

Original Research Paper

High-Protein Solid-State Symbiotic Fermentation of Sauce Residue for Probiotic Feed

^{1,3+}Xinhe Zhao, ²⁺Zhuangzhuang He, ¹Wenwei Lu, ³Yubin Zhao, ¹Yuanda Song and ^{1*}Xiaojie Ren

¹Colin Ratledge Center for Microbial Lipids, School of Agriculture Engineering and Food Science, Shandong University of Technology, Zibo, Shandong, China

²School of Agriculture Engineering and Food Science, Shandong University of Technology, Zibo, Shandong, China

³Luzhou Bio-Chem Technology Limited, Linyi, Shandong, China

Article history

Received: 22-07-2020

Revised: 08-10-2020

Accepted: 09-10-2020

Corresponding Author:

Xiaojie Ren

Colin Ratledge Center for Microbial Lipids, School of Agriculture Engineering and Food Science, Shandong University of Technology, Zibo, Shandong, China
Email: renxiaojie2020@163.com

*Contributed equally to this work and should be considered co-first authors

Abstract: The abuse of antibiotics in animal feed has caused a series of problems in livestock breeding, meat products and the natural environment. In this study, sauce residue was used as the main raw material that includes 25.98% of dry-based crude protein, 3.91% of NaCl, 16.28% of crude fat, 11.06% of the ash and the multi-strain solid-state symbiotic fermentation technology was used to increase the amount of probiotics and the crude protein content of feed. Meanwhile, the addition of functional probiotics improved the feed palatability, while reducing the use of antibiotics further improved the cycle utilization of soy sauce residue. This study systematically optimized the symbiotic conditions of two probiotics, *Lactobacillus bulgaricus* and *Saccharomyces cerevisiae*. This study defined viable cell count and crude protein content as the core targets for analysis and evaluation. Firstly, the optimal carbon source concentration, the temperature and the inoculation process of symbiotic fermentation were determined by single factors; then the symbiotic fermentation conditions (inoculation volume, material-liquid ratio, fermentation time) were optimized by orthogonal test. The results showed that 40 g/L glucose/lactose (50/50%) was the best carbon source for sauce residue fermentation. The optimum fermentation conditions were a ratio of material to water of 1:0.8 and an inoculation volume of 15% (v/w) and a fermentation duration of 48 h. Meanwhile, the study found that multi-step temperature control could effectively improve the corresponding targets. The inoculation with *Saccharomyces cerevisiae* first and then with *Lactobacillus bulgaricus* after 24 h, boosted the probiotic quantity and protein content. In optimized conditions, probiotic viable count reached 7.2×10^8 CFU·mL⁻¹ and the crude protein content increased from 25.98% (dry content) to 38.86%, which is 49.6% increase compared with the raw material. This research could provide a reference for the development of high-protein probiotic feed using sauce residue.

Keywords: Sauce Residue, Probiotic Feed, Symbiosis Fermentation, *Saccharomyces cerevisiae*, *Lactobacillus bulgaricus*

Introduction

In recent years, the abuse of antibiotics in animal feed has severely affected animals, meat products and the environment (Kumar *et al.*, 2005). The long-term feeding of antibiotics to livestock and poultry has caused severe damage to the micro-ecological environment of their digestive tracts (Gu and Peng, 2015). Human health is further affected by human consumption of these

livestock and poultry products (Barton, 2000). Therefore, developing probiotic feeds to replace antibiotics could regulate the health of the animals by boosting their immunity. This is significant for the development of quality livestock and poultry meat products.

Sauce residue (soy sauce residue) is a by-product of soy sauce brewing (Chen *et al.*, 2017). The global annual output of soy sauce residue is more than 100 million tons per year and this causes increasingly serious

environmental pollution problems (Gong *et al.*, 2018). Some manufacturers dispose it by landfill, which results in soil salinization and serious soil pollution (Duan *et al.*, 2019). Sauce residue is rich in nutrients such as protein, fat, etc., which can breed microorganisms (Chen *et al.*, 2017). A high crude fiber content and some anti-nutritional factors of sauce residue make it difficult to be absorbed by the digestive tracts of animals. So, it is difficult to use original sauce residue directly as feed (Asada *et al.*, 2010; Murasawa *et al.*, 2013). With the higher requirement of environmental protection, the reuse of sauce residue is urgently needed.

Orthogonal experimental design is a design method to study multi-factors and multi-levels. According to the orthogonality, some representative points are selected from the comprehensive test. It can judge the significant degree of the influence of factors on the test index. Meanwhile, it can find out the optimal level of test factors and the optimal combination within the test range and estimate the size of the test error. But it cannot examine the interaction between factors when the purpose of the experiment is mainly to find a good horizontal combination. Therefore, in this study, orthogonal experiments were selected to find the optimal symbiotic fermentation conditions with fewer experiments and to find out the significance of symbiotic fermentation conditions on the viable count and crude protein content.

Microbial fermented feed, especially probiotic fermented feed, can produce a large number of metabolites that are beneficial to animals, decomposing the anti-nutritional factors and greatly improving the nutritional value of the feed (Wang *et al.*, 2019). The ability of probiotics to adjust the micro-ecological balance of animal intestines could promote the growth of animals, enhance their immunity and thus reduce the need for antibiotics (Zhao and Dai, 2020). This study used sauce residue as raw materials to establish two probiotic symbiotic fermentation modes of *Lactobacillus bulgaricus* and *Saccharomyces cerevisiae*. The fermentation parameters were optimized based on the symbiotic fermentation technology. This study aimed to increase the quality of fermented probiotic feed and thus provide theoretical reference and data support for the development of high-protein probiotic feed from sauce residue.

Materials and Methods

Materials

Saccharomyces cerevisiae and *Lactobacillus bulgaricus* were preserved at the Comprehensive Laboratory of Food Science and Engineering, Shandong University of Technology, Zibo, China. They are stored in a slant preservation in a refrigerator at 4°C. The raw material used for the experiment was fresh sauce residue provided by Boshan Zhengtang Brewing Factory in Zibo City,

China. The water content of the sauce residue was 66.7%, the pH value was 6.35, the dry-based crude protein content was 25.98% (and the wet-based content was 5.97%), the NaCl content was 3.91%, the crude fat content was 16.28% and the ash content was 11.06%. DeMan-Rogosa-Sharpe (MRS) medium and Yeast Extract Peptone Dextrose (YPD) medium were used for the seed liquid culture of *Lactobacillus bulgaricus* and *Saccharomyces cerevisiae* respectively. Bengal red medium was used to determine the number of viable cell count.

Methods

Selection and Addition of Carbon Source

The carbon source was selected from glucose, maltose, lactose, sucrose and glucose/lactose (50/50%). Twenty (20) g/L of the carbon source and 500 g/L of sauce residue were added in each culture and sterilized at 121°C for 20 min. *Saccharomyces cerevisiae* and *Lactobacillus bulgaricus* were inoculated at 1:1 ratio with inoculation volume of 10% (v/w) and fermented at 32°C (Yan and He, 2012; Zhai *et al.*, 2018). After 48 h, the effective viable count and crude protein content were measured.

Then the optimal concentration of the carbon source was determined by using 10, 20, 30, 40 and 50 g/L respectively for further fermentation.

Research based on Temperature Regulation

Single temperature control: Fermentation was carried out for 48 h at 28, 30, 32, 34 and 36°C respectively, after which the fermentation products were sampled and analyzed.

Two-step temperature rise control: The temperature was controlled at 30°C for the first 24 h of the fermentation process and at 34°C for the next 24 h, after which the fermentation products were sampled and analyzed.

Multi-step temperature rise control: The temperature was controlled at 30°C for the first 12 h (0-12 h) of the fermentation process, at 32°C for the next 12 h (12-24 h), at 34°C for the third duration of 12 h (24-36 h) and at 36°C for the final 12 h (36-48 h), after which the fermentation products were sampled and analyzed.

Research based on the Inoculation Process

Simultaneous inoculation: *Saccharomyces cerevisiae* and *Lactobacillus bulgaricus* were inoculated at 1:1 ratio at the same time; the fermentation conditions were the same as for the 'multi-step temperature rise control' and the fermentation products were sampled and analyzed after 48 h.

Lactobacillus bulgaricus was inoculated first, which was followed after 24 h of culturing by the inoculation of *Saccharomyces cerevisiae*; the fermentation conditions were the same as for the 'multi-step temperature rise control' and the fermentation products were sampled and analyzed after a total inoculation time of 48 h.

Table 1: Factors and levels of orthogonal test

Levels	Material-liquid ratio	Inoculation volume (%)	Fermentation time (h)
1	1:0.8	10	36
2	1:1	15	48
3	1:1.2	20	60

Saccharomyces cerevisiae was first inoculated, which was followed after 24 h of culturing by the inoculation of *Lactobacillus bulgaricus*. The fermentation conditions were the same as for the 'multi-step temperature rise control' and the fermentation products were sampled and analyzed after a total inoculation time of 48 h.

Optimization of the Fermentation Conditions

Using the effective viable count, crude protein and the pH value of the fermentation product as the detection index, the experiments were carried out at various values of the solid-liquid ratios (1:0.6, 1:0.8, 1:1, 1:1.2, 1:1.4), inoculation amounts (5, 10, 15 and 20%), fermentation times (24 h, 36 h, 48 h, 60 h) were carried out. Other culture conditions were adopted such as the multi-step temperature, 40g/L glucose/lactose (50/50%) as the carbon source and the inoculation by *Saccharomyces cerevisiae* first. Based on the above single factor test results, the $L^9 (3^4)$ orthogonal test was carried out. The orthogonal test factors and levels are shown in Table 1. From the orthogonal test results, double index analysis was performed to optimize the fermentation conditions.

Measurement Methods of Related Indicators

The drying differential weight method was used to measure the moisture content, the Soxhlet extraction method was used to determine the crude fat content, the burning weight method was used to determine the ash content and the Kjeldahl determination was used for crude protein measurement (Zhang *et al.*, 2017). The NaCl content of sauce residue was determined by silver nitrate titration. The number of viable cells was determined by the dilution plate counting method (Hou *et al.*, 2018). All data were analyzed by one-way Analysis of Variance (ANAVA), compared by Duncan multiple comparisons at $P < 0.05$ using SPSS and all the experiments were carried out in parallel and expressed as the means \pm Standard Deviation (SD). This part has been revised in the manuscript.

Results and Analysis

Carbon Source Significantly Affected Symbiotic Fermentation

Sauce residue contains lots of crude fiber but lack small molecules of sugar which *Lactobacillus bulgaricus* and *Saccharomyces cerevisiae* can utilize directly (Zhang *et al.*, 2017). So, an external carbon source is

needed to improve cell growth. Several commonly used fermentable sugars were added into the medium, including glucose, maltose, lactose, sucrose and glucose/lactose (50/50%). The effective viable count of probiotics was determined after culturing for 48 h. The results are shown in Figure 1. At 8.32×10^7 CFU·mL⁻¹, the effective viable count of probiotics in the glucose/lactose (50/50%) culture group was highest. In the control group (no external carbon source was added), the number of viable probiotics was only 1.7×10^7 CFU·mL⁻¹. Therefore, with carbon source added, the best expression of the viable probiotics obtained was about 5-fold that of the control group, showing that the combination of lactose and glucose could significantly improve the growth of *Lactobacillus bulgaricus* and *Saccharomyces cerevisiae*. However, when utilizing glucose and lactose as single carbon sources, the number of viable cells obtained were 3.30×10^7 and 7.00×10^7 CFU·mL⁻¹ respectively. Both of these single carbon sources expressed lower viable cells than the combination of lactose and glucose culture. Meanwhile, when maltose and sucrose were separately added, the number of effective viable cells in each case was lower than that when any of glucose and lactose was used as the carbon source. In this study, the cells showed preference for lactose. In fact, after one molecule of lactose was used by the microorganisms, one molecule of glucose and one molecule of galactose were obtained. While *Lactobacillus bulgaricus* prefers to make use of galactose, *Saccharomyces cerevisiae* prefers to use glucose directly. Therefore, the addition of glucose/lactose (50/50%) as the optimal carbon source was beneficial to the growth of both *Saccharomyces cerevisiae* and *Lactobacillus bulgaricus*.

The best carbon source of glucose/lactose (50%/50%) was further optimized by varying the concentrations and the results were shown in Fig. 2. When the concentration of glucose/lactose (50/50%) was 40 g/L, the effective viable count of probiotics was 3.47×10^7 CFU·mL⁻¹, which was the highest obtained for any concentration. The number of viable probiotics in Fig. 2 shows a tendency to rise first as the concentration of carbon source rises, until it reaches a maximum value and then slowly begins to decline, indicating that when the concentration of the carbon source was too low, the requirements for probiotics growth were not met, especially as *Saccharomyces cerevisiae* required a higher carbon source. When the concentration of the carbon source was 50 g/L, the effective viable count of

probiotics decreased, indicating that this mass concentration was too high and inhibited the growth and reproduction of the probiotics. When the mass concentration of glucose + lactose (1:1) was 40 g/L, the crude protein content of the supernatant of the fermentation broth was 3.94%. At the same concentration, the viable count of *Saccharomyces cerevisiae* was also the highest, reaching 2.01×10^7 CFU·mL⁻¹. The growth trend of the viable count of *Saccharomyces cerevisiae* was basically consistent with the growth trend of the crude protein content, which showed that yeast as the main source of fermented cell protein could increase the crude protein content of fermented feed. When the mass concentration was 30

g/L, the viable count of *Lactobacillus bulgaricus* was 1.73×10^7 CFU·mL⁻¹, its highest level, indicating that this concentration was the most suitable for the growth and reproduction of *Lactobacillus bulgaricus*. However, the number of *Saccharomyces cerevisiae* was only 0.88×10^7 CFU·mL⁻¹ at this concentration. The main reason was that the excessively high lactic acid produced by the action of *Lactobacillus bulgaricus* on lactose drastically reduced the pH of the symbiotic environment, which inhibited the growth of yeast and ultimately led to low total probiotics. Therefore, the final experimental results showed that the optimal mass concentration of glucose + lactose (1:1) was 40 g/L.

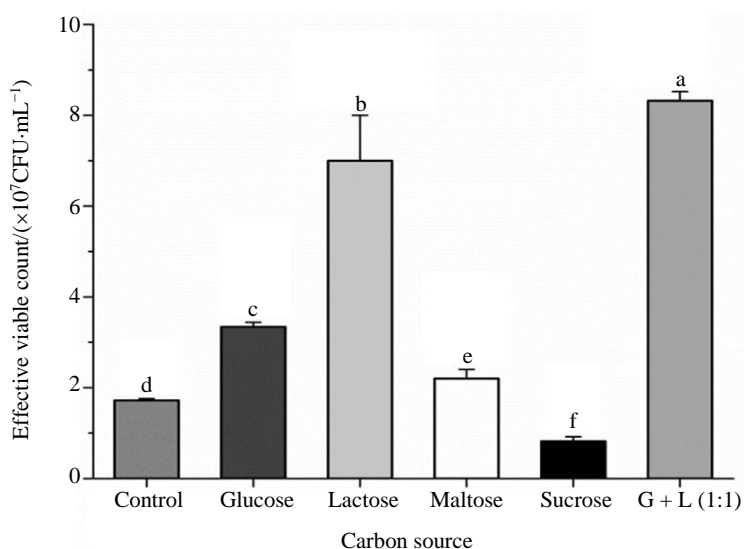


Fig. 1: Effects of different carbon sources on the growth of probiotics in fermented sauce. Control means no carbon source was added; G + L (1:1) represents carbon source with a glucose to lactose ratio of 1:1. The different letters indicate that the comparison of each peak value with the data of its neighboring detection points yielded a significant difference ($P < 0.05$)

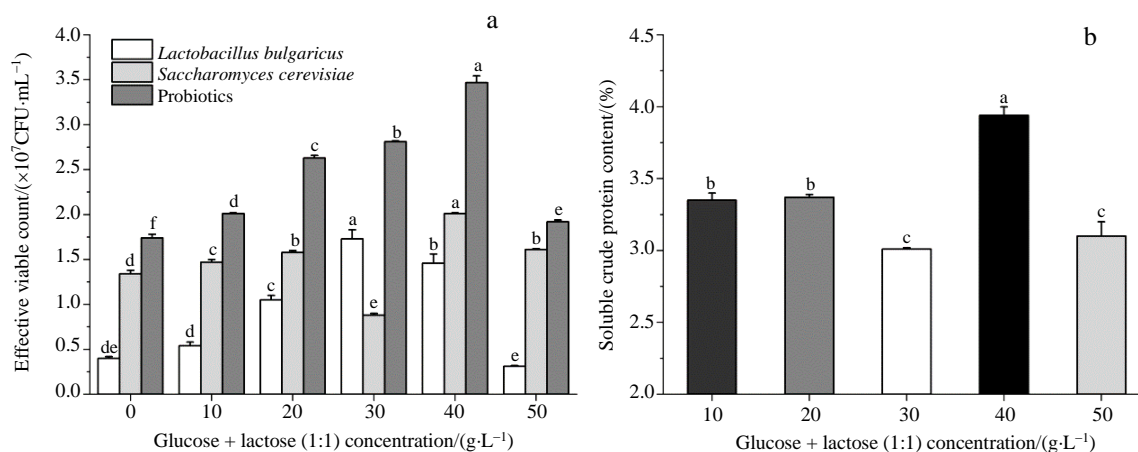


Fig. 2: Effects of different concentrations of glucose + lactose (1: 1) on fermented sauce

Multi-Step Temperature-Controlled Improved Symbiotic Culture

Generally, the suitable growth temperature of *Saccharomyces* is 28~30°C, while that of the *Lactobacillus* is 36~40°C (Han *et al.*, 2014). Since the optimal growth temperatures of *Lactobacillus bulgaricus* and *Saccharomyces cerevisiae* differ significantly, the multi-step temperature method was used to control the fermentation process, mainly to avoid the rapid propagation of the *Lactobacillus*, which would have led to its dominance and the production of a large amount of lactic acid, causing the pH to drop and inhibiting the growth of yeast (Han *et al.*, 2014). In fact, various temperature control methods were tried and the results are shown in Fig. 3. When the temperature was kept constant at 32°C, the number of effective viable count of probiotics was 1.93×10^7 CFU·mL⁻¹. In the multi-step temperature-controlled culture, the effective viable count, at 1.88×10^7 CFU·mL⁻¹, was similar to that of the 32°C constant-temperature culture, but the crude protein content (7.88%) was significantly higher than those of the other groups. The crude protein content of the 32°C constant-temperature culture, at just 6.81%, was of course lower than that of the multi-step temperature culture group. In fact, over low temperature or over high temperature both inhibited cells growth. For example, in the culture of 28 and 36°C, the effective viable count and crude protein content were both lower than those of the 32°C culture and the multi-step temperature controlled fermentation. The rate of cell growth and the crude protein content of the 30 and 34°C constant-temperature cultures fell between the corresponding values for the 28~36°C culture and the multi-step temperature-controlled group. It seems that single temperature control was not suitable for the growth of symbiotic strains in the cultures. In the multi-step temperature-controlled group, the number of effective viable count of *Lactobacillus bulgaricus* was highest (1.51×10^7 CFU·mL⁻¹), which indicated that this method was beneficial to the growth of *Lactobacillus bulgaricus*. Therefore, the optimized culture plan was to select a suitable temperature for *Saccharomyces cerevisiae* growth for a period of time and then raise the temperature to allow the bacteria to multiply in a large amount and to reduce the impact of lactic acid production by *Lactobacillus bulgaricus*. The experimental results showed that temperature-controlled fermentation can be used as the key parameter in the optimization of symbiotic fermentation technology and the multi-step temperature control was the best choice for the symbiotic fermentation of *Saccharomyces cerevisiae* and *Lactobacillus bulgaricus*.

Staggered Inoculation Process Effectively Promoted Cell Growth

In symbiotic fermentation, the characteristics of different strains determine if the symbiosis status of the

strains are different when utilizing different inoculation sequences. Figure 4 showed the effective live count and the crude protein content when the sauce residue was inoculated first with *Lactobacillus bulgaricus* and then inoculated with *Saccharomyces cerevisiae* after 24 h of fermentation (group A); when it was inoculated first with *Saccharomyces cerevisiae* and then inoculated with *Lactobacillus bulgaricus* after 24 h (group B); and when simultaneously inoculated (group C) by the two bacteria. The total number of viable probiotic in group B was significantly higher than in the other groups (7.8×10^7 CFU·mL⁻¹) and the number of *Lactobacillus bulgaricus* was also highest (6.6×10^7 CFU·mL⁻¹). The effective viable count in group A was only 3.8×10^7 CFU·mL⁻¹. Interestingly, the pH of the above two groups did not show much difference after 48 h. Therefore, except that the lactic acid produced by *Lactobacillus bulgaricus* could affect the initial growth of the *Saccharomyces cerevisiae*, a symbiotic enzymatic system plays a key role in such fermentation. For the simultaneously inoculated process (group C), the number of viable probiotic fell between the values for the two staggered inoculation methods. However, the crude protein content for the simultaneous inoculation method, at 26.81%, was higher than for the two staggered inoculation. But it is the method of inoculation first with *Saccharomyces cerevisiae* and then with *Lactobacillus bulgaricus* after 24 h (group B), that can be considered the best method, because it expressed a high value for both the effective cell count and crude protein content.

Optimization of the Symbiotic Fermentation Conditions

Material-Liquid Ratio Experiments

The material-liquid ratio is one of the key factors that affect solid-state fermentation. Using the effective viable count, pH and crude protein as indicators, different material-liquid ratios were optimized. As shown in Fig. 5, when the material-liquid ratio in the sauce residue medium was 1:0.8, the total effective viable count reached a maximum of 3.77×10^8 CFU·mL⁻¹ and both the viable counts of *Saccharomyces cerevisiae* and *Lactobacillus bulgaricus* were significantly higher than the corresponding values for the other ratios. When the material-liquid ratio was 1:1.4, the total effective viable count was as low as 0.45×10^8 CFU·mL⁻¹, just 12% of the viable count for the ratio 1:0.8. In a high material-liquid ratio medium, the nutrients are diluted and the porosity of the matrix is reduced, which in turn reduces the gas concentration of the medium and then inhibits the growth of microorganisms (Zhan *et al.*, 2015). However, if the material-liquid ratio is too low, the swelling degree of the matrix is reduced, which decreases the concentration of the nutrients and the fluidity of the bacteria, thereby also inhibiting the growth of microorganisms (Zhan *et al.*,

2015). For the material-liquid ratio of 1:0.8, the crude protein content of the dry-base of the fermented feed reached a maximum of 36.89%, which is about 57%

higher than the crude protein content for the ratio 1:1.4 (23.52%). Therefore, the material-liquid ratio of 1:0.8 was defined as the optimal ratio.

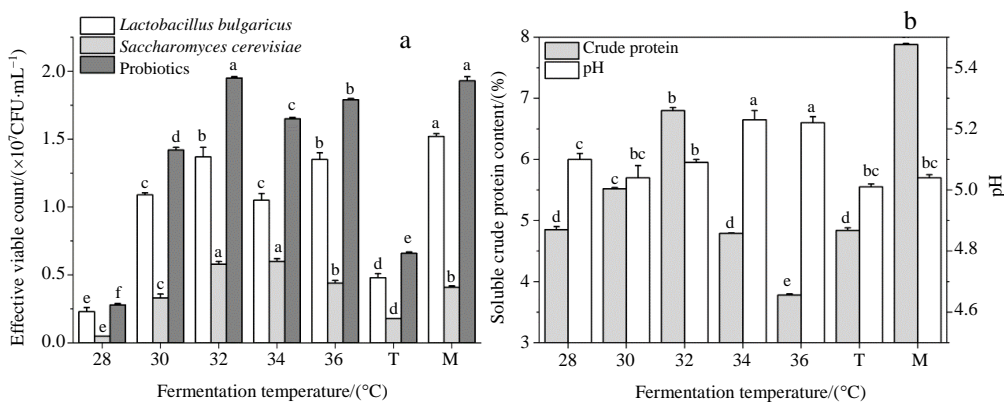


Fig. 3: Effects of temperature control on the fermentation of sauce residue. T represents two-step temperature rise control; M represents multi-step temperature rise control

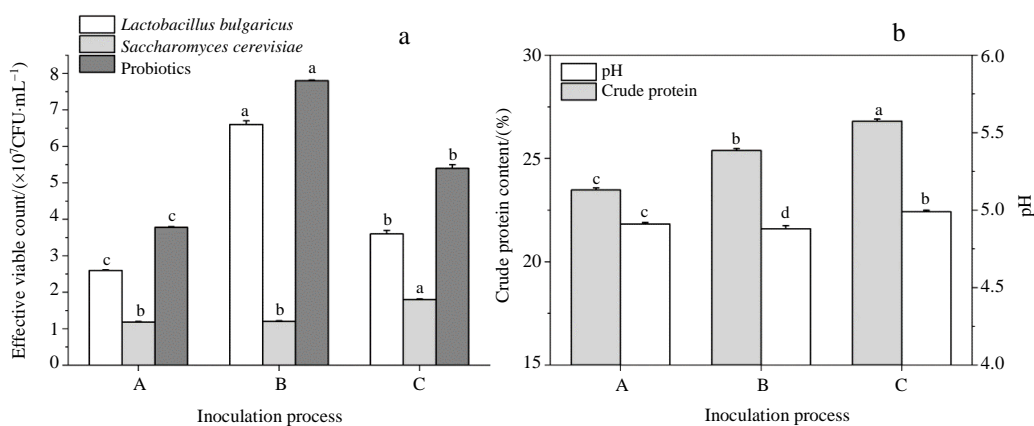


Fig. 4: Effect of probiotic inoculation process on fermented sauce residue. Group A represents sauce residue inoculation first with *Lactobacillus bulgaricus* first and then with *Saccharomyces cerevisiae* after culturing for 24 h; Group B represents inoculation with *Saccharomyces cerevisiae* first and then with *Lactobacillus bulgaricus* after culturing for 24 h; Group C represents simultaneous inoculation

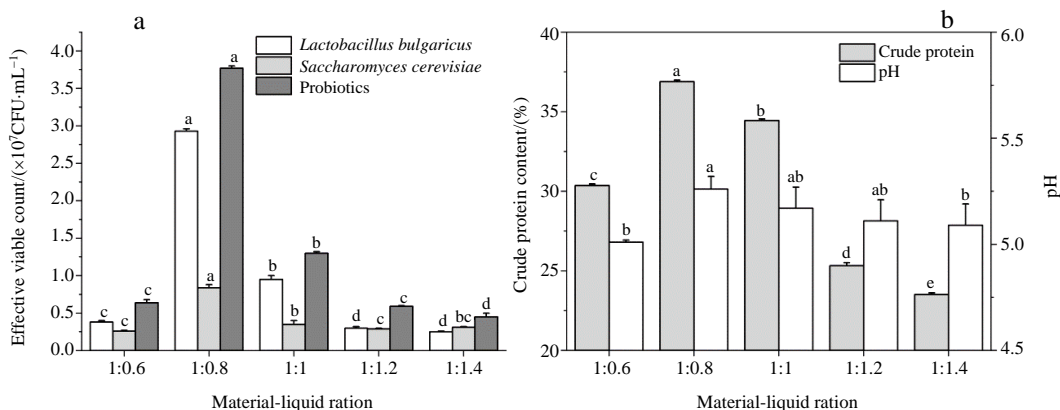


Fig. 5: Effect of material-liquid ratio on fermented sauce residue

The Inoculation Volume Affected Fermentation Conversion Rate and the Effective Viable Count of Probiotics

The inoculation amount can directly affect the fermentation cycle and conversion rate. The viable count, pH value and the crude protein content of the dry-base were tested for the optimization of the inoculation amount; the results are shown in Fig. 6. When the inoculation amount was 15%, the total effective viable count of the ferment (1.93×10^8 CFU·mL⁻¹) was significantly higher than for the other inoculation doses and the number of live *Lactobacillus bulgaricus* was also highest (1.53×10^8 CFU·mL⁻¹). At the same inoculation amount was 15%, the dry-base crude protein content of the fermented feed reached the highest value of 40.42%. But when the inoculation amount was 20%, the effective viable count of the probiotics in the fermented feed started to decrease. This is because when the inoculation amount was too high, the nutrients in the medium were quickly consumed, which inhibited the growth of microorganisms. In addition, the inoculation amount was positively related to the pH value of the fermentation broth. This may be because the inoculum amount was too large, which led to insufficient oxygen and nutrients and to premature decay of probiotics, resulting in pH increase. Meanwhile, *Saccharomyces cerevisiae* produced ethanol under anaerobic condition, which inhibited the growth of *Lactobacillus bulgaricus* and the production of lactic acid. In addition, the excessive inoculation of *Saccharomyces cerevisiae* also affected the fermentation of *Lactobacillus bulgaricus*. Therefore, based on the factors mentioned above, 15% was selected as the optimal inoculation volume.

Effect of Fermentation Time

With increase in symbiotic fermentation time, the total number of viable probiotics, the number of viable *Lactobacillus bulgaricus* and the dry-base crude protein

content all showed increasing trends first and then decreasing trends (Fig.7). After 48 h, the effective viable count of the probiotics in the fermented feed had reached its highest at 1.2×10^8 CFU·mL⁻¹ and the viable count of *Lactobacillus bulgaricus* was 9.5×10^7 CFU·mL⁻¹. After the same time, the dry-based crude protein content was 34.45%. Then, as the fermentation time increased, the number of viable probiotics began to decrease. This may be because as the fermentation time got too long, the nutrients reduced and the bacteria began to decay, leading to a rise in pH and the decrease in the number of viable probiotics. However, the number of viable yeast and pH value showed continuously increasing trends. The growth cycle of yeast was longer than that of *Lactobacillus bulgaricus*. The pH increased after autolysis and decay of the lactobacillus in the late stage, making it suitable for the growth of yeast. Due to the lack of nutrients in the late stage, yeast decomposed proteins as carbon and nitrogen sources for its growth, resulting in the decrease in the crude protein content in the late stage. Therefore, 48 h was the most suitable cultivation time for the symbiotic fermentation (Fig.7).

Orthogonal Test

According to the single-factor experiment on the fermentation conditions (material-water ratio, inoculation amount, fermentation time), the optimal factor level was selected for the L⁹ (3⁴) orthogonal experiment and the range analysis of two indices was carried out for the orthogonal test results (Table 2). Comparison of range sizes in Table 2 shows that the factors that affected the total effective viable count were inoculation amount (B) > material-liquid ratio (A) > fermentation time (C) > error (D), in that order; however, the order of the factors that affected the crude protein content was material-liquid ratio (A) > fermentation time (C) > inoculation amount (B) > error (D).

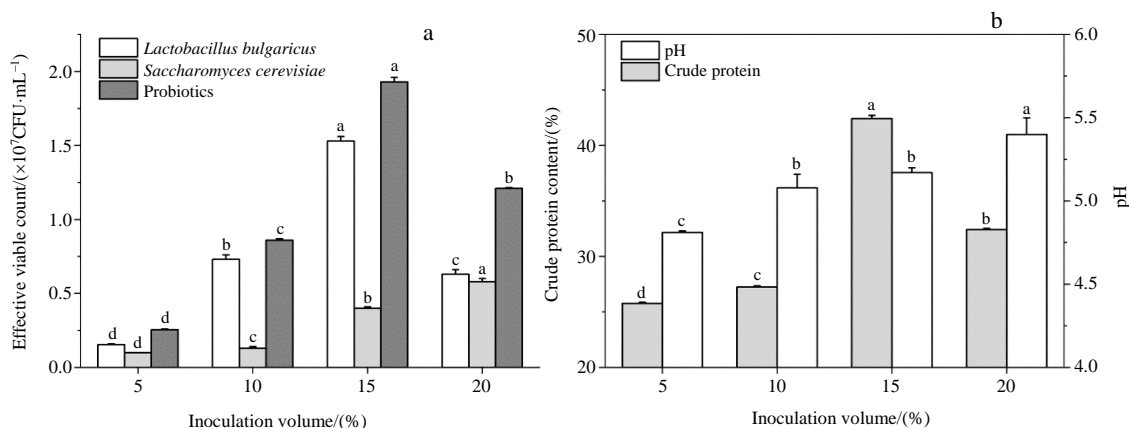


Fig. 6: Effect of probiotic inoculation on fermented sauce residue

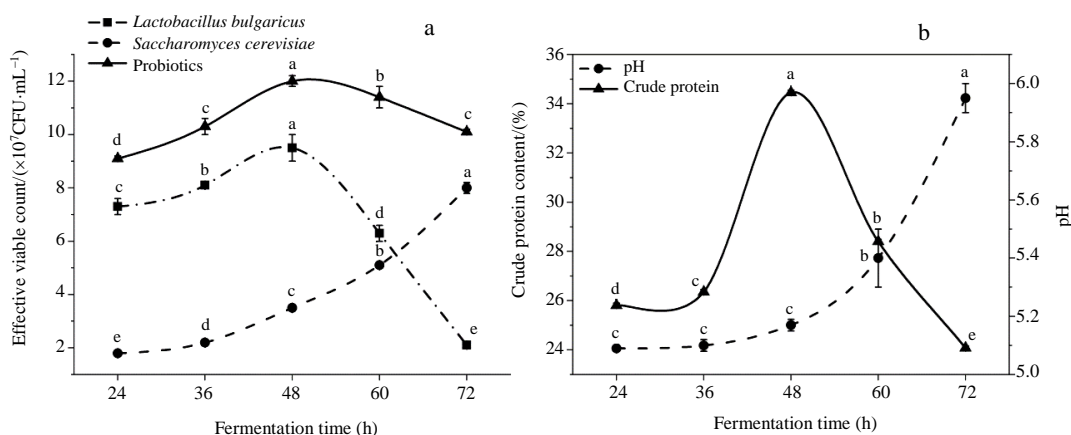


Fig. 7: Effect of fermentation time on fermented feed from sauce residue

Table 2: Results of the orthogonal test

Test group	Material-liquid ratio A	Inoculation volume B	Fermentation time C	Empty column D	Effective viable count/(10 ⁸ CFU·mL ⁻¹)	Crude protein /%
1	1.00	1.00	1.00	1.00	4.20	30.72
2	1.00	2.00	2.00	2.00	7.20	38.86
3	1.00	3.00	3.00	3.00	3.80	29.29
4	2.00	1.00	2.00	3.00	3.00	27.38
5	2.00	2.00	3.00	1.00	5.30	26.93
6	2.00	3.00	1.00	2.00	3.20	20.82
7	3.00	1.00	3.00	2.00	4.10	36.67
8	3.00	2.00	1.00	3.00	4.20	28.11
9	3.00	3.00	2.00	1.00	5.00	30.68
k ₁	5.07	3.77	3.87	4.83		
k ₂	3.83	5.57	5.07	4.83		
k ₃	4.43	4.00	4.40	3.67		
R	1.23	1.80	1.20	1.17		
k ₁	32.96	31.59	26.55	29.44		
k ₂	25.04	31.30	32.31	32.12		
k ₃	31.82	26.93	30.96	28.26		
R	7.91	4.66	5.76	3.86		

This shows that the inoculation amount and the material-liquid ratio had the greatest impact on the effective viable count and crude protein content, respectively. The optimal level was determined according to the value of the single factor K1 and the optimal combination of fermentation conditions was finally determined as A1B2C2: That is the material-liquid ratio was 1:0.8, The inoculation amount was 15%, the fermentation time was 48 h, the effective viable number of probiotics could reach 7.20×10^8 CFU·mL⁻¹ and the dry-based crude protein content could reach 38.86%. After the verification test, the effective viable count was 6.90×10^8 CFU·mL⁻¹ and the dry-based crude protein content was 36.54% ($P < 0.05$).

Discussion

As the main by-product of soy sauce production, about 5.5 million tons of sauce residue was produced per year in China (2014) (Shu *et al.*, 2015). The sauce

residue is rich in protein (25-30%), crude fiber (13-25%), crude fat (7-10%), minerals (8-16%) and other nutrients (Gong *et al.*, 2018; Shu *et al.*, 2015; Zhang *et al.*, 2017). Therefore, it is extremely easy to rot and stink and its use is restricted (He *et al.*, 2014). Many studies were devoted to the secondary extraction of obtain fat, crude protein, dietary fiber, soy isoflavones and other nutrients from sauce residue (Chen *et al.*, 2015; 2014; He *et al.*, 2014; Shu *et al.*, 2015), but the extraction cost was relatively high and the extraction process could affect product quality and cause secondary pollution. There were also studies on the simple development of sauce residue into feed or fertilizer (Cui *et al.*, 2014; Jing *et al.*, 2016), but the feed has poor palatability and can easily cause salt poisoning etc. (Gong *et al.*, 2018). In recent years, research on micro-ecological feed has developed rapidly. The fermentation of feed materials in the presence of probiotics not only improves the nutritional level and degrades the anti-nutritional factors

of the feed, but it also improves the intestinal flora of the animal and produces a variety of probiotic metabolites, which boosts animal immunity (Zhao and Dai, 2020). The raw material for brewing soy sauce contains more protein, which is suitable for the growth of microbial flora. In addition, the sauce residue is rich in isoflavones, which improve immunity, improve lactation, promote growth and improve the quality of livestock and poultry products (Sun *et al.*, 2016). Therefore, the fermentation of sauce residues with various probiotics not only utilizes the nutrients of the sauce residues, but also makes full use of the fermented materials to improve feed palatability. The use of fermented feed could ultimately reduce the need for antibiotics in animal feed and improve the health of animal intestines. Zhang *et al.* (2017) used *Rhodospirillum rubrum* to ferment soy sauce residue into microbial feed, which increased the nutritional content of the soy sauce residue and reduced the amount of toxic substances. Peng *et al.* (2013) used *Aspergillus niger* to degrade hemicellulose in sauce residue and the degradation rate reached 84.28% in 30 days.

In this study, sauce residue was used as raw materials for symbiotic fermentation and after the fermentation, the dry-based crude protein content of the biological feed was potentially 38.86%, which is a 49.6% increase compared with the raw material. Relevant research also showed that microbial fermentation could improve the nutrient level of sauce residue. Bian *et al.* (2012) inoculated *Geotrichum candidum*, *Candida tropicalis*, *Aspergillus niger* (1:1:2) in sauce residue, which increased the crude protein content after fermentation by 23.09%. However, most studies on fermented feed from sauce residue were concerned only with increasing the crude protein content, while neglecting the problem of the bad odor and poor palatability of sauce residue feed. Fermentation of sauce residue in the presence of probiotics not only increases the protein content and the number of live probiotics, but also produces more organic acids such as lactic acid, which gives the sauce residue a unique flavor and significantly boosts its attraction for animals. The metabolites and liquid produced by *Lactobacillus bulgaricus* have a strong bacteriostatic effect on pathogenic microorganisms. *Lactobacillus bulgaricus* and its metabolites contain more Superoxide Dismutase (SOD), which can regulate the immune function of animals (Yu *et al.*, 2018; Zhao and Dai, 2020). Therefore, feed fermented in the presence of *Lactobacillus bulgaricus* not only has high nutritional value and utilization rate, but also is extremely rich in lactic acid. Such feed has a unique smell and easily attracts animals, which could increase the food intake by the animals (Li and Zeng, 2018).

The sauce residue contains about 10~20% of crude fat and the yeast fermentation process can produce lipase and other digestive enzymes, which can convert the

crude fat, non-protein nitrogen, carbohydrates, etc. into microbial protein. In addition, yeast is rich in many essential amino acids, B vitamins and other nutrients and can effectively degrade the anti-nutritional factors (Zhong *et al.*, 2018). It produces coenzyme A, coenzyme I, coenzyme Q, cytochrome C and other biologically active substances (Shi *et al.*, 2017; Zhuang *et al.*, 2020). In this study, we explored symbiotic fermentation by the action of *Lactobacillus bulgaricus* and *Saccharomyces cerevisiae* and the effective number of probiotics potentially reached 7.2×10^8 CFU·mL⁻¹, a result significantly higher than that obtained by (Li *et al.*, 2020), who studied the symbiotic fermentation of palm pulp residue in the presence of beer yeast and *Lactobacillus bulgaricus* and obtained a total number of colonies equal to 0.9×10^8 CFU/g. Therefore, the current study showed that the sauce residue is a basic nutrient more suited to symbiotic fermentation in the presence of *Lactobacillus bulgaricus* and *Saccharomyces cerevisiae*. Li *et al.* also showed that when the fermentation temperature was 30°C, the cell growth and metabolism of the palm kernel residue fermented by the action of beer yeast and *Lactobacillus bulgaricus* were strongest and the total number of colonies was highest. But the optimal temperature for constant-temperature fermentation in the current study was 32°C. This may be because the different matrix composition led to a slight deviation in the optimum temperature for symbiotic fermentation. In addition to the nutrient composition of sauce residue, which limits the increase in the number of probiotics, yeast growth could produce fatty acids and other substances by metabolize that could inhibit the growth of *Lactobacillus bulgaricus*. Meanwhile, *Lactobacillus bulgaricus* could produce lactic acid and other substances during its growth process, which inhibits the growth of yeast and decreases the effective viable count of probiotics during mixed fermentation in the presence of *Lactobacillus bulgaricus* and *Saccharomyces cerevisiae*.

A carbon source is essential for the growth and metabolism of *Lactobacillus bulgaricus* and yeast and it provides the material structure and energy for cell growth and metabolism (Jiang *et al.*, 2018). But sauce residue only contains about 20% of crude fiber and 10% of starch. *Lactobacillus bulgaricus* can convert these macromolecular substances such as starch and protein into small molecular substances like glucose, amino acids and peptides, which are then used by yeast. But the quantity of the small molecular substances often does not meet the needs of microbial growth. Therefore, it is very important to add fermentable sugar that can be directly used by yeast. In this study, it was found that the addition of 40 g/L glucose/lactose (1:1) as the carbon source during sauce residue fermentation had the best effect. In fact, many studies have found that monosaccharide glucose was the best carbon source for

yeast growth (Mao *et al.*, 2018). However, *Lactobacillus bulgaricus* can make better use of lactose decomposition into 1 molecule of glucose and 1 molecule of galactose as the carbon source and can also provide part of the glucose for yeast. This experiment provides theoretical guidance for finding cheap carbon-source raw materials for practical industrial production and raw materials with lactose and glucose as the main substances will be valued.

Conclusion

In this study, sauce residue was used as raw material to establish a relatively complex inoculation process and temperature-controlled mode using symbiotic fermentation technology; the symbiotic fermentation conditions of the two probiotics used were optimized. The experimental results showed that the probiotic viable count and the protein content of probiotics could be effectively increased by the initial inoculation of *Saccharomyces cerevisiae* and then of *Lactobacillus bulgaricus* after 12 h, with 30°C as the initial temperature and an increase of 2°C every 12 h but not exceeding 37°C. By optimizing the fermentation parameters, the best fermentation conditions were determined as glucose/lactose (1:1) of concentration 40 g/L, material-liquid ratio of 1:0.8, inoculation volume of 15% and fermentation time of 48 h. Under these conditions, the effective viable count of probiotics could reach 7.2×10^8 CFU·mL⁻¹ and the dry-based crude protein content could reach 38.86%. This provides the theoretical and data support for the high-value use of sauce residue to produce high-protein probiotic feed.

Acknowledgement

The authors are grateful to the funding support by Chongqing technological innovation and application development key projects (cstc2019jscx-gksbX0113), China Postdoctoral Science Foundation, China (2019M662362 and 2019M650167), Shandong key research and development program-Major scientific and technological projects, China (2019JZZY011005), Innovation Project Special Fund for Post Doctors of Shandong Province, China (201902048).

Author's Contributions

Xinhe Zhao and Zhuangzhuang: Contributed equally and participated in the whole experiment process and also contributed to the interpretation of the results and manuscript preparation.

Wenwei Lu and Yubin Zhao: Participated in part of the experimental design and manuscript preparation. Yuanda Song: Ameliorated the manuscript.

Yuanda Song and Xiaojie Ren: Contributed to the guidance of experimental design and ameliorated the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

References

- Asada, C., Kondo, Y., Sasaki, C., & Nakamura, Y. (2010). Bioconversion of soy sauce residue treated with steam explosion into ethanol by Meicelase and *Mucor indicus*. *Journal of Food Technology*, 8(4), 187-190.
- Barton, M. D. (2000). Antibiotic use in animal feed and its impact on human health. *Nutrition research reviews*, 13(2), 279-299.
- Bian, M., Zong, X., & Zuo, Y. (2012). Process optimization of synergistic fermentation using sauce residue for protein feed. *Feed Research*, 08, 83-85.
- Chen, P., Song, H., Wang, Y., Chen, P., Shen, X., & Yao, S. (2015). Homogeneous acetylation of hemicelluloses from soy sauce residue in imidazolium-based ionic liquid. *Journal of Material Cycles and Waste Management*, 17(3), 574-582.
- Chen, X., Luo, Y., Qi, B., Luo, J., & Wan, Y. (2017). Improving the hydrolysis efficiency of soy sauce residue using ultrasonic probe-assisted enzymolysis technology. *Ultrasonics Sonochemistry*, 35, 351-358.
- Chen, X., Luo, Y., Qi, B., & Wan, Y. (2014). Simultaneous extraction of oil and soy isoflavones from soy sauce residue using ultrasonic-assisted two-phase solvent extraction technology. *Separation and Purification Technology*, 128, 72-79.
- Cui, Y., Dong, X., & Tong, J. (2014). Application of Residue Products of Food and other Manufacture as Feed Resource in China. *Chinese Journal of Animal Nutrition*, (7), 4.
- Duan, Y., Zhou, A., Wen, K., Liu, Z., Liu, W., Wang, A., & Yue, X. (2019). Upgrading VFAs bioproduction from waste activated sludge via co-fermentation with soy sauce residue. *Frontiers of Environmental Science & Engineering*, 13(1), 3.
- Gong, S., Liu, C. L., & Shu, X. G. (2018). Research Progress on Refine Utilization of Soy Sauce Residue. *Journal of Anhui Agricultural Sciences*, 2018(15), 9.
- Gu, L., H, J., & Peng, W. (2015). Add the Impact of Antibiotics on Intestinal Health of Animals.
- Han, X., Lei, P., & Qiao, F. (2014). Research progress on symbiotic between yeasts and *Lactic Acid Bacteria* in fermented dairy products. *Science and Technology of Food Industry*, 35(07), 388-391.
- He, H. F., Yan, J., Lin, H. L., & Liu, Q. H. (2014). Extracting crude fat from soy sauce residue. *Food and Fermentation Industries*, (10), 38.

- Hou, N., Xie, Q., & Lei, C. (2018). Effects of microbial solid-state fermentation on nutritional quality of soybean meal. *China Feed*, 5), 32-34.
- Jiang, Y., Pan, T., & Xi, Z. (2018). Effect of assimilable nitrogen and reducing sugar concentrations of synthetic grape must on the fermentation characteristics of *Saccharomyces cerevisiae*. *Shipin Kexue/Food Science*, 39(2), 131-137.
- Jing, X., Liu, Y., & Lin, H. (2016). Maize growth influenced by conjugated sauce residue oil-coated fertilizer and coating structure analysis. *Jiangsu Journal of Agricultural Sciences*, 32(03), 563-569.
- Kumar, K., Gupta, S. C., Baidoo, S. K., Chander, Y., & Rosen, C. J. (2005). Antibiotic uptake by plants from soil fertilized with animal manure. *Journal of environmental quality*, 34(6), 2082-2085.
- Li, B., Jia, H., & Peer, M. A. (2020). Response surface optimization of preparing palm residues feed by fermentation with *Saccharomyces cerevisiae* and *Lactobacillus*. *China Oils and Fats*, 45(03), 126-134.
- Li, H., & Zeng, Y. (2018). The application of *Lactic acid bacteria* in solid fermentation feed. *China Feed*, 17, 86-89.
- Mao, X., Xia, Y., & Zhang, Y. (2018). Isolation, identification and fermentation characteristics of yeast from Sichuan bran vinegar starter. *Journal of Food Science and Technology*, 36(06), 21-27.
- Murasawa, N., Koseki, H., & Iwata, Y. (2013). Causes of accidents by soy sauce squeezing residue and fish meal. *Journal of Material Cycles and Waste Management*, 15(1), 42-48.
- Peng, L., Liang, M., Huan, F., & Wenjie, W. (2013). Study on degradation of soy sauce residues by *Aspergillus niger*. *Feed Industry*, 2013(16), 10.
- Shi, Y., Du, W., & Yao, J. (2017). Progress in extracting coenzyme Q10 produced by yeast fermentation. *Biotic Resources*, 39(03), 168-174.
- Shu, D., Wang, D., & Song, X. (2015). Extraction methods and process optimization of soy sauce residue protein. *China Brewing*, 34(06), 67-71.
- Sun, X., Meng, X., & Su, J. (2016). Research Progress on Application and Safety of Soybean Isoflavone in Animal Husbandry. *China Animal Husbandry & Veterinary Medicine*, 43(08), 2208-2213.
- Wang, Y., Xie, H., & Zhang, N. (2019). Application and development expectation of microbial fermentation feed. *Journal of Henan Institute of Science and Technology*, 47(06), 49-53.
- Yan, B., & He, Y. F. (2012). Symbiotic fermentation characteristics of lactic acid bacteria and yeast isolated from koumiss in Inner Mongolia. *Food Science*, 7, 1311137.
- Yu, X., Lv, J., & Yu, F. (2018). Research on Antioxidant Enzyme Activities of *Lactic Acid Bacteria* in Different Media. *China Condiment*, 43(01), 39-42.
- Zhai, Y., Zhang, H., & Zhang, L. (2018). Preparation of Biological Feed with Grape Pomace Fermented by *Yeast* and *Lactobacillus*. *Animal Husbandry and Feed Science*, 39(07), 70-75.
- Zhan, T., Ke, F., & Chen, Q. (2015). Effects of fermentation time and material-water ratio on soybean meal fermentation. *Journal of Fujian Agriculture and Forestry University*, 44(02), 193-197.
- Zhang, J., Yuan, J., Zhang, W. X., Zhu, W. Y., Tu, F., Jiang, Y., & Sun, C. Z. (2017). An aerobic detoxification photofermentation by *Rhodospirillum rubrum* for converting soy sauce residue into feed with moderate pretreatment. *World Journal of Microbiology and Biotechnology*, 33(10), 184.
- Zhao, X., & Dai, X. (2020). The mechanism of probiotics' function and its application in animal husbandry. *China Feed*, 06, 21-23.
- Zhong, W., Qi, H., & Zhao, Y. (2018). Application of *Yeast* Preparation to Regulate Ruminant Production and Rumen Ecology. *Chinese Journal of Animal Science*, 54(08), 26-30.
- Zhuang, R., Wang, R., & Qiu, X. (2020). Bioactive substances produced by *Rhodotorula mucilaginosa*: a comprehensive review. *Food Science*, 41(01), 318-329.