RESEARCH ARTICLE

Analysis of *Zygosaccharomyces ruckeri* metabolic characteristics and safety in the field of food processing

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In recent years, the safety of fermented foods has attracted social attention. This study employed fermented soy sauce to analyze the metabolic characteristics and safety of *Zygosaccharomyces ruckeri* in the soy sauce production process. The metabolic characteristics of the strain were analyzed by using gas chromatography during the culture process. The drug resistance experiments were conducted using ketoconazole, voriconazole, *etc*. The safety of Russelt's *saccharomycetes* in the food industry was analyzed using mice as experimental animals. In the analysis of *Zygosaccharomyces ruckeri* growth, the results showed that *saccharomycetes* grew rapidly between 2 h and 24 h, and then gradually stabilized. The main volatile metabolites included alcohols, esters, aldehydes, and other metabolites, and reached their peak after 30 days of the *saccharomycetes* metabolite analysis, while the number of ketones and aldehydes decreased rapidly after 30 days. For non-volatile substances, most of them reached their peak on the 50th day including esters, acids, sugars, and other substances. In addition, the fermentation products were tested, and the contents of biogenic amines and other substances were within the national standard range. The drug resistance experiments showed that *saccharomycetes* demonstrated certain resistance to fluconazole, while the toxicity experiment demonstrated that there were no significant changes in the body weight, blood lipids, and organ development of the mice, which indicated that *Zygosaccharomyces ruckeri* had high safety in the field of food production. The results of this study would provide important technical reference for the application of *Zygosaccharomyces ruckeri* and food safety analysis.

Keywords: *Zygosaccharomyces ruckeri*; food processing; metabolic characteristics; drug resistance; safety.

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Introduction

With the continuous development of the food processing field, more and more microorganisms are used in the fermentation process. Among them, *Zygosaccharomyces ruckeri* is an important microbial resource, and its metabolic characteristics and safety in the field of food processing have attracted much attention [1]. *Schizosaccharomyces pombe* is a unicellular fungus and also a type of *saccharomycetes* with its cells exhibit a conjugated shape during division [2]. *Zygosaccharomyces ruckeri* widely exists in various environments in nature such as water, soil, and plant surfaces [3], which can produce a variety of metabolites including *saccharomycetes* alcohols, organic acids, and odor compounds, *etc*. These metabolites often provide special flavors and aromas to food [4].

Currently, *Zygosaccharomyces ruckeri* is widely used in food processing fields such as bread,

beer, wine, and soy sauce. In the field of soy sauce production, commonly used fungi include lactic acid bacteria, *saccharomycetes*, and *Aspergillus oryzae* [5]. Among them, *Aspergillus* is a filamentous fungus and is currently the main microorganism in soy sauce production, which can secrete a large amount of glycoside hydrolase and protease to provide a nutritional basis for subsequent microorganisms. In the early stage of soy sauce fermentation, the main fermentative ester-producing yeast species is *Zygosaccharomyces ruckeri*, while the main alcohol-producing yeast species in the later stage is *Torulopsis globus* [6]. The early fermentation has a direct impact on the flavor and mouthfeel of soy sauce, and *Zygosaccharomyces ruckeri* plays a key role in it. However, in soy sauce brewing, differences in material ratios and production processes will cause many differences in the food itself [7]. For example, some soy sauce may be too sweet. In addition, soy sauce is also prone to produce harmful substances such as urethane and biogenic amines during fermentation and brewing. When the substance's content reaches a certain level, it will threaten human health. Human oral administration of 8 - 40 mg of histamine can cause mild poisoning symptoms such as vomiting and dizziness. If the dose exceeds 40 mg, moderate poisoning symptoms may occur. Once it reaches 100 mg, it can directly threaten human life. According to Food and Drug Administration (FDA) (Silver Spring, MD, USA) regulations, the histamine content in food needs to be less than 50 mg/kg body weight, and the tyramine content needs to be less than 100 mg/kg body weight. Exceeding food standards can also have an impact on human safety [8].

Zygosaccharomyces ruckeri has a long history in soy sauce brewing, but there are few studies on the safety of *Zygosaccharomyces ruckeri* in the food field. This research analyzed the metabolites produced by *Zygosaccharomyces ruckeri* during the fermentation of soy sauce and the characteristics of *Zygosaccharomyces ruckeri* in the food field through drug resistance and safety tests. The results of this study would provide support for the application and safety assessment of fungi in the field of food processing, thereby ensuring the health and safety of food.

Materials and methods

Preparation of cultural media

The *saccharomycetes* extract peptone glucose culture medium was prepared by dissolving 20 g of anhydrous glucose, 20 g of peptone, and 10 g of *saccharomycetes* powder in 1,000 ml of distilled water followed by sterilization at 120°C. The solid culture medium was prepared by adding 2 g of agar powder to the above liquid medium. The rice koji juice, the product of koji fermentation using rice as the base, was prepared by soaking 30 g of rice overnight in a 1,000 mL of distill water followed by high-temperature sterilization and cooling down to 25°C under sterile environment before inoculating the *Aspergillus oryzae* (National Culture Laboratory, Tianjin, China) four times into it and shaking for 24 hours. The culture was then flat for 8 hours until covered with yellow spores, which was the mature rice koji. After placing the culture in dry environment for 9 hours, it was transferred into an Erlenmeyer flask with the addition of four times distilled water before saccharifying at 55°C for 5 hours. The culture was then boiled for 10 minutes, filtered, and added distill water to set the sugar content to 11 degrees with 2% agar before high temperature sterilization. The blood plate was prepared by mixing 0.5 g of sodium chloride, 2 g of agar, 0.3 g of beef extract, and 1 g of peptone with 100 mL of distill water, sterilizing at high temperature and cooling down to 25°C before adding 10% defibrinated sheep blood under sterile environment and plating on petri dishes.

Koji making and fermentation

The seed koji was made by adding 4 rings of *Aspergillus oryzae* into the culture medium under sterile environment, stirring and accumulating at 29°C, shaking for 72 hours. During the incubation, the spore amount was checked frequently to see

if it met the required amount before drying the cultured koji and store it in dry kraft paper. Daqu was produced from wheat, water, mother koji, and other raw materials by mixing fried wheat and soybean meal (Chengdu Guoniang Food Co., Ltd. Chengdu, Sichuan, China) at a ratio of 6:4. The soybean meal was soaked in hot water for 0.5 h before wheat being added and mixed evenly. The mixture was then wrapped in koji cloth and kept at 121°C for 0.2 h. When the temperature dropped down to 39°C, 3% koji was added and stirred evenly at room temperature. The koji needed to be turned when its temperature reached 38°C in the first 7 to 8 hours before continuing incubation for another 42 hours until the song matured. Sauce fermentation was carried out by mixing 250 ml sauce with 10% fermentation *saccharomycetes* solution and fermenting in Thermo Fisher 371 incubator (Thermo Fisher, Shanghai, China) at 30°C.

***Saccharomycetes* detection**

1 to 2 rings of *Zygosaccharomyces ruckeri* (National Culture Laboratory, Tianjin, China) was inoculated in 30 mL of peptone glucose medium and incubated at 35℃, 150 rpm, for 24 hours before replacing the medium with fresh one and continuing cultivation in 60 mL at the same temperature and environment. 2 mL of activated Lu's conjugated yeast was added to 100 mL of culture medium, while keeping the initial concentration of the yeast at 106 colony forming unit (cfu)/mL and incubating at 30℃, 150 rpm. The cultured *Zygosaccharomyces ruckeri* was taken out every 2 hours to measure the absorbance at 600 nm using Nanodrop One Ultra Micro Ultraviolet Spectrophotometer (Thermo Fisher, Waltham, MA, USA) and plot the yeast growth curve. The direct counting method under a Sanyo BX51 Optical microscope (Sanyo, Osaka, Japan) was also used to detect the amount of yeast in the sauce mash. The solid-phase microextraction-GC-MS method was used to detect the volatile metabolites of *Zygosaccharomyces ruckeri* in the mash juice. 5 mL of the sample was placed in a sealed extraction bottle before the 85 μm US Superco 57329-U automatic solid-phase microextraction head was inserted into the bottle through the extraction handle and extracted at 50°C for 0.5 h. The extraction head was put into Shimadzu LC-20A gas chromatograph (Shimadzu, Kyoto, Japan) for the detection of substance and nonvolatile metabolites from *Zygosaccharomyces ruckeri* [9, 10]. The extracellular metabolites were detected by placing 1 mL of soy sauce sample in a petri dish and drying it by vacuum freezing. 0.5 g of freeze-dried sample was decomposed in dimethylformamide (DMF) (Sigma Aldrich, St. Louis, MO, USA) followed by ultrasonic operation for 0.5 h and vibration for 30 s. The sample was then placed at 80°C for 2 hours and analyzed by a gas chromatograph-mass spectrometer [11].

Detection of fermentation products

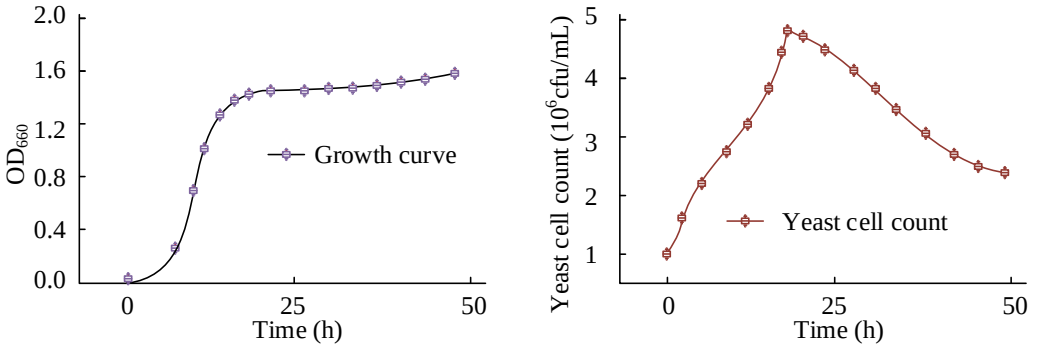
A gas chromatograph-mass spectrometer was used to detect the emission of ethyl carbamate from *Zygosaccharomyces ruckeri* in the mash juice [12]. Carbamate was detected at 200, 400, 600, 800, and 1,000 ng/mL, respectively, and the concentration curve was then obtained.

Drug resistance test

The agar diffusion method was applied for drug resistance test. The antigens of ketoconazole, amphotericin B, voriconazole, and yeast were fixed in the agar, respectively, and incubated at 32℃ to allow them freely diffusion to form antigen-antibody complex with a clear band. The reaction results were observed between two substances to determine the presence of specific antigens or antibodies.

Toxicity test

A total of 40 pathogen free Kunming mice (20 males and 20 females) (Beijing Sibeifu Biotechnology Co., Ltd., Beijing, China), aged from 2 to 3 months, and weighed approximately 18 to 19 g were involved in this study. All animal experimental procedures were approved by the Ethics Committee of Cangzhou Medical College (Cangzhou, Hebei, China) (Approval No. 2023BH-036-01). The experimental animals were divided into 4 groups with 10 mice in each group, including normal control group administered



(a) Growth curve of Lutheran conjugated yeast (b) Yeast colony count in sauce mash

Figure 1. Comparison of the growth status of Lutheran conjugated yeast.

with normal saline and three experimental groups administrated 0.2 mL of 7.5×10^9 cfu/mL, 3.75×10^{10} cfu/mL, and 7.5×10^{10} cfu/mL *Zygosaccharomyces ruckeri* for one week. The living conditions of the mice were recorded, and the tail venous blood samples were collected. The blood biochemical indicators and organ effects were examined including total bilirubin (TBIL), aspartate aminotransferase (AST), glucose (GLU), total protein (TP), and alkaline phosphatase. (ALP), creatinine (CREA), albumin (ALB), total cholesterol (CHO), and triglyceride (TG).

Statistical analysis

SPSS 23.0 (IBM, Armonk, New York, USA) was employed in this study for statistical analysis. The data was expressed as mean ± standard deviations (SD). The student t-test was used for results comparison with *P* value less than 0.05 as the significant difference between groups.

Results and discussion

***Saccharomycetes* growth analysis**

The growth of *Zygosaccharomyces ruckeri* was analyzed at 0, 10, 25, 30, and 50 h. The results showed that, from 2 to 24 h, the growth curve was steep, indicating the growing of *saccharomycetes* during this period. From 25 to 50 h, the optical density (OD) of the *saccharomycetes* gradually weakened, indicating that the saccharomycetes growth was in a relatively stable state (Figure 1a). The colony forming unit (cfu) of *saccharomycetes* was counted with the inoculating concentration of 1×10^6 cfu/mL, and the growth of the *saccharomycetes* were recorded. In the first 25 hours, the *saccharomycetes* demonstrated a rapid growth state with the cfu reaching the maximum value of 4.98×10^6 cfu/mL at the 25th hour before the cfu gradually decreased. At the 50th hour, the cfu was 2.45×10^6 cfu/mL (Figure 1b). The results indicated that, during the growth process, the *saccharomycetes* grew rapidly during the first 25 hours followed by stabilized growth with gradually decreased cfu.

Metabolite analysis of *Zygosaccharomyces ruckeri*

Zygosaccharomyces ruckeri produced a large amount of volatile substances during the growth and development process, which included alcohols, esters, aldehydes, ketones, *etc.* [13] (Figure 2). In the brewing process of soy sauce, alcohols, esters, and acids directly affected the flavor of food. The more alcohols and esters, the better the flavor of soy sauce [14]. The results showed that most volatile substances reached their highest values around 30 days, and then gradually decreased. No toxic substances were found during the tests. *Zygosaccharomyces ruckeri* also metabolized many non-volatile substances during its growth and development, which included organic acids, amino acids, *etc*. These substances were not easily volatilized and had strong thermal stability. The derivatization of

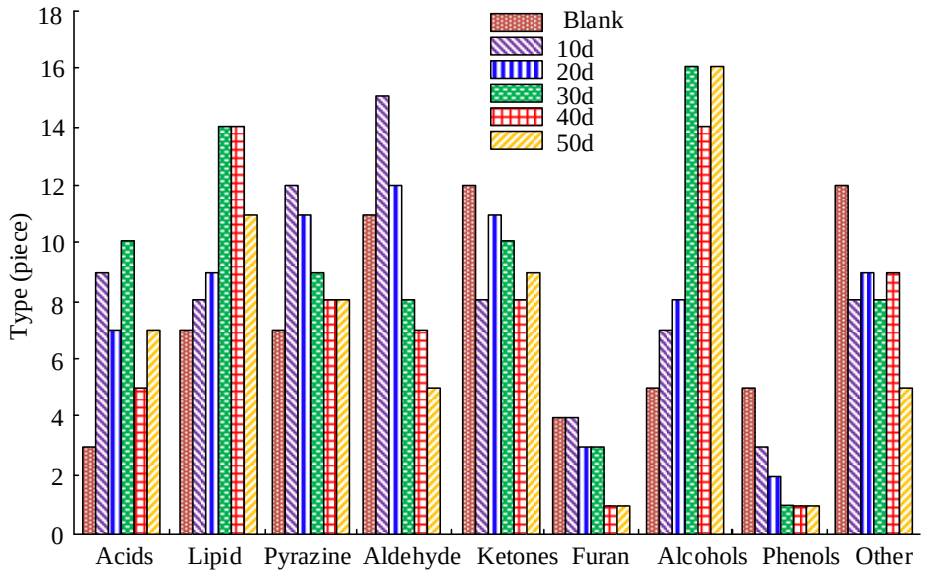


Figure 2. Comparison of volatile substance metabolism.

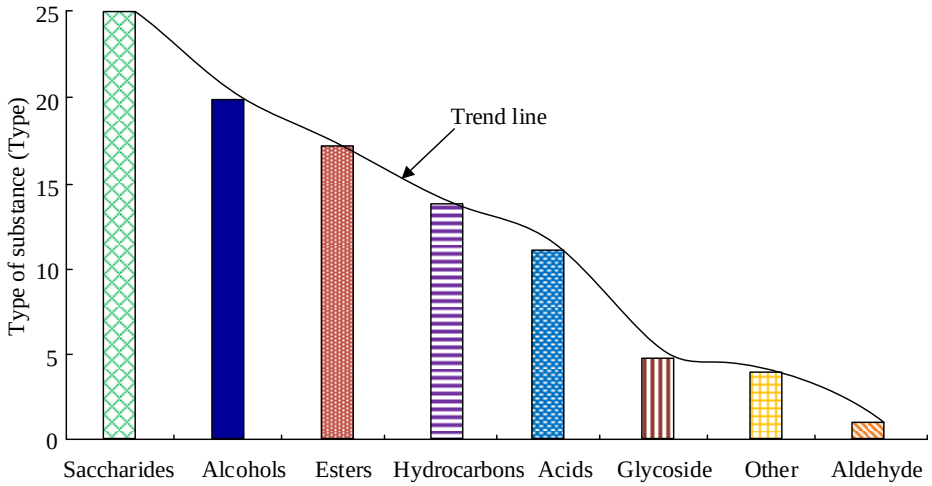


Figure 3. Comparison of internal metabolism in yeast cells.

most substances needed to be completed through silicide reagents [15]. Therefore, silane groups were introduced into cultured molecules and silicon derivatives were detected (Figure 3). The results showed that the derivatives were mainly sugar substances, alcohol substances, ester substances, hydrocarbon substances, and acid substances with 25, 20, 18, 15, and 13 types, respectively. The metabolic composition of non-volatile substances at different periods of time demonstrated that the main substances were esters, sugars, and alcohols. Among them, in the early stage of fermentation, ester substances, acid substances, *etc*. gradually increased, while sugar substances were consumed as nutrients. The number of species of ester substances increased from 10 days to 50 days, reaching 33 on the 50th day followed by sugar substances with the number of types decreased from 10 days to 50 days and remaining at around 27 types. The number of alcohol species changed slightly from 10 days to 50 days, remaining around 24. In

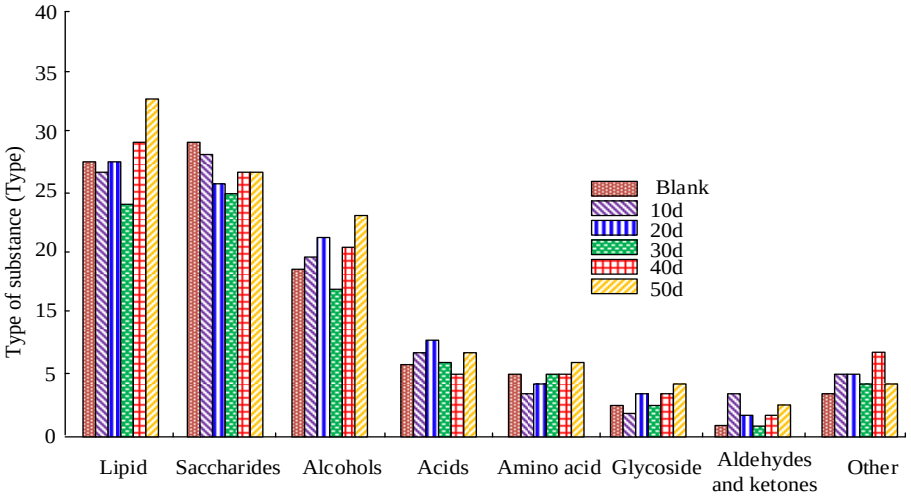


Figure 4. Analysis of non-volatile substance meta.

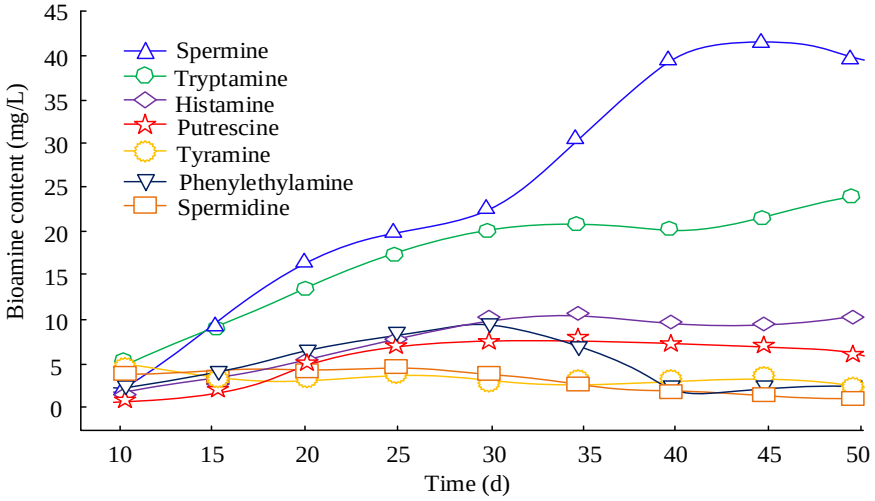


Figure 5. Analysis of biogenic amine content at multiple time points.

addition, the overall proportion of aldehydes was small, maintained between 2 and 3 types (Figure 4). A comprehensive comparison showed that the contents of both volatile and non-volatile metabolites were within the range of national safety standards.

Analysis of substances formed by fermentation

During the fermentation process of food, many microorganisms that secrete amino acid decarboxylase are required to assist in decomposing the food to form amino acids, while producing biogenic amines [16]. Biogenic amines are common organic compounds found in fermented foods. People who ingest large amounts of biogenic amines may experience symptoms like rhinitis, cough, skin itching, difficulty breathing, and even death [17]. The detection of biogenic amines in different growth periods was shown in Figure 5. Since the fermented food cycle was more than a month, microbial collaborative fermentation produced a large amount of biogenic amines during this process, and there were obvious differences in the content of biogenic amines in different periods. In this study, 10 days to 50 days after

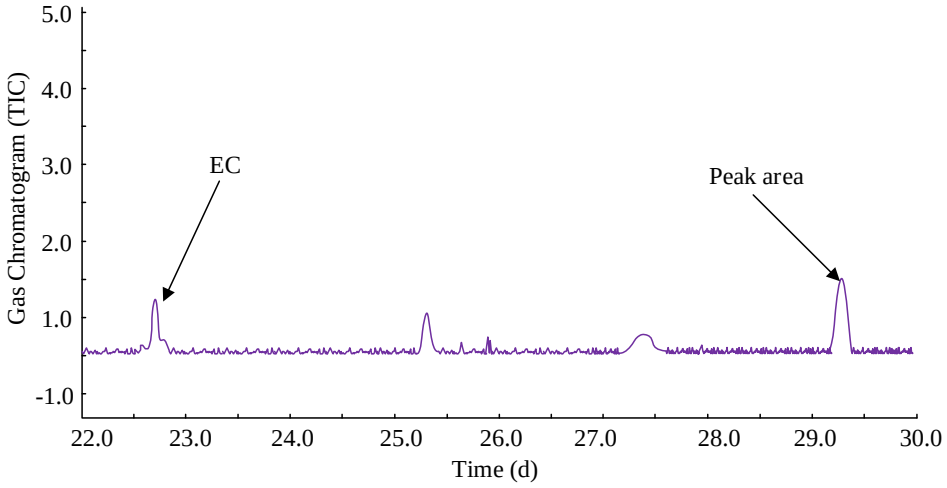


Figure 6. Gas chromatography of EC and internal standard.

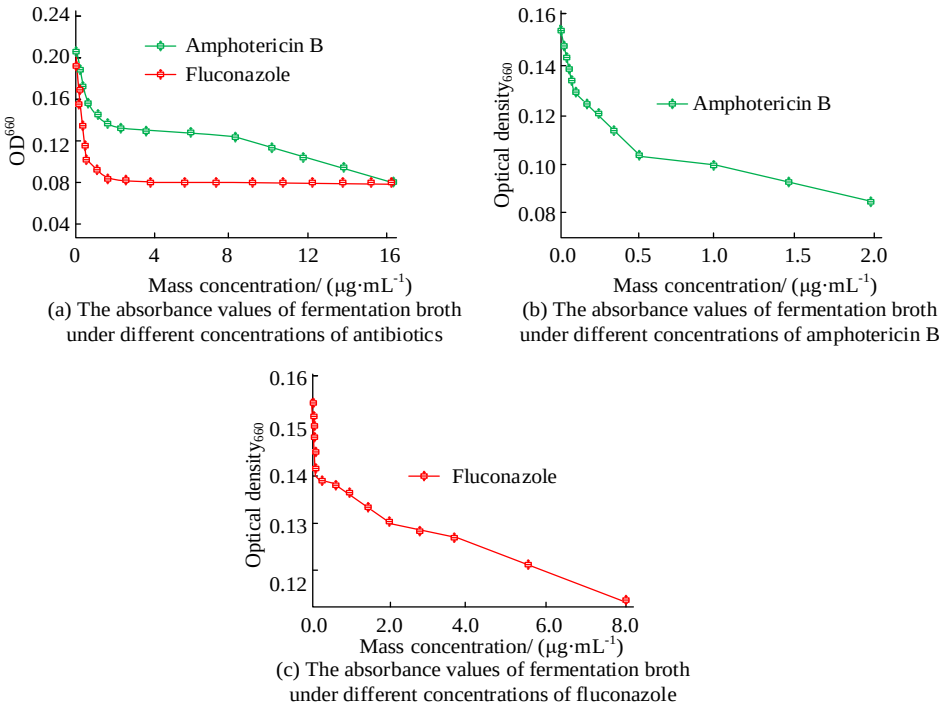
fermentation was selected to detect the biogenic amine contents. Among them, on the 10th day of fermentation, the contents of the seven biogenic amines were all at a low level of 5.0 mg/L. From the 15th to 20th day of fermentation, except for the slow increase of tyramine and spermidine, the contents of other biogenic amines increased significantly, especially spermine and tryptamine. On the 30th day, spermine and tryptamine reached 24.5 mg/L and 19.8 mg/L, respectively. After 30 days, the contents of some biogenic amines began to decrease, including histamine and phenylethylamine. On the 50th day, the main components were spermine and tryptamine, which reached 39.5 mg/L and 24.8 mg/L, respectively. The rest of the contents, from high to low, were histamine, putrescine, tyramine, and spermidine. During the entire fermentation process, the overall changes in tyramine and spermidine were not significant and remained in the range of 2.0 to 5.0 mg/L. During the fermentation process, ethyl carbamate by-product was generated along with the fermented food, which was mainly formed by the combination of amine compounds and ethanol substances [18]. The ethyl carbamate in soy sauce brewing first increased and then gradually decreased with the maximum value of 36.36 μg/L after the 20th day of fermentation. The growth of ethyl carbamate in the early stage of fermentation was related to the formation of ethanol. The decrease in ethanol in the later stage affected its formation, so the ethyl carbamate content gradually decreased. The results of gas chromatogram used equivalence concentration (EC) and peak area to reflect the ethyl carbamate content showed that there was an obvious reaction at 22 days, indicating that the ethyl carbamate content gradually increased. The maximum peak area appeared around 29 days, indicating that the urethane content was the highest at this time (Figure 6).

Drug resistance analysis

The comparative results of drug resistance of *Zygosaccharomyces ruckeri* were shown in Table 1 using the disk diffusion method [19]. Five antibiotics were selected for resistance tests, which included ketoconazole, amphotericin B, voriconazole, nystatin, and fluconazole. Considering that strains had certain resistance to fluconazole, resistance tests could only be used to screen preliminary susceptible yeast. The results demonstrated that, for ketoconazole, nystatin, and voriconazole, they were all sensitive, while amphotericin B was the intermediary resistant and fluconazole was resistance. The absorbances of the fermentation broth under different antibiotics were used to determine the minimum inhibitory concentration (MIC) of *saccharomycetes*. The results showed that amphotericin B and azole drugs had good

Table 1. Comparison of drug resistance under different drugs.

Drugs	Results (mm) (interpretation)	Standard (mm)		
		Intermediary (I)	Resistance (R)	Sensitive (S)
Nystatin	28.5 (S)	15 – 25	≤ 14	≥ 26
Fluconazole	13.2 (R)	15 - 18	≤ 14	≥ 19
Voriconazole	18.25 (S)	14 - 16	≤ 13	≥ 17
Amphotericin B	17.25 (I)	15 – 25	≤ 14	≥ 26
Ketoconazole	29.65 (S)	21 - 27	≤ 20	≥ 28



(a) The absorbance values of fermentation broth under different concentrations of antibiotics

(b) The absorbance values of fermentation broth under different concentrations of amphotericin B

(c) The absorbance values of fermentation broth under different concentrations of fluconazole

Figure 7. Light absorption of fermentation broth with different concentrations.

antifungal effects and were often used in drug resistance experiments. According to the American drug resistance experimental standards, the MICs of fluconazole and amphotericin B are > 4 μg/mL and > 64 μg/mL, respectively [20]. As the concentrations of fluconazole and amphotericin B increased, the absorbance of *Zygosaccharomyces ruckeri* gradually decreased with the inhibitory effect of amphotericin B on *saccharomycetes* more obviously (Figure 7a). When the concentration of amphotericin B was 2 μg/mL, the optical density value dropped to 0.08, and the growth of *saccharomycetes* was 100% inhibited (Figure 7b). Fluconazole also demonstrated a significant inhibitory effect on *saccharomycetes*. When its concentration reached 8 μg/mL, the growth of *saccharomycetes* was also significantly restricted by 80% (Figure 7c). The results indicated that the quality control interval of amphotericin B was between 0.020 and 2 μg/mL, while that of fluconazole was between 0.035 and 8 μg/mL. The MIC of *Zygosaccharomyces cerevisiae* against fluconazole was significantly lower than the US drug resistance test standard.

Toxicity analysis

The body weight changes of the toxicity experimental mice over 30 days showed that mice in each group had no toxic reactions or

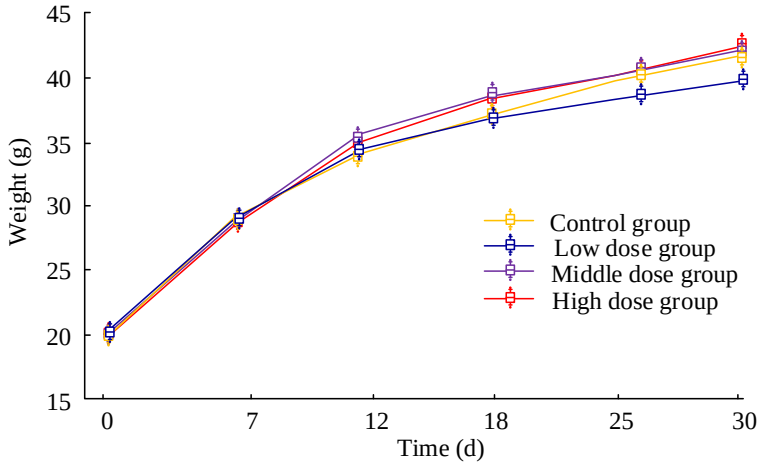


Figure 8. Comparison of experimental mouse mass.

Table 2. Comparison of blood biochemical indicators.

Blood biochemical indicators	Low dose group	Middle dose group	High dose group	Control group	*P
TBIL	10.6 ± 1.28	9.3 ± 123	10.55 ± 1.29*	10.75 ± 1.88	> 0.05
AST	111.62 ± 9.72	107.89 ± 4.25	103.6 ± 3.22*	104.9 ± 6.58	> 0.05
GLU	6.13 ± 1.03	6.26 ± 1.21	6.87 ± 1.16*	6.83 ± 1.19	> 0.05
TP	130.72 ± 5.98	128.974 ± 3.456	136.6 ± 6.27*	122.43 ± 3.25	> 0.05
ALP	154.25 ± 12.15	166.5 ± 18.21	180 ± 15.38*	171 ± 20.18	> 0.05
CREA	26.5 ± 2.41	26 ± 2.38	24.6 ± 1.97 *	27 ± 2.63	> 0.05
ALB	63. 26 ± 7.87	62.8 ± 6.351	68.175 ± 8.92 *	58.1 ± 5.73	< 0.05
GIVE	4.2 ± 0.42	5.2 ± 0.87	5.3 ± 0.93 *	4.5 ± 0.69	< 0.05
TG	6.1 ± 2.11	8.4 ± 3.06	7.01 ± 1.97 *	7.6 ± 2.36	> 0.05

Note: * *P* value was the comparison between the control group and the high-dose group.

deaths. The hair, physical characteristics, activities, and feces of the mice were all examined, and no abnormalities were found. The weight of mice in each group increased significantly, however, there was no statistical weight differences between different groups (P>0.05) (Figure 8). The blood biochemical indicators of experimental mice including TBIL, AST, GLU, TP, ALP, CREA, *etc*. showed that combining different amounts of *Zygosaccharomyces ruckeri* had different effects on the blood biochemical indicators of mice. The high-dose group had a certain improvement in TP and ALP compared with other groups, but no statistical significance compared with the reference group (P >0.05). ALB and CHO increased significantly in the high-dose group compared with the reference group ($P < 0.05$) (Table 2). The blood biochemical indicators could effectively reflect whether the functions of various organs and the lives of mice were affected and provide data support for pathological research. The results suggested that feeding different amounts of *Zygosaccharomyces ruckeri* to mice had no significant effect on the blood lipids of mice. To further analyze the safety impact of *Zygosaccharomyces ruckeri* on mice, multiple organs of the mice were examined

Table 3. Comparison of organ effects in mice.

Group	Low dose group	Middle dose group	High dose group	Control group	*P
Kidney	1.43 ± 0.24	1.33 ± 0.28	1.44 ± 0.28	1.45 ± 0.17*	> 0.05
Spleen	0.32 ± 0.16	0.36 ± 0.15	0.35 ± 0.09	0.32 ± 0.06*	> 0.05
Heart	0.58 ± 0.09	0.56 ± 0.17	0.56 ± 0.12	0.50 ± 0.03*	> 0.05
Thymus	0.55 ± 0.13	0.58 ± 0.22	0.58 ± 0.12	0.54 ± 0.45*	> 0.05
Liver	3.82 ± 0.59	3.86 ± 0.82	4.16 ± 0.58	4.21 ± 0.49*	> 0.05

Note: * *P* value was the comparison between the control group and the high-dose group.

including kidney, spleen, heart, thymus, and liver. The organ index was introduced to reflect the health changes of mouse organs, which was the ratio between the wet weight of organs and body weight. The results showed that the organ index of the medium-dose group decreased, but no significant difference to that of the control group ($P > 0.05$). In the comparison of the spleen, the organ index of the medium-dose group also had the largest change. Compared with other dose groups, the organ index increased, but no statistical significance was found compared with the control group. In addition, comprehensive comparison found that the low-dose group had a certain impact on liver organ index, however, there was still no significant difference compared to control group (Table 3). The results suggested that using different amounts of *Zygosaccharomyces ruckeri* to feed experimental mice would not have a significant impact on the activity and health of the mice. It also showed that, in the toxicity experiment, *Zygosaccharomyces ruckeri* had high safety and no impact on the survival indicators of experimental mice and no obvious toxic and side effects. The results illustrated that *Zygosaccharomyces ruckeri* had certain safety and practical value in the field of food production.

Conclusion

In the modern food industry, *Zygosaccharomyces ruckeri* has a very wide range of applications. The study analyzed the safety of Lu's yeast using traditional soy sauce fermentation experiments. Cultivate experimental strains using sauce and mash as nutrient substrates and analyzing the metabolic products of the strains using gas chromatography to determine the metabolic characteristics of *Zygosaccharomyces ruckeri*, simultaneously, analyzing the drug resistance and safety of *Zygosaccharomyces ruckeri*. Alcohol, esters, acids, sugars, and other substances were detected in the analysis of metabolites, which directly affected the flavor and taste of soy sauce. On the 50th day of fermentation, the biogenic amine content reached its peak and gradually decreased with increasing fermentation time. Ethyl carbamate reached its peak on the 30th day of fermentation and gradually decreased thereafter. In drug resistance analysis, the maximum inhibitory concentration of amphotericin B on *saccharomycetes* was 2 μg/mL, while the maximum inhibitory concentration of fluconazole was 8 μg/mL. A mouse toxicity experiment was conducted, and no abnormal phenomena were observed. The results indicated that *Zygosaccharomyces ruckeri* had a certain level of safety. However, in metabolite detection, some volatile metabolites were severely lost. In the future, research on volatile metabolites should be strengthened to improve the effectiveness of research.

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