

Sleep-promotion of probiotics-fermented *Gastrodia elata* Bl.: Chemical variation and omics exploration.

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Article

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Abstract

Fermentation of Chinese herbal medicines (CHM) could enhance its bioactivities. We hypothesized probiotic-fermented GE with better potential to alleviate insomnia than that of unfermented, thus the changes in chemical composition and the insomnia-alleviating effects and mechanisms of fermented GE on PTZ-induced insomnia zebrafish were explored via HPLC and LCMS, phenotypic, transcriptomic, and metabolomics analysis. The results demonstrated that probiotic fermented GE performed better than unfermented GE in increasing the content of chemical composition, reducing the displacement, average speed, and number of apoptotic cells in zebrafish with insomnia. Metabolomic investigation showed that the anti-insomnia effect was related to regulating the pathways of actin cytoskeleton and neuroactive ligand-receptor interactions. Transcriptomic and RT-qPCR analysis revealed that SFL significantly modulated the expression levels of *neurod1*, *msh2*, *msh3*, *recql4*, *ercc5*, *rad51c*, and *rev3l*, which are mainly involved in neuron differentiation and DNA repair. Collectively, as a functional food, Fermented GE possessed potential for insomnia alleviation.

Introduction

Insomnia is a commonly observed sleep disorder, predominantly defined by challenges in starting sleep and experiencing suboptimal sleep quality, and often manifests as comorbidity with depressive disorders^{1,2}. In the long run, insomnia will seriously affect people's life quality and physical and mental health³⁻⁵. Dysfunctions in the hypothalamic-pituitary-adrenal axis, central neurotransmitter systems, vagal tone, melatonin system function, inflammatory response factors, and limbic cortical system circuits have been proposed by researchers to be the main cause of insomnia⁶. However, further research is required to better elucidate the pathogenesis of insomnia for better treatment. Sleep disorders are often managed using a range of medications, such as benzodiazepines, nonbenzodiazepine omega-receptor agonists, tricyclic antidepressants, selective GABA agents, and antihistamines. Nevertheless, the sedative medications mentioned above may cause serious adverse effects⁷. Overdose deaths involving benzodiazepines increased between 1999 and 2015⁸. Nonpharmacological interventions are currently being recognized as the preferred initial approach for managing chronic insomnia⁹. Functional food or healthcare food can be served as an important approach to relieve insomnia symptoms, such as *Schisandra Chinensis Fructus*, and *Polygalae Radix*. *Gastrodia elata* Bl. (GE) is a botanical specimen originating from China, which holds significant historical significance in the realms of traditional medicine and culinary traditions¹⁰. Phenols, glycosides, adenosine, and its analogs, particularly p-hydroxybenzaldehyde, are the main sources of sedative and hypnotic effects of GE^{11,12}. Interestingly, GABA_A-Rs is a target of benzodiazepines and is known to exert its hypnotic effects by enhancing its function¹³. In addition, GE may also regulate sleep by turning on adenosine A₁/A(2A) receptors and stimulating the VLPO sleep center¹⁴. It has been reported that HBA and PHB possess the capability to induce anxiolytic effects in the context of sedation and hypnosis by interacting with the 5-HT system and γ -GABA system, respectively^{12,15}. Sleep is closely linked to a variety

of metabolic dysfunctions in humans, sufficient sleep is essential for restoring the functions and metabolism of nearly all tissues and cells in the body, promoting optimal metabolic health^{16,17}.

CHM has been used for centuries to treat diseases and is vital for human well-being. Moreover, CHM typically contains low contents of bioactive compounds¹⁸. Fermentation not only leverages the bioactivity of probiotics to change the nutritional composition and bioactive compounds of plant-based foods, but it also has great benefits for gut microbiome composition and the immune system¹⁹. Nevertheless, whether probiotic-fermented GE has the potential to serve as a functional food with insomnia-alleviating effects has not been reported so far.

We found that the probiotics-fermented GE showed a higher content of active ingredients than unfermented GE. In light of the above considerations, it could be reasonably hypothesized that probiotics rely-fermented GE has the potential to be a functional food with insomnia-alleviating effects. Herein, we applied transcriptomic and metabonomic analysis of insomnia Zebrafish treated with fermented GE to explore the essential regulations invoked by GE.

Results

Neuroprotective effect against low dose PTZ-induced mental injury

Locomotor activity in zebrafish is a measured, complicated behavior, and their neural networks resemble those of mammals²⁰. To confirm the effect of GE samples at each treatment stage on animal behavior and nervous system damage, we measured the total travel distance and swimming speed of zebrafish for 30 minutes and 50 minutes, and the amount of apoptotic cells. As shown in Fig. 1A-D, the total locomotion distance and mean speed were significantly increased after PTZ-exposed larvae. The behavioral changes of zebrafish larvae were significantly reversed after pretreatment with GE samples at different processing stages. Nevertheless, there were no statistical differences between the low-dose and high-dose GE samples. In general, PFL and SFL samples had similar effects on the total movement distance and average movement speed of zebrafish, with both being significantly superior to WGE. Obviously, fermentation process enhanced the biofunction of GE to promote sedative and sleeping.

Sleep deprivation has a significant influence on the nervous system. Lack of sleep can cause an imbalance in DNA damage and repair²¹. Anti-apoptotic activity is an important cause for the neuroprotective effects of sleep-related disease drugs²². Apoptosis in the nervous system is an important reflection of nerve damage. The apoptotic cells in zebrafish larvae can be visualized through fluorescent staining using a fluorescence microscope, owing to the transparent nature of their bodies. Apoptotic cells within the nervous system had a green fluorescence following staining with Acridine orange (AO). Figure 1F illustrates a significant presence of apoptotic cells in zebrafish brain of the PTZ group. Different processing stages of the GE sample considerably decreased the number of apoptotic

cells observed in the brain of zebrafish. GE sample effectively inhibited PTZ-induced apoptosis in the larvae brains. Thus, fermented GE provided more nerve protection than unfermented GE.

Main components change in GE fermentation

To investigate the dynamic change of chemical compounds, we comparatively analyzed the GE samples of WGE, PFL, and SFL. The results indicate that the content of four active ingredients (parishin B, C, E, and 4-Hydroxybenzyl alcohol) remained stable from stage WGE to stage PFL. An obvious increase of these active ingredients was found from stage PFL to stage SFL (Fig. 1G). In particular, the concentration of 4-Hydroxybenzyl alcohol, parishin B, C, and E in the SFL stage increased 19.7-, 2.9-, 2.9-, and 2.9-fold, respectively, compared to stage WGE. However, it is worth noting that parishin A and gastrodin showed a brief decline from WGE to PFL, and decreased to 0 from PFL to SFL. Adenosine increased briefly from WGE to PFL stage and then rapidly increased in SFL stage ($245.72 \pm 12.53 \mu\text{g/mL}$), increased 28.9-fold, compared to stage WGE (Fig. 1G and Table S2). During the fermentation process, the contents of seven active ingredients underwent significant changes. Among them, parishin E and gastrodin were not detected in SFL sample. The remaining five active components demonstrated a notable overall increase. These content variations indicated that probiotics fermentation invoked active components GE changing. Similar exploration has also found that probiotics can influence changes in active ingredients^{23,24}. It is therefore that probiotic fermentation could be a useful processing method to enhance the biological functions of ingredients or herbs. However, this enhancement may vary from ingredient material and probiotic strains and more exploration is still needed.

LC-MS/MS Profiling of GE samples at different fermentation stages

To profile chemical component changes in fermented GE, we used LC-MS/MS analysis. Figure 2A presents the aggregation of metabolite overall expressions for each sample. A total of 55 chemical components were detected from positive and negative ions using ESI. ESI positive and negative resources yielded the identification of only three metabolites (Fig. 2B). The results disclosed obvious variations among GE samples at different fermentation stages, as shown in Fig. 2C and 2D. PCA analysis displayed 71% features across samples and each group had strong clustering tendencies and maintained distinct separation from one another.

In the present study, these metabolites were identified in the samples at three distinct processing stages that encompassed nine benzenoids, 16 lipids and lipid-like molecules, one nucleoside, nucleotide, and analog, four organic acids and derivatives, four organic nitrogen compounds, four organic oxygen compounds, 10 organoheterocyclic compounds, seven phenylpropanoids, and polyketides. This information is visually represented in Fig. 2E. Lipids and lipid-like compounds made up the largest group among them, making up 29.09% of the total metabolite composition in terms of relative concentration.

To further understand the impact of fermentation process on the active components in GE, we performed PLS-DA analysis. Hierarchical cluster analysis (HCA) showed how metabolites accumulated

in fermentation processing in Fig. 2E. The Euclidean distance arithmetic was used to cluster the 55 discovered metabolites of GE samples in heat maps, as illustrated in Fig. 2F. The dendrogram classified the metabolites found at various heat processing stages into three groupings. The higher concentration of a specific metabolite in the corresponding sample is indicated by the brighter hue. The HCA heat map revealed more variations in abundance between the PFL and WGE samples than between the PFL and SFL samples, suggesting that the metabolites in the fermented liquid may undergo distinct changes throughout the fermentation phase, and the abundance of PFL and SFL samples is similar, probably because of their fermentation time. Nevertheless, in general, the contents of chemical components before and after fermentation changed dynamically. Compared with WGE, SFL and PFL significantly increased the contents of 24 and 19 compounds respectively. Among them, 12 compounds were detected in both (Fig. S1 A and B). Benzenoids, lipids and lipid-like molecules, organic nitrogen compounds, and organoheterocyclic compounds are the major super classes. This may be one of the main reasons why the PFL and SFL have similar phenotypic results. In addition, SFL also shows unique fermentation advantages compared with PFL. These compounds classes that SFL improved include organic acids and derivatives, benzenoids, lipids and lipid-like molecules, organoheterocyclic compounds, phenylpropanoids, and polyketides. the direct parent compounds are alpha amino acids, benzamides, medium-chain fatty acids, phthalic anhydrides, coumarins and derivatives, cinnamic acids, and stilbenes (Table S3). The VIP score chart shows that the top five compounds are 2,6-dimethylaniline, nicotinamide, 3-methylpyrazole, triclosan, and adenosine. However, the top four compounds have nothing to do with the sleep-promoting activity. The fifth-ranked adenosine, one of the primary active components of GE (Fig. 2G), has a large contribution rate in different processed GE samples, and the research has shown that the adenosine analog could be a potential hypnotic²⁵. From the GE processing efficiency perspective, adenosine may become a new entry point for GE research. Short-chain fatty acids can participate in many physiological functions and are thought to affect sleep through the gut-brain axis pathway²⁶. Herein, we investigated the impacts different stages short-chain fatty acid (SCFA) accumulation, as shown in Fig. 2H. Although only acetic acid and propionic acid were detected. The findings indicated that the content of acetic acid and propionic acid also increased sequentially as the fermentation progressed. It could be seen that changes in chemical composition content were related to fermentation technology, which may be one of the reasons for the change in the phenotypic results.

Metabolomic profiling of insomnia larvae exposed SFL

Insomnia is closely related to changes in metabolic function²⁷. To investigate the metabolomic influence by fermented GE, we then carried out LC-MS/MS profiling on metabolome variation on SFL-treated, insomnia, and blank control groups. As shown in Fig. S2. In the positive ion mode, 203 metabolites were detected, whereas in the negative ion mode, 204 metabolites were discovered. Among them, 66 metabolites were identified in both modes. PC1 and PC2 were able to capture 93% of the sample features, as indicated in Fig. 2B. Furthermore, a considerable change in metabolites across treatments was indicated by the relatively high gap between groups. Each group was predicted by the sPLS-DA analysis in Fig. 3B. As a result, there were significant differences in the metabolic data between the groups when using different treatments. The groups treated with 20 µg/mL SFL(G3) and 80 µg/mL (G4)

showed considerable overlap in PCA and PLS-DA analysis plots. This suggests that the metabolic levels of the two groups were very close to each other.

We then conducted pairwise comparisons to collect metabolites of upregulation or downregulation between G2/ G1, G3/G2 as well as G4/G2, as shown in Fig. 3C-E. The altered metabolites were enumerated and visually shown in the Venn diagram (Fig. 3F and 3G). Four important differential metabolite expression levels were redressed by both low and high-dose SFL, which are the hydoxycholesterol (C00319), propanoylcarnitine (C03017), alpha-linolenic acid (C06427), acetylcholine chloride (C08201). The G2 raised the levels of three metabolites and lowered the levels of one, as seen in Fig. 3H, yet these four differential metabolites were significantly reversed in both G3 and G4 groups.

To gain a better understanding of how SFL exposure induces metabolite changes, we performed metabolic pathway enrichment analysis to explore and visualize metabolic pathways affected by SFL. The enriched pathways observed in Fig. 3I encompass the regulation of actin cytoskeleton (dre04810), neuroactive ligand-receptor interaction (dre04080), glycerophospholipid metabolism (dre00564), alpha-linolenic acid metabolism (dre00592), biosynthesis of unsaturated fatty acids (dre01040). The *p*-value associated with the metabolic path indicates the level of statistical significance on the enrichment of the pathway. The top one significantly enriched pathway is the regulation of actin cytoskeleton (dre04810). Traditional Chinese medicine can improve insomnia by the regulation of actin cytoskeleton, and the aforementioned pathway assumes a critical function in neuroprotection and regulating cell proliferation and differentiation^{28,29}.

Transcriptomic analysis on regulations of SFL

Transcriptomic profiling can provide a system view on mRNA variation influenced by SFL. To evaluate if a given set of genes exhibits statistically significant, consistent difference between two biological states, a computer method was applied. A statistical technique called gene enrichment analysis, also known as functional enrichment analysis is used to find genes that are overrepresented in a large gene set and may be connected to particular disease characteristics³⁰. In this way, we can choose "interesting" genes or pathways for function analysis without having to rely on any arbitrary predefined criterion.

We then used GSEA to investigate the impact of SFL on gene function. GO biological process function annotation was combined to screen the gaps between 15 and 500 genes in the GO entries. Then, the absolute value of the normalized enrichment score (NES) > 1, FDR *q*-value < 0.25, and the nominal *p*-value < 0.05 were deemed as the significantly enriched pathways. These gene sets were ranked from highest to lowest in absolute value of NES, and the top gene sets were randomly selected. Positive and negative NES indicate higher and lower expression in G4 or G2, respectively.

We found that the peripheral nervous system development, neuron differentiation, and DNA repair were higher expressed in the G4 Group (Fig. 3A-B) and (Fig. S3A). On the contrary, the 'Negative regulation of mitochondrial electron transport, cytochrome c to oxygen' and the 'negative regulation of organic acid metabolic process' were higher expressed in G2 Group (Fig. S3B and 33C); The 'neutrophil chemotaxis'

and 'regulation of neurogenesis' were higher expressed in G2 Group (Fig. S3D and S4A). On the contrary, the 'negative regulation of visual perception' was highly expressed in the G1 Group (Fig. S4B). The above results indicated that the related pathways should deserve more attention in further research. Next, 133 genes with significant changes among the groups were screened out on the above-related pathways (Table S5); however, which gene(s) among the 133 genes plays a key role is unclear. Thus, we carry out key driver gene analysis (KDA) to help understand the genes in the main regulatory position among the selected genes.

KDA analysis

A total of 133 genes were selected for investigation, predetermined the number of key genes and linked genes, as well as set the PPI score. Subsequently, a KDA analysis was conducted. We select 133 genes, set the number of key genes and associated genes, and the PPI score, and then perform KDA analysis. As shown in Fig. 4, interestingly, we found ten key genes through KDA; among them, ten key driver genes such as *neurod1*, *rev3l*, *msh2*, *recql4*, *ercc5*, *msh3*, *polh*, *smc5*, and *rad51c*, are involved in the neuron differentiation (GO:0030182) and DNA repair (GO:0006281) pathway, and positively correlated with G4; one gene (*ccl20b*) is involved in the neutrophil chemotaxis (GO:0030593) pathway and is positively correlated with G2 (Fig.S3D); It revealed that SFL improves insomnia caused by changes in neutrophil chemotaxis by activating DNA repair and neuromodulation pathways. These results demonstrate that SFL interferes with the expression of genes in these pathways, which is consistent with previous studies³¹. Hence, SFL may also exhibit important potential for promoting sleep. The correlation heat map obtained by calculating Pearson correlation coefficient between key driving genes and metabolites. As shown in Fig. 5B, the correlation analysis revealed a positive association of seven genes (*neurod1*, *msh2*, *msh3*, *recql4*, *ercc5*, *rad51c*, and *rev3l*) with a metabolite (acetylcholine chloride), and a negative association with three metabolites (linolenic acid, propionylcarnitine, and hydoxycholeic acid). These findings underscore the close relationship between the changes in the insomnia-like phenotype and distinctly different metabolites as well as genes exhibiting differential expression. These observations demonstrate that changes in the insomnia-like phenotype are closely associated with significantly different metabolites and genes.

RT-qPCR validation of key genes

To identify the results of the transcription profile, ten key driver genes were chosen for RT-qPCR verification. However, the *polh* gene was not expressed even after changing the primer sequence, which may be due to the low expression level. As depicted in Fig. 6, a total of seven genes demonstrated contrasting alterations in the G2 and G4 groups, and these genes were found to be associated with enriched pathways. The results demonstrated that the expression pattern of seven genes exhibited concurrence with that ascertained using RNA-seq, hence affirming the dependability of the RNA-seq data. The expression of *neurod1*, *msh2*, *msh3*, *recql4*, *ercc5*, *rad51c*, *smc5*, and *rev3l* genes was downregulated in the G2 group and upregulated after G4, which may play a positive role in alleviating the insomnia symptoms. Combined with the GSEA-enriched pathway, it was observed that the regulation of

SFL on insomnia-like phenotype in zebrafish was related to neuron differentiation and DNA repair pathway, respectively.

Discussion

Sleep is essential for maintaining and restoring many physiological processes in the body^{32,33}. TCM has unique advantages in the treatment of insomnia. Comprehensive transcriptomic and metabolomic analysis showed that there were significant differences between the G1, G2, and G4 groups at the gene and metabolite levels. In the transcriptomics analysis, seven key genes associated with neuron differentiation and DNA repair pathways were identified. These seven genes are key genes in the neurodifferentiation and DNA repair pathway signaling pathway, indicating that SFL affects the expression of genes in this pathway. DNA damage repair increases during sleep and after external stimulation of DNA damage, and DNA damage in zebrafish neurons can trigger sleep, which may be a basic pathway related to insomnia³¹. Metabolomics analysis screened out four significantly different metabolites, including acetylcholine chloride, linolenic acid, propionylcarnitine, and hyodeoxycholic acid. Acetylcholine chloride and linolenic acid are closely related to sleep^{34,35}. Four metabolites are primarily involved in the regulation of actin cytoskeleton, neuroactive ligand-receptor interaction, glycerophospholipid metabolism, alpha-linolenic acid metabolism, and biosynthesis of unsaturated fatty acids pathways. As mentioned earlier in section results, traditional Chinese medicine can improve insomnia through the top-ranked regulation of actin cytoskeleton pathway. These findings strongly suggest that these discovered metabolites and genes may be key regulators in the complex regulatory network of SFL on insomnia zebrafish. The above studies further confirm the preventive effect of SFL on insomnia, primarily attributed to its regulation of the actin cytoskeleton and DNA repair pathways. The findings suggest that the combined effect of significant metabolites and genes may contribute to the improvement of sleep disorders induced by PTZ-induced mental damage.

In conclusion, fermented GE effectively ameliorated PTZ-induced insomnia by reducing total distance and mean velocity of movement and improving neuronal apoptosis. HPLC/LC-MS analysis showed that fermentation treatment significantly increased the content of five major types of compounds, including alkaloids, lipids, diterpenes, anthraquinones, and benzoyl derivatives, which might be responsible for the phenotypic differences. We then elucidated the pharmacological mechanism and potential therapeutic effect of SFL through the comprehensive application of transcriptomics and metabolomics. Our findings provide integrated omics information regarding the effect of fermented GE, a traditional Chinese medicine ferment, on an insomnia model. Our results can be expected to be impactful, as the daily inclusion of fermented GE in a diet could be a dietary strategy to maintain sleep health.

Methods

General

We purchased *Lactobacillus plantarum* from Yaxin Biotechnology in Taiwan. The *Saccharomyces cerevisiae* (active dry yeast) was purchased from Angel Yeast Co.Ltd. Acetic acid bacteria (Hu Niang No. 1.01 Acetic acid bacteria) was purchased from Shanghai Difa Brewine-Biotechnology Co., Ltd. Gas chromatograph (Trace1300; Thermo Fisher Scientific, Waltham, MA, USA). LC-MS analysis was conducted using the 5600+ qTOF ESI MS system, which was manufactured by AB Sciex Pte. Ltd, located in MA, USA. Adenosine, gastrodin, 4-hydroxybenzyl alcohol, parishin A, B, C, and E were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Pentylene-tetrazole (PTZ) was procured from Macklin Biochemical Technology Co. Ltd. (Shanghai, China). Oxazepam was bought from Yimin Pharmaceutical Factory (Beijing, China). AB wild adult zebrafish were procured from Shanghai FishBio Co. Ltd. (Shanghai, China). Energy Chemical Co. Ltd (Shanghai, China) supplied the organic solvents for the UPLC-QE-MS/MS.

Sample preparation of GE at different processing stages

After fresh GE was cleaned, it was put into the fermentation tank, decocted with an appropriate amount of water for 90 min, and filtered to obtain GE extract (WGE). Next, the lactic acid bacteria strain solution was added to the sugar-sweetened WGE at the inoculation rate of 3% (V/V), after anaerobic fermentation at 37 °C for 24 h, And then the yeast strain solution was added. The prime fermentation liquid (PFL) was obtained after anaerobic co-fermentation at 28 °C for 15 days and sterilization at 60 °C for 30 min. Finally, an acetic acid bacteria strain solution was added to the PFL. After about 50 days of aerobic fermentation at 30 °C, the fermentation was completed and the secondary fermentation liquid was prepared. The supernatant of fermentation liquid at different processing stages was collected, and all water was removed by freeze-drying to obtain GE samples at different processing stages.

Zebrafish locomotor behavior investigation

Zebrafish maintenance and embryo collection were carried out according to a reported protocol^{36,37}. The Institutional Animal Care & Use Committee of Northwest A&F University approved the animal experimental protocol (Protocol Permit Number: XN2023-0902, 15 March 2022). The investigation of zebrafish mobility was conducted in accordance with the previously established procedure outlined by our research group³⁷. The zebrafish larvae at 5 dpf were subjected to treatment with varying quantities (20 µg/ml and 80 µg/ml) of WGE, PFL, and SFL samples for 4 hours. The zebrafish groups were individually allocated into separate compartments of a 6-well plate, followed by the addition of 2.5 mM PTZ. Following 10 minutes of adaptation, the larvae were observed and their behavior was recorded for durations of 30 and 50 minutes, respectively. The video was subjected to analysis using ethovision software, which was developed by Noldus Information Technology bv. in the Netherlands.

Assessment of neuronal apoptosis

The method described in the literature was utilized to carry out our experiment³⁸. In brief, zebrafish larvae at 5 dpf were subjected to treatment with varying quantities (20 µg/ml and 80 µg/ml) of WGE, PFL, and SFL samples for 4 hours. The zebrafish were segregated into distinct groups and thereafter

introduced into individual compartments of a 6-well plate. Following this, a concentration of 2.5 mM PTZ was administered to each group for 10 minutes. Following that, the larvae were subjected to two rinses using phosphate-buffered saline (PBS) and subsequently exposed to a concentration of 5 mg/L acridine orange (AO) in a light-restricted setting for 30 minutes. After undergoing three rounds of washing with phosphate-buffered saline (PBS), the subjects were anesthetized with the administration of 0.016% tricaine in culture water. The Nikon SMZ25 fluorescent stereoscope, manufactured by Nikon Corporation in Tokyo, Japan, was used to observe apoptotic nerve cells.

Quantitative analysis of the main medicinal components of fermented GE

Briefly, the fermentation broth (5 ml) was centrifuged in a sterile centrifuge tube at 5,000 r/min for 10 min. The supernatant was collected and filtered through a 0.22 µm nylon filter, and 1 mL of the supernatant was analyzed by high-performance liquid chromatography (HPLC). Detection was performed using an Ultimate3000 HPLC-UV (Thermo Fisher Scientific, USA) with a Century SIL C18 column (250 × 4.6 mm, 5 µm). The mobile phase consisted of 0.1 % phosphoric acid in water for phase A and acetonitrile for phase B. The injection volume was set at 20 µL, the flow rate was 1 mL/min, the column temperature was set at 30 °C, and the detection wavelength was 220 nm for quantification of adenosine, 4-hydroxybenzyl alcohol, parishin B, parishin E, gastrodin, parishin A, and parishin C in WGE, PFL, and SFL. The elution gradient was as follows: 0-10.0 min, 3%-10 % B; 10.0-15.0 min, 10 %-12 % B; 15.0-25.0 min, 12 %-18 % B; 25.0-40.0 min, 18 % B; and 40.0-40.1 min, 18%-3 % B; 40.1-50.0 min, 3 % B.

Metabolite extraction and LC-MS analysis of GE at different processing stages

Three portions of WGE (WGE1, WGE2, WGE3) and PFL (PFL 1, PFL2, PFL3) and SFL (SFL 1, SFL2, SFL3) freeze-dried samples were precisely weighed and were respectively dissolved in MeOH solution to prepare the sample solution with the concentration of 100 µg/mL. UPLC-ESI-MS analysis and metabolite extraction procedures were conducted using the protocols outlined in our previously published works³⁷. Furthermore, the PLS-DA analysis was performed using the Metaboanalyst website (<https://www.metaboanalyst.ca/>, visited on July 2, 2023).

Zebrafish Larvae preparation for transcriptome and metabolism analysis

In brief, the blank group (G1) was given E3 water, the model group (G2) was to add 2.5 mM PTZ based on G1, and the test group (G4) was given SFL (80 µg/ml) for 4h, and then 2.5 mM PTZ was added for 10 minutes. On day five, zebrafish larvae were gathered. Before use, centrifuge tubes were prepared and weighed. Each tube held a single hundred fish, which were then given three rounds of clean water washing before being killed in liquid nitrogen. Lastly, the samples were kept for transcriptomic, metabolomic, and PCR analysis in a refrigerator at −80 °C.

RNA sequencing and data analysis of Zebrafish larvae

Three separate biological replicates of the 5 dpf Zebrafish larvae of the G1, G2, and G4 groups were used. For details of the experiment, please refer to the previous paper of our study group³⁶. The BGI (<http://report.bgi.com>) processed RNA-seq data and carried out gene set enrichment analysis (GSEA) utilizing the Dr. TOM technique, an internal, specially designed data mining system of the BGI. Enriched gene sets were allocated based on a nominal *p*-value of less than 0.05 and an FDR *q*-value of less than 0.25. The use of gene set enrichment analysis (GSEA) was used to uncover trends related to the overrepresentation of biological terms in the transcript.

LC-MS/MS profiling of metabolism

For sample processing methods and condition parameter settings, please refer to the previously published articles by our team^{36,39}. Next, we used the Oebiotech tools, a free online platform for data analysis (<https://cloud.oebiotech.cn/task>), to do pathway enrichment.

Evaluation of short-chain fatty acids

The separation was performed using a Thermo TG-WAXMS capillary column with dimensions of 30 m in length, 0.25 mm in internal diameter, and a particle size of 5 μ m. Gas chromatography equipment was employed for this purpose. The samples were subjected to centrifugation at a velocity of 15,000 revolutions per minute and a temperature of 4 °C for five minutes until a transparent supernatant became visible. Using a 0.45 μ m pore size membrane, the supernatant from various fermentation stages was collected in a 1 mL syringe and filtered. The organic phase was gathered and subjected to the following gas chromatography analysis. The temperature was started at 100 °C and increased gradually over the next three minutes to 150 °C at 5 °C/min, 220 °C at 70 °C/min, and finally, to 220 °C, where it remained for three minutes. The carrier gas, helium, was employed at a flow rate of 2.0 mL/min. The injection volume of the sample was 1 μ L, and it was divided at a ratio of 1:10 at a temperature of 230°C, with an inlet split flow rate of 20 ml/min. The flame ion detector (FID) used for the GC had a temperature of 250 °C. To create the standard curve, the concentration was utilized as the abscissa and the peak area as the ordinate. The content was then computed using the standard curve.

RT-qPCR Analysis

RT-qPCR assays were utilized to validate the extent of gene expression. The three groups (G1, G2, and G4) were conducted consistently, following the sample preparation procedure outlined in Section 2.7. The RNA extraction process utilized AG RNAex Pro RNA kit, manufactured by Accurate Biotechnology (Hunan) Co., Ltd. The retrotranscription reactions were performed using the EVO M-MLV RT Mix Kit (Accurate Biotechnology Co., Ltd., China). The 5X gDNA Clean Reaction Mix reagent contained in the kit was employed to eliminate DNA, whereas the 5X Evo M-MLV RT Reaction Mix was utilized to perform the reverse transcription of RNA into DNA. The sequences of the primers, which were created using the Sheng Gong online primer creation tool, are provided in Table S1. Lastly, the quantification of fluorescence was conducted utilizing the Premix Pro Taq HS qPCR kit, manufactured by Accurate Biotechnology Co.,

Ltd., located in China. The internal reference gene utilized was the β -actin gene from zebrafish. The $2^{-\Delta\Delta CT}$ method was utilized to evaluate the fold change of the genes that were being tested.

Statistical analysis

Statistical analysis was performed using the program GraphPad Prism (version 8.0) developed by GraphPad program Inc. (La Jolla, CA, United States) and the R project (version 4.2.1). The data were presented in the form of mean values together with their corresponding standard deviations. A one-way analysis of variance (ANOVA) was employed to perform the analysis of variations among groups.

Declarations

Credit authorship contribution statement

Chao-Qi Zhang: Formal analysis, Investigation, Data curation, Visualization, Methodology, Writing – original draft. **Xu-Dong Zhang:** Formal analysis, Validation, Data curation. **Yan Wang:** Formal analysis, Validation. **Yi-Han Liu:** Formal analysis. **Cun-Li Zhang:** Writing – review & editing, Funding acquisition. **Qiang Zhang:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization, Methodology, Data curation.

Declaration of Competing Interest

The authors declare that no known financial conflicts or interpersonal links could have influenced the findings and conclusions given in this research.

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Figures

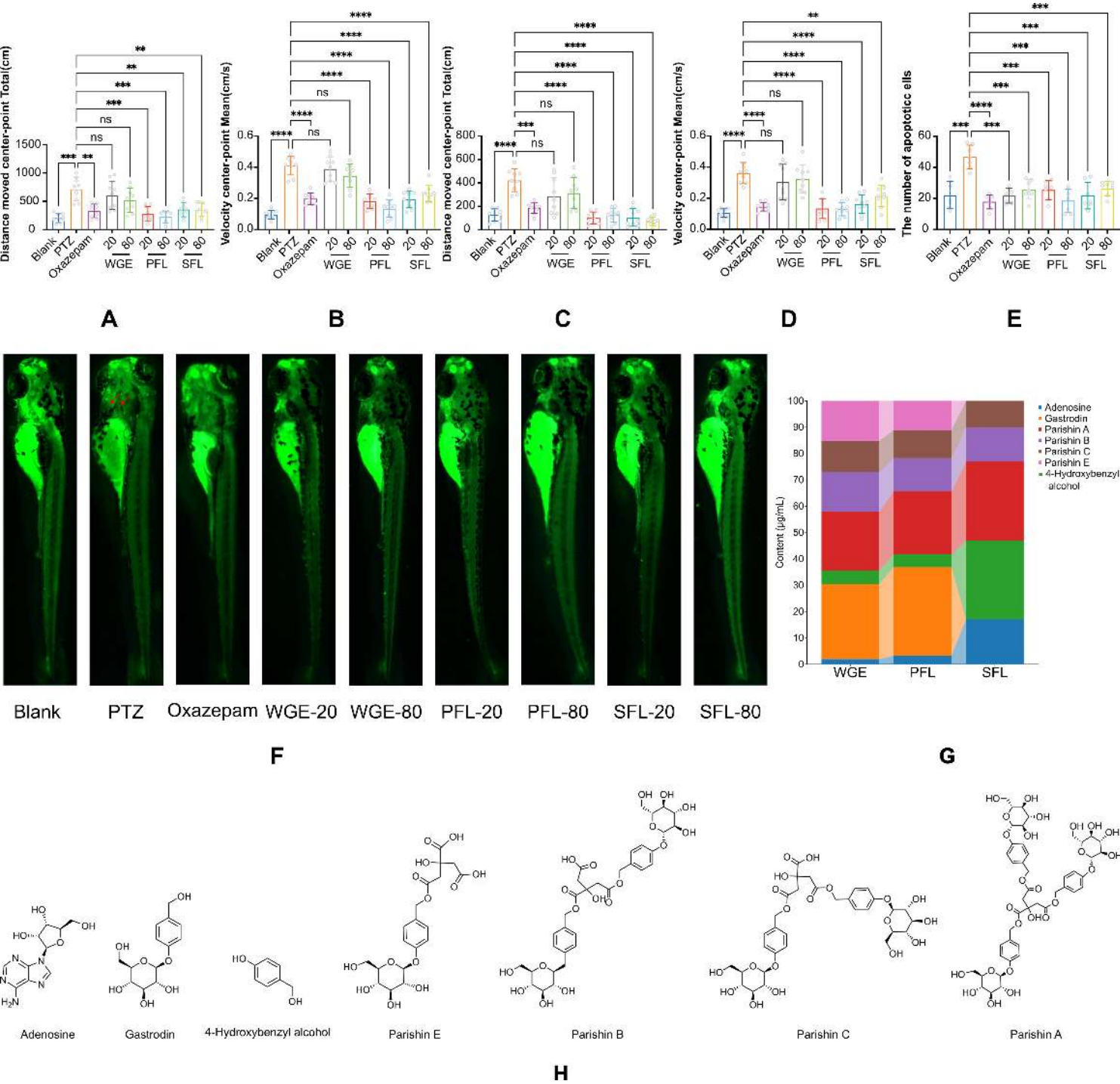


Figure 1

(A-E) Effects of GE samples treated at different stages on PTZ-induced insomnia-like zebrafish behavior. (F) Zebrafish brain cell apoptosis. (G) Dynamic changes of the main chemical constituents of GE samples during fermentation. (H) Chemical structure of the main components.

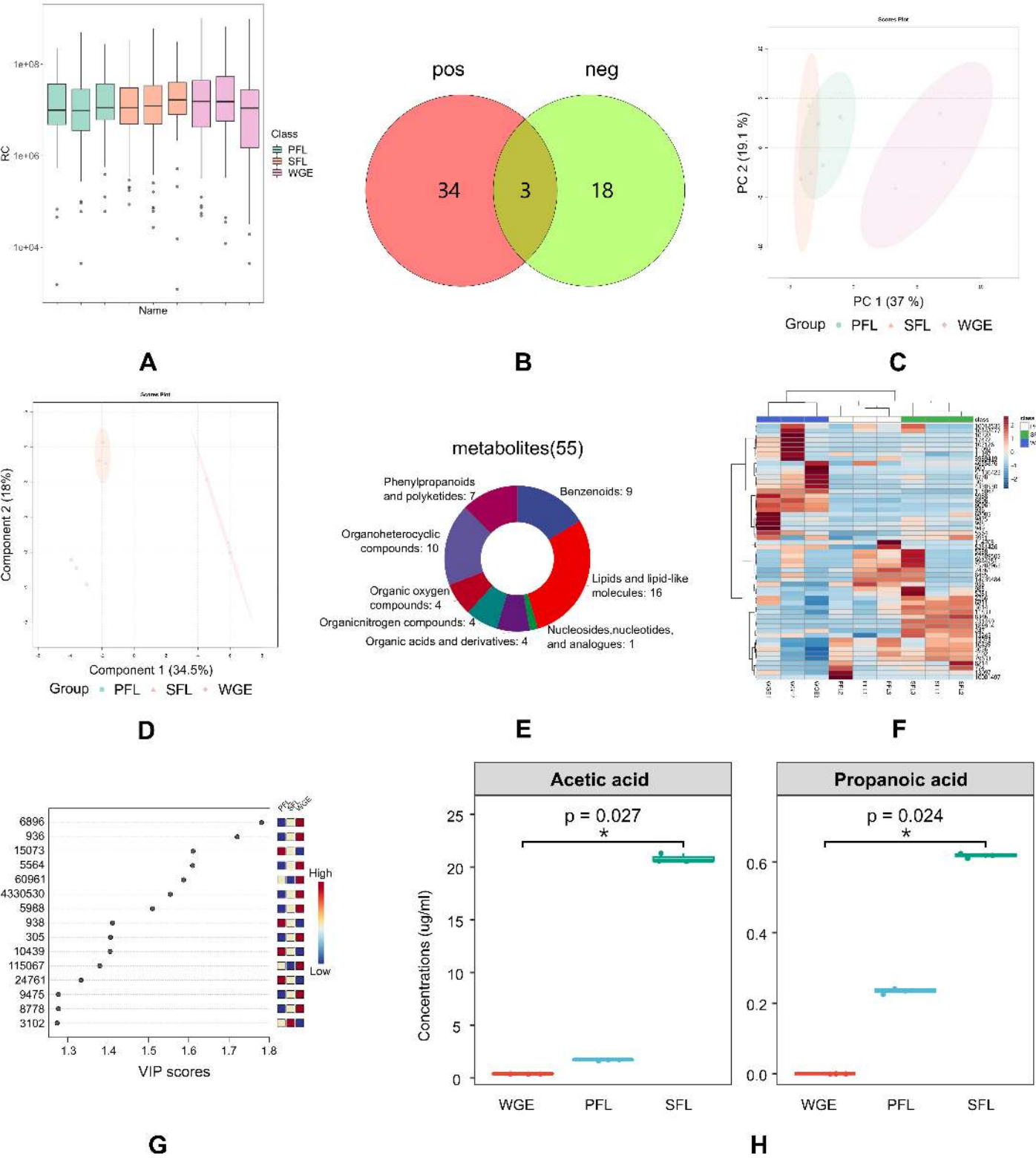


Figure 2

LC-MS/MS analysis of GE samples at different treatment stages. **(A)** The box plot of Component in WGE, PFL, and SFL groups. **(B)** The quantity of chemicals found in both the negative (neg) and positive (pos)

ion modes of the ESI. (C, D) PCA and sPLS-DA examination of samples at different treatment stages. (E) Types and proportions of the compounds identified from WGE, PFL, and SFL. (F) Heatmap visualization. (G) VIP value obtained from the OPLS-DA model of the potential markers in WGE, PFL, and SFL. (H) The changes of SCFAs in samples at different treatment stages.

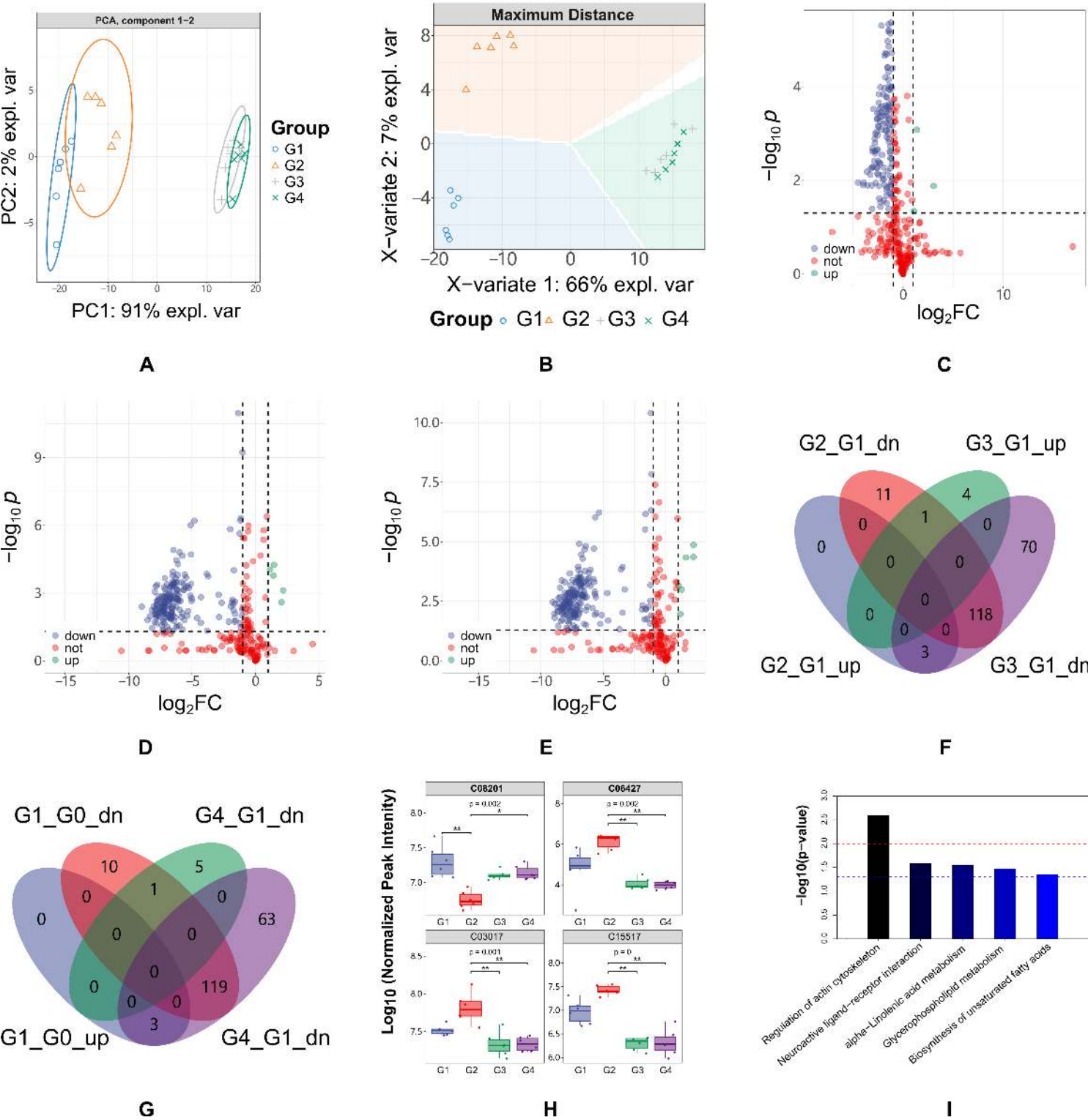


Figure 3

Metabolome analysis of zebrafish. **(A)** PCA and **(B)** sPLS-DA analysis of LC-MS profiles, n = 6 per group. **(C-E)** Differences in metabolites between G1 and G2, G2 and G3, and G2 and G4 are represented by volcano graphs. Every point in the volcano plots is a metabolite. **(F-G)** The number of significantly changed differential metabolites among the three groups of G1, G2, G3 and G4. **(H)** The relative amounts of the important differential metabolites. **(I)** Pathway enrichment.

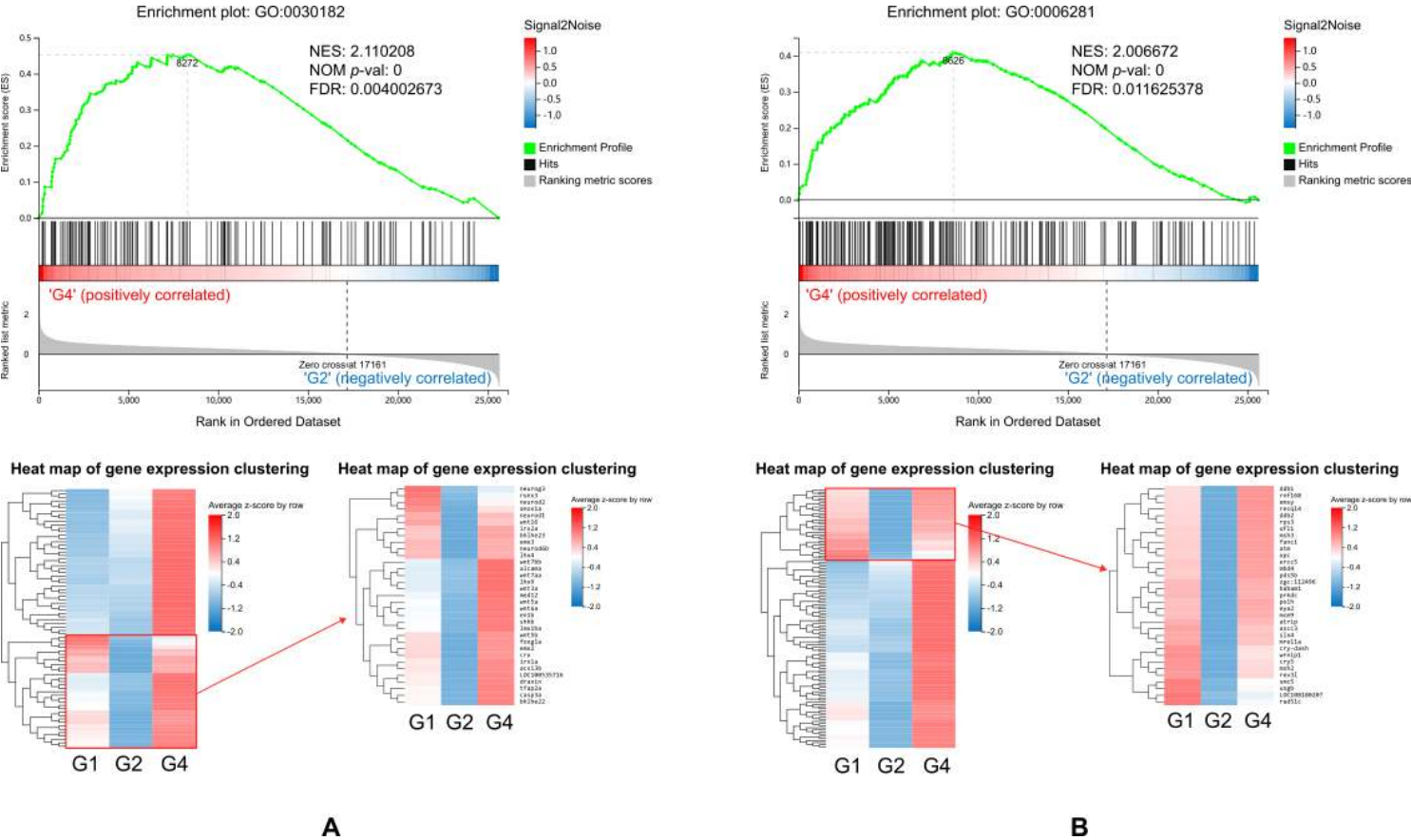


Figure 4

GSEA was performed in the G4 and G2 groups and G2 and G1 groups. In a ranked list of all the genes in the RNA-seq dataset, the GSEA algorithm generates an enrichment score that indicates the extent of overrepresentation at the top or bottom of the list of genes included in a gene set. Positive enrichment scores (ES) indicate gene set enrichment at the top of the ranked list, whereas negative ES indicate gene set enrichment at the bottom of the ranked list. Heatmaps beneath each enrichment plot show the expression heatmap signatures of genes involved in the GSEA analysis, referring to enrichment plots **(A)** to **(B)**. Z scores were used. Left: the clustering of all genes within the pathway. Most sensitive pathways are highlighted at the right (indicated by a red square in panel 4). The red color is used to represent high expression, whereas the blue color is used to represent low expression. The analysis demonstrates that known **(A)** neuron differentiation, and **(B)** DNA repair, are enriched in G4 groups.

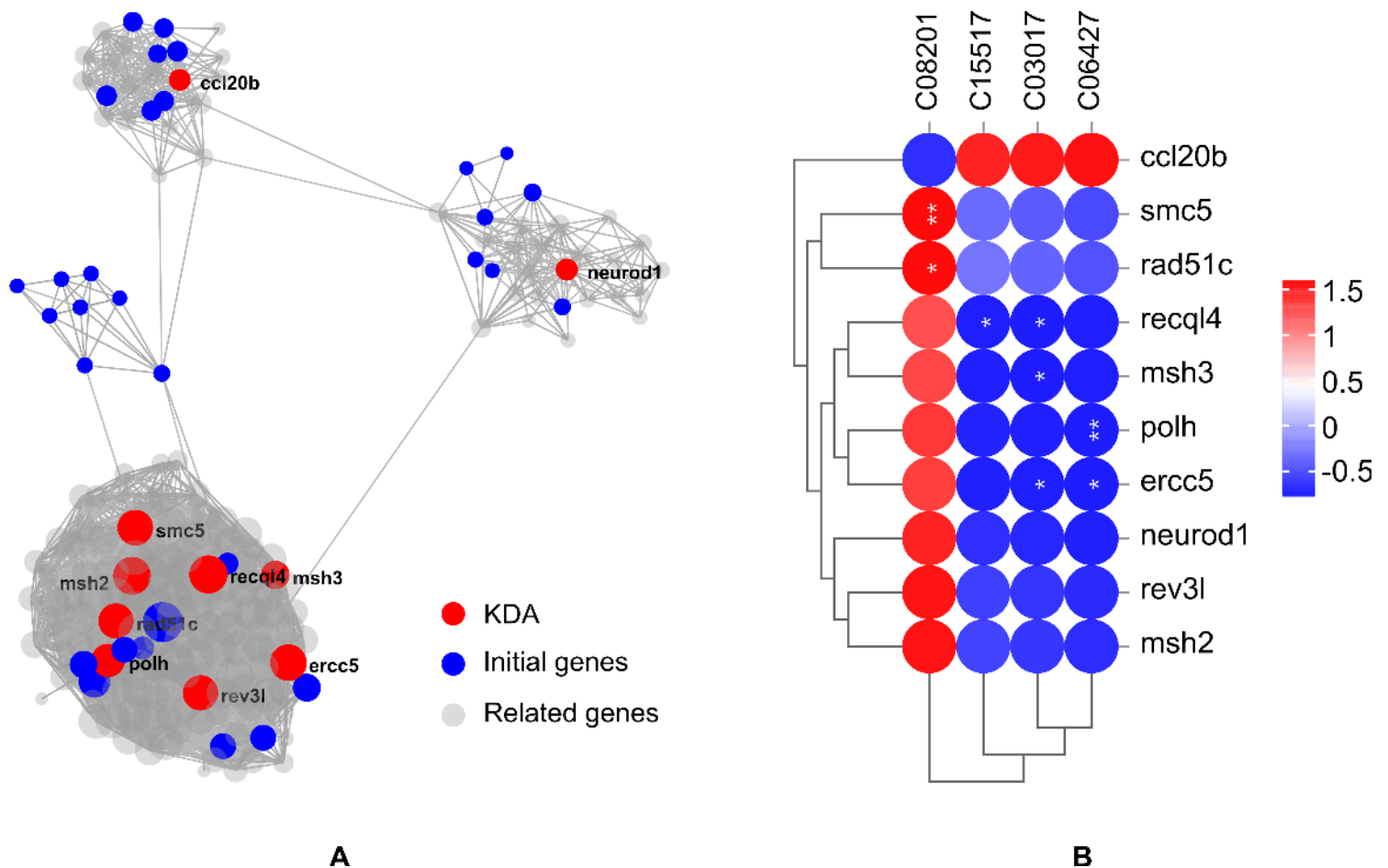


Figure 5

(A) Key driver analysis of all genes differentially expressed in pathways. The genes labeled red are the key driver genes. Parameter interpretation: key gene (KDA) refers to the gene with a key role in the gene set. The key gene may come from the selected gene or from a gene with a PPI relationship with the selected gene under the corresponding PPI score parameter. Initial gene: The non-critical gene (KDA) of the initially selected gene. Related genes: Genes with a PPI relationship with the selected gene (excluding key genes) under the corresponding PPI score parameter. **(B)** Correlation coefficient clustering heat map. The abscissa represents metabolites, the ordinate represents genes, * represents a p value of < 0.05 in the correlation coefficient significance test, and ** represents a p value < 0.01 . The color labels represent Pearson correlation coefficient values.

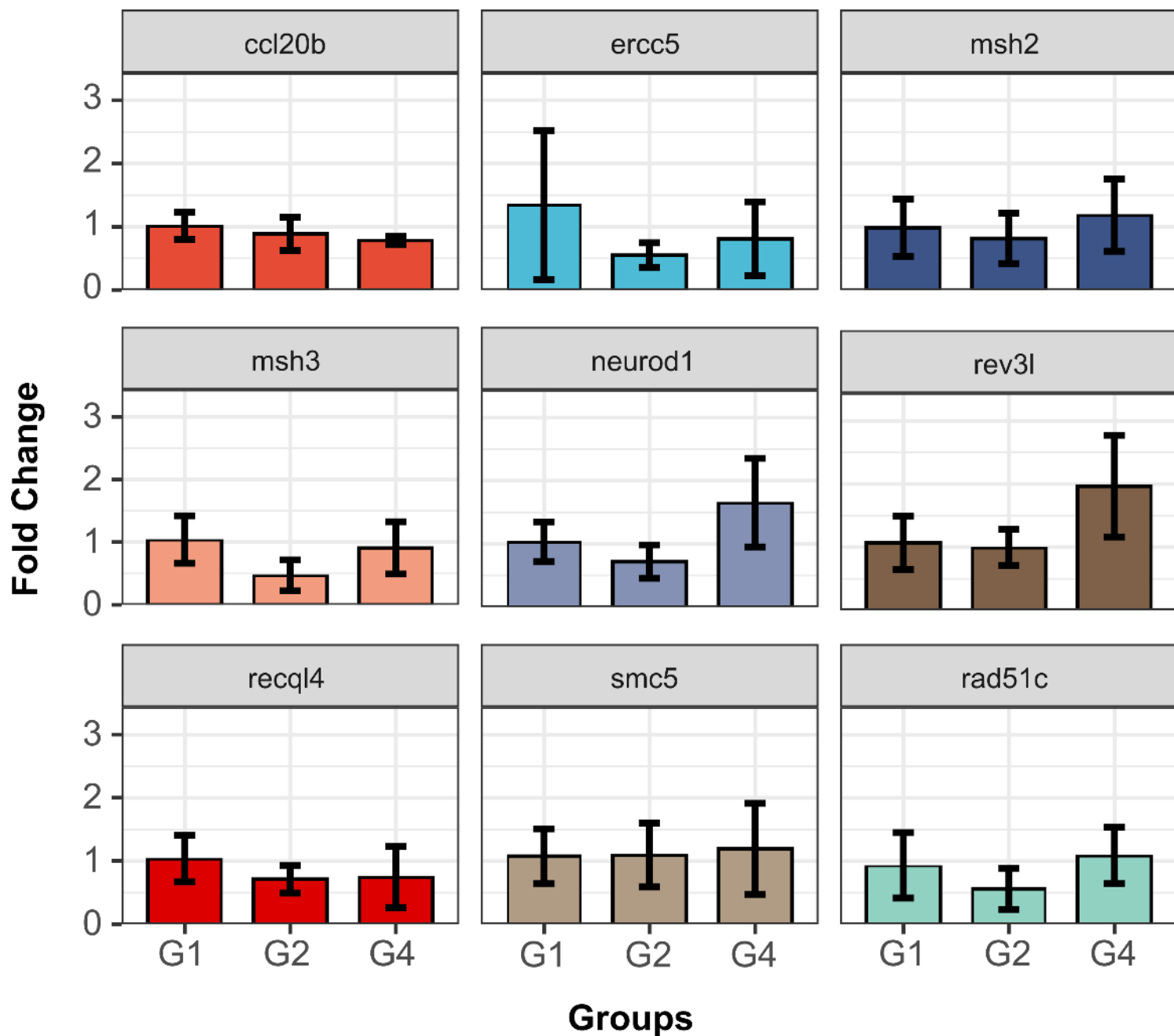


Figure 6

RT-qPCR validation of key genes in zebrafish.

Supplementary Files

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