Survivability of *Lactobacillus rhamnosus* during the Preparation of Soy Cheese

Dong-Mei Liu¹, Li Li²*, Xiao-Quan Yang², Shi-Zhong Liang¹ and Jin-Shui Wang²

¹College of Bioscience and Biotechnology, South China University of Technology, 510640 Guangzhou, PR China
²Research and Development Center of Food Proteins, College of Light Industry and Food Science, South China University of Technology, 510640 Guangzhou, PR China

Received: November 2, 2005
Accepted: March 3, 2006

Summary
The aim of this study was to develop a new probiotic soy cheese on the basis of Chinese sufu. The soy cheese was made from soymilk fermented with soy cheese bacterial starter cultures (DH1 and GH4) and *L. rhamnosus* 6013. After ripening, probiotic soy cheese sensory scores (standard SB/T 10170-93) were compared to the control. The changes in pH, bacterial growth and the survivability of the potential probiotic *L. rhamnosus* 6013 during fermentation and storage at 10 °C were examined. After 6 h of fermentation, *L. rhamnosus* 6013 was capable of growing in soymilk as high as 10⁸–10⁹ CFU/mL. After being stored for 30 days at 10 °C, slight decrease in pH and the viable counts of the strain was noticed. The viable counts of *L. rhamnosus* 6013, DH1 and GH4 were 10⁷, 10⁶ and 10⁶ CFU/g, respectively, after storage for 30 days. The levels of stachyose, raffinose and sucrose in soy cheese were determined by high performance liquid chromatography. The results indicated that *L. rhamnosus* 6013 could utilize the soybean oligosaccharides as carbon sources. In addition, 2–4 % of NaCl had little effect on the survivability of *L. rhamnosus* 6013. It indicated that *L. rhamnosus* 6013 could withstand the technological processing of soy cheese and had no negative effect on the fermentation and the sensory properties of the soy cheese.

Key words: *Lactobacillus casei* ssp. *rhamnosus* 6013, fermentation, survivability, probiotic soy cheese, HPLC

Introduction
Soy products play an important role in prevention of chronic diseases such as menopausal disorder, cancer, atherosclerosis and osteoporosis (1–2). Soymilk is rich in high quality proteins and suitable for the growth of bifidobacteria (3–6) because this product contains sucrose, raffinose and stachyose, used by most of the strains that belong to this genus during fermentation (7). It is difficult for mammals to digest the two latter sugars, which tend to cause flatulence in human body (8). These bacteria appear promising starter cultures for the production of quality fermented soymilk products containing reduced quantities of antinutritional factors.

Preparation of a cheese-like product from soymilk coagulated by lactic acid bacteria gained much interest in the past (9–11). The Chinese cheese (sufu), produced in many different forms by various processes in different localities in China (12–13), occurred there many centuries ago. It is a soft creamy cheese-type product that possesses a characteristic flavour and a relatively high protein level, and it can be used in the same way as cheese (14).
Probiotic lactic acid bacteria are widely used in dairy products but not in soy cheese products. Probiotics are live microbial food ingredients that are beneficial to the health (15). Consumption of probiotic bacteria via food products is an ideal way to reestablish the intestinal microflora balance. Recently, the potential probiotic strains L. gasseri JCM113 (16–17), L. rhamnosus FERM P-15120 and L. paracasei ssp. paracasei FERM P-15121 (18) were used for meat fermentation. The potentially probiotic L. casei LC-01 and probiotic Bifidobacterium lactis Bb-12 were used in fermenting dry sausages (19). However, little information about L. rhamnosus used in soy cheese production is available.

In this study, soy cheese with L. rhamnosus 6013 and two soy cheese strains (DH₁ and GH₂) was made. Moreover, the technological properties of the potentially probiotic L. rhamnosus 6013 during soy cheese processing were examined, as well as some of the technological properties during soymilk fermentation and storage. Apart from that, changes in the sugar content by HPLC, and the effect of NaCl concentration on survivability were evaluated. Sensory evaluation was developed according to the corresponding standard (SB/T 10170-93).

Materials and Methods

Bacterial strains, soymilk, and chemicals

Lactobacillus casei ssp. rhamnosus 6013 (L. rhamnosus 6013) was obtained from China Center of Industrial Culture Collection (CICC). We screened over 250 bacterial strains from Chinese sufu products in our laboratory. After selecting those strains most likely to degrade soy protein and produce proteinase, 10 strains were screened for their ability to ferment soy cheese. The promising strains were further investigated for coagulation in soymilk and flavor in soy cheese. Two strains (DH₁ and GH₂) were used in this study.

Dehulled soybeans were soaked in deionized water (water/bean ratio=6:1) overnight. After rinsing, the soaked beans were ground and soymilk was obtained after sieving through 200 mesh and was sterilized (0.1 MPa, 121 °C, 20 min). The soymilk contained (in g/100 mL): protein 4.80, fat 2.50, sugars 3.50 (sucrose 2.20, stachyose 0.54 and raffinose 0.23) and ash 0.50.

Cultivation of L. rhamnosus 6013 in soymilk

 Cultures of L. rhamnosus 6013 were grown in MRS anaerobically at 37 °C for 48 h. After successive transfers of L. rhamnosus 6013 into the sterilized soymilk at 37 °C for 48 h, this cell suspension was inoculated at 5 % into the sterilized soymilk.

Preparation of soy cheese

The soy cheese fermented by bacteria was made according to the method by Han et al. (20). After cooling the sterilized soymilk to 37 °C, L. rhamnosus 6013, DH₁ and GH₂ were inoculated at 5 % (soy cheese A – L. rhamnosus 6013; soy cheese B – L. rhamnosus 6013 and DH₁; soy cheese C – L. rhamnosus 6013 and GH₂). Soymilk was fully coagulated after 6 h of fermentation, and then incubated at 55–60 °C for 30 min to accelerate coagulation. When the soft curd was transferred to moulds for draining and pressing, cake was cut into cubes of the size 3.0×3.0×1.5 cm. The cubes were placed in jars containing NaCl brine and salted for 3 days at 4 °C. After the brine was drained, the cubes were placed in sealed jars for storage at 10 °C for 30 days.

Microbiological analysis

Samples were taken (at 0, 1, 2, 3, 4, 5 and 6 h) and the growth of bacteria was determined as follows: L. rhamnosus 6013 was determined by plating suitable dilutions on MRS agar, while DH₁ and GH₂ were determined on LB agar. Serial dilutions of each sample in sterile saline solution (γ(NaCl)=9 g/L in deionized water) were prepared. A volume of 0.4 mL of diluted samples was spread onto MRS (Oxoid, England) (21) and LB (Huankai Microbial Sci. & Tech. Co., Ltd., Guangdong) agar plates. Colonies on MRS and LB plates were counted after incubation at 37 and 28 °C, respectively, for 48 h. The numbers of viable cells (expressed in CFU/mL for soymilk and in CFU/g for soy cheese) were the means of four repeated experiments. The pH change during growth was measured.

A mass of 10 g of soy cheese was taken aseptically from a jar at 0, 5, 10, 15, 20, 25 and 30 days, homogenized with 100 mL of sterile deionized water, and decimal dilutions in 0.9 % of sterile saline solution were prepared. The viability of the samples was assessed by the method mentioned above, and the pH value was measured.

Physical and chemical analyses

NaCl content of soy cheese

After the cubes were salted in different NaCl solutions for 3 days at 4 °C, the brine solution was drained and the cubes were placed in sealed jars for storage at 10 °C for 30 days. Soy cheese was taken and homogenized with 100 mL of sterile deionized water, and decimal dilutions in 0.9 % of sterile saline solution were prepared. The viability of the samples was assessed by the method mentioned above, and the pH value was measured.

Measurement of sugars

The HPLC was used to determine the contents of sugars (sucrose, stachyose and raffinose) using Waters Spherisorb NH₂ column (4.6 mm×250 mm, 5 μm). Oper-ational conditions were as follows: mobile phase: 70 % acetonitrile (Kermel Chemical Reagents Development Centre, Tianjin, China) in distilled deionized water; flow rate: 0.6 mL/min; column temperature: 40 °C; a refractive index detector (model 830-RJ, Jasco). Reference sucrose, stachyose and raffinose (Sigma-Aldrich, Shanghai, China) were chromatographed to determine their retention times. Samples were deproteinized (22) and centrifuged for 20 min at 8000 rpm. Supernatant fractions...
were filtered through a 0.45-\(\mu\)m membrane, and then subjected to HPLC analysis. External standards were prepared by diluting specific amounts of sugars in deionized water. Standard curves were constructed for each carbohydrate. Least squares regression analysis was used to derive equations from the values reported for each carbohydrate.

**Sensory evaluation of soy cheese**

Quality standard and examination for fermented bean curd SB/T 10170-93 (13) was used for soy cheese grading. Evaluation was based on five features, such as appearance, colour, flavour, aroma and texture, resulting in a maximum of five points for each characteristic. According to this system, there are four classes of cheese, such as excellent (4.50 to 5.00 points), standard (4.00 to 4.49 points), II class (3.50 to 3.99 points), and III class (under 3.50 points). Soy cheese A, soy cheese B, soy cheese C and control were graded in a blind manner after 30 days of ripening by five specialists familiar with the sensory evaluation of soy cheese from a local sufu manufacturing plant and our center. Control was commonly mould-fermented sufu from China.

**Statistical analysis**

All the tests were done four times and the data were averaged. Standard deviation was also calculated. Microcal Origin V.7.0 was used to evaluate significantly different (\(p<0.05\)) means for each sample.

**Results and Discussion**

**The pH change of soymilk in cheese and growth of L. rhamnosus 6013 during cheese fermentation**

Changes in the pH during fermentation of soy cheese are shown in Fig. 1. The initial pH of all soymilk samples after inoculation was 5.5-5.9. At 37 °C, the fastest pH decline was observed in soy cheese C, while soy cheese A and B showed a slower decrease after 3 h of fermentation. After 6 h of fermentation, the pH values of soy cheese C reached 4.7, whereas those of cheese A and B remained at 5.1–5.2. All the samples were coagulated after 6 h of fermentation. The viable cell counts of \(L.\) rhamnosus 6013 and starters, including pathogens (clostridia, salmonellae) and spoilage bacteria and therefore limited their growth (25). Besides China, many other countries such as Vietnam, Philippines, Thailand and Korea produce various soy cheeses, most of which depend on heat, salts (magnesium sulphate, calcium sulphate or glucono-\(\delta\)-lactone) and lactic acid to produce the gel, but the texture of the final products is often unsatisfactory (23). However, if using lactic acid bacteria for fermentation in soymilk, the slower coagulation might obtain a product with more consistent texture product. The lactic acid bacteria metabolize carbohydrates in soymilk and produce lactic acid that coagulates the soymilk protein. The significant (\(p<0.05\)) decrease in pH causes a decrease in the water binding capacity of the soymilk, which accelerates the coagulation process of soy cheese (6).

**Survivability of L. rhamnosus 6013 in the fermented soymilk during storage**

Fig. 3 shows that pH decreases slightly in these three soy cheese samples during storage. The pH of the soymilk of the soychese A decreased from 4.5 to 4.3, while the pH of the soy cheese B and C decreased significantly (\(p<0.05\)) from 4.8 to 4.4 in both cases. The pH values of these three soy cheese samples remained 4.3–4.4 after 30 days of storage at 10 °C. Han et al. (24) investigated 23 samples of three different types of soy cheese in China and some in the Netherlands, and they found that the pH value was almost between 5.25–7.45. The lower pH of this novel probiotic soy cheese assured the food safety, because the lactic acid disturbed the homeostasis of the bacterial cells, including pathogens (clostridia, salmonellae) and spoilage bacteria and therefore limited their growth (25). Fig. 4 shows that the viable numbers of \(L.\) rhamnosus 6013 in these soy cheeses increased significantly (\(p<0.05\)) from 6.7, 6.6, and 6.5 log (CFU/mL) to 9.0, 8.1 and 8.8 log (CFU/mL), respectively, after 6 h of fermentation. The viable count of \(DH_1\) of soy cheese B, and \(GH_4\) of soy cheese C increased significantly (\(p<0.05\)) from 6.8 and 6.9 log (CFU/mL) to 8.9 and 8.1 log (CFU/mL), respectively. Besides China, many other countries such as Vietnam, Philippines, Thailand and Korea produce various soy cheeses, most of which depend on heat, salts (magnesium sulphate, calcium sulphate or glucono-\(\delta\)-lactone) and lactic acid to produce the gel, but the texture of the final products is often unsatisfactory (23). However, if using lactic acid bacteria for fermentation in soymilk, the slower coagulation might obtain a product with more consistent texture product. The lactic acid bacteria metabolize carbohydrates in soymilk and produce lactic acid that coagulates the soymilk protein. The significant (\(p<0.05\)) decrease in pH causes a decrease in the water binding capacity of the soymilk, which accelerates the coagulation process of soy cheese (6).
mount at least 106 CFU/mL of the bacteria on expiry date

because 108–109 cells is the minimum therapeutic dose

Survivability of bacteria during storage for 30 days at 10 °C

Effects on the quality of cheese.

could withstand the cheese manufacturing process and

Table 1. Effect of NaCl solution concentration on survivability of L. rhamnosus 6013 in soy cheese

<table>
<thead>
<tr>
<th>m/V (NaCl)/% Soy cheese A</th>
<th>Soy cheese B</th>
<th>Soy cheese C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.04±0.01/7.9±0.22</td>
<td>0.04±0.01/7.9±0.08</td>
</tr>
<tr>
<td>2</td>
<td>1.40±0.11/7.9±0.85</td>
<td>1.30±0.15/7.6±0.31</td>
</tr>
<tr>
<td>4</td>
<td>3.00±0.21/7.8±0.25</td>
<td>2.76±0.23/7.5±0.13</td>
</tr>
<tr>
<td>10</td>
<td>8.83±0.27/6.4±0.08</td>
<td>8.37±0.65/6.3±0.29</td>
</tr>
</tbody>
</table>

Changes in the quantities of stachyose, raffinose and sucrose after 6 h of soy cheese fermentation

The behaviour of L. rhamnosus 6013 in soymilk was studied with respect to hydrolysis of sucrose, raffinose and stachyose. It was established by HPLC that the predominant sugars in unfermented soymilk were (in g/L): sucrose 22.00, raffinose 2.30 and stachyose 5.40. The results show that the antinutritional factors raffinose and stachyose can be used by L. rhamnosus 6013. In particular, raffinose was completely metabolized after 6 h (p<0.05), whereas stachyose was significantly (p<0.05) reduced to 2.70, 1.76 g/L and 1.49 g/L in soymilk fermented by L. rhamnosus 6013, L. rhamnosus 6013+DH1 and L. rhamnosus 6013+GH4, respectively. Sucrose concentrations also decreased significantly (p<0.05) from 22.00 to 10.50, 8.45 and 4.38 g/L by L. rhamnosus 6013, L. rhamnosus 6013+DH1 and L. rhamnosus 6013+GH4 respectively. Stachyose and raffinose, the principal oligosaccharides in soymilk, are believed to cause flatulence in humans after the consumption of soybean foods. These oligosaccharides can be hydrolysed by α-galactosidase (7), which is capable of hydrolyzing α-1,6 linked α-galactoside residues. The production of galactosidase by lactic acid bacteria and bifidobacteria has been reported (28,29). Changes in the content

Table 1. Effect of NaCl solution concentration on survivability of L. rhamnosus 6013

<table>
<thead>
<tr>
<th>m/V (NaCl)/% (log (CFU/g))</th>
<th>Soy cheese A</th>
<th>Soy cheese B</th>
<th>Soy cheese C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.04±0.01/7.9±0.22</td>
<td>0.04±0.01/7.9±0.08</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.40±0.11/7.9±0.85</td>
<td>1.30±0.15/7.6±0.31</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3.00±0.21/7.8±0.25</td>
<td>2.76±0.23/7.5±0.13</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>8.83±0.27/6.4±0.08</td>
<td>8.37±0.65/6.3±0.29</td>
<td></td>
</tr>
</tbody>
</table>

α m/V (NaCl)/% /log (CFU/g)
of stachyose and raffinose in soymilk fermented with L. acidophilus or S. thermophilus or with Bifidobacteria have been reported (30). The ability of L. fermentum, L. casei and S. salivarius ssp. thermophilus to utilize soybean oligosaccharides has been evaluated by Garro et al. (22,31, 32). The changes of stachyose, raffinose and sucrose contents were related to the pH drop and fast growth noted in Figs. 1 and 2, respectively. The lactic acid bacteria metabolize carbohydrates in soymilk and produce lactic acid that coagulates the soymilk protein. The L. rhamnosus 6013 inoculated into soymilk can metabolize oligosaccharides and produce organic acids that coagulate the soymilk protein.

Sensory evaluation

When trying to introduce a probiotic strain into soy cheese products, a probiotic strain should withstand the manufacturing process without the loss of viability or negative effect on the sensory properties of the soy cheese. Soy cheese variants with probiotic additive were found to have flavour and texture comparable to the control. Soy cheese B, C and the control were described to be of commercial grade with respect to sensory criteria after 30 days of ripening. The score value of the control was 4.5 points. Soy cheese B and C achieved the grades of 3.7 and 3.9 points, respectively, due to their harder texture and less strong flavour (Fig. 5). The score value of soy cheese A was only 3.3 points because it had a harder consistency and the moisture content was too low (28.65%). The interactions between probiotic and starter do not have an impact on the product sensory properties. In this study, it is possible to produce soy cheese with good sensory properties and good survival of L. rhamnosus 6013 and starters (DH1 and GH1).

Conclusions

The incorporation of probiotics into cheese during processing is expected to result in the functional foods if the cultures maintain viability during ripening and do not adversely affect composition, texture or sensory criteria of the products. L. rhamnosus 6013 can favourably utilize soy oligosaccharides as carbon sources. This strain is capable of withstanding the technological processing of soy cheese. No negative effect on fermentation and the sensory properties of the soy cheese has been observed in this study, which indicates that this strain can be used as a promising starter culture in producing a soy cheese of good taste.

Acknowledgements

This research was supported by Guangdong Provincial Science and Technology Foundation, PR China (Project 2005B10401006).

References


