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Characteristics and correlation of flavor substances and hangover indexes in Chinese baijiu during storage

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ABSTRACT

It is generally believed that there is a great relationship between hangover and the age of Baijiu. However, what factors make Baijiu (stored for a long time) feel better after drinking has not been well explained. In this study, ethanol metabolism, oxidation stress, inflammation and release of inhibitory neurotransmitter were selected as the indicator of hangover. The results showed that the longer the age of Baijiu, the higher the antioxidant and anti-inflammatory levels, the less damage to the liver of mice. In addition, we also found that the longer the age of Baijiu, the faster the ethanol metabolism rate, the smaller the impact on the brain. A correlation analysis on Baijiu ingredients and hangover related indicators was conducted. These results showed that ethyl acetate, n-butanol, n-hexanol, butyl acetate, ethyl octanoate, isovaleric acid, 2-hydroxypropionic acid had a great correlation with all hangover related indicators.

1. Introduction

The Alcohol Hangover Research Group defined the alcohol hangover as "the combination of mental and physical symptoms experienced the day after a single episode of heavy drinking, starting when blood alcohol concentration (BAC) approaches zero" (Verster et al., 2020). The seven most common symptoms of alcohol induced hangover are headache, nausea, thirst, tiredness, dizziness, stomach ache and reduced concentration (Penning et al., 2012; van Schrojenstein Lantman et al., 2017). The symptoms of hangover can cause serious adverse effects on people's daily life (Carpenter and Merrill, 2021; Verster et al., 2014). Abstinence from alcohol or moderation of drinking habits is the only intervention that can prevent alcohol hangover symptoms. However, alcoholic beverages are an important kind of consumer products in daily life, and hangover is also inevitabel. In order to improve the hangover state, the research on the correlation between Baijiu flavor substances and post drinking comfort has been paid more attention in recent years.

Baijiu is a unique distilled liquor in China, which carries a long history and profound cultural heritage. The flavor of Baijiu is mainly formed by volatile (eaters, alcohols, acids, aldehydes, nitrogen-containing, sulfur-containing compounds, and terpenes) and nonvolatile components produced during microbial fermentation. Freshly made Baijiu usually has unpleasant odors and irritants, such as peppery, pungent, grassy, and alcoholic, which are due to the amount of acetaldehyde, acrolein, crotonaldehyde, methanol, free ammonia, alkenes, sulfides (hydrogen sulfide, mercaptan, dimethyl sulfide, etc.), and other compounds (Fan and Qian, 2006). The physicochemical properties changed continuously during aging to provide a high esterifying activity, and aging removed unpleasant flavor compounds and helped to stabilize the flavor compounds in mature Baijiu (Fan et al., 2020). In the past few years, the research on age of Baijiu mainly focused on the variation of cellar storage time and components of Baijiu (He et al., 2022; Wang et al., 2020). Chinese people have the habit of collecting old wine. It was usually thought that the longer it is stored, the better Baijiu taste. However, the relationship between Baijiu composition and hangover degree has not been systematically studied.

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The mechanism leading to hangover is very complex. Existing studies have focused on the biomarkers of alcohol metabolism, oxidative stress and alcohol inflammatory reaction, as potential important determinants of the severity of hangover (Hong, 2016; Mackus et al., 2020; van de Loo et al., 2020). After drinking a lot of alcohol, the overexpression of alcohol dehydrogenase will lead to the accumulation of acetaldehyde, which will produce reactive oxygen species (ROS) and lead to oxidative stress (Lieber, 2004). Alcohol-induced oxidative stress has been proved to be related to antioxidant enzyme damage, including superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GSH) (Tsermpini et al., 2022). At the same time, lipid peroxidation caused by oxidative stress also produces malondialdehyde (MDA) (Erba et al., 2003). Oxidative stress and related cell damage promote inflammation (Reuter et al., 2010), and the increase of pro-inflammatory cytokine tumor necrosis factor in Kupffer cells aggravates inflammation (Lieber, 2004). In addition, there was a significant correlation between the rate of alcohol metabolism and the severity of hangover (Mackus et al., 2020). Alcohol metabolism mainly occurs in the liver. The key enzymes of alcohol metabolism are alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH), and ALDH is the key rate-limiting enzyme of alcohol metabolism (Edenberg, 2007). At present, more and more people pay attention to some changes in the brain after a hangover, especially changes in the content of some neurotransmitters (Daviet et al., 2022; Zhang et al., 2022). After acute intoxication, ethanol can enter the brain tissue due to its molecular structure. Ethanol does not bind to specific targets or receptors in the brain, but its metabolites have a longer psychoactive effect in the human body than ethanol (Rae et al., 2014). Low-dose ethanol entering the brain tissue will be metabolized by cerebellar astrocytes ALDH2 and produce metabolites such as acetic acid, which will increase the level of inhibitory neurotransmitter γ-GABA, enhance the inhibition of rigidity, and damage the ability of balance and coordination (Jin et al., 2021). 5-HT is an important neurotransmitter to maintain normal wakefulness and sleep rhythm, and long-term repeated drinking will lead to a decrease in 5-HT level, and it is more likely to form alcohol-induced dependence (Langen et al., 2002). Alcohol will trigger the activation of microglia, which will reduce the synapses between neurons in the prefrontal cortex of the brain, inhibit nerve transduction and cause anxiety (Socodato et al., 2020). At the same time, microglia are the natural immune cells of the central nervous system and the main medium of neuroinflammation. When activated, they can secrete cytokines that promote inflammation or inflammation and affect the process of disease (Obst et al., 2017).

In recent years, with the development of Baijiu industry, more and more people pay attention to the changes of Baijiu composition during storage, and more and more people pay attention to hangovers. However, few people study the relationship between the changes in the composition of vintage liquor and hangovers. Therefore, this study aims to explore whether Baijiu stored for many years can reduce the degree of hangover, which components in Baijiu are related to the degree of hangover, and provide a reference for the design of Baijiu components and the improvement of Baijiu hangover.

2. Materials and methods

2.1. Materials

Five Chinese Baijiu samples were collected from Jiangsu Yanghe Distillery Co. (Jiangsu, China), unopened and well preserved. The samples with different biological ageing times were named M315 (7 years), M317 (5 years), M319 (3 years), M321 (1years) and M322 (0 years). Dimethyl sulfoxide (DMSO), ethanol, methyl thiazolyl tetrazolium (MTT), reactive oxygen (ROS), and BCA protein were purchased from Beyotime (Beijing, China).

2.2. Volatile compounds analysis

In order to ensure the representativeness of the experimental design and clarify the differences in the main volatile compounds among the five types of baijiu, we analyzed the volatile compounds of liquors via gas chromatography. Agilent 6890 (Agilent, USA) equipped with FID detector. Separation was performed using a CP-Wax 57CB column (50 m × 0.25 mm, 0.25 μm). The injector was set at split mode with a 50:1 split ratio at 270 °C. The oven was preheated to 35 °C for 6 min, ramped up to 60 °C at 6 °C/min for 3 min, ramped up to 80 °C at 4.5 °C/min for 2 min, and finally to 210 °C at 9.5 °C/min. At 1 mL/min, 99.999% pure nitrogen was used as the carrier gas.

2.3. Cell culture

The HepG2 human hepatocellular carcinoma cell line obtained from the Type Culture Collection of the Chinese Academy of Sciences was grown in DMEM containing 10% fetal bovine serum and 100 μg/mL streptomycin and 100 U penicillin in an incubator with 5% CO_2 at 37 °C.

The AML12 (alpha mouse liver 12 cell) line obtained from the Type Culture Collection of the Chinese Academy of Sciences was grown in DMEM/F12 containing 10% fetal bovine serum and 10 μg/mL Insulin and 5.5 μg/mL Transferrin and 5 ng/mL Selenium and 40 ng/mL Dexamethasone and 100 μg/mL streptomycin and 100 U penicillin in an incubator with 5% CO_2 at 37 °C.

The C6 rat glioblastoma obtained from the Type Culture Collection of the Chinese Academy of Sciences was grown in Ham's F-12 K containing 15% house serum and 2.5% fetal bovine serum and 100 μg/mL streptomycin and 100 U penicillin in an incubator with 5% CO_2 at 37 °C.

2.4. Measurement of cellular ALDH activity

HepG2/AML12/C6 cells were seeded in cell culture dishes (6×10^6 cells/100 mm dish). After 48 h, the cells were treated with different types of Baijiu, a group of cells was treated with 2% (v/v) ethanol for 3 h, the ethanol concentration in Baijiu group and ethanol control group was the same. To determine the ALDH enzyme activity, cells were processed according to the manufacturer's protocol.

2.5. Measurement of antioxidant levels

Determination of intracellular antioxidant value using HepG2 cells. The production of ROS was detected by using the fluorescent dye DCFH-DA. HepG2 cells were seeded at 5×10^4 cells/well in a 6-well plate for 40 h, and then, 5% (v/v) ethanol and baijiu (ethanol content diluted to 5%) were added for 4 h. DCFH-DA (10 μm) was added to the cells and incubated for 20 min in the dark. The fluorescence was measured by spectrophotometry at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. To determine the content of CAT, cells were processed according to the manufacturer's protocol.

2.6. Measurement of NO levels

Peritoneal macrophages were seeded at 5×10^4 cells/well in 96-well plates. After 24 h, the cells were treated with 0.25% ethanol (v/v) or baijiu (ethanol content diluted to 0.25%). Each experimental group included 3 double wells. After treatment, 100 μL of supernatant and the same volume of mixed Griess reagent were added to the enzyme-labelled plate. After 10 min, the absorbance was measured at 540 nm.

2.7. Animal experiments

Specific pathogen-free BALB/c mice (male, age 6 weeks, weight 18–22 g) were purchased from Charles River (Nanjing, China). All mice received human care in compliance with institutional guidelines after the experimentation. All mice were maintained in a controlled

environment (20–22 °C, 12 h light/dark cycle) and free access to ultrapure water and fodder. After one week of adaptive feeding, the mice were randomly allocated to seven groups of eight mice: the control group (Ctrl, PBS), ethanol group (EtOH) and baijiu group (M315, M317, M319, M321, M322). To ensure a consistent gavage volume, ethanol was diluted to the same ethanol concentration with baijiu. The gastric perfusion volume of each group is 13 μL/g.

2.8. Biochemical analyses

The activity of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using the ALT and AST Kit (Nanjing Jiancheng, Nanjing, China). The activity of liver and brain alcohol dehydrogenase (ADH) or acetaldehyde dehydrogenase (ALDH) were measured using the ADH Kit (Nanjing Jiancheng, Nanjing, China) or ALDH Kit (Solarbio, Beijing, China). The activity of liver catalase (CAT), reduced glutathione (GSH) and superoxide dismutase (SOD) were measured using the CAT, GSH and SOD Kit (Nanjing Jiancheng, Nanjing, China). The content of liver malondialdehyde (MDA) were measured using MDA Kit (Nanjing Jiancheng, Nanjing, China).

2.9. Histopathological observation

The tissues of liver samples were fixed in 4% paraformaldehyde avoid light. Then the tissue was embedded in paraffin and stained with hematoxylin and eosin (H&E) to assess the histological features of steatosis and inflammation. The tissue of the brain sample is fixed in the light-proof 4% polyformaldehyde, and then the tissue is embedded in the paraffin. After that, antigen repair, serum sealing, microglia labeling with Anti-Iba1 Rabbit pAb, adding the corresponding secondary antibody, DAPI re-staining the nucleus, and finally spontaneous fluorescence quenching, sealing, and taking pictures. The ultraviolet excitation wavelength of DAPI is 330–380 nm, the emission wavelength is 420 nm, and it emits blue light; CY3 excitation wavelength 510–560 nm, emission wavelength 590 nm, emitting red light.

2.10. Statistical analysis

All date were expressed as the mean ± SEM of three independent experiments. The results were statistically analyzed using Student's t-test, and a value of $P<0.05$ was considered to be statistically significant. SPSS 26.0 (IBM, Armonk, NY, USA) was used for calculating Pearson correlation coefficient. The Unscrambler X 10.4 (64-bit) was used for partial least squares analysis.

3. Results

3.1. The influence of baijiu with different storage time on hangover indicators in cell level

Based on the method established by our research group, the evaluation of cell activity can better reflect the degree of Baijiu hangover. We will first conduct the degree of Baijiu hangover in vitro experiment. To evaluate the effect of acute intoxication on acetaldehyde metabolizing enzymes in vitro, we measured the activity of ALDH in HepG2 and AML12 cells. As shown in Fig. 1A and Fig. B, the sample with the longest ages in the Baijiu group (M315) had higher ALDH enzyme activity than the ethanol group, and the ALDH enzyme activity of the Baijiu group showed a certain rule, that was, the ALDH enzyme activity increased with the increase of ages. In order to determine the effect of acute intoxication on ALDH enzyme activity in neurons, we measured the ALDH enzyme activity in C6 exposed to ethanol (Fig. 1C). Compared with the control group, the ALDH enzyme activity of the ethanol group increased, and the ALDH enzyme activity of the Baijiu group was lower than that of the ethanol group, and showed a certain rule, that is, the ALDH enzyme activity decreased with the increase of ages. In order to study the oxidation induced by Baijiu stored in different years in vitro, we measured the ROS level and CAT activity in HepG2 (Fig. 1D). Acute ethanol treatment significantly promoted the production of ROS in HepG2 cells, and the ROS level in the Baijiu group was significantly lower than that in the ethanol group. In addition, we also measured the

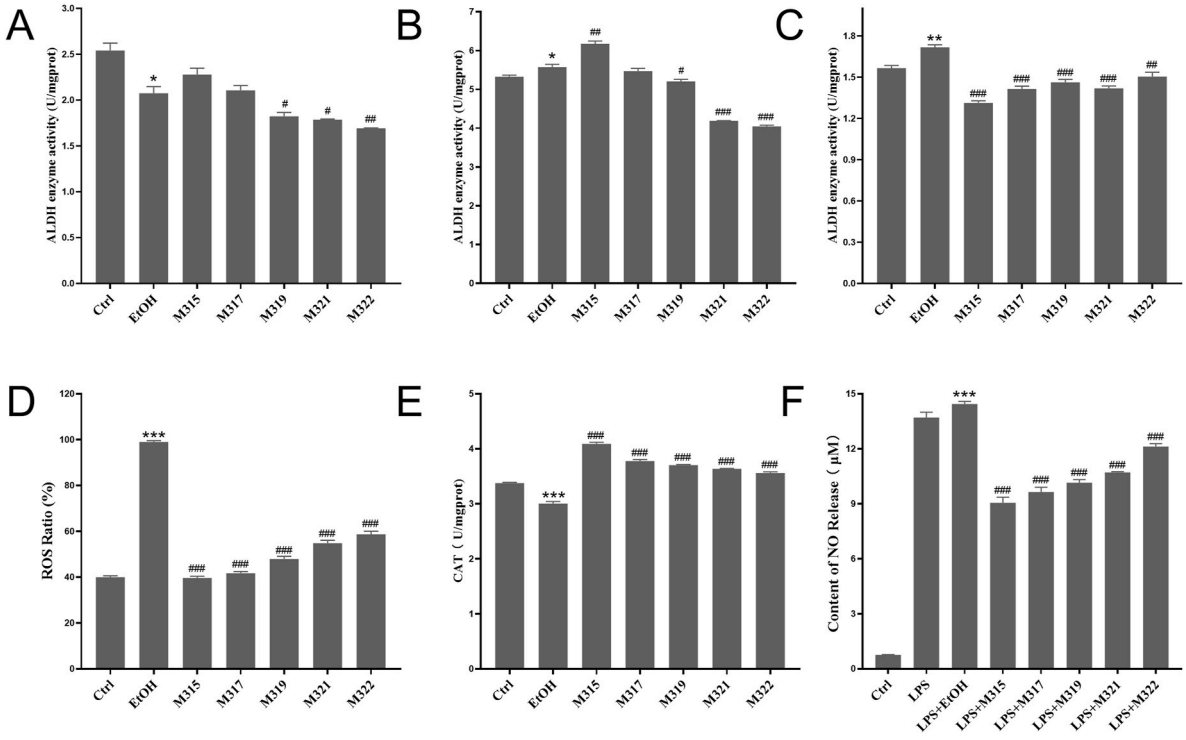

Fig. 1. Hangover related indicators on cell level. (A) ALDH enzyme activity of HepG2, (B) ALDH enzyme activity of AML12, (C)ALDH enzyme activity of C6, (D) The levels of ROS in HepG2, (E) CAT enzyme activity in HepG2, (F) The release of NO in Peritoneal macrophages. The data represent the mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 *vs* the control group; #P < 0.05, ##P < 0.01, ###P < 0.001 *vs* the Ethanol group.

activity of catalase (CAT), which can remove excess ROS in cells (Fig. 1E). Ethanol treatment reduced the CAT activity in HepG2 cells, but the CAT activity in the Baijiu group was higher than that in the control group. Because inflammation is closely related to oxidative stress, we detected the inflammatory reaction of Baijiu and ethanol in cells (Fig. 1F). In the primary mouse peritoneal macrophages, the release of NO in the Baijiu group was far lower than that in the ethanol group. Through the evaluation of cell activity, we found that the longer the Baijiu was stored, the lower the degree of hangover.

3.2. Mice behavior in acute alcohol intoxication model

Alcohol can inhibit the brain reaction, which will affect the related cognitive function and affect the nervous system, thus showing the influence of alcohol on the nervous system in behavior. In order to study the effect of Baijiu ages on the behavior of mice, the stick rotation test and righting reflex test were conducted after intragastric administration to determine the drop time, drunken time and sober up time of mice. As shown in Fig. 2A, the drop time of the mice in the ethanol group was significantly lower than that in the longest ages Baijiu group (M315) in the stick rotation test, indicating that ethanol affected the nervous system of the mice more quickly, and the behavioral ability of the mice was more affected. The results of righting reflex test are expressed by the degree of intoxication, as shown in Fig. 2B, it can be seen that the degree of intoxication of mice in the Baijiu group is significantly lower than that in the ethanol group, and the longer ages of the Baijiu group, the lower the degree of intoxication. Through the animal behavior experiment of mice, our results showed that the longer the age of Baijiu was, the less influence it had on the movement, balance and coordination ability of mice.

3.3. Analysis of liver pathological damage in acute alcohol intoxication model

ALT and AST play an important role in the synthesis and catabolism of amino acids, which are the most sensitive indicators of liver cell damage. Through the analysis of serum AST level (Fig. 3A), it was found that the AST level in the ethanol group was significantly higher than that in the control group, the AST level in the Baijiu group decreased with the increase of Baijiu ages, and the AST level of M315 was significantly lower than that in the ethanol group. Similarly, ALT levels were significantly elevated in the ethanol group compared to the control, reflecting liver damage. Moreover, there was an observed trend of declining ALT levels with increasing age of Baijiu.

H&E staining is one of the commonly used staining methods for histopathological observation. The liver slices of mice were taken for H&E staining observation (Fig. 3C). The results of hepatic cord in the control group were clear, the structure of liver cells was complete, and no obvious degeneration of liver cells was found. In ethanol group, the arrangement of hepatocytes was irregular, the hepatocytes were loose, swollen, watery degeneration, and the chromatin of cells was deepened, the number of binucleate hepatocytes increased. The damage degree of hepatocytes in the Baijiu group was different. M315 was the least damaged, the structure of hepatic cord was clear, there was no obvious necrosis of hepatocytes or inflammatory cell infiltration, and M322 was the most severely damaged, the hepatocytes were in disorder, the cells were swollen, the number of binuclear cells increased, and inflammatory cell infiltration appeared in some areas. Through the detection of biochemical indicators and tissue observation of the mouse liver, we found that the longer the storage years of Baijiu, the less damage to the liver.

3.4. Oxidative stress and inflammatory levels in the liver tissue of acute alcohol intoxication mice

Ethanol can cause oxidative stress in the liver of mice. As shown in Fig. 4A–C, the level of CAT, GSH, and SOD in the liver of mice in the ethanol group is lower than that in the control group. There are certain differences between the levels of antioxidant enzymes of ethanol and five different ages of Baijiu. MDA is the end product of lipid peroxidation of cell membrane, which can cause DNA and protein damage, and is a common marker of intracellular oxidative stress. As shown in Fig. 4D, the content of MDA in the ethanol group is significantly higher than that in the control group, and the content of MDA in Baijiu group is lower with the longer ages. These results indicate that different alcohol components consumed by mice will affect the antioxidant level of the liver. Inflammation is closely related to oxidative stress. To verify whether ethanol can induce inflammatory response in mice, serum levels of IL-1β and TNF-α were measured. The contents of IL-1β and TNF-α in the ethanol group were significantly increased, while the contents in baijiu group were lower than that in the ethanol group, indicating that the inflammatory reaction caused by liquor was lower than that caused by the same concentration of ethanol. Through the detection of liver oxidase system and inflammatory reaction, we confirmed that Baijiu can reduce the oxidative stress and inflammation caused by ethanol to a certain extent.

3.5. Effect of baijiu with different storage time on key enzymes involved in ethanol metabolism in vivo

ADH and ALDH are the main rate-limiting enzymes of ethanol metabolism pathway. It can be seen from Fig. 5A–B that the activities of ADH and ALDH enzymes in the liver ethanol group are higher than those in the normal group, indicating that ethanol can cause the increase of

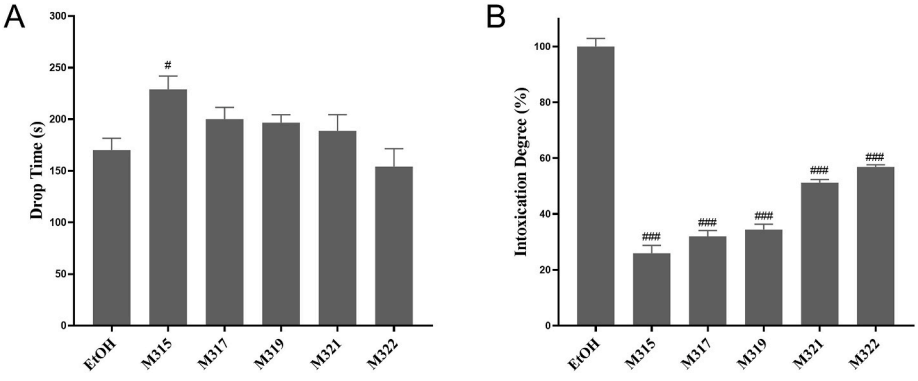

Fig. 2. Mouse Behavior Experiment. (A) Rotating rod experiment, (B) Righting reflex experiment Intoxication Degree = 50 × (Drunken time of ethanol group/Drunken time of Baijiu group + Waken time of Baijiu group/Waken time of ethanol group). The data represent the mean ± SEM of three independent experiments. #P < 0.05, ###P < 0.001 *vs* the Ethanol group.

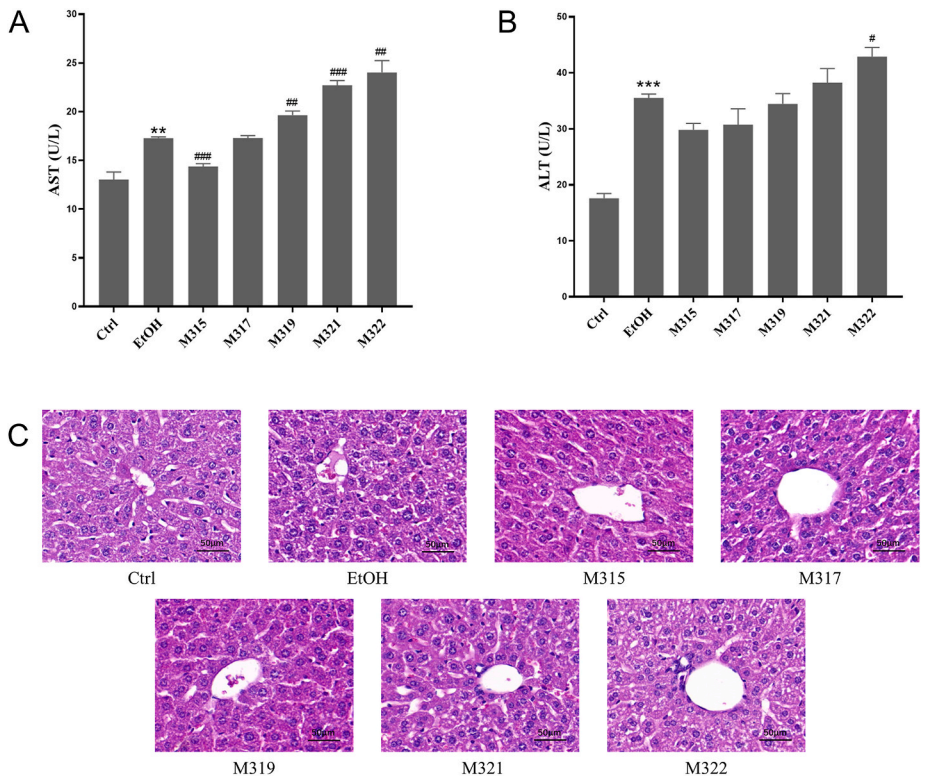

Fig. 3. Characterization of liver injury in mice. (A) The level of AST in serum, (B) The level of ALT in serum, (C) H&E section of mouse liver. The data represent the mean ± SEM of three independent experiments. **P < 0.01, ***P < 0.001 *vs* the control group; #P < 0.05, ##P < 0.01, ###P < 0.001 *vs* the Ethanol group.

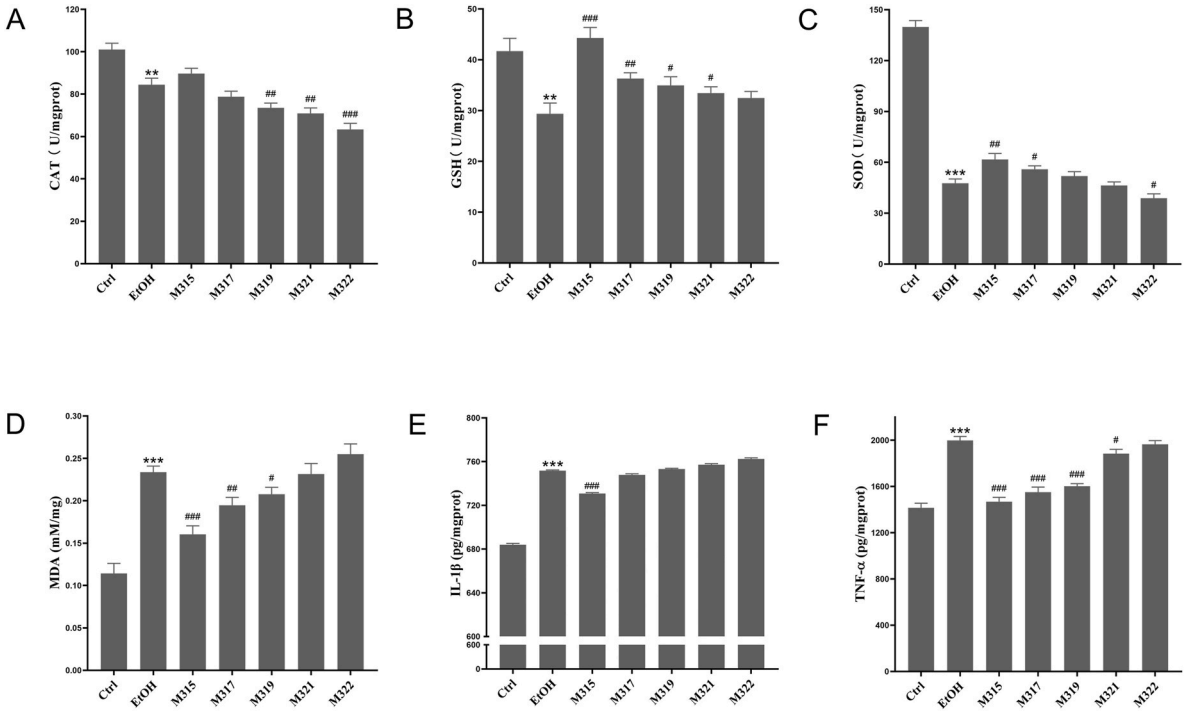

Fig. 4. Oxidative damage and inflammation of mouse liver. The levels of CAT (A), GSH (B), SOD (C), MDA (D), IL-1β (E), TNF-α (F) in mouse livers. The data represent the mean ± SEM of three independent experiments. **P < 0.01, ***P < 0.001 *vs* the control group; #P < 0.05, ##P < 0.01, ###P < 0.001 *vs* the Ethanol group.

alcohol metabolism enzymes to a certain extent. The enzyme activity of metabolic enzymes in the Baijiu group was higher than that in the ethanol group, and showed a certain rule, indicating that the metabolic rate of Baijiu in the body was higher than that of pure ethanol, and the longer the Baijiu year, the higher the metabolic rate. Through the detection of liver alcohol metabolism enzymes, we confirmed that the

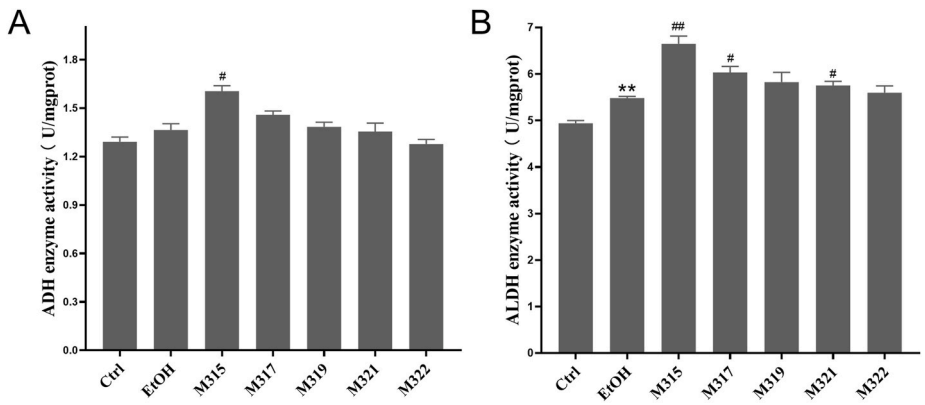

Fig. 5. Ethanol metabolic enzymes in mouse liver. (A) The enzyme activity of ADH in mouse livers, (B) The enzyme activity of ALDH in mouse livers. The data represent the mean ± SEM of three independent experiments. **P < 0.01 *vs* the control group; #P < 0.05, ##P < 0.01 *vs* the Ethanol group.

Baijiu with a long age can be metabolized in the liver faster.

3.6. Effect of baijiu with different storage time on brain tissue

In case of acute intoxication, a small amount of alcohol will enter the brain tissue and eventually be metabolized into acetic acid by ALDH in the cerebellum. Acute intoxication also affects the release of inhibitory neurotransmitters γ-GABA and 5-HT In Fig. 6A–C, we can see that compared with the control group, the activity of ALDH enzyme in the ethanol group increased, the content of γ-GABA increased and the content of 5-HT decreased. For the Baijiu group, ALDH enzyme activity and the content of γ-GABA decreased and the content of 5-HT increased. In M315 group, the activity of ALDH and the content of γ-GABA was the lowest, and the content of 5-HT was the highest, while that of M322 group was the opposite. Ethanol metabolism in the brain will also cause inflammatory reaction in the brain tissue, so we have done the immunofluorescence reaction in the mouse brain tissue to mark the microglia in the brain. As shown in Fig. 6D, compared with the control group, the number of microglia in the ethanol group increased significantly, while the number of microglia in the Baijiu group was lower than that in the ethanol group, and the number of microglia in the M315 group was the smallest. Through the detection of the relevant indicators of the mouse cerebellum, we found that the longer the age of Baijiu, the smaller the impact on the mouse brain tissue.

3.7. Characterization of volatile compounds in five baijiu

We compared the concentrations of 46 important volatile flavor compounds in baijiu as shown in the column chart (Fig. 7A). The content of some components decreased with the increase of years, such as ethyl

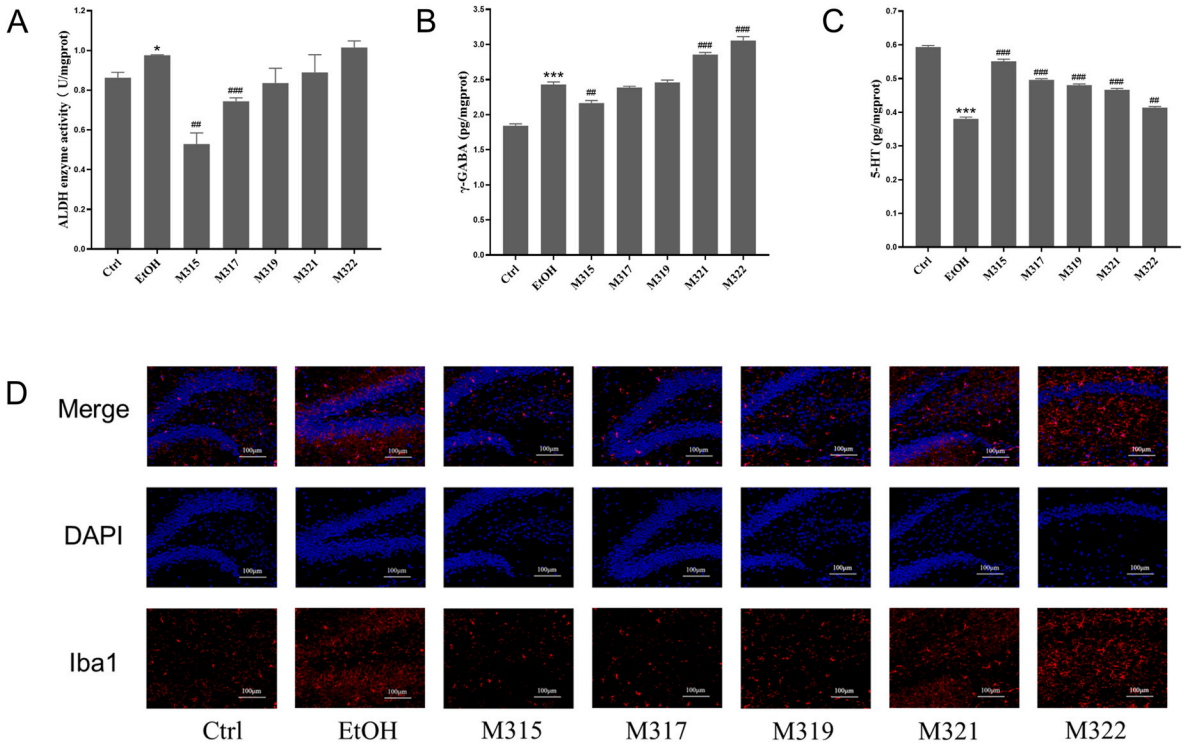

Fig. 6. Related hangover indicators of mouse brain. (A) The enzyme activity of ALDH in mouse cerebellum, (B) The level of γ-GABA in mouse hippocampus, (C) The level of 5-HT in mouse corpus striatum, (D) Immunofluorescence detection of microglia in mouse brain (Blue fluorescence represents nucleus and red fluorescence represents microglia). The data represent the mean ± SEM of three independent experiments. *P < 0.05, ***P < 0.001 *vs* the control group; ##P < 0.01, ###P < 0.001 *vs* the Ethanol group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

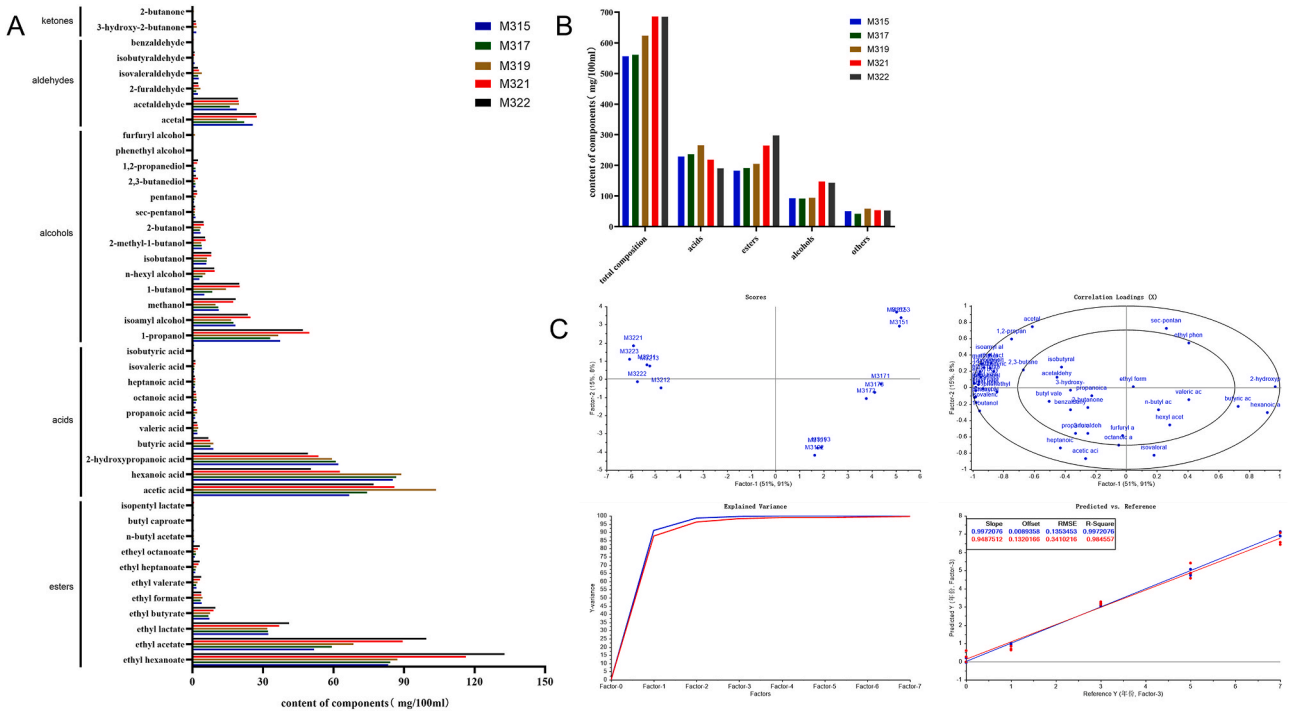

Fig. 7. Analysis of Main Volatile Components in Baijiu with different ages. (A) Main volatile compounds, (B) total compounds, acids, esters, alcohols, others, (C) Partial least squares regression (PLSR) analysis of 46 volatile compounds. The data represent the mean ± SEM of three independent samples.

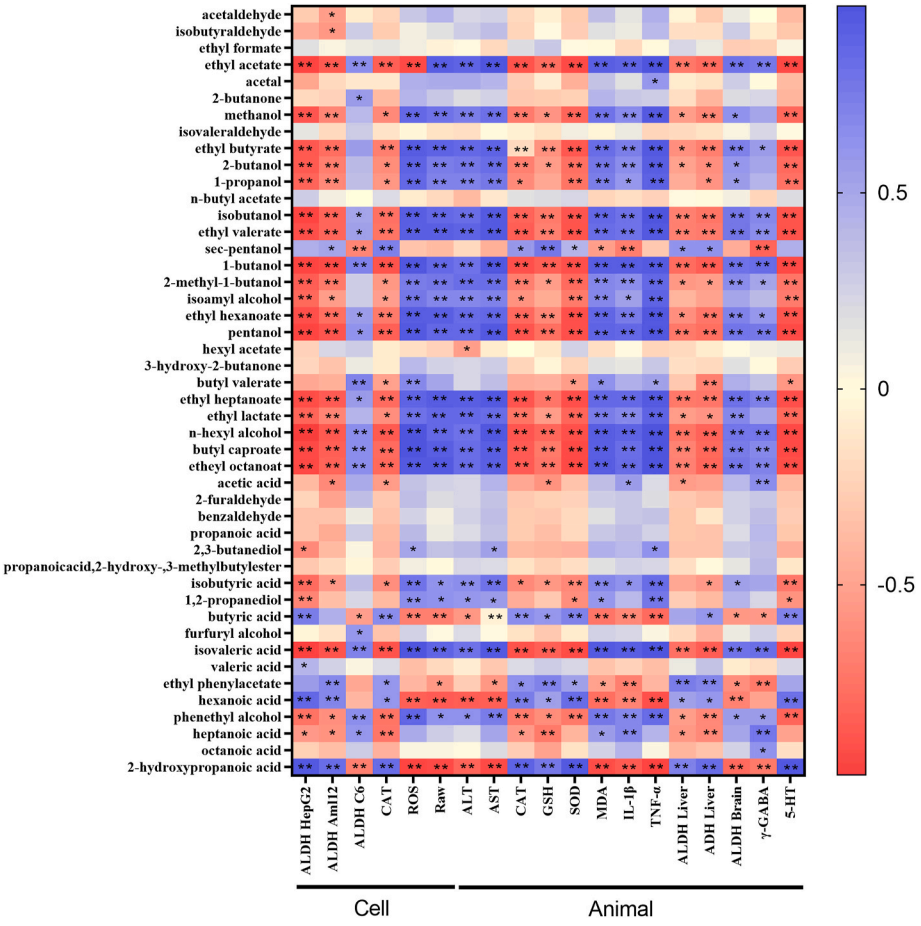

Fig. 8. Correlation analysis of volatile components and hangover related indicators. SPSS was used to calculate the Pearson coefficient of Baijiu composition (n = 15) and hangover related indicators (n = 15), and thermographic analysis was conducted according to Pearson coefficient. *P < 0.05, **P < 0.001.

caproate, ethyl acetate, 1-butanol, n-hexanol and so on. The content of some components increased firstly and then decreased with the increase of years, such as octanoic acid, acetic acid. And the content of 2-hydroxypropanoic acid increased with the increase of years. Then we summarized the total components, acids, esters and alcohols in Baijiu as shown in the column chart (Fig. 7B). We could clearly observe that the longer the year, the less the total components content, and so did the esters and alcohols. In order to further separate the selected baijiu groups in different years, a partial least squares regression (PLSR) model was established, which eliminated the influence of intra group error interference and background noise, and maximized the differences between groups. From the score chart in Fig. 7C, we could see that the samples perform well, indicating that there were significant differences that can be identified between samples. The three important indicators of PLSR model quality, $R^2X = 0.997$, $R^2Y = 0.984$, $Q^2 = 0.955$, the interpretation rate and prediction rate of the model were more than 0.5, indicated that the PLSR model had excellent interpretation performance and sample different prediction performance. By using PLSR to analyze the changes of Baijiu components, we can get some characteristic components related to age in Baijiu.

3.8. Relationship between baijiu components and hangover indicators

In order to clarify the relationship between the components of Baijiu and the degree of hangover, we established the correlation between the components of Baijiu and the related indicators of hangover, including ALDH, ROS, CAT, the release of NO at the cell level and the ethanol metabolism in liver or brain tissue (ALDH, ADH), liver damage (ALT, AST), liver oxidative level (CAT, GSH, SOD, MDA), liver inflammatory level (IL-1β, TNF-α), Brain tissue damage (γ-GABA, 5-HT) at the animal level. The contents of 49 main flavor substances were determined in Baijiu samples, and the contents of 29 substances were significantly related to at least one hangover indicator (Fig. 8). Ethyl acetate, n-butanol, n-hexanol, butyl acetate, ethyl octanoate, isovaleric acid, 2-hydroxypropionic acid were significantly correlated with all the indicators of hangover. In addition, 16 components are significantly related to more than 50% of hangover related indicators.

4. Discussion

In recent years, people have paid more and more attention to the health of drinking. The factors that affect the comfort of Baijiu after drinking include the proportion of acid esters, appropriate non-alcoholic components higher alcohol compounds and the storage year of Baijiu (Wu et al., 2022; Zhang et al., 2021). At present, few people have studied the influence of Baijiu storage years on the degree of hangover. The main purpose of this study is to evaluate the effects of Baijiu ages on alcohol hangover and related health indicators by establishing an acute intoxication model in mice.

A large amount of alcohol ingested in a short time will lead to the accumulation of a large amount of acetaldehyde, which will cause irreversible damage to the liver (Song et al., 2022). ALT and AST are two important transaminases in the liver. When hepatocytes are exposed to ethanol, the hepatocytes are damaged, the permeability of cell membrane is changed, and alanine aminotransferase and aspartate aminotransferase will be released from the cytoplasm into the serum, resulting in an increase in the content of these two transaminases in the serum. At the same time, it will also cause liver tissue lesions, mainly manifested as lenticular hepatocyte necrosis accompanied by neutrophil infiltration, and disordered arrangement of hepatocytes. In this study, the contents of ALT and AST in serum decreased with the increase of baijiu ages, the pathological degree of liver tissue has also been reduced to a certain extent, indicating that the increase of Baijiu ages can reduce the liver damage caused by drinking to a certain extent. This may be because with the increase of baijiu ages, a series of chemical reactions occur in Baijiu, and some bad ingredients in Baijiu volatilize or transform into other ingredients.

The accumulation of acetaldehyde in the liver will stimulate the production of ROS, resulting in oxidative stress and damage to the liver (Ceni et al., 2014; Li et al., 2004). CAT, GSH and SOD are three important antioxidant enzymes in the liver, which can effectively remove various oxidants produced during ethanol metabolism (Rawat et al., 2021). Malondialdehyde is one of the commonly used indicators to measure the degree of oxidative stress. *In vivo*, free radicals act on lipids to produce peroxidation. The final product of oxidation is malondialdehyde, which has cytotoxicity. The results showed that Baijiu stored in five years reduced the activity of antioxidant enzymes in the liver to a certain extent, but the activity of antioxidant enzymes in M315 group was higher than that in ethanol group, and the activity of antioxidant enzymes in M322 group was the lowest, indicating that the longer Baijiu ages, the less oxidative damage to the liver was caused. Ethanol can cause oxidative stress in the liver and damage the liver, thus promoting the production of inflammatory cytokines by hepatocytes (Cohen et al., 2009). The mRNA level of TNF-α and IL-1β in liver indicated that M315 induced inflammation in liver tissue was significantly lower than that in other drinking groups. As the storage time of M3 series Baijiu increases, the level of inflammation in the liver decreases.

Iba1 is the marker of microglia, and the expression level of Iba1 can reflect the number of microglia in the brain tissue. Microglia are innate immune cells in the central nervous system, and activated microglia have phagocytic function. Any neurological disorder can typically lead to inflammation and activation of microglia, resulting in an increase in the number of glial cells. The chronic inflammatory response mediated by microglia is harmful to the body and can cause damage to nerve tissue. Therefore, Iba1 staining can reflect the level of inflammation in brain tissue after drinking alcohol. Our data indicated that the longer M3 series Baijiu is stored, the lower the level of inflammation in brain tissue. In general, prolonging the storage time of Baijiu appropriately can reduce the damage of acute intoxication to the liver and the degree of hangover to a certain extent.

Studies have shown that the rate of alcohol metabolism is an important determinant of the severity of hangovers (Mackus et al., 2020). Most ethanol is metabolized through the oxidation process of the liver (Hyun et al., 2021; I.C. A., 2012). Ethanol metabolism is a two-step process driven by the action of two enzymes. ADH oxidizes ethanol to acetaldehyde, and ALDH oxidizes acetaldehyde to acetic acid. Both ALDH and ADH enzyme activities in cell level and mouse liver showed that the ethanol metabolic rate of the Baijiu group was higher than that of the ethanol group, which suggested that Baijiu was less prone to hangovers. In addition, the ethanol metabolic rate of M315 group was the highest, and that of M322 group was the lowest. The ethanol metabolic rate of other groups also showed a gradient trend with the year of Baijiu, indicating that the longer the year of Baijiu, the lower the degree of hangover.

The molecular structure of ethanol allows ethanol to freely pass through the blood-brain barrier, and brain tissue is exposed to the ethanol environment (Fein and Meyerhoff, 2000). Shiyun Jin et al. showed that the brain can metabolize alcohol and produce acetic acid, and the metabolic enzyme ALDH2 is mainly expressed in astrocytes of human and mouse cerebellum, acetic acid generated in the brain directly causes inhibitory neurotransmitter γ-GABA increases, thus inhibiting balance and motor coordination function (Jin et al., 2021). In addition to increasing the content of γ-GABA in the brain, alcohol also affects the release of neurotransmitter 5-HT (Khom et al., 2020; Oubraim et al., 2022). Some studies have proved that the content of 5-HT in alcohol preference mice is lower than that in nonalcoholic preference mice, that is, 5-HT can avoid alcohol-induced dependence in mice (Lagerspetz, 1972). In this study, we detected the activity of ALDH in the cerebellum of mice, the content of γ-GABA in the hippocampus and the content of 5-HT in the striatum. The results showed that compared with the control group, the activity of ALDH in the cerebellum of the ethanol group increased, the content of γ-GABA also increased, and the content of 5-HT

decreased, which indicated that the metabolism of ethanol in the brain tissue was faster, and more acetic acid was produced at the same time, resulting in the increase of γ-GABA content and the decrease of 5-HT content, consistent with previous research results. At the same time, it can also be seen that the results of the Baijiu group tend to be better, especially the M315 group. The results of the Baijiu group also show a certain law with the Baijiu year, that is, the longer the Baijiu year, the better.

Although the composition of Baijiu is complex, up to thousands of kinds, this study found some main components related to the degree of Baijiu hangover from the complex system, and further exploration of related components is needed in the future. The fusel oil in fermented wine mainly including n-propanol, n-butanol, isobutanol, n-amyl alcohol, isoamyl alcohol, n-octanol, etc. The oxidation and decomposition rate of fusel oil in the body is slower than that of ethanol, which will make the nervous system congest, which is an important factor leading to dry head and mouth after drinking (Lachenmeier et al., 2008). In the research on the correlation between Baijiu ingredients and hangover related indicators, we can see that there is a significant correlation between fusel oil and hangover related indicators. In addition, there are some other compounds such as ethyl acetate, ethyl butyrate, ethyl valerate, isovaleric acid and 2-hydroxypropionic acid are related to hangover related indicators. The principle of 'aged Baijiu is good' is that with the extension of storage time, the content of trace components (mainly acids, esters, non-ethanol alcohols) in Baijiu has changed, and this kind of chemical reaction often presents a similar rule, and our study just reveals the key component indicators.

This study found that in a certain year of Baijiu, the longer the year of Baijiu, the less damage to the liver and the less hangover. This is because Baijiu has undergone a series of chemical changes in the storage process, and some components will also be volatilized, making the components of Baijiu healthier. By analyzing the correlation between Baijiu ingredients and hangover related indicators, we can provide a certain direction for the future development of Baijiu industry. However, the mechanism of its specific components is not clear and needs further study.

5. Conclusion

In conclusion, through *in vivo* and in vitro studies on the degree of Baijiu hangover in different years, we found that the longer the year, the less damage to the liver, the faster the metabolic rate in the liver, and the smaller the impact on brain tissue. In addition, we also conducted a correlation analysis on the changes of Baijiu composition and hangover related indicators, and found that ethyl acetate, n-butanol, n-hexane, butyl acetate, ethyl octanoate, isovaleric acid, etc. in Baijiu were significantly related to Baijiu hangover related indicators, providing a reference for the design of Baijiu ingredients in the future.

CRediT authorship contribution statement

Yuan Dai: Conceived the project, designed experiments, discussed data, and, wrote the manuscript. **Xianyu Fan:** Designed experiments, conducted data analyses, and, wrote the manuscript. **Zhiqing Yang:** Performed assays, Formal analysis, extractions, and, Writing – review & editing. **Lulu Wu:** Performed assays, Formal analysis, extractions, and, Writing – review & editing. **Xinhu Zhou:** Conceived the project, Designed experiments, Funding acquisition. **Xianying Fang:** Formal analysis, Writing – review & editing, and, Writing – review & editing. **Xiangyang Ge:** Directed the overall study and provided final approval for the version to be published. **Linguo Zhao:** Directed the overall study and provided final approval for the version to be published, All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

We declare that no conflict of interest exists in the submission of this manuscript and manuscript is approved by all authors for publication.

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Data availability

Data will be made available on request.

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