

# Comparison of Acetic Acid Production Characteristics of Different Grains in *Baijiu* Brewing

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**Abstract:** To investigate the differences in the production of acetic acid and ethyl acetate during *Baijiu* production using various grain raw materials, including sorghum, glutinous sorghum, rice, glutinous rice, wheat, and corn, with medium-high temperature *Daqu* as the fermentation agent. The results indicate that bacteria were the primary microorganisms responsible for acetic acid production, and yeasts were predominantly responsible for ethyl acetate production. The contents of acetic acid and ethyl acetate were the highest in glutinous sorghum fermented grains, with specific contents of 1.45g/kg and 1.76g/kg, respectively, while the acetic acid was the lowest in rice and glutinous rice fermented grains. Furthermore, microbial community result showed that *Bacilli* and *Pichia* were positively correlated with the production of acetic acid and ethyl acetate, significantly, so *Bacilli* and *Pichia* are the key microorganisms in *Baijiu*. This study provides a theoretical basis for selecting raw materials to control acetic acid and ethyl acetate content in strong-flavor *Baijiu* and offers new approaches for improving *Baijiu* quality.

**Keywords:** *Baijiu*; Raw Materials; Acetic Acid; Ethyl Acetate.

## 1. Introduction

Solid-state fermentation is a unique and traditional technique in Chinese *Baijiu* production that fundamentally differs from liquid fermentation. This distinctive fermentation environment facilitates the formation of complex enzymatic systems and diverse flavor compounds, enhancing the sensory complexity of *Baijiu* through multidimensional biochemical interactions [1, 2]. In the traditional brewing of strong-aroma *Baijiu*, sorghum, rice, glutinous rice, corn, and wheat are commonly used as raw materials. Under the action of complex microorganisms in medium- and high-temperature *Daqu*, these raw materials ferment into various flavors [3]. According to the classification of starch types in sorghum, it can be categorized into two main types: sorghum and glutinous sorghum. Sorghum is characterized by a higher amylose content, a more compact molecular structure, and limited water solubility. Additionally, its protein content exceeds that of glutinous sorghum. In contrast, glutinous sorghum consists entirely of amylopectin, exhibiting a looser molecular arrangement, superior water absorption capacity, and easier gelatinization. These differences result in variations in alcohol yield and flavor profile during the fermentation process of liquor production [4]. Rice contains approximately 70% starch and is characterized by its pure texture and soft structure, which facilitates gelatinization. It also contains relatively low levels of protein, fat, and fiber [5]. Amylopectin is the main component of glutinous rice, exhibiting pronounced stickiness and a tendency to gelatinize upon steaming. During *Baijiu* fermentation, this characteristic may result in an elevated moisture level within the fermented grains [6]. Wheat is abundant in carbohydrates and contains a variety of trace metallic elements in appropriate proportions. It exhibits strong adhesive properties and provides rich nutritional content. During fermentation, it generates a significant amount of heat [7]. The starch in corn is mainly located in the endosperm, and the fat content in the germ is

approximately 5%. Corn is rather tough and requires a considerable amount of time for steaming to fully gelatinize the starch. Excessive fat can expedite the production of organic acids, leading to the development of off-flavors in *Baijiu* and causing cloudiness under low-temperature conditions [8, 9].

This study inoculated the bacteria and yeast enriched in high-temperature *Daqu* into the wort medium, analyzed the key microbial communities that produce acetic acid and ethyl acetate, and conducted solid-state fermentation experiments to reveal the differences in acetic acid and ethyl acetate production and the differences in the community structure of *Daqu* microorganisms caused by different grains during the fermentation process.

## 2. Materials and Methods

### 2.1. Materials and Culture

#### 2.1.1. Materials

Medium-high temperature *Daqu*, malted barley, sorghum, glutinous sorghum, rice, glutinous rice, wheat, and corn were purchased from the market and ground using a hammer mill. The resulting powder was sieved through a 20-mesh sieve to remove large particles, then packed into bags, and stored in a refrigerator at 4°C.

#### 2.1.2. Culture

The preparation method for 1L of malt extract medium is as follows: dissolve 3 g of yeast extract powder, 4 g of peptone, 10 g of anhydrous glucose, and 20 g of malt extract powder in water, then adjust the volume to 1L. The medium should be Sterilized at 121°C for 20 minutes to ensure complete sterilization.

The configuration method of LB medium is as follows: 0.5 g of yeast extract powder, 1 g of peptone, 1 g of sodium chloride, and make up to 1 L with water. Sterilize at 121°C for 20 minutes.

## 2.2. Methods

### 2.2.1. Identification of Acetic Acid and Ethyl Acetate-Producing Microorganisms in Medium-High Temperature *Daqu*

The enrichment method for bacteria in medium and high-temperature *Daqu* is as follows: Sterilize 100 mL of prepared LB medium, cool it to room temperature, and add nystatin to achieve a working concentration of 0.1 mg/L in the medium. Subsequently, add 1 g of medium and high-temperature *Daqu* and incubate at 37 °C with shaking at 150 r/min for 24 hours.

The enrichment method for yeast in medium and high-temperature *Daqu* is as follows: Sterilize 100 mL of prepared malt extract medium, cool it to room temperature, and add chloramphenicol to achieve a working concentration of 1 mg/L in the medium. Then, inoculate 1 g of medium and high-temperature *Daqu* and incubate at 30 °C with shaking at 150 r/min for 24 hours.

Weigh 500g of crushed malt extract and put it in a 5L beaker. Add 2L of hot water and keep the water temperature at around 60°C. Maintain this for 90 minutes. After saccharification is completed, filter it through four layers of gauze and adjust the sugar content to 18 Brix. The fermentation process involves inoculating the yeast and bacteria that have been enriched from medium-high temperature *Daqu*.

### 2.2.2. Brewing Test

Weigh 2 kg of raw materials into a bucket, add 500 g of steamed and cooled rice husks, and pour in 1.2 kg of boiling water at 90°C. Mix thoroughly, cover with gauze, and allow it to soak for 22–24 hours. Subsequently, evenly distribute the raw materials in the steamer and begin steaming once the steam is fully generated, continuing for 30 minutes. After steaming, immediately remove the materials from the steamer while still hot, and add cold water equivalent to 30% of the raw material weight to separate the grains. Mix well and spread out the mixture to cool. When the temperature of the grain decreases to 20–22°C, add 500 g of medium-high temperature *Daqu* powder and mix thoroughly. Adjust the moisture content to 55%. Transfer the mixed *Daqu* grains into the fermentation tank, seal it, and maintain the room temperature at 28°C for fermentation.

Weigh 10 g of dried fermented grains into a conical flask, add 50 mL of sterile water, and soak for 30 minutes. Then filter the mixture and transfer 5 mL of the filtrate to another conical flask for determining the reducing sugar content in the fermented grains using the Fehling reagent method [10].

$$\text{Reducing sugars(\%)} = ((V2 - V1) \times C) / (5 \times 5 / 50) \times 100\%$$

In the formula:

V1 - Volume of standard Fehling solution consumed for glucose determination, mL

V2 - Volume of standard glucose consumed by the sample, mL

C - Concentration of standard glucose, g/mL

Weigh 10 g of dried fermented grains into a conical flask, add 100 mL of sterile water, and allow it to soak for 30 minutes. Filter the mixture and collect 10 mL of the filtrate for determining the acidity of the fermented grains using acid-base neutralization titration [11].

$$\text{Acidity(mmol/10 g)} = c \times V \times 5 / 10 \times 100$$

In the formula:

c - concentration of the standard sodium hydroxide solution, mol/L.

V - volume of the standard sodium hydroxide solution

consumed during titration, mL.

The moisture content in the fermented grains was determined by the hot drying method [12].

$$\text{Water content(\%)} = (m1 - m2) / m1 \times 100\%$$

In the formula:

m1 - The weight of the fermented grains before drying.

m2 - The weight of the fermented grains after drying.

Weigh 5 g of fermented grains and transfer it into a round-bottomed flask. Add 45 mL of 1+4 HCl solution, heat the mixture for 30 minutes, cool it to room temperature, and then dilute to a final volume of 500 mL. Determine the reducing sugar content using the Fehling's reagent method, and subsequently convert the results into equivalent starch content [13].

$$\text{Starch(\%)} = ((V2 - V1) \times C) / (10 \times 5 / 100) \times 100 - C \text{ (Reducing sugars)}$$

In the formula:

V1 - Volume of standard Fehling solution consumed for glucose determination, mL

V2 - Volume of standard glucose consumed by the sample, mL

C - Concentration of standard glucose, g/mL

Take 100 g of fermented grains and put it into a round-bottomed flask. Add 200mL of sterile water and distill off 100 mL. Determine the alcohol content by alcoholometer.

### 2.2.3. Acetic Acid and Ethyl Acetate Detection

Weigh 10 g of dried distiller's grains and transfer them into a conical flask. Add 100 mL of sterile water, allow the mixture to soak for 30 minutes, and then filter. Take 100 µL of the filtrate and transfer it into a 2 mL centrifuge tube. Add 10 µL of formic acid and 390 µL of anhydrous ethanol. Centrifuge at 12,000 r/min for 7 minutes. After centrifugation, collect 200 µL of the supernatant for detection (the injection port temperature is maintained at 230°C, using split injection with a split ratio of 39:1; the initial temperature of the gas chromatography column is set at 50°C and held for 5 minutes, followed by an increase at 5 °C/min to 115 °C and held for 15 minutes, then increased at 10 °C/min to 170 °C and held for 15 minutes) [14, 15].

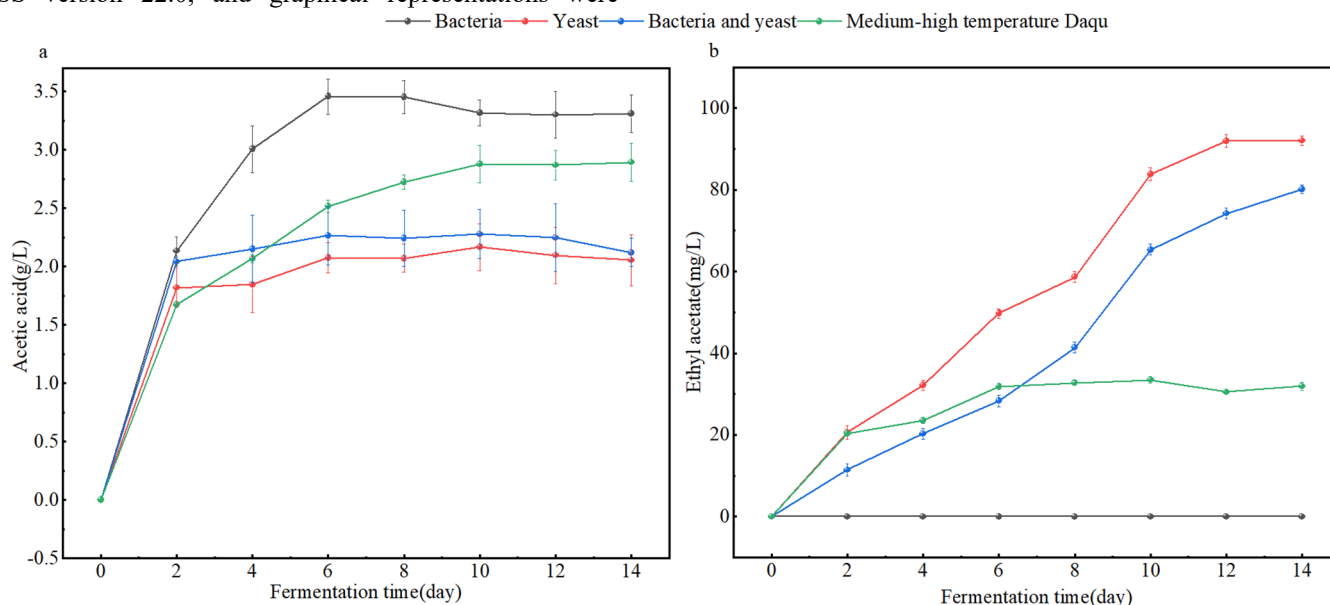
## 2.3. DNA Extraction and PCR Amplification

Microbial community genomic DNA was extracted from samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to manufacturer's instructions. The DNA extract was checked on 1 % agarose gel, and DNA concentration and purity were determined with NanoDrop2000 spectrophotometer (Thermo Scientific, United States). For bacterial community, the bacterial 16S rRNA genes were amplified using the universal bacterial primers 27F (5'-AGRGTTYGATYMTGGCTCAG-3') and 1492R (5'-RGYTACCTTGTTACGACTT-3'). For fungal community, the ITS sequences were amplified using the primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS1R (5'-GTAATTCGATGAAGTAA-3') and ITS4R (5'-TCCTCCGCTTATTGATATGC-3'). Primers were tailed with PacBio barcode sequences to distinguish each sample. Amplification reactions (20- µL volume) consisted of 5 × FastPfu buffer 4 µL, 2.5 mM dNTPs 2 µL, forward primer (5 µM) 0.8 µL, reverse primer (5 µM) 0.8 µL, FastPfu DNA Polymerase 0.4 µL, template DNA 10 ng and DNase-free water. The PCR amplification was performed as follows: initial denaturation at 95 °C for 3 min, followed by 27 cycles of denaturing at 95 °C for 30 s, annealing at 60°C for 30 s and extension at 72 °C for 45 s, and single extension at 72 °C for 10 min, and end at 4 °C (ABI GeneAmp® 9700,

USA). After electrophoresis, The PCR products were purified using the AMPure® PB beads (Pacfic Biosciences, CA, USA) and quantified with Qubit 4.0 (Thermo Fisher Scientific, USA) [16, 17].

## 2.4. Data Analysis

Raw data were sorted and categorized by Excel 2021, while statistical analysis of experimental data was performed with SPSS version 22.0, and graphical representations were



**Fig 1.** Determination of key microorganisms producing acetic acid and ethyl acetate in medium-high temperature *Daqu*.

a the variation in total acid content of the fermentation broth during the microbial fermentation process inoculated with medium-high temperature *Daqu*. b Identification of key microbial communities responsible for ethyl acetate production in medium-high temperature *Daqu*.

To investigate the key microorganisms responsible for acetic acid production in *Baijiu* brewing (Fig. 1a), bacteria enriched from medium-high temperature *Daqu* were inoculated into malt culture media. During fermentation, the concentration of acetic acid in the fermentation broth progressively increased and essentially ceased after 8 days of fermentation. Upon completion of the fermentation process, the fermentation broth inoculated with bacteria exhibited the highest acetic acid content at 3.31 g/L, whereas the fermentation broth inoculated with yeast produced the lowest amount of acetic acid at 2.05 g/L. These results indicate that bacteria are the predominant microorganisms involved in acetic acid production during strong-flavor *Baijiu* fermentation. The ethyl acetate content in each fermentation liquid gradually increased with fermentation time. Ethyl acetate was undetectable in the fermentation broth inoculated with bacteria, likely due to the absence of alcoholic fermentation in the broth, which prevented the esterification reaction. After 14 days of fermentation, the fermentation broth inoculated with yeast exhibited the highest ethyl acetate content at 92.05 mg/L. These results demonstrate that ethyl acetate in strong-flavor *Baijiu* brewing is predominantly synthesized by yeast communities. However, the underlying production mechanism of ethyl acetate in actual strong-flavor *Baijiu* production requires further investigation (Fig. 1b).

## 3.2. Divergence in Physicochemical Properties of Cereal Fermented Grains During Alcoholic Fermentation

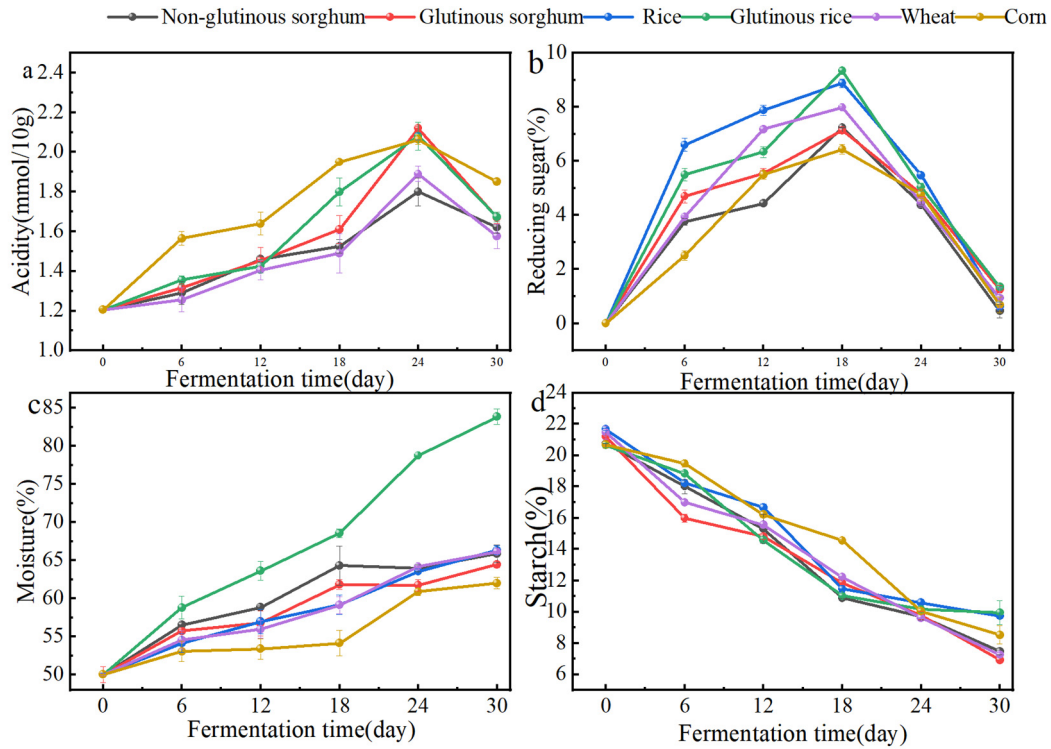
To investigate the changes in various parameters during the fermentation of different grains, this study systematically

created utilizing Origin 2022.

## 3. Results

### 3.1. Identification of Microbial Consortia Responsible for Acetic Acid and Ethyl Acetate Production in Medium-High Temperature *Daqu*

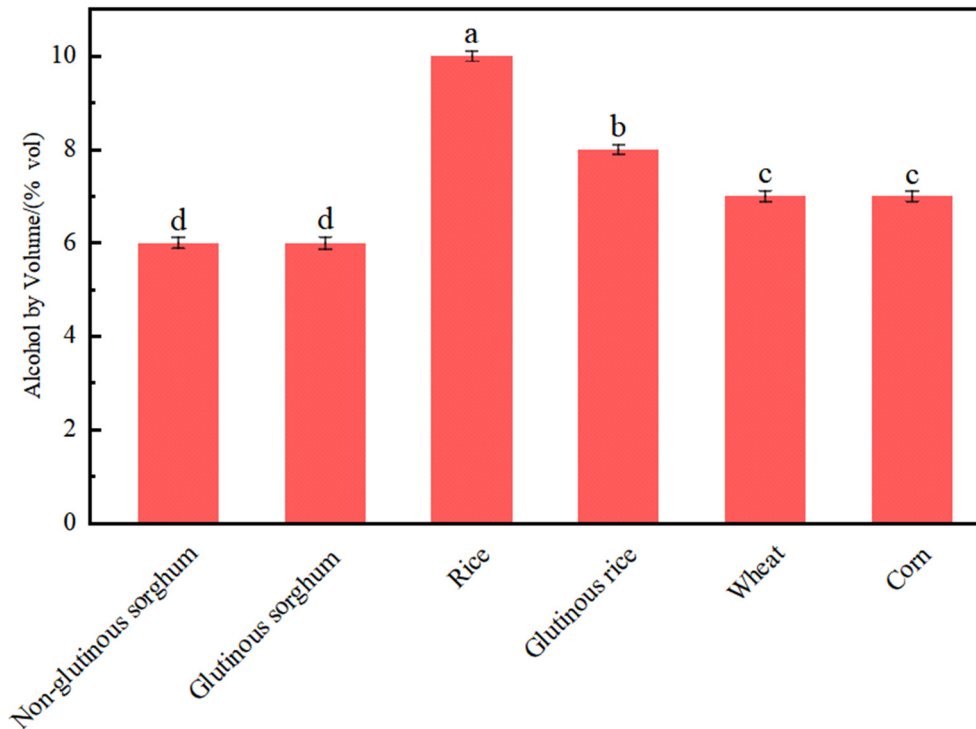
monitored multiple factors throughout the fermentation process. As fermentation progressed, acidity in all fermented grains exhibited a gradual increasing trend, reaching peak values at 24 days. Notably, glutinous sorghum fermented grains demonstrated significantly higher acidity compared to other raw materials, indicating its suitability for bacterial acid fermentation (Fig. 2a). The reducing sugar content initially increased until day 18, presumably due to the action of saccharifying enzymes from *Daqu* and microbial metabolism. Subsequently, it rapidly decreased as microorganisms consumed the sugars, resulting in approximately 2% residual sugar by the end of fermentation, which indicated the completion of the fermentation process (Fig. 2b). The water content in all fermented grain samples progressively increased during the fermentation process. Glutinous rice fermented grains exhibited the most rapid increase, rising to 83.58% by the end of fermentation. Corn fermented grains maintained the lowest water content at 62.09%. These findings highlight the influence of raw materials on microbial activity during fermentation (Fig. 2c). The starch content gradually decreased during the fermentation process, with the most rapid decline observed between 0 and 15 days. Interestingly, glutinous rice fermented grains retained the highest starch concentration at the end of fermentation, potentially due to the inhibition of microbial metabolism caused by the rapid increase in water content (Fig. 2d).



**Fig 2.** Changes in physicochemical Indicators of different grain fermented grains.

a Effect of grain type on acidity variation during fermentation. b Effect of grain type on reducing sugar variation during fermentation. c Effect of grain type on moisture variation during fermentation. d Effect of grain type on starch degradation during fermentation

### 3.3. Analysis of Alcohol Content in Fermented Grains of Different Cereal Grains



**Fig 3.** Ethanol content in different raw material fermentation fermented grains.

Note: Letters in the figure represent significant differences between groups ( $P < 0.05$ ).

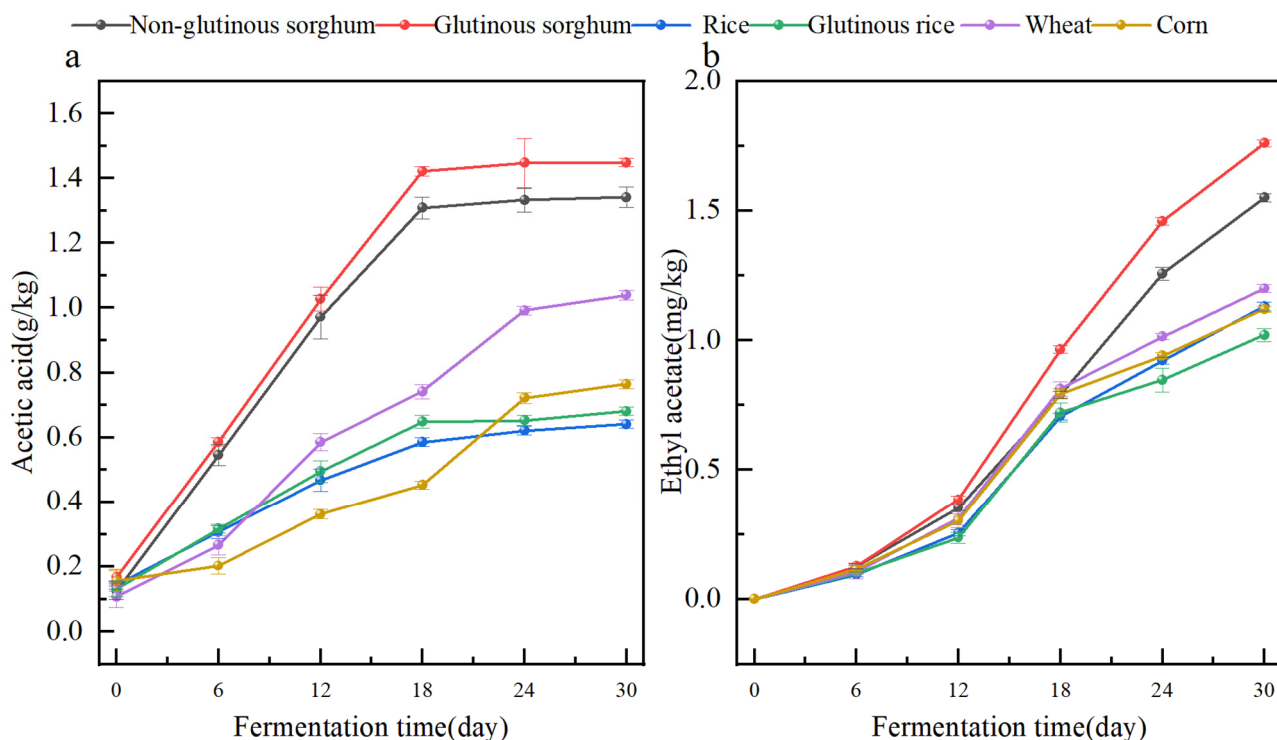
To investigate the differences in alcohol content among fermented grains derived from various types of grains, the alcohol concentration in the fermented grains was measured (Fig. 3). At the end of fermentation, the alcohol content in all

types of fermented grains exceeded 6% vol. Notably, the alcohol content in rice-fermented grains was significantly higher than that in fermented grains derived from other raw materials ( $P < 0.05$ ), reaching up to 10%vol. This indicates

that the fermentation process for all types of fermented grains proceeded successfully. The alcohol content in fermented grains serves as an indicator of fermentation progress and reflects microbial activity levels. An appropriate level of alcohol in fermented grains not only inhibits the growth and reproduction of undesirable microorganisms but also

enhances the production of key flavor compounds.

### 3.4. Analysis of Acetic Acid and Ethyl Acetate Contents in Fermented Grains of Different Cereal Raw Materials



**Fig 4.** Changes in acetic acid and ethyl acetate during the fermentation process of different fermented grains.

a Effect of grain type on acetic acid content during fermentation. b Effect of grain type on ethyl acetate content during fermentation.

To investigate the variations in acetic acid content during the fermentation process of fermented grains prepared from different raw materials (Fig. 4a), it was observed that the acetic acid concentration in all fermented grains exhibited a gradual increasing trend as fermentation progressed, eventually stabilizing on the 18th day of fermentation. Fermented grains made from glutinous sorghum had the highest acetic acid content at 1.45 g/kg, followed by non-glutinous sorghum at 1.34 g/kg. Rice and glutinous rice fermented grains consistently exhibited lower acetic acid levels throughout the fermentation process, with final concentrations of 1.13 g/kg and 1.02 g/kg, respectively, by the end of fermentation. This suggests that rice and glutinous rice are less prone to acetic acid accumulation during *Baijiu* fermentation. A comparative analysis of ethyl acetate changes in fermented grains derived from different grain materials at various fermentation times (Fig. 4b) revealed that as fermentation progressed, ethyl acetate levels in all fermented grains showed a gradually increasing trend, reaching maximum values on the 30th day of fermentation. During the same fermentation period, glutinous sorghum fermented grains exhibited the highest ethyl acetate content, reaching 1.76 g/kg by the end of fermentation, which was significantly higher than that in other fermented grains. Non-glutinous sorghum fermented grains showed the second-highest ethyl acetate content at 1.55 g/kg. These results indicate that both types of sorghum are conducive to ethyl acetate accumulation during *Baijiu* brewing. The differences in acetic acid and ethyl acetate contents in fermented grains derived from

various raw materials may be attributed to the compositional characteristics of the raw materials [16, 17].

### 3.5. Analysis of Bacterial Community Structure in Fermented Grains of Different Cereal Grains

To investigate the impact of different grain types on bacterial communities during fermentation (Fig. 5), the original data obtained from 16S amplicon sequencing identified a total of 30 top-ranked genera. At 15 days of fermentation, the dominant genera included *Lentilactobacillus*, *Acetobacter*, *Acinetobacter*, *Pseudomonas*, and others. After 30 days of fermentation, the dominant bacterial genera in fermented grains from different grain types primarily included *Lentilactobacillus*, *Acinetobacter*, *Lactobacillus*, and others. The relative abundance of *Acetobacter* was significantly higher in the fermented grains at 15 days of fermentation, with its proportion reaching 62% in glutinous sorghum fermented grains. This suggests that glutinous sorghum facilitates the growth of *Acetobacter*, leading to an increase in acetic acid content in the fermented grains. The *Daqu* (starter culture) did not harbor *Acetobacter* but was predominantly colonized by *Acinetobacter*, indicated that the *Acetobacter* in the fermented grains likely originated from the ambient air. This may account for the relatively high acetic acid content observed in solid-state fermented grains. Therefore, attention should be paid to contamination by environmental microbes during commercial production [18, 19].



Fig 5. Dynamics of bacterial community structure at genus level during fermentation in various cereal-based fermented grains

### 3.6. Analysis of Fungal Community Structure at the Genus Level in Fermented Grains from Different Cereals

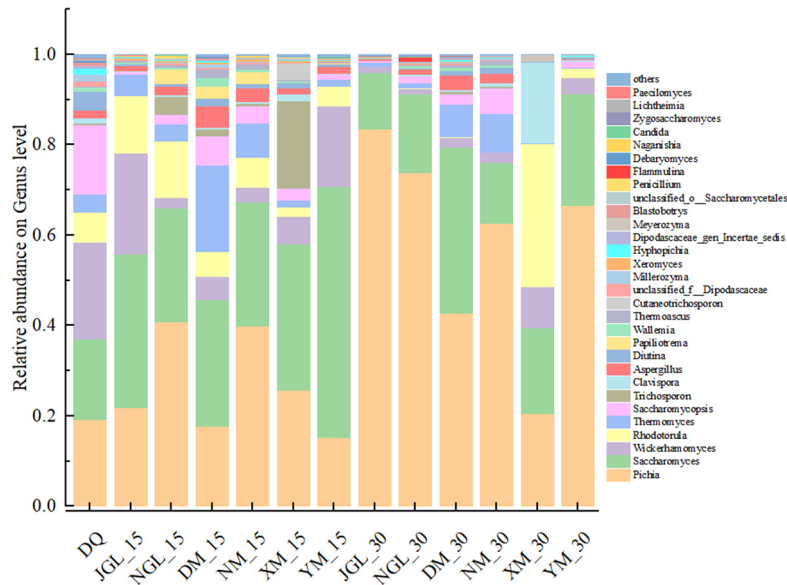


Fig 6. Dynamics of fungal community structure at genus level during fermentation in various cereal-based fermented grains

This study analyzed the fungal community structure at the genus level during the fermentation process of fermented grains (Fig. 6), identifying a total of 31 predominant genera. The dominant genera in the fermented grains were *Pichia*, *Saccharomyces*, *Wickerhamomyces*, and others. After 30 days of fermentation, *Pichia* and *Saccharomyces* emerged as the predominant microbial populations in the fermented grains, comprising 90% of the total relative abundance. *Saccharomyces* is a pivotal microorganism in alcoholic fermentation. Under anaerobic conditions, it converts sugars such as glucose and fructose into ethanol and carbon dioxide, representing the primary pathway for alcohol production during *Baijiu* brewing. Meanwhile, *Pichia* synthesizes various unique flavor compounds during fermentation,

including specific alcohols, esters, and aromatic compounds. These compounds contribute distinctive flavors and aromas to *Baijiu*, thereby enriching its flavor profile. Certain esters produced by *Pichia* exhibit fruity and floral notes, which enhance the complexity and richness of *Baijiu*'s aroma [20, 21].

### 3.7. Correlation Analysis between Fungal Communities and Ethyl Acetate in Fermented Grains from Different Cereals

To analyze the correlation between bacteria, fungi, and acetic acid and ethyl acetate in *Baijiu* brewing (Fig. 7), correlation analysis showed that different strains exhibited significantly different association patterns. Regarding acetic

acid, *Bacilli* showed a positive correlation, while *Acinetobacter*, *Levilactobacillus*, *Staphylococcus*, and others displayed significant negative correlations ( $P < 0.05$ ). Notably, *Weissella*, *Thermoactinomyces*, *Companilactobacillus*, and *Bacillus* exhibited extremely significant negative relationships ( $P < 0.01$ ). For ethyl acetate, *Bacilli* demonstrated significant positive correlation, while other strains showed negative correlations, with *Acinetobacter*, *Pseudomonas*, *Weissella*, and other microorganisms reaching extremely significant negative levels ( $P < 0.01$ ). These results suggest that, except for *Bacilli* which may promote ethyl acetate accumulation, most microorganisms have inhibitory effects on the production of these two compounds. *Pichia* exhibited extremely significant positive correlation with ethyl acetate production, with *Zygosaccharomyces* and *Flammulina* showing significant positive correlations,

indicating these strains may promote ethyl acetate production, while *Wickerhamomyces* and most other strains had significant negative correlations, with *Diutina* and *Dipodascaceae gen Incertae sedis* showing the most significant inhibition. Similarly, *Saccharomycopsis*, *Diutina*, and *Blastobotrys* displayed significant negative correlations with acetic acid, while *Pichia* showed positive correlation. Study has shown that *Pichia* and other yeasts have positive correlations with ester compounds [22]. *Bacillus licheniformis*, a dominant species in high-temperature *Daqu* (starter culture), can produce various volatile compounds when fermenting wheat substrate at different temperatures. Under high-temperature conditions, it has characteristics that enable good production of pyrazines and guaiacol compounds, which can improve the quality of sauce-flavor *Baijiu* [23].

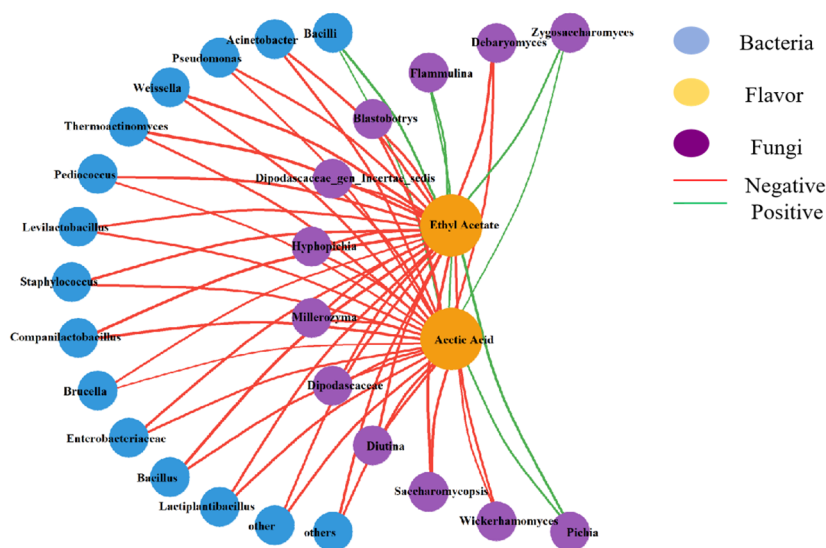


Fig 7. Microbial co-occurrence network analysis of ethyl acetate-producing microorganisms

## 4. Conclusion

This study systematically analyzed the key microbial communities responsible for acetic acid and ethyl acetate production in medium-high temperature *Daqu*, elucidating the differential impacts of various grain raw materials on the formation of acetic acid and ethyl acetate during *Baijiu* brewing. The results demonstrated that bacteria in medium-high temperature *Daqu* act as the predominant microorganisms responsible for acetic acid production during *Baijiu* brewing, while yeast communities represent the primary microbial groups involved in ethyl acetate production. In solid-state fermentation tests, glutinous sorghum fermented grains contained the highest levels of acetic acid and ethyl acetate, as the ingredient of glutinous sorghum caused elevated relative abundance of microorganisms related to acetic acid and ethyl acetate production during fermentation, resulting in increased concentrations of these compounds in the fermentation fermented grains. Sorghum-fermented grains exhibited significantly higher levels of acetic acid and ethyl acetate compared to other experimental groups, suggesting that sorghum generally facilitates the proliferation of microorganisms involved in acetic acid and ester production. In terms of microbial community structure, the relative abundance of *Bacillus* species in glutinous sorghum-fermented grains reached 62%, and both *Bacillus*

and *Pichia* genera demonstrated significant positive correlations with the production of acetic acid and ethyl acetate. This study investigates the impact of raw materials on microbial metabolism during *Baijiu* (Chinese liquor) brewing, aiming to provide a theoretical foundation and innovative strategies for reducing acetic acid and ethyl acetate concentrations in strong-flavored *Baijiu*.

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