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Screening and Fermentation Characteristics of Excellent Boza Strain

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Abstract Boza is a special fermented dairy product in Xinjiang Uyghur Autonomous Region (XUAR). Boza has the characteristics of CO_2 mouthfeel, low sugar content, and dense texture, and is produced from barley, millet, or corn as raw materials using the traditional natural fermentation technology. In this study, six strains each of yeast and lactic acid bacteria isolated from XUAR Boza were used as test strains for single strain selection and composite starter screening. The analytical indicators mainly included ethanol production ability, acid production, tolerance, fermentation force, and growth. Saccharomyces cerevisiae Y8 and Lactobacillus plantarum L9 were selected as the fermentation strains suitable for Boza fermentation. The results will provide scientific inputs for utilization by the Boza fermentation industry.

Keywords: *Boza, yeast, lactic acid bacteria, Screening*

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1. Introduction

Boza is a naturally fermented beverage with low sugar and alcohol content made from thick grains [1,2] such as corn and millet. It is a national fermented beverage of significant nutritional and economic value, produced in the Xinjiang Uyghur Autonomous Region of China. The taste of Boza depends on the production method and raw materials used, as well as on the starter. Owing to the diversity of microorganisms in nature, the fermented product presents various flavors. Currently, screening and optimization of fermenting strains for the production of different fermented beverages is being actively pursued. Research on traditional fermented beverages mostly focuses on the isolation and identification of the flora and strains involved in fermentation. For example, Almeida et al. observed that the main strains in cauini are *Lactobacillus pentosus* and *Lactobacillus plantarum*, which were critical for the industrial production of fermented beverages [3]. Traditional Boza is produced in Bulgaria, Turkey, and other countries. The main raw materials for producing traditional fermented Boza are barley, oats, millet, and corn, and the main microorganisms included yeast and lactic acid bacteria [4]. The vitamins produced by lactic acid bacteria and yeast in Boza are able to increase the nutritional value of the product. Lactic acid bacteria also synthesize antibiotic peptides, which prolong the shelf-life of the product [5]. Boza can also be made into non-alcoholic, sour, fermented, or non-fermented beverages [6,7].

Reports on the microbial components, mainly yeasts, used for Boza fermentation are limited. For example, Heathman et al.reported that microbial components such as L. plantarum, L. brevis, Bacillus cereus, Acetobacter baumannii, and brewer's yeast were present in Boza [8]. Aheberdi et al.identified three strains of S. cerevisiae, five strains of Cryptococcus, and four strains of Candida from the yeasts in Ili Boza [9]. However, studies on the screening and application of the starter for Boza are rare. Thus, the traditional fermented beverages in Xinjiang Uyghur Autonomous Region (XUAR hereafter) can be said to be of "hundreds of productions, and hundreds of products". However, the summary on the characteristics of fermentation on the different of types strain, especially, the bacteria isolated from XUAR Boza were still fragmental. In this study, the yeast and lactic acid bacteria isolated from XUAR Boza were used as test strains, and their fermentation characteristics were investigated for single strain screening. The selected strains were used to inoculate fresh substrate as a starter to ferment Boza, then test strains' fermentation capability. Its physical and chemical properties and sensory attributes were considered indicators for further determining the best Boza starter, thereby providing a scientific basis for the utilization of Boza starter on an industrial scale.

2. Materials and Methods

2.1. Test Strain

Yeast strain: Representative yeast strains were isolated and purified from naturally fermented Boza, including *Saccharomyces cerevisiae* (Y4, Y8, Y19), *Cryptococcus* (Y20), *Pichia* (Y23, Y34), respectively. After culturing on

slants, the strains were placed in a refrigerator at 4°C for storage.

Lactic acid bacteria strain: The lactic acid bacteria strains were isolated and purified from the naturally fermented Boza and included *Weissella* (L1), *Pediococcus* (L2), *Lactococcus* (L8), *L.s plantarum* (L9, L12), and *L. rhamnosus* (L15), which were cultured on slants and placed in a refrigerator at 4 °C for subsequent use.

Media

Yeast extract-peptone-dextrose (YPD) liquid medium, wort agar medium, wort medium; MRS agar medium and MRS broth medium were used.

2.2. Instruments and Equipments

Instruments

AN133 electronic analytical balance (Shanghai Mettler Toledo Instrument Co., Ltd.); MC-SP1105 multi-function induction cooker (Guangzhou Meite Life Electric Manufacturing Co., Ltd.); DL-1 electronic universal furnace (Beijing Yongguangming Medical Instrument Co., Ltd.); HH-S electric thermostat digital display water bath (Shanghai Heng Technology Co., Ltd.); DHP-9162 electric thermostat incubator (Shanghai Mingqiao Precision Instrument Co., Ltd.); YXQ-LS-18SI portable pressure steam sterilization pot (Shanghai Boxun Industrial Co., Ltd. Medical Equipment Factory); SW-CJ-2FD Clean Workbench (Suzhou Antai Air Technology Co., Ltd. bcd-215kam); refrigerator (Qingdao Haier Co., Ltd.); XSP-2CA Microbiology Microscope (Shanghai Youke Instrument Co., Ltd.); UV-1200 UV-visible luminometer (Shanghai Meistar Instrument Co., Ltd.); SNB-2 viscometer (Shanghai Fangrui Instrument Co., Ltd.); PHS-3C acidity meter (Shanghai Dapu Instrument Co., Ltd.) etc.

2.3. Activation of Strains

The yeast strain isolated and purified from the naturally fermented Boza was taken from the refrigerator, using to SW-CJ-2FD Clean Workbench (Suzhou Antai Air Technology Co., Ltd. bcd-215kam) the yeast strain was inoculated into wort agar medium under aseptic conditions, followed by activation for 1-2 times and propagation in YPD liquid medium (cultivation at 30°C for 24 h). Subsequently, it was stored at 4°C.

Eight strains of lactic acid bacteria isolated and purified from the naturally fermented Boza were taken out from the refrigerator and inserted into MRS solid medium under sterile conditions, after which the strains were activated 1 to 2 times and expanded in MRS liquid medium (cultivation at 30°C for 24 h in the DHP-9162 electric thermostat incubator (Shanghai Mingqiao Precision Instrument Co., Ltd.)). After that, they were stored at 4°C.

Screening of excellent strains

2.4. The Tolerance of Individual Strains

Different sugar concentrations (0%, 10%, 20%, 30%, 40%, and 50%) were prepared in YPD [10,11,12] and MRS [13] medium with glucose or sucrose, and different ethanol concentrations were prepared with absolute ethanol (0%, 5%, 10%, 15%, and 20%). The pH was adjusted to six different values (2.0, 2.5, 3.0, 3.5, 4 and 4.5) with HCl (1 mol/L) and NaOH (1 mol/L). After sterilization, the activated yeast liquid with 3% (v/v) inoculum was inoculated into the above liquid medium with different sugar and alcohol concentrations, and of different pH. Cell culture was performed at 28°C in the DHP-9162 electric thermostat incubator (Shanghai Mingqiao Precision Instrument Co., Ltd.). The OD_{600} value was measured and plotted after 48 h.

2.5. Yeast Fermentation Characteristics

Comparison of the gas production performance of yeast

The Duer tube fermentation method was used to seed the yeast of the second generation into the YPD liquid medium test tube [10,14] with built-in Duchenne tube, and the inoculum quantity was 3% (v/v). The culture was incubated at 28°C for 48 h in the DHP-9162 electric thermostat incubator (Shanghai Mingqiao Precision Instrument Co., Ltd.) to observe gas production. The test was repeated several times.

Comparison of yeast fermentation and ethanol production ability

Ten milliliters of fragrant pear juice were dispensed into test tubes and sterilized. The fresh test yeasts were inoculated into the sterilized medium, and cultured at 28°C for 48 h. The mixed liquid in the test tube was connected to a sterile flask and 95 mL of distilled water was added to observe the gas collection in the flask. The weight was measured after every 8 h, and the bottle was shaken beforehand to remove carbon dioxide. The bottle weight gradually decreased with fermentation time until the extent of reduction was less than 0.2 g, which indicated that the fermentation was complete.

To study the ethanol produced by the fermentation of the yeast strain isolated from Boza, the yeast to be tested was inoculated in a liquid wort medium of 10°Bx and fermented at 28°C for 48 h to determine the ethanol content in the fermentation broth [15]. Determination of alcohol content: Density distillation method (according to GB/T15038-2006, the general analysis method for wine and fruit wine) was used.

2.6. Fermentation by Lactic Acid Bacteria

Determination of the growth curve of lactic acid bacteria and evaluation of acid production

The lactic acid bacteria (5% (v/v)) isolated from Boza was activated and inoculated in the MRS liquid medium and cultured at 37°C. The uninoculated MRS liquid medium was used as a blank control. A sample was collected at 0 h, and sampling was performed thereafter after every 2 h to measure the absorbance. The test was repeated several times. After 48 h of incubation, the values were measured and plotted. At the same time, 15 mL of the 0 h samples were taken to measure the pH. Each strain was cultured at 30°C and sampled every 8 h to measure the pH. The mean valuewas calculated (n = 3) [16]. After 48 h of incubation, the measured pH values were plotted.

2.7. Screening of Starter for Multiple Strains

Preparation of Boza-saccharified hydrolyzate:

Barley, hazelnut (black skin), and corn were mixed in 1:2.5:1.5 ratio. This was then mixed with water in 1:3 ratios, followed by stirring and mixing into a paste. Then, gelatinization was performed until the raw materials were cooked. After cooling to 65°C, 1.1% α-amylase (activity > 6000 U/mg) was added for liquefaction. β-amylase (activity > 50 U/mg; 0.01%) and pullulanase (1 U/mg) were added for saccharification. The enzyme was denatured after 3 h.

Comparison of the fermentation characteristics of different starter combinations:

The three selected strains of lactic acid bacteria and yeast were co-fermented for 30 h, and the inoculum of yeast and lactic acid bacteria was 2% (v/v). *S. cerevisiae* (1%) was inoculated first, and then fermented at 30°C for 8 h. Next, the isolated and purified *L. faecalis*, *P. pentosaceus*, *L. rhamnosus*, and *L. plantarum* were inoculated with 1% (v/v) inoculum and fermented at 30°C for 24 h in the electric thermostat incubator. The alcohol content, pH, total acid, viscosity, water holding capacity, and sensory index of the fermentation broth were determined, and the best combination was selected as the starter for Boza brewing [17]. pH was directly Measured with a pH meter. Determination of total acid: Indicator method (according to GB/T15038-2006, the general analysis method for wine and fruit wine) was used. Viscosity was measured using a SNB-2 viscometer.

2.7. Sensory Evaluation of Boza

Sensory evaluation was performed by twelve panelists (three men and five women, 23–50 years of age), who had more than 200 h of previous experience in the sensory evaluation of food products and an additional 20 h training on evaluation of Boza. The sensory evaluation of Boza included a comprehensive score of the taste, odor, mouthfeel, and texture, the total score being 100, Sensory indicators were measured using the same method as mentioned in [18].

3. Results and Discussion

3.1. The Tolerance of Individual Strains

Determination of resistance to sugar concentration. The sugar tolerance ability of the yeast strain isolated from Boza is shown in Figure 1(A). We observed that with the increase in sugar concentration, the growth of each strain first increased and then decreased. The concentration of the bacterial liquid was maximum with favorable growth when the sugar concentration was 30%. When the sugar concentration was 50%, the concentration of strains Y4 and Y8 (*Saccharomyces cerevisiae*) was higher, whereas the growth rates of the other strains were low. The highest concentration of reducing sugars in fermented Boza is generally lower than 30%; hence, these six strains can fulfill the fermentation requirements.

The results regarding the sugar tolerance of the lactic acid bacteria strain isolated from Boza are shown in Figure 1(A). The growth of each strain first increased and then decreased with the increase in sugar concentration. The concentrations of strains L1, L2, L8, L9, and L12 were highest in the presence of 30% sugar with good growth. When the sugar concentration reached 50%, the concentrations of L9 (*Lactobacillus plantarum*) and L15 (*L. rhamnosus*) were high, and the growth rates of the other strains were low. The concentration of reducing sugar in the fermentation Boza is generally less than 30%, and therefore, these six strains met the Boza fermentation requirements.

Determination of alcohol resistance

The results of alcohol tolerance of yeast strains isolated from Boza are shown in Figure 1(B). The growth of each strain decreased gradually with increase in alcohol concentration. The growth of each strain was good when the alcohol concentration was less than 10%. When the alcohol concentration reached 20%, the concentration of Y19 (*Saccharomyces cerevisia*e), Y23 (*Pichia pastoris*), and Y34 (*P. pipien*s) decreased significantly, indicating that their alcohol resistance was low. Y4 (*Saccharomyces cerevisiae*) and Y8 (*Saccharomyces cerevisiae*) strains displayed stronger alcohol resistance. In general, the alcohol content in Boza does not exceed 10% (v/v), and hence all six strains can meet the fermentation requirements.

The results of alcohol tolerance of the lactic acid bacteria strain isolated from Boza are shown in Figure 1(B). The growth of each strain gradually decreased with the increase in alcohol concentration. The strains grew well when the alcohol concentration was less than 5%. When the alcohol concentration reached 10%, the concentration of L2 and L8 strains decreased significantly, indicating that their alcohol resistance was very low. L1, L9, L12, and L15 strains exhibited strong alcohol resistance, and the alcohol content of Boza generally did not exceed 10% (v/v). Therefore, with the exception of the L2 and L8 strains, the other strains met the fermentation requirements.

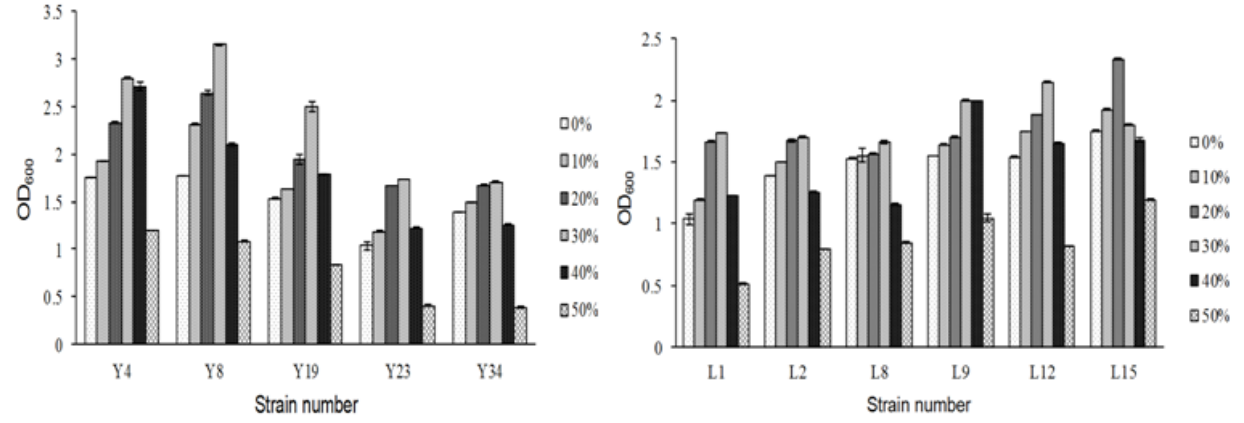
Acid resistance

Determination of acid resistance

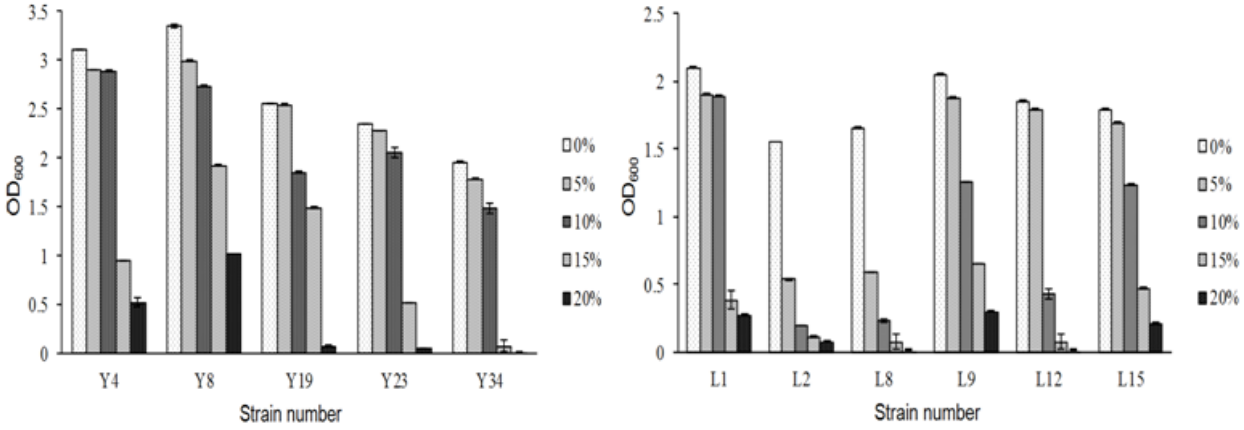
The results of acid resistance of the yeast strains isolated from Boza are shown in Figure 1(C). With the increase in pH, the growth of each strain first increased and then decreased. Five strains namely, Y4, Y8, Y19, Y23, and Y34, grew well within the range of pH 4–5.5. Growth decreased when the pH was lower than 3.5. Y8 and Y19 (*S.s cerevisiae*) grew well when pH was lowered to 3, exhibiting relatively strong acid resistance, whereas the other strains were difficult to grow. The pH of the fermentation Boza is approximately 3.5, and therefore, the strain Y8 met the Boza fermentation requirements.

The results of acid resistance of the lactic acid bacteria strains isolated from Boza are shown in Figure 1(C). The growth of each strain gradually increased with the increase in pH. All six strains grew well in the range of pH 4–5.5. The growth of the strains weakened when the pH was lower than 3.5. The strains L9, L12, and L15 (*L. plantarum* and *L. rhamnosus*) grew well, showing strong acid resistance at pH of 3, whereas the other strains were difficult to grow. The average pH of the human gastric juice is ãpproximately 3 [15], and the ratio of sweetness to acidity of Boza may affect its taste; hence, the pH of 3

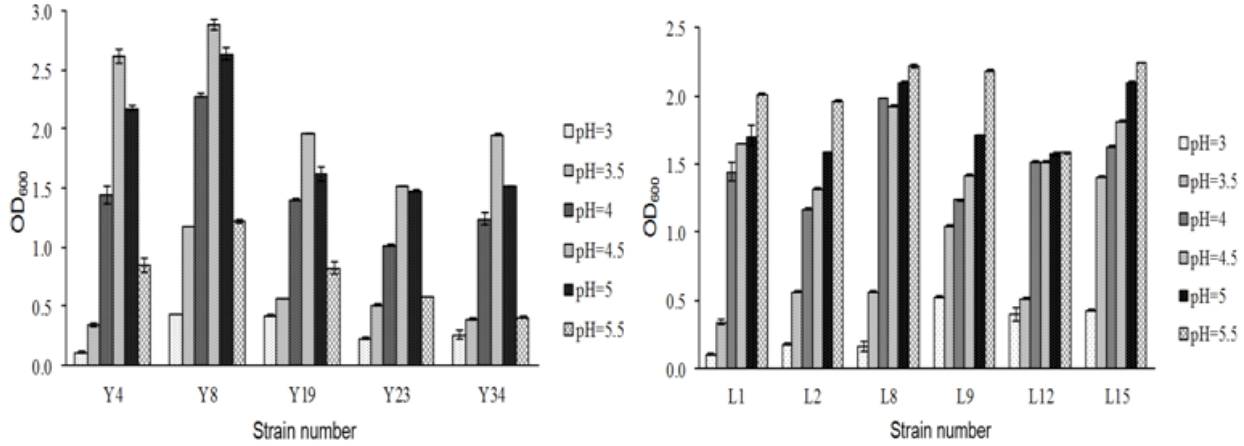
was suitable. Therefore, strains L9, L12, and L15 can meet the Boza fermentation requirements.



(A) Growth of each strain at different sugar concentrations



(B) Growth of each strain at different alcohol concentrations



(C) Growth of each strain at different Ph

Figure 1. Growth of each strain at different sugar concentrations (A), alcohol concentrations (B) and Ph (C)

Table 1. Gas production of yeast strains observed in the Duchenne tube

Strain number	Gas production after 12 h of fermentation	Gas production after 24 h of fermentation	Gas production after 36 h of fermentation	Gas production after 48 h of fermentation
Y4, Y8	+	+	++	++
Y19	++	++	++	++
Y20	-	-	-	-
Y23, Y34	+	+	+	+

Note: -: no gas production, +: 1/3 volume of Duchenne tube, ++: 2/3 volume of Duchenne tube, +++: 3/3 volume of Duchenne tube.

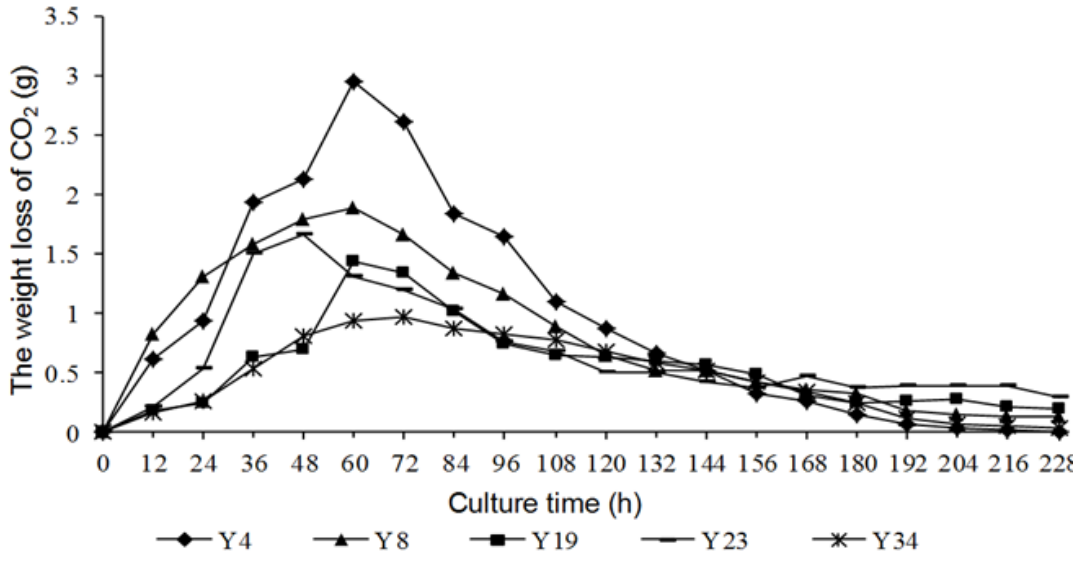
3.2. Yeast Fermentation Characteristics

Comparison of the gas production performance of yeast

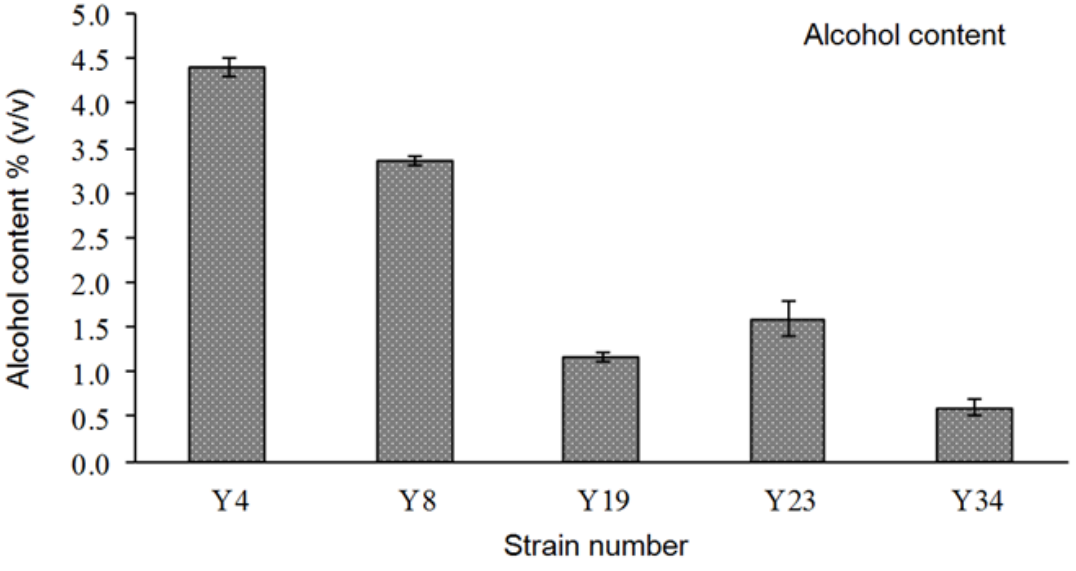
The gas production of the yeast strain isolated from Boza is shown in Table 1. For five strains, Y4, Y8, Y19, Y23, and Y34, the gas volume accounted for 2/3rd of the volume of the Duchenne tube after 48 h. The gas volume of strain Y20 was lesser than 1/3rd the volume of the Duchenne tube, and therefore these strains were discarded.

Comparison of fermentation and ethanol production capacity of yeast

The results of the fermentation and ethanol production by the five yeast strains isolated from Boza are shown in Figure 2(A,B). Within a certain times range, the fermentation of each isolated strain increased at first and then decreased with fermentation time. Y4, Y8, and Y23 showed good fermentation ability. During the logarithmic growth period, the number of viable bacteria increased rapidly; hence, these three strains can effectively inhibit the proliferation of other bacteria. However, the fermentations of Y4 and Y23 were unstable, with more obvious fluctuations. In addition, the amount of ethanol produced by Y4 was higher, and that produced by Y23 was considerably low. Therefore, these two strains were discarded. The fermenting abilities of Y19 and Y34 were too low, along with slow initiation of fermentation and cell proliferation, and low production of carbon dioxide. As their ethanol production capacity was extremely low, these strains were discarded. In summary, Y8 isolated from Boza met the requirements of rapid initiation of fermentation and cell proliferation rate of Boza with good and stable fermentation capacity. The amount of carbon dioxide produced was high, while the amount of ethanol produced was low (3.4%; v/v). Therefore, Y8 was the most suitable strain for the fermentation of Boza.



(A) Fermentation power of yeast



(B) Results of alcohol production of yeast

Figure 2. Fermentation power (A) and results of alcohol production (B) of yeast

3.3. Fermentation by Lactic Acid Bacteria

Growth curve of lactic acid bacteria and determination of acid production performance

The growth curves of the six strains of lactic acid bacteria isolated from Boza are shown in Figure 3. We observed that in a certain time range, the fermentation of the isolated strains increased with fermentation time, indicating that the growth of each strain in the media was good and that it was the best time for lactic acid bacteria to produce lactic acid by metabolizing sugar. L2, L9, and L15 showed strong vigor. During the logarithmic growth period (4 to 14 h), the number of viable bacteria increased rapidly and the pH decreased rapidly, which can effectively inhibit the proliferation of other acid-tolerant bacteria. The number of viable cells decreased after reaching the stationary phase (14 to 20 h), and continued to decrease after entering the decay period (after 20 h). This was because large quantities of nutrients were consumed during the growing and multiplication of lactic acid bacteria, which induced the bacteria to enter the decay phase. As the bacteria still remained in the culture solution after death, the absorbance remained stable. L1, L8, and L12 displayed low vigor, and the logarithmic growth period was from 4 to 16 h. The number of viable bacteria increased slowly till it reached a stable period, after which it again increased slowly and then remained stable without any further increase. Therefore, the L2, L9, and L15 strains can meet the requirements of Boza fermentation. If these strains are inoculated into Boza with the highest number of viable bacteria, they will show the best growth vigor and optimal fermentation characteristics that will ensure the quality of the product [19].

The acid production rate of the strain is an important indicator of the fermentation vigor of the Boza strain. During the fermentation of Boza, the acid was mainly produced by the lactic acid bacteria. Figure 4 shows that strains L9 and L15 produced acid more rapidly than others. After 8 h, the pH reached 4.23 and 4.11, respectively, for L9 and L15. The rapid decrease in pH can effectively inhibit the propagation of acid-tolerant bacteria, thereby reducing the accumulation of harmful metabolites. The other strains produced acid at slightly lower speed, and after 24 h, the pH reached approximately 3.8. Therefore, the L9 and L15 strains can meet the requirements of Boza fermentation. L9 (*L. plantarum*) and L15 (*L. rhamnosus*) inoculated in Boza ensured stable fermentation and imparted the best texture and flavor to Boza [20].

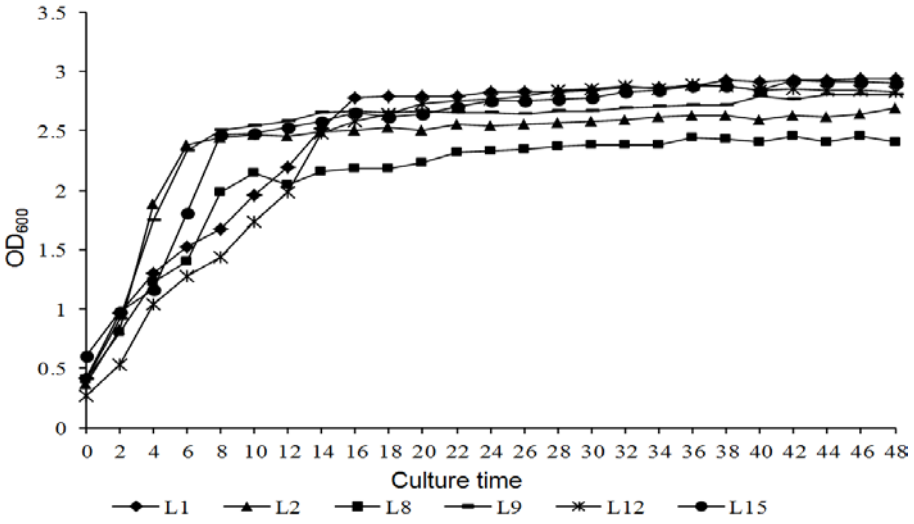


Figure 3. Growth curves of lactic acid bacteria strains

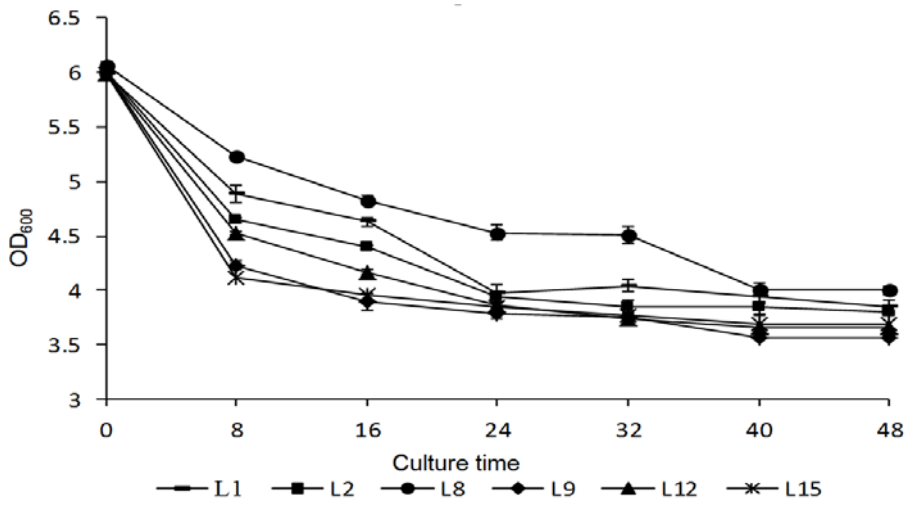


Figure 4. Changes in the pH of lactic acid bacteria in MRS medium

Table 2. Determination of fermentation performance and sensory evaluation results of Boza produced by different fermentation starters

Compound starter	pH value	Total acid (g/L)	Viscosity (Pa.s)	Water holding capacity (%)	Alcohol (% v/v)	Sensory
Group 1: Y8+L2	3.83±0.01	1.9±0.3	6.628±0.47	13.16±0.05	2.9±0.0	87±2.1
Group 2: Y8+L9	3.48±0.20	2.5±0.3	8.990±0.19	10.28±0.12	2.8±0.1	94±1.2
Group 3: Y8+L15	3.61±0.05	2.1±0.1	7.349±0.12	11.57±0.06	2.8±0.0	90±0.5

Note: Y8 was *Saccharomyces cerevisiae;* L2 was *Pediococcus;* L9 was *Lactobacillus plantarum*, and L15 was *Lactobacillus rhamnosus*.

3.4. Starter Screening for Compound Strains

To identify the compound strain starter suitable for Boza fermentation, three groups of compound strains were inoculated in the Boza hydrolysate, and stationary fermentation was performed at 30°C for 30 h. The fermentation performance and sensory evaluation results of the three groups of compound strains are shown in Table 2. We observed that the compound strains of group 2 resulted in the lowest pH and highest total acid, indicating that the strain had stronger acid production ability, while groups 1 and 3 have relatively weaker acid production capacity. Acid is the main contributor to the flavor of Boza. The higher the total acid value, the better the overall flavor of Boza. Thus, group 2 was most suitable for Boza fermentation. The viscosity of Boza produced by the group 2 compound starter was higher and its water loss rate was lower. The Boza viscosity of the group 2 and group 3 was relatively lower, and the water loss rate was correspondingly higher. Based on preliminary judgement, the viscosity of Boza was inversely related to the water loss rate. Boza is a low-alcohol fermented beverage. The alcohol contents of the three groups of compound strains were between 2.8 and 3.0%, indicating that the ethanol contents of the three groups were controlled at a low level.

The taste, odor, mouthfeel, and texture of Boza produced by the group 2 compound starter were good, and the results of overall sensory evaluation (score of 94 points) showed that the color was uniform, and the taste was smooth and robust, with moderate sour and sweet flavor, which accounted for the high sensory score. The scores for Boza produced by the other two groups of composite starters were relatively low, namely 87 and 90 points, respectively.

Based on the above indicators, Boza fermented by the group 2 strains presented good acid production capacity, high viscosity, and low water loss rate, and had superior mellow as well as sweet and sour taste.

Various yeasts are involved in the natural fermentation of Boza, which is unstable and difficult to store. An excellent fermentation strain can reduce the production cost of Boza, and improve the efficiency of the Boza industry and quality of Boza. As a result, characteristics such as rapid initiation of fermentation, high cell proliferation rate, good fermenting capacity, high yield of CO_2 [17], low alcohol content, outstanding flavor, and the ability to exist as symbionts with lactic acid bacteria constitute the fundamental starting points for screening a good starter strain. Interestingly, fermentation of lactic acid bacteria can generate acid [4,5,6]. In this study, six strains each of yeast and lactic acid bacteria isolated from Boza were selected as test strains to screen the single strain and compound strain starter. Finally, the mixed fermenting agents, namely *S. cerevisiae* and *L. plantarum*, were selected. However, the storage of Boza at the end of the fermentation was not tested, which is a limitation of this study. Our results showed that the two strains, *S. cerevisiae* and *L. plantarum*, fulfilled the basic requirements of large-scale Boza production. Thus, further studies should investigate the effect of this mixed strain on the storage stability of Boza.

4. Conclusion

Types of strains and their fermentation characteristics are very important to study and improve the flavor of different fermented foods. Very few works have studied the bacteria isolated from XUAR Boza. This experiment aimed to screen the strains from Boza. In the experiment, six different yeasts and lactic acid bacteria each isolated from Boza were selected for the single strain screening, and the analysis indicators mainly included sugar tolerance, alcohol resistance, acid resistance, bacterial fermentation power, growth, and acid production performance.

One excellent yeast strain and three excellent strains of lactic acid bacteria (Y8, L2, L9, and L15 which indicates *S. cerevisiae*, *P. pentosaceus*, *L. plantaru*m, and *L. rhamnosus*, respectively) were screened. In addition, about the fermentation ability of strains in the XUAR Boza has not been reported by previous works. Four types of fermentation strains were used as the compound starter, and the mixed strains were inoculated into Boza. The physicochemical and sensory indicators were used as reference indicators to screen the compound starter of Boza. In the end, *S. cerevisiae*, which was suitable for the Boza fermentation, was screened, and Y8 (*S. cerevisiae*) and L9 (*L. plantarum*) were used as compound fermentation strains.

The fermented Boza had the better flavor, and this taste were caused by good acid capacity, superior mellow and sweet mixed with sour. Therefore, screening of excellent fermentation strains is necessary to alter natural fermentation to pure or mixed fermentation. Furthermore, the key aroma compounds and non-volatile compounds have to be analyzed during storage of Boza, and the functional characteristics need to be investigated in the future work.

Acknowledgments

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