Preparation and *in vitro* Evaluation of High Performance Fermented Soybean Meal using a mixture of *Lactobacillus plantarum*, *Bacillus subtilis* and *Saccharomyces cerevisiae*

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Abstract

Background: Soybean meal is used in animal feeds as a protein supplement but contains significant amounts of anti-nutritional factors such as trypsin inhibitor that directly inhibit digestion, absorption and utilization of nutrients.

Results: Herein, we used solid state batch fermentation of soybean meal using *Lactobacillus plantarum, Bacillus subtilis* and *Saccharomyces cerevisiae* alone and in combination to alter the content of anti-nutritional compounds in the meal. A systematic analysis identified a temperature of 40°C, water content of 40 % in a 48 h fermentation that optimized the fermentation according to our goals. The digestibility of the meal increased within 36 h and by 48 h, the free amino acid content increased while glycinin, β-conglycinin and trypsin inhibitor significantly decreased and the indigestible sugars stachyose and raffinose were reduced to undetectable levels. These processes correlated with high levels of lactic acid that were responsible for an overall pH decrease.

Conclusions: Bacterial and mixed fermentations increased nutritional content while eliminating anti-nutritional factors and significantly improved the nutritional quality of the soybean meal.

Background

Soybean meal (SBM) is a by-product of oil extraction from soybeans and is a major source of protein in pig diets [1, 2]. Its high protein (43–48%), balanced amino acid and high lysine content (2.5–2.8%) has made it an ideal feed source for food animals [3, 4]. However, there several undesirable anti-nutritional factors that seriously hinder SBM as a foodstuff and is a particular problem for young animals such as piglets [5].

There are four major antigenic proteins in SBM that are deposited into storage vacuoles during soybean seed development. Glycinin, β-conglycinin, α-conglycinin and γ-conglycinin are all members of the glycinin gene family and are potent allergens and immunogens in animals [6]. SBM also contains high levels of sucrose, raffinose (cottonseed sugar) and stachyose and the latter two are only minimally degraded in the small intestine and are fermented by gut microbes to produce gas [7]. This can lead to indigestion, abdominal distension and an increase in gastric cavity osmotic pressure resulting in diarrhea. Therefore, untreated SBM in animal feed is undesirable [8].

A variety of processes have been developed to eliminate these anti-nutritional factors and to improve the nutritional value of SBM [9, 10]. Fermented soybean meal (FSBM) is an ideal substitute for fish meal because it can reduce the incidence of diarrhea in weaned pigs when added to their regular diets [11, 12]. The common microorganisms used for fermentation include Lactobacillus plantarum, Bacillus subtilis and Saccharomyces cerevisiae are permissible feed additives by the Ministry of Agriculture and Rural Affairs of the People's Republic of China (http://www.moa.gov.cn/nybgb/2014/dyq/201712/t20171219_6104350.htm). For example, sourdough fermentation with L. plantarum and other lactic acid bacteria [13] decrease levels of phytic acid (inositol

polyphosphate), condensed tannins, raffinose and trypsin inhibitory activities and result in higher protein digestibility. They also increase lactic acid production that has a significant inhibitory activity on pathogens [14]. The addition of B. subtilis to soybean fermentation mixtures acts to decrease glycinin, β-conglycinin and trypsin inhibitor and increases crude protein content [15]. A novel by-product of this fermentation was recently identified and this bacillopeptidase degrades the Kunitz trypsin inhibitors (KTI) that are present in SBM [16]. A common component of bread fermentation is Saccharomyces cerevisiae and produces high levels of lactic acid, α-amylase and aldehyde decarbonylases that catalyze decarbonylation of fatty aldehydes to alkanes in an anaerobic environment [17]. We wanted to combine the advantages of these strains and use them for a mixed fermentation to improve the quality and utilization of soybean meal as an animal feed. The latter include the removal of anti-nutritional factors while retaining high protein content. We therefore tested bacterial and yeast fermentations in single and mixed cultures and adjusted moisture content, temperature and time to give the optimum fermentation conditions. The objective of this study was to improve the SBM fermentation process to increase its quality and stability for its use as a foodstuff.

Results And Discussion

SBM is a protein source staple in the food and feed industries but the presence of anti-nutritional factors greatly decrease its value [18]. Soybean protein and soybean derived peptides also play important roles in prevention of chronic diseases in animals [19]. In this study, we adopted a biological method to ferment SBM to increase its nutritional value and eliminate anti-nutritional factors and to optimize pH and lactic acid levels.

Single organism fermentations

When we utilized single organism fermentation, glycinin and β-conglycinin decreased in the *Lactobacillus* groups with the greatest reduction in the Lab13 fermentation that degraded glycinin and β-conglycinin to 36.25 mg/g and 47.22 mg/g, respectively (Figure 1A). Oligosaccharide content was lowest in the *Bacillus* fermentations and effectively decreased stachyose and raffinose levels (Figure 1B). The *Lactobacillus* groups also generated a lower pH than *Bacillus* and Yeast fermentations that were equal to controls (Figure 1E). Lactic acid production increased up to 76.32 mg/g for the *Lactobacillus* groups and was greater than *Bacillus* and yeast fermentations (Figure 1D). At the conclusion of these fermentations, the FSBM possessed an acid flavor. The fermentations Lab 2, 8, 13, Bacillus 4 and Yeast w303a also were able to degrade the trypsin inhibitor (Figure 1C). The Yeast w303a fermented soybean meal had a very strong wine flavor. Therefore, Lab 13, Bacillus 4 and Yeast w303a were selected for mixed fermentation.

Fermentation optimization

The optimal ratios of mixed bacteria were determined by that combination that minimized glycinin, β-conglycinin, trypsin inhibitor, stachyose, raffinose and possessed acceptable lactic acid and pH levels. The mixed fermentation test 9 (Lab13: Bacillus 4: w303a) at an initial ratio of 3:5:1, respectively, generated the lowest levels of glycinin, β-conglycinin and trypsin inhibitor from 150, 123.20 and 11.16

mg/g to 49.74, 46 and 4.55 mg/g. Except for tests 5, 6 and 7, the mixed fermentations also displayed significant decreases in stachyose and raffinose where tests 8 and 9 had the greatest decreases (Figure 2B). The lowest content of trypsin inhibitor was found in tests 3 and 9 but declined in all test groups. The pH also decreased in the 2 fermentations compared with controls but were not significant between test groups (Figure 2D). We therefore choose test 9 as the best ratio for mixed fermentation.

Moisture content influenced the amount of glycinin, β-conglycinin and trypsin inhibitor and the levels of these compounds sharply declined at 40% moisture and there were no further increased above this moisture level (Figure 3A). The lowest amounts of these 3 compounds were observed at 40°C and reached 31.59, 27.21 and 3.19 mg/g (Figure 3B). Modifications of the fermentation time resulted in reductions to 28.54, 32.64 and 0.41 mg/g at 48 h but continuing the fermentation did not result in any further significant reductions (Figure 3C).

Protein characteristics of fermented SBM

The protein characteristics of fermented SBM in single and mixed cultures showed increases in the crude protein content from 2.24 to 5.71 % after fermentation for 48 h (Figure 4A). The single fermentation groups Lab13, Yeast w303a and Bacillus4 were not significantly different but mixed culture fermentation generated the highest crude protein content and was only 3 % less and 4.5 % greater than reported in previous studies [20]. These differences were most likely due to increased carbohydrate and protein utilization in mixed culture that can be attributed to *S. cerevisiae* [21]. The crude protein content in the mixed reached levels of 48 % at 48 h after the start of fermentation but this was not significantly different from the starting material (Fig. 7A).

The trend for KOH protein solubility in the 4 fermentation groups was a decrease from 8.14 to 13.57 %. The Lab13 group levels were similar to the mixed group and both were significantly lower than Bacillus4 and Yeast w303a (Figure 4B). Overall, the mixed culture fermentation displayed a steady decrease in KOH protein solubility and plateaued at 36 h. In contrast, TCA protein levels steadily increased to 8 % in 48 h and then also plateaued (Figure 7A). The TCA-soluble protein in mixed culture fermented SBM was nearly 10-fold higher than SBM and the single culture batches were ranked Lab13 > Bacillus4 > Yeast w303a (Figure 4C).

The KOH protein solubility was used to estimate feed quality and values <70 % indicate protein damage while values >85 % indicate undesirable urease activity [22]. Our KOH protein solubility in levels ranged from 76.83 to 81.65 % for our 4 fermentation groups and all fell within the desirable range (Figure 4B). In contrast, our TCA protein solubility was 10-fold greater than reported previously [23] (Figure 4C).

Changes in amino acid, pH and lactic acid levels of fermented SBM

Overall, we found that the soybean protein solubility decreased by a maximum of 76 % which we attribute to heating during fermentation and drying following this process. This decrease indicated a release of a significant quantity of free amino acids that were at levels optimal for protein digestion, absorption and

utilization in animals. The rapid changes in protein levels from 24 to 48 h period correlated with the period of the most active microbial growth as has been found previously [24]. We found increases in the essential amino acids threonine, valine, methionine, isoleucine, leucine, phenylalanine and lysine that increased significantly ($p<0.05$) at 2.23 to 8.33 % in single and >12 % in the mixed culture. Non-essential amino acids showed a slightly greater increase with the mixed culture from 5 to 12% while levels of the remainder were not significantly ($p>0.05$) altered (Table 3). A previous study using a mixed *Aspergillus* - *Lactobacillus* fermentation of SBM increased essential and non-essential amino acids 9 and 9.37 %, respectively [18]. This study and ours generated greater increases for SBM fermentation than had been previously found [23]. A lowering of essential amino acid levels has been attributed to high cell masses when using yeast fermentations [25]. The lysine content in our mixed culture fermentation increased by 10 % similar to previous reports and can be accounted for by microbial degradation or by microbial amino acid biosynthesis [26, 27].

The variations in pH levels we found in our mixed fermentation were most likely driven by *Lactobacillus* because our single Lab13 batch and the mixed group had pH values >3 units lower than the control whereas Bacillus 4 and Yeast w303a declined only slightly (Figure 5A). The lactic acid levels in fermented SBM were 8- to 21-fold greater than SBM with the greatest increases in the *Lactobacillus* groups followed by the mixed culture. The Bacillus4 and Yeast w303a groups were lower than the mixed cultures but were still at significant levels (Figure 5B). In the mixed fermentation, the lactic acid content was maximal at 48 h while the pH decreased sharply over the first 36 h and then remained stable (Figure 7B). The presence of lactic acid improves dietary palatability resulting in increased feeding by animals [7]. The more alkaline mixtures created by the *Bacillus* and *S. cerevisiae* fermentations could be accounted for by ammonia production due to amino acid catabolism as has been shown previously [28, 29]. We found the greatest improvement of 106.5 mg/g lactic acid in the *Lactobacillus* ferment that was higher than for *Aspergillus*-fermented SBM and lower than a mixed fermentation reported previously. High lactic acid levels are correlated with the presence of galactose or sucrose for *Lactobacillus* growth and this would influence dietary palatability [18].

Changes in anti-nutritional factors and *in vitro* digestibility of fermented SBM

Fermentation removes macromolecular protein structures and anti-nutritional factors [24, 27]. The glycinin and β-conglycinin content of our mixed fermentation decreased 82.0 and 70.7 %, respectively (Figure 6A). The trypsin inhibitor content was lowered from 11.16 to between 4 and 0.33 % in all single groups while the mixed fermentation resulted in a 97 % decrease (Figure 7C). Similar to the trend of pH and lactic acid content, changes in glycinin, β-conglycinin and trypsin inhibitor were completed within 48 h (Figure 3C). SBM contains numerous oligosaccharides including the readily-digestible sucrose but stachyose and raffinose are not easily absorbed and cause intestinal bloating [30, 31]. Compared to controls, the content of stachyose and raffinose in Bacillus4 and the mixed cultures both were effectively reduced from 5.79 to near 0.70 with the greatest decrease in the mixed culture (Figure 6B). A qualitative analysis of raffinose and stachyose using HPLC indicated these sugars were at undetectable levels and within 36 h, the metabolic conversion of stachyose and raffinose was almost complete as was found in a

previous report [32] (Figure 7C). However, it is possible that these sugars were decomposed into unidentified oligosaccharides or completely metabolized.

Increasing nutrient bioavailability in many cases is the result of greater metabolic activity of the microorganisms in a fermentation [33]. We found that all 4 of our experimental fermentations improved protein digestibility from 83.71 to 96.21% and the rates were increased from 6 to 48 h (Figure 6D and 7D). The higher digestibility of FSBM was most likely due to the low trypsin inhibitor content and this would also translate into improved growth performance in piglets.

Conclusions

In this study we selected a moisture content of 40% to decrease cost and drying time and anti-nutritional factor content was minimized at 40 °C over 48 h. Using these conditions, we compared the effects of single and mixed fermentations and evaluated the FSBM quality on a more comprehensive scale that has been previously applies in these types of studies. The fermentation of SBM increased its nutritional value and the mixed fermentation improved digestibility and almost completely removed non-metabolizable sugars stachyose and raffinose, and significantly lowered levels of glycinin, β-conglycinin and trypsin inhibitor. Overall, the quality of the fermented SBM was greatly improved and this process should be further examined using the mixed fermentation outlined in this study.

Methods

Strains

B. subtilis (CVCC717) (Chinese Veterinary Microorganism Preservation Management Centre) and *S. cerevisiae* strain w303 YSC1058 (Open Biosystems, Thermo Scientific, Pittsburg, PA, USA) were cultured in our laboratory and used for experiments after reaching early stationary phase. Cultivation of *B. subtilis* used Luria Bertani (LB) medium at 37ºC and *S. cerevisiae* was grown using yeast extract peptone dextrose (YPD) medium at 30ºC. The abbreviations used to designate the fermentation batches were ‘Bacillus, Lab and Yeast’ followed by the test number. The Mongolian Agricultural University provided *L. plantarum* and 19 strains were cultured in de Man, Rogosa and Sharpe (MRS) medium and pH was measured for all strains after culture in an orbital shaker at 37℃ for 24 hours. We chose 5 strains of *L. plantarum* that were further screened for their abilities to generate a low pH in the fermentation liquor and for their bacteriostatic activity (Table S1).

Preparation and optimization of fermented soybean meal

Fermentation was initiated by soaking SBM in distilled water to achieve 30 % moisture content and water-soaked SBM (100 g) was transferred to a 500 mL conical flask and inoculated with 10 mL of culture fluid to achieve 10^8 CFU/g of SBM. SBM mixtures were anaerobically solid-state fermented at 37ºC for 48 h using inocula from each of the three organisms individually and then together using equal cell numbers. The resulting fermented SBM products were dried at 50°C to a moisture content of 10% and then milled

into 0.20 mm particles. A single bacterial fermentation was conducted to identify the strains for mixed fermentation based upon the levels of glycinin, β-conglycinin, trypsin inhibitor, stachyose, raffinose, lactic acid and pH. The experimental design was optimized to determine the ratio of mixed bacteria (Table 1), initial water content, fermentation temperature and time obtained from the single-factor experiments (Table 2).

Chemical analysis

Crude protein content of the starting and fermented material was determined using established methods (Association of Official Analytical Chemists). KOH protein solubility, total amino acid content and trichloroacetic acid (TCA) soluble protein were determined by procedures previously described [34]. Glycinin and β-conglycinin and trypsin inhibitors were determined using a commercial kit (Dragontech Ark Biological Engineering Technology Center, Beijing, China). Measurements of pH were determined using 10 g fermented SBM after homogenization in a blender with 90 mL distilled water. The lactic acid concentration was determined by high performance liquid chromatography (HPLC) as previously described [35]. Briefly, a 2 g fermented SBM sample in 70 mL water was sonicated for 20 minutes at 50°C and then cooled to room temperature. The suspension was centrifuged for 10 min at 1,800 ×g and the supernatant was filtered using a 0.22 μM nylon filter (Millipore, Burlington, MA, USA). The samples were analyzed using an Agilent Technologies 1260 infinity HPLC equipped with a Rezex ROA-Organic Acid H+ (8%) column (300 mm × 7.8 mm, Phenomenex, Torrance, CA, USA) with a column temperature of 25°C. Detection used UV spectroscopy at 210 nm. The mobile phase was acetonitrile: 0.5 % H_3PO_4 buffer (3:97 v: v) at pH 2 with a 20 μL injection volume. Oligosaccharide compositions were analyzed by HPLC as previously described [27]. In brief, 0.2 g samples were added to 2 mL 50% acetonitrile, sonicated for 45 min at 40°C and centrifuged and filtered as per above. The samples were analyzed using an Agilent Technologies 1100 infinity HPLC equipped with an Inertsil NH_2 column (4.6 × 250 mm) (Shimadzu, Kyoto, Japan) at a column temperature of 35°C.

In vitro digestibility was assayed by a previously published method [36] using 1 g sample combined with 15 mL 0.1 M HCl and 150 U pepsin (Sigma, St. Louis, MO, USA) in 150 mL conical flasks. The mixture was shaken at 37°C for 4 h at 150 RPM, and then adjusted to pH 7.0 with 1 M NaOH. 300 U trypsin (Sigma) was combined with 15 mL 0.2 M phosphate buffer (pH 8.0) and the mixture was gently shaken at 37°C for 24 h. After digestion, 10 mL of 10% TCA was added and the mixture was centrifuged at 7150×g for 15 min. The precipitate was washed twice with phosphate buffered saline (PBS) buffer and dried at 60°C to a constant weight. The amount of crude protein in the residue was determined and the protein digestibility was then calculated.

Abbreviations

SBM: Soybean meal; FSBM: Fermented soybean meal; MRS: Rogosa and Sharpe; LB: Luria Bertani; YPD: yeast extract peptone dextrose; AOAC: Association of Official Analytical Chemists; TCA: trichloroacetic acid; HPLC: high performance liquid chromatography; PBS: phosphate buffer saline

Declarations

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Authors' contributions

All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors consent to publish.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Ratio of mixed bacteria in mixed SBM fermentation tests

Bacteria	Test number									
	CK#	1	2	3	4	5	6	7	8	9
Lab13	0	1	1	2	2	3	3	3	3	3
Bacillus 4	0	3	5	3	5	1	2	3	4	5
Yeast w303a	0	5	3	5	3	5	4	3	2	1

Control

Table 2. Initial fermentation test conditions

Level	Factors		
	A. moisture content (%)	B. temperature (^{0}C)	C. time (h)
1	20	25	12
2	30	30	24
3	40	35	36
4	50	40	48
5	60	45	60

Table 3. Amino acid compositions of SBM and fermented SBM

Amino acid	SBM	Lab13	*Bacillus4*	*Yeast w303*	Mix
Asparagine	5.41	5.77	5.64	5.59	5.86
Threonine	2.1	2.19	2.18	2.14	2.21
Serine	2.13	2.55	2.36	2.34	2.56
Glutamine	7.46	7.97	7.91	7.89	7.99
Glycine	1.85	2.18	2.01	1.97	2.26
Alanine	1.95	2.07	2.04	2.01	2.16
Cysteine	0.7	0.81	0.79	0.77	0.94
Valine	1.83	2.08	2.08	2.03	2.18
Methionine	0.7	0.75	0.7	0.66	0.77
Isoleucine	1.79	2.05	1.91	1.86	2.15
Leucine	3.48	3.64	3.52	3.49	3.71
Tyrosine	1.77	1.93	1.88	1.82	1.98
Phenylalanine	2.24	2.48	2.39	2.37	2.54
Lysine	3.08	3.38	3.26	3.18	3.51
Histidine	1.38	1.44	1.35	1.31	1.62
Arginine	3.57	3.84	3.64	3.58	3.96
Proline	1.5	1.73	1.69	1.64	1.77
Total	42.94	46.86	45.35	44.65	48.71

Figures

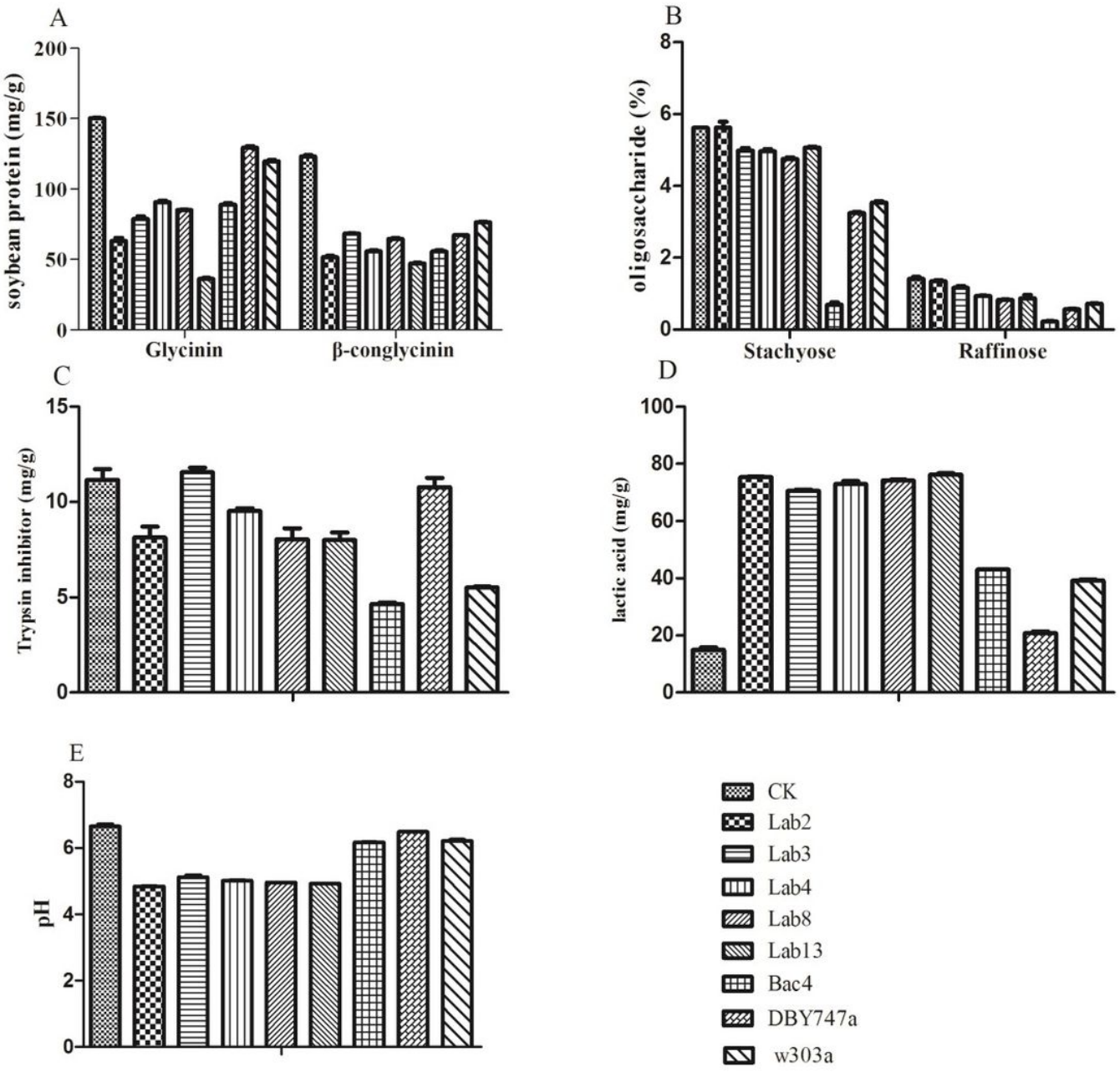

Figure 1

Soybean meal fermentation batches using single microbes. (A) Glycinin and β-conglycinin, (B) Stachyose and raffinose, (C) Trypsin inhibitor, (D) Lactic acid, and (E) pH. CK, control group, soybean meal fermented in the absence of added microorganisms.

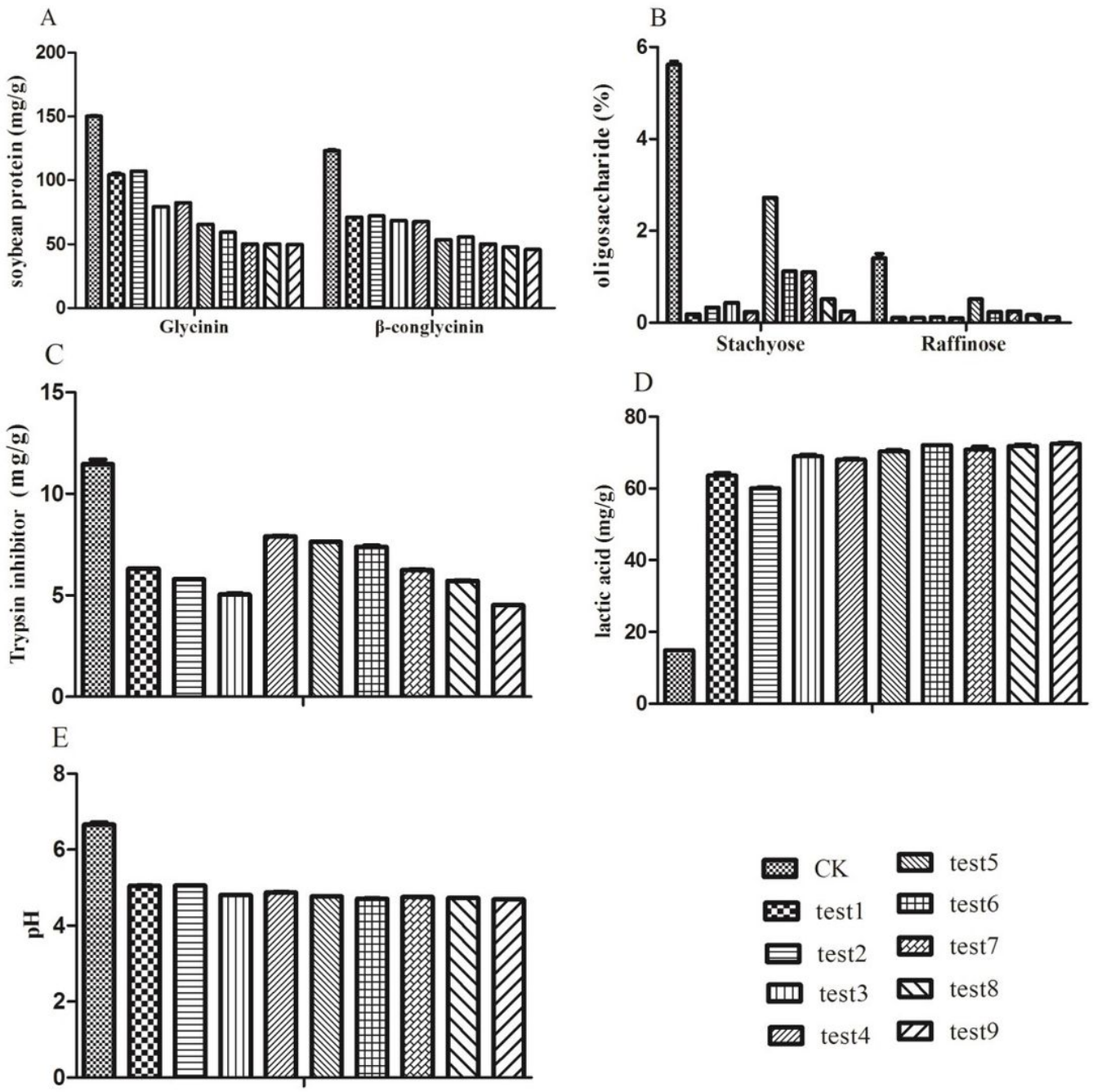

Figure 2

Influence of mixed fermentation on anti-nutritional factor, lactic acid and pH content. (A) Glycinin and β-conglycinin, (B) Stachyose and raffinose, (C) Trypsin inhibitor, (D) Lactic acid and (E) pH. For CK, control (see Fig. 1).

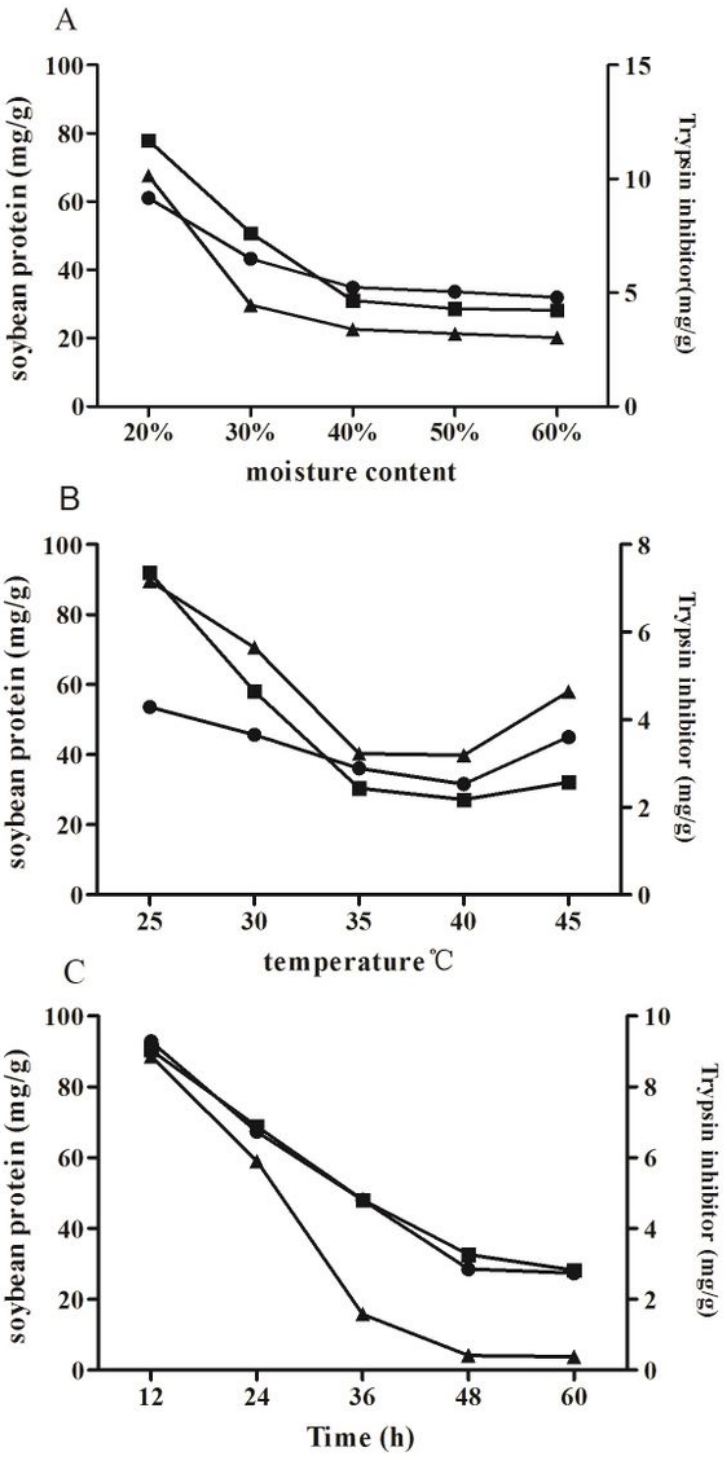

Figure 3

Fermentation optimization. (A) Moisture content, (B) temperature and (C) time. Filled circles, Glycinin; squares, β-conglycinin and triangles, Trypsin inhibitor.

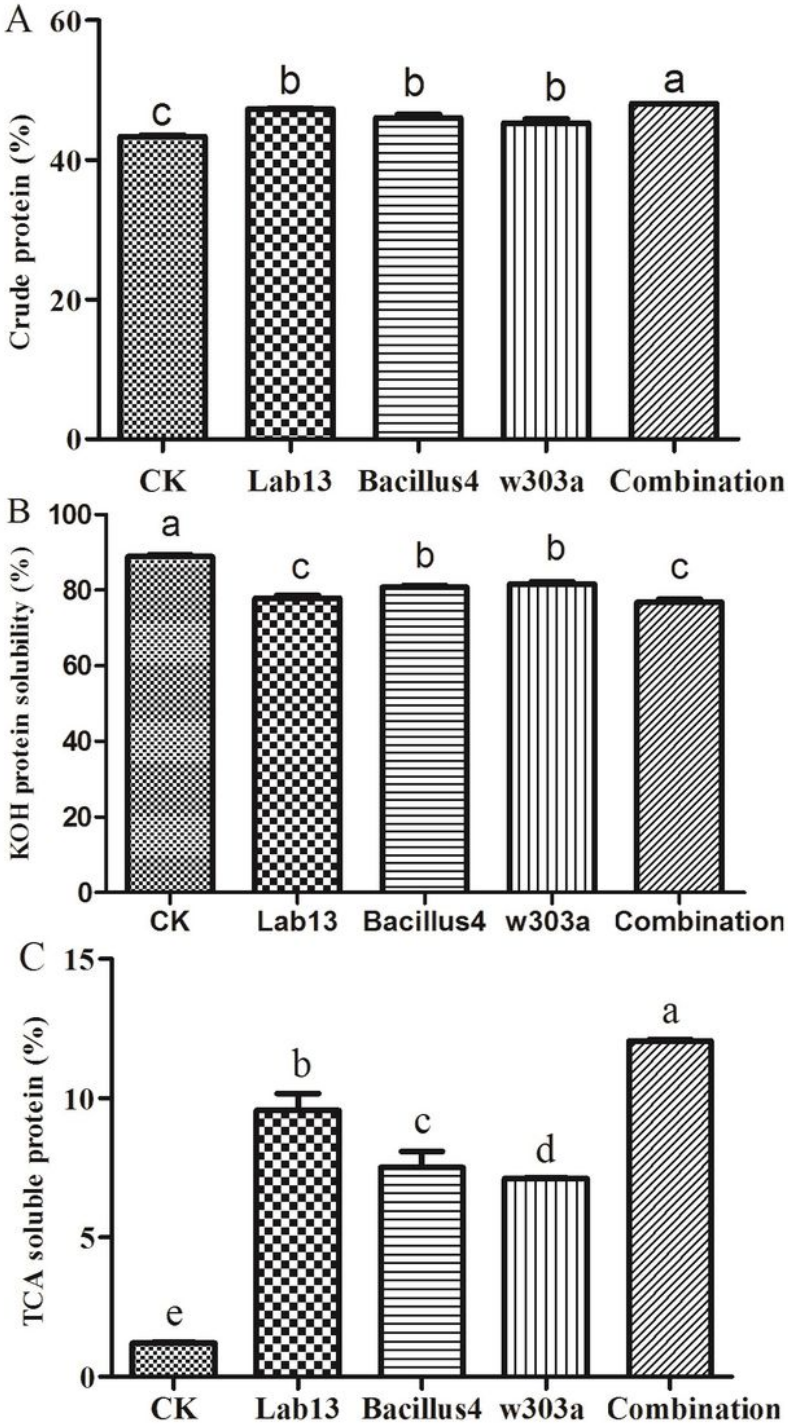

Figure 4

Protein characteristics of fermented SBM. (A) Crude protein, (B) KOH protein solubility and (C) TCA soluble protein. CK, SBM fermented without added microorganisms compared with SBM fermented with Lab13, Bacillus 4 and Yeast w303a and in combination. a, b, c, d, e Means in the same row without the same superscripts are significantly different ($p < 0.05$).

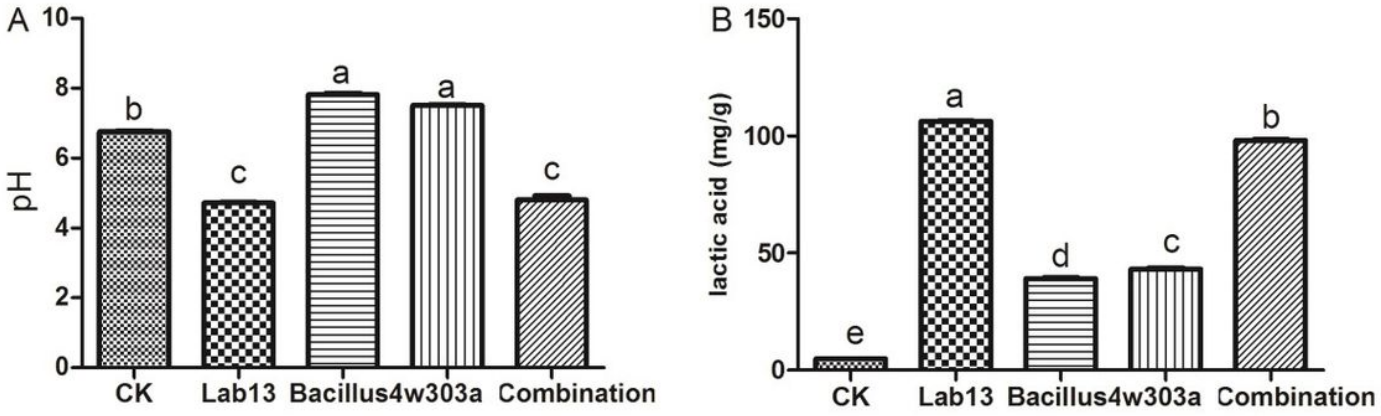

Figure 5

(A) pH and (B) Lactic acid content in fermented SBM. For CK and statistical significance, see Figure 4.

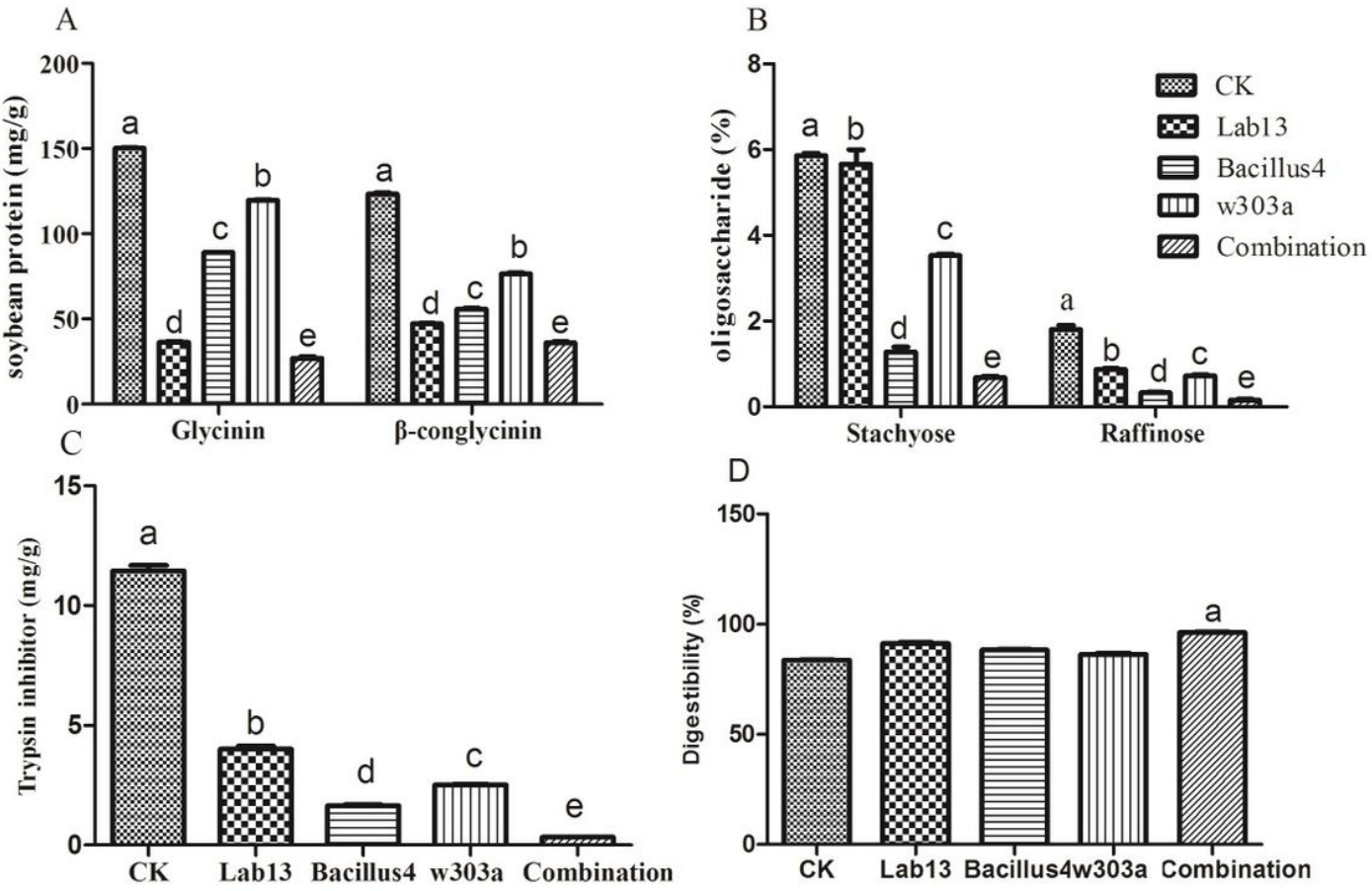

Figure 6

Changes in anti-nutritional factors and in vitro digestibility of FSBM. (A) Glycinin and β-conglycinin, (B) stachyose and raffinose, (C) trypsin inhibitor, and (D) in vitro digestibility of fermented SBM. For CK and

statistical significance, see Figure 4.

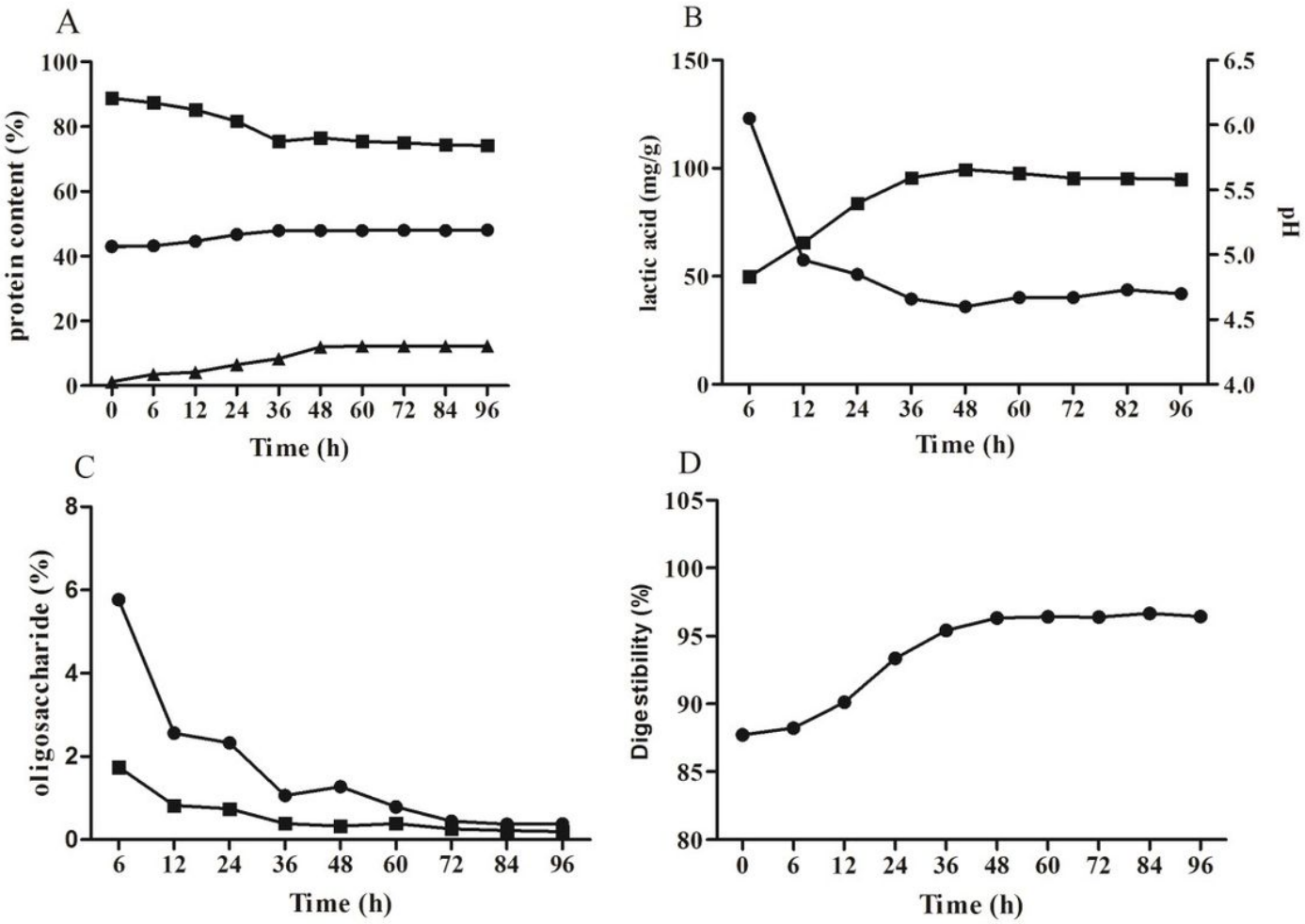

Figure 7

Time dependent changes in anti-nutritional factors and in vitro digestibility of FSBM from mixed batch fermentation. Time series for mixed culture fermentation and levels of (A) Crude protein (filled circles) KOH protein solubility (squares) and TCA preciptable protein (triangles) (B) Lactic acid (filled circles) and pH (squares), (C) Glycinin, β-conglycinin and trypsin inhibitor content (filled circles) and stachyose and raffinose (squares) and (D) Digestibility.