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Research Note

Effect of High-Pressure Processing on Strains of *Enterobacter sakazakii*

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ABSTRACT

Four strains of *Enterobacter sakazakii* were inoculated into tryptic soy broth and reconstituted powdered infant formula and exposed to high-pressure processing. Pressures of 200, 400, and 600 MPa were used for each medium for 1 min. *E. sakazakii* was reduced by more than 6 log (strains A and B) in both media at 600 MPa. Strain B was significantly ($P \leq 0.05$) more pressure resistant than the other strains, with just more than a 3-log reduction at 600 MPa in both media. The reconstituted infant formula has a significant ($P \leq 0.05$) protective effect for certain strains and pressures (strain B at 400 MPa and strain D at 400 and 600 MPa). Differences in log reductions between media (milk and broth) were also observed for certain strains and specific pressures (strain B at 400 MPa and strain D at 400 and 600 MPa; $P \leq 0.05$). This research showed that *E. sakazakii*, when present in reconstituted powdered infant formula, can be submitted to high-pressure processing (600 MPa for 1 min) and achieve log reductions ranging from 3 to 6.84, depending on the strain.

High hydrostatic pressure processing is becoming recognized worldwide as a safe alternative to thermal processing that effectively reduces microbial loads and does not affect organoleptic properties in some products *(2, 11)*.

The effects of high pressure on microorganisms vary greatly, depending on many factors, such as species, strain, medium, temperature, amount of pressure during treatment, and growth phase *(1–3, 7, 9, 11)*. All of these factors are affected by the impact that pressure has on morphology, cell membrane and wall, biochemical reactions, and genetic mechanisms of microorganisms *(8)*.

The effect of high-pressure processing on *Enterobacteriaceae* has been researched extensively *(1, 3, 6, 7, 9, 11, 13, 14)*; however, to our knowledge, no research has been published that examines the effect of pressure on *Enterobacter sakazakii*, a potential emerging pathogen that has been reported to infect neonates that are fed milk-based powdered infant formulas with an outcome that could lead to meningitis, sepsis, cerebritis, and necrotizing enterocolitis *(5, 10, 12)*.

The purpose of this research was to analyze the effects of different pressures (200, 400, and 600 MPa) for 1 min on reconstituted powdered infant formula and trypic soy broth (TSB) inoculated with several serotypes of *E. sakazakii*.

MATERIALS AND METHODS

Strains. *E. sakazakii* strains were purchased from the American Type Culture Collection (Manassas, Va.) (strain A = ATCC 51329; strain C = ATCC 12868; and strain D = ATCC 29004) and Biotrace International Bioproducts (Lyfocult, Bothell, Wash.) (strain B = ATCC 29544). Each strain was reconstituted following instructions from the providers. Next, according to the U.S. Food and Drug Administration (FDA) isolation and enumeration method *(12)*, a loopful of each strain was streaked onto violet red bile glucose agar plates (36°C, 24 h), and typical colony morphology (purple colonies surrounded by purple halo) was visually confirmed. Colonies from each strain were then streaked onto a Trypticase soy agar (TSA; BD Diagnostic Systems, Sparks, Md.) plate at 25°C for 48 to 72 h and checked for yellow pigment formation. Strain identification was biochemically confirmed by the API 20E biochemical identification system (bioMérieux, Basingstoke, UK).

Preparation and inoculation of infant formula and broth. Cans of commercial dehydrated infant formula fortified with iron were purchased from local retail stores. The formula was aseptically rehydrated following instructions on the cans (1 scoop [8.5 g] per 60 ml of water). The milk was prepared in a sterile container with sterile distilled water (90% formulation). Nine milliliters of milk was transferred to sterile tubes and exposed to a heat treatment at 90°C for 10 min to ensure a reduction in the original microbial load while preserving the physicochemical properties of the milk.

TSB (Bacto, BD Diagnostic Systems) was prepared following instructions, and 9 ml was placed in tubes, which were then sterilized (15 min, 121°C).

A colony (TSA plates) from each strain was inoculated into the 10-ml TSB tubes and incubated at 36°C for 19 h (strains A, B, and C) and for 14 h (strain D) to achieve a concentration of 10^8 CFU/ml, which was then diluted to 10^7 CFU/ml. Sterile 9-ml TSB tubes and the heat-treated reconstituted infant formula 9-ml tubes were both inoculated with 1 ml of the above prepared inoculum (10^7 CFU/ml in TSB tubes) to achieve an initial *E. sakazakii* load of 10^6 CFU/ml of each strain and a 100% formula concentration.

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High-pressure processing. Inoculated TSB and reconstituted infant formula were immediately transferred to sterile stomacher bags (4 by 6 in. [10.16 by 15.24 cm], 3 ml; Fisher Scientific, Pittsburgh, Pa.), heat sealed, and then double-bagged with vacuum pouches (6 by 8 in. [15.24 by 20.32 cm] and 8 by 12 in. [20.32 by 30.24 cm]; Prime Source, Bunzl Distributions Inc., St. Louis, Mo.); the latter had been sprayed with chlorine solution (10%; 5 ml) to prevent contamination in case of pouch puncture or leakage. Each sample (infant formula and TSB) of each strain was pressure treated at 200, 400, and 600 MPa for 1 min. The samples were processed with a high-pressure processing unit (Quintus Food Press QFP 35 L-600, Avure Technologies, Inc., Kent, Wash.), which is capable of producing pressures of up to 600 MPa between 5 and 50°C with a pressure vessel volume of 35 liters. The hydrostatic pressurization medium was filtered tap water filled from the bottom. The temperatures in the vessel at the beginning of pressurization for the 200-, 400-, and 600-MPa treatments were 16.7, 12.3, and 8.43°C, respectively, and the temperatures at which the bacteria were exposed under pressure for 1 min in these treatments were 21.8, 23.3, and 25.3°C, respectively ($n = 3$ per pressure).

Microbiological analysis. After pressure treatments, samples were immediately plated. Appropriate dilutions were performed in 0.1% peptone solution, and 1- or 0.1-ml volume dilutions were plated onto TSA plates (duplicate). Plates were incubated at 30°C for 24 h, and representative colonies were counted and confirmed by API 20E.

Statistical analysis. The experiment was repeated three times. Log reductions ($\log N_0 - \log N$) were averaged per treatment and subjected to one-way analysis of variance by Jump software (JMP; SAS Institute, Cary, N.C.). Mean differences were analyzed by the Tukey-Kramer honestly significant difference test.

RESULTS AND DISCUSSION

E. sakazakii was not detected (less than 1/10 g) in the reconstituted infant formula that had been heat treated prior to inoculation of the bacteria, which ensured that the amounts of bacteria present after pressurization came from the inoculation and not from the original microbial population of the infant formula. Edelson-Mammel and Buchanan *(4)* suggested that the presence of *E. sakazakii* in infant formula has been reported to be ca. 1 CFU/100 g of dry formula and that dried infant formula should be prepared with water at 70°C or more to ensure the absence of bacteria per serving size and to maintain the nutritional and physicochemical properties of the milk.

In the present study, pressures at 600 MPa for 1 min (25°C) (Table 1) achieved at least a 6-log reduction in the population of *E. sakazakii* strains A, C, and D in TSB and in the population of strains A and C in reconstituted infant formula. Strain D showed a 5-log reduction in infant formula after treatment, whereas strain B was significantly more pressure tolerant than the other strains in broth and formula ($P \leq 0.05$) (Table 1). However, pressurization at 600 MPa still showed at least a 3-log reduction in strain B. The effect of 200 MPa on the population of all the strains in broth or formula was almost negligible.

As observed in Table 1, some of the strains achieved a 3-log reduction in broth when subjected to 400 MPa (strains A and D). In infant formula, log reductions of 0.5 to 2 log for all the strains were observed, with strains A and D being the most sensitive. It was also observed that at 400 MPa, the populations of strains B and D were more resistant in infant formula ($P \leq 0.05$). Strain D was the only one that showed a significant difference in log reductions when broth and formula were compared at 600 MPa. Gervilla et al. *(7)* showed that ovine milk protected *Escherichia coli* CECT 405 from high pressure, which suggests that inactivation of the bacteria was highly influenced by the composition of the media or food *(9)*.

TABLE 1. *Inactivation of* Enterobacter sakazakii *strains on TSB and reconstituted infant formula with iron after pressurization at 200, 400, and 600 MPa for 1 min*

Pressure (MPa)	*E. sakazakii* strains[a]: A	B	C	D
	TSB			
200	0.87	0.47	0.34	0.88
400	2.96 A[b]	1.68 B	1.44 B	3.73 A
600	6.72 A	3.05 B	6.91 A	6.91 A
	Reconstituted infant formula			
200	0.63 A	0.18 B	0.30 B	0.33 AB
400	1.91 A	0.42 B	0.90 BC	1.65 AC
600	6.84 A	3.11 B	6.44 A	5.04 C

[a] Strain A (ATCC 51329), strain B (ATCC 29544), strain C (ATCC 12868), and strain D (ATCC 29004). Values represent means ($n = 3$); $\log N_0 - \log N$ CFU/ml (initial *E. sakazakii* load before pressurization − survival of *E. sakazakii* after pressurization).

[b] Values with different letters in the same row are significantly different statistically ($P \leq 0.05$).

The log reductions achieved in this study occurred after 1 min of pressure treatment; however, studies on the effects of high pressure on other *Enterobacteriaceae* used a longer exposure to pressure at levels of 300 to 500 MPa *(1–3, 6, 7, 9, 11, 13, 14)*. To our knowledge, there has been no published research that examines the effect of high pressure on *E. sakazakii*; however, from data reported in the articles cited, it appears that the log reductions achieved by *E. sakazakii* were similar to other *Enterobacteriaceae*, even though the treatment occurred only for 1 min and not 10 or 15 min.

Variations in pressure resistance were observed between strains. Strain B showed a significantly greater pressure resistance in broth and formula when compared to the other strains. Some authors have reported variations in pressure resistance between strains that have been caused by different factors. Alpas et al. *(1)* suggested that differences in pressure resistance among strains occur at low temperatures (25°C) and not at 50°C. In the present study, the bacteria were pressure treated at ca. 25°C, and if, indeed, the variations in pressure tolerance existed because of temperature, additional research should be performed with *E. sakazakii* to determine if the pressure tolerance differences between strains show a continuum at higher temperatures. However, some authors have suggested that variations in pressure resistance between strains occur more in the sta-

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tionary phase than in the exponential phase *(2, 11)* because of differences in the stationary-phase–inducible sigma factor RpoS, which controls genes related to protection against stress. In the present study, all the strains had been grown to stationary phase, suggesting that strain B would have a higher *rpoS* activity *(11)*. However, research that relates *rpoS* with the pressure resistance of *E. sakazakii* strains has not been performed. Heat resistance differences among *E. sakazakii* strains have also been observed, which suggests that the bacteria have a simple set of genetic determinants, because the thermoresistance of these strains fell into only two phenotypes *(4)*.

High-pressure processing effectively inactivated some *E. sakazakii* strains in broth and infant formula, especially at 600 MPa for 1 min. More research should be performed that compares different exposure temperatures and times in combination with different pressures to establish the effect on the pressure-resistant strain and to observe what is responsible for this increase in resistance.

According to the Food and Agricultural Organization/World Health Organization *(5)*, researchers are encouraged to search for treatments that can reduce existing *E. sakazakii* populations in reconstituted powdered infant formula. This initial study of the effect of high pressure on *E. sakazakii* showed that at least a 3-log reduction in the most resistant strain and a 6-log reduction in the other strains could be achieved. More research is required to study the mechanism of high-pressure inactivation of *E. sakazakii* in order to gain an understanding of why certain strains are more pressure tolerant than others and to seek technologies and components that might act synergistically to reduce the pressure tolerance of this group of microorganisms.

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