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Catechins promoted *Enterococcus faecalis* to alleviate related indices of nonalcoholic steatohepatitis mice induced by high-fat diet

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ABSTRACT: This study provides different opinion for exploring the mechanism of catechin (CAT) relieving nonalcoholic steatohepatitis (NASH), it is more innovative to explore from the perspective of intestinal microorganism. Through *in vitro* fermentation experiments, CAT could improve the abundance of *Enterococcus*, and *Enterococcus faecalis* (EF) accounts for the vast majority of *Enterococcus* in human gut. The experimental results *in vivo* showed that EF group and CAT+EF group could reduce the body weight, liver weight and epididymal fat weight of NASH mice, and improve the changes of serum and liver indexes. HE staining observation showed that these two groups have greatly improved the fatty degeneration, balloon degeneration and necrotic focus caused by NASH. The alleviation of CAT+EF group was more obvious. Results of targeted metabonomics showed that CAT could promote EF to produce more methyl palmitate (MP, C16:0), which plays a great role in relieving NASH. Our results indicated that EF could alleviate NASH and CAT+EF group had better alleviation may due to more production of MP by EF. This study provides a new idea for CAT to alleviate NASH.

Keywords: Catechin; *In vitro* fermentation; *Enterococcus faecalis*; Nonalcoholic steatohepatitis; Targeted metabonomics; Methyl palmitate

1. Introduction

Polyphenols are rich in grains.[1, 2] Flavonoids in polyphenols mainly exist in grains in free form, and catechin (CAT) is the main flavonoid.[3] CAT is rich in purple rice, highland barley and other grains. However, the bioavailability of CAT *in vivo* is very poor.[4] About two-thirds of CAT reaches the colon, where it is degraded by microbial enzymes to produce a series of metabolites.[5, 6] Studies have shown that[7] CAT affects intestinal microorganism composition after metabolic reaction, thus improving the overall intestinal health and helping to improve metabolic disorders. Therefore, it is of great significance to explore the interaction between CAT and intestinal microorganisms. At present, the research on CAT mainly focuses on the separation and purification of CAT,[8] the exploration of its antioxidant function and structural characteristics,[9] and the study on the alleviation of some symptoms. The research on relieving symptoms includes: the prevention of obesity;[10, 11] the resistence on atherosclerosis;[12] improvement of oxidative renal

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injury in rats;[13] the relieving effect on NASH.[14, 15] The influence of CAT on individual microorganism is rarely involved, so it is interesting to study CAT on improving some symptoms from the perspective of microorganism in depth.

Intestinal microorganism is the largest and complex system of human body, which is closely related to human life and health. At present, a dense microbial community consisting of $10^{11} \sim 10^{14}$ cells is defined in human intestine.[16] *Enterococcus faecalis* (EF) is the most common intestinal microorganism in *Enterococcus*,[17] which often exists in healthy people and is also a kind of lactic acid bacteria.[18] EF is a facultative anaerobic gram-positive bacterium and a member of mammalian intestinal flora. It can inhibit the growth of other pathogenic bacteria[18], thus regulating the intestinal microenvironment and human immunity .[19, 20] EF is a normal flora in human intestine, which can tolerate bile acid and gastric acid. Besides, EF can colonize in intestine. At present, EF is widely used in the fields of medicine and food.[21] Studies have shown that EF can alleviate obesity in mice, among which acyl-CoA thioesterase (ACOT) in EF may mediate the biosynthesis of myristic oleic acid (MA), so that mice can reduce obesity by increasing energy metabolism, activating brown adipose tissue and promoting beige adipose formation.[22] EF can also be used as a starter culture.[23] At present, no one has specifically explored the mitigation effect of EF on NASH.

Nonalcoholic steatohepatitis (NASH) is a chronic inflammatory disease of the liver, which is characterized by fatty degeneration of hepatocytes, balloon degeneration, diffuse mild inflammation of hepatic lobules and other diseases caused by no history of excessive drinking or no alcohol intake.[24] Among patients with nonalcoholic fatty liver disease, nonalcoholic steatohepatitis accounts for 41.4% ~ 54.0%, but at present, the pathogenesis of nonalcoholic steatohepatitis is still unclear,[25] and the progress in prevention, inhibition and treatment of nonalcoholic steatohepatitis is slow. The pathological symptoms of animal model with high-fat diet are similar to those of human nonalcoholic steatohepatitis. At present, there were many researches showed CAT can alleviate NASH, but few of them involved the interaction with intestinal microorganisms.[14, 15] According to the research, lactic acid bacteria in the intestine have the potential function of regulating NASH.[26] Therefore, it is of great significance to explore the mitigation of NASH by CAT from the perspective of microorganisms.

At present, nonalcoholic fatty liver disease (NAFLD) generally increases at the same time as obesity and diabetes. NASH is an important stage of NAFLD. However, studies have shown that intestinal microorganisms will affect the lipid level and lipid metabolism in blood and tissues of mice and humans.[27] Some studies also shown that there is a certain relationship between the intestine and the liver, and the theory of "intestine-liver axis" is put forward. The theory of "intestine-liver axis" may be that intestinal microorganisms indirectly affect the development of NAFLD through signal pathways mediated by their components and metabolites.[28]

In this paper, firstly, we used human feces to ferment CAT *in vitro*, and found the most significant strain. Then explored the alleviation of CAT on NASH through specific intestinal microorganism of EF.

Finally, we preliminarily explored the possible mechanism, which provides a new idea for CAT as a prebiotic to relieve NASH. We will continue to explore the interaction between CAT and EF *in vitro* and *in vivo* from the perspective of quorum sensing（QS）.

2. Materials and methods

2.1 Materials and reagents

Catechin (CAT, MFCD00150865, purity ≥99.9%) was purchased from Shanghai McLean Biochemical Technology Co., Ltd. *Enterococcus faecalis* (EF, BNCC 194756) was purchased from Beijing Beina Chuanglian Biotechnology Research Institute. C57BL/6J mice (male) were purchased from Shanghai Jiesijie Experimental Animal Co., Ltd. 60% fat-purified feed for mice（20% protein, 20% carbohydrate, 60% fat）, irradiation maintenance feed and compressed wood shavings padding were all purchased from Jiangsu Collaborative Pharmaceutical Bioengineering Co., Ltd. Antibiotics （ampicillin 1g/L, neomycin sulfate 1g/L, metronidazole 1g/L, vancomycin 500mg/L）[29, 30] were purchased from Shanghai Yuanye Biotechnology Co., Ltd. Triglyceride (TG) assay kit (specification: 96T), total cholesterol (TC) assay kit (specification: 96T), high-density lipoprotein cholesterol (HDL-C) assay kit (specification: 96T), low-density lipoprotein cholesterol (LDL-C) assay kit (specification: 96T), γ -glutamyltransferase (γ-GT/GGT) test box (specification: 96T), superoxide dismutase (SOD) assay kit (specification: 96T), malondialdehyde (MDA) assay kit (specification: 96T), alkaline phosphatase (ALP/AKP) determination kit (specification: 96T), alanine aminotransferase (glutamic-pyruvic transaminase /ALT/GPT) test box (specification: 96T), aspartate aminotransferase (glutamic-oxaloacetic transaminase /AST/GOT) test kit (specification: 96T) and reduced glutathione (GSH) test kit (specification: 96T) were purchased from Nanjing Jiancheng Institute of Bioengineering. *In vitro* fermentation samples and targeted metabolomics samples were sent to Shanghai Meiji Biomedical Technology Co., Ltd. for testing. Samples of liver tissue and epididymal adipose tissue were sent to Shanghai Lanyao Biotechnology Service Center for detection and observation.

2.2 In vitro fermentation

The fecal samples were collected from three healthy volunteers, aged 21-25, who had no history of intestinal tract and had not used any drugs within 3 months before sampling. Samples of 30 g feces from three volunteers were sent to the laboratory within 1h after sampling, frozen in liquid nitrogen and stored in a refrigerator at -80 ℃. After thawing, the feces were evenly mixed, 10 g was mixed with the prepared sterile PBS buffer at a ratio of 1:9, swirled for 3 min, and filtered with four layers of sterile gauze to obtain a fecal sample solution.[31]

The experimental group of *in vitro* fermentation was divided into two groups. One group was the control group, which was fermented without adding CAT, and the other group was fermented with adding CAT. Weigh 300 mg of CAT monomer, put it into an ultra-clean table in advance, add 180 mL of sterilized basal medium, mix it evenly with vortex vibration, add 20 mL of fecal sample solution, and put it into an

anaerobic incubator at 37 ℃ for fermentation with the control group. There were 6 parallel groups in control group and CAT group respectively.[32]

After 48 h of fermentation, 12000 g of fermentation broth was centrifuged for 10 min (4 ℃) by a centrifuge, and the remaining precipitate was frozen in a refrigerator at -80 ℃ after removing the supernatant, and then sent to Shanghai Meiji Biomedical Technology Co., Ltd. for detection.

2.3 Animal Experiments

According to the guide of "Standard for Laboratory Animals in China", animal experiments are carried out in accordance with internationally effective guidelines and schemes, and approved in advance by the Animal Ethics Committee. Fifty C57BL/6 male mice were prepared for the experiment (6 weeks old, weighing 18-20 g), which were raised under standard conditions (temperature of (25±2) ℃, humidity of (60±5)%, light/dark cycle of 12 hours), during which they ate and drank freely. After a week's adaptation, the mice were randomly divided into five groups as shown from Fig. 1, with ten mice in each group: (1) normal diet (CON), (2) high-fat diet induced NASH (NASH), (3) NASH feeding induced by high-fat diet for 9 weeks, antibiotic drinking water for 2 weeks, EF gavage for 4 weeks (2.5×10^8 CFU/ml bacterial suspension was given once a day) (NASH+ABX+EF), (4) NASH feeding induced by high-fat diet for 9 weeks, antibiotic drinking water for 2 weeks, gavage of the mixture of CAT and EF for 4 weeks (2.5×10^8 CFU/ml bacterial suspension was mixed with 80 mg/kg.d CAT) (NASH+ABX+CAT+EF), CON and NASH groups were given the same amount of normal saline every day. At the eighth week, two animals were randomly killed in the CON group and the NASH group, and the weight of mice, liver weight, epididymal fat weight, serum TG, TC, HDL-C, LDL-C and HE staining were measured to observe the liver tissue, so as to judge whether the NASH model was successful. After the animals were fed, they were weighed, and the blood of mice was collected from eyeball and tissue samples were dissected. The mice were killed by cervical dislocation, and the tissue samples were quickly frozen in liquid nitrogen and stored in a refrigerator at 80 ℃.[33]

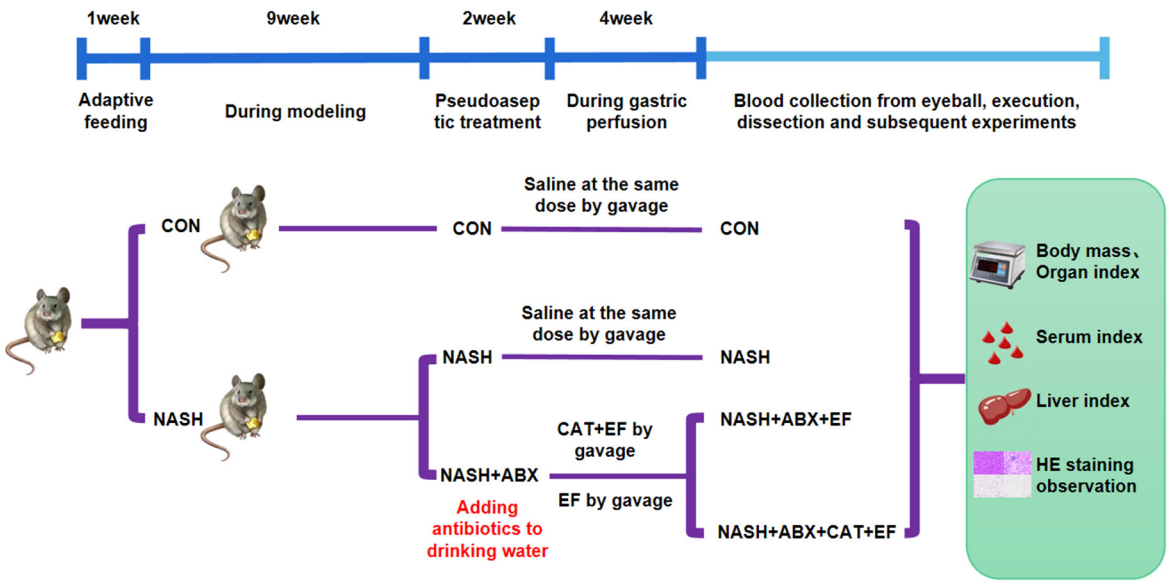

Fig. 1 Animal experiment design.

2.4 Body weight and organ index

During the experiment, the diet, defecation and death of mice were observed, and the weight of mice was weighed every week and recorded. After the mice were killed, the fat of heart, liver, spleen, kidney and epididymis was taken out, washed with normal saline, then dried with filter paper, weighed and calculated the organ index. The fat lobule of liver and epididymis was preserved and observed by HE staining. The remaining liver was put into a freezing tube and kept in a refrigerator at -80 ℃ for later use.[34]

Organ index (%) = organ mass (g) / mice weight (g) ×100%

2.5 Biochemical indexes of liver and serum

After the mice were killed, the livers were taken out and placed in the same position on a ruler to compare the sizes of the livers. A small part of the liver was taken to determine TG, GSH, ALT, AST, ALP, SOD and MDA, and the kit was used for determination. The remaining liver was put into a freezing tube and kept in a refrigerator at -80 ℃ for later use. The detection indexes mainly included four blood lipids, TG, TC, HDL-C, LDL-C, serum liver function indexes, ALP, ALT and AST, as well as γ-GT and GSH, which were determined by the kit.[35]

2.6 HE staining observation

After the mice were killed, 1 g fatty tissues of liver and epididymis were cut, washed with normal saline and put into 5% paraformaldehyde fixed solution, and three samples were sent to each group for parallel detection.[35]

2.7 Targeted metabonomics assay

Targeted metabonomics assay group was divided into two groups, one was the control group (CON), and the other was the experimental group (CAT), in which CAT was added to the bacterial solution, and the concentration of CAT was set at 1mg/mL, and 6 samples in each group were parallel. After 24 hours of culture, the microorganisms and the culture medium were separated by a centrifuge tube, and the thalli were collected into a 15mL centrifuge tube, then added with a precooled 10mL PBS solution, centrifuged at 8000×g for 5-10min, and then stored in a refrigerator at -80 ℃ after the sludge was discarded.[34] After that, it was sent to Shanghai Meiji Biomedical Technology Co., Ltd. for testing.

2.8 Statistical analysis

The data in this study were expressed by the average standard deviation (n≥6) of each sample. SPSS 21 was used for regression analysis and other statistical analysis, and Duncan's multiple comparison test was used for analysis. GraphPad Prism 8.0 software was used to draw the graph, and all the data were statistically analyzed by one-way ANOVA. * indicates the comparison among CON group, NASH group and gavage group (*$P < 0.05$, **$P < 0.01$, *** $P < 0.001$).

3. Results and discussion

3.1 In vitro fermentation results of CAT

At present, it is very effective to explore the biological activity of polyphenols through *in vivo* intervention, but it is time-consuming and laborious. Therefore, *in vitro* fermentation experiment, which is more convenient and ethically unrestricted, is used for preliminary exploration. *In vitro* fermentation of human feces is helpful to better study the relationship between CAT and intestinal microorganisms, and *in vitro* experiment can fully explain the influence of polyphenols on intestinal microorganisms. Some researchers have explored the effects of polyphenols-rich substances on intestinal microorganisms through *in vitro* fermentation, such as green tea and some grains. At present, the research results show that tea polyphenols rich in catechins can regulate the co-metabolism process of host-intestinal flora through the intervention of intestinal microorganisms, so as to improve the disease. However, there are few specific studies on the effects of CAT on single bacteria. Our research aims to screen out the bacteria most affected by CAT through *in vitro* fermentation, so as to carry out further research.

As shown from Fig. 2, the relative abundance of intestinal microorganisms in CAT group has changed greatly compared with that in CON group. On the genus level, compared with the CON group, the decreased flora include *Escherichia*, *Romboutsia*, *Phascolarctobacterium*, *Paeniclostridium*, etc., while the CAT group significantly increased the relative abundance of several groups of flora, such as *Enterococcus*, *Eggerthella*, *Blautia*, etc. (Fig. 2A). Also can be seen from the figure, the addition of CAT could significantly improve the abundance of *Enterococcus* (Fig. 2B). From the comparative analysis results of this two groups, adding CAT could especially improve the abundance of *Enterococcus* in the intestine (Fig. 2C).

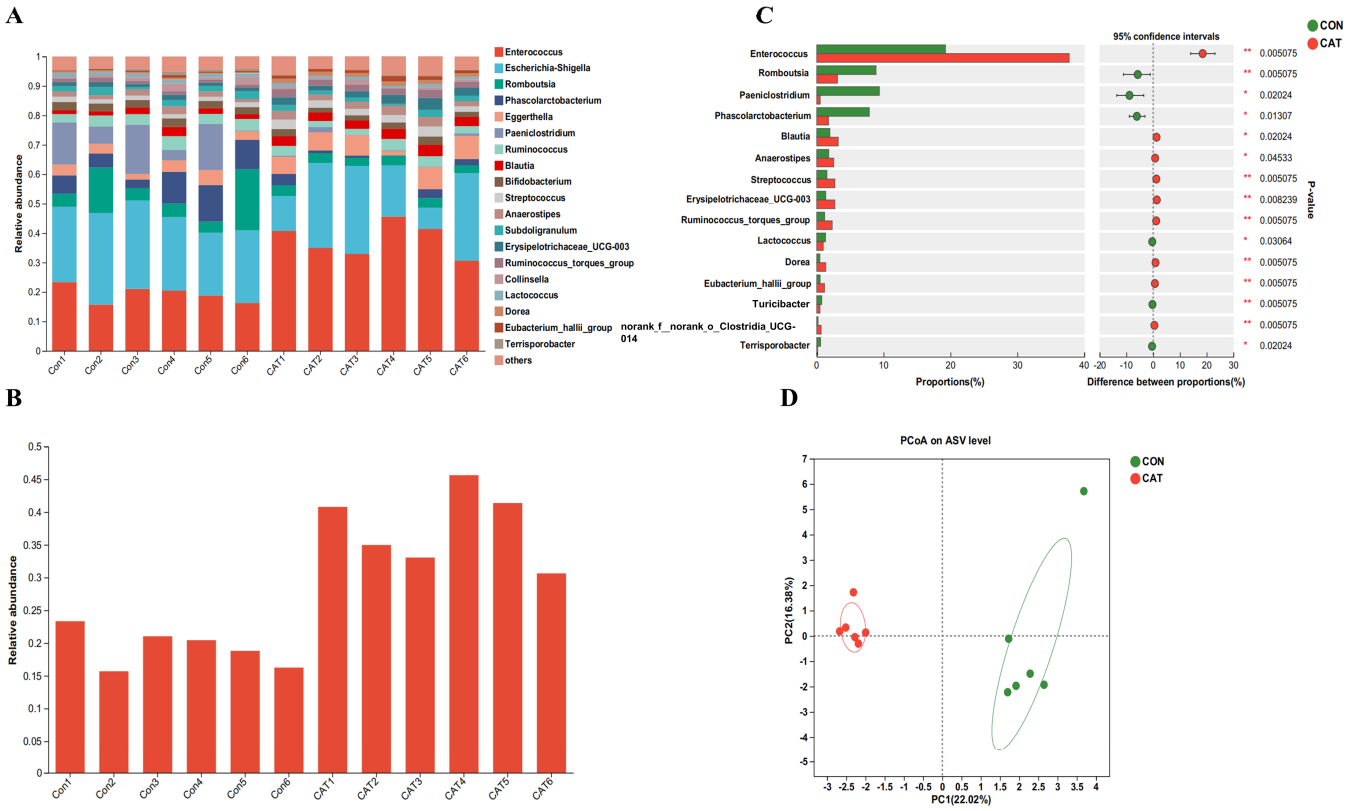

Fig. 2 Effect of CAT on human intestinal flora during 48h *in vitro* fermentation. (A) Composition and relative abundance of genus-level microorganisms. (B) Difference in relative abundance of Enterococcus. (C) Comparative analysis results of the two groups. (D) Diversity of microbial communities.

Compared with CON group, the flora of CAT group was obviously separated. In the study of *in vitro* fermentation on human intestinal microorganism flora by CAT, we can see the similarity between the samples from the PCoA diagram (Fig. 2D). The closer the samples were, the stronger the similarity was. The structural similarity between CON group and CAT group was very small, with obvious differences. A comprehensive analysis of the two groups showed that the overall structure of intestinal bacteria has changed significantly after adding CAT polyphenols. There was no significant difference in intestinal flora at the phylum level. At the genus level, CAT could significantly increase the abundance of *Enterococcus*, and the data was not accurate to the species level, which was consistent with other research results.[36, 37]

3.2 Effects of EF, CAT+EF on body weight and organ index of mice

During the feeding process, the mice were in a good mental state, with no special secretions in the mouth and nose, no abnormal diet, normal urination and defecation, and no mice death. The appetite of mice in CON group was more exuberant. Compared with other groups, each mice consumed 1-2 g more basic feed every day on average.

At the beginning of the experiment, there was no significant difference in the weight of mice in each group. According to Fig. 3A, at 9 weeks after mice modeling, the weight of NASH group was 30% higher than that of CON group. In addition, one mice in NASH group and CON group was killed, and the liver tissue was stained with HE and the blood lipid was measured. Together with the results of NAFLD activity score (NAS score), the NASH modeling of mice was successful. Most of *Enterococcus* is EF, and few others are *Enterococcus faecium* and *Enterococcus avium*. Therefore, EF was selected in combination with CAT for the alleviation of NASH. At present, the establishment of pseudo-germfree mice model is mainly used to study the relationship between diseases and intestinal microorganisms. Compared with the control group, there is no difference in physiological changes, growth and reproduction between the pseudo-germfree mice and the normal mice. Compared with the sterile mice, the experimental results of the pseudo-germfree mice model and the sterile mice are basically the same. In addition, the pseudo-germfree mice is more economical and convenient, and it is a good model for studying intestinal flora. Compared with NASH group, intragastric administration of EF and CAT+EF could significantly reduce the body weight of mice. This is positively related to the results obtained by previous studies on CAT alleviating NASH. [38]

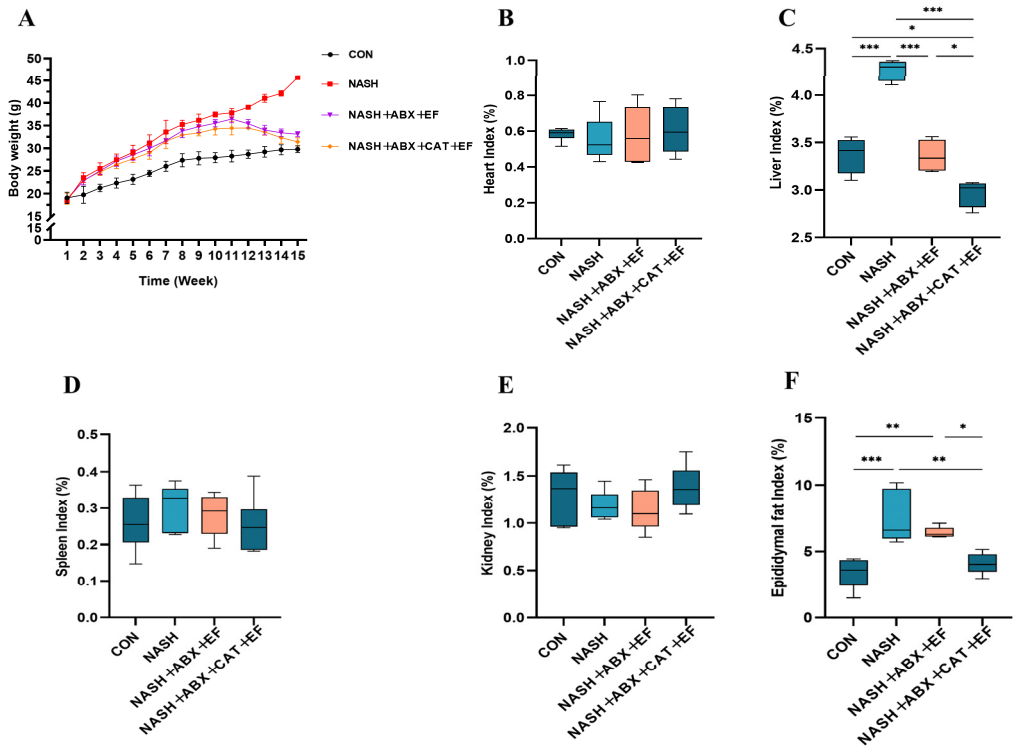

Fig. 3 Comparison of weight and organ index of NASH mice model induced by high-fat diet. (A) Changes of body weight of mice. (B-F) Comparison of fat index of heart, liver, spleen, kidney and epididymis in each group.

Organ index generally reflects the pathological changes of mice. Compared with healthy mice, organ index decreases, indicating organ atrophy, while organ index increases, which may be due to hypertrophic changes or edema. There was no abnormality in all organs of normal mice. In NASH group, the liver volume of mice increased, the color was yellow, the section was greasy, and some of them had yellow-white lipid precipitation. The epididymal fat volume also increased. There was no obvious change in other organs.[35]

According to Fig. 3B-F, the analysis of organ index of each mice showed that the liver index of mice can be significantly reduced by gavage of EF and CAT+EF ($P < 0.001$), and the epididymal fat index of mice can be significantly reduced by gavage with CAT+EF ($P < 0.01$). There was a difference between gavage with CAT+EF and gavage with EF alone ($P < 0.05$), which showed that CAT+EF decreased the liver index and epididymal fat index of mice more obviously than that of EF alone. There was no significant difference in other organ indexes among the groups. Therefore, it was considered that CAT+EF has a better effect on relieving NASH than EF.

3.3 Effects of EF, CAT+EF on biochemical indexes of mice liver and serum

The pathological changes of mice can be reflected by the changes of liver volume and color. Compared with the CON group, the liver volume of model group was larger and the color was lighter. Compared with the model group, the liver volume of mice in EF, CAT+EF groups were smaller (Fig. 4A). The determination results of TG and GSH in liver were shown in Fig. 4B-C.[39, 40] Compared with the CON group, the liver TG in model group showed an upward trend ($P < 0.01$) and GSH showed a significant downward trend ($P < 0.001$). Compared with the model group, the TG of mice fed with CAT+EF decreased

($P < 0.001$), while the GSH of mice fed with EF and CAT+EF increased ($P < 0.001$). Among them, compared with the EF group, the TG of mice in CAT+EF group decreased ($P < 0.05$), and GSH increased more significantly ($P < 0.01$). As shown in Fig. 4D-H, they were results of measuring liver SOD, MDA, ALT, ALP and AST. Compared with the control group, SOD and MDA in the model group showed a downward trend ($P < 0.001$), while ALT, ALP and AST showed an upward trend ($P < 0.01$). Compared with the model group, SOD in EF group and CAT+EF group increased ($P < 0.001$), MDA in CAT+EF group increased ($P < 0.01$), ALT, ALP and AST in CAT+EF group all decreased ($P < 0.01$). In all, CAT+EF group showed better effect than EF group in indexes of TG, GSH, SOD, MDA, ALP to relieve NASH.

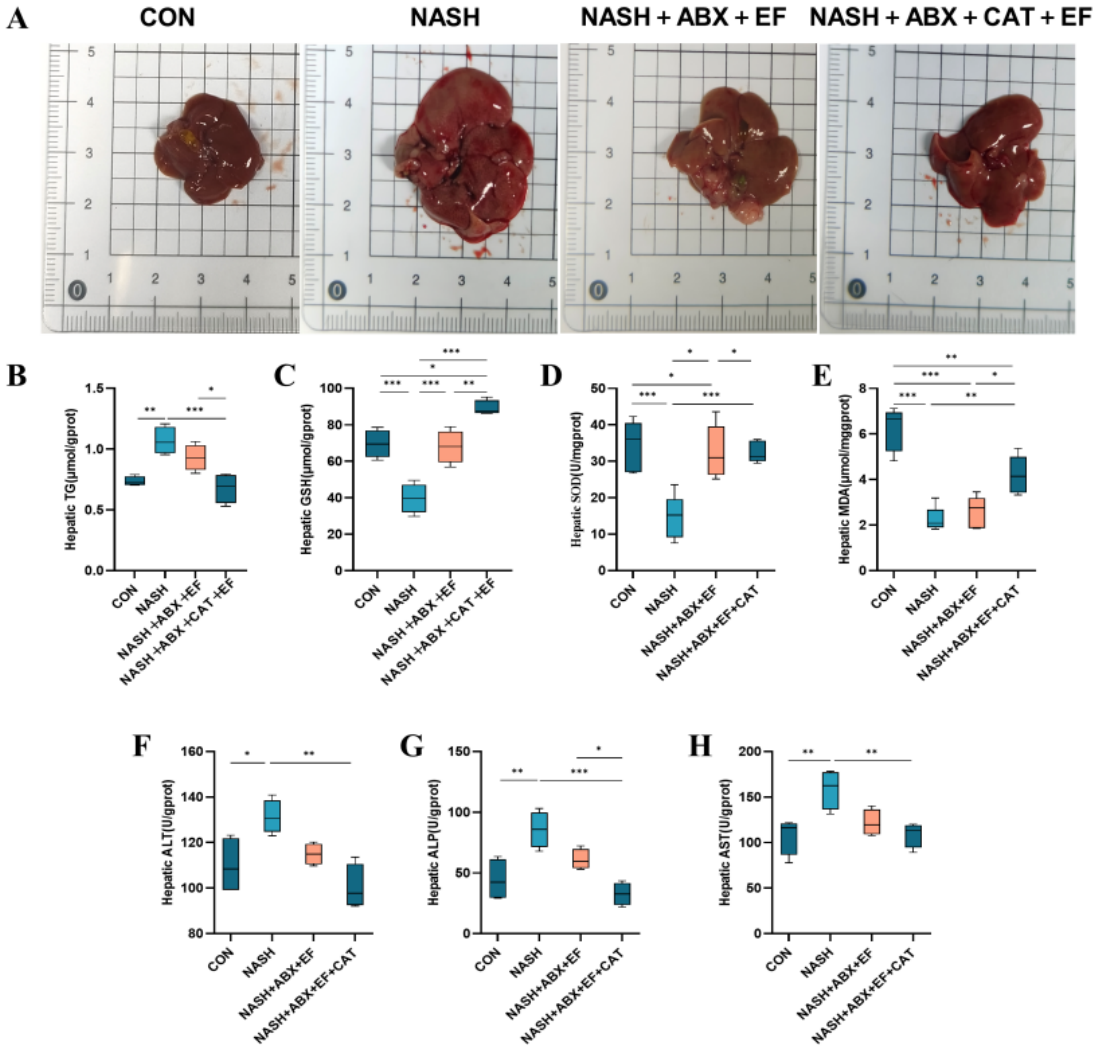

Fig. 4 Comparison of liver tissue and liver indexes in NASH mice model induced by high-fat diet. (A) Liver tissue of mice in each group. (B) Triglyceride (TG) concentration of in liver tissue. (C) Glutathione (GSH) concentration in liver tissue. (D) Superoxide Dismutase (SOD) concentration of in liver tissue. (E) Malondialdehyde (MDA) concentration of in liver tissue. (F) Alanine aminotransferase (ALT) concentration of in liver tissue. (G) Alkaline phosphatase (ALP) concentration of in liver tissue. (H) Aspartate aminotransferase (AST) concentration of in liver tissue.

As seen in Fig. 5, compared with CON group, the serum biochemical indexes TC and LDL-C level in NASH group were significantly increased（$P < 0.001$）, TG level was on the rise ($P < 0.05$), and HDL-C level was significantly decreased ($P < 0.01$). Compared with NASH group, the indexes of LDL-C in mice fed with EF and CAT+EF decreased significantly ($P < 0.001$), among which CAT+EF group had a better

effect on regulating serum biochemical indexes than EF. Compared with NASH group, the indexes of TG and TC of mice in CAT+EF group were decreased ($P < 0.05$, $P < 0.01$), and the index of HDL-C was significantly increased ($P < 0.001$), but there was no difference in the indexes of TC and TG in the EF group. To sum up, the serum indexes showed that CAT+EF group has a better effect of relieving NASH than EF alone. Studies have shown that[39] green tea polyphenols can reduce the serum TG and TC indexes of NASH mice.

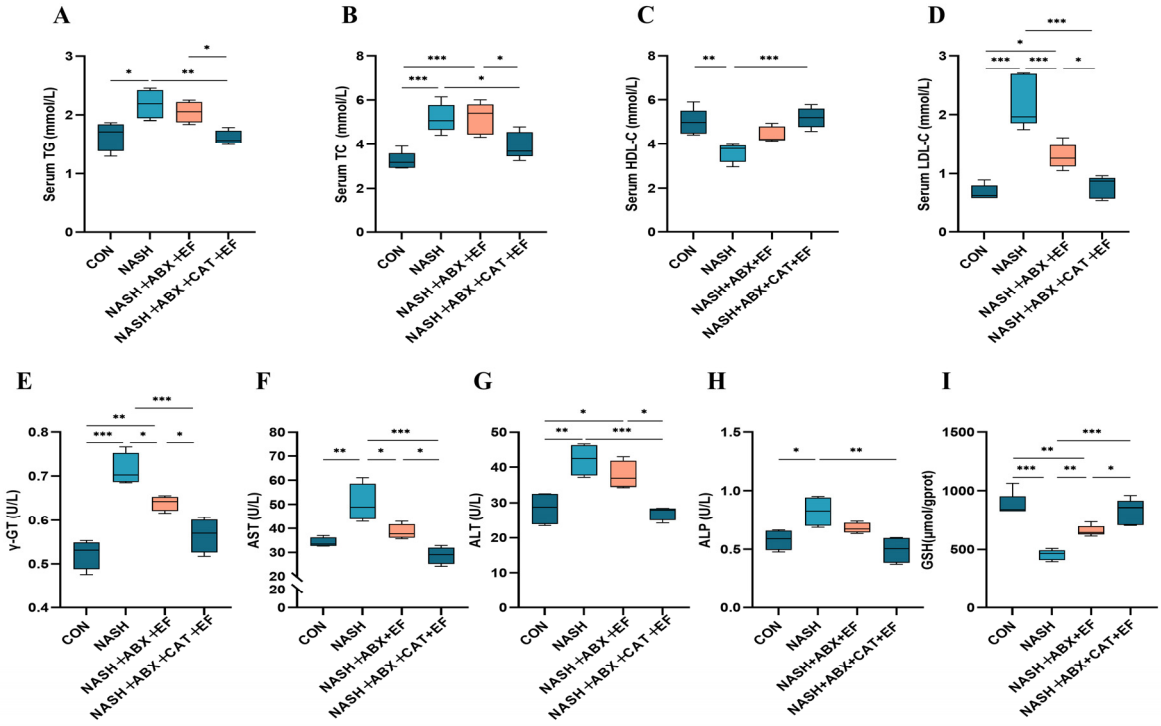

Fig. 5 Blood biochemical detection of NASH mice model induced by high-fat diet. (A) Serum triglyceride (TG) concentration. (B) Serum total cholesterol (TC) concentration. (C) Serum high density lipoprotein cholesterol (HDL-C) concentration. (D) Serum low density lipoprotein cholesterol (LDL-C) concentration. (E) Serum γ -glutamyltransferase (γ-GT) concentration. (F) Serum alkaline phosphatase (ALP) concentration. (G) Serum alanine aminotransferase (ALT) concentration. (H) Serum aspartate aminotransferase (AST) concentration. (I) Serum glutathione (GSH) concentration.

It can be seen from Fig. 5 that the indexes of γ-GT, ALT and AST of serum of mice in NASH group were significantly higher than those in CON group ($P < 0.01$, $P < 0.001$), and the index of ALP was higher than that in CON group ($P < 0.05$), while the index of GSH in serum of mice in NASH group was significantly lower than that in CON group ($P < 0.01$). From the above data, NASH model was successfully established. Compared with NASH group, serum γ-GT, ALT and AST in EF group and CAT+EF group were decreased ($P < 0.05$, $P < 0.001$), and serum GSH showed an upward trend ($P < 0.01$, $P < 0.001$). Some researchers also showed that tea polyphenols rich in CAT could effectively alleviate the phenomenon of excessive ALT in NASH mice.[39] Compared with single gavage of EF, there were differences of γ-GT, ALT, AST and GSH in CAT+EF group ($P < 0.05$), and the effect of CAT+EF was much better than that of EF (Fig. A and C-E). At present, studies have shown that,[14] green tea catechins can reduce the AST index of Nash mice, which shows the same trend. Our results showed that intragastric administration of EF and CAT+EF all alleviated NASH induced by high-fat diet, while CAT+EF group had a better effect than EF group.

3.4 Effects of EF, CAT+EF on HE staining of mice

From the results of HE staining in liver tissue, we can see that in CON group, the liver structure was clear, arranged radially with the central vein as the center, the size of hepatocytes was normal, the nucleus of hepatocytes was located in the center of cells, and the cytoplasm was uniform, and there was no steatosis, balloon degeneration, necrosis focus and other phenomena (Fig. 6A). In the model group, the structure of liver cells was disordered, the size of hepatocytes was uneven, the hepatocyte nucleus was pushed to one side, the cytoplasm was uneven, and spherical lipid droplets with different sizes appeared in the cytoplasm. Large lipid droplets filled the whole cell and pushed the nucleus to one side, and a large number of balloon-like degeneration, hepatocyte necrosis and local inflammatory cell infiltration can be seen, which showed that NASH mice model was successful (Fig. 6B).

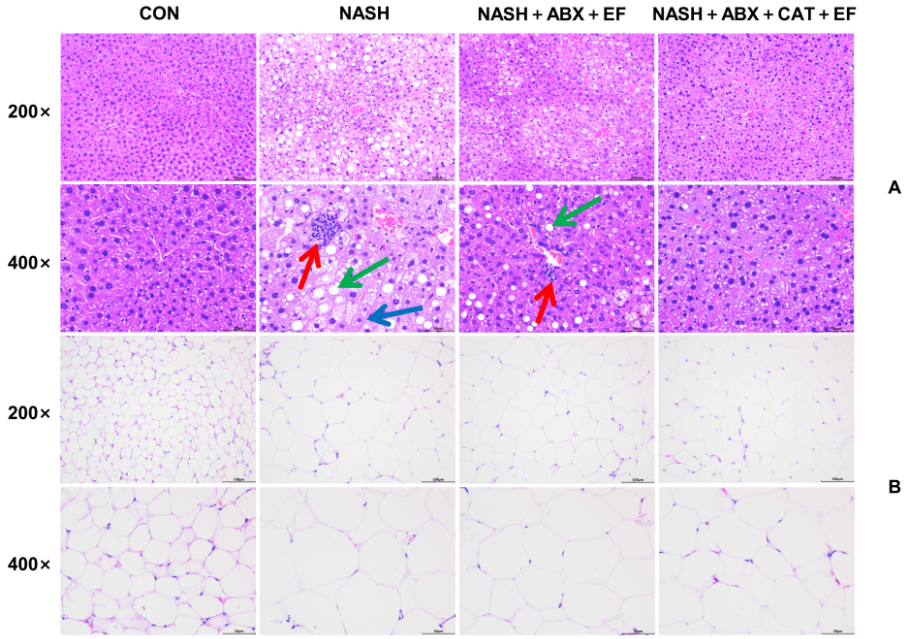

Fig. 6 HE staining observation of NASH mice model induced by high-fat diet. Hepatic lobule (A) and epididymal adipose tissue (B) of normal control mice（CON）. Hepatic lobule (A) and epididymal adipose tissue (B) of NASH model mice（NASH）. Hepatic lobule (A) and epididymal adipose tissue (B) of mice in EF group（NASH+ABX+EF）. Hepatic lobule (A) and epididymal adipose tissue (B) of mice in CAT+EF group（NASH+ABX+CAT+EF）. In the figure, the red arrow points to the necrotic focus area, the blue arrow points to the balloon-like degeneration area, and the green arrow points to steatosis.

In EF group, the structure of liver cells recovered to some extent, and the liver cells showed different degrees of fatty degeneration, balloon degeneration was relieved, and inflammatory cells infiltrated locally (Fig. 6C). In CAT+EF group, the structure of most hepatocytes basically recovered, with the central vein as the center, which arranged radially, the size of hepatocytes was relatively normal, the nucleus of hepatocytes was located in the center of cells, with uniform cytoplasm, and less steatosis, which was greatly improved compared with the group of EF, with a little balloon degeneration and inflammatory cell infiltration (Fig. 6D). Other studies have shown that intragastric administration of CAT can alleviate the balloon degeneration and inflammatory cell infiltration in the liver caused by NASH.[39]

From the results of HE staining of epididymal adipose tissue, we can see that in CON group, the epididymal adipose tissue structure was clear, and the epididymal adipose cells were the smallest, while in

the model group, the epididymal adipose cells enlarged, which was much larger than that in CON group and slightly larger than that in two intragastric administration group. In all, EF and CAT+EF group could alleviate NASH induced by high-fat diet in mice, and CAT+EF group was better than EF alone.

3.5 Effect of CAT on targeted metabonomics of EF

In order to explore why the role of CAT+EF group was better than that of EF group, we analyzed the metabonomics of CAT on EF. As can be seen from Fig. 7A, the addition of CAT could promote EF to produce more methyl stearate (C18:0), but C18:0 was mostly studied as biodiesel. Although adding CAT can also promote EF to produce more methyl oleate (C18:1n9c) and methyl linoleate (C18:2n6c), but C18:1n9c and C18:2n6c were not related with NASH, so they is no need to further study C18:0, C18:1n9c and C18:2n6c. Research has shown that MP (C16:0) can alleviate NASH by up-regulating the expression of α(PPARα), promoting the expression of β-oxidized protein and gene, and inhibiting the expression of TNF-α, MCP-1, TGF-β1 and Colla1.[41] It was considered that the better alleviation of CAT+EF group on NASH may be related to promoting the production of MP by EF.

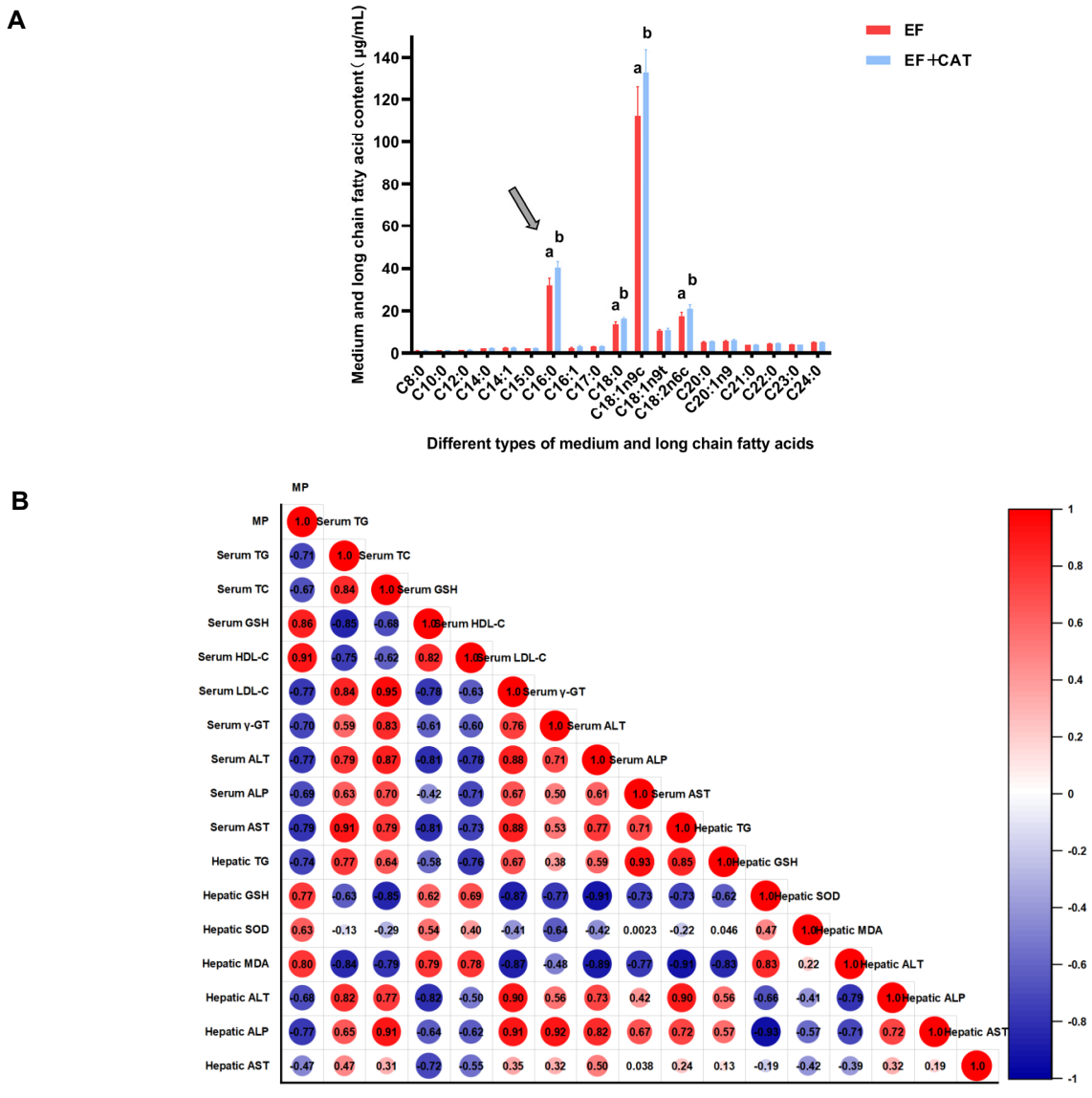

Fig. 7 Metabonomics results of CAT targeting EF and correlation analysis between MP and biochemical indexes. (A)Metabonomics results of CAT targeting EF. (B)Correlation analysis between MP and biochemical indexes.

We have done some correlation analysis between MP and above biochemical indexes. As can be seen from Fig. 7B, MP was positively correlated with serum indexes of GSH, HDL-C, liver indexes of GSH, SOD and MDA (r = 0.63~0.91), among which, it was highly positively correlated with serum indexes GSH and HDL-C, and negatively correlated with serum indexes of TG, TC, LDL-C, γ-GT, ALT, ALP, AST and liver indexes of TG, ALT, ALP, AST (r = -0.79~-0.47). Our data in Fig. 4 and Fig. 5 also showed that compared with EF group, CAT+EF group can better reduce serum TG, TC, LDL-C, γ-GT, AST, ALP, ALT and liver TG, AST, ALT, ALP, better improve serum HDL-C, GSH and liver GSH, SOD and MDA. Therefore, it can be seen from the correlation that MP content was related with these biochemical indexes, which proved that CAT may promote EF to produce MP, thus playing a better role in alleviating NASH.

How does CAT regulate EF to produce more MP needs further experiments. At present, we are conducting *in vitro* verification experiments to explore whether CAT promoted EF to produce MP is related to the quorum sensing system of bacteria, and preliminarily proved the existence of QS system by adding signal molecules from outside in Fig.S1. It was indicated that CAT can promote production of MP by regulating the AI-2-mediated QS system of EF. Because CAT could up-regulated QS related gene of *LuxS* and *pfs*, and the genes of *fabZ1*, *fabZ2* and *fabI* related to the production of long-chain fatty acids in EF. This is the preliminarily mechanism of CAT alleviating NASH from the perspective of intestinal microorganism.

4. conclusions

In vitro fermentation was used to explore the effect of CAT on human intestinal microorganisms. CAT could change the composition of intestinal microorganisms and significantly increase the abundance of *Enterococcus*. The NASH mice model was established by using high-fat diet, and whether the alleviating effect on NASH by CAT was related to intestinal microorganism of EF was explored. EF group and CAT+EF group all could reduce the body weight and organ index of NASH mice. In addition, these two groups could basically improve the changes of serum and liver indexes and HE staining observation also showed that two groups had greatly improved the fatty degeneration, balloon degeneration and necrotic focus caused by NASH. Results of targeted metabonomics indicated that CAT could promote EF to produce more methyl palmitate (C16:0), which plays a great role in relieving NASH. Therefore, we think that the decrease of NASH by CAT was related with EF, which may due to the fact that CAT promoted EF to produce MP, thereby exhibiting better relief effects. At present, the clinical treatment of NASH is limited to the treatment of NASH by drug targets, or the alleviation of NASH by small molecular active substances, and rarely involves intestinal microorganisms, but we discovered that CAT can promote EF to alleviate NASH more effectively. These findings may provide different insight for exploring the mechanism of CAT alleviating NASH. In addition, we can further explore the relationship between CAT and EF *in vitro* and *in vivo* from the perspective of QS.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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