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Protease-producing lactic acid bacteria with antibacterial properties and their potential use in soybean meal fermentation

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Abstract

Background: As an adequate plant protein raw material, soybean meal has attracted extensive attention because of its high protein content, abundant amino acids essential for animals, and many functional nutrients. Feed fermentation is a new way to improve nutrient absorption of animals by inoculating microorganisms, which can also play a probiotic role. In order to screen the lactic acid bacteria with remarkable protease-producing performance and excellent growth and fermentation characteristics, 1000 strains isolated from different materials were evaluated in this study. After primary and re-screening, 22 strains both with protein clear zone diameters larger than 15.00 mm (including the diameter of a hole puncher 10.00 mm) and protease activities greater than 20.00 U/mL were screened out for physiological and biochemical tests, as well as antimicrobial performance tests.

Results: Strains P (Pig) 15 and ZZUPF (Zhengzhou University Pig Fecal) 95, which have excellent physiological and biochemical characteristics, as well as good inhibition abilities for *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, and *Micrococcus luteus*, were selected and used as fermenting agents for 3- and 30-day (d) fermentation of soybean meal. The analysis of microorganisms, fermentation quality, and chemical composition during fermentation revealed that all lactic acid bacteria addition groups had lower harmful bacteria and pH value, as well as higher lactic acid bacteria content and lactic acid level compared with CK and protease-treated groups. These effects were even better especially after 30 days of fermentation.

Conclusions: This study indicated that the selected strains *Lactiplantibacillus plantarum* subsp. *plantarum* P15 and *Enterococcus faecalis* ZZUPF95 could be considered as potential probiotics for fermenting soybean meal for further research.

Keywords: Lactic acid bacteria, Soybean meal, Solid-state fermentation, Microbial content, Fermentation quality

Background

As an important plant protein resource in animal nutrition, soybean meal (SBM) has high protein level, relatively low price, and balanced amino acid profile [1]. However, the nutritional value of SBM is restricted by various macromolecular protein and anti-nutritional factors, which can disrupt or hinder the digestion and use of nutrients, and have a negative impact on the health and growth performance of animals [2,

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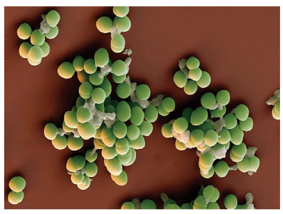
Graphical abstract

Protease-producing lactic acid bacteria with antibacterial properties and their potential use in soybean meal fermentation



Selective and identification of LAB strains producing protease

Solid-state fermentation of fermented soybean meal with selected strain

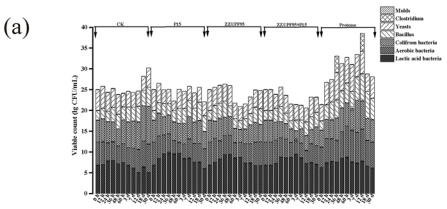


Fig. 1. Microbial content changes during the process of soybean meal fermentation.

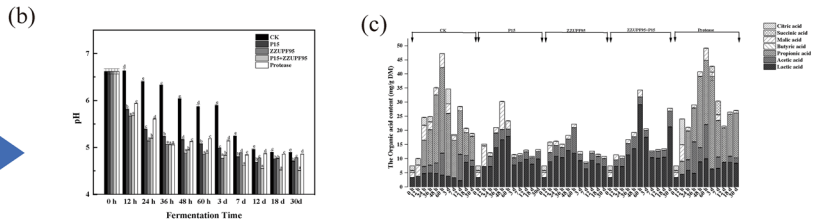


Fig. 2. Analysis of fermentation quality in various stages of fermented soybean meal (FM%). b, pH; c, organic acid content.

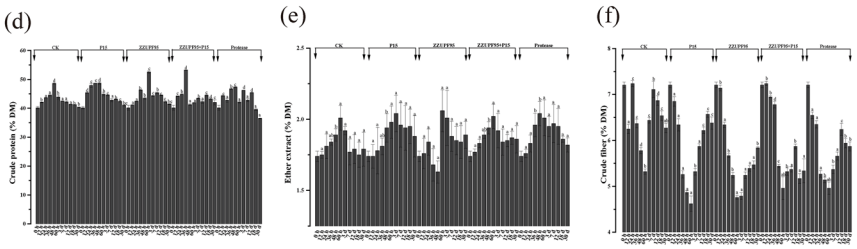


Fig. 3. Chemical composition of fermented soybean meal (DM%). d, CP; e, EE; f, CF.

After fermentation, the quality of soybean meal was improved significantly

Conclusions:

This study indicated that the selected strains *Lactiplantibacillus plantarum* subsp. *plantarum* P15 and *Enterococcus faecalis* ZZUPF95 could be considered as potential probiotics for fermenting soybean meal for further research.

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3]. To solve this problem, various methods of processing such as heating, soaking, enzymatic hydrolysis, and microbial solid-state fermentation are used for degrading macromolecular proteins and eliminating anti-nutritional factors [4]. Among these, heating can only make part of the heat-unstable antigenic protein decompose, and it may destroy the amino acids, vitamins, and other nutrients in the feed, this reducing the nutritional value of SBM and protein digestibility. Chemical treatment is prone to reagent residues [5]. Compared with physical and chemical methods, the application of biotechnology, including enzyme and fermentation, can eliminate or suppress anti-nutritional factors in SBM, as well as reduce damage and impact on the nutrient content of the feed. Moreover, it introduces no residues of harmful substances [6]. But purified enzyme has high cost and there are many different manufacturers and types of enzyme preparations on the market, with different standards and a wide range of products [7]. Fermentation, which can metabolize high-molecular-weight proteins into smaller, biologically active peptides or amino acids

through the production of protease, is beneficial to animal digestion and absorption, improves the nutrient conversion rate of feed, alleviates the impact of large-molecule proteins on the structure of animal small intestine villi, promotes the proliferation of beneficial intestinal bacteria, and inhibits the growth of harmful bacteria. It improves animal growth performance and the rearing environment by reducing fecal odors [8, 9]. But the fermentation process alone is time-consuming, lossy, and inefficient, and the proliferation of miscellaneous bacteria is difficult to control [10]. Therefore, it is essential to add probiotics to aid fermentation.

Lactic acid bacteria (LAB), as one of the most frequently used probiotics, can secrete diverse active metabolites [11]. Thus, they are often used in the production of fermented soybean-based food. It has been reported that LAB are associated with the biodegradation of fatty substances for improving protein digestibility and maintaining the balance of intestinal flora [8]. These properties may be attributed to enzymes produced by LAB, such as lipase, protease, and antimicrobial proteins [12]. The fermentation of SBM by LAB not only degrades anti-nutritional factors, but also oligosaccharides such as raffinose and stachyose in SBM through the production of α-galactosidase [13], and could produce lactic acid to increase organic acid levels, lower pH, and improve feed flavor and palatability [14]. Moreover, it converts glycosidic soy isoflavones into free forms, blocks the free-radical reaction chain, reduces the formation of free radicals, improves the antioxidant capacity of the animal body, and protects the body from free radicals [15]. LAB with high intestinal adhesion can also produce antibacterial proteins and block the attachment of harmful microorganisms in the intestine [16, 17], and it plays an active role in immunoregulation and maintenance of intestinal flora balance [18, 19]. All of the above research indicate that LAB could be used as inoculants of solid-state fermentation by specific enzyme-producing and bacteriostatic properties, *Lactiplantibacillus* (*L.*) *plantarum*, *L. casei*, *L. paracasei*, *L. acidophilus*, *L. salivarius*, *Enterococcus* (*E.*) *faecium*, and *Pediococcus pentosaceus* have been used in SBM fermentation.

At present, the fermentation time for SBM is generally 3 days (d), and it still has problems such as high pH value and insufficient antibacterial ability. Furthermore, SBM fermentation often focuses on degradation of protein and the elimination of anti-nutritional factors, but little information is related to the effects of LAB on the microbiology, fermentation characteristics, and chemical composition during the fermentation process. In response to these issues, this study aimed to select LAB strains on the basis of capacity to biosynthesize proteases first. Physiological and biochemical tests and identification of these LAB were carried out. Strains that performed well in the probiotic test were then applied to SBM fermentation, and the microbiology community, fermentation quality, and chemical components of fermented soybean meal (FSBM) were evaluated.

Materials and methods

LAB strains and indicator bacteria

One thousand LAB strains isolated from piglet intestine, corn and wheat stovers, dairy products, feed grass silage, total mixed ration (TMR) and stored in the lab were used in the study. All LAB isolates were recovered by incubation in de Man Rogosa Sharpe (MRS) broth at 30 ℃ for 48 h. *Staphylococcus aureus* ATCC 6538^T, *Bacillus subtilis* ATCC 19217^T, *Escherichia coli* ATCC 11775^T, *Listeria monocytogenes* ATCC 51719^T, *Pseudomonas aeruginosa* ATCC 15692^T and *Micrococcus luteus* ATCC 4698^T were used as indicator bacteria and incubated in Luria Bertani (LB) medium at 37 ℃ for 24 h.

Screening of LAB strains producing protease

Preliminary screening

Agar well diffusion technique was used as preliminary screen of LAB strains producing protease [20]. LAB suspensions were prepared from colonies grown on MRS broth overnight at 30 °C. Firstly, 25 mL skim milk agar medium was incubated into each sterilized petri plate and left it to solidify at room temperature, after that, wells of 10.00 mm diameter were completed using a sterile cork borer, and then, 200 µL of each LAB suspension was placed in well one by one and plates were incubated for 72 h at 30 °C. The diameter of the transparent zone was measured after incubation to determine the protease production capacity. Comparison of the transparent zone among different LAB strains and strains had relatively larger zone would be selected for further research.

Re-screening

Re-screening was determined by protease activity metabolized from LAB and measured by the Folin–phenol method [21]. In the CK (blank control) tube, a testing solution (1.0 mL) was mixed with trichloroacetic acid solution (TCA) reagent (2.0 mL), while the testing solution (1.0 mL) was mixed with casein reagent (1.0 mL) and both were held at 40 °C for 10 min. After then, TCA reagent (2.0 mL) and casein reagent

(1.0 mL) were added into the control tube and sample tube, respectively, and 1.0 mL supernatant was taken after centrifugation. Then, Na_2CO_3 solution and Folin–phenol reagent were added and held at 40 °C for 20 min, and absorbance was measured at a wavelength of 680 nm. By combining the results of initial screening and re-screening, LAB with greater protein transparent circle diameter and protease activity would be selected for the next experiment.

Selection and identification of LAB strains producing protease

Environmental stress tolerance assay

The effect of temperature, pH, and osmotic pressure on selected LAB after re-screening was examined. To study the effect of temperature on LAB, they were cultured in MRS at 30 °C for 24 h, and 100 µL of each bacterial suspension was added into 10 mL of MRS broth and incubated in different temperatures (4, 10, 45 and 50 °C). The growth was evaluated by measuring OD_{600} after 24 h of incubation without shaking.

In order to examine the tolerance of strains to different pHs, 100 µL bacterial suspensions prepared as above was added into 10 mL of MRS broth and adjusted using NaOH (0.5 M) or HCl (0.5 M) to different pH values (3.0, 3.5, 4.0, 4.5, 8.0, 9.0 and 10.0), and incubated at 37 °C for 24 h. Then the absorbance of each solution was measured at 600 nm by the spectrophotometer [22].

To examine the resistance of LAB to osmotic stress, strains were resuspended in MRS broths, modified with the addition of NaCl (3.0 and 6.5%). On the other hand, bacterial solutions were added into the MRS broth with 0.2% bile salts and incubated at 37 °C for 4 h for the determination of bile salt tolerance, and MRS broth without bile salt was used as control [23]. Whether the LAB grows or not was determined according to the plate counts on MRS agar after 24 h incubation by referring to Wang et al. [24].

Identification by 16S rRNA gene sequencing and recA gene amplification

Molecular identification of LAB was carried out by means of 16S rRNA gene sequencing, and PCR amplification was performed following Pang et al. [25] and sequenced by sequencing service (MGI Tech Co., Ltd, Beijing, China). The 16S rRNA genes of selected strains were amplified by PCR using the 27 F (5′-AGAGTTT GATCCTGGCTCAG-3′) and 1492 R (5′-GGTTACCTT GTTACGACTT-3′) universal primer sets. The resulting sequences were compared by Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih. gov/) database.

L. plantarum cluster, including *L. casei*, *L. paraplantarum*, *L. pentosus*, *L. plantarum* subsp. *plantarum* and *L. plantarum* subsp. *argentoratensis*, was distinguished by *rec*A gene amplification according to the procedure by Pang et al. [26].

Simulated gastrointestinal test

The survival of LAB in simulated gastrointestinal conditions was determined according to the method as previously described by Wang et al. [27] with modifications. Briefly, for the simulated gastric fluid (SGF), 3.5 g/L pepsin was suspended in 0.2% (w/v) sterile NaCl solution and adjusted pH to 2.0, made the total volume of the solution up to 100 mL and filtered through a 0.22-µm filter membrane. For the simulated intestinal fluid (SIF), 1 g/L trypsin, 18 g/L bile salt from ox, and 11 g/L $NaHCO_3$ were suspended in 0.2% (w/v) sterile NaCl solution, adjusted the pH of the solution to 6.8, and filtered through a 0.22 µm filter membrane. LAB suspension was added to 20 mL SGF and incubated for 3 h, as for SIF was 4 h. After 0, 3 and 7 h of culture, the number of viable colonies was determined by MRS agar plate counting method.

Antimicrobial activity

The inhibitory effect of LAB against pathogenic microorganisms was determined by agar well diffusion method [20]. Indicator strains *Staphylococcus aureus* ATCC 6538^T, *Bacillus subtilis* ATCC 19217^T, *Escherichia coli* ATCC 11775^T, *Listeria monocytogenes* ATCC 51719^T, *Pseudomonas aeruginosa* ATCC 15692^T, and *Micrococcus luteus* ATCC 4698^T were grown in LB broth at 37 °C in shaker for 18 h and LAB were cultured in MRS broth at 30 °C for 24 h. The activated LAB suspension was centrifuged at 6000 rpm for 5 min to obtain the supernatant, and the pH was adjusted to 7.0. The indicator strain was dispersed on the surface of LB agar plate, wells of diameter 10.00 mm were bored and 200 µL supernatant was loaded into each well. The zones of inhibition were measured after incubation at 37 °C for 24 h.

Carbohydrate utilization patterns

Thirty-four common carbon sources including glycerol, d-arabinose, l-arabinose, ribose, d-xylose, adonitol, β-methyl-xyloside, galactose, l-glucose, d-fructose, l-mannose, l-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, salicin, cellobiose, maltose, lactose, melibiose, saccharose, trehalose, melezitose, d-raffinose,

starch, glycogen, xylitol, β-gentiobiose, d-turanose, d-lyxose, d-tagatose and d-fucose were used to detect carbohydrate utilization patterns of LAB strains producing protease by replacing the carbon source in turn in the MRS liquid medium. According to instruction of Bergey's manual of systematic bacteriology, glucose in MRS medium was replaced in equal quantities by 34 carbon sources, and whether the strain could use the carbon source was determined by detecting the colony growth on MRS after 48 h of incubation.

Growth curve measurement

The selected LAB strains were inoculated into 50 mL of MRS liquid medium with 2% inoculum, and cultured anaerobically at 37 °C for 24 h. The absorbance of the strain at 600 nm was measured every 2 h and the growth curve was drawn.

Solid-state fermentation and analysis of fermented soybean meal

Solid-state fermentation

Commercially SBM (Inner Mongolia Xing'an Feed Co. Ltd., China) was used as fermentation material, and two LAB strains with the best protease-producing ability and excellent characteristics were added as inoculant. There were a total of 5 groups as following: CK, SBM (solid-to-liquid (non-sterile water) ratio was 1:1, (v/v), all groups same); A group, SBM plus strain A ($OD_{600}=0.8$, next same); B group, SBM plus strain B; A + B group, SBM plus strains A + B and acid protease group, SBM plus acid protease (Beijing Solaibao Technology Co., Ltd., China). LAB and acid protease inoculations were both added at 2%, and 100 g mixture were added to sealing bags and vacuumized after mixing, respectively. Each treatment had 3 replicates and all bags were fermented at room temperature.

Microbiological analysis

On the 0, 12, 24, 36, 48, 60, 72 h and 7, 12, 18 and 30 d of fermentation, 10 g FSBM was added to 90 mL sterilized distilled water, and the mixture was serially diluted to 10^{-1}, 10^{-3} and 10^{-5} in sterilized water, respectively. Yeast and mold colonies were inoculated on potato dextrose agar (PDA) at 30 ℃ for 48 h, LAB colonies on MRS medium at 37 ℃ for 48 h, coliform bacteria, *Clostridium*, bacilli and aerobic bacteria were incubated on eosin methylene blue (EMB), *Clostridium* enrichment medium (CLO) agar and nutrient agar (NA) at 37 ℃ for 48 h, respectively. Colonies were counted to determine the numbers of viable microorganisms, and values are represented as colony-forming units (CFU)/g fresh matter (FM) [28].

Determination pH and organic acids

The pH of FSBM was measured by glass electrode pH meter (Mettler-Toledo Instruments (Shanghai) Co. Ltd., China), and organic acids were determined by HPLC (Waters 2695 HPLC system, Waters Technology (Shanghai) Co., Ltd. China) using a Symmetry C18 column (4.6 × 250 mm, 5 μm). The mobile phase was 25.4% vitriol with a flow rate of 0.6 mL/min, and the column temperature was 55 °C, and absorbance of the elution was monitored at 214 nm.

Measurement of chemical composition

Crude protein (CP), ether extract (EE), crude fiber (CF) were determined using the AOAC standard method [29]. The samples were dried in a forced-air oven at 65 ℃ for 48 h to constant weight, and the automatic Kjeldahl nitrogen, ether extract and crude fiber analyzer were used for determination, respectively.

Statistical analysis

In order to follow the principles of scientific experimentation, three repeated treatment groups were set up for each experiment to reduce the error caused by the randomness of the experiment. The results were processed for analysis of variance by SPSS software (version 21.0). The significant difference was determined at the $p<0.05$ using Duncan's multiple range tests.

Results

Screening of LAB strains producing protease

The primary screening of 1000 LAB strains from different sources by the disc diffusion method yielded 60 strains with protein clear zone diameter larger than 15.00 mm (the diameter of hydrolysis zone including that of the diameter well was 10.00 mm), as well as 33 strains from which those with protease activity greater than 15.00 U/mL were selected by re-screening with the Folin method. Table 1 presents 22 strains both with protein clear zone diameters larger than 15.00 mm and protease activities greater than 15.00 U/mL selected for further research. Of these, P (Pig) 15, P24, ZZUPF (Zhengzhou University Pig Fecal) 94, and ZZUPF95 produced protein clear zone diameters larger than 20.00 mm and protease activities greater than 20.00 U/mL.

Table 1 Primary and secondary screening of protease-producing lactic acid bacteria

Separation source	Strains number	Initial screening diameter (mm) ± SD	Re-screening protease activity (U/ml) ± SD
Feces of healthy weaned piglets	P3	16.93 ± 0.23	16.24 ± 0.22
	P8	20.03 ± 0.16	15.23 ± 0.15
	P12	16.40 ± 0.25	16.20 ± 0.32
	P13	15.60 ± 0.15	15.90 ± 0.18
	P14	17.86 ± 0.13	16.26 ± 0.32
	P15	24.10 ± 0.24	24.30 ± 0.14
	P22	15.40 ± 0.22	16.10 ± 0.24
	P24	23.60 ± 0.32	25.60 ± 0.22
Alfalfa silage	ZZUPF92	18.40 ± 0.18	17.60 ± 0.24
	ZZUPF94	22.10 ± 0.32	21.20 ± 0.16
	ZZUPF95	24.60 ± 0.17	27.40 ± .0.31
	ZZUPF145	21.30 ± 0.25	19.80 ± 0.25
Corn silage	LAO49	19.20 ± 0.35	19.15 ± 0.24
	LAO53	22.02 ± 0.18	19.40 ± 0.31
	LAO56	16.39 ± 0.21	21.30 ± 0.25
	LAO60	16.10 ± 0.20	16.20 ± 0.24
	LA066	16.30 ± 0.26	15.60 ± 0.18
Wheat silage	MB56	18.03 ± 0.16	19.32 ± 0.11
	MB66	18.13 ± 0.35	15.68 ± 0.09
	MB72	21.63 ± 0.18	17.52 ± 0.12
	MB73	18.36 ± 0.32	16.85 ± 0.42
	MB76	16.05 ± 0.19	16.21 ± 0.15

Results were expressed as mean ($n = 3$). All data in the table are averaged

The diameter of inhibition zone including that of hole puncher (10.00 mm)

Selection and identification of LAB strains producing protease

Physiological and biochemical test

As shown in Table 2, strains P15, P24, ZZUPF94, ZZUPF95, LAO (Laos) 49, LAO53, LAO56, and MB (Mongolia bread) 66 could grow within all temperature gradients (from 4 to 50 °C). As for NaCl tolerance, P13, P15, P24, ZZUPF94, ZZUPF95, LAO49, LAO53, LAO56, LAO60, and MB66 could grow at 3.0% and 6.5% NaCl concentrations. For tolerance to acid and alkali, these ten strains could grow from pH 3.0 to 10.0. To sum up, P15, P24, ZZUPF94, ZZUPF95, LAO49, LAO53, LAO56, and MB66 could be carried out in the subsequent experiments.

The viable count of the 8 selected LAB strains, P15, P24, ZZUPF94, ZZUPF95, LAO49, LAO53, LAO56,and MB66, in 0.2% bile salt within 4 h is shown in Fig. 1. It can be seen that in the whole test period, viable count of P15, P24, ZZUPF94, and ZZUPF95 were higher than 5.00 lg CFU/mL compared with the CK, while those of MB66 were lower than 4.00 lg CFU/mL; LAO49, LAO53, and LAO56 did not grow well enough at certain times. Combining the analysis of 4 h survival results, P15, P24, ZZUPF94, and ZZUPF95 were identified as the next experimental strains for the gastrointestinal (GI) simulation experiment.

16S DNA gene sequence analysis and recA gene multiple detection

Comprehensive physiological and biochemical results and strains P15, P24, LAO49, LAO53, LAO56, ZZUPF94, ZZUPF95, and MB66 were chosen for 16S DNA gene sequences analysis. Phylogenetic trees constructed from these eight strains based on the evolutionary distance determined by the neighbor-joining method are shown in Figs. 2 and 4.

As shown in Fig. 2, LAO49, LAO53, and LAO56 in Fig. 2 were also placed in the cluster of the genus *Lactobacillus*, and they could be identified as *L. reuteri*, *L. fermentum*, and *L. amylovorus*, which are all supported by 100% bootstrap values. Strains P15, P24, and ZZUPF94 placed in the *L. plantarum* cluster including *L. casei*, *L. paraplantarum*, *L. pentosus*, *L. plantarum* subsp. *plantarum*, and *L. plantarum* subsp. *argentoratensis* could not be distinguished by 16S DNA sequencing. Therefore,

Table 2 Physiological and biochemical analysis of protease-producing lactic acid bacteria strains

Isolates	Growth at temperature (°C)				Growth in NaCl (w/v, %)		Growth at pH						
	4.0	10.0	45.0	50.0	3.0	6.5	3.0	3.5	4.0	4.5	8.0	9.0	10.0
P3	−	+	w	−	−	−	w	−	+	+	+	w	−
P8	−	+	w	w	+	−	−	−	+	++	+	w	−
P12	−	+	+	−	+	w	w	+	+	+	+	+	−
P13	−	+	+	+	+	+	+	+	+	++	+	+	+
P14	−	+	+	w	+	w	−	+	+	++	+	w	−
P15	+	++	+	+	+	+	+	+	+	++	+	+	+
P20	−	+	−	−	−	−	−	−	+	+	+	+	w
P22	−	+	−	−	+	−	−	−	+	+	+	+	+
P24	+	+	+	+	+	+	+	+	+	++	++	+	+
ZZUPF92	−	++	+	−	w	+	+	+	+	+	+	+	+
ZZUPF94	+	++	+	+	+	+	+	+	+	++	+	+	+
ZZUPF95	+	++	+	+	+	+	+	+	+	++	+	+	+
ZZUPF145	−	+	+	w	w	−	w	w	+	+	+	+	+
LAO49	+	+	+	+	+	+	+	+	+	+	+	+	+
LAO53	+	+	++	+	+	+	+	+	+	++	+	+	+
LAO56	+	+	+	+	+	+	+	+	+	+	+	+	+
LAO60	−	+	+	+	+	+	−	w	+	+	+	+	w
LAO66	−	+	+	w	+	−	w	+	+	+	+	−	−
MB56	−	+	+	+	+	−	−	+	+	+	+	+	−
MB66	+	+	+	+	+	+	+	+	+	+	+	+	+
MB72	−	+	+	+	+	w	+	+	+	+	+	−	−
MB73	−	+	+	w	+	w	−	+	+	+	+	−	−
MB76	+	+	+	w	+	w	−	+	+	+	+	+	−

All representative strains were positive for Gram stain and negative for catalase reaction

For fermentation type, all representative strains are homofermentative except LAO56, which was heterofermentative

++grow well; + could grow; − could not grow; *w* could weakly grow

the *rec*A gene PCR amplification experiment was carried out to distinguish the species of these three strains.

From the results of *rec*A gene multiple analysis in Fig. 3, five type strains of *L. plantarum* cluster *L. casei* JCM 16167^T, *L. paraplantarum* JCM 12533^T, *L. pentosus* JCM 1558^T, *L. plantarum* subsp. *plantarum* JCM 1149^T, and *L. plantarum* subsp. *argentoratensis* JCM 16169^T were placed in lanes 1, 2, 3, 4, and 5, respectively, while strains P15, P24, and ZZUPF94 to be identified were placed in lanes 6, 7, and 8, respectively. From the strips, lanes 6, 7, and 8 produced 318 bp amplification products that are the same as lane 4 (*L. plantarum* subsp. *plantarum*) while other type strains did not.

In Fig. 4, strains ZZUPF95 and MB66 were placed in the *Enterococcus* and *Weissella* (*W.*) clusters, with the species *E. faecalis* NBRC 100480^T and *W. cibaria* LMG 17699^T being the most closely related species, respectively. Combined with 16S DNA sequence similarities, these two strains were identified as *E. faecalis* and *W. cibaria*.

16S DNA sequence GenBank login numbers of P15, P24, LAO49, LAO53, LAO56, ZZUPF94, ZZUPF95, and MB66 were MT635042, MW020292, MW020294, MW020295, MW020295, MW020295, MW020290, and MW020291, respectively.

Simulated GI experiment

The growth situations of the selected protease-producing LAB strains P15, P24, ZZUPF94, and ZZUPF95 in the SGF within 3 h are presented in Fig. 5. After 3 h growth in the artificial gastric juice, there was no significant difference ($p > 0.05$) in viable count among P15 (7.81 lg CFU/mL), P24 (7.56), ZZUPF94 (7.80), and ZZUPF95 (7.81). On the contrary, after 3 h culturing in SIF, the viable count of P15, P24, ZZUPF94, and ZZUPF95 were different, 6.24, 5.72, 5.51, and 6.40 lg CFU/mL, respectively.

Antibacterial activity

As shown in Table 3, P15, and ZZUPF95 had different degrees of inhibition of six indicator bacteria. Among

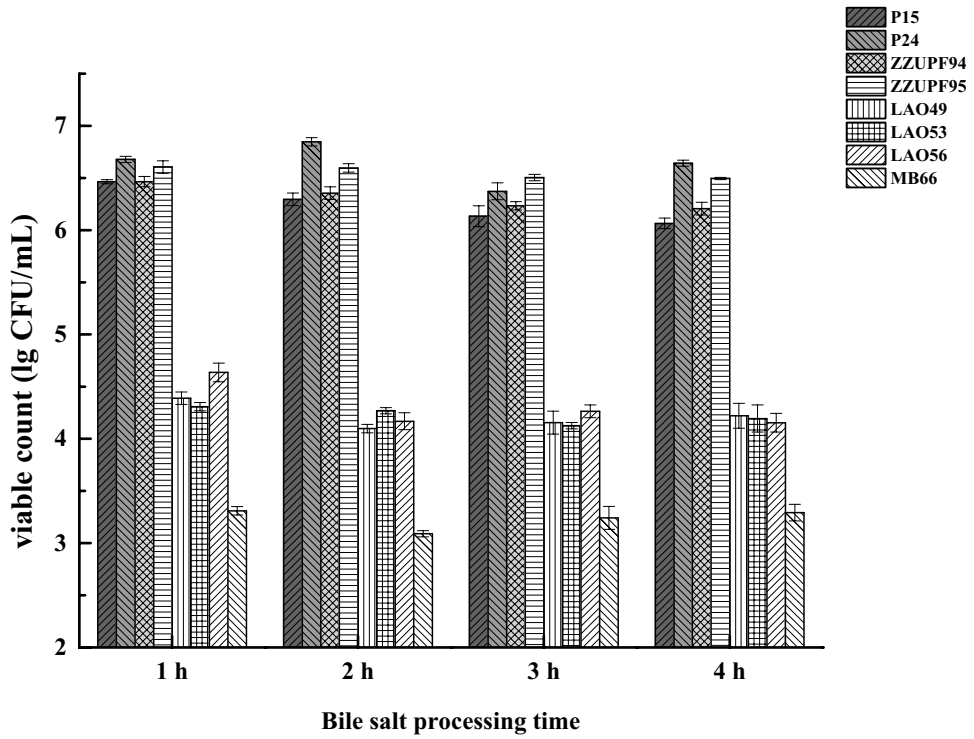

Fig. 1 Bile salt tolerance of representative protease-producing LAB

them, the neutralizing supernatant of P15 and ZZUPF95 showed significant inhibitory effect on *Escherichia coli* ATCC 11775^{T}, and the inhibition zone diameters were 14.00 and 16.00 mm, respectively. The inhibitory activities of P24 on *Pseudomonas aeruginosa* ATCC 15692^{T}, *Listeria monocytogenes* ATCC 51719 ^{T}, and *Salmonella enterica* ATCC 43971^{T} manifested as diameters of the inhibition zone were 11.00, 12.00, and 11.00 mm, respectively. ZZUPF95 also had an inhibitory effect on *Bacillus subtilis* ATCC 19217^{T} and *Salmonella enterica* ATCC 43971^{T}, with 15.00 and 16.00 mm inhibition zone diameters, respectively. Thus, P15 and ZZUPF95 were used for further testing.

Carbohydrate utilization patterns

Carbohydrate utilization patterns of P15 and ZUPF95 are shown in Table 4. The results indicated that P15 could use galactose, d-glucose, d-fructose, d-mannose, l-sorbose, rhamnose, mannitol, sorbitol, maltose, lactose, melibiose, saccharose, and trehalose as carbon sources, and that ribose and β-gentiobiose could be weakly used. Other than that, ribose, d-xylose, adonitol, d-glucose, d-mannose, l-sorbose, rhamnose, mannitol, sorbitol, cellobiose, maltose, melibiose, saccharose, trehalose, glycogen, and β-gentiobiose could be used by ZZUPF95.

Growth curve

Bacterial growth and its dynamics can be studied by plotting the cell growth (absorbance) versus the incubation time (Fig. 6). The lag phases of P15 and ZZUPF95 were in the period of 0 to 4 h. After that, both P15 and ZZUPF95 started the logarithmic phase at 5 h, while P15 ended at 9 h and ZZUPF95 at 12 h. Two LABs then entered the steady phase and both ended at 24 h.

In summary, P15 and ZZUPF95 were selected for SBM fermentation. P15 was assigned to group A, ZZUPF95 to group B, and P15 + ZZUPF95 to group A + B.

Microbial content, fermentation quality, and chemical composition of fermented soybean meal

Microbial content analysis

Analyses of various microorganisms in FSBM samples are demonstrated in Fig. 7. Within 30 d fermentation, there was no mold detected in any of the groups, and *Clostridium* was only detected in the protease group, with 4.01 lg CFU/mL at 36 h and 4.30 lg CFU/mL at 12 d. For LAB,the P15 group increased from 24 to 60 h, and the ZZUPF95 group from 36 to 48 h were all up to 9.00 lg CFU/mL. After fermentation for 60 h, the colony number of LAB in the P15 + ZZUPF95 group reached 9.00 lg CFU/mL, followed by the CK and protease groups

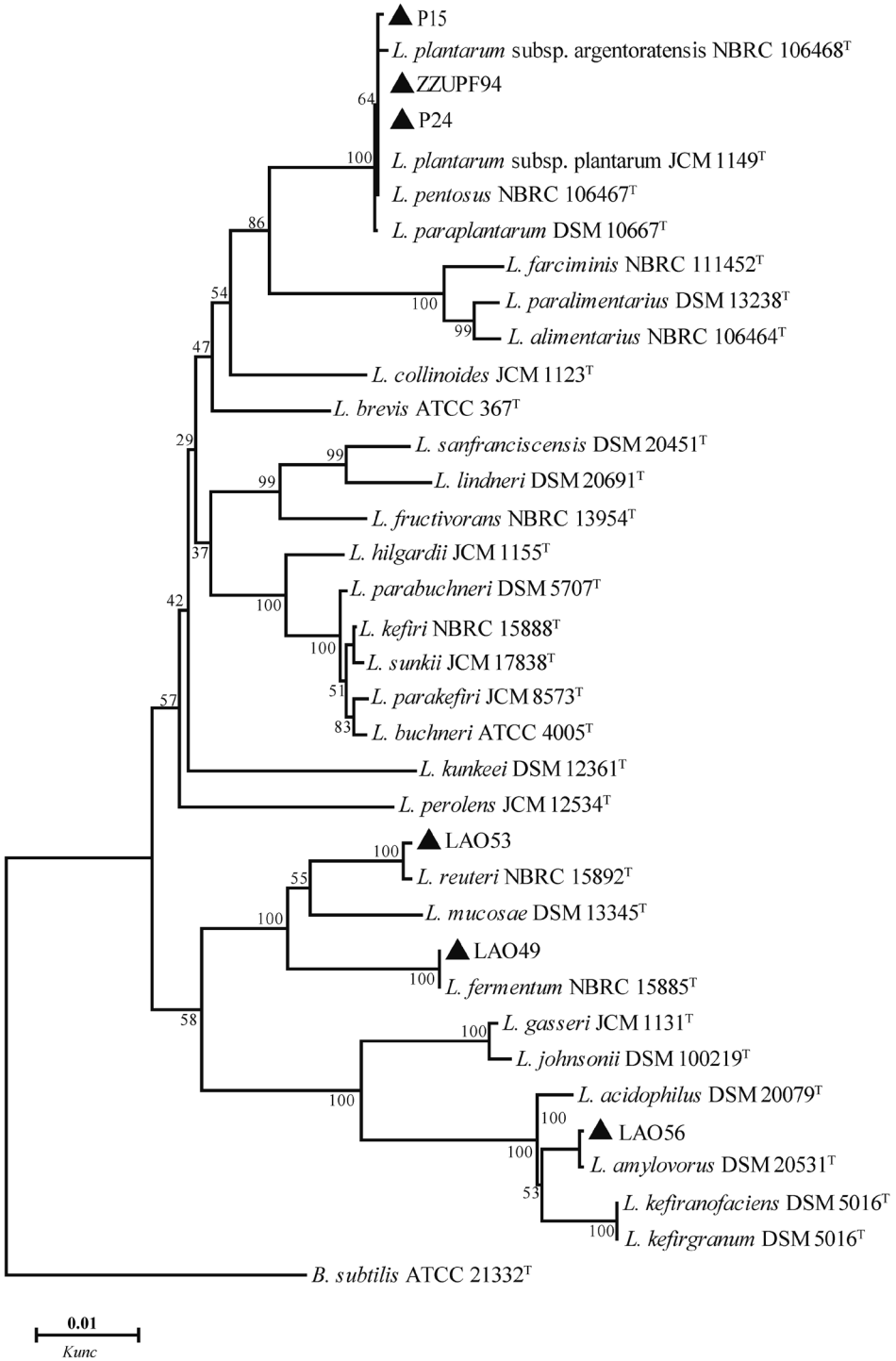

Fig. 2 Phylogenetic tree of selected protease-producing rod strains. Bootstrap values for 1000 replicates are shown at the nodes of the tree. *B. subtilis* is used as an outgroup. The bar indicates 1% sequence divergence. *L. Lactobacillus*, *B. Bacillus*. *Knuc* nucleotide substitution rate. Evolutionary distance usually marked on the branch presenting the degree of clade variation, the shorter the clade, the smaller the difference and the closer the evolutionary distance

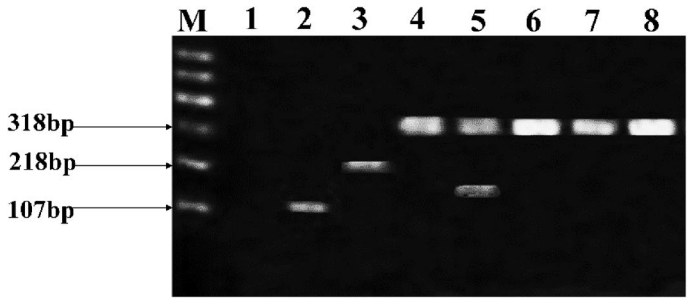

Fig. 3 Analysis of recA gene amplification products. Lane M contained a 2000 bp PLUS DNA ladder. Lanes 1, 2, 3, 4 and 5, PCR amplification products from *L. casei* JCM 16167^T (negative control), *L. paraplantarum* JCM 12533^T, *L. pentosus* JCM 1558^T, *L. plantarum* subsp. *plantarum* JCM 1149^T and *L. plantarum* subsp. *argentoratensis* JCM 16169^T, respectively; lanes 6, 7 and 8, PCR amplification product from P15, P24 and ZZUPF94

at only 7.00 lg CFU/mL. As for aerobic bacteria, the P15, ZZUPF95, and P15 + ZZUPF95 groups decreased to 3.00 lg CFU/mL during 48 h to 3 d, 60 h to 3 d, and 48 h to 7 d, respectively. On the contrary, the CK and protease groups were above 4.00 lg CFU/mL throughout the whole fermentation period. The protease group, in particular, reached 6.00 lg CFU/mL at 48 h and 7.00 lg CFU/mL from 60 h to 12 d. The same situation appeared in the protease group for coliform bacteria, which achieved to 7.00 lg CFU/mL at 3 d, while the highest were 5.00 lg CFU/mL in the other four groups. Moreover, three LAB-treated groups decreased to 3.00 lg CFU/mL. Regarding *Bacillus*, amounts reached 10^4 from 24 h and continued until the end of fermentation. Yeasts in the protease group reached 6.00 lg CFU/mL during fermentation from 36 h to 3 d. After 30 days of fermentation, the count of viable LAB in each group decreased to varying degrees, including 5.00 lg CFU/mL in the CK group, 6.00 lg CFU/mL in the LAB addition groups, and the highest in the ZZUPF95 group, which was 6.15 lg CFU/mL. In addition, the LAB addition groups inhibited other pathogenic bacteria, especially coliform bacteria, after 30 days of fermentation; compared with the CK and protease groups, the viable number of coliform bacteria decreased to 3.00 lg CFU/mL.

Measurement of pH and organic acids

Changes in pH and organic acid content in FSBM during the 30 d fermentation period are presented in Fig. 8. For pH, ZZUPF95 (4.88) and P15 + ZZUPF95 (4.94) decreased to below 5.00 for only 48 h, the P15 group took 3 d (4.98), while the protease group took 7 d (4.84), and the CK group took 12 d (4.96). At the end of the fermentation, both the ZZUPF95 and P15 groups reached 4.70; P15 + ZZUPF95 even reached 4.51, significantly lower than the other groups ($p < 0.05$).

In the results for organic acid determination, butyric acid was only detected in the protease at 18 d. The lactic acid content showed a considerable increase, and each group reached its peak at 48 h (CK and P15 groups) or 60 h (ZZUPF95, P15 + ZZUPF95, and protease groups) fermentation. Acetic acid only in the protease group exceeded 1.0. Propionic acid in the CK and protease groups were detected and both reached the highest value at 3 d. After 30 days of fermentation, the lactic acid content of the P15 + ZZUPF95 group was higher than that in other groups, reaching 21.25 mg/g, while the propionic acid content in the protease and CK groups was still at a high level, with the protease group reaching 15.88 mg/g.

Chemical composition

The effects of fermentation on the nutritional changes in CP, EE, CF, and matrix water content are in Fig. 9. For the CP content, P15 and protease reached 48.72% and 47.34% from 0 to 48 h. ZZUPF95 achieved its peak value at 60 h as 49.56% after 3 d of fermentation, and P15 + ZZUPF95 reached the ceiling value. Meanwhile, the EE content of FSBM in the P15, ZZUPF95, P15 + ZZUPF95, and protease groups increased to 2.04%, 2.06%, 2.02%, and 2.04%, respectively. The CF content showed a decrease, which decreased to 4.62%, 4.76%, 4.96%, and 4.96%, respectively. After 30 d of fermentation, the protease group had the most significant reduction in CP ($p < 0.05$). There was no significant difference in EE among all groups ($p > 0.05$); the P15 + ZZUPF95 group had the most significant degradation in CF ($p < 0.05$).

Discussion

SBM is a by-product of soybean after oil extraction with CP content 40–50%. It is rich in amino acids, especially lysine, which is easily deficient in other plant feeds, and so it is suitable as an ideal plant protein feed. However, the presence of macromolecular proteins and a variety of anti-nutritional factors in SBM can directly affect the digestion and absorption of protein, as well as reduce the

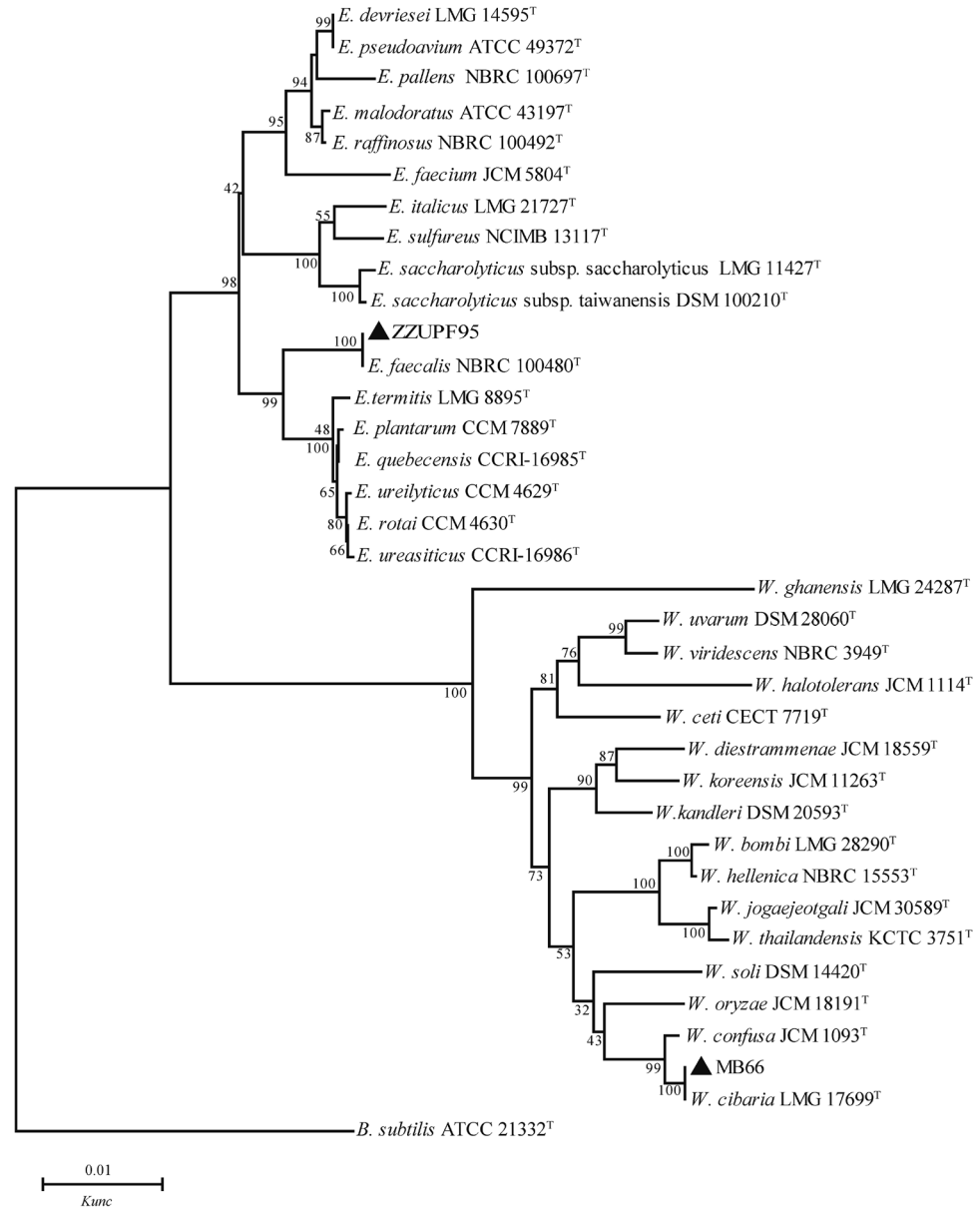

Fig. 4 Phylogenetic tree of selected protease-producing cocci strains. Phylogenetic tree showing the relative positions of isolates ZZUPF95 and MB66 as referred by the neighbor-joining method of complete 16S DNA sequences. Bootstrap values for 1000 replicates are shown at the nodes of the tree. *B. subtilis* is used as an outgroup. The bar indicates 1% sequence divergence. *E. Enterococcus*, *W. Weissella*, *B. Bacillus*, *Knuc* nucleotide substitution rate

growth performance and value of animals [30, 31]. Studies have shown that in addition to using oligosaccharides to produce lactic acid to lower the pH and inhibit the growth of harmful bacteria of fermented feed [14, 32], there is growing evidence that LAB have the property of degrading protein during the fermentation process [28, 33]. On the basis of these studies, 22 strains both with protein clear zone diameters larger than 15.00 mm and protease activities greater than 15.00 U/mL were screened from 1000 strains by the Oxford cup method of primary screening and Folin reagent method of re-screening in this study.

Whether probiotics can perform their function initially depends on their capacity to survive in the natural host defense and to reproduce in the gastrointestinal tract, since the pH of the gastric juice is normally around 3.0, that is to say, acid tolerance is a prerequisite for probiotics to be able to function in the gut [34]. Therefore,

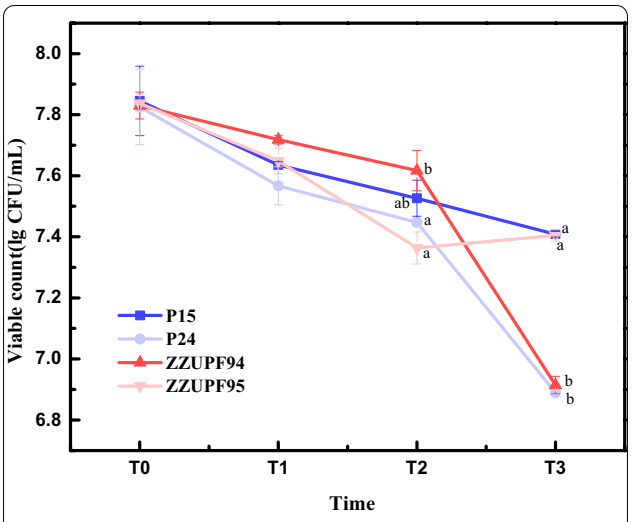

Fig. 5 Survival of representative protease-producing LAB strains in simulated gastrointestinal fluids. Gastric juice *T0* viability at the beginning of gastric juice, *T1* viability after simulation of gastric conditions; intestinal juice *T2* viability at the beginning of gastric juice, *T3* viability after simulation of enteric conditions

eight isolates grew at pH 3.0–10.0. They were initially shown to be possibly tolerant of gastric acid and pass through the stomach into the intestinal tract and were selected from 22 strains. They also grew well at 4–50 °C since the activity of probiotics added to feed is easily affected by temperature in the process of feed preparation [35]. Moreover, they could survive in NaCl solution with concentrations of 3.0% and 6.5%. The above indicates that these eight strains hold better physiological and biochemical characteristics. Sensitivity to bile is an important indicator for screening probiotics because tolerance to bile salts is a prerequisite for bacterial colonization and metabolism in the host gut. Among the eight selected LAB strains in this study, the growth of P15, P24, ZZUPF94, and ZZUPF95 in bile salt solution with a concentration of 0.2% was not inhibited.

Strain identification is the prerequisite for understanding the habits, metabolism, and pathogenicity of bacteria and hence for effective application [27]. On the basis of evolutionary distances of their 16S rDNA sequences by the neighbor-joining method, eight selected strains were placed in the cluster composed of the genera *Lactobacillus*, *Weissella*, and *Enterococcus*. Since *L. pentosus* and *L. plantarum* species differed only by 2 bp and could not be identified at the species level on the basis of 16S rRNA gene sequence [24], comprehensive unique *rec*A gene multiple analysis, P15, P24, and ZZUPF94 were assigned to *L. plantarum* subsp. *plantarum*.

High growth vigor and acid production rates are important characteristics for the screening of good LAB. To survive and colonize the gastrointestinal tract, functional LAB needs to tolerate not only the low pH of gastric acid and different osmotic pressure concentrations, but also high bile salt concentrations [36]. In the present study, LAB isolates P15, ZZUPF94, and ZZUPF95 still held high viability after treatment with gastric juice for 3 h, with 99.60%, 99.62%, and 99.65% survival rates, respectively, which are similar to the research of Joghataei et al. [37], who showed that *L. fermentum* FH19 exhibited a 96% survival rate after 3 h in simulated gastric juice. As for SIF, after 4 h treatment, the survival ratios of P15 and ZZUPF95 were 84% and 93%, respectively. Wang et al. [27] found that *L. plantarum* subsp. *plantarum* ZA3 had a 98.88% survival ratio after the SIF phase.

FSBM is in the rapid development stage, and the technology varies. The basic use of open fermentation results in serious contamination by miscellaneous bacteria and inconsistent quality. Therefore, probiotics with a broad spectrum of bacterial inhibition are required for fermentation, and LAB have been the focus of bioprotective bacteria screening because of their ability to produce metabolites (organic acids, bacteriocins, and cyclic peptides) with antibacterial activity and to inhibit the growth of spoilage bacteria through microbial community sensing and competition [38, 39]. LAB strains P15, and ZZUPF95 exhibited broad-spectrum activity against a wide range of microorganisms, including Gram-positive and -negative bacteria. In addition, P15 had marked

Table 3 Analysis of antibacterial experiment results

Isolates	Indicator bacteria					
	Pseudomonas aeruginosa	*Listeria monocytogenes*	*Micrococcus luteus*	*Bacillus subtilis*	*Escherichia coli*	*Salmonella enterica*
P15	+	+	+	+	++	+
P24	+	+	−	−	+	−
ZZUPF94	−	−	−	+	−	−
ZZUPF95	+	+	+	++	++	++

Diameter of inhibition zone: + 8.00−12.00 mm; + + 12.00−16.00 mm; + + + 16.00−20.00 mm; −no inhibition zone was detected; the diameter of inhibition zone including that of hole puncher (10.00 mm)

Table 4 Carbohydrate utilization patterns of P15 and ZZUPF95

Substrate	P15	ZZUPF95	Substrate	P15	ZZUPF95
Glycerol	−	−	Salicin	−	−
d-Arabinose	−	−	Cellobiose	−	+
l-Arabinose	−	−	Maltose	+	+
Ribose	w	+	Lactose	+	−
d-Xylose	−	+	Melibiose	+	+
Adonitol	−	+	Saccharose	+	+
β-Methyl-xyloside	−	−	Trehalose	+	+
Galactose	+	−	Melezitose	−	−
d-Glucose	+	+	d-Raffinose	−	−
d-Fructose	+	−	Starch	−	−
d-Mannose	+	+	Glycogen	−	+
l-Sorbose	+	+	Xylitol	−	−
Rhamnose	+	+	β-Gentiobiose	w	+
Dulcitol	−	−	d-Turanose	−	−
Inositol	−	−	d-Lyxose	−	−
Mannitol	+	+	d-Tagatose	−	−
Sorbitol	+	+	d-Fucose	−	−

+ positive; − negative; *w* weakly positive

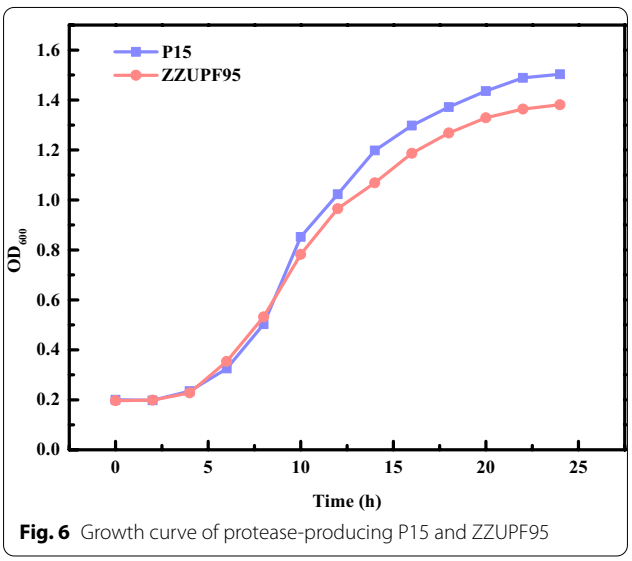

Fig. 6 Growth curve of protease-producing P15 and ZZUPF95

inhibitory effects on *Escherichia coli*, which causes diarrhea in piglets, and ZZUPF95 has a relatively great effect on the elimination of *Salmonella enterica*, which causes gastroenteritis. The results are similar to those of Ziadi et al. [20], who reported the strain *L. plantarum* F3 showed antagonistic activity against *Escherichia coli*, with an inhibition zone diameter of 11.00 mm by the same agar well diffusion method. In addition, *L. plantarum* strains MJM60319, MJM60298, and MJM60399 found by Palaniyandi et al. [40] showed good but varying levels of antimicrobial activity against the various pathogens.

Microbiology analysis of FSBM in this study found that LAB was the predominant microbe and, and no mold was found in any of the groups throughout the fermentation period. Moreover, in both LAB groups, P15 and ZZUPF95 had an inhibitory effect on undesirable microorganisms, including aerobic bacteria, coliform bacteria, bacilli, and facultative anaerobe yeast. Aerobic bacteria, mold, and yeast are spoilage microorganisms that can cause the decomposition of proteins, carbohydrates, and fats. They not only cause energy loss of feed, but also enter the digestive and respiratory tracts, invade the internal tissues, and cause livestock and poultry food contamination. *Clostridium* spp. is the main species causing spoilage of protein feed because of its potential to cause DM loss and butyric acid production, thereby promoting the growth of less-acid-resistant putrid microorganisms and thus resulting in reduced feed intake [41]. In this study, no *Clostridium* was found in other groups except for that protease-treated for 36 h and 12 d. This is further evidence that enzymatic treatment may pose the risk of contamination and energy loss for the fermentation feed.

Among the various parameters used to estimate feedstuff quality, pH is an important parameter because low pH could prevent the growth of harmful organisms and increases the production of carbohydrates that can be effectively degraded [42, 43]. According to this study, LAB addition groups had the fastest rate of reducing the pH, which indicates fast and high acid production. This may be due to the fact that in the early stage of fermentation, the nutrition of LAB in FSBM is sufficient, LABs grow rapidly and produce abundant organic acids such as lactic acid. The result is consistent with findings that the addition of LAB to the fermentation process ensures a fast and vigorous fermentation [44], and it echoes previous better inhibition of harmful bacteria in the LAB group.

Organic acids such as lactic acid and acetic acid can inhibit the growth of pathogenic bacteria and enhance the utilization of minerals and nutrients [45]. Furthermore, organic acid production increases chewing rate and helps eliminate total hydrogen ions in the stomach and intestine [46]. In this study, the increase in lactic acid content was the most significant the LAB groups ($p < 0.05$), which may also be one of the reasons for the great broad-spectrum antibacterial performance and lower pH of these groups. Butyric acid produced by the metabolism of pathogenic bacteria *Clostridium* can cause feed spoilage. This was only detected in the protease group, consistent with the result that *Clostridium* was only found in the protease group. Low concentrations of propionic acid can control the proliferation of yeast and mold, but a high level is pungent and may irritate

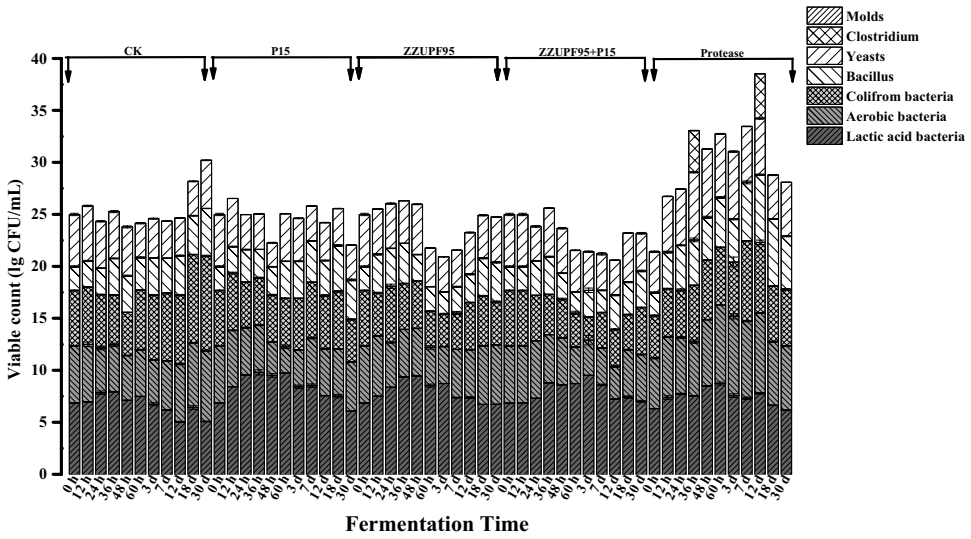

Fig. 7 Microbial content changes during the process of fermented soybean meal

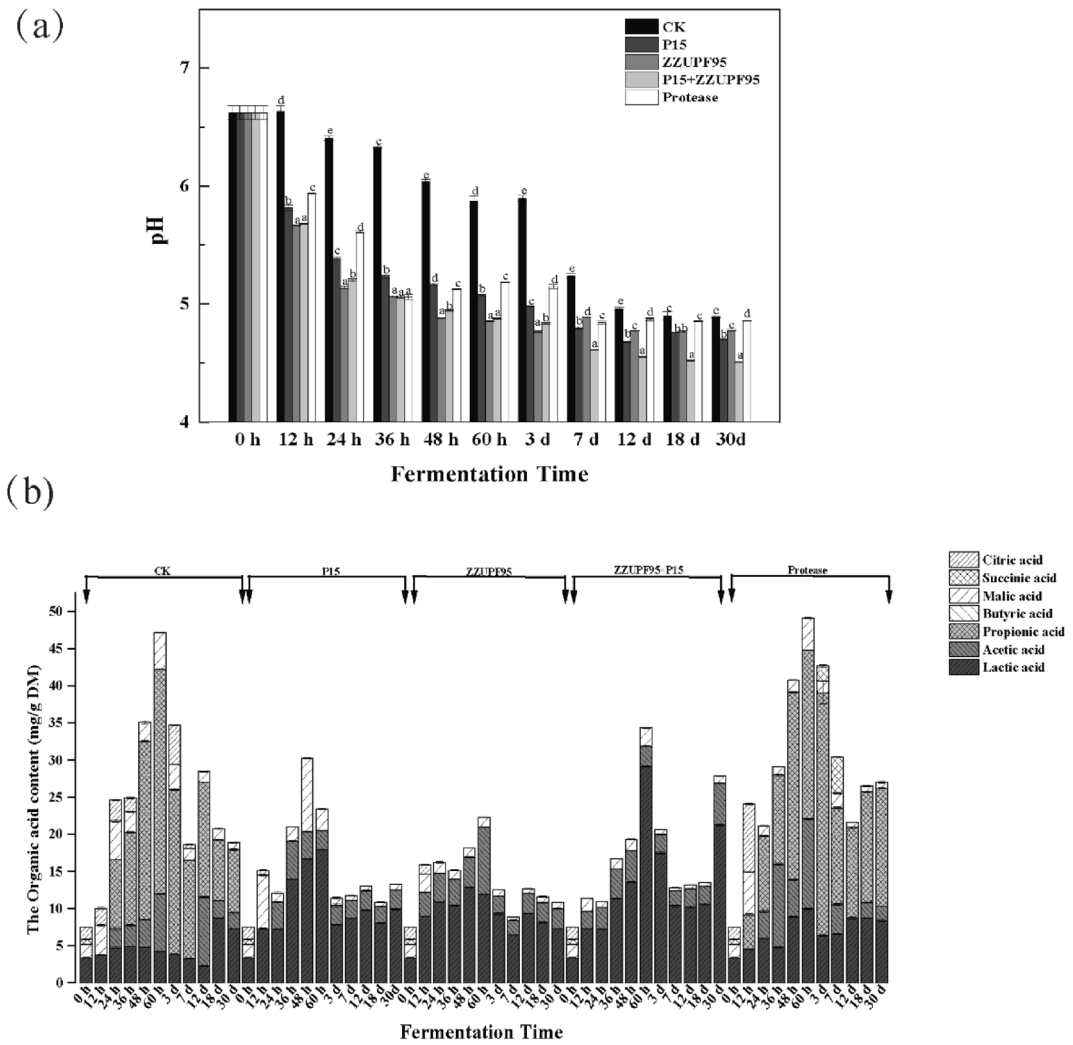

Fig. 8 Analysis of fermentation quality in various stages of fermented soybean meal (FM%). **a** pH; **b** organic acid content

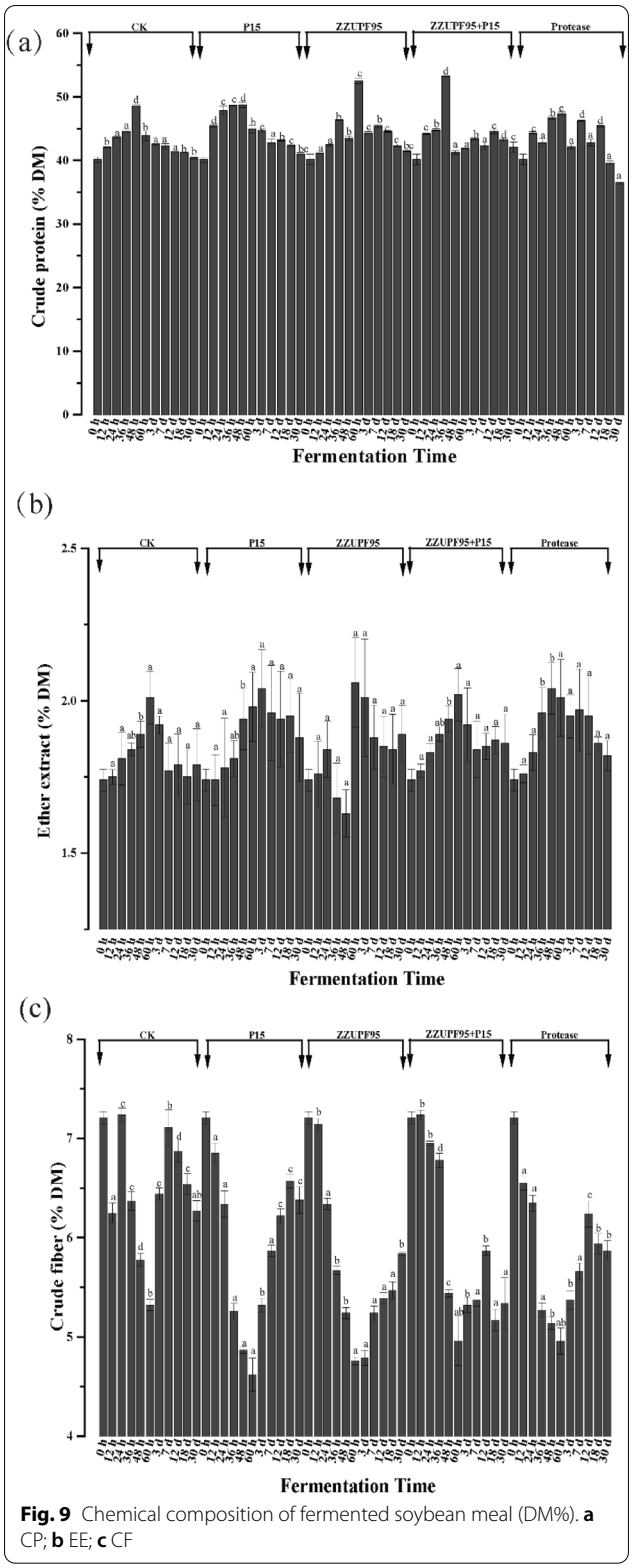

Fig. 9 Chemical composition of fermented soybean meal (DM%). **a** CP; **b** EE; **c** CF

the respiratory tract and esophagus of both humans and animals. Therefore, compared with protease group, LAB addition groups could inhibit the growth of pathogenic bacteria.

For chemical composition analysis, CP in P15 and ZZUPF95 increased to 5.72% and 15.31% from 0 h to 3 d, respectively, which may be related to microorganisms that could convert carbohydrates, fats, and other complex proteins into CP. The CP content in LAB addition groups were significantly higher than that in CK group and protease group at 60 h. Similarly, Chi et al. [47] found the protein content of SBM increased significantly after the fermentation of *L. acidophilus* and *L. plantarum*. In this study, EE content of FSBM in all treated groups increased to at least 2.02%. As one of the important nutrients in the growth and development of livestock and poultry, EE can not only supply and store energy, but an important component of the body cells of livestock and poultry, can also promote the absorption of fat-soluble vitamins and improve animal growth performance [48, 49]. Conversely, CF contents decreased to 17.39% and 25.62%, and this phenomenon occurred in the protease group. The CF was probably degraded by cellulolytic and hemicellulolytic proteases, which were secreted by the LABs. Santos et al. [50] have also detected a much lower CF content of SBM after fermentation. Animals such as pigs do not secrete cellulase or hemicellulase in stomach and small intestine and have very limited ability to digest cellulose and hemicellulose, which is only accomplished by microbial fermentation in the cecum and colon [51]. Thus, feeds containing large amounts of CF generally with a coarse texture and poor palatability are difficult to digest. Moreover, it contains other nutrients in the cell wall, which prevents them from coming into contact with various proteases and reduces their digestibility [52]. Therefore, CF degraded in FSBM probably by enzymes secreted by LABs may have the effect of improving the palatability of SBM.

Conclusions

L. plantarum subsp. *plantarum* P15 and *E. faecalis* ZZUPF95, with the greatest protease production abilities, had excellent probiotic properties. They were selected from 1000 LAB strains and used as agents for SBM fermentation. Compared with SBM as well as CK and protease groups of FSBM, LAB groups showed reduced pH, inhibition of pathogenic bacteria, and degradation of CF; whereas lactic acid, CP, and EE increased, and these effects were even more pronounced after 30 d of

fermentation. *L. plantarum* subsp. *plantarum* P15 and *E. faecalis* ZZUPF95 might be considered as potential feed additives to improve the quality of FSBM. As opposed to protease treatment, these two strains may have less protease content and weaker protease activity, but they have excellent attributes in terms of microbiology, fermentation characteristics, and chemical composition of FSBM. Hence, the next step will be to add LAB and protease together to SBM for fermentation.

Abbreviations
LAB: Lactic acid bacteria; SBM: Soybean meal; MSSF: Microbial solid-state fermentation; FSBM: Fermented soybean meal; TMR: Total mixed ration; MRS: De Man Rogosa Sharpe; LB: Luria Bertani; PDA: Potato dextrose agar; EMB: Eosin methylene blue; CLO: *Clostridium*; NA: Nutrient agar; TCA: Trichloroacetic acid solution; BLAST: Basic local alignment search tool; *L.*: *Lactobacillus*; *Lactiplantibacillus plantarum*: *L. plantarum*; SGF: Simulated gastric fluid; SIF: Simulated intestinal fluid; CP: Crude protein; EE: Ether extract; CF: Crude fiber; FM: Fresh matter.

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Author contributions
Designed experiments, H.P.; carried out experiments, H.M. and H.Y.; analyzed experimental results, H.M., W.W., G,Q., Z.T., L.W., G.W. and Y.W.; wrote and edited the manuscript, H.M. and H.P. All authors read and approved the final manuscript.

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Availability of data and materials
All datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interest.

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