Whole soybean as probiotic lactic acid bacteria carrier food in solid-state fermentation

ShanTing Zhang, Yan Shi, ShuLi Zhang, Wei Shang, XueQin Gao, HaiKuan Wang*

Key Laboratory of Industrial Fermentation Microbiology, Ministry of Education, College of Biotechnology, Tianjin University of Science and Technology, No. 29, 13th Avenue TEDA, Tianjin, People’s Republic of China

A R T I C L E   I N F O

Article info
Received 26 October 2013
Received in revised form 18 December 2013
Accepted 20 December 2013

Keywords:
Whole soybean
Solid-state fermentation
Lactic acid bacteria
Bacillus subtilis natto

A B S T R A C T

For the purpose of preparing lactic acid bacteria (LAB) carrier food, the solid-state fermentation of whole soybean was performed using Bifidobacterium animalis 937, Lactobacillus casei Zhang and Lactobacillus plantarum P-8 mixed with Bacillus subtilis natto, respectively. The physicochemical properties, the amino nitrogen content and peptide molecular weight distribution of the fermented whole soybean products were examined during this process. After 48 h of fermentation, the viable counts of the three samples were 1.41 × 10⁸ CFU/g (B. animalis 937), 1.74 × 10⁹ CFU/g (L. casei Zhang) and 2.19 × 10¹⁰ CFU/g (L. plantarum P-8), with the pH declined rapidly from 6.32 to 5.78, 5.60 and 5.44 at the early stage of the fermentation and increased to 6.71, 6.47 and 6.60 at the later stage of the fermentation. The fermentation caused a sharply increase in the content of the free amino nitrogen from 99.7 μmol/g to 301.9 μmol/g, 390.1 μmol/g and 529.1 μmol/g in the solid fermented soybean products, due to the multiplication of microorganism and the effect of enzyme system. Furthermore, the levels of soybean peptide with molecular weight less than 1000 Da increased 30.7%, 71.2% and 81.3% relative to that of the control group at 48 h. The result of the present work implied that whole soybean fermented by LAB can provide the different probiotics for the host, and there is potential to develope nutritious fermented soybean products.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Soybean is an excellent source for nutrition that include plant protein, oligosaccharides, V₈, V₆ and mineral substance (Dajanta, Chukeatirote, & Apichartsrangkoon, 2012). Acceptance of soybean protein products has increased because of the low cost and high nutritional quality for human consumption and also as protein source for animal meals (Frias, Song, Martinez-Villaluenga, Gonzalez De Mejia, & Vidal-Valverde, 2008). Soybean is also a crucial protein source for Asian people (Ojokoh & Wei, 2011). For example, soybean curd and soymilk are their favorite food (Amadou, Le, Shi, & Jin, 2011; Feng, Eriksson, & Schnurer, 2005). It has been shown that the production of soybean have some functional compound which can reduce the risk of cardiovascular diseases and cancers (Amadou, 2011). In addition, fermented soybean products such as black bean sauce, natto and tempeh are people’s daily diet (Juan & Chou, 2010; Lv, Guo, & Yang, 2009). After fermentation, the nutrition inhibitory factor in soybean was eliminated. Under the action of the microbial enzyme, insoluble macromolecular substances such as protein, fat and carbohydrate were degraded into polypeptides, fatty acids and oligopeptides, which can improve the nutrition utilization of soybean.

Lactic acid bacteria (LAB) and Bacillus subtilis natto as probiotics can exert different health effects on the consumers, which leads to the developing of certain functional foods (Molina, Médici, Font de Valdez, & Taranto, 2012). LAB are well known to be major beneficial microflora in human intestine, and they have been widely utilized to manufacture fermented soymilk products and other types of foods (Kim, Lee, & Yoo, 2012). For example, it can reduce the soy flour immunoreactivity by fermenting with Lactobacillus plantarum by decreasing the IgE immunoreactivity (Frias, Song, Martinez-Villaluenga, Gonzalez De Mejia, & Vidal-Valverde, 2008; Nguyen, Guyot, Icard-Vernière, Rochette, & Loiseau, 2007). Lactobacillus casei as a significant member of LAB can survive naturally in the intestinal tract to regulate the gut microorganism and reduce the risk of cancer, and also be used for fermenting soymilk (John, Nampoothiri, & Pandey, 2007). In addition, soybean also has natural oligosaccharide, such as raffinose, stachyosea, and sugar, and they have some unique characteristics to make Bifidobacterium proliferating, to improve the Bifidobacterium viable count, and to reduce harmful...
bacteria, so as to improve the quality of soybean fermentation products (Han, Ebert, Zhao, Li, Zhang, & Tian, 2005). *Bacillus subtilis* is the most broadly used strain for soybean fermentation, which can increase antioxidative activity, anti-allergic activity and fibrinolytic function of the soybean (John, Nampoothiri, & Pandey, 2007; Juan, Wu, & Chou, 2010; Kwon, Lee, Lee, Chang, & Chang, 2000).

Many studies suggested that LAB was widely used in the fermentation of soybean derived products, such as sufu (a Chinese fermented soybean food), soybean flour and soy milk (Georgetti et al., 2009; Han, Cao, Rombouts, & Nout, 2004; Marazza, Nazareno, de Giori, & Garro, 2012). However, to our knowledge, the whole soybean fermented by LAB mixed with *B. subtilis* natto as probiotics carrier food was not investigated. Besides, the solid-state fermentation possesses several biotechnological advantages, such as higher fermentation productivity, higher end-concentration of products, higher product stability, lower catabolic repression, cultivation of microorganisms specialized for water-insoluble substrates and lower demand on sterility (Holker, Hofer, & Lenz, 2004). After the solid-state fermentation of the whole soybean, the products can be lyophilized directly without centrifugation. As a result, the solid-state fermentation of the whole soybean is a more economical and simple fermentation technology in order to produce probiotics carrier food.

In our research, the solid-state fermentation of whole soybean was performed using *Bifidobacterium animalis* 937, *L. casei* Zhang and *L. plantarum* P-8 mixed with *Bacillus subtilis* natto, respectively. The physicochemical properties, the amino nitrogen content and peptide molecular weight distribution of the fermented whole soybean products were examined during this process.

2. Material and methods

2.1. Microorganisms

The microorganism, *L. casei* Zhang and *L. plantarum* P-8 were obtained from Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University (Inner Mongolia, PR China), which had been considered as new probiotic strains (Bao, Wang, & Zhang, 2012; Bao, Zhang, & Li, 2012; Wang, Zhang, Zhang, Wei, Bao, Zhang, Sun, Postnikoff, Meng, & Zhang, 2012; Wu, Zhang, Sun, Wu, Yue, Meng, & Zhang, 2011) isolated form traditional fermented foods in Inner Mongolia of China. *B. animalis* 937 was isolated from the feces of a healthy child in our laboratory and *B. subtilis* natto was isolated from our laboratory from a traditional fermented food (natto) collected from Heilongjiang, China (Wang, Zhang, Sun, & Dai, 2013).

2.2. Inoculum preparation

For inoculum preparation, *L. casei* Zhang and *L. plantarum* P-8 were grown in MRS broth at 37°C. About 15 ml nutrient agar medium was poured into plates prior to incubation at 37°C for 48 ± 2 h. Total Viable counts of *L. plantarum* P-8, *L. casei* Zhang and *B. animalis* 937 were made using a pour plate method and MRS agar after serial dilution in maximum recovery diluents. A pre-prepared test sample (1 ml) of 10⁻⁵, 10⁻⁶ and/or 10⁻⁷ dilution was transferred into a sterile petri dish, in triplicate, and warm (45 ± 2°C) sterile plate count MRS agar (15 ml) was mixed with the inoculums. Cultures were incubated anaerobically at 37°C for 48 ± 2 h. Total Viable counts of *B. subtilis* natto was made using spread plate technique. About 15 ml nutrient agar medium was poured into plates prior to use. A pre-prepared test sample (0.1 ml) of 10⁻⁶, 10⁻⁷ and/or 10⁻⁸ dilution was transferred to the surface of nutrient agar medium, the inoculums was spread evenly over the entire surface of the agar by a rotary twirling motion of the plate under the rod. Cultures were incubated at 37°C for 24 ± 2 h. The colonies were then counted and expressed as logarithmic colony forming units per gram (lg CFU/g) of the sample.

2.3. Soybean fermentation

The soybeans (Northeast soybean, Heilongjiang, PR China) were cleaned and soaked in the water (pH 6.5) overnight at 4°C and then the soaking water was discarded. The soybeans were sterilized at 115°C for 25 min and cooled to 24°C and then were inoculated with 10⁶ CFU/g of *L. plantarum* P-8, *L. casei* Zhang and *B. animalis* 937 mixed with 10⁶ CFU/g of *B. subtilis* natto, respectively. The fermentation process was carried out at 37°C for 48 h and the fermentation of *B. animalis* 937 mixed with *B. subtilis* natto was cultured with anaerobic conditions. The samples were taken for analysis after 0, 24, 28, 32, 36 and 48 h of the fermentation.

2.4. Microbiological analysis (total viable bacterial count)

Total viable counts were determined during the fermentation. The fermented soybeans (10 g) were homogenized with 90 ml of the sterilized physiological saline (0.85%). Serial dilutions were prepared in sterilized physiological saline and 1 ml of appropriate dilutions was poured in triplicate plates for total viable count. Total viable counts of *L. plantarum* P-8, *L. casei* Zhang and *B. animalis* 937 were made using a pour plate method and MRS agar after serial dilution in maximum recovery diluents. A pre-prepared test sample (1 ml) of 10⁻⁷, 10⁻⁶, and/or 10⁻⁵ dilution was transferred into a sterile petri dish, in triplicate, and warm (45 ± 2°C) sterile plate count MRS agar (15 ml) was mixed with the inoculums. Cultures were incubated anaerobically at 37°C for 48 ± 2 h. Total Viable counts of *B. subtilis* natto was made using spread plate technique. About 15 ml nutrient agar medium was poured into plates prior to use. A pre-prepared test sample (0.1 ml) of 10⁻⁶, 10⁻⁷, and/or 10⁻⁸ dilution was transferred to the surface of nutrient agar medium, the inoculums was spread evenly over the entire surface of the agar by a rotary twirling motion of the plate under the rod. Cultures were incubated at 37°C for 24 ± 2 h. The colonies were then counted and expressed as logarithmic colony forming units per gram (lg CFU/g) of the sample.

2.5. Physicochemical analysis

2.5.1. The determination of pH

10 g of the fermented soybeans (wet weight) were homogenized in a blender with 90 ml of distilled water for 30 s and the pH value of the suspension was measured with an FE20 pH meter (Mettler-Toledo, Shang Hai, PR China).

2.5.2. The determination of free amino nitrogen

10 g of the fermented soybeans (wet weight) were homogenized in a blender with 90 ml of distilled water for 30 s. The free amino nitrogen of the homogenate was extracted at 4°C for 24 h, centrifuged at 7000 rpm for 15 min and the supernatant was reserved. The free amino nitrogen was determined according to the ninhydrin method (Abernathy, Spedding, & Starcher, 2009).

2.5.3. The determination of molecular weight distribution

Fermented soybeans (10 g) were homogenized with 50 ml of distilled water and incubated at 4°C for 4 h, centrifuged at 4500 rpm for 10 min at 4°C and then the supernatant was passed through 0.45 μm Millipore filters (Minisart, Sartorius, Germany). The filtered supernatant was collected, lyophilized and stored at −20°C until further used.

1.0 g freeze-dried samples was dissolved in 99 ml mobile phase (50 mM phosphate buffer containing 0.15 M NaCl, pH = 7.2). The sample was loaded onto GE Superdex™ Peptide 10/300 GL column (i.d. 10 × 300 mm), eluted with mobile phase at a flow rate of 0.5 ml/min and monitored at 220 nm. A molecular weight calibration curve was obtained from the following standards: Cytochrome C (12,500 Da), Trasylol (6512 Da), Glutathione Disulfide (615 Da), Reduced Glutathione (310 Da) and Glycine (75 Da).
2.6. Statistical analysis

All experiments were performed in triplicate, and the results were expressed as means ± standard deviation. Data were tested for statistical significance by the Statistical Analysis System software (SAS 9.00; SAS Institute Inc. NC, USA).

3. Results and discussion

3.1. Microbial counts during fermentation

Microbiological monitoring of the fermented soybean was co-inoculated with individual LAB species and *B. subtilis* natto. As shown in Figs. 1-3, the population of the LAB increased remarkably from 24 h to 36 h, and then entered a stationary phase. The addition of *B. subtilis* natto resulted in an increase in the number of viable cells of the LAB. As it was reported, *B. subtilis* natto can produce catalase, which exhibit a similar growth-promoting effect on lactobacilli (Hosoi, Ametani, Kiuchi, & Kaminogawa, 2000). After 48 h of the fermentation, the viable count of the sample fermented by *L. plantarum* P-8 mixed with *B. subtilis* natto was $2.19 \times 10^{10}$ CFU/g, which is higher than that of the samples fermented by *B. animalis* 937 mixed with *B. subtilis* natto and *L. casei* Zhang mixed with *B. subtilis* natto with the viable count of $1.41 \times 10^8$ CFU/g and $1.74 \times 10^9$ CFU/g, respectively. The results indicated that the fermented whole soybean could serve as LAB probiotic carrier food effectively. Besides, fermentation could decrease soy immunoreactivity (Frias, Song, Martinez-Villaluenga, Gonzalez De Mejia, & Vidal-Valverde, 2008) and increase fibrinolytic enzyme (nattokinase) activity proportionately (Kim, Hwang, & Lee, 2010).

3.2. The pH values

At the initial stage of the fermentation, the microbiological monitoring of the fermented samples revealed that the LAB was able to dominate soybean fermentation and the production of the lactic acid resulted in the decrease of pH (Fig. 4). The pH values of the samples fermented by *B. animalis* 937, *L. plantarum* P-8 and *L. casei* Zhang mixed with *B. subtilis* natto declined rapidly from 6.32 to 5.78, 5.60 and 5.44, respectively. At the later period of the soybean fermentation, *B. subtilis* natto could largely hydrolyze soy proteins leading to release of amines and ammonia and a rise of pH value. The pH of the three samples increased to 6.71, 6.47 and 6.60 at the end of the fermentation. As compared with other fermented soybean foods, due to its characteristic of the musty odor and slimy appearance, natto (the sterilized whole soybean was only fermented by *B. subtilis* natto) was not so popular and widely consumed, even though it was well known in Japan (Weng & Chen, 2010). The lowest pH of the mixture slurries (rice/soybean slurries) fermented by *L. plantarum* A6 was 3.79 (Nguyen, Loiseau, Icard-Vernière, Rochette, Trèche, & Guyot, 2007), which was too sour for the consumer to accept. The results of our study indicated that the combination of the LAB and *B. subtilis* natto in the fermentation could improve the odor and the slimy appearance of the fermented whole soybean by maintaining at an appropriate pH value and providing acidic taste.

3.3. Free amino nitrogen

The results of the microbial counts during the fermentation showed that LAB grew well with *B. subtilis* natto. The content of the free amino nitrogen (Fig. 5) sharply increased at the middle and
Later stages of the fermentation with the multiplication of microorganism and the effect of enzyme system, and reached 301.9 μmol/g (B. animalis 937), 390.1 μmol/g (L. casei Zhang) and 529.1 μmol/g (L. plantarum P-8) at the end of the fermentation, increased 202.8%, 291.3% and 430.7% relative to that of the control group (the control group was not inoculated with LAB and B. subtilis natto). As it was reported that the soybean meal was fermented by L. plantarum with the total amino acids increasing significantly (p < 0.05) by Song, Frías, Martinez-Villaluenga, Vidal-Valdeverde, & de Mejia (2008).

The solid-state fermentation of whole soybean can cause a significant increase in protein solubility, available amino acid and in vitro digestibility. It is well known that the hydrolysis of protein in fermented soybean is greatly dependent upon microbial strain, substrate and moisture content (Kim, Lee, & Yoo, 2012). Such as, in the process of the natto, the most nonessential amino acids and essential amino acids are found to increased after 48 h of fermentation (Weng & Chen, 2010). And the level of total free amino acids of the soy protein extract hydrolyzed by the enzyme of the LAB also increases (Aguirre, Garro, & de Gioria, 2008). In our study, the greatly increase of the content of free amino nitrogen of the whole soybean fermented by LAB mixed with B. subtilis natto demonstrated that the enzyme system of LAB and B. subtilis natto could work well to hydrolyze the soybean protein.

Fig. 4. The effect of LAB and B. subtilis natto on the pH in the solid-state fermentation of the whole soybean. The samples were taken for analysis after 0, 24, 28, 32, 36 and 48 h. Data were expressed as mean ± SD from three independent experiments. — — Whole soybean was fermented by B. animalis 937 mixed with B. subtilis natto. — — Whole soybean was fermented by L. casei Zhang mixed with B. subtilis natto. — — Whole soybean was fermented by L. plantarum P-8 mixed with B. subtilis natto.

Fig. 5. The effect of LAB and B. subtilis natto on the content of the free amino nitrogen in the solid-state fermentation of the whole soybean. The samples were taken for analysis after 0, 24, 28, 32, 36 and 48 h. Data were expressed as mean ± SD from three independent experiments. — — Whole soybean was fermented by B. animalis 937 mixed with B. subtilis natto. — — Whole soybean was fermented by L. casei Zhang mixed with B. subtilis natto. — — Whole soybean was fermented by L. plantarum P-8 mixed with B. subtilis natto.

Fig. 6. The molecular weight distribution of the fermented whole soybean with LAB and B. subtilis natto after 24 h of the fermentation. Data were expressed as mean ± SD from three independent experiments. — — Control group was not inoculated with LAB and B. subtilis natto. — — Whole soybean was fermented by B. animalis 937 mixed with B. subtilis natto. — — Whole soybean was fermented by L. casei Zhang mixed with B. subtilis natto. — — Whole soybean was fermented by L. plantarum P-8 mixed with B. subtilis natto.

Fig. 7. The molecular weight distribution of the fermented whole soybean with LAB and B. subtilis natto after 48 h of the fermentation. Data were expressed as mean ± SD from three independent experiments. — — Control group was not inoculated with LAB and B. subtilis natto. — — Whole soybean was fermented by B. animalis 937 mixed with B. subtilis natto. — — Whole soybean was fermented by L. casei Zhang mixed with B. subtilis natto. — — Whole soybean was fermented by L. plantarum P-8 mixed with B. subtilis natto.
3.4. Molecular weight (MW) distribution

The size of peptides is known to be a significant factor to assess the nutritional value of soybean product (Song et al., 2008). Fig. 6 showed that the levels of peptide with MW less than 1000 Da in the samples fermented by L. casei Zhang mixed with B. subtilis natto, L. plantarum P-8 mixed with B. subtilis natto for 24 h increased 5.0% and 12.9%. The sample fermented by B. animalis 937 mixed with B. subtilis natto decreased 4.6% relative to that of the control group, because the production of proteases was poor at the initial stage of the fermentation, the protein of the soybean was only partly hydrolyzed and the most peptides were utilized by B. animalis. The levels of peptide with MW more than 10,000 Da (Fig. 6) in the samples decreased 14.5% (B. animalis 937 with B. subtilis natto), 20.5% (L. plantarum P-8 with B. subtilis natto) and 22.3% (L. casei Zhang with B. subtilis natto) with the hydrolysis of proteases.

After 48 h of the fermentation, the levels of peptide with MW less than 1000 Da (Fig. 7) in the three samples fermented by B. animalis 937, L. casei Zhang and L. plantarum P-8 mixed with B. subtilis natto increased 30.7%, 71.2%, and 81.3% relative to that of the control group. And the levels of peptide with MW more than

---

Fig. 8. Chromatograms of molecular weight calibration curve, control group and the fermented whole soybean. (A) Chromatograms of molecular weight calibration curve: Cytocrome C (12,500 Da), Trasyol (6512 Da), Glutathione Disulfide (615 Da), Reduced Glutathione (310 Da) and Glycine (75 Da). (B) Control group (whole soybean was not fermented by B. animalis 937 mixed with B. subtilis natto). (C) Whole soybean was fermented by B. animalis 937 mixed with B. subtilis natto. (D) Whole soybean was fermented by L. casei Zhang mixed with B. subtilis natto. (E) Whole soybean was fermented by L. plantarum P-8 mixed with B. subtilis natto.
10,000 Da in the three samples decreased 34.3%, 75.2%, and 77.2%, respectively. And the molecular weight distribution of peptide in the samples fermented by L. animalis 937, L. casei Zhang and L. plantarum P-8 mixed with B. subtilis natto was showed in Fig. 8. As it was reported that the soybean protein meal fermented by L. plantarum Lp6 increased the amount of low molecular weight peptides (Amadou, Le, Shi, Gbadamosi, Kamara, & Jin, 2011). Therefore, the effect of LAB and B. subtilis natto on the levels of peptide with MW less than 1000 Da of the fermented whole soybean could lead to the development of the nutritious and functional fermented soybean products.

4. Conclusion

This study presented a series of the fermented whole soybean foods by using LAB mixed with B. subtilis natto in solid-state fermentation, which had received recognition as a potential biotechnological process without causing serious environmental pollution. Due to the microbial autolysis and enzyme chemistry reaction, the whole soybean fermented by LAB mixed with B. subtilis natto could provide different probiotics for the host and had the capacity to improve nutritional and functional properties. The results also indicated that the viable count, the content of free amino nitrogen and the level of peptide with MW less than 1000 Da in the sample fermented by L. plantarum P-8 mixed with B. subtilis natto were the highest compare with L. animalis 937 mixed with B. subtilis natto and L. Casei Zhang mixed with B. subtilis natto. In conclusion, a novel probiotic LAB carrier food was provided by using LAB mixed with B. subtilis natto to ferment the whole soybean in the solid-state fermentation.

Acknowledgments

The authors wish to acknowledge the financial support provided by the National Natural Science Foundation of China (No. 30900961) and Tianjin Social Science Planning Program (No. TJGLWT11-08).

References