Firmicutes* and declined that of *Bacteroidetes* were reported to be positively related with liver injury³⁴⁾. Increased fecal proportion of F/B was reported in D-gal-induced aging model³⁵⁾, that is consistent with our results. Herein, astaxanthin and *H. pluvialis* residue notably decreased F/B, relative levels of *Firmicutes* and increased *Bacteroidetes* in the feces of D-gal treated mice. *H. pluvialis* also displayed similar trend. The results indicated the

protective roles of the supplements on gut microbiota dysbiosis in aging mice. At genus levels, *norank_f__Muribaculaceae*³⁶⁾, *Lactobacillus*³⁷⁾, *unclassified_f__Lachnospiraceae*, *norank_f__Lachnospiraceae*³⁸⁾, *Lachnospiraceae_UCG-006*³⁹⁾, *Alistipes*⁴⁰⁾, *Streptococcus*⁴¹⁾, *Rikenellaceae_RC9_gut_group*⁴²⁾, *unclassified_f__Prevotellaceae*⁴³⁾ are SCFAs-producing bacteria, which are beneficial for liver health and are reported to be negatively associated with hepatic oxidative stress, inflammation and damage⁴⁴⁾. *Norank_f__norank_o__Clostridia_UCG-014*⁴⁵⁾, and *Prevotellaceae_UCG-001*⁴⁶⁾ are beneficial bacteria, that are reported to exert anti-inflammatory function and are inversely correlated with pro-inflammatory cytokines, like IL-6 and TNF- α . In this study, D-gal administration led to significantly declined relative abundance of *norank_f__Muribaculaceae*, *Lachnospiraceae_UCG-006*, *Streptococcus*, and *Alistipes*, supporting the microbial dysbiosis in gut of D-gal-fed mice. D-gal treatment also led to a notable enhanced abundance of *Lactobacillus* in the feces. As a normally recognized beneficial bacteria, the enrichment of *Lactobacillus* in feces of D-gal injected mice might lying in the specificity of the model, which was also reported in other literature⁴⁷⁾. In addition, the three supplements reversed the elevation of *Lactobacillus*. Noteworthily, we found astaxanthin, *H. pluvialis* and *H. pluvialis* residue distinctly modified the gut microbial genus. Astaxanthin mainly enhanced fecal beneficial genus *unclassified_f__Lachnospiraceae*, *norank_f__Lachnospiraceae*, *norank_f__norank_o__Clostridia_UCG-014*, *Prevotellaceae_UCG-001*, *unclassified_f__Prevotellaceae*. *H. pluvialis* increased *norank_f__Lachnospiraceae* and *Lachnospiraceae_UCG-006*, while *H. pluvialis* residue primarily enriched *Streptococcus* and *Rikenellaceae_RC9_gut_group*. The findings revealed the protective effects of the three supplements on D-gal-induced on intestinal microbiota dysbiosis and the effects of astaxanthin seems more remarkable. The attenuating roles of astaxanthin on gut microbiota dysbiosis were reported in fatty liver mice¹⁵⁾, that are in line with our findings. Our study was the first to report the ameliorative roles of astaxanthin on aging related intestinal dysbiosis. We are also the first to reveal the regulative effects of *H. pluvialis* and *H. pluvialis* residue on gut microbiota. We also measured the microbial metabolites. The microbial metabolites, primarily SCFA and LPS are crucial mediators of gut-liver axis¹²⁾. SCFAs are beneficial for liver health through maintaining the intestinal integrity, down-regulating NF- κ B mediated inflammatory pathway⁴⁸⁾ and activating Nrf2 mediated anti-oxidant pathway⁴⁹⁾. SCFAs supplementation was reported to ameliorate liver injury in various liver disease models⁵⁰⁾. Astaxanthin, *H. pluvialis* and *H. pluvialis* residue markedly elevated fecal levels of SCFAs, and the results are in line with the restored SCFAs-producing bacteria aforementioned. Astaxanthin was reported to enhance fecal produc-

tion of SCFAs in corticotropin-releasing factor (CRH)-induced intestinal hypermotility rats⁵¹⁾ and *in vitro* fermentation model⁵²⁾. The results are consistent with our findings. LPS could induce liver injury by activating hepatic TLR-4/NF- κ B inflammation pathways¹⁴⁾ and suppressing Nrf2/Keap1 antioxidant pathway⁵³⁾. LPS also destroy gut barrier to facilitate the penetration of LPS into circulation¹⁴⁾. In the current study, *H. pluvialis* and *H. pluvialis* residue dramatically decreased fecal levels of LPS and a decreasing trend was observed in astaxanthin tread mice, supporting the protective roles of the three components on intestinal dysbiosis. Interestingly, astaxanthin displayed the most remarkable on serum levels of LPS, which might be due to the remarkable improvement effect of astaxanthin on integrity of intestinal barrier⁵⁴⁾. In addition, correlation analysis also revealed that the altered intestinal bacteria and the microbial metabolites were significantly associated with most of the indices regarding liver function, oxidative stress and inflammation. Collectively, our results proved that the suppression of astaxanthin, *H. pluvialis* and *H. pluvialis* residue on D-gal induced liver injury was related with their regulation of intestinal microbiota and microbial metabolites.

5 Conclusion

In conclusion, the current work demonstrated that dietary astaxanthin, *H. pluvialis* and *H. pluvialis* residue supplementation ameliorated aging related liver injury, including damaged liver structure, declined liver function and elevated hepatic oxidative stress and inflammation. The potential mechanisms (Fig. 7) were related with the modulation of gut microbiota composition and regulation of microbial metabolites production, like SCFAs and LPS. Our results lead a novel insight into the mechanisms of astaxanthin and *H. pluvialis* in preventing aging related hepatic injury and provide evidence for exploiting of *H. pluvialis* residue as a functional ingredient for the treatment of liver diseases. Future studies will further clarify the effects and mechanisms of dominant components of *H. pluvialis* residue on liver injury, which are expected to provide a reference for the high-value utilization of *H. pluvialis* resources.

Author Contributions

Conceptualization, Y. Z. and X. G.; methodology, Y. Z., C. G. and Y. J.; software, Y. Z.; validation, X. G., Q. Z., Y. S. and F. C.; formal analysis, Y. Z., C. G. and M. Z.; investigation, C. G. and M. Z.; resources, X. G., Q. Z. and Y. S.; data curation, C. G.; writing—original draft preparation, Y. Z. and C. G.; writing—review and editing, X. G. and Q. Z.; supervision, X.

G. and Q. Z. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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