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레토르트 곡물 두유 내 *Bacillus cereus*, *Escherichia coli* O157:H7, *Listeria monocytogenes*의 내열특성

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Thermal Resistance Characteristics of *Bacillus cereus*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* in a Multi-grain Soy Milk Product

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Abstract This study determined the thermal resistance of *Bacillus cereus*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* in multi-grain soymilk and proposes processing conditions that meet the national standard for retort food products in Korea. D and z values were calculated from thermal inactivation kinetic curves after heating at 55, 60, and 65°C. The D value for *B. cereus* at 55°C was the highest (22.8 min), followed by that for *E. coli* O157:H7 (18.8 min) and *L. monocytogenes* (17.6 min). At 60-65°C, the order was *L. monocytogenes* ($D_{60\text{-}65^{\circ}C}$=3.4-0.9 min), *E. coli* O157:H7 (3.0-0.3 min), and *B. cereus* (1.2-0.3 min). The z values for these species were 5.2, 5.5, and 7.7°C, respectively. The Korean national standard for retort food products was achieved by thermal processing at 124±2°C for 0.3-2.2 min. This study provides useful data for ensuring both the microbiological safety and product quality of multi-grain soymilk products.

Keywords: multi-grain soymilk, foodborne pathogenic bacteria, thermal resistance, retort food product

Introduction

Soy milk is produced by soaking dried soybeans in water and then grinding them with or without other grains. It is a popular alternative to cow's milk due to the reported beneficial effects of plant proteins on nutrition and health (1). Soy beans have a variety of health-promoting effects, including reducing cholesterol (2), lowering blood pressure (3), and preventing diseases such as cancer (4), diabetes (5), and obesity (6). As some consumers tend to prefer soy milk to cow's milk for these reasons, food manufacturers have released various types of new commercial products (7).

Microbial contamination of raw materials and unsanitary food processing environments may affect the microbiological quality of the final product (8,9). Because soy milk has a rather complicated manufacturing processes (i.e., soaking, shelling, grinding, boiling, filtration, and homogenization), either in the presence or absence of a number of other ingredients (e.g., barley, brown rice, corn, soybean, or wheat), it is likely to contain microbial contaminants such as spoilage or pathogenic bacteria. Indeed, several pathogens (including *Bacillus cereus*, *Salmonella* spp., and *Staphylococcus aureus*) have been isolated from various soy milk products (10-12). In addition, the long-term survival and growth of *Escherichia coli* O157:H7 and *Listeria monocytogenes* in soy milk and cow's milk have been reported previously (13-15). In China, 3,936 students and 260 teachers suffered from infectious disease after drinking soy milk, and a 13-year-old girl died in 2003 (16). As the microbiological safety of soy milk is a growing concern for consumers, it is important to specify appropriate processing conditions for these commercial products.

Modern food consumers prefer soy milk supplemented with various ingredients (e.g., black bean, black sesame, or oatmeal) as a breakfast substitute or a diet food; therefore, the market for these products has expanded (17). Supplementation changes not only the nutritional value of the soy milk product, but also its physicochemical characteristics such as viscosity and heat conductivity (18); thus, it is generally considered that the nutritional value and physicochemical characteristics of multi-grain soy milk are significantly different from those of common soy milk. In addition, both inherent and added nutrients (e.g., plant proteins, isoflavone, taurine, or oligosaccharides) may affect its biological activity and resistance to external stresses. For instance, Anderson *et al*. (19) and Gibson (20) reported that adding dextrose or sucrose to products increases the heat resistance of the micro-organisms present in these products. Kenney and Beuchat (21)

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also reported that a higher fat content results in increased thermal resistance of *L. monocytogenes*. Thus, because the ability of microorganisms to survive thermal processing may be affected by the abundance of nutrients in foods, it is important to investigate the heat resistance characteristics of various foodborne pathogens and ascertain appropriate thermal processing conditions to ensure the microbiological safety of that product.

Here, *B. cereus*, *E. coli* O157:H7, and *L. monocytogenes* were selected as potential microbiological hazards of multi-grain soy milk products, because *B. cereus* has been frequently isolated from raw soybeans, grains, and related-products (22,23), and *E. coli* O157:H7 and *L. monocytogenes* are major causative microorganisms of foodborne illness outbreaks associated with the consumption of commercial milk products (24-27). In addition, D'Aoust *et al.* (28) found that *E. coli* O157:H7 had the strongest resistance to thermal inactivation among three foodborne pathogens (i.e. *Campylobacter* spp., *E. coli* O157:H7, and *Yersinia enterocolitica*).

Therefore, this study aimed to devise appropriate thermal processing conditions that inactivate *B. cereus*, *E. coli* O157:H7, and *L. monocytogenes* in commercial multi-grain soy milk products and that meet the national standards for retort food products described in the Korea Food Code, by examining the thermal resistances of these microorganisms in these products.

Materials and Methods

Suspensions of the target microorganisms

Three strains of *B. cereus* (ATCC 10876, 13061, and 14579), *E. coli* O157:H7 (ATCC 35150, 43889, and 43890), and *L. monocytogenes* (ATCC 19111, 19115, and 19117) were used in this study. All test strains were obtained from the Food Microbiology Culture Collection at Korea University (Seoul, Korea) and stored at −80°C in tryptic soy broth (TSB, Difco, Detroit, MI, USA) supplemented with glycerol (20%). The stock microorganisms were sub-cultured monthly in tryptic soy agar (TSA, Difco). Each strain was inoculated into TSB and incubated at 30°C (*B. cereus* and *L. monocytogenes*) or 37°C (*E. coli* O157:H7) for 24 h. The cultured strains of each bacterial species were then combined into a sterile 50 mL centrifuge tube and washed twice with sterile 0.85% saline solution at 3,000×*g* (Centra-CL2, IEC, Needham Heights, MA, USA) for 15 min.

Preparation of soy milk sample

The multi-grain soy milk sample used in this study was a commercially produced retort pouch product and comprised 22 ingredients: adlay, barley, black rice, black soybean, brown rice, brown rice oil, corn, dried chestnut, embryo bud of brown rice, fructo-oligosaccharide, glutinous millet, glutinous rice, honey, millet, non-glutinous rice, refined sugar, rye, salt, sesame, sorghum, wheat, and white soybean. All of the ingredients were produced and made in Korea. The semi-final product (the product obtained immediately prior to sterilization) was supplied by the manufacturing company (Doctorsoy Inc., Yangju, Korea) on the day of the experiment (July to August, 2012). The viscosity of this product was measured using a cone/plate viscometer (cone #CP-40; LVT, Brookfield, Stoughton, MA, USA) with stirring at 12 rpm at room temperature.

Before the experiment, the absence of target bacteria (*B. cereus*, *E. coli* O157:H7, and *L. monocytogenes*) was confirmed by qualitative microbiological analysis, according to the method described in the Korea Food Code (2012), with minor modifications. Briefly, 25 mL of product were poured into stomacher bags containing 225 mL of enrichment broth, homogenized at 230 rpm for 2 min, and incubated under the following conditions: *B. cereus*, TSB supplemented with polymyxin at 30°C for 24 h; *E. coli* O157:H7, modified TSB at 35-37°C for 24 h; and *L. monocytogenes*, *Listeria* enrichment broth (Difco) at 30°C for 24 h. To check that the target bacteria were present in the enriched samples, each enriched sample was streaked onto selective media and incubated as follows: *B. cereus*, mannitol-egg yolk-

Table 1. Bacterial strains used in this study

Bacteria	Strain	Application	Sources
Bacillus cereus	ATCC 10876	-[a]	Contaminated flask
	ATCC 13061	Produces penicillinase beta-lactamase I	-
	ATCC 14579	Produces restriction endonuclease Bce14579I Food testing	-
Escherichia coli O157:H7	ATCC 35150	-	Feces, human
	ATCC 43889	Quality control strain for biosynthetic products Produces Shiga-like toxin II	Feces of patient with hemolytic uremic syndrome, North Carolina, USA
	ATCC 43890	Produces Shiga-like toxin I	Human feces, California, USA
Listeria monocytogenes	ATCC 19111	Media testing Enteric research Food testing	Poultry, England
	ATCC 19115	Quality control strain Enteric research	Human
	ATCC 19117	Enteric research	Sheep, USA

[a]No information is provided by the American Type Culture Collection.

polymyxin agar (MYP, Difco) supplemented with 50% egg yolk and antimicrobial vial P (Difco) at 30°C for 24 h; *E. coli* O157:H7, Sorbitol MacConkey Agar with Cefixime and Tellurite (CT-SMAC, Difco) at 37°C for 24 h; and *L. monocytogenes*, modified Oxford agar supplemented with modified Oxford antimicrobic supplement (mOxford, Difco) at 30°C for 24 h. Only target pathogen-free samples were used for the subsequent experiments.

Bacterial survival during heat treatment

Prior to the experiment, glass culture tubes (18 mm inside diameter×150 mm length) containing 9.9 mL of target pathogen-free multi-grain soy milk were placed in shaking water baths set at 55, 60, and 65°C (VS-1205SW1, Vision Scientific Co. Ltd., Daejeon, Korea) for pre-warming. Cell suspensions of the target pathogens were separately inoculated into the prepared samples to yield approximate 7-8 log colony forming units (CFU)/mL per inoculum. Heat treatment was performed with regular shaking at 100 rpm, and the survival of each pathogen was confirmed at appropriate time intervals (four time points) and heating temperatures (i.e., *B. cereus*: 30 min at 55°C, 1 min at 60°C, and 15 s at 65°C; *E. coli* O157:H7: 15 min at 55°C, 1.5 min at 60°C, and 15 s at 65°C; and *L. monocytogenes*: 10 min at 55°C, 2 min at 60°C, and 30 s at 65°C). These pathogen-specific time points and heating temperatures were determined according to the results of previous studies on the general thermal resistance of each of these pathogens in laboratory media or foodstuffs (29-31).

The following procedure was conducted to confirm the number of survivors in each of the treated samples. Briefly, each sample was serially diluted 10-fold in sterile 0.85% saline and then spread-plated in duplicate on MYP (*B. cereus*), CT-SMAC (*E. coli* O157:H7), or mOxford (*L. monocytogenes*) agar medium. The plates were then incubated at 30°C (*B. cereus* and *L. monocytogenes*) or 37°C (*E. coli* O157:H7) for 24 h, and the number of typical colonies on each plate was counted and expressed as a log value (log CFU/mL). Each experiment was repeated three times.

Determination of D- and z- values and thermal death time (TDT)

The data for each individual pathogen at each of the treatment temperatures were used to plot a thermal death curve (TDC; log population of survivors against heating time). The linear fit for the plot was determined using a linear regression application in Sigma Plot software (version 10, Systat Software, Richmond, CA, USA). The D value at each temperature was obtained by calculating the inverse negative of the slope for the log population of survivors (D value= −1/slope). The $\log_{10}$ D values were then plotted against temperature and the z values were calculated from the curve (i.e., z value= −1/slope of the $\log_{10}$ D versus temperature curve).

Korea national standard for sterilization processing of retort food products is defined as 'heat treatment at 120°C for 4 min or higher level of heating condition' (Korea Food Code, 2015). The TDT at a specific temperature (T) and z value for each pathogen required to achieve this standard was calculated using following equation:

$$TDT_T = 10^{\log(4) + \frac{120 - T}{z}}$$

Statistical analysis

Statistical analyses were performed using SAS software version 9.1 (SAS Institute, Inc., Cary, NC, USA). Data from triplicate experiments were compared using analysis of variance (ANOVA). Statistical significance was determined using Tukey's studentized range test. A $p<0.05$ was considered significant.

Results and Discussion

Survival of target pathogens in a multi-grain soy milk product after heating

The viscosity of the multi-grain soy milk product ranged from 19-20 cP, which was higher than that of a normal soy milk product that did not contain any supplementary ingredients (4-5 cP) (18). The initial populations of *B. cereus*, *E. coli* O157:H7, and *L. monocytogenes* in the inoculum were 6.4-6.5, 7.1-7.3, and 6.4-6.5 log CFU/mL, respectively. The TDCs at 55, 60, and 65°C were generated by fitting the data to the log linear model, with $R^2>0.9$; thus the models fitted the data well. Figure 1 shows the survival of each pathogen in the multi-grain soy milk after heating at 55, 60, or 65°C. Heating at 55°C for 30 min reduced the *B. cereus* and *L. monocytogenes* populations by 1.9 and 2.1 log CFU/mL, respectively; however, the same treatment reduced the *E. coli* O157:H7 population by <1 log CFU/mL (Fig. 1A). At 60°C, the viable cell count for *B. cereus* was reduced by up to 3.0 log CFU/mL within 4 min; however, *E. coli* O157:H7 and *L. monocytogenes* survived (4.5-5.2 log CFU/mL), even after heating for 6 min (Fig. 1B). More than 4 log CFU/mL of *L. monocytogenes* endured heating to 65°C for 2 min, whereas heat treatment for 1 min at the same temperature reduced the number of *B. cereus* and *E. coli* O157:H7 by up to 2.9 and 3.9 log CFU/mL, respectively (Fig. 1C).

Farber *et al*. (32) reported that heating a milk product inoculated with *L. monocytogens* to 64.5-66°C for <17 s significantly reduced the population by 3.1-3.7 log CFU/mL, resulting in 1.2-1.8 log CFU/mL of *L. monocytogens* remaining after this procedure. D'Aoust *et al*. (28) also reported that *E. coli* O157:H7 in a fluid milk was completely inactivated after pasteurization at >64.5°C for 16.2 s. Thus, the thermal resistances of these micro-organisms are higher in multi-grain soy milk than in cow's milk.

Thermal resistance characteristics of the target pathogens in a multi-grain soy milk product

Table 2 shows the $D_{55\text{-}65°C}$ values and z values for *B. cereus*, *E. coli* O157:H7, and *L. monocytogenes* in the multi-grain soy milk product. $D_{55°C}$ was highest for *B. cereus* (22.8 min), followed by *E. coli* O157:H7 (18.8 min) and *L. monocytogenes* (17.6

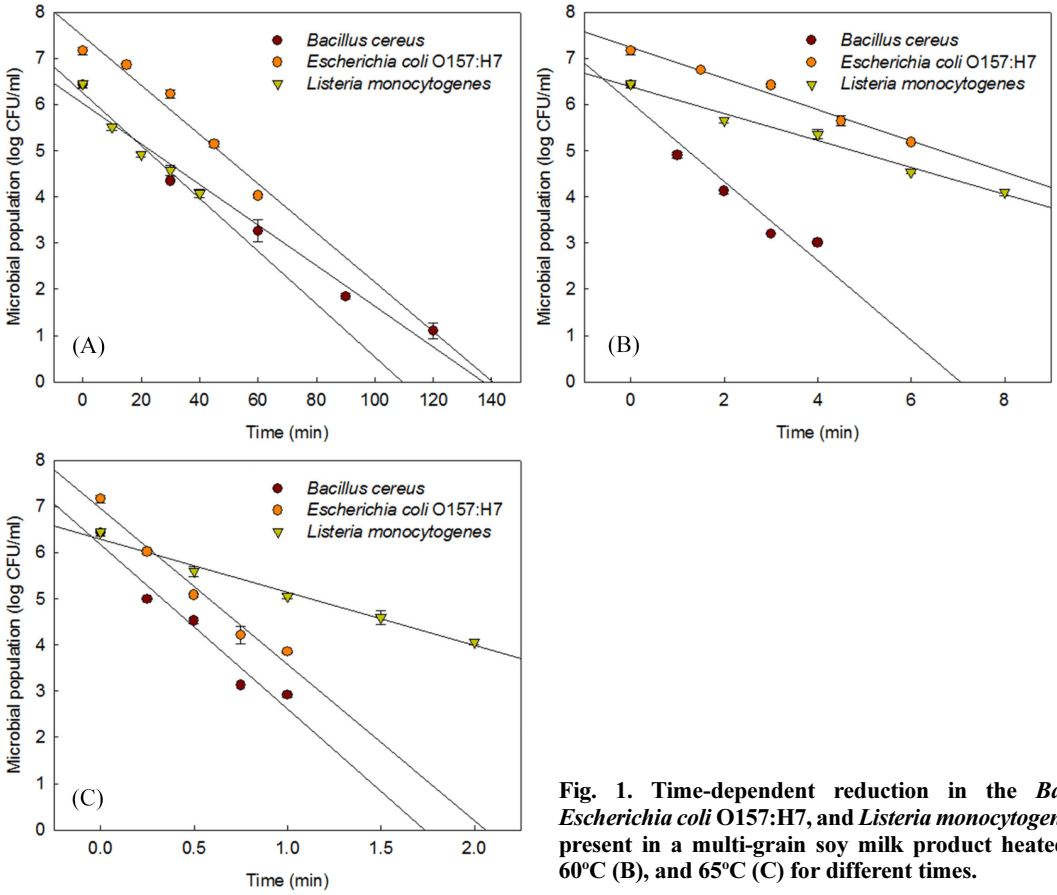

Fig. 1. Time-dependent reduction in the *Bacillus cereus*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* populations present in a multi-grain soy milk product heated at 55°C (A), 60°C (B), and 65°C (C) for different times.

min) (p<0.05). When heated at 60°C, the thermal resistance of *L. monocytogenes* (D value=4.5 min) was higher than that of *E. coli* O157:H7 (3.0 min) and *B. cereus* (1.2 min). Heating at 65°C rapidly inactivated the target pathogens, resulting in D values of 0.3-0.9 min. The D value versus heating temperature curves were generated by fitting the data to a log linear model, with R^2>0.95 (Fig. 2). The calculated z values are shown in the right-hand column of Table 2. The z values for *B. cereus*, *E. coli* O157:H7, and *L. monocytogenes* in the multi-grain soy milk product were 5.2, 5.6, and 7.7°C, respectively, meaning that *B. cereus* was more susceptible to changes in heating temperature than either *E. coli* O157:H7 or *L. monocytogenes* (p<0.05).

Previous studies calculated D values for these pathogens in a variety of food samples. Byrne *et al*. (29) reported that the $D_{55°C}$ and $D_{60°C}$ values for *B. cereus* in pork luncheon roll were only 6.4 and 1.0 min, respectively (z value=6.6). Juneja and Marmer (33) examined *E. coli* O157:H7 in various ground meat products (lamb, turkey, and pork) at 55-65°C, and reported the following $D_{55\text{-}65°C}$ values: 11.9-0.4 min for lamb (z value=6.5), 11.5-0.3 min for turkey (z value=6.9), and 11.5-0.3 min for pork (z value=6.5). When *E. coli* O157:H7-inoculated ground beef was heated to the same temperature, the D values were 21.1-0.4 min (z value=6.0), while those in ground chicken (11.8-0.4 min) were similar to those of lamb, turkey, and pork (z value=6.8) (30). McCormick *et al*. (31) reported that the $D_{60°C}$ and $D_{65°C}$ for *L. monocytogenes* in a packaged low-fat ready-to-eat turkey bologna were 2.1 and 0.3 (z value=4.4), respectively. Farber (34) reported that D values for *L. monocytogenes* in ground meat at 56-62°C ranged from 13.2-1.0 min, resulting in a z value of 4.9.

Here, we found that the $D_{55°C}$ values for *B. cereus* (22.8 min), *E. coli* O157:H7 (18.7 min), and *L. monocytogenes* (17.6 min) in a multi-grain soy milk product were higher than those for meat

Table 2. D and z values for *Bacillus cereus*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* in a multi-grain soy milk product at 55, 60, and 65°C

Bacteria	D values (min) at temperature below (R^2)			Z values (°C)
	55°C	60°C	65°C	
Bacillus cereus	22.80±0.85^{B} (0.97)	1.17±0.02^{A} (0.94)	0.28±0.00^{A} (0.95)	5.24±0.04^{A} (0.96)
Escherichia coli O157:H7	18.74±0.16^{A} (0.95)	2.97±0.17^{B} (0.98)	0.30±0.02^{A} (0.97)	5.55±0.08^{A} (0.99)
Listeria monocytogenes	17.60±1.02^{A} (0.97)	3.44±0.06^{C} (0.98)	0.87±0.05^{B} (0.98)	7.66±0.27^{B} (0.99)

$^{A-C}$Values denoted by different superscript capital letters in the same column are significantly different (p<0.05).

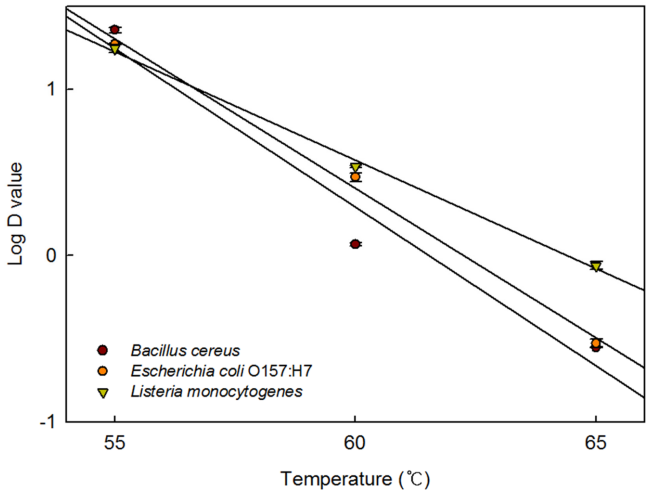

Fig. 2. Temperature-dependent-changes in the thermal resistance (D values) of *Bacillus cereus*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* in a multi-grain soy milk product.

products examined in the previous reports mentioned above. The $D_{60°C}$ values also tended to be higher. In general, when heating a solid-liquid mixture, the rate of heat transfer in the liquid part is higher than that in the solid part. Sandeep and Puri (35) showed that during the heating procedure, the temperature of the liquid fraction increases first. This heat is then transferred to the solid particles by convection. Thus, it is generally believed that the thermal resistance of a microorganism is higher in solid products than in liquid products. By contrast, in this study, the thermal resistances of *B. cereus*, *E. coli* O157:H7, and *L. monocytogenes* were higher than those in solid products such as meat products. This may be attributed to the higher content in solid substances, such as the powder of various grains (adlay, barley, black rice, dried chestnut, embryo bud of brown rice, sesame, wheat), in multi-grain soy milk, which may decrease the heat conductivity of this product, thereby diminishing the thermal inactivation of the pathogenic bacteria present. Therefore, it can be concluded that target pathogens contaminating multi-grain soy milk are more difficult to kill by pasteurization at low temperatures (55-60°C) than those in a normal soy milk product that does not contain various grains.

TDT for industrial retort processing

Using the calculated z values, we next calculated the TDT required to achieve the national standard for sterilization of retort food products (120°C, 4 min) using an industrial steam retort processor (122±2°C) (Table 3). *L. monocytogenes* required the longest time (0.7-2.2 min) to achieve this standard (122-126 °C; $p<0.05$), whereas *E. coli* O157:H7 and *B. cereus* required 0.3-1.7 and 0.3-1.8 min, respectively. In other words, *L. monocytogenes* had the highest thermal resistance, followed by *E. coli* O157:H7 and *B. cereus*.

However, if this product is contaminated with more than one species of pathogenic bacteria or with the heat resistant endospores of *B. cereus*, the thermal resistance values of the target microorganism (i.e., D value, z value, and thermal death time for industrial retort processing) may be altered.

Table 3. Thermal death time required for an industrial retort processor to meet a mandatory regulation regarding retort products (120°C, 4 min)

Temp (T, °C)	Thermal death time at T (min)		
	Bacillus cereus	*Escherichia coli* O157:H7	*Listeria monocytogenes*
122	1.66±0.01 ^{A}	1.75±0.02 ^{B}	2.19±0.05 ^{C}
123	1.07±0.01 ^{A}	1.15±0.02 ^{B}	1.62±0.05 ^{C}
124	0.69±0.01 ^{A}	0.76±0.02 ^{A}	1.20±0.05 ^{B}
125	0.44±0.01 ^{A}	0.50±0.01 ^{B}	0.89±0.05 ^{C}
126	0.29±0.01 ^{A}	0.33±0.01 ^{A}	0.66±0.04 ^{B}

$^{A-C}$Values denoted by different superscript capital letters in the same row are significantly different ($p<0.05$).

Conclusion

This study suggests that major foodborne pathogenic bacteria (*B. cereus*, *E. coli* O157:H7, and *L. monocytogenes*) in a multi-grain soy milk consisting of 22 ingredients had relatively higher thermal resistance than in other liquid food products. We attribute this to the higher fluid viscosity of the multi-grain soy milk compared with that of plain soy milk. In particular, *L. monocytogenes* had the strongest thermal resistance followed by *E. coli* O157:H7 and *B. cereus*. The data presented in this study will be useful for soy milk manufacturers and will enable them to design appropriate sterilization processes (methods and target microorganisms) and procedures (specific conditions: temperature and time) to control potential microbiological hazards present in commercial soy milk products. In addition, these data can be used practically to set the critical limit for the multi-grain soy milk product-specific critical control point (commercial sterilization) in the Hazard Analysis Critical Control Point (HACCP) system.

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