Short communication: Effects of high-pressure processing on the inactivity of *Cronobacter sakazakii* in whole milk and skim milk samples

Rui Jiao,*† Jina Gao,* Yinxiang Li,* Xiyan Zhang,* Maofeng Zhang,* Yingwang Ye,*†¹ Qingping Wu,† and Hongying Fan‡

*Center of Detection and Control of Foodborne Risk Factors, School of Food Science and Engineering, Hefei University of Technology, Hefei, 230009, China

†State Key Laboratory of Applied Microbiology, South China (the Ministry–Province Joint Development), Provincial Key Laboratory of Microbiology Culture Collection and Application, Guangdong Institute of Microbiology, Guangzhou, 510070, China

‡School of Public Health and Tropical Medicine, Southern Medical University, Guangzhou, Guangdong Province, 510515, China

ABSTRACT

Powdered infant formula is considered as the main transmission vehicle for *Cronobacter sakazakii* infections including meningitis, septicemia, and necrotizing enterocolitis. The effects of high-pressure processing treatment on inactivation of *C. sakazakii* ranging from 100 to 400 MPa for 3.0, 5.0, and 7.0 min in whole milk and skim milk were studied. Significant differences in inactivation of *C. sakazakii* were observed in milk samples under different pressures for 3 to 7 min compared with untreated samples, and *C. sakazakii* was not detected after 400 MPa for 3 min. The lethality rates of *C. sakazakii* cells in whole and skim milk with an initial level of 10^4 cfu/mL after 100 and 200 MPa treatments were not significantly different, but relatively higher lethality rates were found in whole milk after 300 MPa treatment than in skim milk. Finally, the scanning electron micrographs indicated that cellular envelope and intracellular damage of *C. sakazakii* cells were apparent after 300 and 400 MPa for 5.0 min compared with the untreated cells, and a progressive increase of injured cells with increased pressure treatment was found. It was concluded that *C. sakazakii* was sensitive to high-pressure processing treatment and that high-pressure processing treatment with 400 MPa for 3.0 min can be used to control *C. sakazakii* contamination in milk samples.

Key words: high-pressure processing, *Cronobacter sakazakii*, inactivation, whole milk, skim milk

Short Communication

The genus *Cronobacter* is a group of foodborne pathogens, including *C. sakazakii*, *C. malonaticus*, *C.*

turicensis, *C. muytjensii*, *C. condimenti*, *C. universalis*, and *C. dublinensis*, that cause meningitis, septicemia, and necrotizing enterocolitis in neonates (Iversen et al., 2008; Joseph et al., 2012). Epidemiologic surveys indicated that powdered infant formula was the main transmission vehicle for *Cronobacter* infections (Biering et al., 1989; van Acker et al., 2001; FAO/WHO, 2008). In addition, Xu et al. (2014) reported that *C. sakazakii* was the most frequent species within the genus of *Cronobacter* from powdered milk samples. Under various environmental stresses, several studies have shown that *Cronobacter* species have unusual abilities to survive (Gurtler and Beuchat, 2005; Gurtler et al., 2005; Lin and Beuchat, 2007; Shaker et al., 2008; Ye et al., 2014). Consequently, the control of *C. sakazakii* is of importance for ensuring microbial safety of milk samples.

High-pressure processing (HPP) is a promising alternative method to conventional thermal pasteurization for improving food safety due to the inactivation of foodborne pathogens (Yang et al., 2012; Yao et al., 2014; Sheen et al., 2015); minimizing the loss of nutrients, such as vitamins, and flavor; and maintaining the quality of food products (Kaletunç et al., 2004; Lee and Kaletunç, 2010). High-pressure processing has been approved to be applied in food processing procedures by the US Food and Drug Administration and the USDA. In recent years, HPP has been used to inactivate the important foodborne pathogens such as *Staphylococcus aureus* in milk or meat products (Yao et al., 2014), pathogenic *Escherichia coli* (Sheen et al., 2015), *Salmonella* (Wang et al., 2013a), *Vibrio parahaemolyticus* (Wang et al., 2013b), and *Listeria monocytogenes* (Munoz-Cuevas et al., 2013) in various food samples. However, little research has focused on the inactivation of HPP on *C. sakazakii* in milk, which is considered the main transmission vehicle.

The current study was to determine the effects of HPP on inactivation of *C. sakazakii* with different inoculum levels into whole milk and skim milk samples.

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¹Corresponding author: yeyw04@mails.gucas.ac.cn

In addition, the morphological changes in HPP-treated *C. sakazakii* cells was also evaluated using a scanning electron microscope.

The type strain ATCC 29544 was incubated into tryptic soy broth for 16 h at 37°C and different concentrations (3.2×10^5 and 1.7×10^3 cfu/mL) were obtained using the 10-fold method. Then, 1.0 mL of *C. sakazakii* suspension (10^5 and 10^3 cfu/mL) was incubated with 9 mL of whole milk and skim milk, respectively. Thus, the whole milk and skim milk samples containing 10^4 or 10^2 cfu/mL were prepared. The whole milk and skim milk samples incubated with *C. sakazakii* were treated with HPP using 100, 200, 300, and 400 MPa for 0 min (control samples), 3.0, 5.0, and 7.0 min. Each experiment was performed in triplicate.

After HPP treatment, the survival of *C. sakazakii* was counted using the 10-fold dilution method. The lethality rate was determined as the decreasing percentage between colony counts of the control (untreated) and treated milk samples. Each experiment was replicated 3 times.

Under 100 to 400 MPa pressure treatment for 3.0, 5.0, and 7.0 min, a significant difference ($P < 0.05$) of survival of *C. sakazakii* under the holding times was observed (Table 1). In addition, different inactivation under 200 to 400 MPa treatments was extremely significant ($P < 0.01$) compared with untreated whole milk samples. The inactivation effect of HPP was significantly enhanced by increased pressure intensity ($P < 0.05$), and cell counts were not detected at 400 MPa for 3.0 min. Similar results of survival of *C. sakazakii* in skim milk samples after HPP treatment are shown in Table 1. Yao et al. (2014) reported that *S. aureus*

in sterile saline and minced meat samples can survive after 450 MPa for 15 min, but no *S. aureus* can be detected after 550 MPa for 3.0 min. Less than 0.5 log cfu/mL of *S. aureus* survived after 500 MPa for 5.0 min in buffered peptone water with an initial inoculum of 10^4 cfu/mL (Yuste et al., 2004). Wuytack et al. (2002) found no significant reduction of *S. aureus* after 400 MPa for 15 min in PBS buffer. Munoz-Cuevas et al. (2013) reported that reduction of *Listeria monocytogenes* in nonselective medium was about 3 log cfu/mL after 400 MPa for 3.0 min. Gram-negative bacteria might be more sensitive to HPP treatment due to the structure and composition of the outer membrane. For example, the high hydrostatic pressure (300 MPa for 10 min) could extensively inactivate *Vibrio parahaemolyticus* from 10^9 cfu/mL to no viable cells (Wang et al., 2013b). After high hydrostatic pressure (350 MPa for 5.0 min), the number of viable *Salmonella enterica* decreased from 10^9 cfu/mL to no viable cells (Wang et al., 2013a). Consequently, inactivation of HPP on different foodborne bacteria was species or strain dependent.

In addition, the food matrix also played important roles in the inactivation of bacteria (Alpas et al., 2003). In this study, lethality rates of *C. sakazakii* in whole milk and skim milk were also analyzed. In Table 2, the lethality rates of *C. sakazakii* cell in whole and skim milk with an initial level of 10^4 cfu/mL after the 100 and 200 MPa treatment were not significantly different ($P > 0.05$). In addition, the total tendency of lethality rates in whole milk was lower than in skim milk after 100 and 200 MPa treatment for 3.0 and 5.0 min, indicating that whole milk provided a certain protective effect for inactivation of *C. sakazakii* under relatively

Table 1. Survival of *Cronobacter sakazakii* in milk samples after treatment with high-pressure processing

		Survival of <i>C. sakazakii</i>			
Sample	Treatment time (min)	100 MPa	200 MPa	300 MPa	400 MPa
Whole milk	0	$3.2 \pm 0.30 \times 10^{4A,a}$	$3.2 \pm 0.30 \times 10^{4A,a}$	$3.2 \pm 0.30 \times 10^{4A,a}$	$3.2 \pm 0.30 \times 10^{4A,a}$
	3	$2.5 \pm 0.19 \times 10^{4A,bcd}$	$1.7 \pm 0.11 \times 10^{4BCD,bc}$	$0.8 \pm 0.01 \times 10^{3BC,bc}$	0
	5	$2.3 \pm 0.20 \times 10^{4A,cd}$	$1.2 \pm 0.05 \times 10^{4CD,cd}$	$0.3 \pm 0.02 \times 10^{3C,c}$	0
	7	$2.1 \pm 0.03 \times 10^{4A,d}$	$0.8 \pm 0.04 \times 10^{4D,d}$	0	0
	0	$4.6 \pm 0.30 \times 10^{2A,a}$	$4.6 \pm 0.30 \times 10^{2A,a}$	$4.6 \pm 0.30 \times 10^{2A,a}$	$4.6 \pm 0.30 \times 10^{2A,a}$
	3	$3.9 \pm 0.01 \times 10^{2ABC,bc}$	$1.9 \pm 0.30 \times 10^{2BCD,bcd}$	$0.7 \pm 0.30 \times 10^{2BC,bc}$	0
	5	$3.5 \pm 0.03 \times 10^{2BC,cd}$	$1.5 \pm 0.30 \times 10^{2CD,cd}$	$0.2 \pm 0.10 \times 10^{2C,c}$	0
	7	$3.3 \pm 0.04 \times 10^{2C,d}$	$1.2 \pm 0.25 \times 10^{2D,d}$	0	0
Skim milk	0	$3.2 \pm 0.15 \times 10^{4A,a}$	$3.2 \pm 0.15 \times 10^{4A,a}$	$3.2 \pm 0.15 \times 10^{4A,a}$	$3.2 \pm 0.15 \times 10^{4A,a}$
	3	$2.2 \pm 0.14 \times 10^{4BCD,bcd}$	$1.5 \pm 0.07 \times 10^{4BC,b}$	$6.9 \pm 0.06 \times 10^{3B,b}$	0
	5	$2.1 \pm 0.06 \times 10^{4CD,cd}$	$1.1 \pm 0.08 \times 10^{4CD,cd}$	$4.2 \pm 0.02 \times 10^{3C,c}$	0
	7	$1.9 \pm 0.08 \times 10^{4D,d}$	$9.2 \pm 0.06 \times 10^{3D,d}$	$1.2 \pm 0.01 \times 10^{3D,d}$	0
	0	$5.3 \pm 0.45 \times 10^{2A,a}$	$5.3 \pm 0.45 \times 10^{2A,a}$	$5.3 \pm 0.45 \times 10^{2A,a}$	$5.3 \pm 0.45 \times 10^{2A,a}$
	3	$3.3 \pm 0.25 \times 10^{2ABC,bcd}$	$1.6 \pm 0.25 \times 10^{2BCD,bcd}$	$0.8 \pm 0.30 \times 10^{2BC,bc}$	0
	5	$2.9 \pm 0.40 \times 10^{2BC,cd}$	$1.1 \pm 0.20 \times 10^{2CD,cd}$	$0.3 \pm 0.10 \times 10^{2C,c}$	0
	7	$2.6 \pm 0.04 \times 10^{2C,d}$	$0.6 \pm 0.15 \times 10^{2D,d}$	0	0

^{a-d}Within the same column, different superscripts indicate significant differences ($P < 0.05$).

^{A-D}Within the same column, different superscripts indicate significant differences ($P < 0.01$).

lower pressure. Interestingly, significant differences of lethality rates ($P < 0.05$) were observed in whole milk and skim milk after 300 MPa for 3.0, 5.0, and 7.0 min, and higher lethality rates were found in whole milk than in skim milk. These contradictory findings might be related to the interaction between structure or compositions of outer membrane and fat after HPP, but a detailed mechanism remains to be revealed. Previous studies have indicated that the food matrix has a protective effect on the microorganism against high pressure (Panagou et al., 2007; Yao et al., 2014). A comparison between the survival curves in phosphate buffer and gilt-head seabream showed a clear protective effect of the food matrix on the resistance of *Pediococcus damnosus*, especially at 500 and 550 MPa (Panagou et al., 2007). After high hydrostatic pressure of 350 and 450 MPa for 3.0, 6.0, 9.0, 12, and 15 min, the inactivation of *Staphylococcus aureus* in sterile saline was higher than in minced meat (Yao et al., 2014). However, no significant differences in inactivation of *E. coli* were observed in cooked ham homogenized with water (3:1) and in phosphate buffer after 500 MPa for 10 min (Garriga et al., 2002).

Furthermore, *C. sakazakii* cells were prepared from pressure-treated and untreated cell suspensions via centrifugation at $5,000 \times g$ for 10 min at room temperature after washing the pellet twice in 0.1 M phosphate buffer (pH 7.4). The subsequent steps were described by Wang et al. (2013a). Finally, the morphological changes of *C. sakazakii* after HPP were analyzed by performing scanning electron microscopy (Hitachi S4700, Hitachi, Tokyo, Japan) at 15 kV voltage, and photomicrographs were obtained.

From Figure 1, scanning electron microscopy indicated that morphological changes of cell structure such

as cytomorphosis and cellular crimp were observed after 300 and 400 MPa for 5.0 min, whereas untreated cells exhibited intact cell structure and clear cell wall of *C. sakazakii*. In addition, a progressive increase of injured cells occurred with increased pressure treatment, which was consistent with the survival of *C. sakazakii* under 300 and 400 MPa. After 300 MPa treatment, the structure of some cells was intact, whereas *C. sakazakii* cells were completely injured under 400 MPa. In *V. parahaemolyticus*, some cell disruptions were caused by 300 MPa treatment. Similar results were observed in *E. coli* and *S. aureus* by transmission electron microscopy as described by Yang et al. (2012). In addition, some bud scars were caused and the membrane integrity of cells was lost after HPP treatment in this study, suggesting that the cellular wall and outer membrane might be the main target of HPP. Hayakawa et al. (1994) suggested that protein denaturation might be the main mechanism for inactivation by HPP treatment. Moreover, metabolic activity and membrane potential of foodborne pathogens decreases after HPP treatment (Ritz et al., 2001). We inferred that inactivation of *C. sakazakii* after HPP treatment was associated with cell disruption, and simultaneously the release of cytoplasmic content caused the loss of viability of cells and denaturation of functional components of cells such as enzymes and structural proteins.

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Table 2. The comparison of lethality rate (%) between whole and skim milk after high-pressure processing (HPP) treatment

HPP	Treated time (min)	10 ⁴ cfu/mL		10 ² cfu/mL	
		Whole milk	Skim milk	Whole milk	Skim milk
100 MPa	3	21.88 ± 5.93 ^{A,a}	34.46 ± 4.31 ^{A,a}	15.22 ± 0.22 ^{A,a}	37.74 ± 4.72 ^{A,b}
	5	28.13 ± 6.25 ^{A,a}	36.62 ± 1.85 ^{A,a}	23.91 ± 0.65 ^{A,a}	45.28 ± 7.55 ^{A,a}
	7	34.34 ± 0.94 ^{A,a}	40.31 ± 2.46 ^{A,a}	28.26 ± 0.87 ^{A,a}	50.94 ± 0.75 ^{B,b}
200 MPa	3	46.88 ± 3.44 ^{A,a}	55.08 ± 2.15 ^{A,a}	58.70 ± 6.52 ^{A,a}	69.81 ± 4.72 ^{A,a}
	5	62.50 ± 1.56 ^{A,a}	66.15 ± 2.46 ^{A,a}	67.39 ± 6.52 ^{A,a}	79.25 ± 3.77 ^{A,a}
	7	75.00 ± 1.25 ^{A,a}	71.69 ± 1.85 ^{A,a}	73.91 ± 5.43 ^{A,a}	88.68 ± 2.83 ^{A,a}
300 MPa	3	97.50 ± 0.31 ^{A,a}	78.77 ± 1.85 ^{B,b}	84.78 ± 6.52 ^{A,a}	84.91 ± 5.66 ^{A,a}
	5	99.06 ± 0.63 ^{A,a}	87.08 ± 0.62 ^{B,b}	95.65 ± 2.17 ^{A,a}	94.34 ± 1.89 ^{A,a}
	7	100	96.31 ± 0.31 ^{A,a}	100	100
400 MPa	3	100	100	100	100
	5	100	100	100	100
	7	100	100	100	100

^{a,b}Different superscripts in the same row indicate significant differences ($P < 0.05$).

^{A,B}Different superscripts indicate significant differences ($P < 0.01$).

Figure 1. Scanning electron micrographs of *Cronobacter sakazakii* cells. (A) Untreated cells, (B) cells treated at 300 MPa for 5 min, and (C) cells treated at 400 MPa for 5 min.

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