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Screening and Identification of the Strain *Pediococcus acidilactici* and Its Application in Fermentation of Corn–Soybean Meal Uncooked Materials

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Abstract: Cost and contamination are the bottleneck problems for the replacement of antibiotics with fermented feed. A strain of lactic acid bacteria was isolated and screened from acidified apple juice and identified as *Pediococcus acidilactici* using morphological, physiological, and biochemical tests and 16S rDNA sequence analysis. The strain was combined with *Bacillus subtilis* and *Saccharomyces cerevisiae* to ferment raw corn–soybean meal, and the two-stage fermentation process was optimized with a single-factor test, orthogonal test, and response surface methodology. Compared with unfermented raw material, the protein content of fermented feed was increased by 5.21 percentage points ($p < 0.05$) and the total amino acids were increased by 3.7 percentage points ($p < 0.05$), making it rich in amino acids essential for pigs. The high-throughput sequencing results showed that, at the species level, the highest relative abundances of bacteria in the fermentation system were those of *Pediococcus acidilactici* and *Bacillus subtilis*, and the most abundant fungi was *Saccharomyces cerevisiae*. No pathogenic bacteria, such as *Salmonella*, were detected in the fermentation system. This paper provides a feasible scheme for cheap preparation of an alternative to antibiotics, fermented feed, with uncooked raw materials. It has positive significance for promoting high-value utilization of agricultural and sideline products and improving feed cost-effectiveness.

Keywords: *Pediococcus acidilactici* identification; uncooked material fermentation; synergistic solid-state fermentation; high-throughput sequencing

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1. Introduction

Fermented feed refers to biological feed containing microorganisms or their metabolites produced by fermentation technology using raw feed materials and microorganisms permitted by the relevant laws and regulations [1,2]. Fermented feed can provide an effective way to utilize the byproducts of agricultural and sideline products, increase the nutritional quality of feed, improve the utilization efficiency of feed, reduce breeding costs, and inhibit intestinal pathogens in animals [3–5]. Since the European Union completely banned the use of antibiotics in animal diets in 2006, and especially after announcement No. 194 of the Ministry of Agriculture of China, antibiotics and other drug feed additives have been officially withdrawn from the Chinese feed market, and fermented feed, an alternative to antibiotics, has attracted much attention [1,2,6].

The fermentation of feed can be divided into two types: submerged fermentation (SmF) and solid-state fermentation (SSF). The main difference between the two is that SSF has a lower ratio of material to water, while that of SmF is between 1:1.5 and 1:4 [7]. SSF uses a solid medium with a certain water content as the substrate. Under a certain temperature, through the growth and metabolism of microorganisms, biological enzymes or metabolites are produced that degrade macromolecular organic matter in the medium and increase its biological value [2,8,9]. Compared with SmF, SSF saves energy consumption to

remove moisture later, and the feed products are easy to store and transport. Moreover, the raw material cost for SSF is low due to the possibility of using agro-industrial residues or byproducts as nutrient sources, and the sterility requirements are low because of the low water activity in the raw materials [10–13].

Traditional SSF needs to employ the routine procedures, including sterilization of the medium, cooling, inoculating, and fermentation under aseptic conditions, which makes the actual production process cumbersome and laborious, leading to long operation times and increases in costs. Therefore, some researchers have tried to use non-sterilized raw materials—namely, fermentation with uncooked materials—to reduce the cost of SSF and shorten the preparation time. Wu P. used distilled water to configure soybean meal medium, and the SSF feed was fermented at 55.33 °C by *Geobacillus stearothermophilus*. Compared with the unfermented soybean meal, the crude protein of fermented soybean meal increased by 5.26% and the crude fiber decreased by 14.63% [14]. Zhang Y. configured soybean meal medium with distilled water; it was aerobically fermented in a barrel while sterile air was input for the first 48 h and then fermented in a sealed barrel for the second 48 h. The whole fermentation process was carried out with *Saccharomyces cerevisiae*, *Bacillus subtilis*, *Lactobacillus plantarum*, and *Lactobacillus reuteri*. The total amounts of protein and amino acid were improved [15]. Xin N. et al. used *Bacillus natto* and *Aspergillus oryzae* to ferment soybean meal and, by comparing sterilized raw materials, it was found that there was a contamination problem in the uncooked raw materials fermentation [16]. Lin C. et al. used sterilized water to configurate solid-state fermentation raw materials, and the macromolecular proteins were degraded after mixed fermentation with *Bacillus amyloliticus*, *Lactobacillus plantarum*, and *Saccharomyces cerevisiae* for 120 h [17]. Although some progress has been made in the fermentation of uncooked raw materials, due to the large differences between the strains and the complex metabolisms of the fermentation system, there are still several problems, such as high cost, tedious operation, and pathogen contamination, which affect such aspects of the process as the water standard for raw materials, fermentation process control, fermentation temperature requirements, etc., in actual research and application. Therefore, further research is needed in this regard.

The strains commonly used in solid-state fermentation are *Bacillus subtilis*, *Saccharomyces cerevisiae*, and lactic acid bacteria, which themselves are safe and harmless for long-term use with animals [1,8]. *Bacillus subtilis* can produce protease, amylase, cellulase, phytase, and many other hydrolases and degrade glycinin, β-conglycinin, phytic acid, and other anti-nutritional factors; increase the content of small peptides; improve the nutritional composition; and promote the digestion and absorption rates of protein, starch, fiber, fat, and minerals in raw materials [18–20]. Yeast protein is produced after yeast cell multiplication and can increase the beneficial components, including yeast cells and their metabolites, in the feed, and the comprehensive properties of yeast protein are better than those of other bacterial proteins [21,22]. *Pediococcus acidilactici* is a kind of probiotic that can produce lactic acid, bacteriocin, and cyclic dipeptide [23,24]. It can also optimize the intestinal microflora structure of pigs, promote intestinal development, improve the serum biochemical indexes of pigs, enhance the antioxidant capacity of pigs, and promote the growth of pigs [23–26]. Protein is the core component of feeds and the main factor affecting their cost [27]. Soybean meal is one of the most widely used sources of protein in pig production, and its bioavailability is affected by the fact that it contains many anti-nutritional factors [16,18]. Soybean antigenic proteins (mainly glycinin and β-conglycinin) provoke delayed hypersensitivity in weaned piglets, which results in damage to the intestinal structure and diarrhea in piglets [28,29]. Trypsin inhibitor (TI) inhibits the hydrolysis of intestinal protease and stimulates the pancreas to secrete excessive pancreas enzymes, resulting in digestive and metabolic disorders in pigs [30]. However, in previous research, the antigen proteins from soybean meal were found to be degraded, and crude protein and amino acid contents increased in fermented soybean meal [18,22,27]. After feeding on fermented soybean meal, piglets' average daily gain, immune protein content, and intestinal microflora abundance increased and the ratio of feed to gain decreased significantly; furthermore, the

feed intake of pregnant sows was increased and constipation and lactating performance in sows were improved [1,4,13,18,21,27]. Synergistic fermentation with microbial strains effectively utilizes the metabolic characteristics of each strain, inhibits intestinal pathogens, degrades anti-nutritional factors in soybean meal, increases protein content, and reduces the cost of protein use. Solid-state fermentation is one of the effective ways to solve the cost-effectiveness problem affecting uncooked material fermentation of feed.

In this paper, uncooked corn–soybean meal materials were fermented synergistically with *Pediococcus acidilactici*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* under non-sterilization conditions through a two-stage process, aiming to find a balance between the raw material treatment and the microbial metabolism. The fermented feed was rich in the essential amino acids and umami amino acids and did not contain pathogenic bacteria, giving it good taste and palatability. This study provides a feasible scheme for the cheap preparation of fermented feed for pigs, which could be used to replace antibiotics and has positive significance for promoting the upgrading of pig feed, efficient utilization of soybean meal resources, and improving feed cost-effectiveness.

2. Materials and Methods

2.1. Materials

The strains *Pediococcus acidilactici* (CGMCC No. 24220), *Bacillus subtilis* (CGMCC No. 9660), and *Saccharomyces cerevisiae* (*Saccharomyces cerevisiae* Y-06) were isolated and preserved in the grain engineering laboratory of the College of Food Sciences, Northeast Agricultural University, Harbin, China. Soybean meal and corn were purchased from the local market (Harbin, China). The composition of the soybean meal was as follows: crude protein (38.06 ± 0.77%), crude fat (1.55 ± 0.25%), moisture (9.02 ± 0.87%), ash (6.83 ± 0.21%). The composition of the corn meal was as follows: crude protein (7.01 ± 0.03%), crude fat (4.75 ± 0.02%), moisture (13.23 ± 0.68%), ash (0.83 ± 0.05%).

2.2. Culture Medium

De Man, Rogosa, Sharpe (MRS) medium: peptone 1%, beef extract 0.5%, yeast extract 0.4%, glucose 2%, tween-80 0.1%, $K_2HPO_4{\cdot}7H_2O$ 0.2%, $CH_3COONa{\cdot}3H_2O$ 0.5%, $C_6H_{17}N_3O_7$ (Triammonium citrate) 0.2%, $MgSO_4{\cdot}7H_2O$ 0.02%, $MnSO_4{\cdot}4H_2O$ 0.005%, pH 6.2, agar 1.5%.

Complete culture (CM) medium: beef extract 1%, peptone 1%, glucose 0.5%, NaCl 0.5%, pH 7.2, agar 1.5%.

Potato dextrose agar (PDA) medium: potato concentrate 20%, glucose 2%, agar 1.5%.

Lactic acid bacteria isolation medium: MRS medium with 2% calcium carbonate added.

Peptone, beef extract, yeast extract, glucose, and agar powder were from Aoboxing Biotechnology Co., Ltd., Beijing, China. Tween-80 was from Solarbio Science and Technology Co., Ltd., Beijing, China. The other reagents were from Zhiyuan Chemical Reagent Co., Ltd., Tianjin, China.

For the medium used in the physiological and biochemical tests of the strains, refer to Dong Xiuzhu's Handbook for the Systematic Identification of Common Bacteria [31].

2.3. Methods

2.3.1. Screening of High-Yield Lactic Acid Bacteria Strains

Under aseptic conditions, 1 mL of acidified apple juice was added into a conical flask with 20 mL sterilized normal saline (9 g/L NaCl) and some glass beads and shaken well. Then, after gradient dilution to 10^{-6}, 1 mL of each gradient sample liquid was injected into the sterilized empty dishes, respectively. Then, 15 mL~20 mL/plate of the sterilized separated medium was poured, allowed to cool at about 45 °C, shaken well horizontally, inverted after solidification, and then put into a constant temperature incubator at 37 °C for 48 h. The first 10 single colonies with larger transparent circles around them were selected and inoculated in sterilized semi-solid MRS medium for culturing at 37 °C for 48 h. Then, each strain was inoculated in the MRS liquid medium at 37 °C for 24 h,

respectively, transferred again, and the process was repeated twice. Lactic acid content was determined with a lactic acid detection kit (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China), and the strain with the highest lactic acid production was selected as the strain for subsequent identification.

2.3.2. Strain Identification

Morphological Identification

The fresh slant culture was selected for Gram staining and microscopic morphology observation (B302 Biological Optical Microscope, Chongqing Aote Optical Instrument Co., Ltd., China; SU 8010 Field Emission Scanning Electron Microscope, Hitachi, Japan). The morphology of single colonies on the plate was observed and identified.

16S rDNA Sequence Analysis

The cells in the slant of the identified strain were picked up and denatured in 50 µL of TaKaRa microbial cracking buffer (Code No. 9164, Dalian Bao Biological Co., Ltd., Dalian, China) at 80 °C for 15 min, and the centrifuged supernatant was used as the PCR template. The TaKaRa 16S rDNA Bacterial Identification PCR Kit (Code No. RR176, Dalian Bao Biological Co., Ltd.) was used for PCR amplification of the full length of the 16S rDNA and the universal primers (27 F and 1492 R) were used to amplify the full length of the 16S rDNA with PCR. The PCR amplification system totaled 50 µL: 1 µL denaturing solution, 25 µL PCR premix, 0.5 µL forward primer (20 pmol/µL), 0.5 µL reverse primer (20 pmol/µL), and 23 µL dd H_2O. PCR amplification conditions were as follows: 94 °C 5 min; 94 °C 1 min, 55 °C 1 min, 72 °C 1.5 min, 30 cycles; 72 °C 5 min. The PCR, product purification, and 16S rDNA sequencing were all completed by Dalian Bao Biological Co., Ltd.

2.3.3. Lactic Acid Determination

The lactic acid test kit was produced by Nanjing Jiancheng Bioengineering Research Institute.

2.3.4. Selection of Solid-State Mode for Raw Fermentation

The soybean meal was crushed through a 40 mesh sieve. The ratio of corn meal to soybean meal = 3:1 (w/w), 20 g/sample. The initial water addition proportion was 50% (v/v). Tap water was used to prepare the solid-state fermentation medium for corn–soybean meal, and the following was the same. The mixed raw materials were respectively loaded into plastic bags and solid-state fermentation was carried out in the following three fermentation modes. The inoculated size of each strain was 5% (v/w). The seed liquid concentration was adjusted to OD_{600nm} 1.0. The three fermentation modes were as follows:

Pattern 1: The three strains, *Pediococcus acidilactici* SLB-04, *Saccharomyces cerevisiae* Y-06, and *Bacillus subtilis* YSJB-30, were inoculated in the raw materials at the same time and then the fermentation was undertaken in a plastic bag, which had a mouth with a cotton plug, for 48 h;

Pattern 2: First, the strains *Pediococcus acidilactici* SLB-04 and *Bacillus subtilis* YSJB-30 were inoculated in the raw materials at the same time, and the sealed fermentation was undertaken in a plastic bag for 24 h. Then, the strain *Saccharomyces cerevisiae* Y-06 was inoculated again, and the fermentation proceeded in the plastic bag with a mouth with a cotton plug for 24 h;

Pattern 3: First, the strain *Pediococcus acidilactici* SLB-04 was inoculated in the raw materials and the sealed fermentation was undertaken in the plastic bag for 24 h. Then, the strains *Saccharomyces cerevisiae* Y-06 and *Bacillus subtilis* YSJB-30 were inoculated at the same time again and the fermentation proceeded in the plastic bag with a mouth with a cotton plug for 24 h.

The pH value of the fermented feed was determined, and the best fermentation mode was selected according to the sensory score combined with color, odor, and microscopic examination of the fermented feed. Each experiment was repeated three times. The sensory analysis team consisted of 10 sensory evaluators (5 males and 5 females, mean

age: 22) who were professionally trained members with relevant professional knowledge and strong aroma recognition abilities. GB/T 16291.1-2012 General Guidelines for the Selection, Training and Management of Evaluators in Sensory Analysis Part 1: Selecting Evaluators was used as a reference for the selection and training of sensory evaluators. Sensory evaluation was carried out in a standard sensory laboratory at room temperature (20 °C). All samples were numerically coded randomly and stored in sterile, dry Petri dishes. Evaluators were instructed to rest for 2 min between each evaluation to avoid olfactory fatigue. In accordance with the scoring method and standard, each index of the fermented feed with the three different fermentation modes was scored. The standards for sensory evaluation are shown in Table 1.

Table 1. Sensory evaluation criteria for fermentation mode.

Item	Score	Scoring Rubric
Appearance	20	Bright color, uniform particles, no mildew particles (14~20) Darkening, relatively uniform particles, fewer mildew particles (7~13) Darker, uneven particles, more moldy particles (0~6)
Aroma	20	Rich aroma, prominent aromas of fruit and wine, odorless (14~20) Weak aroma, aromas of fruit and wine, basically odorless (7~13) Flat without aroma, no aroma of fruit or wine, smelly (0~6)
Contaminated with bacteria or not	20	Basically no contamination (14~20) Moderate contamination (7~13) Heavy contamination (0~6)
pH range	40	pH < 5.5 (27~40) 5.5 < pH < 6.5 (13~26) pH > 6.5 (0~12)

2.3.5. Optimization of Solid-State Fermentation Conditions in the First Stage—The Single-Factor Tests for Acid Production of *Pediococcus acidilactici*

The single-factor experiments for the water addition proportion: fermentation substrate 20 g (corn meal: soybean meal = 3:1 (w/w)); water addition proportions of 70%, 80%, 90%, 100%, and 110%; inoculation amount (OD_{600nm} 1.0, the following was the same) of 6%; initial pH value 4.5. The lactic acid content of the fermented feed was determined after a fermentation time of 48 h. Each experiment was repeated three times and the average value was used.

The single-factor experiments for the inoculum size: the fermentation substrate was 20 g (corn meal: soybean meal = 3:1; the water addition proportion was 100%; the initial pH value was 4.5; the inoculation sizes were 2%, 4%, 6%, 8% and 10%, respectively; and the lactic acid content of the fermented feed was determined after a fermentation time of 48 h. Each experiment was repeated three times, and the average value was used.

The initial pH experiments: fermentation substrate 20 g (corn meal:soybean meal = 3:1); the water addition proportion was 100%; the inoculation size was 6%; the initial pH values were 3.8, 4.1, 4.4, 4.7, 5.0, and 5.3, respectively; and the lactic acid content of the fermented feed was determined after a fermentation time of 48 h. Each experiment was repeated three times, and the average value was used.

2.3.6. Optimization of Solid-State Fermentation Conditions in the First Stage—The Orthogonal Test for Acid Production of *Pediococcus acidilactici* SLB-04

The orthogonal experimental design is shown in Table 2.

Table 2. The orthogonal experimental factors and levels.

Level	A (Initial pH)	B (Water Addition Proportion, %)	C (Inoculum Size, %)
1	4.1	80	6
2	4.4	90	8
3	4.7	100	10

2.3.7. Optimization of Solid-State Fermentation Conditions in the Second Stage—The Single-Factor Experiments

The single-factor experiments for fermentation temperature: the total inoculation amount was added at 10% to the seed culture solution (*Bacillus subtilis*:*Saccharomyces cerevisiae* = 1:1) and, for the second stage, the fermentation substrate in the plastic bag with a mouth with a cotton plug was treated with temperatures of 25 °C, 28 °C, 31 °C, and 34 °C, respectively. The fermentation proceeded for 48 h for the static culture, and then the fermented feed was taken out and baked at 60 °C until the weight was constant. The protein content of the fermented feed was determined.

The single-factor experiments for inoculum size: with a seed culture solution ratio of *Bacillus subtilis* to *Saccharomyces cerevisiae* of 1:1, the total inoculated amounts were 6%, 8%, 10%, and 12%, respectively. The fermentation in the second stage proceeded at 31 °C for 48 h, and the fermented feed was taken out and baked at 60 °C until the weight was constant. The protein content of the fermented feed was determined.

The single-factor experiments for inoculation ratio: the seed culture solution ratios of *Bacillus subtilis* to *Saccharomyces cerevisiae* were 3:7, 5:5, 7:3, and 8:2, respectively, and the total inoculation amount was added to the second-stage fermented feed at 10%. The fermentation proceeded at 31 °C for 48 h, and the fermented feed was taken out and baked at 60 °C until the weight was constant. The protein content of the fermented feed was determined.

2.3.8. Response Surface Optimization of the Conditions of the Second Stage of Solid-State Fermentation

Based on the single-factor experiments, a response surface test was designed in Design-Expert 12.0 to optimize the solid-state fermentation conditions in the second stage. The response surface analysis tests were designed with protein content as the response value and the factor-level coding table is shown in Table 3.

Table 3. Independent variable levels.

Code Level	A Inoculation Proportion	B Fermentation Temperature/°C	C Inoculum Size/%
+1	3:7	31	6.0
0	5:5	34	8.0
−1	7:3	37	10.0

2.3.9. Determination of Protein Content in Raw Materials

Standard Curve Drawing

Casein was accurately weighed and dissolved in 0.05 mol/L sodium hydroxide solution to prepare the protein standard solution (5.0 mg/mL). Standard protein solutions of 0.0 mL, 0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL, and 1.0 mL were supplemented with distilled water to 1.0 mL, respectively. Then, 4 mL biuret reagent was separately added, mixed well, and left at room temperature for 30 min, and the absorbance was measured at 540 nm. The standard curve is drawn in Figure S1. The regression equation was as follows:

$$Y = 0.25557x - 0.00162, \quad (1)$$

$$r^2 = 0.9985. \quad (2)$$

The Determination of Protein Content with the Biuret Method

Next, 0.2 g of the tested, dried sample was taken and the protein content was determined with the biuret method [32]. The protein content was calculated with a regression equation. This was repeated three times for each sample and the results averaged.

$$\text{sample protein (\%)} = [\text{the protein content obtained from the regression equation(mg)/sample weight}] \times 100\% \times \text{casein purity} \quad (3)$$

Casein Purity Determination

The protein content of the casein samples was determined using the GB/T6432-2018 Kjeldahl nitrogen determination method, and casein purity was calculated.

2.3.10. Salmonella Detection

Salmonella detection was undertaken according to the Chinese national standard GB/T13091-2018. Each sample was analyzed three times and the product with three negative test results was qualified.

2.3.11. Detection of Microbial Abundance in Fermented Feed

The samples were sent to Novogene BioInformation Co., Ltd. High-throughput sequencing with the PacBio platform was used to analyze the relative abundance of the microbial community in the fermentation system at the species and genus level.

DNA extraction and PCR amplification: after sample pretreatment [33], the STE method was used to extract bacterial genomic DNA, and the GP1 extraction method was used to extract fungal genomic DNA [34]. Bacterial 16S and the fungal ITS1 of the rDNA were amplified using forward primers (27 F; AGAGTTTGATCCTGGCTCAG, ITS 9 munngs TACACACCGCCCGTCG) and a reverse primer (1492 R: GNTACCTTGTTACGACTT, ITS 4 ngsUni CCTSCSCTTANTDATATGC), respectively. Amplicons were then sequenced using a PacBio platform at Novogene Biological Information Technology Co., Ltd., Beijing, China.

Sequencing data processing: the data from PacBio were transferred, and the short and long sequences were filtered through CCS and raw reads were obtained (SMRT Link v 7.0) [35]. Then, primer excisions and simple sequence repeat (SSR) removal were carried out to obtain valid data for subsequent analysis; i.e., clean reads were obtained [36].

OTU clustering and species notes: the sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity [37]. OTUs of 16S rRNA gene amplicons were mapped to the Silva database and OTUs of ITS amplicons were mapped to the Unite database to obtain taxonomic information for the sequences [38–41]. The representative sequences of OTUs were then annotated by species. The top 10 species with maximum abundance were selected to generate a columnar graph of relative abundance.

2.3.12. Statistical Method

The statistical analysis was repeated three times ($n = 3$) for each sample and the results averaged. Means ± standard deviations are reported, and means were separated by Duncan multiple comparisons. Differences were accepted as statistically significant at $p < 0.05$. The response surface design and analysis were carried out in Design-Expert 13.0 (Stat-Ease, Inc., Minneapolis, MN, USA). The means and standard deviations were calculated in Microsoft Excel software (Microsoft Corporation, Seattle, WA, USA). The significance of the differences was analyzed by multiple comparison in SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Drawing was undertaken in Origin8.0 software (Origin Lab Corporation, Northampton, MA, USA).

3. Results

3.1. Screening of the Strains Producing Lactic Acid

Many studies have shown that the application of lactic acid bacteria in fermented feed can produce organic acids (mainly lactic acid), reduce the pH value of the feed, and inhibit pathogenic bacteria, such as Escherichia coli, Salmonella, and mold, in feed [29,42]. A lactic acid concentration above 150 mmol/L can inhibit endogenous pathogens [43]. Du Dongxiao et al. used a plate medium with bromocresol purple added to screen strains with high acid production from soil [44]. However, since acid-producing strains may produce volatile substances, such as acetic acid and ethanol, in addition to lactic acid, it is advisable to select the strains producing high amounts of lactic acid. In accordance with this idea, in this study the first ten strains of acid-producing bacteria with larger diameters for their transparent rings were screened out with the isolation medium at the initial screening, and then the lactic acid production capacity of each strain was determined by re-screening. The results are shown in Table 4. The strain SLB-04 in the table had the highest lactic acid content, so strain SLB-04 was finally selected for the subsequent test.

Table 4. The data for the lactic acid production of strains at the re-screening.

Strain number	SLB-01	SLB-02	SLB-03	SLB-04	SLB-05
Lactic acid concentration, g/L	10.2	17.1	15	21	19.7
Strain number	SLB-06	SLB-07	SLB-08	SLB-09	SLB-10
Lactic acid concentration, g/L	16	18.3	15.8	15.5	11.9

3.2. Identification of Lactobacillus Strains

3.2.1. Morphological Identification

For the colony morphology, the structures of strain SLB-04 were observed under a light microscope and scanning electron microscope (Figure S2). On the surface of the plate medium, the colony of strain SLB-04 was small and milky white with a round bulge. The colony edge was smooth and clear and no pigment was secreted. The strain was Gram-positive. Under the electron microscope, the cells of strain SLB-04 were spherical with a diameter of about 0.6 nm and appeared in pairs. Single cells were rare and usually divided to form tetrads in two vertical directions, and they did not show chain formation, spores, or flagella around them. The strain grew in a facultatively anaerobic manner in the medium. The morphology of the plate colony and the morphology of the bacteria under the optical microscope were completely consistent with those described in previous studies [44], which preliminarily proved that the strain SLB-04 may have been *Pediococcus acidilactic*.

3.2.2. Physiological and Biochemical Identification Tests

Some physiological and biochemical test results for strain SLB-04 are shown in Table 5. Comparing the characteristics of *Pediococcus* in Bergey's Bacterial Identification Manual and Common Bacterial Systematic Identification Manual, it was found that the results were consistent [31,45], and it was further concluded that the strain might be *Pediococcus acidilactici* of the *Pediococcus* genus.

3.2.3. 16S rDNA Sequence Analysis

The 16S rDNA PCR of strain SLB-04 was performed. After the PCR product was purified, the strain's 16S rDNA was sequenced by Dalian Bao Biological Co., Ltd. BLAST aligning with the NCBI database showed that *Pediococcus acidilactici* SLB-04 had 99.71% homology with *Pediococcus acidilactici* DSM 20,284 (GenBank: NR_042057) for the 16S ribosomal RNA gene partial sequence. This sequence was compared with the 16S rDNA sequences of other strains of the genus *Pediococcus* and non-*Pediococcus* lactic acid bacteria in NCBI. MEGA 6.06 software (Mega Limited, Auckland, New Zealand) was used to

construct phylogenetic trees with the maximum likelihood method and adjacency method, respectively (Figure S3 and Figure 1). This showed that the strain SLB-04 and *Pediococcus acidilactici* DSM 20,284 were both in the same branch, and the boot values after comparison were 100% and 99%, respectively. The higher the boot value is, the more reliable the results obtained are. Therefore, SLB-04 belongs to *Pediococcus acidilactici.* The Genbank accession number of the sequence of the 16S rRNA gene of the *Pediococcus acidilactici* SLB-04 strain is OP059631.1.

Table 5. Comparison of some physiological and biochemical test results for strain SLB-04 and *Pediococcus acidilactici*.

Item		SLB-04	*Pediococcus acidilactici*
Gram staining		+	+
Motility		−	−
Catalase test		−	−
Methyl red test		+	+
Cytochrome test		−	−
Sugar or alcohol fermentation test	Galactose	+	+
	Xylose	+	+
	Sucrose	+	+
	Glucose	+	+
	Maltose	−	−
	Mannitol	−	−
	Lactose	−	±
	Trehalose	±	+
	Arabinose	±	+
	Dextrin	−	−
	Amylose	−	−
Growth pH	pH 4.2	+	+
	pH 7.5	+	+
	pH 8.5	+	d
Growth NaCl	4%	+	+
	6.5%	+	+
	18%	−	−
Growth temperature	35 °C	+	+
	40 °C	+	+
	50 °C	+	+

Note: +, positive; −, negative; “d” indicates that 11–89% of strains were positive.

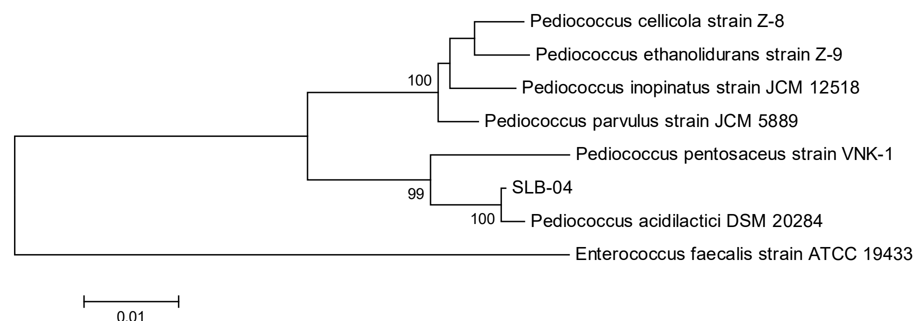

Figure 1. The 16S rDNA phylogenetic tree constructed on the basis of the adjacency method.

Based on the results of the morphological, physiological, and biochemical tests, as well as the 16S rDNA sequence analysis, SLB-04 was preliminarily identified as *Pediococcus acidilactici*. The strain was stored at the China Microbiological Culture Preservation Center (CGMCC), no. 24220.

3.3. Selection of Solid-State Mode for Raw Fermentation

The sensory evaluation results for the three fermentation modes are shown in Table 6. It can be seen in the table that the four sensory evaluation indexes all showed significant differences ($p < 0.05$), indicating that the different types of fermentation modes had strong influences on the fermentation results. Among them, the differences in the product odor, pH value, and appearance were the most obvious. The pH value of model C was the lowest and the smell was the most acidic; the appearance of the fermented product was bright, uniform, and without mold pollution. To understand the situation of bacterium infection in the fermentation more clearly, after the observation of the appearance, the sample was viewed under an optical microscope to determine whether there were non-mycotic bacteria in the fermentation products. It was found that, in both mode B and mode C, there were bacteria other than the three kinds of artificial inoculating strains, which might have been other bacteria growing in the fermentation system. The main problem facing uncooked raw fermentation is how to prevent the endogenous contamination of the fermentation system, especially by *E. coli*, *Salmonella*, and other pathogenic bacteria. *Pediococcus acidilactici* can produce bacteriocin to inhibit *Listeria monocytogenes*, *Shigella*, and *Clostridium perfringens* [23,46,47]. The results showed that the lactic acid, acetic acid, and total acid contents in the fermentation system of *Pediococcus acidilactici* were positively correlated with the antibacterial activity [24]. Zhang Yiran et al. reported that *Bacillus subtilis* MA139 could secrete antimicrobial peptides that kill *Escherichia coli*, *Salmonella*, and *Staphylococcus aureus*, but they have no effect on the growth and metabolism of yeast and lactic acid bacteria [48]. In this experiment, the bacteriostatic characteristics of *Pediococcus acidilactici* and *Bacillus subtilis* were used to antagonize pathogenic bacteria in the fermentation system. As *E. coli*, *Salmonella*, and other intestinal pathogenic bacteria cannot tolerate pH levels below 4.5, to effectively inhibit pathogenic bacteria in the fermented feed, rapid initial lactic acid production was required [49–51]. Since *Pediococcus acidilactici* can simultaneously produce bacteriostatic substances and lower pH, it was more conducive to inhibiting the growth of pathogenic bacteria such as *E. coli* and *Salmonella*. Therefore, model C was determined to be the best model for complex probiotic fermentation; that is, *Pediococcus acidilactici* was inoculated first for closed fermentation and then *Saccharomyces cerevisiae* and *Bacillus subtilis* were inoculated simultaneously for static fermentation.

Table 6. The results of the sensory evaluation of fermentation modes.

Sample	Appearance	Odor	Bacteria or Not	pH	Total Score
A	5.9 ± 0.94^{c}	2.4 ± 0.92^{c}	2.7 ± 0.64^{b}	6.8 ± 1.17^{c}	17.8 ± 3.67^{c}
B	8.5 ± 1.5^{b}	10.8 ± 1.17^{b}	11 ± 1.34^{a}	16.9 ± 1.81^{b}	47.2 ± 5.82^{b}
C	15.6 ± 1.36^{a}	17.2 ± 0.98^{a}	8.9 ± 1.3^{a}	32.8 ± 2.56^{a}	74.5 ± 6.2^{a}

Note: Experiments were performed in triplicate (n = 3). The data are expressed as the means ± SD. Different shoulder-marked letters in the same column indicate significant differences ($p < 0.05$).

3.4. Pediococcus Acidilactici Fermentation Test

Although it has been reported in the literature that pediocin can be produced by *Pediococcus acidilactici* [52,53], it is more commonly used for food preservative or fermented silage [54–56] and rarely for solid-state fermentation of raw corn–soybean meal. As a facultative anaerobic fermentation bacterium, *Pediococcus acidilactici* is a micro-aerobic microorganism with a strong bacteriocin production ability under micro-oxygen conditions [52], and its SSF should be carried out in a closed environment. Therefore, in this experiment, the bag was filled with the raw materials and then closed static SSF was adopted. Low moisture content in a solid-state fermentation medium results in high substrate porosity, which is conducive to air penetration, leading to relatively abundant oxygen in the substrate. High moisture content in a solid-state fermentation medium results in low substrate porosity, which, in turn, prevents air penetration and an anaerobic environment is created. At the same time, the water content in the substrate, the water activity, and the mass transfer rate between microbial cells increase, which is conducive to the generation

of microbial metabolites [3,10]. As shown in Figure 2, in the single-factor experiments for the water addition amount, the lactic acid content in the system gradually increased with the increase in the amount of water added in the solid medium, and the increase in lactic acid content was not significant when the water addition amount was less than 90%. When the water addition amount exceeded 90%, the lactic acid content in the system increased significantly. When the water addition amount reached 110%, although the lactic acid content was relatively the highest, the actual state of the medium was semi-solid, which was not conducive to the subsequent fermentation. Taking into account the actual situation, it was judged appropriate to choose the 100% water addition amount for the experiment.

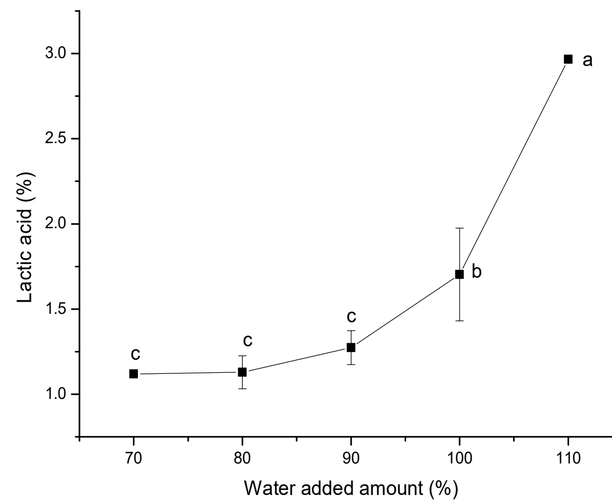

Figure 2. The single-factor experiments for the water addition amount with *Pediococcus acidilactici*. Experiments were performed in triplicate (n = 3). Sites not sharing a common letter showed significant differences ($p < 0.05$).

Excessive inoculation size leads to excessive growth of bacteria in the early stage of fermentation and consumes a lot of nutrients, which is not conducive to the accumulation of metabolic substances. If the inoculation size is too small, the microbial growth in the system will be slow, and the fermentation cycle will be prolonged [57]. As shown in Figure 3, with the increase in inoculation size, lactic acid production first increased and then decreased. This was because, with the increase in inoculation size in the fermentation system, the initial numbers of lactic acid bacteria cells and the cell proliferation of *Pediococcus acidilactici* increased and, thus, the production of lactic acid and metabolites by the bacteria also increased. However, when the inoculation size exceeded 8%, the metabolic acid production in the system no longer increased due to the proliferation of cells and the consumption of nutrients in the system. The lactic acid yield reached its maximum when the inoculation size was 8%, so 8% was the optimal inoculation size.

pH affects the function of membrane proteins by affecting the charge distribution on the cells, changes the absorption of nutrients, and ultimately affects the growth of the cell itself and the accumulation of metabolites [57]. As shown in Figure 4, with the increase in initial pH, lactic acid production in the fermentation system increased first and then decreased. When the initial pH was 4.4, lactic acid production was relatively the highest. As intestinal pathogenic bacteria, such as *Escherichia coli* and *Salmonella*, cannot tolerate pH below 4.5 [51], the growth of intestinal pathogenic bacteria would be inhibited in systems with pH values lower than 4.4, and such systems would be more conducive to the growth of *Pediococcus acidilactici*. When the pH value of the system was above 4.6, the growth of various bacteria, including intestinal pathogenic bacteria, affected the proliferation of *Pediococcus acidilactici* cells, leading to a greater decline in lactic acid production. However, too low an initial pH value could also affect the membrane protein function, the intake of

nutrients, the cell proliferation, and the acid production of *Pediococcus acidilactici*. Therefore, the optimal initial pH was 4.4.

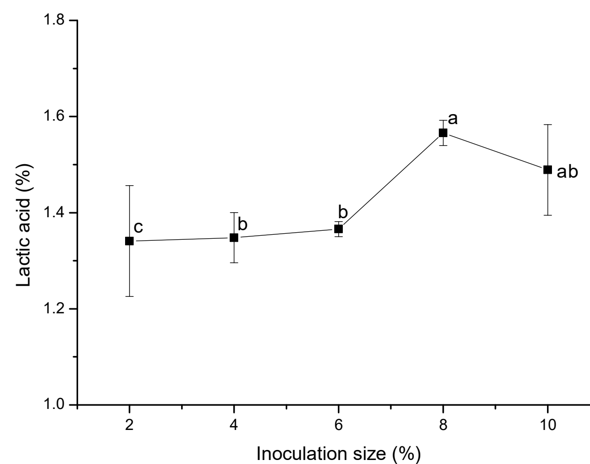

Figure 3. The single-factor experiments for *Pediococcus acidilactici* inoculum size. Experiments were performed in triplicate (n = 3). Sites not sharing a common letter showed significant differences ($p < 0.05$).

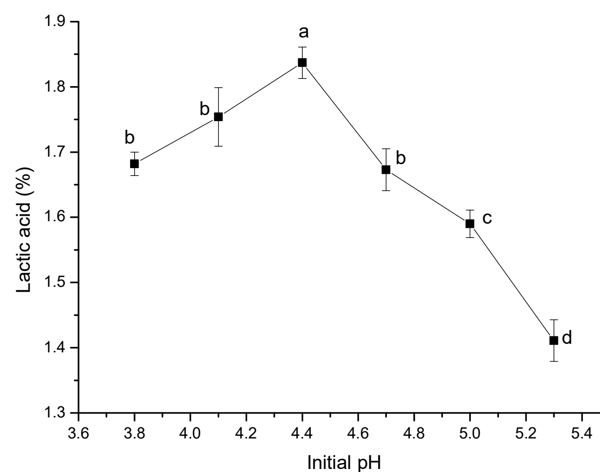

Figure 4. Initial pH single-factor experiments with *Pediococcus acidilactici.* Experiments were performed in triplicate (n = 3). Sites not sharing a common letter showed significant differences ($p < 0.05$).

The orthogonal test of the fermentation conditions of *Pediococcus acidilactici* is described in Table 7. The factors influencing lactic acid yield in the fermentation system were as follows: initial pH value > water addition amount > empty column > inoculation amount. The extremum difference analysis showed that the optimal process conditions were $A_2B_3D_1$; that is, initial pH 4.4, 100% water addition amount, and 6% inoculation size. Table 8 shows the variance analysis of the orthogonal experiment. The table shows that the major factors affecting lactic acid yield were as follows: initial pH value > water addition amount > empty column > inoculation size. The initial pH value and water addition amount had significant effects on lactic acid production, while the empty column and inoculation size had no significant effects on lactic acid production. Furthermore, $MS_{\text{inoculation size}} < 2\,MS_{\text{empty column}}$. Therefore, the sum of the squares of deviation and freedom for the inoculation size were merged with the sum of the squares of deviation and freedom for the blank column, respectively, and then transformed into the sum of the squares of deviation and freedom for the error term, respectively, to improve the sensitivity of the *F* test. Considering the economic cost and other factors, the optimal process conditions were pH 4.4, 100% water addition amount, and 6% inoculation size.

Table 7. Orthogonal test of fermentation conditions for *Pediococcus acidilactici*.

Test Number	A (Initial pH)	B (Water Addition Amount)	C (Empty Column)	D (Inoculation Size)	Lactic Acid %
1	1 (4.1)	1 (80%)	1	1 (6%)	1.50 ± 0.01
2	1 (4.1)	2 (90%)	2	2 (8%)	1.53 ± 0.005
3	1 (4.1)	3 (100%)	3	3 (10%)	1.59 ± 0.003
4	2 (4.4)	1 (80%)	2	3 (10%)	1.65 ± 0.001
5	2 (4.4)	2 (90%)	3	1 (6%)	1.67 ± 0.002
6	2 (4.4)	3 (100%)	1	2 (8%)	1.75 ± 0.003
7	3 (4.7)	1 (80%)	3	2 (8%)	1.43 ± 0.002
8	3 (4.7)	2 (90%)	1	3 (10%)	1.45 ± 0.005
9	3 (4.7)	3 (100%)	2	1 (6%)	1.57 ± 0.001
k1	1.54	1.53	1.57	1.58	
k2	1.69	1.55	1.58	1.57	
k3	1.48	1.64	1.56	1.56	
R	0.21	0.11	0.02	0.02	
Order of importance			A > B > C = D		
Optimal level	A2	B3		D1	
Optimal combination			$A_2B_3D_1$		

Note: Experiments were performed in triplicate (n = 3). The data are expressed as the means ± SD.

Table 8. Variance analysis of *Pediococcus acidilactici* fermentation test.

Source of Variation	Sum of Squares of Deviations	Degrees of Freedom	Mean Square	*F* Value	*F* Critical Value	Significance
Initial pH	0.068	2	0.034	136.000	6.94	*
Water addition amount	0.020	2	0.010	40.000	6.94	*
Empty column	0.001	2	0.0005	2.000	6.94	
Inoculation size	0.000	2	0.000	0.000	6.94	
Error	0.001	4	0.00025			

Note: * indicates significant difference, $F_{0.05(2,4)} = 6.94$, F value > $F_{0.05}$, $p < 0.05$.

3.5. Optimization of Solid-State Fermentation Conditions in the Second Stage

With different strains, inoculation proportions, and culture temperatures, the bacteria body protein content of the fermented feed will differ, which will affect the feeding effect in animals [22]. Therefore, it is necessary to optimize the fermentation feed preparation process to obtain higher bacteria body protein content at lower cost and thus achieve higher cost benefits. The purpose of the second stage of the SSF was to use *Bacillus subtilis* to degrade macromolecular substances in raw material, which is conducive to the proliferation of *Saccharomyces cerevisiae*, and thus increase the protein content in fermented feed. Therefore, the inoculation ratio of *Bacillus subtilis* to *Saccharomyces cerevisiae*, inoculation size, and SSF temperature were the main factors affecting the protein content. Firstly, the single-factor experiments for the inoculation ratio, inoculation size, and SSF temperature were respectively conducted, and the results are shown in Figures 5–7. In previous research, *Bacillus subtilis* could grow on raw materials consisting of grains and beans and secrete protease, cellulase, amylase, lipase, and other hydrolytic enzymes, especially those that show strong neutral protease and alkaline protease activity. Furthermore, the raw organic matter was decomposed, and the digestion and absorption rate of animals were improved [18,42,58,59]. It has been shown that inoculated probiotics, such as *Saccharomyces cerevisiae*, *Bacillus subtilis*, and lactic acid bacteria, can convert non-protein nitrogen in soybean meal into bacteria body protein with higher nutritional value during the cell proliferation process, improve the nutritional structure of raw materials, and increase the protein content of raw materials [59–62]. As shown in Figure 6, protein content in the fermentation system initially increased with the increase in the *Bacillus subtilis* inoculation ratio. When the ratio

of the two strains was 5:5, the protein content was the highest. When the proportion of *Bacillus subtilis* inoculated exceeded that of *Saccharomyces cerevisiae*, the protein content in the system began to decrease. With certain nutrients in the system, the proportion of *Bacillus subtilis* increased appropriately, which was conducive to its metabolism and production of enzymes and decomposition of starch, protein, and other macromolecular substances in the fermentation system; such changes are useful for yeast proliferation and to increase yeast protein. However, when the proportion of *Bacillus subtilis* was too high, the cell proliferation led to consumption of excessive nutrients, which is not conducive to the production of enzymes and the degradation of macromolecular substances, so the protein content in the fermentation system began to decline again. Therefore, the optimal inoculation ratio for the single-factor experiment was *Bacillus subtilis*: *Saccharomyces cerevisiae* = 5:5.

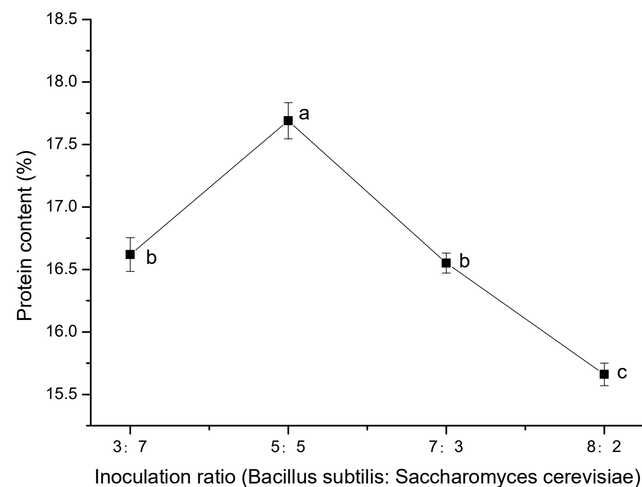

Figure 5. The single-factor experiments for the inoculating proportion in the second stage of the solid-state fermentation. Experiments were performed in triplicate (n = 3). Sites not sharing a common letter showed significant differences ($p < 0.05$).

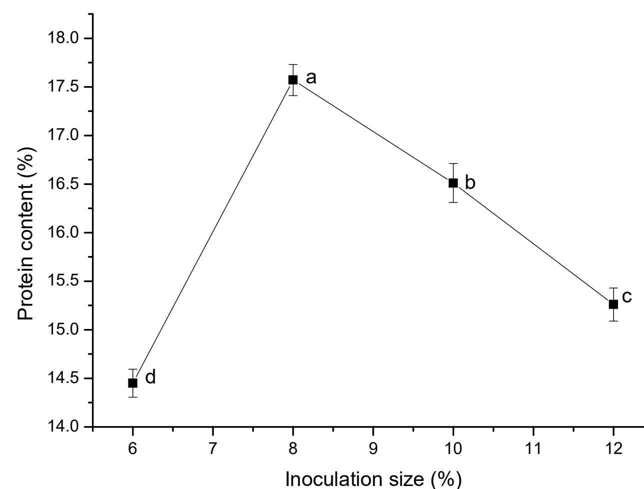

Figure 6. The single-factor experiments for inoculation size in the second stage of the solid-state fermentation. Experiments were performed in triplicate (n = 3). Sites not sharing a common letter showed significant differences ($p < 0.05$).

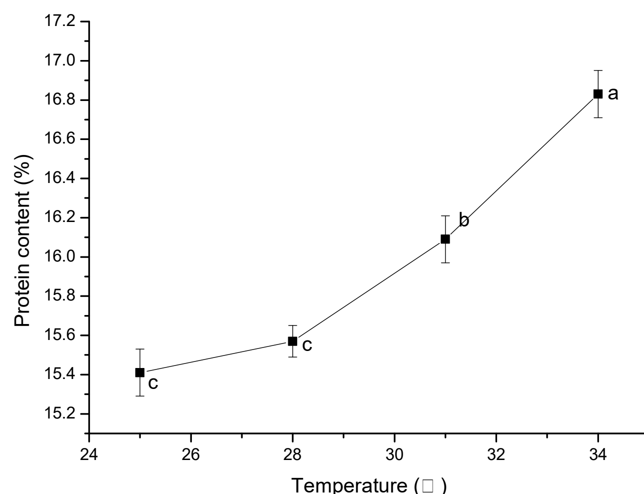

Figure 7. The single-factor experiments for the fermentation temperature in the second stage of the solid-state fermentation. Experiments were performed in triplicate (n = 3). Sites not sharing a common letter showed significant differences ($p < 0.05$).

The inoculation size affected the proliferation rate and metabolite yield of bacteria [57]. As shown in Figure 6, protein content increased as the inoculation size increased from 6% to 8%. When the inoculation size exceeded 8%, the protein content tended to decline due to excessive proliferation of cells and excessive consumption of nutrients in the system. It can be seen that a larger inoculation size is not always better for fermented feed, which is consistent with the results published by Guo Ruyi et al. [57]. Therefore, the optimal inoculation size for the single-factor experiment was 8%.

The optimum growth temperatures for the different microorganisms differ. The optimum growth temperature for *Bacillus subtilis* is 37 °C, and that of *Saccharomyces cerevisiae* is between 28 and 31 °C. High temperature is not conducive to the growth of *Saccharomyces cerevisiae*, but *Bacillus subtilis* can grow well at 40 °C since it has strong heat resistance [58,59]. Considering the temperature growth characteristics of *Bacillus subtilis* and *Saccharomyces cerevisiae*, the temperature range of 25 °C–34 °C was selected in the study. As shown in Figure 7, the protein content in the system increased with the increase in culture temperature. This was because, with the increase in temperature, the exudation of soluble substances and the efficiency of enzymatic hydrolysis in the system increased, so *Bacillus subtilis and Saccharomyces cerevisiae* could obtain more available substances and the protein content of the bacteria and yeast in the system increased. In the range of temperatures used, the optimum culture temperature was 34 °C. Based on the single-factor experiments, a response surface methodology (RSM) was used to further optimize the SSF conditions in the second stage. The experiment results are shown in Table 9 and the variance analysis results are shown in Table 10. The p value of 0.0082 of the regression model in Table 10 was less than 0.01, indicating that the regression relationship between the independent variable and the response value was extremely significant ($p < 0.01$). The p value for the lack of fit was 0.0689, indicating that the difference between the lack of fit and the pure error was not significant. According to the F values in Table 10, the contribution rates of the independent variable factors were as follows: A > B > C; that is to say, inoculation ratio > fermentation temperature > inoculation size. The inoculation ratio had the most significant effect on the protein content of the fermented feed, followed by the fermentation temperature, and the inoculation size had the least significant relative effect. In the model, the first-order term of the independent variable and the interaction between the independent variables had no significant influence on the response value. In the model, the quadratic term of the independent variable fermentation temperature had a very significant influence on the response value ($p < 0.01$), and the quadratic term of the independent variable inoculation size had a significant influence on the response value ($p < 0.05$).

Table 9. The design and results of the response surface methodology for the second stage of the SSF.

Run	Inoculation Ratio (A)	Fermentation Temperature/°C (B)	Inoculation Size (C)	Protein Content (%)
1	−1 (7:3)	0 (34)	1 (6.0)	17.27 ± 0.011
2	0 (5:5)	0 (34)	0 (8.0)	17.88 ± 0.034
3	0 (5:5)	0 (34)	0 (8.0)	17.87 ± 0.023
4	1 (3:7)	0 (34)	−1 (10.0)	18.98 ± 0.021
5	0 (5:5)	0 (34)	0 (8.0)	17.98 ± 0.008
6	0 (5:5)	1 (31)	1 (6.0)	18.45 ± 0.034
7	0 (5:5)	0 (34)	0 (8.0)	18.66 ± 0.012
8	1 (3:7)	1 (31)	0 (8.0)	15.37 ± 0.013
9	−1 (7:3)	0 (34)	−1 (10.0)	17.95 ± 0.034
10	0 (5:5)	−1 (37)	−1 (10.0)	15.68 ± 0.009
11	1 (3:7)	0 (34)	1 (6.0)	19.65 ± 0.052
12	0 (5:5)	1 (31)	−1 (10.0)	17.53 ± 0.024
13	−1 (7:3)	−1 (37)	0 (8.0)	14.77 ± 0.033
14	0 (5:5)	−1 (37)	1 (6.0)	16.21 ± 0.041
15	1 (3:7)	−1 (37)	0 (8.0)	15.51 ± 0.031
16	0 (5:5)	0 (34)	0 (8.0)	18.72 ± 0.023
17	−1 (7:3)	1 (31)	0 (8.0)	14.88 ± 0.011

Table 10. Variance analysis of the response surface methodology for the second stage of the SSF.

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p*-Value	Significant
Model	34.38	9	3.82	7.18	0.0082	**
A	2.69	1	2.69	5.06	0.0592	
B	2.06	1	2.06	3.88	0.0897	
C	0.26	1	0.26	0.49	0.5076	
AB	0.016	1	0.016	0.029	0.8687	
AC	0.46	1	0.46	0.86	0.3854	
BC	0.038	1	0.038	0.072	0.7968	
A^2	2.68	1	2.68	5.03	0.0598	
B^2	22.12	1	22.12	41.61	0.0003	**
C^2	4.53	1	4.53	8.53	0.0223	*
Residual	3.72	7	0.53			
Lack of fit	2.98	3	0.99	5.38	0.0689	Not significant
Pure error	0.74	4	0.18			
Cor total	38.10	16				
R^2			0.9023			

Note: * and ** indicate significant difference and very significant difference, respectively ($p < 0.05$ and $p < 0.01$).

Design-Expert was used to analyze the variance in the experimental data, and the quadratic regression model was established:

$$Y = 18.22 - 0.58A - 0.51B - 0.18C - 0.11AB + 0.063AC + 0.097BC - 0.8A^2 - 2.29B^2 + 1.04C^2 \quad (4)$$

The correlation coefficient of the regression model R^2 = 0.9023, indicating that 90.23% of the response value changes were related to the inoculation ratio, fermentation temperature, and inoculation amount, and the prediction was good.

The optimal process parameters for the model obtained by software optimization are shown in Table 11: at an inoculation ratio of *Bacillus subtilis* to *Saccharomyces cerevisiae* (v/v) of 3.8:6.2, fermentation temperature of 33.52 °C, and inoculation size of 6%, the protein content of the fermented feed was the highest, and the predicted value was 19.66%. As the fermentation temperature of 33.52 °C was not easy to operate, the fermentation temperature was adjusted to 33.5 °C. The verification tests were carried out with the

optimized process parameters and the protein content of the fermented feed was measured as 19.87%, which was basically consistent with the predicted value. Therefore, this proved that the model was reliable. Compared with unfermented raw material, the protein content of the fermented feed was increased by 5.21 percentage points and 35.74% ($p < 0.05$). Hou Nannan et al. used soybean meal, corn meal, and bran as raw materials; solid-state fermentation was conducted with lactic acid bacteria and yeast added for 72 h, and the crude protein content of the fermented feed was 6.07% higher than that of the control [61]. Shi Changyou et al. fermented a mixture of soybean meal, corn meal, and bran with *Bacillus subtilis* and *Enterococcus faecium* in two-stage solid-state fermentation. In the first stage, *Bacillus subtilis* underwent aerobic fermentation for 24 h, and then anaerobic fermentation was conducted with *Enterococcus faecalis* added again for 48 h. Compared with the control, the crude protein in the fermented feed increased by 3.58 percentage points ($p < 0.05$) [29]. In this study, the feed fermented by the compound probiotics had an obvious fruity taste, sweetness, and a bright appearance, and the sensory properties of the fermented feed were consistent with descriptions of high-quality fermented feed [61].

Table 11. The optimization results for the feed fermented with the compound probiotics.

Index	Value
The inoculation ratio (*Bacillus subtilis* to *Saccharomyces cerevisiae* (*v*/*v*))	3.8:6.2
Fermentation temperature (°C)	33.5
Inoculation size (%)	6
Protein content of raw materials (%)	14.66 ± 0.270
Predicted protein content of fermented feed (%)	19.66
Actual protein content of fermented feed (%)	19.87 ± 0.121
The percentage increase in protein content (%)	5.21

3.6. The Results of High-Throughput Sequencing for the Detection of the Relative Abundance of Bacterial Microflora in the Fermentation System

The fermentation samples were collected at 0 h (before inoculation), 48 h (after the second stage of inoculation), and 96 h and sent to Novogene Bioinformation Technology Co., Ltd., respectively, for third-generation high-throughput sequencing using the PacBio platform. The results of the 16S rDNA bacterial microflora abundance detection are shown in Figure 8 and Table S2, and the results of the ITS fungal microflora abundance detection are shown in Figure 9 and Table S3.

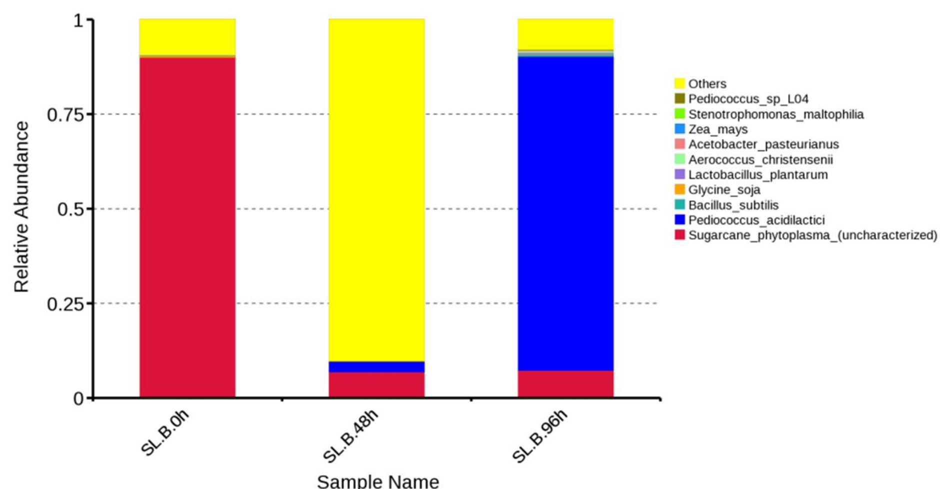

Figure 8. The histogram of the relative abundance of bacterial species at the species level. Abscissa (left to right): 0 h, 48 h, and 96 h fermentation samples; ordinate: relative abundance of species. "Others" represents the sum of the relative abundances of all species outside the top ten.

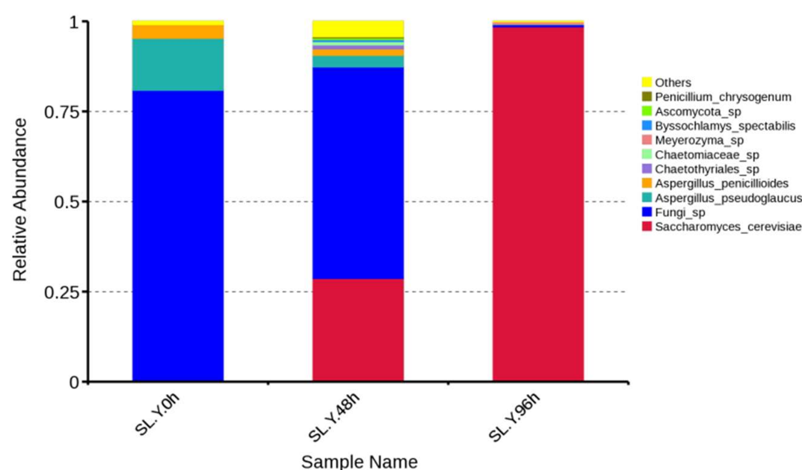

Figure 9. The histogram of the relative abundance of fungal species at the species level. Abscissa (left to right): 0 h, 48 h, and 96 h fermentation samples; ordinate: relative abundance of species. "Others" represents the sum of the relative abundances of all species outside the top ten.

As shown in Figure 8, the original bacterial communities in the uninoculated raw feed system were, in order, *Sugarcane phytoplasma* (uncharacterized), "Others", *Glycine soja*, and *Zea mays*, but the relative abundances of the latter two were low. After being fermented with *Pediococcus acidilactici* for 48 h, the bacterial communities in the fermented system with relative abundances that increased were "Others" and *Pediococcus acidilactici*. The relative abundances of the communities *Sugarcane_phytoplasma* (uncharacterized) and *Glycine soja* decreased, while *Zea mays* disappeared. It could be seen that the initial distribution of the bacterial community in the system was changed by the fermentation of *Pediococcus acidilactici* in corn–soybean meal for 48 h, and the relative abundance of *Sugarcane phytoplasma* (uncharacterized) was reduced and that of "Others" increased. After the cooperative fermentation of *Bacillus subtilis* and *Saccharomyces cerevisiae* for 96 h, *Pediococcus acidilactici* was the dominant bacterial community with the highest relative abundance. Among the top ten, the communities with relative abundances that increased were, in turn, *Bacillus subtilis*, *Lactobacillus plantarum*, *Aerococcus christensenii*, *Acetobacter pasteurianus*, *Stenotrophomonas maltophilia*, and *Pediococcus sp*_L04 (from high to low). The community with relative abundances that decreased was "Others", while the relative abundances of *Sugarcane phytoplasma* (uncharacterized) and *Glycine soja* remained basically stable. Lian Mengran compared the community structure of soybean meal with that of fermented soybean meal and found that the original community in soybean meal, unidentified *Cyanobacteria*, decreased significantly after fermentation. The relative abundances of *Lactobacillus*, *Pediococcus*, *Enterococcus*, and *Staphylococcus* and the diversity of bacterial communities increased after fermentation, which was similar to the change in the trend for bacterial communities in this study [63].

As shown in Figure 9, the original fungi communities with high relative abundances in uncooked corn–soybean meal without inoculation were *Fungi sp.*, *Aspergillus pseudoglaucus*, *Aspergillus penicillioides*, and "Others". After 48 h fermentation with *Pediococcus acidilactici*, the communities with decreasing relative abundances were *Fungi sp.*, *Aspergillus pseudoglaucus*, and *Aspergillus penicillioides*. The fungi communities with increased relative abundance included *Saccharomyces cerevisiae*, "Others", *Chaetothyriales sp.*, *Chaetomiaceae sp.*, *Byssochlamys spectabilis*, *Ascomycota sp.*, and *Penicillium chrysogenum*. Therefore, the inoculation of *Pediococcus acidilactici* had a great impact on the distribution of mycoflora in the system, and the relative abundance of *Fungi sp.* decreased the most, while the diversity of mycoflora increased. After fermentation with *Bacillus subtilis* and *Saccharomyces cerevisiae* for 96 h, the absolute dominant community of mycoflora in the sample was *Saccharomyces cerevisiae*, and the relative abundances of all the top ten fungi that appeared after 48 h, except *Saccharomyces cerevisiae*, declined significantly or they disappeared. Meanwhile, a new microflora, *Meyerozyma sp.*, appeared in the fermentation system. Shi Changyou

et al. studied the two-stage fermentation of corn–soybean meal with *Bacillus subsubis* and *Enterococcus faecium*, and the level of mold in the 96 h fermented feed dropped below the detection limit, which was similar to the results in this study [29].

The fermented feed was sent to the Agricultural Products Testing Center at Heilongjiang Academy of Agricultural Sciences for *Salmonella* detection, but no *Salmonella* was detected (Table S4).

4. Discussion

There are two main problems in replacing antibiotics with fermented feed. One is the preparation cost of the fermented feed, and the other is the efficacy of the fermented feed. The use of uncooked raw materials to prepare fermented feed can simplify the process of fermentation feed preparation and reduce the production cost, but how the metabolism of probiotics can be used to avoid the contamination of endogenous pathogenic bacteria is the key to the success of uncooked raw feed fermentation. The strain of *Bacillus subtilis* used in this paper was a strain that had been previously screened in the laboratory and proved to inhibit *Listeria monocytogenes*, *Salmonella*, and the other intestinal pathogenic bacteria in in vitro tests and to produce protease to degrade macromolecular proteins into low-molecular-weight oligopeptides (published separately). Van Winsen et al. reported that a lactic acid concentration in feed above 150 mmol/L could inhibit endogenous pathogenic bacteria [43]. In the first stage of fermentation, the *Pediococcus_acidilactici* screened in this study could produce 194 mmol/L lactic acid, so the growth of endogenous pathogenic bacteria could be prevented in the first stage of uncooked raw feed fermentation. Wang Gang et al. isolated *Pediococcus_acidilactici* P9 from fermented vegetables to inhibit *Listeria monocytogenes* and *Shigella* [23]. Nieto-Lozano J.C. et al. demonstrated in situ that a bacteriocin produced by *Pediococcus acidilactici* inhibited *Listeria monocytogenes* and *Clostridium perfringens* on the surface of raw Spanish meat [47]. James T. H. et al. isolated and purified the pediocin PA-1 produced by *Pediococcus acidilactici* PAC-1.0. It contained two disulfide bonds and was a 44 amino acid polypeptide with a molecular weight of 4629 Da, and it could inhibit a variety of Gram-positive bacteria, including *Listeria monocytogenes* [46]. After fermentation of raw corn–soybean meal by *Pediococcus acidilactici* in the first stage, the microflora distribution in the raw system of corn–soybean meal changed, the abundances of original adverse bacteria and mold in the raw materials were reduced, and the diversity of mycoflora increased, which may have derived from lactic acid and unknown metabolites produced by proliferating *Pediococcus acidilactici*. The unknown metabolites may have been unknown bacteriocins, which needs to be verified in future tests. When *Bacillus subtilis* and *Saccharomyces cerevisiae* continued to be inoculated in the system for fermentation, the proliferation of *Pediococcus_acidilactici*, *Saccharomyces cerevisiae*, and *Bacillus subtilis* was promoted due to the synergistic effects between the strains, and the growth and reproduction of a variety of fungal communities was inhibited. This showed that the synergistic effects of the three probiotics could help ferment raw materials to form favorable microflora. Therefore, the rapid production of lactic acid in the early stage of uncooked raw fermentation and the synergistic effects between fermentation strains were the key factors in the inhibition of the production of endogenous pathogenic bacteria, highlighting the potential of the procedure to replace the traditional sterilization process and reduce production costs.

Protein is always the core raw material in feed. Increasing the protein content in feed can increase the nutritional value of fermented feed and increase the cost benefit of the feed [21,27]. In this study, through cooperative fermentation with *Pediococcus_acidilactici*, *Saccharomyces cerevisiae*, and *Bacillus subtilis*, the protein content in the fermented feed increased by 5.71 percentage points ($p < 0.05$). The fermented feed was sent to the Agricultural Product Testing Center of Heilongjiang Academy of Agricultural Sciences to test for 17 amino acids (Table S1, separately published). Among the ten amino acids necessary for pigs, all except for tryptophan were detected, and their respective contents increased to varying degrees after fermentation ($p < 0.05$). Among the amino acids necessary for

pigs, lysine, methionine, and threonine, which are often lacking in pig feed, increased by 0.12, 0.13, and 0.16 percentage points, respectively ($p < 0.05$). Chi Chunhua et al. reported that the methionine contents in fermented soybean meal (FSBM) fermented with *Bacillus amyloliquefaciens* U304, *Lactobacillus acidophilus*, and *Lactobacillus plantarum* were 22.2%, 3.5%, and 7.4% higher than those in unfermented raw materials (soybean meal (SBM)), respectively. The methionine content in FSBM fermented with *Saccharomyces cerevisiae* CJ1697 decreased by 11.1%. The lysine content in FSBM fermented with *Bacillus amyloliquefaciens* U304 was the same as that in unfermented SBM, but the lysine content in the other three strains (*Lactobacillus amyloliquefaciens*, *Lactobacillus plantarum*, *Saccharomyces cerevisiae*) after fermentation was lower than that in unfermented raw materials [18]. However, in this study, the methionine content (0.48%) in fermented feed was 37% higher than that in unfermented raw material (0.35%) ($p < 0.05$), and the lysine content (0.98%) was 14% higher than that in unfermented raw material (0.86%) ($p < 0.05$), indicating significant differences between different strains, and essential amino acids increased significantly in mixed fermentation compared with single-strain fermentation. This may have been due to the synergistic effects of the three probiotics in the fermentation system.

Studies have shown that the intake of fermented feed probiotics can have beneficial effects on the intestinal tracts of animals, and *Saccharomyces cerevisiae* cells, cell fragments, and their metabolites can improve the immunity of animals [64]. Protease, amylase, and phytase produced by *Bacillus subtilis* and their enzymolysis products are beneficial to the digestion and absorption of animals, and the probiotics and metabolites provided by fermented feed can promote animal growth [21,65]. In addition, yeasts in animals' guts can bind enterobacteria to the surface of yeast cells, thus blocking the binding of these bacteria to the gut epithelium [66]. The high-throughput sequencing of 96 h fermented feed samples in this experiment showed that the relative abundance of the microflora *Saccharomyces_cerevisiae* accounted for 98.48%, and *Pediococcus_acidilactici* (82.95%) and *Bacillus_subtilis* (0.52%) were the dominant bacterial microflora. In conclusion, if the fermented feed in this study, which contained a variety of probiotics and their metabolites, were added to the base diet as an alternative to antibiotics, it would play a beneficial role in promoting animal growth.

5. Conclusions

In this study, the isolated and identified strain *Pediococcus_acidilactici* was used to ferment uncooked corn–soybean meal synergistically with *Bacillus subtilis* and *Saccharomyces cerevisiae* in a two-stage fermentation process. In the first stage, the fermentation parameters of *Pediococcus_acidilactici* were optimized: initial pH 4.4, water addition amount 100%, and inoculation size 6%. In the second stage, the parameters of the cooperative SSF of *Bacillus subtilis* and *Saccharomyces cerevisiae* were optimized: inoculation proportion (*Bacillus subtilis*: *Saccharomyces cerevisiae* (v/v)) = 3.8:6.2, fermentation temperature 33.5 °C, and inoculation size 6%. Compared with the unfermented raw material, the protein content of 96 h fermented feed increased by 5.21 percentage points ($p < 0.05$), and nine kinds of essential amino acids were increased to different degrees, respectively ($p < 0.05$). The results of high-throughput sequencing showed that, at the species level, *Pediococcus_acidilactici* and *Bacillus subtilis* were the main microflora in the fermented system in terms of bacteria, while *Saccharomyces cerevisiae* was dominant in terms of fungi. The diversity of probiotic bacteria increased in the fermentation product, and no pathogenic bacteria, such as *Salmonella*, were detected. Favorable microbial communities could be formed in the fermented system, which enhanced the probiotic characteristics of the feed. In sum, by screening *Pediococcus_acidilactici* and optimizing the multi-strain cooperative fermentation process, it was found that uncooked raw material fermentation could increase the nutritional value of feed, inhibit the contamination of pathogenic bacteria in the fermented system, save costs and time in the preparation of fermented feed, and improve the cost efficiency of fermented feed, providing an effective scheme to solve the cost and contamination problems in the

preparation of uncooked material fermented feed and promoting the practical application of fermented feed instead of antibiotics.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9040383/s1, Table S1: Amino acid composition of the fermented feed; Table S2: The relative abundance data for the top ten bacteria at the species level; Table S3: The relative abundance data for the top ten fungi at the species level; Table S4: The results of the Salmonella test; Figure S1. The standard curve for proteins obtained with the biuret method. Figure S2. Morphological picture of strain SLB-04. Figure S3. 16S rDNA phylogenetic tree con-structed on the base of maximum likelihood method.

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