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γ-Aminobutyric acid alleviates litchi thaumatin-like protein-induced inflammation and reduces gut microbial translocation

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ABSTRACT

Previous research reported litchi thaumatin-like protein (LcTLP) could lead to inflammation, which is a factor causing the adverse reactions after excessive intake of litchi. As a main amino acid in litchi pulp, γ-aminobutyric acid (GABA) was found with anti-inflammatory effect. Therefore, this study aimed to investigate the effects of GABA on LcTLP-induced inflammation through RAW264.7 macrophages and C57BL mice models. *In vitro* study showed GABA could effectively regulate the level of inflammatory cytokines (interleukin (IL)-1β, IL-6, IL-10, and prostaglandin E2) and Ca^{2+} in cells, and inhibit the phosphorylation of p65, IκB, p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK). These results indicate GABA alleviated inflammation through nuclear factor-κB and mitogen-activated protein kinase pathway signaling pathways. *In vivo* experiment was performed to verify the anti-inflammatory effect of GABA, and the results demonstrated that GABA reduced the inflammation and oxidative stress in the liver of LcTLP-treated mice, as it down-regulated the pro-inflammatory cytokines, malondialdehyde, aspartate transferase, and alanine transaminase. The relative expression of phosphorylated p38, JNK and ERK in mice liver with GABA treatment were reduced to 65%, 39% and 80% of the control group, respectively. Furthermore, GABA treatment enriched probiotic bacteria and decreased pathogenic bacteria in mice gut, which reveals GABA could effectively reduce the translocation of gut microbiota.

1. Introduction

Litchi (*Litchi chinensis* Sonn.) belongs to the Sapindaceae family, and is a famous fruit in tropics and subtropics due to its unique taste and flavor. As the edible part of litchi, the pulp is rich in nutritions, including polysaccharides, polyphenols and γ-aminobutyric acid (GABA) etc., which have attracted a lot of attentions due to their health benefits. However, for a small number of individuals, excessive intake of litchi may result in adverse reactions, such as oral ulcer, tongue swelling, and sore throats etc.[1-2]. These symptoms are regarded as the typical manifestation of 'Shanghuo', which is a widely accepted concept in Chinese traditional medicine. Although there is no specific clinical index to assess this pathological process, the symptoms are similar to inflammation, which is related to disorder in homeostasis and immune system of human body trigered by external stimulus[3].

Our previous research reported a thaumatin-like protein in litchi (LcTLP) is likely partially responsible for the inflammation syptoms after consuming litchi, as it promoted pro-inflammatory cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-1β and transforming growth factor (TGF)-β1) in mouse RAW264.7 macrophages[4]. Futher study confirmed LcTLP led to inflamation in mice liver by activating nuclear factor (NF)-κB and mitogen-activated protein kinase pathways (MAPKs) signaling pathways, and induced dysbiosis of gut microbiota, which has been reported related to inflammation in liver[5-6]. Theoretically, the presence of proinflammatory component like LcTLP is likely to result in inflammation reactions in human

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body; however, there is an interesting phenomenon that in reality the majority of consumers have never experienced these adverse reactions, and only a minority of individuals have 'Shanghuo' issues. Therefore, it was speculated that there might be anti-inflammatory components in litchi pulp, which could protect human body from suffering from these adverse reactions induced by LcTLP.

GABA, known for its neuromodulatory effects, is widely distributed throughout the biological world[7]. It was found that GABA is relatively abundant in litchi pulp, with a content of 1.7–3.5 mg/g fresh weight, which is significantly higher than in other fruits[8]. In recent years, there have been studies reporting GABA possess anti-inflammatory effect. *In vitro* study suggested that GABA pretreatment could inhibit the production of pro-inflammatory cytokines in LPS-stimulated RAW264.7 macrophages[9]. Further study revealed the ability of GABA in regulating inflammation was achieved through NF-κB signaling pathway[10]. *In vivo* study also confirmed the anti-inflammatory activity of GABA, and found it could alleviate liver inflammation in pig[11]. Based on these, it was hypothesized GABA could alleviate the inflammation-related adverse reactions induced by LcTLP, which has not yet been investigated to date.

In this study, LcTLP-treated RAW264.7 macrophages and C57BL mice were used as the *in vitro* and *in vivo* inflammatory models. The inflammatory markers (IL-6 and IL-1β etc.), signaling pathways (NF-κB and MAPK) and the gut microbiota were analyzed to understand the ameliorative effect of GABA on LcTLP-induced inflammation and elucidate the underlying mechanisms. The outcomes of this study would help in unveiling the roles of GABA and LcTLP in developing inflammation reactions after consuming litchi, and would be beneficial for further ultilization of GABA in functional foods.

2. Materials and methods

2.1 Materials and reagents

Fresh litchi was obtained from local orchard in Guangdong, China. GABA (99% purity) was purchased from Sigma Biotechnology Co., Ltd. (St. Louis, USA). Mouse RAW264.7 macrophages were obtained from the cell bank of the Chinese Academy of Science (Shanghai, China). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco/Invitrogen Co. (Carlsbad, CA). Enzyme-linked immunosorbent assay (ELISA) kits for IL-6, IL-1β, and TNF-α were purchased from Neobioscience Biological Technology Co., Ltd. (Jiangsu, China). Aspartate transaminase (AST), alanine aminotransferase (ALT), malondialdehyde (MDA) and superoxide dismutase (SOD) kits were purchased from Nanjing Jiancheng Biological Technology Co., Ltd. Rabbit monoclonal antibodies were obtained from Cell Signaling Technology (San Diego, USA). All other chemical reagents were analytical grade.

2.2 Preparation of LcTLP

LcTLP was prepared using a previous described method[4]. Briefly, litchi pulp was defatted with acetone, then litchi protein was extracted using potassium phosphate buffer. The litchi crude protein was purified with different saturation ammonium sulfate. The precipitate was collected, dialyzed and lyophilized to obtain purified LcTLP.

2.3 In vitro experiment

2.3.1 Cell viability assay

CCK-8 assay was used to determine the toxicity of GABA and LcTLP on RAW264.7 macrophages. The cells at logarithmic phase were seeded in 96-well plates with a density of 1×10^5 cells/well for 24 h. After removing the culture medium, the cells were treated with different concentrations of GABA (25, 50, 100, 200, 400, 800 and 1 600 μg/mL) for 24 h of incubation. Then the cells were mixed with CCK-8 reagent at 37 °C for 3 h and the absorbance was assayed at 450 nm using a microplate reader.

2.3.2 Determination of nitric oxide (NO)

RAW264.7 macrophages at logarithmic phase were seeded in 96-well plates with a density of 1×10^5 cells per well for 24 h. Five treatment groups were included, including NC group (cells were not treated with samples), LcTLP group (cells were treated with 100 ng/mL of LcTLP for 24 h), G20, G100 and G500 groups (cells were pre-treated with 20, 100 and 500 μg/mL of GABA for 2 h and then stimulated with LcTLP for 24 h). NO production in supernatant was determined with Griess regent by a microplate reader at 540 nm.

2.3.3 Determination of inflammatory cytokines

The cell treatment was in agreement with NO assay. The production of IL-6, prostaglandin E2 (PGE2) and IL-10 in RAW264.7 macrophages was determined using ELISA kits and the mRNA expression of IL-1β was analyzed by real time quantitative polymerase chain reaction (qPCR).

2.3.4 Western blot

RAW264.7 macrophages were plated into 6-well plates with a density of 1×10^6 cells per well. After 2 h of GABA pre-treatment, cells were stimulated with 100 ng/mL of LcTLP for 2 h. The supernatant was discarded and each well was mixed in 150 μL of cell lysis solution (1% PMSF, 1% protease inhibitor, and 2% phosphatase inhibitors) to extract the total protein in cells. After the protein content was determined, the lysate was electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at a current of 80/120 V, and the protein was transferred to the polyvinylidene difluoride (PVDF) membrane by 200 mA current. This PVDF membrane was blocked by 5% BSA and subsequently immersed in primary antibodies at 4 °C for 12 h. Finally, the membrane was treated with the corresponding secondary antibody and was exposed by a gel imager (Amersham Imager 600, GE, USA). The results were analyzed and quantized by Image J software.

2.3.5 Determination of calcium concentration in RAW264.7 cells

RAW264.7 macrophages (5×10^3 cells/well) were plated into 6-well plates and were treated with or without GABA (500 μg/mL) for 2 h. Subsequently, the cells were stimulated by 100 ng/mL of LcTLP for 6 h. After removing the supernatant, cells were incubated with 8 μmol/L of Fluo-4/AM (Dojindo, Mashiki, Japan) and 0.05%

Pluronic F-127 (Invitrogen) for 30 min. The cells were washed with HBSS (Buffered solution without Ca^{2+}) and fixed by 4% paraformaldehyde for 10 min. Finally, the cell nucleus was marked by 4′,6-diamidino-2-phenylindole (DAPI). Ca^{2+} was determined by a fluorescence microscope (Axio Observer A1, Carl Zeiss, Germany).

2.4 In vivo experiments

2.4.1 Animal experimental design

Thirty male C57BL/6J mice ((20.0 ± 1.0) g) were obtained from Sijiajingda Biotechnology Co., Ltd. (Guangdong, China). The animals were freely given water and standard chow under a controlled temperature of 25 °C with a 12 h light/dark cycle for 7 days. All animal experiments were performed following the line of legislation and ethical guidelines of the People's Republic of China and were approved by the Ethics Committee of Experimental Animal Care at South China Agricultural University (permit No. SCXK2018-0002).

All mice were randomly divided into 5 groups (n = 6), including NC group, LcTLP group and 3 GABA groups (G10, G50, and G200). Mice in all groups were administrated with water or samples by intragastric administration. NC group: mice were given with 0.2 mL distilled water once a day. LcTLP group: mice were daily given with 0.2 mL LcTLP (100 mg/kg). Three GABA groups (G10, G50 and G200): mice were administered with 100 mg/kg of LcTLP mixed with 10, 50, and 200 mg/kg of GABA. Animal experimental design was shown schematically in Fig. 1. All mice were weighed daily. On day 9, the liver, serum, and faeces of mice were collected after mice were killed.

2.4.2 Biochemical analysis

The serum was obtained after the centrifugation of blood. The liver tissue (0.2 g) of mice was homogenized in phosphate buffered solution (1.8 mL). After centrifugation, the supernatant was collected for further determination. AST and ALT in serum and MDA and SOD in the liver were determined by commercially available kits (Jiancheng Biotech, Nanjing, China).

2.4.3 Inflammatory cytokines analysis

The liver tissue (0.3 g) was homogenized in phosphate buffered solution (2.7 mL). After centrifugation, the supernatant was collected for inflammatory cytokines assays. The levels IL-6, IL-1β, and TNF-α in liver were evaluated by ELISA kits.

2.4.4 Histological analysis

The liver tissue of mice was fixed in 4% paraformaldehyde and embedded in paraffin. Section with a thickness of 4 μm was prepared and stained with hematoxylin and eosin (H&E). The sections were observed with an inverted biological microscope.

2.4.5 Western blot analysis of the key proteins on MAPK signaling pathway

The key proteins on MAPK signaling pathway in liver of mice were accessed by Western blot. The liver tissue (0.2 g) was homogenized in 0.8 mL of RIPA lysis buffer (PMSF and enzyme inhibitor) at 4 °C for 30 min to extract the protein. The experimental parameters of electrophoresis and Western blotting were in accordance with 2.3.4.

2.4.6 16S rRNA sequencing

The extraction of the DNA in mice stool was performed using DNA Stool kit (Tiangen Biotech Co., Ltd., Beijing, China). 16S rRNA sequence in V3−V4 region was amplified with the primer of 5′-ACTCCTACGGGAGGCAGCAG-3′ and 5′-GGACTACHVGGGTWTCTAAT-3′. After PCR amplification, the products were further purified and were conducted on the Illumina MiSeq platform (Illumina, SD, USA).

2.5 Statistical analysis

All data were represented as mean ± standard deviation. Statistical significance was measured by one-way variance analysis (ANOVA) and Duncan's multiple range test (SPSS 25.0). $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1 Effects of GABA on cell viability and inflammatory mediators

CCK-8 assay was used to determine the toxicity of GABA in cells. The results were shown in Fig. 2A. There was no significant difference in cell viability with the increase in GABA concentration (0–1 600 μg/mL), suggesting GABA at concentration lower than 1 600 μg/mL has no toxicity on RAW264.7 macrophages and could be used for further experiment.

NO production results were shown in Fig. 2B. LcTLP treatment (100 ng/mL) caused excessive NO production in cells, indicating LcTLP-induced *in vitro* inflammatory model was successfully

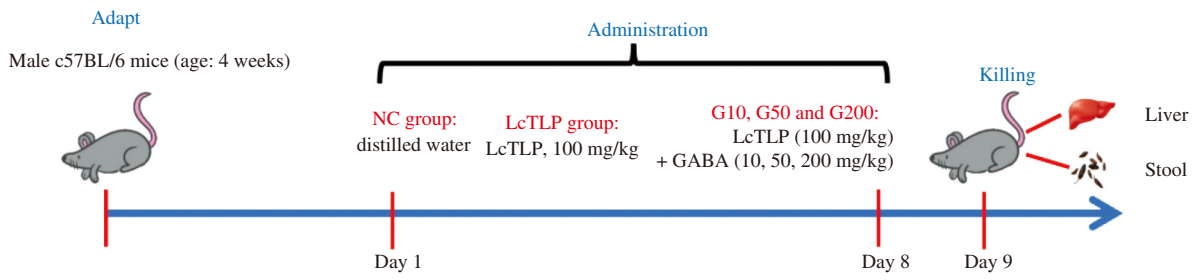

Fig. 1 Animal experimental design.

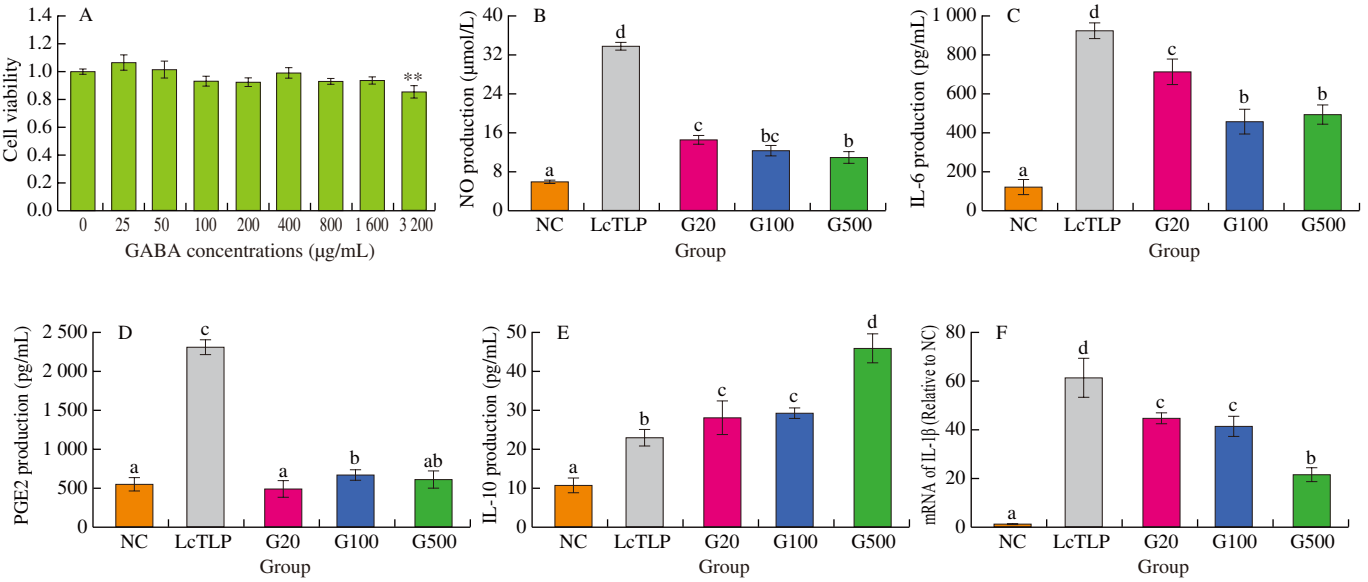

Fig. 2 Effects of GABA on cell viability and inflammatory mediators. (A) Toxicity test of GABA and LcTLP; (B) NO test, NO in cell supernatant was determined after 2 h of GABA pretreatment and 24 h of LcTLP stimulation; (C-E) Expression of IL-6, PGE2 and IL-10, determined by ELISA method; (F) mRNA expression of IL-1β. The significant difference was represented by different lowercase letters ($P < 0.05$), and ** represented significant difference compared with 0 μg/mL of GABA group ($P < 0.01$).

established. When cells were pretreated with 20, 100 and 500 μg/mL of GABA, NO production was significantly decreased from 33.85 μmol/L (LcTLP group) to 14.58, 12.34 and 10.82 μmol/L, respectively ($P < 0.05$). These results preliminarily show the inhibitory effect of GABA on LcTLP-induced inflammation.

Inflammatory cytokines including TNF-α, IL-6 and IL-10 are important signals reflecting the degree of inflammation in cells. The impacts of GABA on inflammatory cytokines were shown in Figs. 2C-F. LcTLP significantly increased the production of IL-6, PGE2, IL-10 and IL-1β in cells ($P < 0.05$). When cells were pretreated with GABA, the level of IL-6 was significantly suppressed by 20, 100 and 500 μg/mL of GABA, with values reducing from 923 pg/mL (LcTLP group) to 713.3, 457.8 and 492.9 pg/mL, respectively (Fig. 2C). The levels of PGE2 in all the three GABA treated groups were decreased by over 70% in comparison with LcTLP group (Fig. 2D). As determined by qPCR, IL-1β production was down-regulated in 3 GABA groups, and it in G500 group was only 32% of LcTLP group (Fig. 2F). IL-10 was an anti-inflammatory cytokine, its expression in cells was positively correlated with GABA concentration and was significantly increased with GABA treatment ($P < 0.05$). These results suggested GABA could regulate the inflammatory cytokines in LcTLP-induced RAW264.7 macrophages, which preliminarily indicated GABA inhibit the inflammation. A published study reported similar efficacy of GABA on the model of LPS-stimulated RAW264.7 cells, showing GABA pre-treatment significantly down-regulated the levels of IL-1β and TNF-α[8].

3.2 Effects of GABA on NF-κB and MAPK signaling pathways in vitro

Previous study indicated LcTLP led to inflammation through activating NF-κB and MAPKs signaling pathways in RAW264.7 macrophages[6]. Therefore, in order to unveil the inflammatory mechanism, the effects of GABA on these two upstream inflammatory pathways were further analyzed. The key proteins on the pathways were determined and the results were summarized in Fig. 3. As compared with the NC group, LcTLP enhanced the expression of p-p65, while 500 μg/mL of GABA significantly decreased its expression ($P < 0.05$). All the 3 concentrations of GABA effectively reduced LcTLP-induced phosphorylation of IκB, and 100 μg/mL was the optimal dose for inhibiting phosphorylation of IκB (Figs. 3A and C). In fact, the phosphorylated level of p65 and IκB reflects the degree of NF-κB pathway activation. When cells are subjected to external stimulus, IκBs is phosphorylated and degraded, subsequently NF-κB p65 is also phosphorylated and translated into the nucleus to activate downstream inflammation[12-13]. Therefore, these results indicate GABA could inhibit the activation of NF-κB pathway. This is in agreement with a previous report showing 2 h of GABA treatment effectively inhibited the activation of NF-κB pathway in human islet cells and lymphocytes[14].

For MAPK pathway, the phosphorylated levels of p38, JNK and ERK in NC group were very low. After LcTLP treatment, the expressions of p-p38, p-JNK and p-ERK were dramatically increased. GABA treatment dose-dependently decreased the expressions of p-JNK and p-ERK (Figs. 3B and D), which in cells were respectively decreased by 55% and 52% after 500 μg/mL of GABA treatment compared with LcTLP treatment. The expression of p-p38 was reduced by over 50% in G100 and G500 groups in comparison with LcTLP group ($P < 0.05$), although there was no significant difference in G20 group. MAPKs including p38, JNK and ERK participate in regulating NF-κB transcription, JNK and p38 are also certified as the motivators of IκBα degradation[15]. Therefore, these results indicated GABA could exert anti-inflammatory effect through MAPKs signaling pathways.

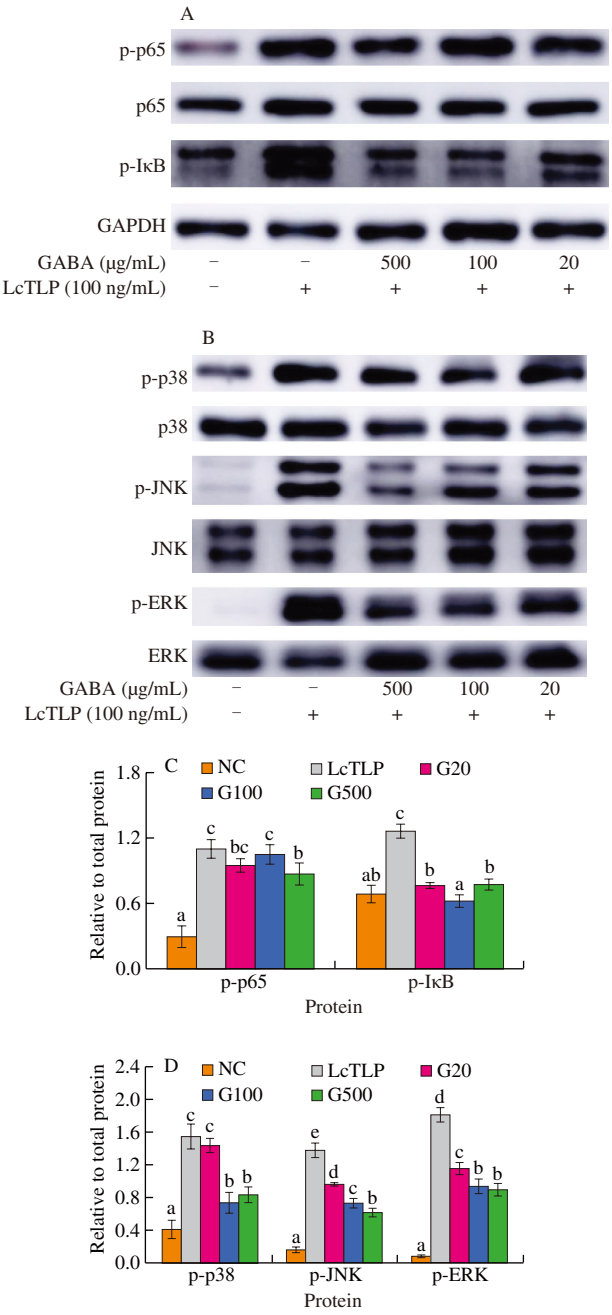

Fig. 3 The analysis of NF-κB and MAPKs signaling pathways. (A-B) Expression of key proteins on NF-κB (p65 and IκB) and MAPKs (p38, JNK and ERK) pathways determined by Western blot; (C-D) Expression ratio of target protein to total protein or GAPDH as calculated by grayscale values.

3.3 *Effects of GABA on calcium ion, iNOS and COX-2*

iNOS and COX-2 are important enzymes involving in the productions of NO and PGE2[16], their expression was determined by Western blot and exhibited in Fig. 4. GABA pretreatment (20, 100 and 500 μg/mL) could inhibit iNOS overexpression caused by LcTLP, and the relative expression of iNOS (the ratio of iNOS and GAPDH) was significantly decreased from 0.74 to 0.43, 0.56 and 0.53, respectively ($P < 0.05$). For COX-2, its expression was down-regulated by GABA in a dose-dependent manner, and the maximum inhibitory rate was reached 26%. The determination of iNOS and COX-2 expression showed similar trends with NO and PGE2 (Fig. 2).

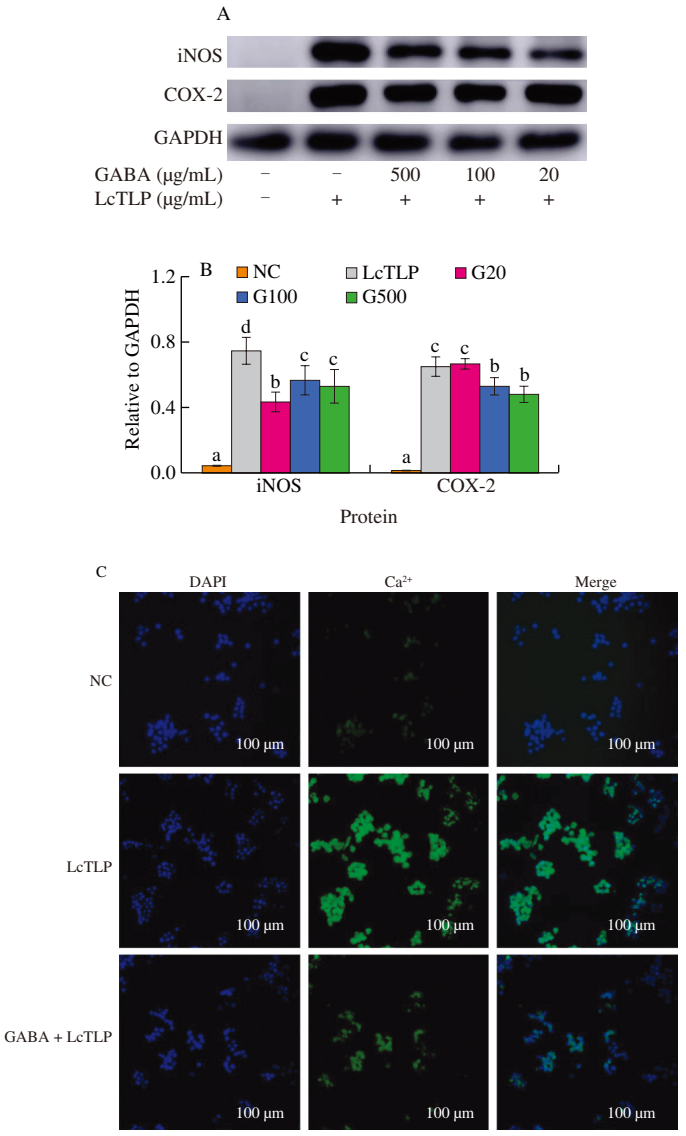

Fig. 4 Determination of intracellular Ca^{2+}, iNOS and COX-2. (A) Expression of iNOS and COX-2 determined by Western blot; (B) Expression ratio of the target protein to GAPDH as calculated by the grayscale value; (C) Fluorescence intensity of intracellular Ca^{2+} observed by fluorescence microscope, cell nucleus was stained by DAPI (blue) and Ca^{2+} was labelled with Fluo-4/AM (green).

Calcium ion (Ca^{2+}) is a signal molecule in cells associated with inflammation, and its enrichment can result in the activation of NF-κB and MAPK pathways[17]. Intracellular Ca^{2+} was detected by immunofluorescent staining, and the results were shown in Fig. 4C. Compared with the NC group, the fluorescence intensity of Ca^{2+} was enhanced by LcTLP. However, when cells were pretreated with GABA (500 μg/mL), the fluorescence intensity was obviously weakened, revealing GABA might intervene the Ca^{2+} channel in RAW264.7 macrophages.

COX-2 and iNOS can be up-regulated with the activation of NF-κB pathway and subsequently accelerating inflammation development[18]. Previous research indicated NF-κB activation and COX-2 overexpression are parallel events occurring in the patients with stomatitis[19], this is also a symptom during 'Shanghuo'[20]. Therefore, COX-2 might be a potential factors resulting in this adverse reaction. According to our results, high-dose GABA suppressed the

overexpression of COX-2 (Fig. 4), suggesting GABA might contribute to ameliorate LcTLP-caused adverse reaction after consuming litchi.

GABA A receptor ($GABA_AR$) is a Cl^- channel, which can regulate the transmembrane movement of Ca^{2+} *via* altering membrane potential[21]. Interestingly, $GABA_AR$ has been also detected in macrophages[22]. Therefore, based on the results in this study, speculating GABA receptor-mediated Ca^{2+} channel might be the target of GABA to exert the anti-inflammatory effect. Zhang et al.[23] proved that GABA could inhibit the phosphorylation of NF-κB by activating $GABA_AR$ in during the inflammatory process. This result partially supports our hypothesis.

3.4 *Effects of GABA on the body weight of mice*

Based on the results of *in vitro* study, GABA was proved with inhibitory effect on LcTLP-induced inflammation in cells. To further investigate this anti-inflammatory effect of GABA *in vivo*, mice were intragastricly treated with 100 mg/kg of LcTLP to establish model. Fig. 5 showed the alteration in body weight of mice after intragastric administration of GABA. On day 1, there was no difference among the 5 groups (Fig. 5A). After LcTLP treatment, the body weight was significantly decreased. The average weight of mice in LcTLP group were 20.1 g on day 1, and it was decreased by 1.07 g on day 3. Compared with the LcTLP group (2.39%), the weight growth rate of mice treated with 10 mg/kg of GABA was significantly increased to 6.38% ($P < 0.05$), however those of G50 and G200 groups were similar to LcTLP group. No apparent trend was observed in the present study regarding the influences of GABA on body weight of mice.

3.5 *Effect of GABA on liver function and oxidative stress*

The effects of GABA on mice liver were investigated, as previous study confirmed LcTLP-induced inflammation was mainly observed in liver[5]. The results were shown in Fig. 6A. The liver of LcTLP-administrated mice showed obvious vacuolation, inflammatory infiltrates and morphology damage. GABA administration eliminated inflammatory infiltrates, and the vacuolation of hepatic cell was alleviated with GABA dose increasing.

AST and ALT in serum are commonly used to reflect the live function, and MDA and SOD in liver are important indices to evaluate the oxidative damage. The present results showed AST and ALT levels in mice of NC group were 138.6 and 58.9 U/L, respectively, and both were increased by more than 3 times after LcTLP administration. The enzyme activities of AST and ALT were decreased in 3 GABA groups in a dose-dependent manner, and the values were 254.5 and 112.2 U/L in G200 group, respectively (Figs. 6B and C). Furthermore, GABA could significantly up-regulate the activity of SOD in liver to normal level ($P < 0.05$), and there was no difference between 3 dose groups. For MDA, the content was increased from 4.1 (NC) to 13.9 (LcTLP) nmol/mg pro. GABA administration at 50 and 200 mg/kg significantly inhibited the overexpression of MDA ($P < 0.05$), whose activities were reduced to 10.04 and 8.02 nmol/mg pro,

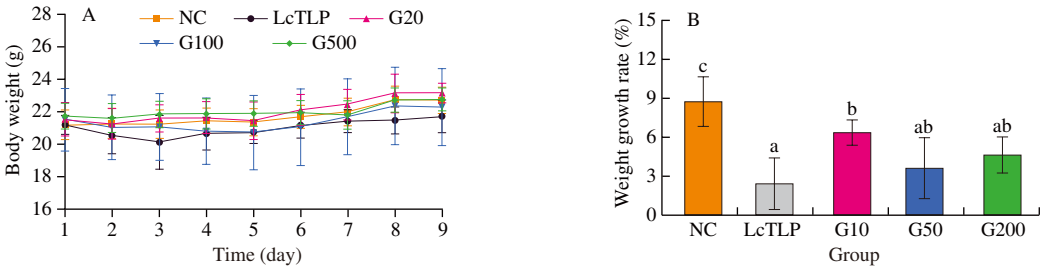

Fig. 5 Alterations of body weight. (A) Variation tendency of mice body weight in 5 groups; (B) Body weight growth rate of mice during the administration. Data were presented as the means ± SD of 6 mice, analyzed by ANOVA and Tukey's post hoc test ($P < 0.05$).

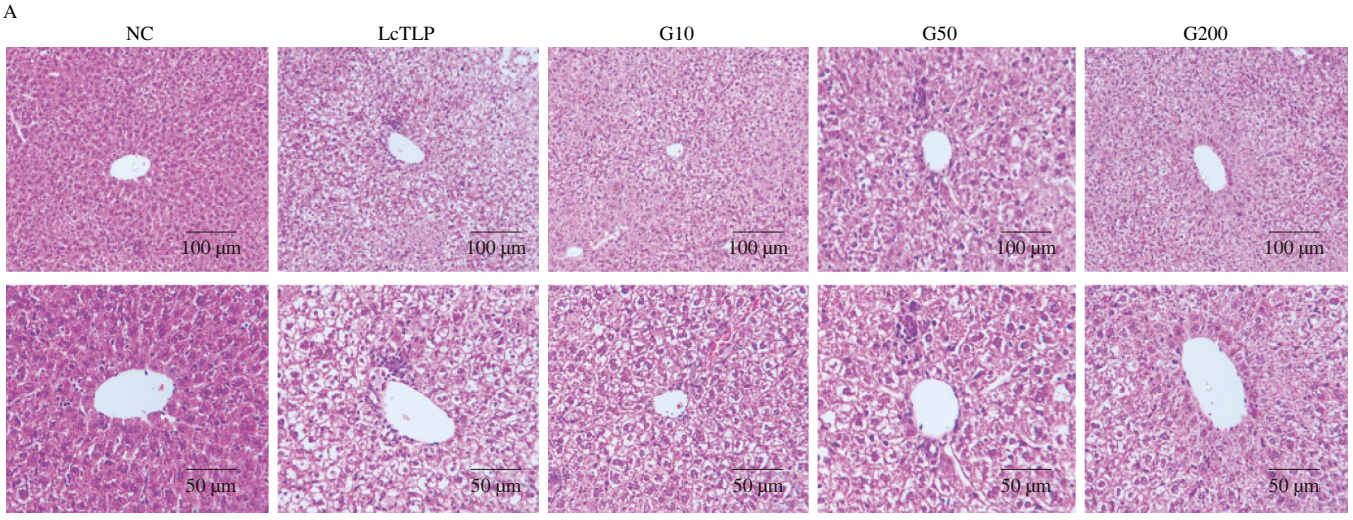

Fig. 6 Histological analysis of liver and biochemical indices determination. (A) Hepatic H&E staining; (B-E) Determination of AST, ALT, SOD and MDA level in liver, respectively. Data were presented as the means ± SD, analyzed by ANOVA and Tukey's post hoc test ($P < 0.05$).

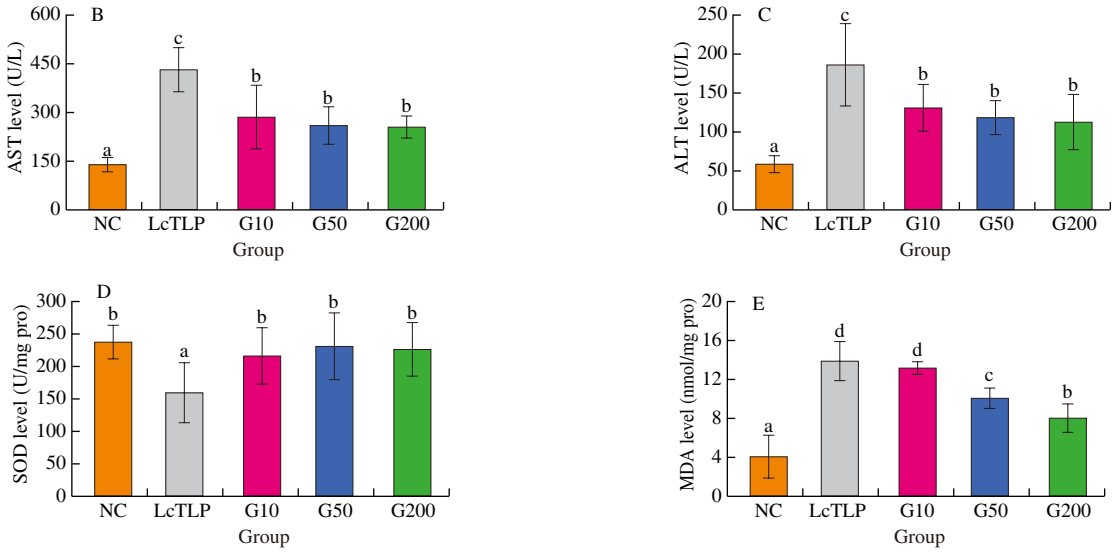

Fig. 6 (*Continued*)

respectively (Figs. 6D and E). These results indicated GABA improved the liver function of mice. Similarly, a *Lactobacillus* strain with the capacity to generate GABA effectively ameliorated the liver function in diabetic mice, including inhibited the up-regulation of AST in serum[24].

Excessive MDA in liver can decrese the activtiy of antioxidase, and SOD is demonstrated with the capacity on eliminating superoxide radical and hydrogen peroxide[25]. Previous study proved GABA possesses antioxidant activity, it is used as the allogenic substance to prevent oxidative damage[26]. According to our results, it was speculated that GABA protected liver *via* releasing the oxidative stress.

3.6 *Effects of GABA on liver inflammation*

Research indicated there is an interaction between oxidative stress and inflammation. Therefore, the inhibited effects of GABA on inflammation *in vivo* were further verified, and the results were shown in Fig. 7. The results indicated LcTLP could up-regulate the expressions of IL-6, IL-1β and TNF-α. However, IL-6 and TNF-α showed significant decrease in G50 and G200 groups ($P < 0.05$) as compared with LcTLP group, and all three doses of GABA inhibited the overexpression of IL-1β (Figs. 7A-C). These results indicated GABA could effectively ameliorate LcTLP-induced inflammatory reaction in liver. Pro-inflammatory cytokines are important factors for maintaining liver health. TNF-α is a critical pathogenic mediator in patients with liver injury[27], IL-6 is related to the regeneration and reconstruction[28], and IL-1β can induce hepatocyte damage and death[29]. The enrichment of pro-inflammatory cytokines might lead to damage of hepatic tissue, which was also consistant with previous results (Fig. 6).

The expressions of phosphorylated p38, JNK and ERK in liver were also determined, with results displayed in Figs. 7D and E. Compared with the NC group, phosphorylated p38, JNK, and ERK were significantly enriched in LcTLP group. The expression of p-p38 was inhibited in G10 and G50 groups, and p-JNK was dose-dependently down-regulated after GABA treatment. Furthermore, only 50 mg/kg of GABA could significantly inhibit the increase of p-ERK expression ($P < 0.05$). These results showed similar trends with *in vitro* experiments, indicating GABA could inhibit LcTLP-induced the phosphorylation of p38, JNK and ERK. The phosphorylation of MAPK's members (p38, JNK and ERK) can activate the inflammation in liver, and they were identified as pro-apoptotic proteins involving in the development of liver injury[30-31]. Our results (Fig. 7) supported GABA could inhibit the phosphorylation of MAPKs, which is in agreement with previous literature showing GABA reduced the expression of p-p38 and attenuated oxidative damage in mouse with liver injury induced by TNF-α and *D*-gal[32].

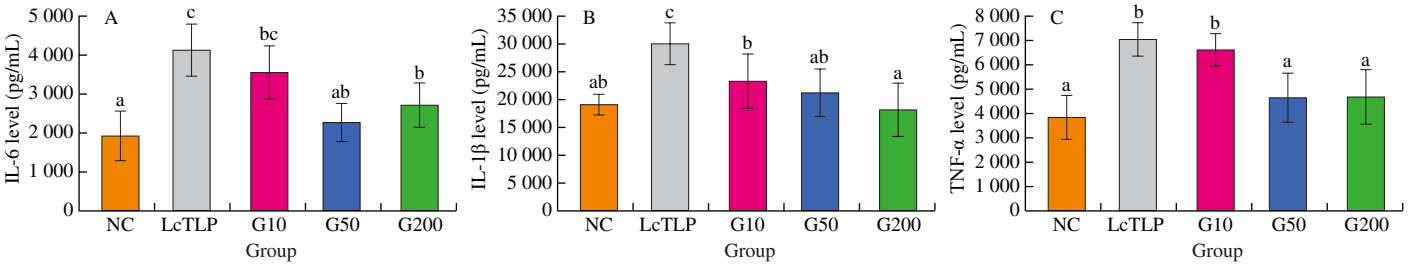

Fig. 7 Inflammatory cytokines and MAPK signaling pathway in liver. Determination of inflammatory cytokines, including (A) IL-6, (B) IL-1β, and (C) TNF-α; (D) Protein expression of p38 (p-p38), ERK (p-ERK), JNK (p-JNK) and GAPDH performed by Western blot; (E) Expression ratio of the target protein (p-p38, p-JNK and p-ERK) to total protein calculated by the grayscale value. Data in histograms were presented as the means ± SD, analyzed by ANOVA and Tukey's post hoc test ($P < 0.05$).

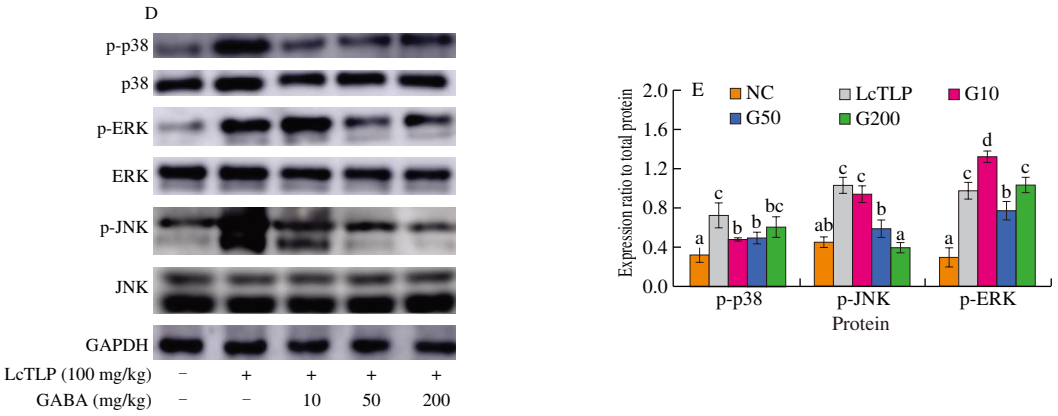

Fig. 7 (*Continued*)

3.7 *Effects of GABA on gut microbiota*

There is a close connection between liver and gut, and many researches confirmed liver inflammation is generally accompanied by gut microbial translocation[33]. Therefore, the effects of GABA on gut microbiota in mice were determined. Rank-abundance curve based on Operational Taxonomic Units (OTU) level can reflect the abundance of species in gut microbiota, and the result was shown in Fig. 8A. The number of OTUs in each sample leveled off gradually with the increase of sequencing amount, indicating the subsequent results were reliable. The results of principal coordinates analysis (PCoA) were shown in Fig. 8B, it suggested there were apparent difference in gut microbiota composition among NC, LcTLP, and GABA (G10, G50 and G200) groups. The total OTUs in LcTLP group were significantly reduced compared with NC group, while three doses of GABA drastically increased total OTUs numbers (Figs. 8C and D), reaching 538–545. Simpson and Shannon indices were analyzed to determine α-diversity of the gut microbiota (Figs. 8E and F). GABA treatment at 50 and 200 mg/kg up-regulated Simpson index compared with LcTLP group ($P < 0.05$), and only 50 mg/kg of GABA could attenuate LcTLP-induced up-regulation of Shannon index ($P < 0.05$).

Gut microbiota is a dynamic ecosystem, its disorder can cause the leakage of gut-derived products. These detrimental substances are transmitted from the enteric cavity to the liver *via* the hepatic portal vein, and resulting in inflammatory reactions[34]. This connection is designated the 'gut-liver axis'. Therefore, more in-depth analysis on gut microbiota was carried out at genus level (Fig. 9). LcTLP intervention decreased the abundances of *Lactobacillus*, *Bifidobacterium* and *Dubosiella*. After GABA administration, the decreases in *Lactobacillus* and *Dubosiella* were significantly ameliorated in G10 and G200 groups ($P < 0.05$), while there was no significant effect on *Bifidobacterium*. *Lactobacillus* and *Bifidobacterium* are the acknowledged probiotics in the gut, they can enhance the intestinal barrier to stabilize the intestinal microenvironment and prevent pathogenic bacteria outflow[35]. It is supported *Lactobacillus* and *Bifidobacterium* may protect the

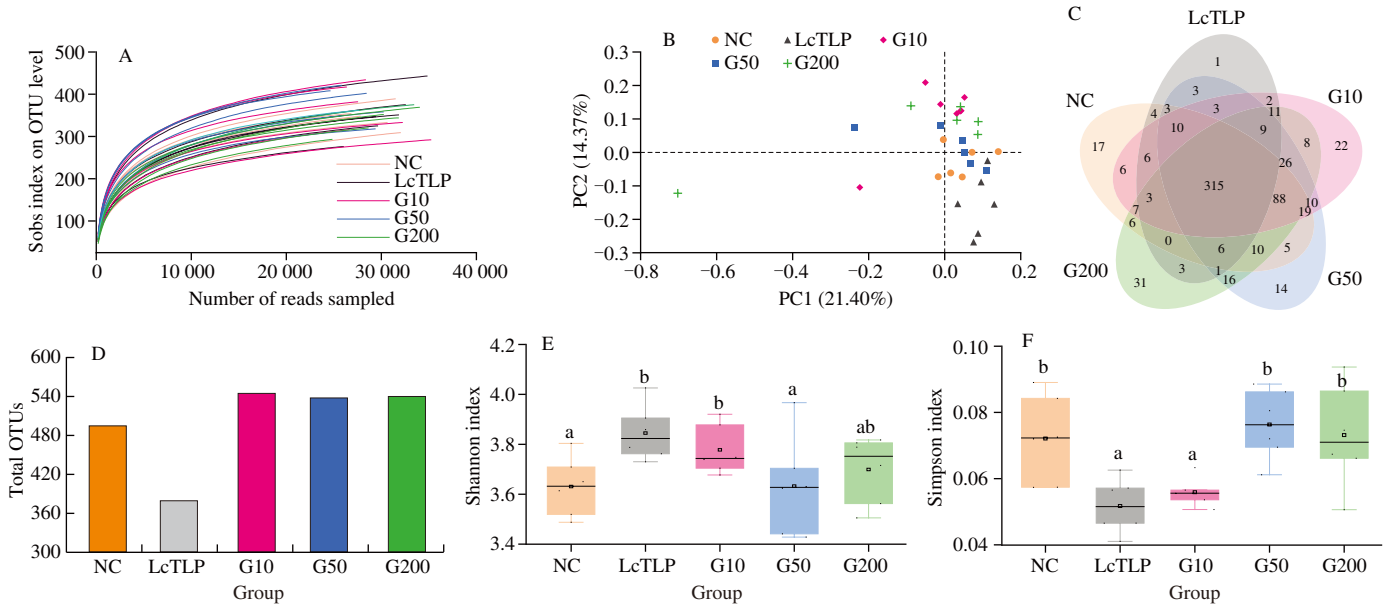

Fig. 8 Intestinal microbiota analysis. (A) Dilution curve of Sob index; (B) β-Diversity analysis of gut microbiota using PCoA; (C) Venn plot, reflecting the diversity of gut microbiota at OTU level; (D) Comparison of the total OTU numbers in mice feces among 5 groups; (E-F) Shannon index and Simpson index from diversity analysis of GABA-treated gut microbiota respectively. Data were presented as the means ± SD, analyzed by ANOVA and Tukey's post hoc test ($P < 0.05$).

liver through the gut-liver axis. Based on the results, speculating *Lactobacillus* might be an important target of GABA *in vivo*. *Dubosiella* is a beneficial bacterium negatively correlated with liver inflammation[36], and it was restored by GABA in our results. Furthermore, researches indicated *Faecalibaculum* is negatively related to the expression of intestinal intercellular connexin proteins, and it can exacerbate liver injury though impairing the gut barrier[37]. *Parasutterella*, a genus of Betaproteobacteria, is an opportunistic pathogen and participates in acute hepatic damage[38]. For *Marvinbryantia*, which is positively correlated with hepatitis virus in liver, revealing it may be a potential pathogenic bacterium[39]. These three bacteria in gut are the negative factors on liver health. The results indicated *Marvinbryantia*, *Parasutterella*, and *Faecalibaculum* in gut were enriched after LcTLP treatment. In contrast, *Faecalibaculum* and *Marvinbryantia* in 3 GABA groups were decreased to NC level ($P < 0.05$), and *Parasutterella* was significantly reduced by 10 mg/kg for GABA treatment group. These results suggest GABA could regulate the composition and relative abundance of gut microbiota.

In fact, it is proved GABA can exert multiple biological function *via* acting on gut microbiota. Yu et al.[40] indicated GABA decreased the level of NO and iNOS in prefrontal cortex through regulating the relative abundance of gut microbiota. This study supported the present results. The regulatory effect of GABA on gut microbiota may be connected with the attenuation in liver inflammation, as the existence of gut-liver axis. Furthermore, previous study proved GABA receptor can be expressed in intestinal tissue[41]. Therefore, GABA might activate the receptor in gut to relieve LcTLP-induced inflammation in liver. Based on these, it was proposed the regulatory effect of GABA *in vivo* is performed through the bidirectional association between gut and liver.

4. Conclusion

This is the first study to investigate the suppressive effects of GABA on LcTLP-induced inflammation. *In vitro* results indicated GABA modulated NO and inflammatory cytokines production in RAW264.7 macrophages through NF-κB and MAPK signaling

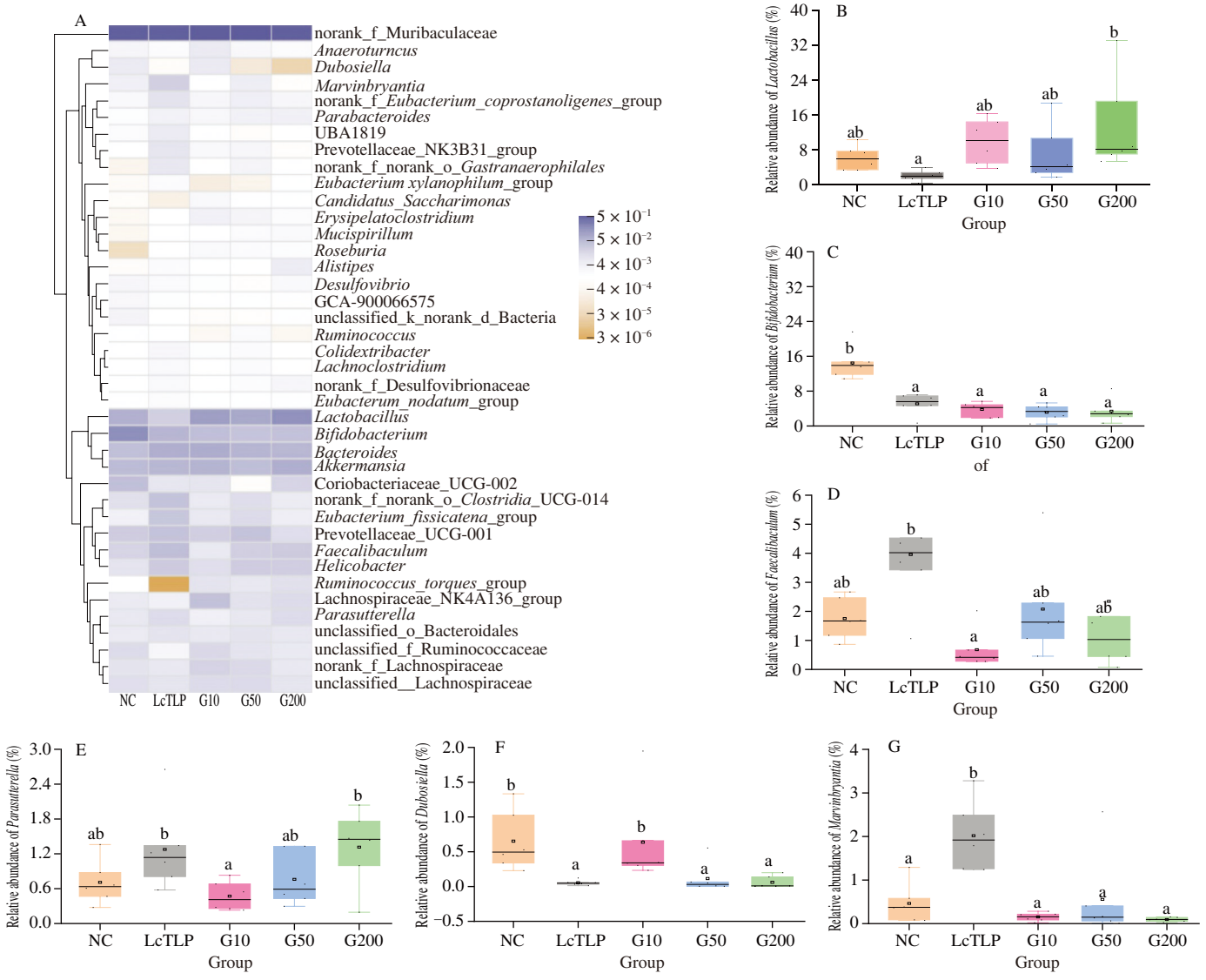

Fig. 9 Analysis of gut microbial composition at genus level. (A) Heat map, top 40 bacteria in relative abundance at genus level; Relative abundance of (B) *Lactobacillus*, (C) *Bifidobacterium*, (D) *Faecalibaculum*, (E) *Parasutterella*, (F) *Dubosiella* and (G) *Marvinbryantia* in 5 groups.

pathways, and inhibited the enrichment of Ca^{2+}. It suggested GABA could inhibit the inflammation *in vitro*. More in-depth analysis was carried out through *in vivo* experiment, the results showed GABA reversed the enhancement of AST and ALT activities in serum of LcTLP-administrated mice and suppressed the production of pro-inflammatory cytokines in liver through MAPK signaling pathway. Based on the connection between gut and liver, gut microbiota was determined and the results indicated GABA enriched probiotic bacteria and decreased pathogenic bacteria in gut of mice. This study might provide explanations for the phenomenon that LcTLP-induced inflammation was rarely observed in the majority of individuals after intake of litchi. These findings could provide theoretical direction for litchi processing and preservation, and contribute to the utilization of GABA in functional foods.

Conflict of 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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References

[1] L. Zhao, K. Wang, K. Wang, et al., Nutrient components, health benefits, and safety of litchi (*Litchi chinensis* Sonn.): a review, Compr. Rev. Food Sci. Food Saf. 19 (2020) 2139-2163. https://doi.org/10.1111/1541-4337.12590.

[2] S. Hoppe, S. Neidhart, K. Zunker, et al., The influences of cultivar and thermal processing on the allergenic potency of lychees (*Litchi chinensis* Sonn.), Food Chem. 96 (2006) 209-219. https://doi.org/10.1016/j.foodchem.2005.02.020.

[3] H. Rongrong, K. Hiroshi, *Shanghuo* syndrome in traditional Chinese medicine, World Sci. Technol. 10 (2008) 37-41. https://doi.org/10.1016/S18763553(09)60024-7.

[4] H. Chen, K. Wang, H. Xiao, et al., Structural characterization and pro-inflammatory activity of a thaumatin-like protein from pulp tissues of *Litchi chinensis*, J. Agric. Food Chem. 68 (2020) 6439-6447. https://doi.org/10.1021/acs.jafc.0c01320.

[5] K. Wang, W. Li, K. Wang, et al., Litchi thaumatin-like protein induced the liver inflammation and altered the gut microbiota community structure in mice, Food Res. Int. 161 (2022) 111868. https://doi.org/10.1016/j.foodres.2022.111868.

[6] S. Zeng, K. Wang, G. Wu, et al., Time-specific ultrasonic treatment of litchi thaumatin-like protein inhibits inflammatory response in RAW264.7 macrophages *via* NF-κB and MAPK transduction pathways, Ultrason. Sonochem. 95 (2023) 106355. https://doi.org/10.1016/j.ultsonch.2023.106355.

[7] A. James, T. Yao, H. Ke, et al., Microbiota for production of wine with enhanced functional components, Food Sci. Hum. Wellness. 12 (2023) 1481-1492. https://doi.org/10.1016/j.fshw.2023.02.008.

[8] Z.C. Wu, Z.Y. Yang, J.G. Li, et al., Methyl-inositol, γ-aminobutyric acid and other health benefit compounds in the aril of litchi, Int. J. Food Sci. Nutr. 67 (2016) 762-72. https://doi.org/10.1080/09637486.2016.1198888.

[9] D.K. Han, H. Young, H.J. Lee, et al., Wound healing activity of gamma-aminobutyric acid (GABA) in rats, J. Microbiol. Biotechn. 10 (2007) 1661-1669.

[10] Y.Y. Wang, S.P. Sun, H.S. Zhu, et al., GABA regulates the proliferation and apoptosis of MAC-T cells through the LPS-induced TLR4 signaling pathway, Res. Vet. Sci. 118 (2018) 395-402. https://doi.org/10.1016/j.rvsc.2018.04.004.

[11] S. Zhang, J. Zhao, J. Hu, et al., Gama-aminobutyric acid (GABA) alleviates hepatic inflammation *via* GABA receptors/TLR4/NF-κB pathways in growing-finishing pigs generated by super-multiparous sows, Anim. Nutr. 9 (2022) 280-290. https://doi.org/10.1016/j.aninu.2022.02.001.

[12] C. Ji, Z. Zhang, J. Chen, et al., Immune-enhancing effects of a novel glucan from purple sweet potato *Ipomoea batatas* (L.) Lam on RAW264.7 macrophage cells *via* TLR2- and TLR4-mediated pathways, J. Agric. Food Chem. 69 (2021) 9313-9325. https://doi.org/10.1021/acs.jafc.1c03850.

[13] G.Z. Dong, E.T. Oh, H. Lee, et al., Beta-lapachone suppresses radiation-induced activation of nuclear factor-kappaB, Exp. Mol. Med. 42 (2010) 327-334. https://doi.org/10.3858/emm.2010.42.5.034.

[14] G.J. Prud'homme, Y. Glinka, O. Udovyk, et al., GABA protects pancreatic beta cells against apoptosis by increasing SIRT1 expression and activity, Biochem. Biophys. Res. Commun. 452 (2014) 649-654. https://doi.org/10.1016/j.bbrc.2014.08.135.

[15] J.M. Han, E.K. Lee, S.Y. Gong, et al., *Sparassis crispa* exerts anti-inflammatory activity *via* suppression of TLR-mediated NF-κB and MAPK signaling pathways in LPS-induced RAW264.7 macrophage cells, J. Ethnopharmacol. 231 (2019) 10-18. https://doi.org/10.1016/j.jep.2018.11.003.

[16] H.M. Wang, L. Fu, C.C. Cheng, et al., Inhibition of LPS-induced oxidative damages and potential anti-inflammatory effects of *Phyllanthus emblica* extract *via* down-regulating NF-κB, COX-2, and iNOS in RAW 264.7 cells, Antioxidants 8 (2019) 270. https://doi.org/10.3390/antiox8080270.

[17] M. Li, P. Xi, Y. Xu, et al., Taurine attenuates *Streptococcus uberis*-induced bovine mammary epithelial cells inflammation *via* phosphoinositides/Ca^{2+} signaling, Front. Immunol. 10 (2019) 1825. https://doi.org/10.3389/fimmu.2019.01825.

[18] B.R. So, T.T. Bach, J.H. Paik, et al., *Kmeria duperreana* (Pierre) Dandy extract suppresses LPS-induced iNOS and NO *via* regulation of NF-kappaB pathways and p38 in Murin macrophage RAW 264.7 Cells, Prev. Nutr. Food Sci. 25 (2020) 166-172. https://doi.org/10.3746/pnf.2020.25.2.166.

[19] M. Sawhney, N. Rohatgi, J. Kaur, et al., Expression of NF-κB parallels COX-2 expression in oral precancer and cancer: association with smokeless tobacco, Int. J. Cancer. 120 (2007) 2545-2556. https://doi.org/10.1002/ijc.22657.

[20] M.H. Pan, S.R. Zhu, W.J. Duan, et al., "Shanghuo" increases disease susceptibility: modern significance of an old TCM theory, J. Ethnopharmacol. 250 (2020) 112491. https://doi.org/10.1016/j.jep.2019.112491.

[21] T. Alejandre-García, J.G. Peña-del Castillo, A. Hernández-Cruz, $GABA_A$ receptor: a unique modulator of excitability, Ca^{2+} signaling, and catecholamine release of rat chromaffin cells, Pflug. Arch. Eur. J. Phy. 470 (2018) 67-77. https://doi.org/10.1007/s00424-017-2080-1

[22] L. Januzi, J.W. Poirier, M.J.E. Maksoud, et al., Autocrine GABA signaling distinctively regulates phenotypic activation of mouse pulmonary macrophages, Cell Immunol. 332 (2018) 7-23. https://doi.org/10.1016/j.cellimm.2018.07.001.

[23] S. Zhang, J. Zhao, J. Hu, et al., Gama-aminobutyric acid (GABA) alleviates hepatic inflammation *via* GABA receptors/TLR4/NF-κB pathways in growing-finishing pigs generated by super-multiparous sows, Anim. Nutr. 9 (2022) 280-290. https://doi.org/10.1016/j.aninu.2022.02.001.

[24] A. Abdelazez, H. Abdelmotaal, S.E. Evivie, et al., Verification of *Lactobacillus brevis* tolerance to simulated gastric juice and the potential effects of postbiotic gamma-aminobutyric acid in streptozotocin-induced diabetic mice, Food Sci. Hum. Wellness 11 (2022) 165-176. https://doi.org/10.1016/j.fshw.2021.07.017.

[25] X. Xu, W. Wang, L. Lin, et al., Liraglutide in combination with human umbilical cord mesenchymal stem cell could improve liver lesions by modulating TLR4/NF-kB inflammatory pathway and oxidative stress in T2DM/NAFLD rats, Tissue Cell 66 (2020) 101382. https://doi.org/10.1016/j.tice.2020.101382.

[26] Y. Wang, Z. Luo, X. Huang, et al., Effect of exogenous γ-aminobutyric acid (GABA) treatment on chilling injury and antioxidant capacity in banana peel, Sci. Hortic. 168 (2014) 132-137. https://doi.org/10.1016/j.scienta.2014.01.022.

[27] R.F. Schwabe, D.A. Brenner, Mechanisms of liver injury. I. TNF-alpha-induced liver injury: role of IKK, JNK, and ROS pathways, Am. J. Physiol. Gastrointest. Liver Physiol. 290 (2006) G583-589. https://doi.org/10.1152/ajpgi.00422.2005.

[28] X. Jin, T.A. Zimmers, E.A. Perez, et al., Paradoxical effects of short- and long-term interleukin-6 exposure on liver injury and repair, Hepatology 43 (2006) 474-484. https://doi.org/10.1002/hep.21087.

[29] Y. Shen, S.A. Malik, M. Amir, et al., Decreased hepatocyte autophagy leads to synergistic IL-1β and TNF mouse liver injury and inflammation, Hepatology 72 (2020) 595-608. https://doi.org/10.1002/hep.31209.

[30] I.G. Ko, J.J. Jin, L. Hwang, et al., Polydeoxyribonucleotide exerts protective effect against CCl_4-induced acute liver injury through inactivation of NF-κB/MAPK signaling pathway in mice, Int. J. Mol. Sci. 21 (2020) 7894. https://doi.org/10.3390/ijms21217894.

[31] G. Qi, Y. Mi, R. Fan, et al., Nobiletin protects against systemic inflammation-stimulated memory impairment *via* MAPK and NF-κB signaling pathways, J. Agric. Food Chem. 67 (2019) 5122-5134. https://doi.org/10.1021/acs.jafc.9b00133.

[32] T. Hata, F. Rehman, T. Hori, et al., GABA, γ-aminobutyric acid, protects against severe liver injury, J. Surg. Res. 236 (2019) 172-183. https://doi.org/10.1016/j.jss.2018.11.047.

[33] N. Ohtani, N. Kawada, Role of the gut–liver axis in liver inflammation, fibrosis, and cancer: a special focus on the gut microbiota relationship, Hepatol. Commun. 3 (2019) 456-470. https://doi.org/10.1002/hep4.1331.

[34] G. Wang, R. Pan, X. Liang, et al., Perfluorooctanoic acid-induced liver injury is potentially associated with gut microbiota dysbiosis, Chemosphere 266 (2021) 129004. https://doi.org/10.1016/j.chemosphere.2020.129004.

[35] Z. Zhao, L. Chen, Y. Zhao, et al., *Lactobacillus plantarum* NA136 ameliorates nonalcoholic fatty liver disease by modulating gut microbiota, improving intestinal barrier integrity, and attenuating inflammation, Appl. Microbiol. Biotechnol. 104 (2020) 5273-5282. https://doi.org/10.1007/s00253-020-10633-9.

[36] J. Miao, L. Guo, H. Cui, et al., Er-Chen decoction alleviates high-fat diet-induced nonalcoholic fatty liver disease in rats through remodeling gut microbiota and regulating the serum metabolism, Evid. Based Complement. Alternat. Med. 2022 (2022) 6221340. https://doi.org/10.1155/2022/6221340.

[37] R. Duan, K. Huang, X. Guan, et al., Tectorigenin ameliorated high-fat diet-induced nonalcoholic fatty liver disease through anti-inflammation and modulating gut microbiota in mice, Food Chem. Toxicol. 164 (2022) 112948. https://doi.org/10.1016/j.fct.2022.112948.

[38] D. Fang, D. Shi, L. Lv, et al., *Bifidobacterium pseudocatenulatum* LI09 and *Bifidobacterium catenulatum* LI10 attenuate *D*-galactosamine-induced liver injury by modifying the gut microbiota, Sci. Rep. 7 (2017) 8770. https://doi.org/10.1038/s41598-017-09395-8.

[39] X. Li, S. Wu, Y. Du, et al., Entecavir therapy reverses gut microbiota dysbiosis induced by hepatitis B virus infection in a mouse model, Int. J. Antimicrob. Agents. 56 (2020) 106000. https://doi.org/10.1016/j.ijantimicag.2020.106000.

[40] L. Yu, X. Han, S. Cen, et al., Beneficial effect of GABA-rich fermented milk on insomnia involving regulation of gut microbiota, Microbiol. Res. 233 (2020) 126409. https://doi.org/10.1016/j.micres.2020.126409.

[41] N. Hyland, J. Cryan, A gut feeling about GABA: focus on $GABA_B$ receptors, Front. Pharmacol. 1 (2010) 124. https://doi.org/10.3389/fphar.2010.00124.