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Upcycling of Chinese Nong-Flavor Baijiu Distiller's Grains Through Solid-State Fermentation by Microbial-Enzyme Synergy

Lin Qiao [1], Kai Wang [1,2,3], Xu Xin [1], Weiwei Wang [1], Yongwei Wang [1], Junxun Li [2], Qingming Cao [3], Kuanbo Liu [1] and Aike Li [1,*]

[1] Key Laboratory of Grain and Oil Biotechnology, Academy of National Food and Strategic Reserves Administration, Beijing 100037, China; ql@ags.ac.cn (L.Q.); 17735200209@163.com (K.W.); xx@ags.ac.cn (X.X.); www@ags.ac.cn (W.W.); wyw@ags.ac.cn (Y.W.); lkb@ags.ac.cn (K.L.)
[2] Shandong Taishan Shengliyuan Group Co., Ltd., Tai'an 271000, China; li_junxun@sina.com
[3] College of Food Science and Engineering, Central South University of Forestry & Technology, Changsha 410004, China; cqm2000cn@163.com
* Correspondence: lak@ags.ac.cn

Abstract

Chinese Baijiu distiller's grains are by-products of the Chinese Baijiu brewing process, characterized by high water content, high acidity, and high fiber content, which make them unsuitable for animal feed, especially for monogastric animals. This study investigated the possibility of increasing the feed value of Nong-flavor Baijiu distiller's grains (NFBDGs) for monogastric animals via solid-state fermentation by microbial-enzyme synergy. Experiments evaluated microbial growth, pH variation, improvement of crude protein (CP), true protein (TP), and acid-soluble protein (ASP), degradation of crude fiber (CF), acid detergent fiber (ADF), and neutral detergent fiber (NDF). The results indicated that *Ligilactobacillus salivarius* CRS23, *Bacillus subtilis* YLZ7, *Saccharomyces cerevisiae* CJM26, and xylanase were identified for the fermentation of NFBDGs. When the initial moisture content of NFBDGs was 60% and the initial pH was 3.4, under the conditions of aerobic fermentation at 37 °C for 4 days, the pH of NFBDGs increased from 3.49 to 6.04, the contents of CP and TP increased by 33.59% and 31.21%,,, respectively, while the contents of CF, ADF, and NDF decrease by 35.44%, 20.53%, and 25.02% respectively. The nutritional value of NFBDGs was significantly improved after microbial-enzyme synergistic fermentation, providing a new approach for their application as feed.

Keywords: Nong-flavor Baijiu distiller's grains; solid-state fermentation; microbial-enzyme synergistic fermentation; upcycling; xylanase

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1. Introduction

Chinese Baijiu is one of the six major distilled liquors in the world, which is a beverage liquor made from grains, such as corn, sorghum, and wheat as the main raw materials, using Daqu, Xiaoqu, or Fuqu, and distiller's yeast as saccharifying and fermenting agents, and through steaming, saccharification, fermentation, distillation, aging, and blending. It has 12 flavor types, mainly including Nong-flavor, Qing-flavor, and Jiang-flavor [1,2]. Solid-state fermentation is the main process for Baijiu production; after fermentation, rice husks need to be added and mixed with fermented grains for distillation to produce Baijiu [3]. Baijiu distiller's grains are by-products and main wastes of the solid-state fermentation process of Baijiu, with an annual output of 30 million tons in China [1,3]. Baijiu distiller's grains have high water content and high acidity, which make them difficult

to transport and store; if not handled in a timely manner, they are prone to mildew and odor, causing environmental pollution [3]. Currently, Baijiu distiller's grains are mainly used for composting, the extraction of active substances, energy production, or directly dried as feed [2]. Among all uses, the cost–benefit ratio of using them as animal feed is higher due to their rich nutritional components [2]. However, approximately 45% rice husks or chaff are added during Baijiu brewing, resulting in high cellulose content and low digestibility of Baijiu distiller's grains, which are particularly unsuitable for monogastric animals. Microbial fermentation technology can improve the nutritional value of Baijiu distiller's grains, while increasing probiotic microbial protein and active metabolites, significantly enhancing their feed value [4–6].

To produce high-quality fermented Baijiu distiller's grains suitable for feed, it is necessary to both screen optimal fermentation strains and optimize the supporting process technology. Strains used for fermenting Baijiu distiller's grains mainly focus on four types of microorganisms: yeasts, lactic acid bacteria, bacilli, and molds. Among them, common yeasts include *Candida utilis*, *Saccharomyces cerevisiae*, and *Candida tropicalis* [4,7,8]; common lactic acid bacteria include *Lactobacillus casei*, *Lactobacillus plantarum*, and *Lactobacillus buchneri* [6,9]; common bacilli include *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus coagulans* [10–12]; and common molds include *Trichoderma viride*, *Geotrichum candidum*, *Aspergillus niger*, *Rhizopus* sp., and *Trichoderma koningii* [6,7,13].

When optimizing the fermentation process of Baijiu distiller's grains, parameters to be considered usually include strain combination ratio, auxiliary material addition (e.g., wheat bran), initial pH, fermentation temperature, and inoculation amount. Many studies have shown that it is difficult to achieve comprehensive improvement in nutritional quality by single-strain fermentation in solid-state feed fermentation; it is necessary to exert the synergistic symbiosis and complementary advantages of mixed strains to maximize fermentation efficiency [10,14], which is consistent with the research on fermented Baijiu distiller's grains. Fan et al. [6] used *Lactobacillus casei*, *Candida utilis*, *Trichoderma viride*, *Geotrichum candidum*, *Aspergillus niger*, and *Rhizopus* sp. to ferment Nong-flavor and Jiang-flavor Baijiu distiller's grains, respectively; when all six strains were inoculated at 0.2% to ferment Baijiu distiller's grains, the crude fiber (CF) degradation rate and true protein (TP) increase degree of both types of Baijiu distiller's grains were optimal. Zhang et al. [12] optimized the fermentation conditions of mixed-strain solid-state fermentation of Baijiu distiller's grains, and obtained the optimal auxiliary material addition and fermentation conditions through orthogonal design: 10% wheat bran, 5% corn flour, 5% rapeseed meal, 1.5% urea, 0.7% potassium dihydrogen phosphate, pH 5, 50% water content, and fermentation at 30 °C for 72 h. Fan et al. [7] optimized the auxiliary material ratio and fermentation conditions under mixed-strain fermentation of Baijiu distiller's grains; the results showed that when wheat bran was used as the auxiliary material with an addition of 5%, mixed microbial agent inoculation amount of 10%, initial pH of 3.70, and with fermentation at 30 °C for 5 d, all tested indicators of Baijiu distiller's grains reached the optimal level.

Traditional fermented feed processes widely use beneficial microorganisms, such as lactic acid bacteria, yeasts, bacilli, and molds for pre-digestion of raw materials. Although the produced fermented feed has a low cost and a pollution-free production process [15], with most anti-nutritional factors eliminated [16,17] and probiotics producing bioactive factors (e.g., organic acids and aromatic substances) to improve feed palatability and animal intestinal health during fermentation [18,19], simple probiotic fermentation of feed often fails to meet expected fermentation indicators. The main reasons include the limitations of microbial strains themselves [15], antagonism between strains [18], and uncontrollable fermentation processes [20]. In such cases, adding enzyme preparations can often overcome the shortcomings of microbial fermentation. Currently, there are few reports on microbial-

enzyme synergistic fermentation of Baijiu distiller's grains, but many reports on other raw materials. Xie et al. [20] optimized the process parameters of microbial-enzyme synergistic fermentation of rapeseed meal; the results showed that through synergistic fermentation of rapeseed meal by *Bacillus subtilis*, *Aspergillus elegans*, and neutral protease, under the conditions of initial pH 6.5, neutral protease addition of 200 U/g dry rapeseed meal, and fermentation at 35 °C for 2 d, the small peptide content reached 12.519%, which was 27.1% higher than that of mixed-strain fermented rapeseed meal, and the glucosinolate degradation rate reached 67.79%. Zhou et al. [21] improved the feed quality of peanut meal by first adding cellulase, phytase, and acid protease for enzymatic hydrolysis, and then adding *Lactobacillus brevis* to ferment the enzymatically hydrolyzed peanut meal for 48 h. The results showed that the crude protein (CP) content of peanut meal treated with microbial-enzyme synergistic treatment increased by 4.2 percentage points compared with raw peanut meal and 3.8 percentage points compared with only fermented peanut meal; the acid soluble protein (ASP) increased by 15.5 percentage points compared with raw peanut meal, the peptide content increased from the initial 1.6% to 15.7%, the total acid content increased from 0.6% to 4.7%, and the hydroxyl radical scavenging rate increased by 2.6 times compared with raw peanut meal. All indicators were significantly higher than those of only enzymatically hydrolyzed or only fermented peanut meal.

To address the shortcomings of high acidity and high fiber content of Nong-flavor Baijiu distiller's grains (NFBDGs) and enhance their feed quality, this study screened probiotic strains for fermenting NFBDGs, optimized the strain combination and fermentation conditions, and, on this basis, further optimized the microbial-enzyme synergistic fermentation process to reduce the lignocellulose content of NFBDGs. This study aims to increase the pH and protein content of fermented NFBDGs and reduce their lignocellulose content, laying a foundation for their application as animal feed.

2. Materials and Methods

2.1. Experimental Materials

A total of 5 yeast strains, 3 *Bacillus* strains, and 5 lactic acid bacteria strains used in the experiment were isolated, identified, and preserved by the authors' laboratory. Detailed information on the strains is shown in Table S1. Enzyme preparations (β-mannanase, cellulase, pectinase, and xylanase) were all provided by Beijing Xindayang Technology Development Co., Ltd., Beijing, China. Fresh NFBDGs were provided by Beijing Zhongnong Lifeng Biotechnology Co., Ltd., Beijing, China.

2.2. Strains Culture

All yeast strains were streaked on Yeast Extract Peptone Dextrose (YPD) solid medium plates (10 g/L peptone, 5 g/L yeast extract, 20 g/L glucose, pH 6.2–6.5, 20 g/L agar) and cultured at 30 °C for 2 d. All *Bacillus* strains were streaked on Luria–Bertani (LB) solid medium plates (10 g/L peptone, 5 g/L yeast extract, 10 g/L sodium chloride, pH 7.2–7.4, 20 g/L agar) and cultured at 37 °C for 1 d. All lactic acid bacteria strains were streaked on De Man, Rogosa, and Sharpe (MRS) solid medium plates (10 g/L peptone, 5 g/L beef extract, 4 g/L yeast extract, 20 g/L glucose, 1 mL/L Tween 80, 2 g/L $K_2HPO_4 \cdot 7H_2O$, 5 g/L $CH_3COONa \cdot 3H_2O$, 2 g/L triammonium citrate, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L $MnSO_4 \cdot 4H_2O$, pH 6.2–6.4, 20 g/L agar) and cultured at 37 °C for 2 d.

2.3. Screening of Strains for Fermenting NFBDGs

The yeast strains were inoculated into a 100 mL conical flask containing 30 mL YPD liquid medium, cultured at 30 °C and 180 rpm for 2 d. The *Bacillus* strains were inoculated

into a 100 mL conical flask containing 30 mL LB liquid medium, cultured at 37 °C and 180 rpm for 1 d. The lactic acid bacteria strains were inoculated into a 100 mL conical flask containing 80 mL MRS liquid medium, statically cultured at 37 °C for 1 d. After the cultivation of all strains, the viable count of the fermentation broth was determined. For the screening of strains for fermenting NFBDGs, a certain amount of NFBDGs was weighed into a 1 L beaker, and the enrichment broth of the candidate strains was added to the NFBDGs at 10^6 CFU/g fresh NFBDGs, mixed evenly, and sealed. For aerobic fermentation, the loading amount of NFBDGs for all samples was 100 g; for anaerobic fermentation, the loading amount of NFBDGs for all samples was 470 g. Aerobic fermentation was sealed with 4 layers of gauze, and anaerobic fermentation was sealed with 4 layers of gauze and 1 layer of kraft paper. During aerobic fermentation, the mixture was stirred every 12 h, while no stirring was needed for anaerobic fermentation. The samples of NFBDGs fermented by bacilli and lactic acid bacteria were cultured at 37 °C for 3 d, and the samples fermented by yeasts were cultured at 30 °C for 3 d. After fermentation, the mixture was stirred evenly, and the viable count of the fermented NFBDGs was determined.

2.4. Optimization of Strain Combination for Solid-State Fermentation of NFBDGs

The experimental method of solid-state fermentation was the same as the aerobic fermentation in Section 2.3. The sensory, mold growth, pH, viable count, dry matter recovery rate (DMR), CP, TP, ASP, CF, acid detergent fiber (ADF), and neutral detergent fiber (NDF) of NFBDGs before and after fermentation were determined. The different strain combinations and their fermentation conditions for NFBDGs are shown in Table 1, with three replicates for each combination.

Table 1. Different strain combinations and their fermentation conditions of Nong-flavor Baijiu distiller's grains (NFBDGs).

Strain Combination	Fermentation Mode	Temperature (°C)	Time (d)
CRS23	Aerobic	37	3
YLZ7	Aerobic	37	3
CJM26	Aerobic	30	3
CRS23+YLZ7	Aerobic	37	3
CJM26+YLZ7	Aerobic	30	3
CJM26+CRS23	Aerobic	30	3
CJM26+YLZ7+CRS23	Aerobic	30	3

Note: CRS23—*Ligilactobacillus salivarius* CRS23; YLZ7—*Bacillus subtilis* YLZ7; CJM26—*Saccharomyces cerevisiae* CJM26. The same below.

2.5. Optimization of Fermentation Conditions for Solid-State Fermentation of NFBDGs

Orthogonal design by SPSS 27.0 was used for the optimization of fermentation conditions. Fermentation time (A), fermentation temperature (B), moisture content of NFBDGs (C), and initial pH of NFBDGs (D) were used as the investigation factors to explore their effects on the mixed-strain fermentation of NFBDGs. The changes in sensory, mold growth, pH, viable count, DMR, CP, TP, ASP, CF, ADF, and NDF contents of NFBDGs before and after fermentation were detected. The experimental factors and levels are shown in Table 2, and the orthogonal experimental design is shown in Table 3.

According to the orthogonal experimental design table, the NFBDGs were pretreated to achieve the water content and pH required by the experimental design. For the adjustment of water content, the initial moisture content of NFBDGs was 60%. To obtain NFBDGs with initial water contents of 40% and 50%, the NFBDGs was spread evenly on the ceramic plate and weighed after the ceramic plate was weighed, and then the ceramic plate with NFBDGs was placed in a 60 °C blast oven. The NFBDGs and the ceramic plate were weighed every 0.5 h until the moisture content of NFBDGs reached 50% and 40%. The drying ceased, and

the NFBDGs were collected and stored at 4 °C for later use. For the adjustment of pH, the initial pH of the NFBDGs was 3.43. To obtain NFBDGs with initial pH values of 5.0 and 7.0, 9 mL of sterile water was added to 1 g of NFBDGs and mixed evenly. Subsequently, 0.01 g of sodium bicarbonate powder was added to the resulting NFBDG suspension, and the pH was measured. The above operation was repeated until the pH of the NFBDGs reached the target value. Then, verification experiments with 10-fold and 100-fold increases in the weight of NFBDGs were carried out according to the same addition amount.

Table 2. Factors and levels of orthogonal test for mixed-culture fermentation of NFBDGs.

Factors	Level		
	Level 1	Level 2	Level 3
Fermentation time (A) (d)	2	3	4
Fermentation temperature (B) (°C)	25	30	37
Moisture content (C) (%)	40	50	60
Initial pH (D)	3.43	5.00	7.00

Table 3. Orthogonal design for mixed-culture fermentation of NFBDGs.

Run	Fermentation Time (d)	Fermentation Temperature (°C)	Moisture Content (%)	Initial pH
1	4	30	60	3.43
2	4	37	40	5
3	3	25	60	5
4	3	37	50	3.43
5	3	30	40	7
6	2	37	60	7
7	2	25	40	3.43
8	4	25	50	7
9	2	30	50	5

To verify the orthogonal experiment, fermentation was carried out under the optimal process conditions obtained from the orthogonal experiment. The DMR, CP, TP, ASP, CF, ADF, and NDF of NFBDGs before and after fermentation were determined.

2.6. Screening of Enzyme Preparations for Microbial-Enzyme Synergistic Fermentation of NFBDGs

The enzyme preparations used included β-mannanase (A), cellulase (B), pectinase (C), and xylanase (D), with initial enzyme activities of 200,000 U/g, 10,000 U/g, 30,000 U/g, and 200,000 U/g, respectively. In total, 100 g of fresh NFBDGs was weighed into a 1 L beaker. The enrichment broth of the 3 strains (Section 2.3) was added to the NFBDGs at 10^6 CFU/g fresh NFBDGs, and the 4 enzyme preparations were added at 200 U/g dry NFBDGs. The mixture was stirred evenly and sealed with 4 layers of gauze. Fermentation was carried out at 37 °C for 4 d; during fermentation, the mixture was stirred every 12 h. A total of 14 groups of experiments were conducted for enzyme preparation combinations, including single-enzyme groups, two-enzyme combinations, three-enzyme combinations, and four-enzyme combinations, with three replicates for each group. The CF, ADF, and NDF of the NFBDGs before and after fermentation were determined.

2.7. Analytical Methods

The viable count of the microbial enrichment broth was determined by inoculating 0.5 mL of enrichment broth of different strains into a 10 mL centrifuge tube containing 4.5 mL of sterile physiological saline. The suspension was mixed evenly with a vortex oscillator (VORTEX-GENIE 2, Scientific Industries Co., Ltd., Wilmington, DE, USA) and

subjected to a 10-fold gradient dilution. Then, 100 µL of the diluted bacterial solution with an appropriate dilution degree was spread on Bengal Red medium plates (5 g/L peptone, 10 g/L glucose, 1 g/L potassium dihydrogen phosphate, 0.5 g/L magnesium sulfate, 0.033 g/L Bengal red, 0.1 g/L chloramphenicol, 20 g/L agar) for yeast counting, and on LB solid medium plates for *Bacillus* counting, followed by cultivation in a constant temperature incubator at 30 °C and 37 °C for 1–2 d, respectively. The viable count of lactic acid bacteria was determined by a 10-fold gradient dilution pour plate method; the gradient dilution procedure was the same as above. Subsequently, 1 mL of the diluted bacterial solution of appropriate dilution was added to the plate, followed by pouring MRS solid medium, and then cultivation in a 37 °C constant temperature incubator for 2 days.

The viable count of NFBDGs before and after fermentation was detected by weighing 5 g of NFBDGs into a 300 mL conical flask containing 45 mL of sterile physiological saline and an appropriate amount of glass beads. The mixture was shaken at 30 °C and 180 rpm for 30 min. Then, a 10-fold gradient dilution was performed, and the appropriate dilution degree was selected for the viable count. The counting method was the same as that for the enrichment broth. For *Saccharomyces cerevisiae* CJM26 and *Bacillus subtilis* YLZ7, the number of colonies exhibiting morphological similarity to the target strains was counted. For acid-producing bacteria (APB) counting, MRS solid medium containing 5 g/L $CaCO_3$ was used. After solidification, the plate was placed in a 37 °C incubator for 2 d, and the number of colonies with calcium-dissolving circles was counted.

The NFBDGs before and after fermentation were subjected to sensory evaluation [22]. The growth of molds in NFBDGs before and after fermentation was detected using Bengal Red medium plates. Specifically, the NFBDGs samples were diluted 10-fold with sterile physiological saline, and 100 µL of the diluted suspension was pipetted and spread onto the Bengal Red medium plates, followed by incubation at 28 °C for 5 days. The growth of molds was graded according to the colony count: "-" was assigned when no colony grew on Bengal Red medium plates; "+" was assigned when the colony number ranged from 1 to 10; "++" for 10 to 50 colonies; "+++" for 50 to 100 colonies; and "++++" for more than 100 colonies. The NFBDGs before and after fermentation were dried at 60 °C for 24 h in a blast oven (DGG-9240B, Shanghai Senxin Experimental Instruments Co., Ltd., Shanghai, China), and subsequently were crushed to pass through a 1 mm sieve for the determination of CF, NDF, and ADF; the samples were further crushed to pass through a 0.425 mm sieve for the determination of CP, TP, and ASP. The moisture, CP, CF, and NDF were analyzed in accordance with the National Standards of the People's Republic of China: GB/T 6435-2014 [23], GB/T 6432-2018 [24], GB/T 6434-2022 [25], and GB/T 20806-2022 [26]. The ASP and ADF were determined in accordance with Industry Standard of the People's Republic of China: NY/T 3801-2020 [27] and NY/T 1459-2022 [28]. Moisture content was measured by heating the samples to a constant weight at 105 °C in an electric forced-air oven (DHG-9140AL, Beijing Luxi Technology Co., Ltd., Beijing, China), and then the dry matter content was calculated. The CP, TP, and ASP contents were determined using an automatic Kjeldahl apparatus (KjeltecTM8400, Foss, Hillerød, Denmark). The CF, NDF, and ADF were determined using a polyester mesh bag-automated fiber analyzer (A2000I, ANKOM, Macedon, NY, USA). For dry matter recovery rate (DMR), the weight and moisture content of NFBDGs before and after fermentation were determined, and DMR was calculated according to Equation (1):

$$\mathrm{DMR} = \frac{\text{Weight of fermented sample} \times \text{Dry matter content of fermented sample}}{\text{Weight of unfermented sample} \times \text{Dry matter content of unfermented sample}} \quad (1)$$

The determination of true protein (TP) was as follows: 75 mL of distilled water was added to a 250 mL beaker with 1 g of sample, and the mixture was heated until boiling.

Deionized water was added during heating to maintain the aqueous solution volume (no less than 75 mL). After boiling for 30 min, the mixture was cooled. Then, 2 mL of 2.5% sodium hydroxide solution was added and stirred thoroughly, followed by the slow addition of 5 mL of 10% copper sulfate solution with stirring. The above operation was repeated three times, adding 6 mL of sodium hydroxide solution and 5 mL of copper sulfate solution at the same concentrations each time. The mixture was left to stand for aging overnight. The precipitate was filtered through medium-speed quantitative filter paper, and washed with hot water above 80 °C for 7–10 times until no precipitate or turbidity was observed when the filtrate was tested with 5% barium chloride solution. The precipitate, together with the filter paper, was dried at 75 °C for 1 h. The dried mixture was detected in accordance with the CP determination method. A blank test was conducted simultaneously. The pH of NFBDGs before and after fermentation was determined by adding 1 g of sample into 9 mL of sterile water, and the pH value was measured with a pH meter.

2.8. Statistical Analysis

Data processing was conducted using Microsoft Excel (v14.0, Microsoft Excel 2010) to compute group means, standard deviations. Statistical significance was assessed via one-way analysis of Variance (ANOVA) followed by Duncan's multiple range test utilizing JMP 10 software (SAS Inc., Cary, NC, USA). Orthogonal design, ANOVA, and Least Significant Difference (LSD) test were conducted using SPSS software (v 27.0, IBM Inc., New York, NY, USA). Results were expressed as mean ± standard deviation, with a significance threshold set at $p < 0.05$.

3. Results and Discussion

3.1. Effect of Different Strains on Fermented NFBDGs

Since both yeasts and lactic acid bacteria are facultative anaerobes, aerobic and anaerobic solid-state fermentation were conducted separately for the two types of strains. Bacilli are strictly aerobic; therefore, only aerobic solid-state fermentation was conducted for them. The screening results of yeast showed that the *Saccharomyces cerevisiae* CJM26 had the largest △log of viable count before and after aerobic fermentation of NFBDGs, with the best growth status (Table 4). However, the △log of viable count of the five yeast strains before and after anaerobic fermentation of NFBDGs was all <0, indicating that yeasts could not grow in NFBDGs under anaerobic conditions (Table 4). This might be attributed to the fact that the five yeast strains belonging to ascomycete yeasts produce ethanol under anaerobic conditions, which, together with the residual ethanol in NFBDGs, exerts a feedback inhibition effect on these five yeast strains [29].

All three bacillus strains could grow in NFBDGs under aerobic conditions, among which *Bacillus subtilis* YLZ7 had the best growth status (Table 4). All five lactic acid bacteria strains could grow in NFBDGs under aerobic conditions, among which *Ligilactobacillus salivarius* CRS23 had the best growth status (Table 4), but none could grow in NFBDGs under anaerobic conditions. Currently, there are few reports on fermenting Baijiu distiller's grains with lactic acid bacteria alone. This study attempted to screen lactic acid bacteria capable of growing in NFBDGs; the results showed that all five candidate lactic acid bacteria strains could grow under aerobic conditions but not under anaerobic conditions. This might be related to the indigenous microorganisms in NFBDGs: under anaerobic conditions, some indigenous microorganisms could grow preferentially to become dominant bacteria or inhibit the growth of inoculated lactic acid bacteria, while under aerobic conditions, such microorganisms do not become dominant bacteria, allowing for the inoculated lactic acid bacteria to grow normally.

Table 4. Screening of yeast, bacillus, and lactic acid bacteria strains for the solid-state fermentation of NFBDGs (CFU/g).

Strains	Fermentation Mode	0 d	3 d	Δlog
ADM	Aerobic	6.9×10^5	1.1×10^7	1.20
SC17-1		1.1×10^6	9.3×10^7	1.93
CJM26		5.6×10^5	6.3×10^7	2.05
FJM13		1.0×10^6	$<1.0 \times 10^7$	--
FJM12		1.3×10^6	2.5×10^7	1.28
ADM	Anaerobic	6.1×10^5	$<1 \times 10^5$	--
SC17-1		8.5×10^5	3.1×10^5	−0.43
CJM26		8.8×10^5	$<1.0 \times 10^5$	--
FJM13		1.3×10^6	$<1.0 \times 10^5$	--
FJM12		9.6×10^5	2.0×10^5	−0.68
YLZ7	Aerobic	5.53×10^6	6.0×10^8	2.03
PYB2		2.4×10^6	8.4×10^7	1.54
CYB44		6.6×10^6	2.8×10^7	0.63
CRS23	Aerobic	1.2×10^6	7.6×10^8	2.74
CRS33		8.9×10^5	1.8×10^8	1.95
CRS2-1		1.4×10^6	2.0×10^8	1.85
CRS5-1		2.1×10^6	2.3×10^8	1.79
CRS5-2		2.1×10^6	3.1×10^8	2.0
CRS23	Anaerobic	5.53×10^6	$<1.0 \times 10^5$	--
CRS33		5.8×10^6	$<1.0 \times 10^5$	--
CRS2-1		6.6×10^6	$<1.0 \times 10^5$	--
CRS5-1		2.6×10^6	$<1.0 \times 10^5$	--
CRS5-2		7.5×10^6	2.0×10^5	−1.57

Note: Δlog—the logarithmic value of the average viable count at 3 d minus the logarithmic value of the average viable count at 0 d. ADM—*Candida utilis* ADM; SC17-1—*Saccharomyces cerevisiae* SC17-1; FJM13—*Saccharomyces cerevisiae* FJM13; FJM12—*Saccharomyces cerevisiae* FJM12; PYB2—*Bacillus amyloliquefaciens* PYB2; CYB44—*Bacillus subtilis* CYB44; CRS33—*Enterococcus faecium* CRS33; CRS2-1—*Ligilactobacillus salivarius* CRS2-1; CRS5-1—*Ligilactobacillus salivarius* CRS5-1; CRS5-2—*Ligilactobacillus salivarius* CRS5-2.

3.2. Effect of Strain Combination on Fermented NFBDGs

3.2.1. Sensory Evaluation, Mold Growth, and pH of NFBDGs Fermented by Different Strain Combinations

The above research results showed that *Ligilactobacillus salivarius* CRS23, *Bacillus subtilis* YLZ7, and *Saccharomyces cerevisiae* CJM26 were suitable for fermenting NFBDGs, and aerobic fermentation was adopted. This section compared the effects of different combination modes of the three strains on fermented NFBDGs. As shown in Table 5, the odor and color of NFBDGs deepened after fermentation. The NFBDGs fermented by *Bacillus subtilis* YLZ7, *Saccharomyces cerevisiae* CJM26, *Ligilactobacillus salivarius* CRS23+*Bacillus subtilis* YLZ7, *Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7, and *Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7+*Ligilactobacillus salivarius* CRS23 showed a rotten smell, and the color change was more obvious, generally darker than the unfermented NFBDGs. Among them, the combination of *Ligilactobacillus salivarius* CRS23+*Bacillus subtilis* YLZ7+*Saccharomyces cerevisiae* CJM26 had the darkest color after fermentation, which was dark brown. In terms of texture, compared with the unfermented NFBDGs, the fermented product's viscosity increased, resulting in a caking phenomenon, which might be attributed to the increase in crude protein or polysaccharide substances in NFBDGs during fermentation. After feed raw materials are fermented, the color and texture of the raw materials often change significantly. Xuan et al. [16] used single strains and mixed strains for the solid-state fermentation of cottonseed meal; the fermented materials

generally turned dark brown, with increased viscosity and a caking phenomenon, and the fermented cottonseed meal inoculated with *Bacillus subtilis* had a rotten smell, which was consistent with the results of this study. Yang et al. [3] fermented Baijiu distiller's grains with *Aspergillus oryzae* and *Aspergillus awamori*, and found that this fermentation process promoted the browning of distilled spent grain, leading to an increase in dark-colored substances. These outcomes were also consistent with the results of this study.

Table 5. Sensory evaluation, mold growth, and pH of NFBDGs after fermentation with different strain combinations.

Groups	Texture	Color	Odor	Mold	pH
CRS23	Clumps	Tawny	Slight sour taste	+	6.39 ± 0.12
YLZ7	Clumps	Dark	Slight rotten smell	++	6.20 ± 0.01
CJM26	Clumps	Dark yellow	Slight rotten smell	—	5.64 ± 0.23
CRS23+YLZ7	Clumps	Brown	Strong rotten smell	+	6.40 ± 0.15
CJM26+YLZ7	Clumps	Brown yellow	Slight rotten smell	—	5.63 ± 0.03
CJM26+CRS23	Clumps	Brown	Slight alcoholic aroma	—	5.49 ± 0.04
CJM26+YLZ7+CRS23	Clumps	Dark brown	Rotten smell	—	5.43 ± 0.02
Unfermented NFBDGs	Loose	Yellow	Odorless	—	3.31 ± 0.02

Note: "+" indicates 1–10 colonies grown on Bengal Red medium plates; "++" indicates 10–50 colonies; "—" indicates no colony growth on Bengal Red medium plates.

The pH of all fermentation groups increased after fermentation. Among them, the pH of the groups fermented by *Saccharomyces cerevisiae* CJM26, *Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7, *Saccharomyces cerevisiae* CJM26+*Ligilactobacillus salivarius* CRS23, and *Saccharomyces cerevisiae* CJM26+*Ligilactobacillus salivarius* CRS23+*Bacillus subtilis* YLZ7 was between 5.4 and 5.6, and the pH of the other fermentation groups was between 6.2 and 6.4. This indicated that alkaline metabolites accumulated during the fermentation of NFBDGs. Generally, it is believed that the pH of materials that are solid-state fermented by lactic acid bacteria decreases due to the acid production by lactic acid bacteria. However, the pH of all experimental groups inoculated with lactic acid bacteria in this study increased at the end of fermentation, which was inconsistent with the research of Mei et al. [30]. The reason might be that the NFBDGs used in this study were not sterilized, which contained residual microbial communities from the Baijiu brewing process. When artificially selected strains were inoculated, the growth of the exogenous strains changed the indigenous microbial community in NFBDGs, promoting the growth of microorganisms capable of producing a large amount of alkaline metabolites, resulting in an increase in pH. The results of this study were also inconsistent with the report of Wang et al. [31], who employed sodium bicarbonate to adjust the initial pH of Baijiu distiller's grains to 6–7; the pH decreased to 5–6 after mixed-strain fermentation.

In addition, due to the high water content of fresh Baijiu distiller's grains (up to 60%), they are prone to mold during storage. Therefore, the mold growth of NFBDGs in each treatment group during fermentation was detected. The results showed that the NFBDGs in the groups *Ligilactobacillus salivarius* CRS23, *Bacillus subtilis* YLZ7, and *Ligilactobacillus salivarius* CRS23+*Bacillus subtilis* YLZ7 had varying degrees of mold growth at the end of fermentation, while no mold appeared in the other treatment groups, indicating that these three combinations could not inhibit the mold growth of NFBDGs during fermentation.

3.2.2. Microbial Growth of NFBDGs Fermented by Different Strain Combinations

As shown in Table 6, whether single-strain fermentation or mixed-strain fermentation was conducted, APB, *Bacillus subtilis* YLZ7, and *Saccharomyces cerevisiae* CJM26 grew in NFBDGs after 3 d of fermentation. Among them, APB grew the best; the viable count reached above 1×10^9 CFU/g after single-strain fermentation and two-strain combination (*Ligilactobacillus salivarius* CRS23+*Bacillus subtilis* YLZ7, *Ligilactobacillus salivarius* CRS23+*Saccharomyces cerevisiae* CJM26) fermentation, and 8.8×10^8 CFU/g after three-strain combination fermentation. The growth performance of *Saccharomyces cerevisiae* CJM26 was inferior to that of APB; the viable count reached above 1×10^8 CFU/g after single-strain fermentation, two-strain combination (*Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7, *Saccharomyces cerevisiae* CJM26+*Ligilactobacillus salivarius* CRS23) fermentation, and three-strain combination fermentation. The viable count of *Bacillus subtilis* YLZ7 after three-strain combination fermentation was 8.1×10^7 CFU/g, and the viable count after single-strain fermentation and two-strain combination (*Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7, *Ligilactobacillus salivarius* CRS23+*Bacillus subtilis* YLZ7) fermentation was above 1×10^6 CFU/g, which increased compared with the initial viable count. The viable count of *Bacillus subtilis* YLZ7 after single-strain fermentation was lower than that after strain screening, while the viable count of *Ligilactobacillus salivarius* CRS23 and *Saccharomyces cerevisiae* CJM26 after single-strain fermentation was higher than that after strain screening. This was possibly due to the unstable composition of the NFBDG raw materials used; although the Baijiu distiller's grains were discarded from the same Baijiu distillery, the composition of those from different batches was not completely consistent.

Table 6. Viable counts of NFBDGs before and after fermentation with different strain combinations (CFU/g).

Groups	Viable Counts Before Fermentation			Viable Counts After Fermentation		
	APB	CJM26	YLZ7	CRS23	CJM26	YLZ7
APB	8.2×10^5			2.7×10^9		
YLZ7			9.1×10^5			1.1×10^6
CJM26		9.3×10^5			2.1×10^8	
CRS23+YLZ7	1.0×10^6		2.3×10^6	2.6×10^9		9.4×10^6
CJM26+YLZ7		7.8×10^5	2.0×10^6		1.5×10^8	6.7×10^6
CJM26+CRS23	1.5×10^6	1.8×10^6		1.6×10^9	2.1×10^8	
CJM26+YLZ7+CRS23	1.2×10^6	1.2×10^6	8.8×10^5	8.8×10^8	2.5×10^8	8.1×10^7

Note: APB—acid-producing bacteria.

3.2.3. Variations in Nutritional Components of NFBDGs Fermented by Different Strain Combinations

The contents of CP, TP, ASP, CF, ADF, and NDF in unfermented NFBDGs and fermented NFBDGs by different strain combinations were determined, respectively, to judge whether the fermented NFBDGs could meet the requirements of feed raw materials. Tables 7 and 8 indicate the dry matter (DM)-based nutritional components of the samples and the results considering the concentration effect, respectively. The calculation formula of the concentration effect was: DM basis content of nutritional component × DMR (Table 7). The reason for calculating the concentration effect data was to consider the concentration increase in nutritional components caused by DM loss during the aerobic fermentation of NFBDGs. By comparing the data converted by the concentration effect, the absolute changes in various nutritional indicators can be reflected more intuitively. Currently, in the previous reports on solid-state fermentation of Baijiu distiller's grains, few researchers have considered the impact of the concentration effect when comparing the changes in nutritional components before and after fermentation, which has led to inaccurate conclusions.

Table 7. Contents of crude protein (CP), true protein (TP), acid-soluble protein (ASP), crude fiber (CF), acid detergent fiber (ADF), and neutral detergent fiber (NDF) on a dry matter (DM) basis of NFBDGs and dry matter recovery rate (DMR) of different treatment groups after fermentation with different strain combinations.

Groups	CP %	TP %	ASP %	CF %	ADF %	NDF %	DMR
CRS23	15.64 ± 0.13^{D}	13.40 ± 0.34^{E}	7.26 ± 1.50^{A}	35.39 ± 0.92^{BC}	61.37 ± 2.24^{A}	69.63 ± 0.70^{AB}	0.78 ± 0.01
YLZ7	17.38 ± 0.54^{A}	15.54 ± 0.38^{AB}	7.64 ± 0.61^{A}	37.12 ± 0.67^{AB}	59.91 ± 1.02^{AB}	72.33 ± 4.18^{A}	0.78 ± 0.04
CJM26	16.42 ± 0.53^{CD}	15.71 ± 0.32^{A}	6.77 ± 0.13^{ABC}	33.05 ± 0.24^{DE}	55.14 ± 0.41^{CD}	65.31 ± 4.44^{BC}	0.82 ± 0.03
CRS23+YLZ7	17.31 ± 0.39^{AB}	15.51 ± 0.29^{ABC}	6.54 ± 0.38^{ABC}	37.82 ± 0.95^{A}	60.29 ± 0.24^{AB}	66.98 ± 6.51^{AB}	0.78 ± 0.01
CJM26+YLZ7	16.55 ± 0.15^{BC}	14.79 ± 0.26^{CD}	6.00 ± 0.07^{BC}	34.09 ± 1.35^{CD}	57.09 ± 3.10^{BC}	66.69 ± 2.80^{BC}	0.85 ± 0.01
CJM26+CRS23	15.69 ± 0.65^{D}	14.30 ± 0.39^{D}	6.64 ± 0.91^{ABC}	35.69 ± 2.36^{BC}	59.03 ± 1.18^{ABC}	68.20 ± 1.08^{AB}	0.81 ± 0.01
CRS23+CJM26+YLZ7	17.10 ± 0.73^{ABC}	15.25 ± 0.84^{ABC}	6.58 ± 0.54^{ABC}	30.81 ± 1.31^{F}	51.71 ± 2.59^{DE}	66.03 ± 1.52^{BC}	0.86 ± 0.06
Unfermented NFBDGs	12.74 ± 0.35^{E}	10.55 ± 0.65^{F}	5.76 ± 0.08^{C}	31.29 ± 1.06^{EF}	48.51 ± 0.88^{E}	61.58 ± 0.75^{C}	--

Note: Within each column, values with different superscripts indicate significant differences, $p < 0.05$; values with the same superscripts indicate no significant differences, $p > 0.05$.

Table 8. Contents of CP, TP, ASP, CF, ADF, and NDF considering the concentration effect of NFBDGs of different treatment groups after fermentation with different strain combinations.

Groups	CP %	TP %	ASP %	CF %	ADF %	NDF %
CRS23	12.27 ± 0.27^{D}	10.51 ± 0.34^{E}	5.70 ± 1.23^{A}	27.76 ± 1.03^{BC}	48.11 ± 1.25^{A}	54.61 ± 1.02^{B}
YLZ7	13.61 ± 0.45^{BC}	12.18 ± 0.64^{BCD}	5.98 ± 0.26^{A}	29.09 ± 1.51^{ABC}	46.93 ± 1.82^{A}	56.69 ± 4.53^{AB}
CJM26	13.55 ± 0.88^{BC}	12.95 ± 0.40^{AB}	5.58 ± 0.13^{A}	27.26 ± 1.18^{C}	45.47 ± 1.45^{AB}	53.93 ± 5.37^{B}
CRS23+YLZ7	13.65 ± 0.24^{BC}	12.24 ± 0.26^{BCD}	5.16 ± 0.30^{AB}	29.82 ± 0.48^{AB}	47.56 ± 0.95^{A}	52.85 ± 5.36^{B}
CJM26+YLZ7	14.18 ± 0.11^{AB}	12.67 ± 0.35^{ABC}	5.14 ± 0.08^{AB}	29.21 ± 1.59^{ABC}	48.93 ± 3.44^{A}	57.16 ± 3.26^{AB}
CJM26+CRS23	12.74 ± 0.51^{CD}	11.62 ± 0.46^{D}	5.39 ± 0.65^{A}	28.98 ± 1.71^{ABC}	47.95 ± 0.93^{A}	55.41 ± 1.18^{B}
CRS23+CJM26+YLZ7	14.83 ± 1.52^{A}	13.19 ± 0.63^{A}	5.68 ± 0.18^{A}	26.74 ± 2.96^{C}	44.88 ± 5.27^{AB}	57.20 ± 4.39^{AB}
Unfermented NFBDGs	12.74 ± 0.35^{CD}	10.55 ± 0.65^{E}	5.76 ± 0.08^{A}	31.29 ± 1.06^{A}	48.51 ± 0.88^{A}	61.58 ± 0.75^{A}

Note: Within each column, values with different superscripts indicate significant differences, $p < 0.05$; values with the same superscripts indicate no significant differences, $p > 0.05$.

Table 7 shows the DM basis contents of various nutritional components. As for CP, the content in the *Bacillus subtilis* YLZ7 group was the highest, but there was no significant difference with the *Ligilactobacillus salivarius* CRS23+*Bacillus subtilis* YLZ7 and *Ligilactobacillus salivarius* CRS23+*Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7 groups ($p > 0.05$), and it was 36.43% higher than that of unfermented NFBDGs. As for TP, the content in the *Saccharomyces cerevisiae* CJM26 group was the highest, but there was no significant difference with the *Bacillus subtilis* YLZ7, *Ligilactobacillus salivarius* CRS23+*Bacillus subtilis* YLZ7, and *Ligilactobacillus salivarius* CRS23+*Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7 groups ($p > 0.05$), and it was 48.99% higher than that of unfermented NFBDGs. As for ASP, the content in the *Bacillus subtilis* YLZ7 group was the highest, but there was no significant difference with the *Ligilactobacillus salivarius* CRS23, *Saccharomyces cerevisiae* CJM26, *Ligilactobacillus salivarius* CRS23+*Bacillus subtilis* YLZ7, *Saccharomyces cerevisiae* CJM26+*Ligilactobacillus salivarius* CRS23, and *Ligilactobacillus salivarius* CRS23+*Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7 groups ($p > 0.05$), and it was 32.63% higher than that of unfermented NFBDGs. The determination of the three protein-related indicators mainly aimed to investigate whether the protein-related indicators of NFBDGs had positive changes after fermentation. CP reflected the nitrogen content of Baijiu distiller's grains and was an important indicator for animal feed raw materials; the higher the crude protein content, the more suitable it was for providing protein nutrition for feed. TP, a bioactive natural protein molecule that can be effectively digested and absorbed by organisms to participate in metabolism [32], refers to the natural protein in crude protein where amino acids are linked by peptide bonds. The content and quality of TP in feed directly affect the growth, development, and health of animals; therefore, the higher the TP content, the more suitable the raw material is for animal feed. The increase in TP content in this study should be due to the conversion of non-protein nitrogen substances

into microbial protein by the microorganisms inoculated into NFBDGs during growth, which can reflect the proliferation of microorganisms in NFBDGs. ASP refers to the protein-related substances that can be dissolved in a certain concentration of acid solution (such as trichloroacetic acid, hydrochloric acid, sulfuric acid, etc.), usually low-molecular-weight proteins, peptides, and free amino acids. Therefore, the content of ASP reflects the content of low-molecular-weight proteins, peptides, and free amino acids in Baijiu distiller's grains; the increase in these substances often comes from the degradation products of macromolecular proteins by proteases, and the higher the content, the stronger the ability of microorganisms to decompose macromolecular proteins during fermentation [33].

CF mainly reflects the sum of insoluble components in the cell wall of plant feed raw materials, usually including cellulose, hemicellulose, and lignin; these components are generally difficult to be digested and utilized by monogastric animals in the digestive tract and become anti-nutritional factors, affecting the digestion and absorption of raw materials by animals [34]. ADF refers to the insoluble residue of plant feed raw materials after boiling with acid detergent (cetyl sulfate), mainly including cellulose, lignin, and acid-insoluble ash. For monogastric animals, excessively high ADF content will reduce the energy of the feed [35]. NDF refers to the insoluble residue of plant feed raw materials after boiling with neutral detergent (sodium dodecyl sulfate solution with pH ≈ 7.0, SDS), mainly including cellulose, hemicellulose, lignin, and acid-insoluble ash. Similarly, for monogastric animals, excessively high NDF content will reduce the energy of the feed; lignin in NDF is a difficult-to-digest component, which affects the absorption of nutritional components by monogastric animals [35]. As for CF and ADF, the content of the *Ligilactobacillus salivarius* CRS23+*Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7 group was the lowest, but there was no significant difference with the unfermented NFBDGs ($p > 0.05$). As for NDF, the content of the *Saccharomyces cerevisiae* CJM26 group was the lowest, but there was no significant difference with the *Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7 group, *Ligilactobacillus salivarius* CRS23+*Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7 group, and unfermented NFBDGs ($p > 0.05$).

Table 8 shows the contents of various nutritional components considering the concentration effect during fermentation. As for crude protein, the content of the *Ligilactobacillus salivarius* CRS23+*Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7 group was the highest, but there was no significant difference with the *Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7 group ($p > 0.05$), and it was 16.41% higher than that of unfermented NFBDGs. As for TP, the content of the *Ligilactobacillus salivarius* CRS23+*Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7 group was also the highest, but there was no significant difference with the *Saccharomyces cerevisiae* CJM26 and *Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7 groups ($p > 0.05$), and it was 25.02% higher than that of unfermented NFBDGs. As for ASP, there was no significant difference between all fermentation groups and unfermented NFBDGs ($p > 0.05$), and the ASP content of the *Bacillus subtilis* YLZ7 group was the highest (5.98%). As for CF, the content of the *Ligilactobacillus salivarius* CRS23+*Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7 group was the lowest, but there was no significant difference with the single-strain fermentation groups, *Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7 group, and *Saccharomyces cerevisiae* CJM26+*Ligilactobacillus salivarius* CRS23 group ($p > 0.05$), and it was 14.5% lower than that of unfermented NFBDGs. As for ADF, the content of the *Ligilactobacillus salivarius* CRS23+*Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7 group was the lowest, but there was no significant difference with other inoculated groups and unfermented NFBDGs ($p > 0.05$). As for NDF, the content of the *Ligilactobacillus salivarius* CRS23+*Bacillus subtilis* YLZ7 group was the lowest, but there was no significant difference with other inoculated groups ($p > 0.05$), and it was 14.18% lower than that of unfermented NFBDGs.

The above results indicate that for the increase in the three protein indicators, the optimal strain combination was *Ligilactobacillus salivarius* CRS23+*Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7; for the decrease in the three cellulose indicators, the *Ligilactobacillus salivarius* CRS23+*Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7 group had a significant effect on CF reduction in NFBDGs, and had a decreasing trend on ADF and NDF. Based on a comprehensive analysis of the determination results, the optimal strain combination for fermenting NFBDGs was confirmed as *Ligilactobacillus salivarius* CRS23+*Saccharomyces cerevisiae* CJM26+*Bacillus subtilis* YLZ7.

Wang et al. [31] first screened *Bacillus subtilis* RLI2019, *Lactobacillus plantarum* DPH, and *Saccharomyces cerevisiae* E9 suitable for fermenting Baijiu distiller's grains from 11 candidate strains based on the increase in probiotic count, decrease in pathogenic bacteria count, reducing sugar content, and pH of Baijiu distiller's grains after single-strain solid-state fermentation. Then, the three screened strains were used in a two-step mixed-strain fermentation of Baijiu distiller's grains, i.e., aerobic fermentation at 37 °C for 60 h, followed by anaerobic fermentation for 120 h. After fermentation, the CP of Baijiu distiller's grains increased by 6.87%, ASP increased by 21.89%, and ADF and NDF decreased by 10.15% and 7.54%, respectively, which was consistent with the results of this study. Yu et al. [13] first conducted single-strain solid-state fermentation of Baijiu distiller's grains to screen the strains with the highest CP content of fermented Baijiu distiller's grains from seven *Trichoderma koningii* strains and two *Geotrichum candidum* strains, which were *Trichoderma koningii* T8 and *Geotrichum candidum* G1, respectively. Then, combined with *Aspergillus niger* A11, mixed-strain fermentation of Baijiu distiller's grains was carried out; the results showed that when the three strains were inoculated at a ratio of 2:2:3 (v/v), the CP content of fermented Baijiu distiller's grains was the highest (29.6%). Zhang et al. [8] investigated the growth of candidate strains via spot inoculation on Baijiu distiller's grains medium plates, performed single-strain and mixed-strain solid-state fermentation of Baijiu distiller's grains, and determined the changes in crude protein, true protein, and CF before and after fermentation to screen the optimal strain combination for the fermentation. The results showed that the optimal strain combination for fermenting Baijiu distiller's grains was *Geotrichum candidum*, *Candida tropicalis*, and *Trichoderma viride* with an inoculation ratio of 1:1:1 (v/v). Compared with unfermented Baijiu distiller's grains, the CP increased by 24.5%, TP increased by 53.18%, and CF decreased by 26.65% on a DM basis. Fan et al. [6] used *Lactobacillus casei*, *Candida utilis*, *Trichoderma viride*, *Geotrichum candidum*, *Aspergillus niger*, and *Rhizopus oryzae* as candidate strains to screen the optimal strain combination for fermenting NFBDGs. The results showed that when the five strains were inoculated into NFBDGs at an equal ratio of 0.2% and fermented for 6 d, the TP content increased the most (16.82%).

It can be seen that most studies have reported that the microbial combinations for fermenting Baijiu distiller's grains were mainly composed of molds, supplemented by *Candida tropicalis* and *Candida utilis*, while lactic acid bacteria and *Bacillus subtilis* were not the main fermentation strains, which is inconsistent with the results of this study. The reason is that many molds can produce a large amount of cellulase and easily utilize the nutrients of Baijiu distiller's grains for growth. However, there are also shortcomings in using molds to ferment Baijiu distiller's grains: on the one hand, many molds have not been proven suitable for feed production, for example, *Trichoderma viride*, *Trichoderma koningii*, and *Geotrichum candidum*; on the other hand, during industrial production, fermentation with molds requires cumbersome fermentation operations and post-treatment processes, and the sensory performance of fermented Baijiu distiller's grains is not pleasant. Therefore, in this study, molds were not used as candidate strains when screening strains for fermenting NFBDGs.

3.3. Effect of Fermentation Process Conditions on Fermented NFBDGs

3.3.1. Sensory Evaluation, Mold Growth, and pH of NFBDGs Fermented by Different Fermentation Process Conditions

The orthogonal design method was used to optimize the process conditions of mixed-strain fermentation for NFBDGs. As shown in Table S2, the NFBDGs in Treatments 1, 2, 3, 4, 6, and 9 showed caking after mixed-strain fermentation, with obvious caking in Treatments 3 and 9. The strains decomposed macromolecular substances in NFBDGs to produce substances, such as polypeptides and polysaccharides, during the growth process; at the same time, these substances bonded to water molecules to increase the surface tension inside the fermentation matrix, which in turn increased the viscosity of the fermentation system and caused caking [36,37]. The NFBDGs in Treatment 4 had the strongest rotten smell. The NFBDGs in Treatment 3 had the darkest color after fermentation. At the end of fermentation, compared with the initial fermentation state, the pH of NFBDGs in all treatment groups increased; among them, Treatment 8 had the highest pH (8.73), while the pH of Treatments 3 and 9 increased slightly, both around 4.8. The pH of most treatment groups was between 5 and 6. A large amount of mold was observed in Treatments 1, 2, 4, and 6 at the end of fermentation; only a small amount of mold was found in Treatments 3 and 4, while no mold was produced in Treatments 7, 8, and 9 at the end of fermentation.

3.3.2. Microbial Growth of NFBDGs Fermented by Different Fermentation Process Conditions

The three strains were used for solid-state fermentation of NFBDGs at an inoculation amount of 10^6 CFU/g fresh NFBDGs. The initial viable count of the three strains in different treatments was calculated based on the viable count of the enrichment broth and the inoculation volume. The viable count of the APB, *Saccharomyces cerevisiae* CJM26, and *Bacillus subtilis* YLZ7 in different treatments at the end of fermentation is shown in Table S3. For APB, Treatment 3 exhibited the highest viable count at the end of fermentation, increasing from 6.7×10^6 to 1.1×10^{10} CFU/g. Except for Treatment 2 and Treatment 8, the viable count of APB in all other treatment groups increased to varying degrees compared with that before fermentation. For *Bacillus subtilis* YLZ7, the viable count in Treatment 1 and Treatment 4 did not increase but dropped below 1×10^4 CFU/g and 1×10^6 CFU/g after fermentation, respectively. Only in Treatment 2 did it increase from 1.1×10^6 to 2.5×10^8 CFU/g, while the viable counts in the remaining treatment groups all increased to varying degrees. For *Saccharomyces cerevisiae* CJM26, the viable count in Treatment 8 was lower than the initial level at the end of fermentation, indicating no microbial growth. The viable count in Treatment 2 increased slightly. In Treatments 4 and 5, the viable count rose from 1.1×10^6 CFU/g to 1.6×10^7 and 6.9×10^7 CFU/g, respectively. The viable counts in the remaining Treatments all exceeded 1×10^8 CFU/g.

3.3.3. Variations in Nutritional Components of NFBDGs Fermented by Different Fermentation Process Conditions

Tables 9 and 10 show the DM basis contents and the contents considering the concentration effect of nutritional components of fermented NFBDGs in different treatment groups, respectively. To obtain more accurate analysis results, range analysis and variance analysis were conducted on the detection results, considering the concentration effect (Table 10), with outcomes presented in Tables 11 and 12.

Table 9. Contents of CP, TP, ASP, CF, ADF, and NDF on DM basis of NFBDGs and DMR of different treatment groups after orthogonal test fermentation.

Treatment No.	CP %	TP %	ASP %	CF %	ADF %	NDF %	DMR
1	13.73 ± 0.18	11.39 ± 1.16	5.62 ± 0.68	23.28 ± 1.17	39.18 ± 2.43	49.55 ± 2.49	0.81 ± 0.01
2	13.09 ± 0.49	11.21 ± 0.44	5.91 ± 0.38	25.38 ± 0.58	45.15 ± 1.30	52.50 ± 2.54	0.83 ± 0.07
3	13.52 ± 0.58	11.10 ± 0.05	8.08 ± 0.55	25.59 ± 0.50	42.87 ± 1.99	50.82 ± 0.75	0.86 ± 0.02
4	13.79 ± 0.81	11.30 ± 1.12	7.66 ± 0.45	26.95 ± 1.22	44.61 ± 5.42	55.00 ± 3.11	0.85 ± 0.09
5	12.82 ± 0.23	10.56 ± 0.29	7.24 ± 0.77	27.70 ± 1.76	41.21 ± 2.60	59.52 ± 1.69	0.87 ± 0.04
6	12.53 ± 1.03	11.80 ± 0.52	7.61 ± 0.49	22.99 ± 1.68	40.73 ± 3.84	45.16 ± 2.59	0.87 ± 0.06
7	13.30 ± 0.15	11.82 ± 0.14	6.99 ± 0.14	26.08 ± 1.00	45.74 ± 1.20	51.43 ± 2.33	0.91 ± 0.02
8	11.31 ± 0.22	9.85 ± 0.26	4.20 ± 0.16	22.72 ± 1.52	39.82 ± 1.94	44.97 ± 0.65	0.90 ± 0.01
9	13.44 ± 0.20	11.99 ± 0.06	5.31 ± 0.30	25.93 ± 2.11	48.18 ± 1.57	58.45 ± 1.92	0.91 ± 0.00

Table 10. Contents of CP, TP, ASP, CF, ADF, and NDF considering the concentration effect of NFBDGs of different treatment groups after orthogonal test fermentation.

Treatment No.	CP %	TP %	ASP %	CF %	ADF %	NDF %
1	11.11 ± 0.07	9.21 ± 0.79	4.55 ± 0.61	18.84 ± 1.00	31.69 ± 1.71	40.08 ± 1.68
2	10.82 ± 0.59	9.27 ± 0.47	4.91 ± 0.71	21.01 ± 1.53	37.38 ± 3.09	43.37 ± 1.83
3	11.62 ± 0.28	9.54 ± 0.31	6.95 ± 0.66	22.02 ± 1.06	36.89 ± 2.68	43.70 ± 1.15
4	11.77 ± 1.13	9.65 ± 1.34	6.52 ± 0.36	23.00 ± 2.30	38.26 ± 7.36	46.96 ± 5.08
5	11.22 ± 0.67	9.24 ± 0.27	6.35 ± 0.90	24.27 ± 2.55	36.01 ± 1.93	52.04 ± 1.53
6	10.87 ± 0.39	10.25 ± 0.42	6.63 ± 0.78	19.94 ± 0.29	35.42 ± 3.80	39.23 ± 2.13
7	12.17 ± 0.11	10.81 ± 0.20	6.39 ± 0.12	23.85 ± 0.71	41.83 ± 0.43	47.07 ± 2.83
8	10.18 ± 0.23	8.86 ± 0.31	3.78 ± 0.13	20.43 ± 1.22	35.84 ± 2.05	40.46 ± 0.51
9	12.26 ± 0.15	10.94 ± 0.05	4.85 ± 0.28	23.66 ± 1.99	43.97 ± 1.38	53.34 ± 1.78

Table 11. Range analysis results of orthogonal test (considering the concentration effect).

Indexes	Factors					Analysis Results
	K Value	A	B	C	D	
CP	k1	11.76	11.32	11.40	11.68	Influence order
	k2	11.53	11.53	11.40	11.57	A > D > B > C
	k3	10.70	11.15	11.20	10.75	Optimal level
	R	1.06	0.38	0.20	0.92	$A_1B_2C_2D_1$
TP	k1	10.66	9.74	9.77	9.89	Influence order
	k2	9.48	9.79	9.82	9.92	A > D > C > B
	k3	9.11	9.72	9.67	9.45	Optimal level
	R	1.55	0.07	0.15	0.47	$A_1B_2C_2D_2$
ASP	k1	5.96	5.71	5.88	5.82	Influence order
	k2	6.61	5.25	5.05	5.57	A > C > B > D
	k3	4.41	6.02	6.05	5.59	Optimal level
	R	2.20	0.77	1.00	0.25	$A_2B_3C_3D_1$
CF	k1	22.48	22.10	23.04	21.90	Influence order
	k2	23.10	22.26	22.37	22.23	A > C > B > D
	k3	20.09	21.31	20.26	21.55	Optimal level
	R	3.00	0.94	2.78	0.68	$A_3B_3C_3D_2$
ADF	k1	40.40	38.19	38.41	37.26	Influence order
	k2	37.06	37.22	39.35	39.41	A > C > D > B
	k3	34.97	37.02	34.67	35.76	Optimal level
	R	5.43	1.17	4.69	3.66	$A_3B_3C_3D_3$
NDF	k1	46.54	43.74	47.50	44.70	Influence order
	k2	47.57	48.48	46.92	46.80	C > A > B > D
	k3	41.30	43.19	41.00	43.91	Optimal level
	R	6.26	5.30	6.49	2.89	$A_3B_3C_3D_3$

Range analysis revealed the factor influence order and optimal combinations for each key index: CP had an influence order of A > D > B > C (fermentation time > initial pH > fermentation temperature > moisture content), optimal combination $A_1B_2C_2D_1$; TP had an influence order of A > D > C > B (fermentation time > initial pH > moisture content > fermentation temperature), optimal combination $A_1B_2C_2D_2$; ASP had an influence order of A > C > B > D (fermentation time > moisture content > fermentation temperature > initial pH), optimal combination $A_2B_3C_3D_1$; CF had an influence order of A > C > B > D (fermentation time > moisture content > fermentation temperature > initial pH), optimal

combination $A_3B_3C_3D_2$; ADF had an influence order of A > C > D > B (fermentation time > moisture content > initial pH > fermentation temperature), optimal combination $A_3B_3C_3D_3$; and NDF had an influence order of C > A > B > D (moisture content > fermentation time > fermentation temperature > initial pH), optimal combination $A_3B_3C_3D_3$ (Table 11).

Table 12. Variance analysis results of orthogonal test (considering the concentration effect).

Factors	Mean Square	F Value	Significance	Index
A	2.819	10.638	<0.001	CP
B	0.322	1.214	0.32	Factor order A > D > B > C
C	0.123	0.465	0.635	Optimal combination
D	2.289	8.639	0.002	$A_{1/2}B_{1/2/3}C_{1/2/3}D_{1/2}$
A	5.925	17.095	<0.001	TP
B	0.013	0.038	0.963	Factor order A > D > C > B
C	0.054	0.156	0.857	Optimal combination
D	0.619	1.787	0.196	$A_1B_{1/2/3}C_{1/2/3}D_{1/2/3}$
A	11.442	34.744	<0.001	ASP
B	1.345	4.085	0.034	Factor order A > C > B > D
C	2.579	7.833	0.004	Optimal combination
D	0.178	0.54	0.592	$A_2B_{1/3}C_{1/3}D_{1/2/3}$
A	22.636	9.143	0.002	CF
B	2.3	0.929	0.413	Factor order A > C > B > D
C	18.895	7.632	0.004	Optimal combination
D	1.047	0.423	0.661	$A_3B_{1/2/3}C_3D_{1/2/3}$
A	67.636	6.193	0.009	ADF
B	55.286	5.062	0.018	Factor order A > B > D > C
C	3.502	0.321	0.73	Optimal combination
D	30.385	2.782	0.089	$A_{2/3}B_{1/2/3}C_3D_{1/3}$
A	101.549	17.703	<0.001	NDF
B	116.343	20.282	<0.001	Factor order B > A > C > D
C	76.292	13.3	<0.001	Optimal combination
D	20.112	3.506	0.052	$A_3B_{1/3}C_3D_{1/3}$

Variance analysis results were consistent with range analysis for most indices, with details as follows:

- CP—influence order A > D > B > C; main factors A and D ($p < 0.01$, extremely significant), secondary factors B and C ($p > 0.05$); optimal combination $A_{1/2}B_{1/2/3}C_{1/2/3}D_{1/2}$;
- TP—influence order A > D > C > B; main factor A ($p < 0.01$, extremely significant), secondary factors B, C, D ($p > 0.05$); optimal combination $A_1B_{1/2/3}C_{1/2/3}D_{1/2/3}$;
- ASP—influence order A > C > B > D; main factors A and C ($p < 0.01$, extremely significant) and B ($p < 0.05$, significant), secondary factor D ($p > 0.05$); optimal combination $A_2B_{1/3}C_{1/3}D_{1/2/3}$;
- CF—influence order A > C > B > D; main factors A and C ($p < 0.01$, extremely significant), secondary factors B and D ($p > 0.05$); optimal combination $A_3B_{1/2/3}C_3D_{1/2/3}$;
- ADF—influence order A > B > D > C (inconsistent with range analysis); main factors A ($p < 0.01$, extremely significant) and B ($p < 0.05$, significant), secondary factors C and D ($p > 0.05$); optimal combination $A_{2/3}B_{1/2/3}C_3D_{1/3}$;
- NDF—influence order B > A > C > D (inconsistent with range analysis); main factors A, B, C ($p < 0.01$, extremely significant), secondary factor D ($p > 0.05$); optimal combination $A_3B_{1/3}C_3D_{1/3}$ (Table 12).

Combining the results of range analysis and variance analysis, the optimal combinations of the four fermentation conditions for mixed-strain fermentation of NFBDGs were as follows: $A_1B_2C_2D_1$ for CP content improvement, $A_1B_2C_2D_2$ for TP content improvement, $A_2B_3C_3D_1$ for ASP content improvement, $A_3B_3C_3D_2$ for CF degradation, and $A_3B_3C_3D_3$ for both ADF and NDF degradation. Based on the significant differences in each factor at different levels and the principle of prioritizing the reduction in the three fiber indices, the optimal levels of the four factors were further analyzed as follows.

For fermentation time (Factor A), the optimal level for increasing both CP and TP contents was Level 1, while the optimal level for enhancing ASP content was Level 2. However, there was no significant difference between Level 1 and Level 2 of fermentation time in terms of CP content improvement ($p > 0.05$). Thus, Level 2 could also be designated as the optimal level for CP elevation. Therefore, the optimal fermentation time for protein content enhancement was either Level 1 or Level 2. In contrast, for the degradation of CF, ADF, and NDF, the optimal level of fermentation time was consistently Level 3. Given that the primary objective of NFBDGs fermentation is to degrade cellulose and improve the digestibility of the NFBDGs, Level 3 was ultimately determined as the optimal fermentation time.

For fermentation temperature (Factor B), the optimal level for increasing both CP and TP contents was Level 2, and the optimal level for enhancing ASP content was Level 3, but there were no significant differences among the three levels of fermentation temperature in terms of CP and TP content improvement ($p > 0.05$). Therefore, Level 3 could be designated as the optimal level of fermentation temperature for increasing the contents of all three protein fractions. Meanwhile, Level 3 was also the optimal level of fermentation temperature for the degradation of the three fiber components. On this basis, Level 3 was ultimately determined as the optimal fermentation temperature. The significance of the three levels of moisture content (Factor C) in increasing the three protein contents and degrading the three fiber contents was consistent with that of fermentation temperature. Thus, Level 3 was also determined as the optimal moisture content.

For initial pH (Factor D), the optimal level for increasing both CP and ASP contents was Level 1, while the optimal level for enhancing TP content was Level 2. However, there was no significant difference between Level 1 and Level 2 of initial pH in terms of CP content improvement ($p > 0.05$). Moreover, no significant differences were observed among the three levels of initial pH with respect to the elevation of TP and ASP contents ($p > 0.05$). Therefore, Level 1 could be designated as the optimal initial pH for protein content enhancement. In terms of fiber degradation, the optimal level of initial fermentation pH was Level 2 for CF breakdown, and Level 3 for the degradation of both ADF and NDF. Nevertheless, no significant differences were detected among the three levels of initial pH in CF degradation ($p > 0.05$), and no significant differences were found between Level 1 and Level 3 in the degradation of ADF and NDF ($p > 0.05$). Thus, Level 1 could also be identified as the optimal initial pH for fiber degradation. On the basis of the above analysis, Level 1 was ultimately determined as the optimal initial pH.

In light of the foregoing analysis, the optimal combination of fermentation conditions for mixed-strain solid-state fermentation of NFBDGs was determined as $A_3B_3C_3D_1$: fermentation time of 4 d, fermentation temperature of 37 °C, water content of 60% (i.e., initial moisture content of NFBDGs), and initial pH of 3.43 (i.e., initial pH of NFBDGs). These results indicated that the three strains used in this study for fermenting NFBDGs can tolerate the acidic environment and high moisture content of NFBDGs under mixed action. In this way, there is no need to add alkaline substances to adjust the pH of raw NFBDGS or pre-dry the raw NFBDGS to reduce water content during industrial production, which significantly reduces production costs and simplifies the production process. Although a fermentation temperature of 37 °C was not conducive to the growth of *Saccharomyces cerevisiae* CJM26, it was beneficial to the degradation of lignocellulose in NFBDGs.

3.3.4. Verification Experiment

A batch of verification experiments was performed according to the optimal process from the orthogonal experiment, with results shown in Tables 13 and 14. Table 13 shows the contents of various nutritional components on a DM basis. Under the optimal process,

mixed-strain fermentation of NFBDGs significantly increased the contents of CP, TP, and ASP compared with unfermented NFBDGs ($p < 0.05$): CP rose from 12.77% to 15.84%, TP from 9.02% to 14.22%, and ASP from 6.85% to 8.33%. In contrast, CF and NDF contents were also significantly increased ($p < 0.05$), while the ADF content showed no significant change ($p > 0.05$), which may be attributed to the concentration effect during fermentation. As shown in Table 14, under the optimal process, after mixed-strain fermentation of NFBDGs, there was no significant difference in CP and ASP contents considering the concentration effect compared with unfermented NFBDGs ($p > 0.05$), while the TP content, considering the concentration, was significantly increased ($p < 0.05$), which was 26.16% higher than that of unfermented NFBDGs. The contents of CF, ADF, and NDF, considering the concentration, were significantly decreased compared with unfermented NFBDGs ($p < 0.05$), with decreases of 12.38%, 21.29%, and 15.4%, respectively. In addition, under the optimal process, the pH of fermented NFBDGs increased from 3.43 to 5.87. The results of the verification experiment were consistent with expectations.

Table 13. Contents of CP, TP, ASP, CF, ADF, and NDF on DM basis of NFBDGs and DMR before and after optimal-process fermentation.

Groups	CP %	TP %	ASP %	CF %	ADF %	NDF %	DMR
Optimal process	15.84 ± 0.94^{A}	14.22 ± 0.59^{A}	8.33 ± 0.94^{A}	38.59 ± 0.87^{A}	56.77 ± 0.55^{A}	70.04 ± 1.95^{A}	0.88 ± 0.04
Uufermented NFBDGs	12.77 ± 0.14^{B}	9.02 ± 0.08^{B}	6.85 ± 0.25^{B}	35.22 ± 1.20^{B}	57.67 ± 0.53^{A}	66.19 ± 1.59^{B}	--

Note: Within each column, values with different superscripts indicate significant differences, $p < 0.05$; values with the same superscripts indicate no significant differences, $p > 0.05$.

Table 14. Contents of CP, TP, ASP, CF, ADF, and NDF considering the concentration effect of NFBDGs before and after optimal-process fermentation.

Groups	CP %	TP %	ASP %	CF %	ADF %	NDF %
Optimal process	12.67 ± 0.77^{A}	11.38 ± 0.54^{A}	6.66 ± 0.74^{A}	30.86 ± 0.93^{B}	45.39 ± 0.43^{B}	56.00 ± 1.71^{B}
Uufermented NFBDGs	12.77 ± 0.14^{A}	9.02 ± 0.08^{B}	6.85 ± 0.25^{A}	35.22 ± 1.20^{A}	57.67 ± 0.53^{A}	66.19 ± 1.59^{A}

Note: Within each column, values with different superscripts indicate significant differences, $p < 0.05$; values with the same superscripts indicate no significant differences, $p > 0.05$.

Zhang et al. [12] used an orthogonal design to optimize the auxiliary material ratio, addition of urea and potassium dihydrogen phosphate, initial fermentation pH, and initial moisture content of Baijiu distiller's grains for mixed-strain fermentation of Baijiu distiller's grains. The strains used were *Geotrichum candidum*, *Aspergillus oryzae*, *Trichoderma viride*, and *Bacillus subtilis* with an inoculation ratio of 1:1:1:1 (v/v) and an inoculation amount of 10%. Under the optimal conditions, after fermentation at 30 °C for 72 h, compared with the unfermented Baijiu distiller's grain base material, the TP content increased by 57.85%, the CF content decreased by 42.39%, and the contents of ADF and NDF decreased by 31.95% and 27.73%, respectively. Fan et al. [7] investigated the effects of wheat bran addition, loading amount, initial pH, fermentation time, fermentation temperature, and inoculation amount on the mixed-strain solid-state fermentation of Baijiu distiller's grains through single-factor experiments and orthogonal experiments. The results showed that after the pretreatment of Baijiu distiller's grains by steam explosion, mixed-strain solid-state fermentation was conducted with *Saccharomyces cerevisiae*, *Candida utilis*, *Lactobacillus plantarum*, *Bacillus subtilis*, *Trichoderma koningii*, *Aspergillus niger*, and *Myceliophthora thermophila*. Under the conditions of a 5% wheat bran addition, a 80 g loading amount, an initial pH of 3.70, a fermentation duration of 5 d, a temperature of 30 °C, and a 10% inoculation amount, compared with unfermented Baijiu distiller's grains, the fermented Baijiu distiller's grains exhibited a 24.46% increase in CP content, a 45.03% reduction in CF content, a niacin content

of 1.21 mg/g distiller's grains, and a 71.73% rise in acetoin content. Dai et al. [5] optimized the conditions for fermenting Baijiu distiller's grains with the cellulose-degrading strain *Cohnella xylanilytica* T5 through single-factor and orthogonal experiments, which included a fermentation temperature of 33 °C, a fermentation time of 5 d, an inoculum amount of 25%, and a material-to-water ratio of 1:1.5. Compared with unfermented Baijiu distiller's grains, the CP and TP contents of the fermented group increased by 19.68% and 30.38%, respectively, and the contents of NDF, ADF, and CF decreased by 31.68%, 27.69%, and 7.95% respectively. Mei et al. [30] optimized the addition of auxiliary materials (corn flour, wheat bran, and rapeseed meal), initial pH, fermentation time, and inoculation amount required for solid-state fermentation of Baijiu distiller's grains through single-factor experiments, and further optimized the initial pH, fermentation time, and inoculation amount through orthogonal experiments. The results showed that the optimal conditions for the mixed-strain fermentation of Baijiu distiller's grains with *Aspergillus niger*, *Enterococcus faecalis*, *Lactobacillus plantarum*, and *Saccharomyces cerevisiae* were an initial pH of 6, an inoculation amount of 8%, a fermentation time of 5 d, and a temperature of 28 °C. Under these conditions, compared with the auxiliary-added Baijiu distiller's grains before optimization, the CP content increased by 15.93%, the NDF content decreased by 29.81%, and the ADF content decreased by 29.77%.

The above results reported by different studies were consistent with the trend changes of the corresponding nutritional components of fermented NFBDGs in this study. However, some results reported in these studies were better than those of this study, which may be attributed to the following reasons: first, different strains were used—this study employed lactic acid bacteria, yeasts, and bacilli, whose protein-producing and cellulose-degrading capabilities are slightly weaker than those of molds such as *Geotrichum candidum*, *Aspergillus oryzae*, *Aspergillus niger*, and *Trichoderma viride*; second, the fermentation substrate in this study was only Baijiu distiller's grains without any auxiliary materials or inorganic nutrients added, whereas the aforementioned studies all supplemented Baijiu distiller's grains with auxiliary materials (e.g., wheat bran, corn flour, rapeseed meal, and urea), and the sources of Baijiu distiller's grains also varied; third, the concentration effect caused by DM loss was not considered when calculating the changes in nutritional components before and after fermentation. Even so, the strains used in this study did not involve the impact of mold mycelia and spores on the post-treatment of products and feed processing. They could directly treat fresh Baijiu distiller's grains without pre-drying to reduce water content, adjusting pH, or adding additional auxiliary materials. The fermentation process was more suitable for industrial production, featuring simple operation and low cost. If it is necessary to further reduce the lignocellulose content, relatively low-cost enzyme preparations can be added to replace the role of cellulose-degrading molds.

3.4. Effect of Microbial-Enzyme Synergistic Fermentation Process on Fermented NFBDGs

3.4.1. Sensory Evaluation, Mold Growth, and pH of NFBDGs Fermented by Mixed Strains with Different Enzymes Added

To further reduce the lignocellulose content of NFBDGs, β-mannanase (A), cellulase (B), pectinase (C), and xylanase (D) were added based on mixed-strain fermentation, and the effects of different enzyme preparation combinations on mixed-strain fermentation of NFBDGs were compared. As shown in Table S4, all microbial-enzyme synergistic fermentation combinations exhibited distinct fermentation states in terms of sensory evaluation, indicating that the addition of different enzyme preparations and their combinations did not interfere with the fermentation of NFBDGs by the mixed strains. Under the 14 enzyme preparation combination conditions, mold growth was detected during fermentation in most experimental groups supplemented with enzyme C: the group with C added alone showed the most mold growth, followed by the A + C combination group; slight mold

growth was also observed in the C + D and A + C + D combination groups. This suggested that the addition of pectinase promoted mold growth in NFBDGs. Although slight mold growth was also observed in the B + D combination group, no mold was found in the groups with enzymes B or D added alone. At 4 d of fermentation, the pH of NFBDGs in all experimental groups was significantly higher than the initial pH; except for the B + D, A + C + D, and A + B + C + D groups (pH ranging from 5 to 6), the pH of NFBDGs in the remaining groups increased to more than 6.

3.4.2. Variations in Fiber Content of NFBDGs Fermented by Mixed Strains with Different Enzymes Added

The contents of CF, ADF, and NDF in unfermented NFBDGs and those fermented with different enzyme preparation combinations were determined, respectively. Tables 15 and 16 present the detection results of the samples' nutritional components on a DM basis and considering the concentration effect, respectively. As shown in Table 15, as for CF, group D had the lowest content, which had no significant difference with groups C and A + C ($p > 0.05$), but was significantly lower than that of unfermented NFBDGs ($p < 0.05$). As for ADF, group D had the lowest content, which had no significant difference with groups B + D and C + D ($p > 0.05$), but was significantly lower than that of unfermented NFBDGs ($p < 0.05$). As for NDF, the group B + C had the lowest content, followed by groups A + B, A + D, D, and A + C; these five experimental groups all had significantly lower NDF content than unfermented NFBDGs ($p < 0.05$).

Table 15. Contents of CF, ADF, and NDF on DM basis of NFBDGs and DMR of different treatment groups before and after mixed-strain fermentation with different enzyme preparations.

Groups	CF %	ADF %	NDF %	DMR
A	32.71 ± 1.76^{FG}	52.66 ± 0.39^{CD}	65.35 ± 0.14^{A}	0.80 ± 0.01
B	35.09 ± 0.68^{DE}	50.15 ± 0.59^{EF}	63.86 ± 1.77^{BC}	0.82 ± 0.02
C	30.80 ± 0.50^{HI}	53.68 ± 0.79^{BC}	56.48 ± 0.47^{EFG}	0.93 ± 0.04
D	30.61 ± 0.54^{I}	44.06 ± 0.82^{I}	55.52 ± 0.73^{G}	0.79 ± 0.05
A + B	33.72 ± 0.54^{EFG}	54.40 ± 0.71^{B}	53.82 ± 1.18^{H}	0.83 ± 0.01
A + C	30.61 ± 0.69^{I}	50.86 ± 0.93^{E}	56.16 ± 1.52^{FG}	0.90 ± 0.01
A + D	34.83 ± 1.50^{E}	52.25 ± 1.00^{D}	53.95 ± 0.95^{H}	0.86 ± 0.04
B + C	38.30 ± 0.48^{BC}	49.20 ± 0.66^{FG}	50.44 ± 0.34^{I}	0.91 ± 0.01
B + D	35.33 ± 0.77^{DE}	44.23 ± 0.54^{I}	62.23 ± 0.65^{D}	0.92 ± 0.04
C + D	32.36 ± 0.29^{GH}	44.13 ± 0.73^{I}	64.59 ± 0.38^{AB}	0.89 ± 0.00
B + C + D	41.78 ± 1.85^{A}	48.37 ± 0.93^{GH}	62.43 ± 1.01^{CD}	0.91 ± 0.03
A + C + D	38.71 ± 0.30^{B}	57.87 ± 0.93^{A}	62.94 ± 0.52^{CD}	0.89 ± 0.03
A + B + C	35.01 ± 0.65^{DE}	50.77 ± 0.88^{E}	57.95 ± 0.81^{EF}	0.87 ± 0.02
A + B + D	34.31 ± 1.41^{EF}	47.40 ± 1.39^{H}	56.43 ± 0.61^{EFG}	0.81 ± 0.02
A + B + C + D	32.82 ± 0.88^{FG}	49.56 ± 0.86^{EFG}	57.37 ± 0.22^{EF}	0.80 ± 0.02
Uufermented NFBDGs	36.70 ± 0.25^{CD}	47.38 ± 0.77^{H}	57.78 ± 0.55^{E}	--

Note: Within each column, values with different superscripts indicate significant differences, $p < 0.05$; values with the same superscripts indicate no significant differences, $p > 0.05$. A—β-mannanase; B—cellulase; C—pectinase; D—xylanase.

Table 16 showed the contents of various nutritional components considering the concentration effect. As for CF, group D had the lowest CF content, which had no significant difference with groups A and A + B + C + D ($p > 0.05$), but was significantly lower than that of unfermented NFBDGs ($p < 0.05$). As for ADF, except for groups C, A + C, and A + C + D, the remaining experimental groups exhibited significantly lower ADF content than unfermented NFBDGs ($p < 0.05$), with group D having the lowest value. As for NDF, group D had the lowest NDF content, which had no significant difference with groups A + B, A + D, B + C, A + B + D, and A + B + C + D ($p > 0.05$), but was significantly lower than that of unfermented NFBDGs ($p < 0.05$).

Based on a comprehensive analysis of the above results and considering the production cost of microbial-enzyme synergistic fermentation, xylanase was determined as the optimal enzyme preparation for the microbial-enzyme synergistic fermentation of NFBDGs. Under this condition, the degradation rates of CF, ADF, and NDF in the fermented NFBDGs were 33.73%, 26.11%, and 23.64%, respectively, compared with unfermented NFBDGs.

Table 16. Contents of CF, ADF, and NDF considering the concentration effect of NFBDGs of different treatment groups before and after mixed-strain fermentation with different enzyme preparations.

Groups	CF %	ADF %	NDF %
A	26.20 ± 1.37^{GH}	42.19 ± 0.92^{DE}	52.35 ± 0.87^{B}
B	28.63 ± 1.41^{EF}	40.89 ± 0.85^{EF}	52.09 ± 2.34^{B}
C	28.62 ± 1.32^{EF}	49.86 ± 1.58^{A}	52.47 ± 1.81^{B}
D	24.32 ± 1.43^{H}	35.01 ± 2.04^{H}	44.12 ± 2.68^{C}
A + B	27.96 ± 0.48^{FG}	45.11 ± 0.28^{C}	44.63 ± 0.68^{C}
A + C	27.58 ± 0.78^{FG}	45.82 ± 0.87^{BC}	50.61 ± 2.16^{B}
A + D	30.06 ± 1.08^{E}	45.14 ± 2.35^{C}	46.59 ± 2.18^{C}
B + C	35.04 ± 0.17^{BC}	45.03 ± 1.06^{C}	46.15 ± 0.16^{C}
B + D	32.58 ± 0.82^{D}	40.80 ± 1.38^{EF}	57.43 ± 2.75^{A}
C + D	28.64 ± 0.18^{EF}	39.07 ± 0.58^{FG}	57.18 ± 0.25^{A}
B + C + D	38.02 ± 2.72^{A}	43.99 ± 1.76^{CD}	56.81 ± 2.95^{A}
A + C + D	34.60 ± 1.03^{CD}	51.72 ± 1.30^{A}	56.27 ± 2.40^{A}
A + B + C	30.77 ± 0.61^{E}	44.75 ± 0.71^{CD}	50.58 ± 0.76^{B}
A + B + D	27.90 ± 1.75^{FG}	38.51 ± 0.40^{G}	45.85 ± 0.68^{C}
A + B + C + D	26.23 ± 1.00^{GH}	39.61 ± 1.21^{FG}	45.85 ± 1.07^{C}
Uufermented NFBDGs	36.70 ± 0.25^{AB}	47.38 ± 0.77^{B}	57.78 ± 0.55^{A}

Note: Within each column, values with different superscripts indicate significant differences, $p < 0.05$; values with the same superscripts indicate no significant differences, $p > 0.05$. A—β-mannanase; B—cellulase; C—pectinase; D—xylanase.

In this study, the microbial-enzyme synergistic process was optimized under the optimal conditions of mixed-strain fermentation. Thus, parameters such as enzyme hydrolysis temperature, time, and pH of the enzyme preparations were not optimized; only the type and combination of the added enzyme preparations were evaluated.

3.4.3. Sensory Evaluation, Mold Growth, pH, and Nutritional Component Changes in NFBDGs by Microbial-Enzyme Synergistic Fermentation

The results in Table S5 showed that after microbial-enzyme synergistic fermentation of NFBDGs with *Ligilactobacillus salivarius* CRS23, *Bacillus subtilis* YLZ7, *Saccharomyces cerevisiae* CJM26, and xylanase, the fermented product exhibited caking, was dark in color, and showed no mold growth; the pH increased from 3.49 to 6.04. Meanwhile, the viable count of APB increased from 1.4×10^6 CFU/g to 2.5×10^8 CFU/g, that of *Bacillus subtilis* YLZ7 increased from 9.5×10^5 CFU/g to 2.7×10^7 CFU/g, and that of *Saccharomyces cerevisiae* CJM26 increased from 8.0×10^5 CFU/g to 1.8×10^7 CFU/g (Table S6).

Table 17 presents the contents of the samples' nutritional components on a DM basis. Under the optimal microbial-enzyme synergistic fermentation process, compared with unfermented NFBDGs, the contents of CP, TP, and ASP of fermented NFBDGs were significantly increased ($p < 0.05$): the CP content increased from 11.7% to 15.63%, the TP content increased from 11.12% to 14.59%, and the ASP content increased from 5.43% to 6.84%. Compared with unfermented NFBDGs, the contents of the three types of lignocellulose were significantly decreased ($p < 0.05$), which should be due to the added xylanase promoting the degradation of lignocellulose in NFBDGs. Table 18 presents the contents of the samples' nutritional components considering the concentration effect. Under the optimal condition,

there was no significant difference in ASP contents between fermented and unfermented NFBDGs ($p > 0.05$), while the CP and TP contents were significantly higher ($p < 0.05$). The contents of CF, ADF, and NDF were significantly reduced compared with unfermented NFBDGs ($p < 0.05$), with decreases of 35.44%, 20.53%, and 25.02%, respectively.

Table 17. Contents of CP, TP, AP, CF, ADF, and NDF on DM basis of NFBDGs and DMR before and after microbial-enzyme synergistic fermentation.

Groups	CP %	TP %	ASP %	CF %	ADF %	NDF %	DMR
Fermented NFBDGs	15.63 ± 0.33 ^{A}	14.59 ± 0.27 ^{A}	6.84 ± 0.16 ^{A}	27.64 ± 0.41 ^{B}	47.75 ± 1.36 ^{B}	58.55 ± 0.56 ^{B}	0.81 ± 0.02
Uufermented NFBDGs	11.70 ± 0.12 ^{B}	11.12 ± 0.39 ^{B}	5.43 ± 0.16 ^{B}	34.90 ± 1.09 ^{A}	48.95 ± 0.19 ^{A}	63.64 ± 0.56 ^{A}	--

Note: Within each column, values with different superscripts indicate significant differences, $p < 0.05$; values with the same superscripts indicate no significant differences, $p > 0.05$.

Table 18. Contents of CP, TP, AP, CF, ADF, and NDF considering the concentration effect of NFBDGs before and after microbial-enzyme synergistic fermentation.

Groups	CP %	TP %	ASP %	CF %	ADF %	NDF %
Fermented NFBDGs	12.74 ± 0.41 ^{A}	11.89 ± 0.42 ^{A}	5.57 ± 0.25 ^{A}	22.53 ± 0.83 ^{B}	38.90 ± 0.80 ^{B}	47.72 ± 1.31 ^{B}
Uufermented NFBDGs	11.70 ± 0.12 ^{B}	11.12 ± 0.39 ^{B}	5.43 ± 0.16 ^{A}	34.90 ± 1.09 ^{A}	48.95 ± 0.19 ^{A}	63.64 ± 0.56 ^{A}

Note: Within each column, values with different superscripts indicate significant differences, $p < 0.05$; values with the same superscripts indicate no significant differences, $p > 0.05$.

Liu et al. [38] studied the production of feed protein from Luzhou Laojiao Baijiu distiller's grains via microbial-enzyme synergistic fermentation: first, three strains with high lignocellulase productivity (*Trichoderma reesei*, *Aspergillus niger*, and *Penicillium*) were isolated and screened from soil samples, and the lignocellulase produced by these strains exhibited complementarity. Next, the enzyme production conditions of the three strains were optimized using response surface methodology, and a crude enzyme solution was prepared. After fermenting Luzhou Laojiao Baijiu distiller's grains under the optimal conditions, the degradation rates of reducing sugar, cellulose, and hemicellulose reached 27.88%, 19.64%, and 10.88%, respectively. Subsequently, *Saccharomyces cerevisiae*, *Candida utilis*, and *Rhodotorula benthica* were inoculated for fermentation, with the true protein content of the Baijiu distiller's grains increased to a maximum of 53.49%. The approach of the report was exactly opposite to that of this study: this study first optimized the optimal process parameters for strain fermentation of NFBDGs, then added different enzyme preparations, and also showed that the microbial-enzyme synergistic treatment had a better effect.

Heng et al. [15] optimized the strains and their combinations, protease combinations, material ratio (soybean residue to soybean meal), inoculation amount, fermentation temperature, and fermentation time required for fermenting the composite material of soybean residue and soybean meal. The results showed that first, *Bacillus subtilis* was inoculated for aerobic fermentation for 12 h, and then *Saccharomyces cerevisiae*, *Lactobacillus plantarum*, and *Lactobacillus rhamnosus* were inoculated for anaerobic fermentation for 11 d. Subsequently, the 50% enzyme preparation (0.65% acid protease +0.65% papain) and 15% wheat middlings were added to the mixture, followed by fermentation at 34 °C. At the end of fermentation, the small peptide content of the fermented product increased from 7.35% to 39.58%. The addition of the two proteases significantly promoted microbial growth, amylase secretion, reducing sugar utilization, and organic acid production during fermentation. Lin et al. [18] used *Lactobacillus fermentum*, *Saccharomyces cerevisiae*, *Bacillus subtilis*, xylanase, β-glucanase, mannanase, cellulase, and pectinase to perform composite

fermentation on corn cobs. After fermentation, compared with unfermented corn cobs, the fermented product had a darker color, produced an odor similar to wine and lactic acid, showed increased contents of CP, calcium, and phosphorus, and decreased contents of DM, crude fat, NDF, ADF, and reducing sugar. Compared with corn cobs fermented by strains alone, the composite fermented product exhibited higher viable count, protein content, and cellulose degradation rate, as well as significantly lower contents of DM, crude ash, and reducing sugar. The results of the above two studies were similar to those of this study.

4. Conclusions

Strains suitable for fermenting NFBDGs were screened. Through the optimization of strain combination, fermentation conditions, and microbial-enzyme synergistic fermentation process, the fermentation process parameters of NFBDGs were obtained: the fermentation strains included *Ligilactobacillus salivarius* CRS23, *Bacillus subtilis* YLZ7, and *Saccharomyces cerevisiae* CJM26, with an addition amount of 10^6 CFU/g fresh NFBDGs each; the enzyme preparation was xylanase, with an addition amount of 200 U/g dry NFBDGs; fresh NFBDGs were used for aerobic fermentation at 37 °C for 4 d. Compared with unfermented NFBDGs, on a DM basis, the CP content increased from 11.7% to 15.63%, the TP content increased from 11.12% to 14.59%, the ASP content increased from 5.43% to 6.84%, the CF content reduced from 34.9% to 27.64%, the ADF content reduced from 48.95% to 47.75%, and the NDF content reduced from 63.64% to 58.55%. After considering the concentration effect, the contents of CF, ADF, and NDF decreased by 35.44%, 20.53%, and 25.02%, respectively. The pH of NFBDGs increased from 3.49 to 6.04. After microbial-enzyme synergistic fermentation, the nutritional components and physicochemical properties of NFBDGs were significantly improved, making them more suitable for use as feed raw materials.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation12010013/s1, Table S1: Strains information of yeast, bacillus and lactic acid bacteria; Table S2: Sensory evaluation, molds growth and pH of NFBDGS after orthogonal test fermentation; Table S3: Viable counts of NFBDGS before and after orthogonal test fermentation (CFU/g); Table S4: Sensory evaluation, molds growth and pH of NFBDGS after mixed-strain fermentation with different enzyme preparations; Table S5: Sensory evaluation, molds growth and pH of NFBDGS after microbial-enzyme synergistic fermentation; Table S6: Viable counts of NFBDGS before and after microbial-enzyme synergistic fermentation (CFU/g).

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