Heliyon 10 (2024) e26167

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

# Efficacy of *Jiuzao* polysaccharides in ameliorating alcoholic fatty liver disease and modulating gut microbiota

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ARTICLE INFO

*Keywords:*
*Jiuzao* polysaccharides
Alcoholic fatty liver disease
Zebrafish larvae
Gut microbiotas

ABSTRACT

*Jiuzao,* the residue from *Baijiu* production, has shown radical scavenging properties in prior investigations, suggesting its potential as a hepatoprotective agent against acute liver damage. This study reveals that *Jiuzao* polysaccharides ameliorated liver morphological damage in zebrafish larvae afflicted with alcoholic fatty liver disease (AFLD), as evidenced by Oil red O, H&E, and Nile red staining. These polysaccharides notably modulated antioxidant enzyme levels and lipid peroxidation components. The real-time quantitative polymerase chain reactions analyses illustrated the significant impact of *Jiuzao* polysaccharides on genes integral to ethanol and lipid metabolism. The 16 S rRNA results showed that *Jiuzao* polysaccharides could improve the intestinal flora in zebrafish larvae exposed to ethanol. In summary, *Jiuzao* polysaccharides efficaciously mitigate liver lipid accumulation, enhance ethanol metabolism, and reduce oxidative stress by downregulating genes involved in AFLD development. They also regulate the changes in gut microbiota, providing further protection against acute alcoholic liver insult in zebrafish larvae.

## 1. Introduction

Alcoholic Liver Disease (ALD), primarily manifesting initially as Alcoholic Fatty Liver Disease (AFLD), results from chronic excessive alcohol consumption. This condition can progressively escalate to alcoholic hepatitis, hepatic fibrosis, and eventually cirrhosis. In advanced cases, pronounced alcoholism can lead to hepatocyte necrosis and consequent liver failure [1]. The imperative need for efficacious hepatoprotective agents and nutritional adjuncts is evident, aiming to mitigate the AFLD-induced physiological damage and curb the onset of ALD.

Polysaccharides, owing to their radical scavenging abilities, emerge as prospective candidates for acute liver injury amelioration. They not only mitigate the depletion of antioxidative molecules induced by oxidative stress but also inhibit oxidative perturbations of lipids, enzymes, and nucleic acids, thereby ensuring the integrity of cellular membrane and organelle integrity [2]. Zhang et al. [3] and

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https://doi.org/10.1016/j.heliyon.2024.e26167
Received 29 September 2023; Received in revised form 2 February 2024; Accepted 8 February 2024
Available online 13 February 2024

Wu et al. [4] demonstrated that polysaccharides protect both normal human liver cells and HepG2 cells against ethanol-induced damage. *Jiuzao,* the residual byproduct of grain alcohol obtained through solid fermentation and distillation, is a rich source of bioactive components that remain largely unexplored. Among these, we identified the beneficial effects of Laowuzeng *Jiuzao* polysaccharide (LJP) against reactive oxygen species generation induced by $H_2O_2$ in zebrafish larvae, thereby slowing cellular demise [5]. Under normal physiological conditions, the liver also removes enterogenous bacteria, fungi, and other toxins from the gut. However, hepatic dysfunction disrupts the gut-liver axis, particularly the gut microbiota, triggering significant alterations in intestinal microecology and impairing intestinal barrier function. This result leads to the systemic influx of intestinal microbes and their metabolites, hyperactivating the host immune system, and thereby eliciting aberrant immune responses [6]. Yet, the intricate interplay between gut microbes and AFLD remains to be elucidated. However, the effect of *Jiuzao* polysaccharide on AFLD remains unreported.

Zebrafish exhibit an approximate 87% genetic homology with humans, paralleling human drug metabolic systems [7]. By 5 days post-fertilization (dpf), all digestive organs of zebrafish larvae reach maturity. The potential for evaluating drug induced hepatotoxicity in zebrafish culminates within a week, positioning them as ideal candidates for hepatotoxic assessments and hepatoprotective screening [8]. Ethanol exposure in zebrafish larvae substantially modulates the hepatic expressions of alcohol dehydrogenase and cytochrome P450 isoforms [7]. It disrupts cholesterol homeostasis, leading to deformities in nascent zebrafish embryos. In contrast, in more advanced embryonic stages, ethanol promotes lipogenesis *via* the cholesterol regulatory element-binding protein, leading to fatty disease. Typically, a 24-h ethanol regimen in zebrafish larvae results in lipid denaturation in over 60% of the cases, causing hepatocellular dysfunction [7]. These characteristics render zebrafish larvae an effective model for studying ethanol-induced AFLD and its impact on gut microbes.

This study aimed to explore the potential role of *Jiuzao* polysaccharide in preventing alcoholic fatty liver disease (AFLD) and to provide a theoretical basis for its application in high-value reuse and functional foods. Utilizing zebrafish larvae as experimental subjects, the 16 S rRNA gene sequencing and RT-qPCR technology were employed to assess the impact of *Jiuzao* polysaccharide on the intestinal microbiota and host genes of zebrafish larvae for the first time. This investigation laid the foundation for future studies on the activity of *Jiuzao* polysaccharides.

## 2. Materials and methods

### 2.1. Materials

Phosphate buffered saline (PBS), 0.5% Oil Red O, Nile red, 4',6-diamidino-2-phenylindole (DAPI) at 10 μg/mL, Triton X-100, and the Hematoxylin and Eosin (H&E) staining kit were acquired from Solarbio Technology Ltd. (Beijing, China). Propylene glycol, glycerol, ammonia solution, acetone, tricaine, and the reverting blue solution were sourced from Macklin (Shanghai, China). Paraformaldehyde (PFA) was procured from J&K Scientific Ltd. (Shanghai, China). Xylene, hydrochloric acid ethanol differentiation solution, and neutral resin were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All reagents employed were of analytical grade.

### 2.2. Polysaccharide extraction

*Jiuzao* was procured from Hebei Hengshui Laobaigan Liquor Co., Ltd., specifically the Laowuzeng *Baijiu* and Sanpaijing *Baijiu* workshops in 2021 [9,10]. Polysaccharides from *Jiuzao* were isolated as outlined previously [5]. Both LJP and Sanpaijing *Jiuzao* polysaccharide (SJP) underwent cold water extraction, ethanol precipitation, deproteinization, and purification *via* DEAE Sepharose Fast Flow and Sephadex G50 columns. Molecular weight determination, methylation, and nuclear magnetic resonance analysis indicated LJP predominantly comprised mannose with a molecular weight averaging 32,402 g/mol. While SJP was processed using a similar extraction protocol, its structure is yet to be characterized.

### 2.3. Zebrafish maintenance and treatment

AB strain wildtype zebrafish embryos, as well as transgenic *Tg (apo14:EGFP) embryos*, were supplied by EzeRinka Biotechnology Co., Ltd. Zebrafish larvae thrived under a 14 h light/10 h dark regime at 28 °C, following the protocol set by the Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (*Danio rerio*, Eugene: Univ. of Oregon Press).

### 2.4. Ethanol-induced toxicity in zebrafish larvae

Six-day post-fertilization (dpf) wildtype AB strain zebrafish larvae were segregated into five ethanol treatment groups: control, 87.5 mM, 175 mM, 350 mM, and 750 mM, and allocated to six-well plates. Each treatment tier, including the control, consisted of three replicate subsets ($n = 25$ each). To mitigate ethanol evaporation, the plates were sealed with Parafilm and incubated at 28 °C. Mortality was promptly addressed, and post-32-h survival rates were computed using the formula: survival rate (%) = (final zebrafish larvae count/initial count) × 100. Following treatment, larvae underwent microscopy and were subjected to Oil Red O staining.

### 2.5. Experimental plan for zebrafish larvae

Fig. 1 illustrates the zebrafish larvae experimental layout. Four dpf larvae were allocated into two primary sets. The control

segment was immersed solely in the zebrafish culture solution, whereas the model cohort was exposed to 350 mM ethanol post-48-h standard culturing. Control larvae were further categorized into 4 subgroups (n = 50 each): C_1 (zebrafish culture medium), C_2 (0.05 mg/mL vitamin E (VE)), C_3 (0.20 mg/mL LJP), and C_4 (0.20 mg/mL SJP). Conversely, model larvae were divided evenly among: M Group (standard culture solution), V Group (0.012, 0.025, 0.05 mg/mL VE concentrations), LJP Group (0.05, 0.1, 0.2 mg/mL LJP), and SJP Group (0.05, 0.1, 0.2 mg/mL SJP). After a 48-h incubation, a 350 mM ethanol solution was introduced for an additional 32 h. Each group had three replicates with 50 healthy larvae.

### 2.6. Microscopy of larvae liver morphology

After ethanol and polysaccharide treatment, 176 hour post-fertilization (hpf) wild-type AB zebrafish larvae were anaesthetized using a 0.02% Tricaine solution. To ensure precise orientation-with aligned eyes and body segments-the liver development was visually assessed. Larval imaging was conducted using an optical microscope (Nikon, Tokyo, Japan).

### 2.7. Zebrafish larvae Oil Red O staining procedure

The procedures were performed with slight modifications as described above [8]. Wild-type AB strain zebrafish larvae were anaesthetized with a 0.02% tricaine solution and underwent fixation in 4% PFA at 4 °C overnight. Following three PBS washes, larvae experienced a graded dehydration series with propylene glycol concentrations of 20%, 40%, 80%, and 100%. Each dehydration step lasted 15 min at ambient temperature. Lipid droplet staining was subsequently achieved using a 0.5% Oil red O solution in acetone, maintained in the dark at room temperature for 12 h. Excess dye was efficiently removed using sequential immersion in 100% and 80% propylene glycol solutions, and residual background staining was cleared with PBS. Hepatic morphological alterations and lipid droplet accumulation were examined microscopically, with three representative images retained. Image J software facilitated the quantification of liver sizes, translating them into grayscale values to assess hepatic steatosis severity. The steatosis reduction percentage is given by:

Liver steatosis reduction (%) $= \frac{V_2 - V_3}{V_2 - V_1} \times 100\%$.

where $V_1$, $V_2$, and $V_3$ represent the grayscale values of the control, model, and sample groups, respectively.

### 2.8. H&E staining protocol

The procedures were performed as previously described [11]. Wild-type AB strain zebrafish larvae were preserved in 4% PFA at

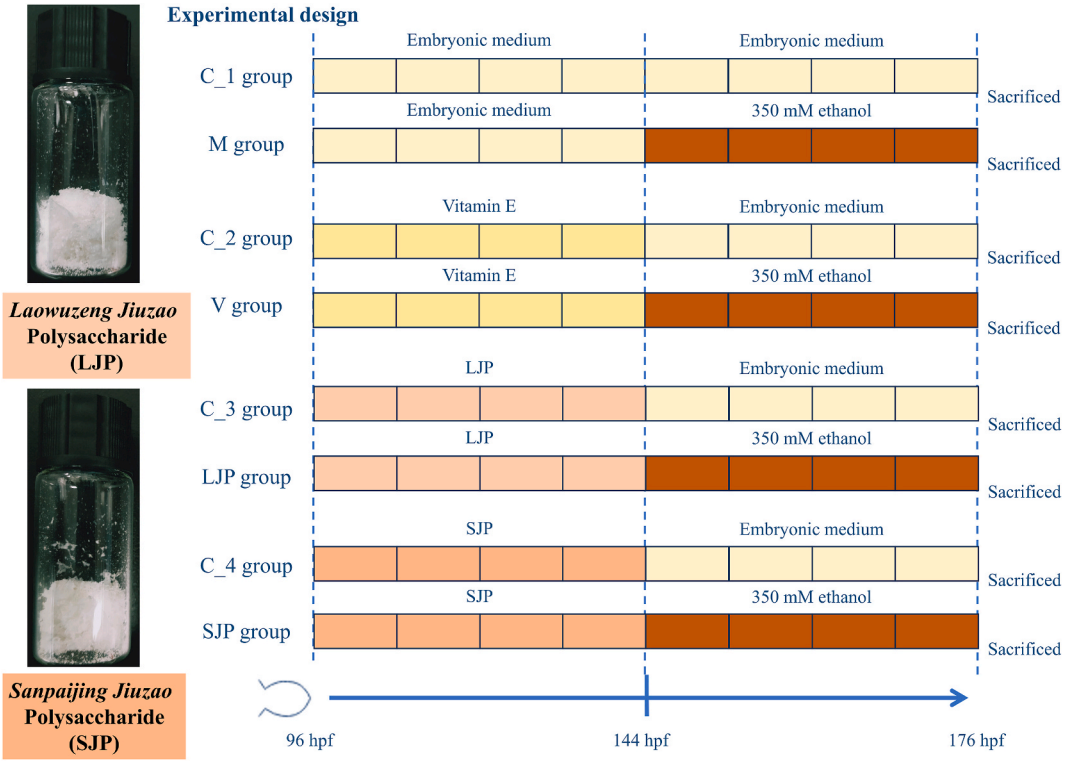



**Fig. 1.** Experimental design for zebrafish larvae.

4 °C overnight. An increasing concentration ethanol series followed for dehydration, then xylene immersion, and finally paraffin embedding. The resulting samples were sectioned at thicknesses ranging from 3 to 5 μm. These sections underwent H&E staining, and were subsequently examined under an optical microscope.

### 2.9. Nile red and DAPI staining technique

Nile red staining was performed as described previously [12]. *Tg* transgenic zebrafish larvae, targeted for alcoholic fatty liver prevention *via Jiuzao* polysaccharides, were anaesthetized using a 0.02% tetracaine solution. An overnight fixation in 4% PFA was followed by triple PBS washes. Larvae were subsequently transferred to 96-well plates, exposed to a citric acid solution (with 0.1% Triton) for 2 h at 65 °C, and washed thrice with PBS. After a 20-min DAPI staining period in the dark at ambient temperature, three additional PBS washes were conducted. Larvae were then exposed to a 0.5 μg/mL Nile red dye solution, prepared from a primary 0.5 mg/mL acetone solution diluted in 75% glycerol. This final staining step lasted 30 min, executed in the dark at room temperature. Larval examination and imaging were accomplished using a fluorescence microscope (Nikon, Tokyo, Japan).

### 2.10. Enzymatic assays in ethanol-induced fatty liver disease in zebrafish larvae

The alterations in enzyme activity due to ethanol-induced fatty liver disease in zebrafish larvae were assessed using commercial kits as per the guidelines provided by Beijing Solarbio Science & Technology Co., Ltd., China. Zebrafish larvae were homogenized in Tris-buffer under chilled conditions and subsequently centrifuged at 8000 g for 10 min at 4 °C. Utilizing the supernatant, we quantified the activities of key antioxidant enzymes—superoxide dismutase (SOD) and catalase (CAT)—as well as evaluating the levels of lipid peroxides, including glutathione (GSH) and malondialdehyde (MDA). Protein quantification was performed using the bicinchoninic acid assay.

### 2.11. Isolation of RNA and real-time quantitative polymerase chain reaction (RT-qPCR) protocol

The procedure was prepared by a previous method with slight modifications [11]. From each experimental group, 150 zebrafish larvae were randomly selected, rinsed thrice with sterile water, and allocated to 1.5 mL nuclease-free centrifuge tubes (50 larvae per tube, resulting in 3 tubes per group). RNA extraction from these larvae was performed using the Trizol reagent (Invitrogen, USA) in adherence to the manufacturer's protocol. The isolated RNA was subsequently reverse-transcribed employing the PrimsScriptTM RT-PCR Kit (Takara). RT-qPCR amplification was conducted on the ABI 7500 real-time PCR system (Life Technology, USA) utilizing the SBYR Green kit (Takara Biotechnology, Inc). Primer sequences employed in this study are listed in Table 1. Ribosomal protein P0 (rpp0) served as the reference gene. Amplification efficiencies for both the target gene and the internal control were consistent, with a marginal deviation below 5%. Quantitative data analysis was conducted using the $2^{-\Delta\Delta Cr}$ computational approach.

### 2.12. Analysis of microbial composition

From each designated group, 150 zebrafish larvae were meticulously selected, rinsed thrice with sterile water, and placed into 1.5 mL nuclease-free centrifuge tubes (50 larvae per tube, resulting in 3 tubes per group). Genomic DNA, encompassing the entirety of the microbial community within the zebrafish larvae, was extracted utilizing the DNeasy PowerSoil Pro kit (MoBio Laboratories, Carlsbad, CA, USA). DNA concentration and purity assessments were performed *via* NanoDrop2000. The 16 S rRNA gene V3–V4 region was selectively amplified through primers 338F and 806R. After purification, amplicons underwent equimolar pooling and were subjected to paired-end sequencing on an Illumina MiSeq PE300/NovaSeq PE250 platform (Illumina, San Diego, USA) according to Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) protocols. Sequenced reads were deposited in the NCBI Sequence Read Archive (SRA). By utilizing UPARSE version 7.1, operational taxonomic units (OTUs) were clustered with a 97% similarity threshold, while chimeras were identified and removed. Sequence species were annotated using the RDP classifier with a comparison threshold of 70% within the Silva 16 S rRNA database (version 138).

**Table 1**
Sequences of the primers.

| Target gene | Forward primer (5′ to 3′) | Reverse primer (5′ to 3′) |
|---|---|---|
| cyp2y3 | 5′-TATTCCCATGCTGCACTCTG-3′ | 5′-AGGAGCGTTTACCTGCAGAA-3′ |
| cyp3a65 | 5′-AAACCCTGATGAGCATGGAC-3′ | 5′-CAAGTCTTTGGGGATGAGGA-3′ |
| hmgcra | 5′-CTGAGGCTCTGGTGGACGTG-3′ | 5′-ATCGGTTGCGGTCTGAAAAT-3′ |
| fasn | 5′-GAGAAAGCTTGCCAAACAGG-3′ | 5′-GAGGGTCTTGCAGGAGACAG-3′ |
| chop | 5′-AGGAAAGTGCAGGAGCTGAC-3′ | 5′-CTCCACAAGAAGAATTTCCTCC-3′ |
| gadd45αa | 5′-TGGCTTTGTTTGTGGGACTT-3′ | 5′-TGGAAAACAGTCCACTGAGA-3′ |
| nrf2 | 5′-CTCCAAACCTCCGTTCACCA-3′ | 5′-GTCGTCTACGGGCAGATTGA-3′ |
| gpx1a | 5′-AAGGAGAAGCTTCCTCAGCC-3′ | 5′-GAGATGTCATTCCTGCACACG-3′ |
| edem1 | 5′-GACAGCAGAAACCCTCAAGC-3′ | 5′-CATGGCCCTCATCTTGACTT-3′ |
| rpp0 | 5′-CTGAACATCTCGCCCTTCTC-3′ | 5′-TAGCCGATCTGCAGACACAC-3′ |

### 2.13. Data and statistical methodology

Data are represented as the mean ± standard error of the mean, with analysis conducted using SPSS 20.0. Results from Oil red O staining and RT-qPCR were evaluated through one-way ANOVA, followed by Duncan's post hoc test for multiple comparisons and the

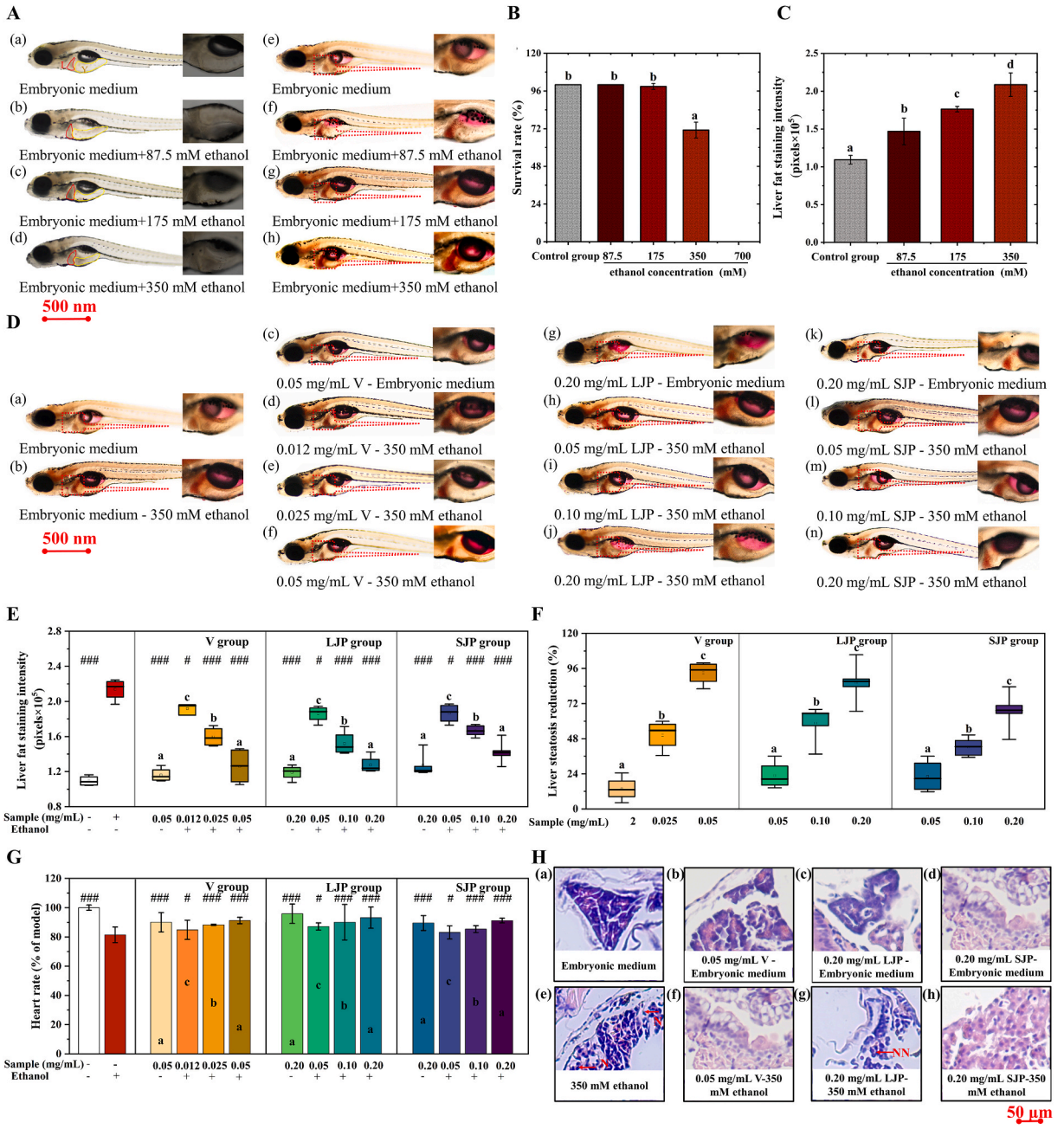



**Fig. 2.** Micrograph snapshots (a–d) and comprehensive whole-mount Oil red O staining images (e–h) of zebrafish larvae liver across varied ethanol gradients (A). Denotations: L-hepatic zone; Y-yolk sac area. A circumscribed liver, enhanced for visual clarity, delineates lipid droplet accumulation utilized in steatosis scoring. Survival rates of zebrafish larvae across ethanol concentration gradients (B). Grayscale intensity quantifications of larval liver exposed to varying ethanol gradients (C). Representative imagery of whole-mount Oil red O staining in zebrafish larvae (D), alongside hepatic fat staining intensities (E), reduction in hepatic steatosis (F), cardiac rate metrics (G). Analyses were conducted using ImageJ software. Prototypical histopathological snapshots of zebrafish larval livers (H) with annotations: NN-normative nucleus; NP-nuclear peripheral alignment; V-vacuolation; K-karyolysis. Data represented as mean ± SD. Significance markers: #<0.05, ##<0.01, ###<0.001 when compared against the model group, evaluated *via* student's *t*-test. Different lowercase letters denote significant differences. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

unpaired 2-tailed Student's *t*-test. Disparities were considered significant when $p < 0.05$. Visualization was facilitated using Origin 2021 software.

## 3. Results and discussion

### 3.1. AFLD model configuration

Microscopic examination of zebrafish larvae post-ethanol exposure revealed hepatic fatty accumulation, further substantiated by whole-mount Oil red O staining (Fig. 2A). While the control group displayed a transparent crescent-shaped liver with a completely absorbed yolk sac, ethanol-treated zebrafish larvae displayed a distorted oval-shaped liver, notable hepatomegaly, and a protracted yolk sac absorption [13]. As illustrated in Fig. 2B, no mortality was evident for ethanol concentrations of 0, 87.5 mM, and 175 mM. However, at 350 mM ethanol, the larval survival rate decreased to 71.11% ± 5.09%, with observable pallor in some specimens. A concentration of 750 mM ethanol proved lethal to all larvae. Grayscale analysis (Fig. 2C) of the liver in the control group showed that the value was 109,323.50 ± 5764.90. This value surged significantly ($p < 0.05$) with escalating ethanol concentrations. Based on survivability metrics, a 32-h exposure to 350 mM ethanol was determined to be optimal for establishing the alcoholic fatty liver model.

### 3.2. Comprehensive Oil Red O staining analysis

Fig. 2D (c, g, k) illustrates the Oil red O staining of zebrafish larvae, revealing that both VE and *Jiuzao* polysaccharides exerted negligible impacts on larval liver morphology. However, progressive concentrations of VE, LJP, and SJP corresponded to a diminished liver size in AFLD-afflicted zebrafish larvae. This underscores the efficacy of VE and *Jiuzao* polysaccharides in counteracting ethanol-

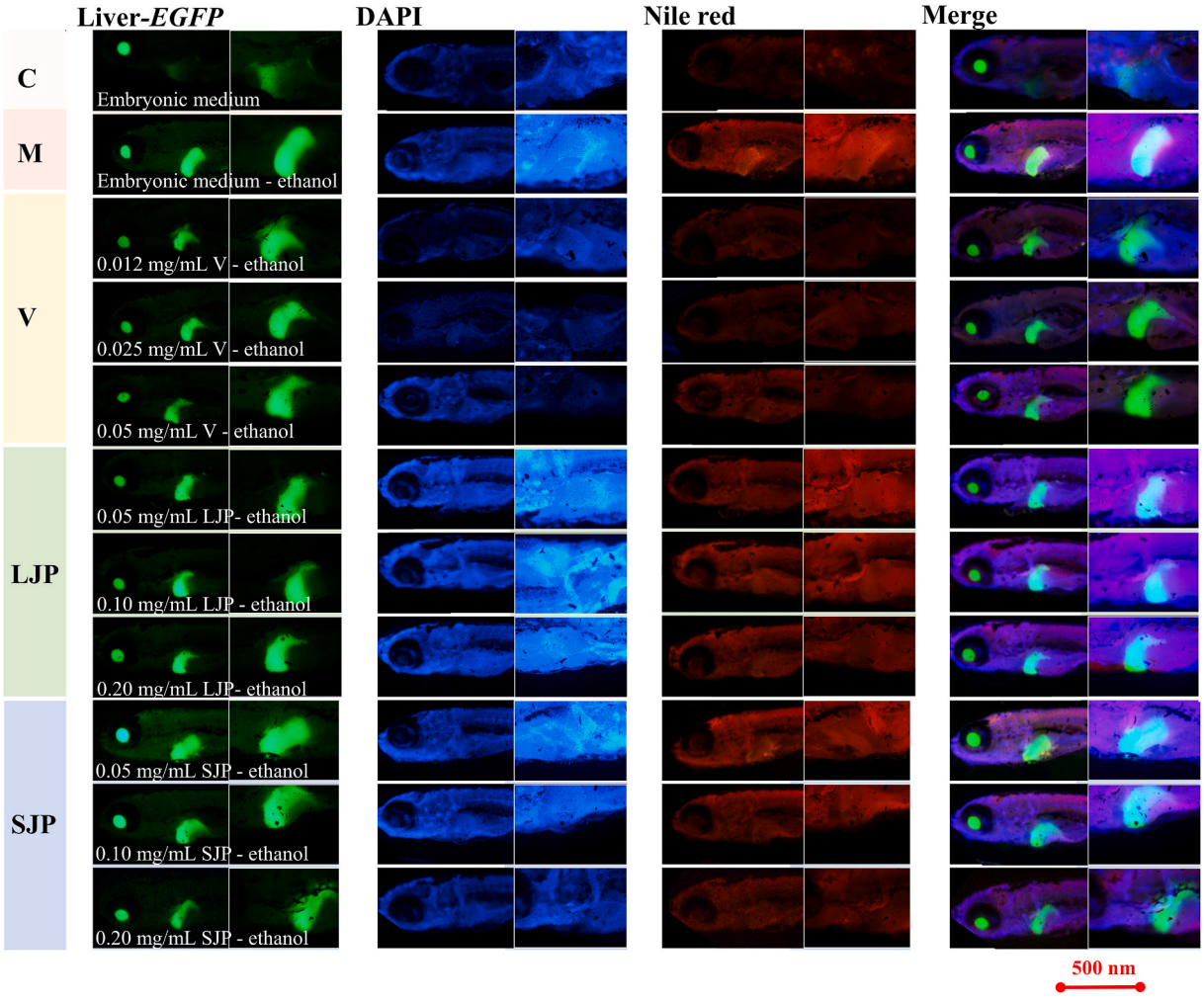



**Fig. 3.** Illustration of Nile red staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

induced hepatic enlargement in the larvae.

Quantitative assessment of the staining was conducted *via* grayscale interpretations using Image J software. As presented in Fig. 2E, significant differences were observed between the M group and the 0.025, 0.05 mg/mL V cohorts, the 0.01, 0.02 mg/mL LJP batches, and the SJP assemblage ($p < 0.001$). Fig. 2F demonstrates that varying dosages of VE, LJP, and SJP significantly modulated hepatic steatosis reduction ($p < 0.05$). Specifically, the steatosis mitigation reached 93.07% ± 7.96%, 86.31% ± 13.80%, and 66.90% ± 12.91% for the 0.05 mg/mL V, 0.20 mg/mL LJP, and 0.20 mg/mL SJP groups respectively, indicating dose-responsive alleviation of ethanol-prompted hepatic steatosis in zebrafish larvae ($p < 0.05$).

### 3.3. Cardiac frequency analysis

Fig. 2G presents the cardiac frequency data for zebrafish larvae. Compared to the C_1 batch, the M group showcased a cardiac downturn to 81.47% ± 0.05% ($p < 0.001$). Following administration of 0.012, 0.025, and 0.05 mg/mL VE, the cardiac rate increased to 84.89 ± 0.07%, 88.32 ± 0.004%, and 91.25 ± 0.02% respectively, denoting significant dose-correlation ($p < 0.05$). Similarly, after 0.05, 0.10, and 0.20 mg/mL LJP treatment, rates increased to 87.16 ± 0.02%, 90.03 ± 0.12%, and 93.21 ± 0.07% respectively, also signifying dose-dependent improvements ($p < 0.05$). Concurrently, SJP dosages of 0.05, 0.10, and 0.20 mg/mL led to cardiac rates of 83.12 ± 0.04%, 85.38 ± 0.02%, and 91.19 ± 0.02% respectively, emphasizing a robust dose relationship ($p < 0.05$). These observations affirm the capability of VE, LJP, and SJP to rehabilitate cardiac frequencies in zebrafish larvae' exhibiting alcoholic fatty liver attributes.

### 3.4. Hepatic histopathological analysis

Histological evaluations through H&E staining revealed significant changes in the hepatic architecture of zebrafish larvae (Fig. 2H). Hepatocytes in the C_1 group(a) exhibited a pristine morphology, characterized by distinct margins and nuclei without the presence of fat vacuoles. The hepatic cords manifested consistent configurations, the lobules were discernible, hepatocytes maintained uniform dimensions, and both cytoplasmic and nuclear integrities remained intact [14]. VE (c) and *Jiuzao* polysaccharides (e and g) imparted no notable hepatic alterations in the larvae as evidenced in Fig. 2H. Consequently, VE, LJP, and SJP were devoid of overt hepatotoxic effects on zebrafish larvae. Contrastingly, ethanol exposure led to pronounced hepatotoxic effects in larvae, typified by nuclear peripheral localization, hepatocyte swelling, and ambiguous boundaries. Concurrently, interstitial cell volume increased, with ensuing hepatic fatty vacuolar degeneration (Fig. 2H (b)). A rise in cytoplasmic fatty vacuole dissolution was noted, enhancing lipid droplet accumulation within hepatocytes. Preventive treatments involving VE, LJP, and SJP induced minor hepatic structural changes, aligning with native zebrafish larvae hepatic morphology.

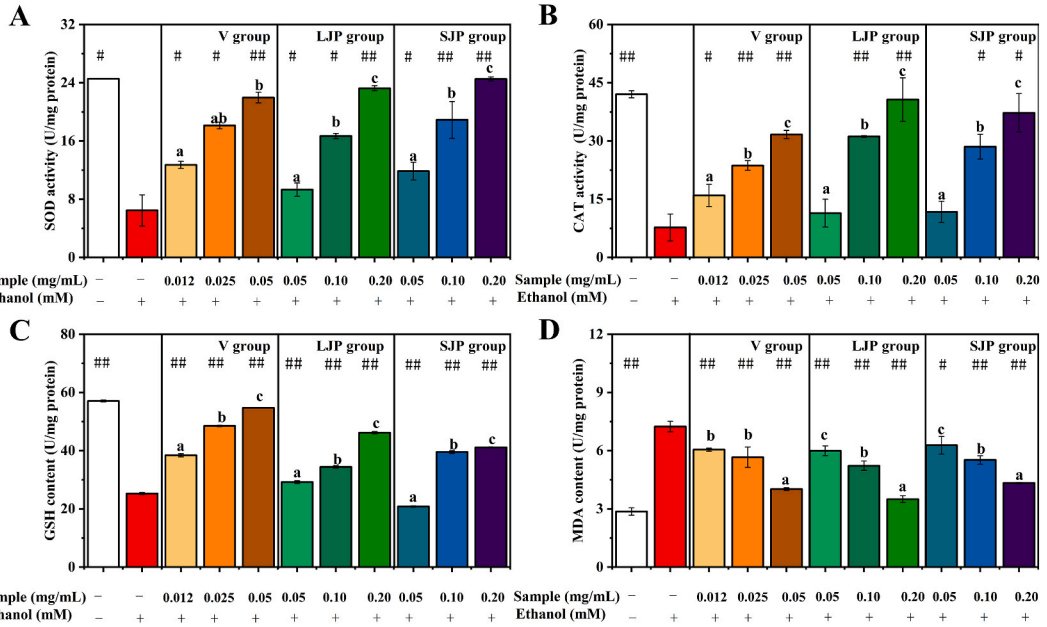



**Fig. 4.** Influence of *Jiuzao* polysaccharides on enzymatic activities in alcoholic fatty liver-afflicted zebrafish larvae. (A) SOD. (B) CAT. (C) GSH. (D) MDA. Data presented as mean ± SD. #<0.05, ##<0.01, ###<0.001 compared to the model group, analyzed *via* student's *t*-test. Different lowercase letters denote significant differences.

### 3.5. Nile red and DAPI staining interpretation

Recent findings suggest that ethanol exposure exacerbates hepatic tissue damage and apoptotic cell death in zebrafish larvae (Fig. 3). This detrimental effect was notably reduced upon administration VE, LJP, and SJP to zebrafish larvae. The overarching conclusion highlights the effectiveness of *Jiuzao* polysaccharides in reducing lipid accumulation, tissue damage, and cellular mortality, supporting observations from Oil red O and H&E staining assays.

### 3.6. Enzymatic activity correlation in alcoholic fatty liver-afflicted zebrafish larvae

Ethanol-induced oxidative stress and lipid peroxidation are crucial in causing hepatocellular damage. As depicted in Fig. 4A and B, compared to the C_1 group, there was a significant decrease in hepatic antioxidant enzymatic activities (SOD and CAT) within the M group, recorded at 6.45 ± 2.14 U/mg protein and 7.72 ± 3.46 U/mg protein, respectively ($p < 0.05$). Conversely, the V, LJP, and SJP groups considerably improved liver enzyme activities. Notably, 0.20 mg/mL SJP showed increased SOD activity compared to 0.20 mg/mL LJP, despite a lower CAT activity.

Critical indicators for lipid peroxidation, namely GSH and MDA levels, are illustrated in Fig. 4C and D. Compared to the C_1 group, the M group experienced a significant decrease in GSH levels, falling to 25.27 ± 0.32 U/mg protein ($p < 0.05$). Conversely, GSH concentrations increased by 53.83%, 45.27%, and 38.58% following treatment with 0.05 mg/mL VE, 0.20 mg/mL LJP, and 0.20 mg/mL SJP, respectively. Excessive ethanol exposure raised hepatic MDA levels, an effect significantly reduced by VE or *Jiuzao* polysaccharide pre-treatment. Consequently, *Jiuzao* polysaccharides and VE impart protective attributes against AFLD.

### 3.7. Ethanol metabolic and lipid metabolic genes

In Fig. 5A, mRNA expression of cyp2y3 and cyp3a65 in the M group exceeded that in the C_1 group by 3.57 and 3.48-fold, respectively. This increase ($p < 0.001$) potentially drives oxidative stress and lipid deterioration in zebrafish larvae liver [14]. In contrast, the V, LJP, and SJP groups exhibited demonstrated significant downregulation of these genes compared to the M group ($p < 0.001$), implicating the role of *Jiuzao* polysaccharides in enhancing ethanol metabolic genes in zebrafish larvae, which may reduce toxic build-up, thereby preventing alcoholic fatty liver formation.

Fig. 5B illustrates that the M group exhibited a notable increase in hmgcra and fasn mRNA levels ($p < 0.001$). Conversely, the

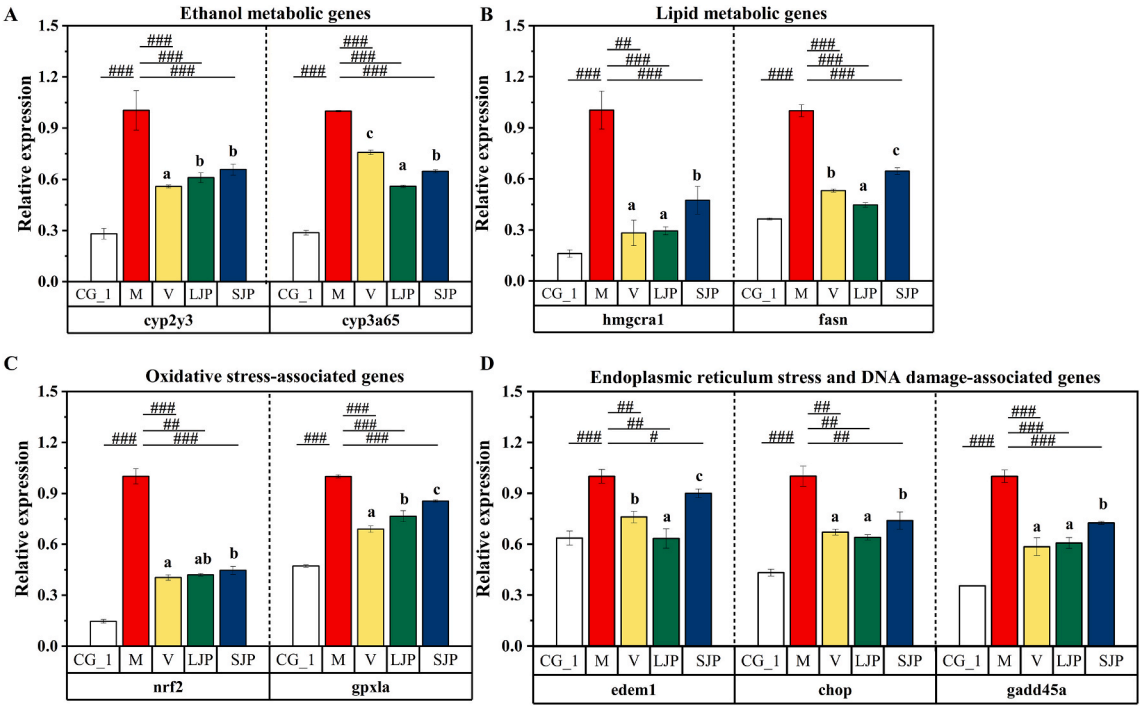
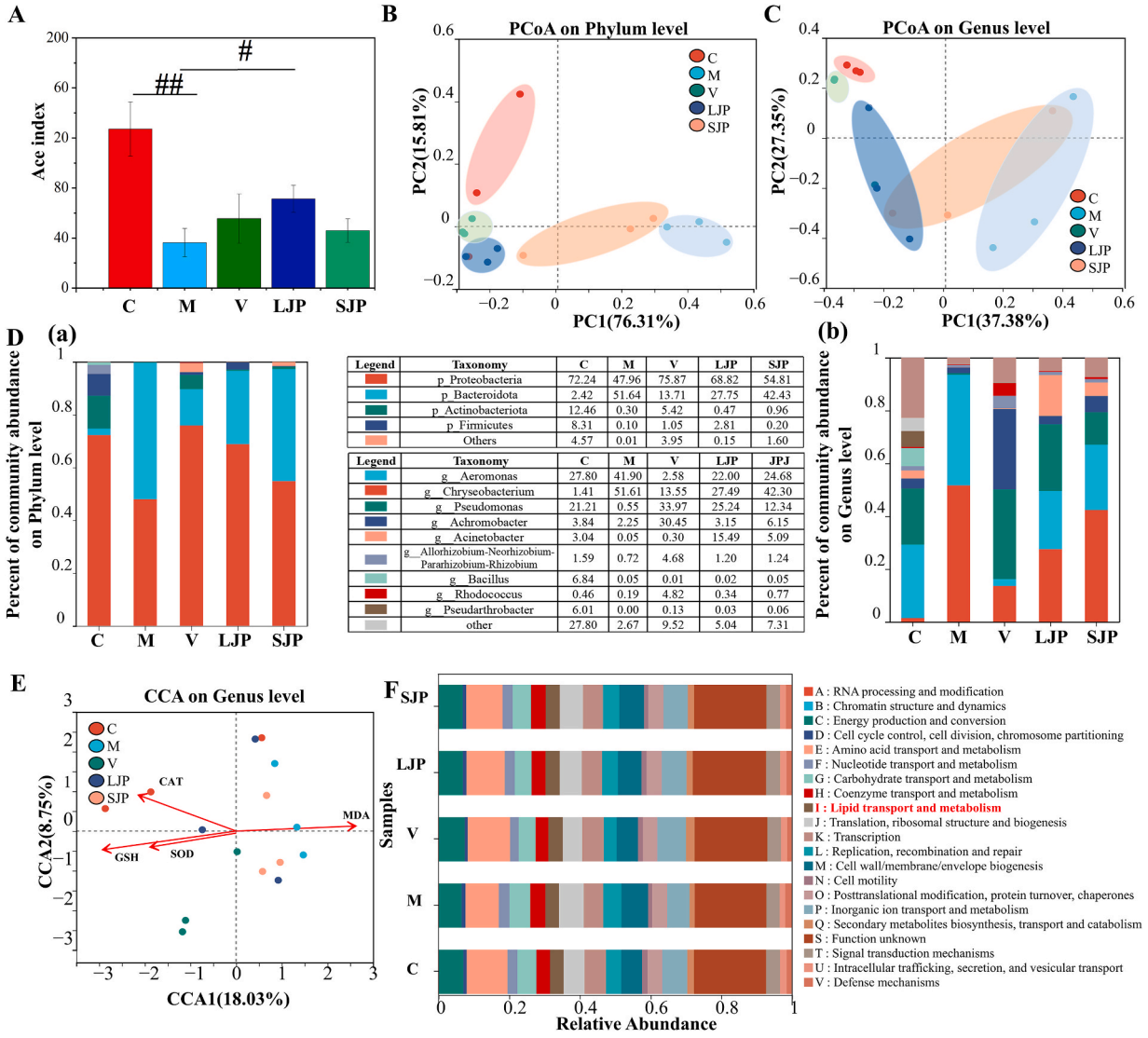



**Fig. 5.** *Jiuzao* polysaccharides mitigate ethanol-induced disturbances and lipid metabolism, reduce oxidative and endoplasmic reticulum stress, and counteract DNA damage in zebrafish larvae. RT-qPCR elucidated the expression patterns of ethanol metabolism-associated genes, cyp2y3 and cyp3a65 (A). RT-qPCR depicted lipid metabolism-associated genes, hmgcra and fasn (B). RT-qPCR of oxidative stress markers, nrf2 and gpxla, was carried out (C). The expression of endoplasmic reticulum stress and DNA damage-associated genes, edem1, chop, and gadd45αa, were assessed (D). Data are expressed as mean ± SD. #<0.05, ##<0.01, ###<0.001 denote significance against the model group as determined by the student's *t*-test. Different lowercase letters denote significant differences.

polysaccharide-treated groups displayed a decline in the expression of these genes ($p < 0.001$). Such findings suggest that *Jiuzao* polysaccharides may regulate lipid metabolism, sustain lipid equilibrium, and thus prevent ethanol-induced hepatic steatosis in zebrafish larvae.

From Fig. 5C, it is evident that the nrf2 and gpx1a mRNA levels in the M group were increased by 6.82 and 2.12 times, respectively, compared to the C_1 group. Nonetheless, the VE and *Jiuzao* polysaccharide treatments significantly reduced the expression of these genes ($p < 0.001$). This implies the potential of *Jiuzao* polysaccharides in reducing liver damage through the downregulation of gpc1a and gpx1a mRNA.

Regarding Fig. 5D, endoplasmic reticulum stress and DNA damage-associated genes, including edem1, chop, and gadd45αa, were accentuated in the M group. Notably, both VE and *Jiuzao* polysaccharides treatment notably repressed the expression of these genes expression ($p < 0.05$). This indicates *Jiuzao* polysaccharides could curtail endoplasmic reticulum stress and DNA damage, limiting ethanol-driven hepatocyte apoptosis and conferring liver protection. Collectively, *Jiuzao* polysaccharides potentially modulate alcoholic fatty liver progression in zebrafish larvae, acting upon genes tied to ethanol metabolism, lipid regulation, oxidative distress, endoplasmic reticulum stress, and DNA damage.




| Legend | Taxonomy | C | M | V | LJP | SJP |
|---|---|---|---|---|---|---|
| | p_Proteobacteria | 72.24 | 47.96 | 75.87 | 68.82 | 54.81 |
| | p_Bacteroidota | 2.42 | 51.64 | 13.71 | 27.75 | 42.43 |
| | p_Actinobacteriota | 12.46 | 0.30 | 5.42 | 0.47 | 0.96 |
| | p_Firmicutes | 8.31 | 0.10 | 1.05 | 2.81 | 0.20 |
| | Others | 4.57 | 0.01 | 3.95 | 0.15 | 1.60 |

| Legend | Taxonomy | C | M | V | LJP | JPJ |
|---|---|---|---|---|---|---|
| | g_Aeromonas | 27.80 | 41.90 | 2.58 | 22.00 | 24.68 |
| | g_Chryseobacterium | 1.41 | 51.61 | 13.55 | 27.49 | 42.30 |
| | g_Pseudomonas | 21.21 | 0.55 | 33.97 | 25.24 | 12.34 |
| | g_Achromobacter | 3.84 | 2.25 | 30.45 | 3.15 | 6.15 |
| | g_Acinetobacter | 3.04 | 0.05 | 0.30 | 15.49 | 5.09 |
| | g_Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium | 1.59 | 0.72 | 4.68 | 1.20 | 1.24 |
| | g_Bacillus | 6.84 | 0.05 | 0.01 | 0.02 | 0.05 |
| | g_Rhodococcus | 0.46 | 0.19 | 4.82 | 0.34 | 0.77 |
| | g_Pseudarthrobacter | 6.01 | 0.00 | 0.13 | 0.03 | 0.06 |
| | other | 27.80 | 2.67 | 9.52 | 5.04 | 7.31 |

**Fig. 6.** Alleviation of ethanol-induced gut dysbiosis by *Jiuzao* polysaccharides. Genus-level Ace index (A). Phylum-level PCoA (B). Genus-level PCoA (C). Relative abundance histograms of gut microbial phyla and genera (D (a and b)). Genus-level RDA (E). COG functional classification (F). #<0.05 and ##<0.01 versus the model group employing student's *t*-test.

### 3.8. Gut microbiota diversity in zebrafish larvae

The gut microbial profiles of zebrafish larvae underwent 16 S rRNA gene sequencing across the C group, M group, 0.05 mg/mL V group, 0.2 mg/mL LJP group, and 0.2 mg/mL SJP group. This was conducted to discern the impact of *Jiuzao* polysaccharides on gut microbial composition in zebrafish larvae affected by AFLD. From 15 zebrafish larvae samples, 239,223 valid sequences were extracted, yielding an average sequence length of 429 bp following adjustment by the minimum sample sequence (7888).

Fig. 6A reveals that, compared to the C group, the Ace index of the M group experienced a significant reduction ($p < 0.01$), indicating decreased community richness in zebrafish larvae post-ethanol administration. Nonetheless, following intervention with VE, LJP, and SJP, the Ace index observed an upturn compared to the M group. These findings suggest that ethanol exposure perturbs gut microbial equilibrium of zebrafish larvae, yet *Jiuzao* polysaccharides could modulate this microbial composition. Principal coordinates analysis (PCoA) insights into intersample species compositional resemblance (Fig. 6B and C) highlighted that LJP, SJP, V, and M group profiles diverged notably from the C group, underscoring the alterations in gut microbial composition post-ethanol challenge. Furthermore, both the LJP and SJP groups displayed marginal deviations from the C group, insinuating their modulatory prowess over the gut microbiota in zebrafish larvae afflicted with alcoholic hepatic damage.

At the phylum level, microbiota composition analysis (Fig. 6D (a)) showed that Proteobacteria and Bacteroidota predominated in zebrafish larvae. In the M group, there was a reduction in the abundance of Proteobacteria, Actinobacteriota, and Firmicutes, concomitant with an increase in Bacteroidota. Ethanol primarily promoted Bacteroidota growth in zebrafish larvae, suppressing the proliferation of Proteobacteria, Actinobacteriota, and Firmicutes. In contrast, the V, LJP, and SJP groups increased the presence of Proteobacteria, Actinobacteriota, and Firmicutes, and attenuated Bacteroidota. Notably, the LJP group closely resembled the C group. This indicates *Jiuzao* polysaccharides predominantly influence Proteobacteria and Bacteroidota in zebrafish larvae gut, with LJP offering superior microbiota modulation compared to SJP. The V group manifested a decline in Firmicutes compared to the M group, similar to the C group, which implies that VE predominantly impacts Firmicutes in zebrafish larvae gut.

At the genus level, microbiota composition (Fig. 6D (b)) disclosed dominant genera in zebrafish larvae as *Aeromonas, Chryseobacterium*, *Pseudomonas*, *Achromobacter*, and *Acinetobacter*. In the C group, *Aeromonas* and *Pseudomonas* had relative abundances of 27.80% and 21.21% respectively. Compared to the C group, the M group exhibited an increased abundance of *Aeromonas* and *Chryseobacterium*, with reduced levels of *Pseudomonas*, *Achromobacter*, *Acinetobacter*, and *Rhodococcus*. Ethanol appeared to predominantly modulate these six genera, suggesting their potential role in the onset of alcoholic fatty liver in zebrafish larvae. Versus the M group, both LJP and SJP groups decreased *Aeromonas* and *Chryseobacterium* and increased *Pseudomonas, Achromobacter, Acinetobacter, and Rhodococcus*, indicating their potential roles in modulating alcoholic fatty liver through these genera. LJP demonstrated superior efficacy over SJP in these modulations, indicating its enhanced capability in regulating gut microbiota in zebrafish larvae. Lipid transport and metabolic functions, inferred from 16 S rRNA gene sequencing (Fig. 6F), exhibited increased relative abundances in the V, LJP, and SJP groups. Redundancy analysis (RDA), employed to gauge the correlation between oxidative damage in zebrafish larvae and gut microbiota (Fig. 6E), ascertained that SOD, CAT, MDA, and GSH considerably influenced gut microbiota of zebrafish larvae. In summary, LJP and SJP are potent modulators of the microbial community in zebrafish larvae with alcoholic liver diseases, affecting the development of alcoholic fatty liver, with LJP likely offering.

## 4. Discussion

The escalating demand for benign natural antioxidants, devoid of negative repercussions, for liver disease prevention and intervention has become palpable. The research emphasizes the potential for ameliorating AFLD by mitigating oxidative stress. Hence, this investigation augments the evidence that *Jiuzao* polysaccharides prophylaxis can protect against ethanol-induced hepatic toxicity by strengthening antioxidant defenses and reducing hepatic lipid accumulation. Detailed histological assessments, including Oil Red O, H&E, and Nile red staining, revealed the restorative capability of *Jiuzao* polysaccharides and VE against ethanol-driven hepatic lipidosis. Li et al. [15] similarly reported that purple sweet potato polysaccharide reduced irregular hepatocyte patterns and excessive lipid vacuoles. Concurrently, we noted decreased activities of SOD and CAT, diminished GSH levels, and increased MDA content within the livers of M group zebrafish larvae, aligning with Lu et al.'s research that connects ethanol exposure to hepatic oxidative stress [16,17]. Chronic ethanol consumption can debilitate hepatic mitochondrial functions, reduce peroxidase activities, and impede lipid oxidation, culminating in lipid retention [3]. Furthermore, *Jiuzao* polysaccharides significantly influenced antioxidant enzymes (SOD and CAT) and lipid peroxidation markers (GSH and MDA) in zebrafish larvae subjected to ethanol-induced hepatic distress, primarily by countering oxidative stress.

The etiology of ALD predominantly lies in the direct cytotoxicity of ethanol and its derivatives to hepatocytes, compounded by the injury from oxidative stress [18]. Ethanol is metabolized in liver cells to acetaldehyde, mediated by enzymes like alcohol dehydrogenase, catalase, and cytochrome P450 enzyme 2E1 (cyp2e1), and subsequently to acetic acid *via* acetaldehyde dehydrogenase. This metabolic pathway generates free radicals, leading to mitochondrial impairment, metabolic anomalies, and lipid accumulation within hepatocytes [18]. Ethanol metabolites compromise intestinal barrier function by disrupting mucosal and tight junction protein expressions, leading to increase intestinal permeability [19]. The resultant permeability ushers in pathogenic bacteria, lipopolysaccharides, and pro-inflammatory intestinal metabolites into the systemic circulation, exacerbating liver inflammation, amplifying oxidative stress, and furthering liver injury.

Cytochrome P450 family 2 subfamily E member 1 (cyp2e1) and cytochrome P450 family 3 subfamily A (cyp3a65) are crucial enzymes in managing oxidative stress during ethanol metabolism, representing salient genes in zebrafish hepatic function [20,21]. The present research revealed that *Jiuzao* polysaccharides modulate the transcriptional activity of cyp2e3 and cyp3a65 mRNA, potentially

mitigating oxidative stress inflictions in zebrafish larvae. HMG Coenzyme A reductase a (hmgcra) and fatty acid synthase (fasn) respectively govern cholesterol and fatty acid biosynthesis [22–24]. Thus, *Jiuzao* polysaccharides appear to downregulate hmgcra and fasn expressions, thereby controlling cholesterol moderation and fatty acid biogenesis [25]. Glutathione peroxidase 1 (gpx1a) facilitates the conversion of GSH to its oxidized form (GSSG), subsequently transforming noxious peroxides into benign hydroxyl compounds, preserving cellular membrane integrity against oxidative perturbations. *Jiuzao* polysaccharides, by modulating nrf2 and gpx1a expressions, might counteract oxidative derangements, ameliorating hepatic distress. Notably, the transcription levels of edem1, chop, and gadd45ga were attenuated upon *Jiuzao* polysaccharides exposure, implying their potential to curtail ethanol-induced endoplasmic reticulum stress in the liver, thereby minimizing cell apoptosis, lipid sequestration, and DNA impairments [24,26].

Evidence suggests that gut microbiota dysbiosis and its metabolic byproducts critically influence liver pathologies *via* the "gut-liver" axis [27]. In this investigation, ethanol was observed to suppress the proliferation of Proteobacteria, Actinobacteria, and Firmicutes, while increasing Bacteroidetes. This shift led to a microbial imbalance in zebrafish larval intestines, resulting in hepatic inflammation and lipid accumulation [28]. Prophylactic application of *Jiuzao* polysaccharides enhanced the prevalence of Proteobacteria, Actinobacteria, and Firmicutes, yet reduced Bacteroidetes growth. Furthermore, it optimized the Firmicutes to Bacteroidetes ratio, suggesting *Jiuzao* polysaccharides' beneficial modulation of gut microbiota in zebrafish larvae with alcoholic fatty liver symptoms. The study identified bacteria such as *Pseudomonas*, *Chryseobacterium*, *Aeromonas*, and *Exiguobacterium* as significant players in inflammation and tissue infections [29,30]. *Jiuzao* polysaccharide administration increased *Pseudomonas* levels while decreasing *Aeromonas* and *Chryseobacterium*. Such alterations suggest *Jiuzao* polysaccharides' capability to orchestrate gut microbial shifts, thereby potentially influencing energy, lipid metabolism, and hepatic determinants, which in turn modulate alcoholic fatty liver development.

This investigation elucidated variances in the effects of LJP and SJP polysaccharides on alcoholic fatty liver development, with the LJP polysaccharide demonstrating superior efficacy. These disparities may stem from distinct gene modulations by LJP and SJP polysaccharides in zebrafish larvae afflicted by alcoholic hepatic damage, potentially influenced by the *Baijiu* distillation technique. The traditional distillation of *Baijiu* is segregated into two primary procedures: "Laowuzeng" and "Sanpaijing". The former involves combining and steaming residual *Jiuzao* with fresh grains for fermentation [31], whereas the latter discards *Jiuzao* post 3 fermentation and distillation cycles [32]. Compared with the Laowuzeng *Jiuzao*, the nutrients in Sanpaijing *Jiuzao* used up completely, polysaccharide and other low content of bioactive substances. Moreover, LJP is predominantly composed of mannose [5]. Mannan polysaccharides, recognized as innate immune stimulants, bolster both non-specific and targeted immune responses [33]. Documented to exhibit potent antiviral immunity [34], they can also decrease gastrointestinal tryptophan and serotonin levels, improving the gut environment [35]. Thus, the efficacy of SJP in mitigating alcoholic hepatic impairments in zebrafish larvae seems inferior to LJP.

## 5. Conclusion

The prophylactic potential of *Jiuzao* polysaccharides against AFLD was elucidated using the zebrafish AFLD model. Microscopic assessments, Oil red O and H&E stainings analyses revealed that *Jiuzao* polysaccharides could mitigate ethanol-induced hepatic lipid accumulation in a dose-responsive manner. In fluorescent zebrafish larvae liver models subjected to Nile red staining, *Jiuzao* polysaccharides demonstrably attenuated liver lesions, lipid droplet accumulation, tissue degradation, and cellular apoptosis. Upon evaluating genes pivotal to AFLD manifestation, *Jiuzao* polysaccharides were identified to suppress these genes, consequently curbing AFLD development. Furthermore, AFLD zebrafish larvae gut microbiota assessments depicted that LJP ameliorated the gut microbiota, particularly modulating Proteobacteria, Bacteriodota, Actinobacteriota, Firmicutes, *Pseudomonas*, *Chryseobacterium*, *Aeromonas*, *Achromobacter*, and *Acinetobacter*. *Jiuzao* polysaccharides could protect liver injury caused by enhancing antioxidant defense, reducing lipid accumulation in liver and affecting intestinal microbial composition. The findings of this research provide theoretical support for the use of *Jiuzao* polysaccharides in the prevention of AFLD, highlighting the potential broad applications of *Jiuzao* in functional foods. The study revealed that various types of *Jiuzao* polysaccharides exert differing effects on AFLD prevention. Therefore, it is essential to further explore the relationship between polysaccharide structure and biological activity. Moreover, it is necessary to conduct a detailed examination of the principal signaling pathways and mechanisms that underlie the contribution of *Jiuzao* polysaccharides to the prevention of AFLD.

## Ethics statement

The Institutional Animal Care and Use Committees of Beijing Technology and Business University approved all experimental designs involving zebrafish (Approval No.2023-32).

## Funding

This work was financially supported by the Study of the technology and its application on the brewing of Laobaigan type Baijiu with multiple grains [grant number 20327107D].

## CRediT authorship contribution statement

**Qing Li:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ronghong Pei:** Writing – review & editing, Visualization, Supervision, Methodology, Conceptualization.

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## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Fuping Zheng reports financial support was provided by Hebei Hengshui Laobaigan Liquor Co., Ltd. If there are other authors, they 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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