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Effects of fermentation and sterilization on quality of soybean milk

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Abstract

Soybean milk is a derivative product from soybean that has been known for its functionality effects on human. However further development is needed to improve its organoleptic quality. Fermentation is known for reducing beany flavor on soybean milk, on the other hand diminishing its shelf life. In this study, the effects of soybean milk fermentation using *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, and post-fermentation sterilization on quality of soybean milk were investigated. Three soybean milk products, i.e. soybean milk (SM), fermented soybean milk (FSM), and sterilized fermented soybean milk (SFSM) were tested for their physico-chemical properties, proximate analysis, amino acid composition, vitamin B_{12} level, antioxidant activity, and organoleptic test. Our results showed that fermentation in FSM effectively reduced pH and total soluble solids, increased viscosity, changed amino acid composition, and increased antioxidant activity compared to those of SM. Interestingly, FSM had higher contents of methionine and cysteine than SM. Meanwhile, post-fermentation sterilization in SFSM changed the texture of fermented soybean milk, degraded heat-sensitive compounds, and reducing the antioxidant activity. However, organoleptic profiles for both FSM and SFSM showed a slight decrease in consumer acceptance compared to that of SM. In summary, fermention and sterilization treatments may contribute to the increased quality of soybean milk.

Introduction

Soybean (*Glycine max* (L.) Merr.) is economically the most important bean in the world, providing vegetable protein for millions of people and ingredients for hundreds of chemical products and a potential source of bioactive peptides (Vij *et al*., 2011). Soybean mostly contains protein (~40%) and other compounds, such as carbohydrates, isoflavones, saponins, phytic acid, and fibers. Soy protein has been reported for its health benefits, including reducing cardiovascular disease risk factors, menopausal symptoms, weight loss, arthritis, brain function, and enhancing exercise performance.

Soybean milk is a water extract of whole soybeans. It contains water soluble proteins and carbohydrates, and most of the oil of the soybeans. Soybean milk does not contain either lactose or cholesterol. Those features have made soybean milk as an alternative protein source, especially for people who have lactose intolerance syndrome (Hajirostamloo, 2009). However, soybean milk has limited consumer acceptance because of its undesirable or “beany” aftertaste. Several methods have been developed to reduce the aftertaste, such as: heating, deodorizing technique, and addition of sweetener and flavor (Nabulsi *et al*., 2014). Fermentation using lactic acid bacteria (LAB) can also reduce the beany flavor of soybean milk. Fermented soybean milk has become popular because it appears to reduce cardiovascular disease, contribute to weight loss, mitigate arthritic symptoms and improve brain function (Nabulsi *et al.,* 2014). By fermentation, proteins are degraded into shorter peptides, thus relieving protein allergy problem and serving as a good source of bioactive peptides (Vij *et al*., 2011).

Although fermented soybean milk offers many benefits, however, the main issue is the instability of product. Fermented beverages, included fermented soybean milk, undergo post acidification (Macbean, 2010). This process will reduce the product’s pH and also the sensory appeal if the yogurt cultures continue to produce significant amounts of acid after packaging and final cooling processes. Maintaining a good cold chain is necessary to reduce post acidification. However, cold chain takes more cost than conventional storage and only extends the shelf life until monthly. This challenge brings an idea to develop sterilized fermented soybean milk with functional ingredients that are produced by LAB without post acidification

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and longer shelf life. Therefore, the objective of the research was to investigate the effect of fermentation and post-fermentation sterilization to the quality of soybean milk, including proximate and amino acids composition, vitamin B_{12}, antioxidant activity, and organoleptic profile.

Materials and Methods

Preparation of soybean milk, fermented soybean milk, and sterilized fermented soybean milk

Soybean was purchased from Indonesian Association of Tofu and Tempe Home Industry (Bogor, Indonesia). Soybean was soaked for 8 hours than blended with water (80^{o}C) at water: bean ratio of 6:1. The slurry was strained through cheese cloth and the filtrate was sterilized using the autoclave at 115^{o}C for 10 minutes, followed by cooling treatment at room temperature to obtain soybean milk (SM). For fermented soybean milk (FSM), the starter cultures used one ml of MRS broth contained S. thermophilus and one ml of MRS broth contained L. bulgaricus, added into 250 ml of SM. Each ml of broth contained around 4×10^7 colonies. The incubation was done at 37^{o}C for 20 hours and FSM was produced. FSM was then sterilized at 115^{o}C for 10 minutes to obtain sterilized fermented soybean milk (SFSM). Viability of LAB in FSM was determined using total plate count in MRS agar and Gram staining (Beveridge, 2001; Atlas, 2004).

Physico-chemical analysis

All SM, FSM, and SFSM products were analyzed for physico-chemical properties. Total soluble solids were analyzed using refractometer (Hand Held Refractometer 95000-002 Alla France, Chemillé, France). The pH value was determined by using pH meter (pH Testr10, Thermo Scientific, Massachusetts, USA). Viscosity was measured by using viscometer (DV-E Viscometer Brookfield, Massachusetts, USA).

Proximate, amino acids, and vitamin B_{12} analysis

All SM, FSM, and SFSM products were quantified for their proximate, amino acid composition, and vitamin B_{12}. Proximate was analyzed using the protocol of National Standardization Agency of Indonesia (1992). Amino acids were analyzed based on the method of Aristoy and Toldra (2004). Vitamin B_{12} level was measured by the method of Zhang *et al.* (2009).

Antioxidant activity analysis

Antioxidant activity was analyzed using DPPH scavenging activity assay according to the method of Rani and Pradeep (2015). The assay mixture contained 0.03 ml of 1.0 mM of DPPH radical solution in ethanol, 0.24 ml of 99% of ethanol, and 0.03ml of sample solution. The mixture was rapidly mixed and after 30 minutes of incubation in room temperature, the absorbance of the solution was measured at 517 nm. Ascorbic acid 1.0 mM was used as standard. Inhibition of free radical DPPH was calculated according to the formula:

$$\% \text{ of inhibition} = \left[\frac{(\text{abs of control} - \text{abs of sample})}{\text{abs of control}}\right] \times 100$$

Organoleptic test

Organoleptic test against all products were conducted according to the method of Ma *et al.* (2015). The hedonic test used 30 untrained panelists. The score ranged from 1 (very dislike) to 6 (very like). The parameters of test included appearance, taste, aroma, and texture.

Statistical analysis

The results were expressed as mean ± standard deviation (SD). Data underwent analysis of variance (ANOVA). The significant differences were analyzed further using LSD test.

Results

Viabilities of LAB in fermented soybean milk

The result of Gram staining (data not shown) showed that FSM contained Gram positive bacteria, including coccus *(S. thermophilus)* and bacillus *(L. bulgaricus)* bacteria. The viabilities of BAL in FSM were around 4×10^7 CFU/ml.

Physico-chemical properties

Table 1 showed different profile of physico-chemical properties among all products (SM, FSM, and SFSM). Total soluble solids and pH were decreased after fermentation, while the viscosity was increased. Post-fermentation sterilization was found to coagulate the protein in FSM and cause the tofu-like texture in SFSM (Figure 1). Due to its coagulated form, SFSM was homogenized before analyzed.

Proximate, amino acids, and vitamin B_{12} analysis

Table 2 showed the results of proximate, amino acids, and vitamin B_{12} from all products (SM, FSM, and SFSM). Fermentation and post-fermentation sterilization did not significantly affect proximate composition. The fermentation decreased some amino acids, e.g serine, glycine, threonine, and alanine. In FSM, methinone and cystein levels were increased up to 50% after fermentation treatment.

Table 1. Physico-chemical properties of soybean milk (SM), fermented soybean milk (FSM), and sterilized fermented soybean milk (SFSM)

Parameter	SM	FSM	SFSM
Color	Brownish	White	Brown
Form	Liquid	Curd	Coagulated
Total soluble solids (°Bx)	4.0	3.0	3.0 (after homogenized)
pH	6.7	5.7	5.7
Viscocity (cP)	5.6	14.6	11.3 (after homogenized)

Figure 1. The appearance of soybean milk (SM; left), fermented soybean milk (FSM; center), and sterilized fermented soybean milk (SFSM; right).

After sterilization in SFSM, methionine content was decreased, while cystein content was slightly increased. Unfortunately, vitamin B_{12} was not detected from all products.

Antioxidant activity

Figure 2 showed the effect of antioxidant activity from all products (SM, FSM, and SFSM). FSM showed a 3-fold higher antioxidant activity compared to those of SM and SFSM. Sterilization treatment caused the decreased level of antioxidant activity in SFSM.

Organoleptic profile

Table 3 and Figure 3 showed that most panelists prefer FSM than SM in appearance, mouthfeel, taste, and aroma. Compared to FSM, SFSM showed a significant difference in aroma, but not in appearance, mouthfeel, and taste.

Discussion

Soybean milk has been recognized as an alternative protein source with beneficial effects on health, but it has limitation in consumer acceptance due to its undesirable aftertaste. Fermentation of soybean milk with LAB offers a promising alternative to reduce the after taste effect and also increase its nutrition contents and functionalities. Our results showed that FSM was rich in LAB (*S. thermophilus* and *L. bulgaricus*) with viability of 4×10^7 CFU/ml. Codex (2010) requires yogurt to have LAB minimal $4\text{x}10^6$ CFU/ml, indicating that FSM contained higher viability of LAB than the requirement in yogurt product. This result showed that formulation of soybean milk and the fermentation process were sufficient to produce fermented soybean milk that meet yogurt requirement. In commercial production whereas sucrose and other ingredients will be added, the fermentation process might be more efficient. Skim milk also could be added to improve the efficiency of bacterial growth because lactose is main carbon source for LAB (Widyastuti *et al*., 2014).

Table 2. Profiles of proximate content, amino acid composition, and vitamin B_{12} level on soybean milk (SM), fermented soybean milk (FSM), and sterilized fermented soybean milk (SFSM)

Parameter	SM	FSM	SFSM
Water content (%)	94.96	95.19	95.52
Ash (%)	0.41	0.5	0.55
Total fat (%)	0.83	0.79	0.78
Total protein (%)	1.57	1.51	1.82
Total carbohydrate (%)	2.23	2.01	1.33
Histidine (ppm)	623.11	437.11	363.27
Threonine (ppm)	711.70	582.56	513.08
Leucine (ppm)	1057.20	1116.19	801.88
Lysine (ppm)	849.12	1128.28	760.59
Valine (ppm)	604.95	574.43	402.50
Isoleucine (ppm)	616.81	615.72	462.95
Phenylalanine (ppm)	1016.01	923.47	633.00
Tryptophan (ppm)	98.29	87.97	48.22
Methionine (ppm)	107.48	153.88	111.02
Cysteine (ppm)	139.13	188.64	203.84
Proline (ppm)	726.72	758.71	593.51
Tyrosine (ppm)	723.50	572.70	427.52
Aspartic acid (ppm)	1400.21	1530.29	1178.55
Glycine (ppm)	1024.26	763.02	628.40
Arginine (ppm)	1137.13	1112.13	1226.76
Alanine (ppm)	685.24	644.61	750.46
Glutamic acid (ppm)	2308.97	2872.13	2286.95
Serine (ppm)	1763.92	843.32	668.79
Vitamin B12 (mg/100 ml)	Not detected	Not detected	Not detected

LAB can be mainly divided into two groups based on the end products formed during the fermentation of carbohydrates. Homofermentative LAB such as *Streptococcus* and *Lactobacillus* produce lactic

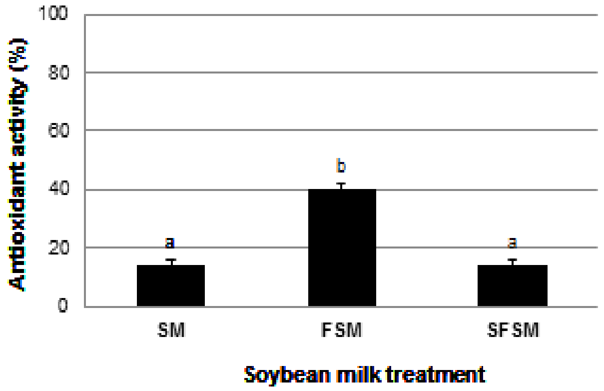

Figure 2. Antioxidant activity of soybean milk (SM), fermented soybean milk (FSM), and sterilized fermented soybean milk (SFSM) by DPPH assay. Note: different superscripts indicate significant differences between treatments at $\alpha = 0.05$ using LSD test.

acid as the sole product of glucose fermentation (Gemechu, 2015). Heterofermentative LAB such as Weissella produce equimolar amounts of lactic acid, CO_2, and ethanol (Widyastuti *et al*., 2014). Soybean milk, instead of lactose, contains sucrose, stachyose, raffinose, glucose and fructose, providing probiotic bacteria many choices of low fermentable substrates (Brandao *et al.,* 2013). Production of lactic acid and other acids could reduce pH value of soybean milk. Reduction of pH from SM to FSM showed that LAB had done some activities in converting SM become FSM (Table 1). Normally LAB converts lactose in milk into organic acid, mostly lactic acid (Widyastuti *et al*., 2014). Considering lactose as the main substrate for LAB, usually researchers add lactose from skim milk into the soybean milk to increase the activity of LAB. In this research, pure soybean milk was used to make sure that the results come from soybean milk fermentation only, not from other ingredients. Therefore, the pH reduction was not as drastically as of the mixture of soybean and dairy milk. Horackova *et al*. (2015) found that acidity of fermented dairy milk would be higher than fermented soybean milk.

Raffinose and stachyose, as main carbohydrates of soybean, are known related to flatulence. The absence of α-galactosidase in human causes those undigested oligosaccharides pass into the large intestine where they are fermented by bacteria with the production of gas (Cruz *et al*., 1981). In fermented soybean milk, raffinose and stachyose are utilized into simple sugars, mainly galactose, that is easily absorbed. The viscous texture of fermented milk is supported by production of exopolysaccharides (EPS), as viscosifying agent, produced by *S. thermophilus* (Rawson and Marshal 1997). EPS interact with the protein content of milk forming matrixes that increase the viscosity of products. This interaction might be reducing soluble carbohydrates and proteins content. Table 1 also showed that fermentation increased the viscosity and reduced total soluble solids of FSM comparing to SM. Those alteration showed the production of EPS by *S. thermophilus* which also normally happened in dairy yogurt. Figure 1 showed the coagulation of SFSM. It happened because the presence of organic acids makes protein to be more heat-sensitive (Obiegbuna *et al.* 2014). Further research is needed to find equilibrium point where all microorganisms get sterilized without changing the texture of FSM.

Jung *et al.* (2016) found that serine, glycine, threonine, alanine, aspartic acid in fermented soybean milk by Weissela koreensis would be higher than original soybean milk. Table 2 showed different result in amino acids composition. The fermentation decreased serine, glycine, threonine, and alanine. Only aspartic acid slightly increased after fermentation. The difference might come from different cultures that were used. Interestingly, FSM showed higher amount of methionine and cysteine where soybean milk lacked of those. The lack of methionine, which is an essential amino acid for human, is one of popular issues in developing soybean food. People used to get methionine from animal protein like chicken breast, beef, tuna, milk, and eggs (McCarty *et al*., 2008), while cysteine can be converted from methionine (Dinkins *et al*., 2001). Since soybean itself contains low amount of methionine and cysteine, improving nutritional values of these amino acids has to be integrated with plant engineering. Dinkins *et al.* (2001) also developed soybean with high content of sulfur amino acids by overexpressing zein protein in soybean plant. Serine was decreased until half of its origin. It is known that other LAB, such as *L. plantarum* could deaminate serine into ammonia, acetate acid, and formic acid (Liu *et al*., 2003). To examine if the same mechanism happens in this kind of fermentation, further research is needed. Those compounds were probably responsible for a large different of organoleptic profile between fermented soybean milk and dairy yogurt.

Analysis of vitamin B_{12} showed that there was no vitamin B_{12} found in all products (SM, FSM, and SFSM) (Table 2). In contrast, previous study by Molina *et al.* (2012) stated that soybean milk fermentation with *L. reuteri* CRL 1908 increased vitamin B_{12} content in mice serum. Tempeh as a fermented soyfood is also known as a potential source of vitamin B_{12} produced by *Klebsiella pneumoniae* (Ayu *et al*., 2014). These results indicated that *L. bulgaricus* and *S. thermophilus* are not vitamin B_{12}-producing bacteria, like *L. reuteri* in fermented

Table 3. Organoleptic profiles on soybean milk (SM), fermented soybean milk (FSM), and sterilized fermented soybean milk (SFSM)

Samples	Appearance	Taste	Aroma	Mouthfeel
SM	3.9±1.3 [b]	3.5±1.2 [b]	3.9±1.5 [c]	4.0±1.3 [b]
FSM	2.7±1.2 [a]	2.4±1.7 [a]	2.1±1.0 [a]	3.1±1.5 [a]
SFSM	2.6±1.3 [a]	2.5±1.4 [a]	2.7±1.5 [b]	2.8±1.3 [a]

Note: Different superscripts in each column indicate significant differences between treatments at α = 0.05 using LSD test. Score 1: dislike very much; score 6: like very much.

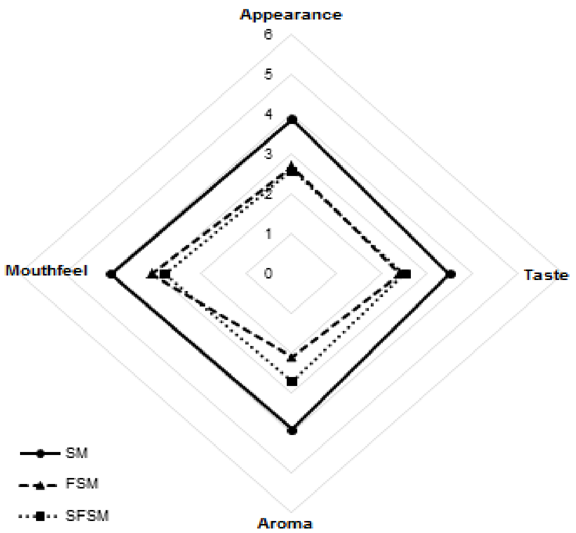

Figure 3. Organoleptic profiles on soybean milk (SM), fermented soybean milk (FSM), and sterilized fermented soybean milk (SFSM). Score 1: dislike very much; score 6: like very much.

soybean milk or *K. pneumoniae* in tempeh. However, the different result might also be caused by difference method that being used. Molina *et al.* (2012) used electrochemiluminiscence immunoassay (ECLIA) that has high sensitivity in detecting vitamin B_{12} (pg/ml), while in this study we employed ultra-performance liquid chromatography tandem mass-spectrometry (UPLC-MS) that only can detect vitamin B_{12} in mg/100 ml. Nonetheless, optimization (e.g. micronutrients addition) can be observed in further research.

Figure 2 showed that FSM exerted antioxidant activity up to 3-fold increasing after fermentation compared to that of unfermented one (SM). It may be due to the increasing of isoflavones in aglycone form during fermentation process. The antioxidant activity may come from many compounds in products, including isoflavones. It is known that isoflavones protect cells from the damaging effects of free radicals (Vij *et al*., 2011). Main isoflavones in soybean are in glucosides form. In fermented soy food like miso, natto, soy sauce, or fermented soymilk, isoflavone glucosides are hydrolyzed using α-glucosidases into aglycone form. Isoflavones in aglycone form are absorbed faster and in higher amounts than their glucosides in humans (Izumi *et al.*, 2000). Wang *et al.* (2006) also found that fermented soybean milk by LAB and bifidobacteria showed antioxidant activity while the unfermented showed none. The alteration from isoflavones glucosides into isoflavones aglycone have been reported by Chien *et al*. (2006). In soybean milk fermented by LAB and bifidobacteria, total glucosides decreased from 49.68 µg/mL to 14.49 µg/mL, while total aglycones increased from 21.91 µg/mL to 59.01 µg/mL. However, Stintzing *et al.* (2006) also found that isoflavones in aglycone forms are mostly degraded by environmental factors, particularly heat and acids. This report is in line with our results, where SFSM only exerted less antioxidant activity compared to that of FSM. SFSM was also produced by sterilization treatment at high temperature (Figure 2).

Organoleptic profile of FSM showed the significant difference compared to dairy yogurt and the undesirable taste compared to the unfermented one of SM (Table 3 and Figure 3). It is known that flavors in dairy-yogurt consisted of more than 90 compounds, mainly acetaldehydes and lactic acid (Routray and Mishra, 2011). These flavors are related to symbiosis between LAB in cow milk. *L. bulgaricus* is used in yogurt production alongside *S. thermophilus*, and both spesies are synergistic. During the initial phase, *L. bulgaricus* stimulates the growth of *S. thermophilus* by releasing amino acids, such as valine, leucine, histidine, and methionine from milk proteins, while *S. thermophilus* promotes the growth of *L. bulgaricus* by producing amounts of folic and formic acids. Thus, the mutual stimulation of both species in the mixed culture significantly increase the amount production of lactic acid and aromatic compounds compared to that of single culture. In contrast, there is no lactose to be digested in soybean milk. Without lactose content, fermentation of soybean milk produces the different composition of volatile compounds and/or organic acids in comparison to dairy yogurt.

Soybean milk had limited consumer acceptance because of its undesirable or "beany" aftertaste due to the presence of hexanal and pentanal (Nabulsi *et al.*, 2014). These aldehydes are formed mainly by hydroperoxidation of polyunsaturated fatty acids catalyzed by lipoxygenase. Development of fermented soybean milk was aimed to reduce the beany flavor. During fermentation, hexanal and pentanal are metabolized by LAB into lactic acid and diacetyl (Beckmann *et al.*, 2009). It will be interesting to see the profile of volatile compounds in fermented soybean milk in further research. Low scores that given to FSM might come from increased pH and viscosity (Table 3). Nevertheless, this result made commercial formulation easier because panelists could not detect any beany flavor either from FSM or SFSM. Developing sweet taste also might increase consumer acceptance. Barus and Wijaya (2011) reported sweet taste at fermented food like tape is desirable. Adding sucrose and flavor will make an acceptable FSM or SFSM commercial product.

Conclusion

Our results suggest that fermentation for soybean milk reduced pH and total soluble solids, increased viscosity, changed amino acids composition like increasing methionine and cysteine content, and increased antioxidant activity. Post-fermentation sterilization for soybean milk changed the texture of fermented soybean milk, degraded heat-sensitive compounds, and reducing the antioxidant activity of fermented soybean milk. Post-fermentation sterilization may delay post acidification that lead to extend product shelf life.

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