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Thermal tolerance of *Cronobacter sakazakii* and *Cronobacter pulveris* in reconstituted infant milk formula

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Abstract

Background: *Cronobacter* sspecies are the most significant foodborne pathogen in infant milk formula (IMF). These pathogens have been incriminated in severe forms of neonatal meningitis, sepsis, and necrotizing enterocolitis with a high mortality rate.

Aim: This study was performed to elucidate the effect of heat stress on *Cronobacter* spp. (*C. sakazakii* and *C. pulveris*) in reconstituted IMF (RIMF).

Methods: The reconstituted formula was inoculated with five *C. sakazakii* isolates and four *C. pulveris* isolates separately. The nine isolates of *Cronobacter* spp. were heated in RIMF at 48°C, 52°C, 56°C, 60°C, 64°C, and 66°C. The *D*- and *z*-values were determined by using linear regression analysis.

Results: The *D*-values of all isolates of *C. sakazakii* (CS1, CS3, CS4, CS5, and CS6) at 48°C, 52°C, 56°C, 60°C, 64°C, and 66°C were in the ranges 7.29–23.47, 2.77–15.50, 0.62–1.04, 0.62–1.02, 0.62–1.00, 0.62–1.00 minutes, respectively; while, the *z*-values extended from 2.50°C to 4.28°C. The *D*- values of *C. pulveris* isolates (CP1, CP2, CP3, CP4) were in the ranges 7.60–22.32, 1.42–8.45, 0.62–1.08, 0.62–0.78, 0.62–0.78, 0.62–0.79 minutes at 48°C, 52°C, 56°C, 60°C, 64°C, 66°C, respectively and the calculated *z*-values ranged from 3.33°C to 4.89°C.

Conclusion: This study may contribute to improving the understanding of the behavior of *C. sakazakii* and *C. pulveris* isolates in RIMF at various heat stress temperatures and may participate in the effective control of these pathogens in infant food production.

Keywords: *Cronobacter sakazakii*, *Cronobacter pulveris*, Thermo-tolerance, *D*-value, *z*-value, Infant milk formula.

Introduction

Breast milk is usually the most important source of nutrition for newborns and is essential for survival, immunological development, health, and early growth in life (Baker *et al.*, 2021). Alternatively, non-breast-fed newborns can be fed on commercial powdered infant milk formula (IMF) that provides a nutritious alternative to breast milk (Costa *et al.*, 2020). Infant formula is considered to be a microbiologically high-quality product, however, some microorganisms have been identified in neonatal infections associated with infant formula feeding (Garbaj *et al.*, 2015). Moreover, powdered infant formula (PIF) manufacturing relies on the blending of many soluble substances and additives into dried base powder. This factor may be considered a critical health risk and serve as a main source of foodborne pathogens that transmit to neonates (Yemiş and Delaquis, 2020). Based on some epidemiological studies on the incidence of neonatal infections, *Cronobacter* spp., particularly *C. sakazakii*, is the most important foodborne pathogens in PIF (Al-Holy *et al.*, 2010; Yemiş and Delaquis, 2020). Surveillance studies have identified *Cronobacter* spp. from different environments and foods such as milk powder, cereal formula, various dairy products, dried foods, meat products, spices, vegetables, rice, plants, etc. (Osaili *et al.*, 2009). This ubiquitous microorganism, Gram-negative, non-spore-forming bacilli has been reported to cause septicemia, meningitis, sepsis, and necrotizing enterocolitis in newborns at low infection doses (Chauhan *et al.*, 2020). In addition, *C. sakazakii* has the ability to withstand and tolerate sever stress conditions for instance high osmotic pressure and extreme dehydration (low water activity) compared

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to other *Cronobacter* spp. (Bai *et al.*, 2019; Zhang *et al.*, 2020). Reconstituted infant formula is known to provide an optimal environment for the growth of these pathogens, survive slight heat stress during preparation, and pose a significant potential risk to newborns (Xu *et al.*, 2015). Moreover, some microbiological studies revealed that *Cronobacter* spp. has the ability to grow rapidly for long periods of time at room temperature in baby bottles (Al-Holy et al., 2009; Azwai et al., 2022). Therefore, World Health Organization (WHO)/FAO suggested that PIF should be reconstituted with water at a temperature of at least 70°C (Zhang *et al.*, 2020). However, this target temperature may be difficult to adjust during home reconstitution of PIF or may not be suitable for direct feeding of neonates. Additionally, the PIF has different reconstituted instructions from different sources which may recommend lukewarm water with an alternative advised temperature (Yang *et al.*, 2015). As a result, serious concerns have been raised about the safety of infant formula during the feeding of neonates worldwide. As C. sakazakii and *C. pulveris* (recently, Stephan et al. (2014), proposed reclassifying *C. pulveris* to Franconibacter pulveris) were previously detected in dairy and meat products sold in the Libyan market (Garbaj *et al.*, 2017); therefore, *C. sakazakii* and *pulveris* needed to be evaluated for their resistance to heat stress which, is one of the most important factors affecting the behavior of these isolates in reconstituted IMF (RIMF). In contrast, from too many studies on the thermo-tolerance of *Cronobacter* spp., there is hardly any data available on variability and behavior of single isolate of *C. sakazakii* in rehydration PIF in countries such as Libya. Therefore, this study was performed to demonstrate the effect of heat stress on *Cronobacter* spp. (*C. sakazakii* and *C. pulveris* isolated from food of animal origin from Libya) in RIMF.

Materials and Methods

Bacterial strains used in this study

The nine strains of *Cronobacter* spp. that have been used in this study (Table 1), were obtained from Foodborne Libyan Type Bacterial Collection (FLBC), these strains were isolated from raw milk, powdered (IMF), some dairy products, and meat from different localities in Libya and stored in cryobeads banking system at −80°C (Garbaj *et al.*, 2017). A single bead was streaked with a wire loop under the aseptic condition on Nutrient agar (NA; CM0003, Oxoid, UK) and incubated at 37°C for 24 hours. Cells from single colonies of each strain were streaked on Tryptone Soya agar slants (TSA; Oxoid, UK), incubated at 37°C for 24 hours, and kept refrigerated until they were used.

Inoculum preparation

The bacterial cultures were separately transferred from TSA slants to Brain Heart Infusion broth ((BHI; CM0225, Oxoid, UK). The strains were transferred individually into sterilized test tubes containing 10 ml BHI and incubated at 37°C for 24 hours. Within this stage, the bacteria are considered to be in the early stationary phase, which is known as the most resistant phase to antibiotics.

Preparation of RIMF

IMF was purchased from local supermarkets and pharmacies in Tripoli, Libya. The recommended amount of powder was added and reconstitution of IMF in sterile distilled water was carried out according to the manufacturer's guideline (two scoops equal 8.6 g per 60 ml of warm water). Ten milliliters (ml) of RIMF were added into sterile test tubes and sterilized at 121°C for 15 minutes to get rid of the background microorganisms.

Thermo-tolerance of Cronobacter spp.

Thermo-tolerance of *Cronobacter* spp. was tested according to Al-Holy *et al.* (2009). The reconstituted milk was prepared and inoculated with *Cronobacter* spp., as follows: IMF was reconstituted according to the manufacturer's instructions. About 10 ml samples of RIMF were sterilized at 121°C for 15 minutes. The isolate was transferred from TSA slants into (BHI) broth and incubated at 37°C for 24 hours. Ten-fold serial dilution of the BHI culture was performed in buffered peptone water solutions (RM 001-500G HI media, India) by adding 1 ml from cultured BHI onto 99 ml of buffered peptone water dilution. A portion of

Table 1. *Cronobacter* strains used in this study.

No.	FLBC Code	Isolate code	*Cronobacter* spp.	Isolate source
1	10305.2	CS1	*C. sakazakii*	Raw Camel's milk
2	10456	CS3	*C. sakazakii*	Cereal baby food
3	2204.2	CS4	*C. sakazakii*	Meat
4	6404.2	CS5	*C. sakazakii*	Fermented milk
5	6208.2	CS6	*C. sakazakii*	Meat
6	10322.1	CP1	*C. pulveris*	Raw cow's milk
7	10324.1	CP2	*C. pulveris*	Raw cow's milk
8	10429	CP3	*C. pulveris*	Maasora cheese
9	10492	CP4	*C. pulveris*	Baby milk

100 µl from each dilution was then aseptically plated (0.1 ml in duplicate) onto (TSA) (Oxoid CM 131) and incubated at 37°C for 24 hours (stationary phase).
Finally, the dilution that had a microbial load of 10^8 CFU/ml was used for the inoculation of the prepared RIMF.
Heat treatment was conducted in a water bath at 48°C, 52°C, 56°C, 60°C, 64°C, and 66°C. The tubes were immersed completely in the heated water bath where the temperature was controlled and adjusted at the target temperature. Following each heat treatment, each tube was then removed from the water bath and immersed immediately into ice slush. At each heat treatment, 100 µl from the inoculated IMF was taken in duplicate at different time points: 0, 3, 6, 9, 12, 15, and 18 minutes. Quantification of the colonies was then performed using the overlay method (Al-Holy *et al.*, 2008). The samples were spread-plated on TSA supplemented with 0.1% (w/v) of sodium pyruvate. The plates were then incubated for 2 hours at 37°C and a thin layer (8 ml) of violet red bile agar (HIMIDIA M 581, India) was overlaid onto TSA and the plates were re-incubated for an additional 22 hours at 37°C. Standard regression analysis was performed by log-linear models in Excel (Microsoft 2010) and the *D*-value was determined by taking the negative reciprocal of the slope. The *z*-value was calculated using linear regression of the log *D*-values at six temperatures: 48°C, 52°C, 56°C, 60°C, 64°C, and 66°C.

Statistical analysis

Data were presented as mean ± SD. The decimal reduction time (*D*-value), the time required at a certain temperature to reduce a specific microbial population, and the *z*-value (the number of degrees of temperature change necessary to change the *D*-value) were determined by using Microsoft Excel (log-linear models).

Results

The microbial quantity of each strain showed relatively similar values, it ranged from 10^7 to 10^9 CFU/ ml. Table 2 shows decimal reduction times (*D*-value and *z*-value) of *C. sakazakii* and *C. pulveris* strains inoculated in RIMF. In this study, *D*-values were calculated at six different temperatures. Results in Table 2 revealed that the *D*-values of all strains of *C. sakazakii* (CS1, CS3, CS4, CS5, and CS6) at 48°C, 52°C, 56°C, 60°C, 64°C, and 66°C were in the ranges 7.29–23.47, 2.77–15.50, 0.62–1.04, 0.62–1.02, 0.62–1.00, 0.62–1.00 minutes, respectively, while, the *z*- values ranged from 2.50°C to 4.28°C. Results represented in Table 2 also revealed the *D*-values of all strains of *C. pulveris* (CP1, CP2, CP3, CP4) were in the ranges 7.60–22.32, 1.42–8.45, 0.62–1.08, 0.62–0.78, 0.62–0.78, 0.62–0.79 minutes at 48°C, 52°C, 56°C, 60°C, 64°C, and 66°C, respectively, whereas, the *z*- values were ranged from 3.33°C to 4.89°C. Figures 1 and 2 illustrated the decimal reduction time curve (*D*-values) of all *C. sakazakii* and *C. pulveris* isolates in RIMF at 48°C–66°C. While atypical thermal death time curve of the same isolates of C. sakazakii and *C. pulveris* in RIMF at 48°C –66°C was shown in Figure 3.

Discussion

IMF product has been the focus of studies on the inactivation and inhibition of *Cronobacter* as the primary vehicle of contamination (Muytjens *et al.*, 1983). The thermal and non-thermal electromagnetic radiation effects were thought to be involved in the mechanism of microbial killing (Najdovski *et al.*, 1991). The thermal resistance of *Cronobacter* spp. is considered a vital parameter likely assisting infection of infant formula. Thus, the extent of thermo-tolerance between different *Cronobacter* strains was evaluated by determining the thermal resistance rate by calculating the *D*-value and *z*-value of each strain. According to our findings, significant differences in thermal resistance were observed between the isolates (Table 2). It has been reported that the D_{48} value ranged from 7.29 minutes for CS5 to 23.47 minutes for CS1, and the D_{52} value ranged from 2.77 minutes for CS4 to 15.50 minutes for CS3. This is in agreement with some

Table 2. *D*- and *z*-values of tested *Cronobacter* strains.

	***D* - value (minute)**						
Strains	**48°C**	**52°C**	**56°C**	**60°C**	**64°C**	**66°C**	***z*-value (°C)**
CS1	23.47	11.39	0.62	0.63	0.63	0.63	2.50
CS3	12.97	15.50	0.62	0.63	0.63	0.63	3.26
CS4	23.25	2.77	1.04	1.02	1.00	1.00	4.28
CS5	7.29	9.15	0.96	0.62	0.62	0.62	3.87
CS6	8.65	9.15	0.90	0.63	0.63	0.63	3.75
CP1	7.60	7.41	0.76	0.62	0.62	0.62	3.99
CP2	10.42	1.42	0.62	0.62	0.63	0.63	4.89
CP3	20.79	5.49	0.74	0.77	0.76	0.75	3.58
CP4	22.32	8.45	1.08	0.78	0.78	0.79	3.33

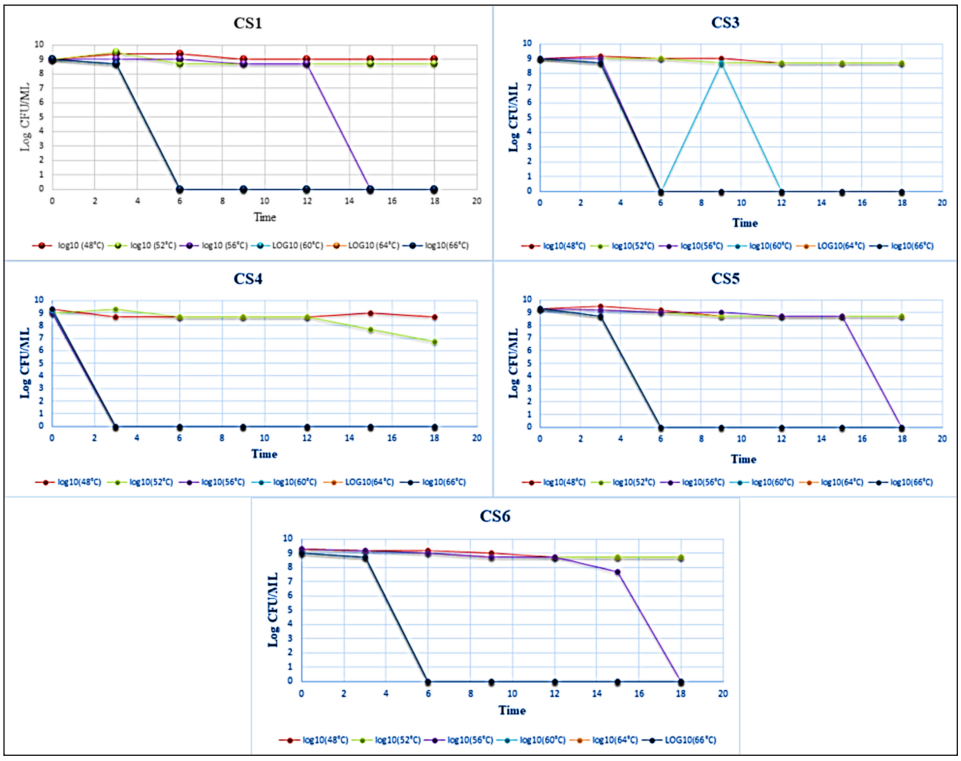

Fig. 1. Thermal inactivation of *C. sakazakii* strains (CS1, CS3, CS4, CS5 and CS6) at 48°C, 52°C, 56°C, 60°C, 64°C, and 66°C in RIMF.

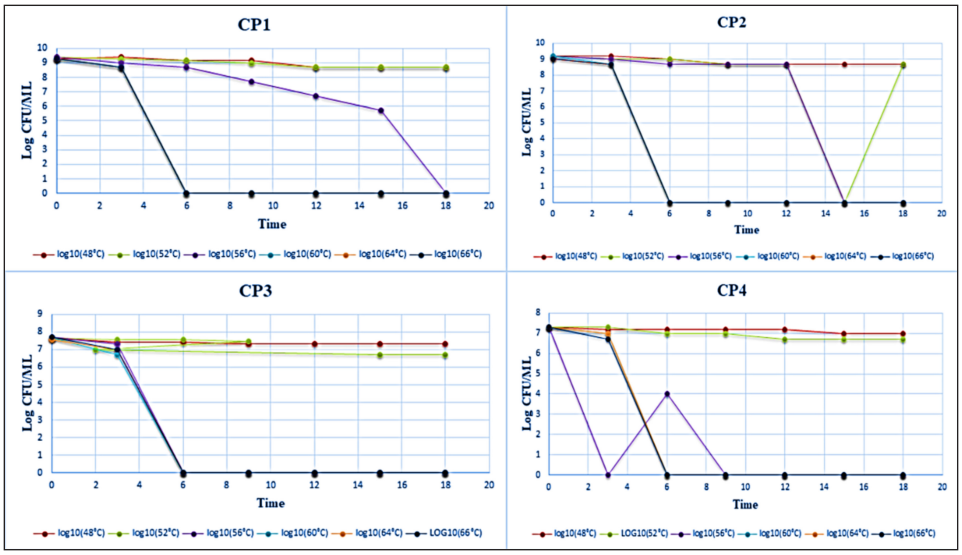

Fig. 2. Thermal inactivation of *C. pulveris* strains (CP1, CP2, CP3 and CP4) at 48°C, 52°C, 56°C, 60°C, 64°C, and 66°C in RIMF.

studies that reported this pathogen had a different and wide range of heat resistance (Edelson-Mammel and Buchanan, 2004; Al-Holy *et al.*, 2009). Moreover, D_{60} ranged from 0.62 minutes for CS5 to 1.02 minutes for CS4 in this current study, which is in agreement with (Walsh *et al.*, 2011) who found that D_{60} ranged from 0.10 to 0.73 minutes. However, D_{60} of *Cronobacter* isolates was 5.74 minutes which indicates a high heat resistance (Awadallah *et al.*, 2018). This may be due to the examined *Cronobacter* strains being isolated from animal feces. These results revealed that it is relatively difficult to compare *D*-values among researchers. This could be attributed to the fact that the bacterial thermo-tolerance can generally be influenced by several

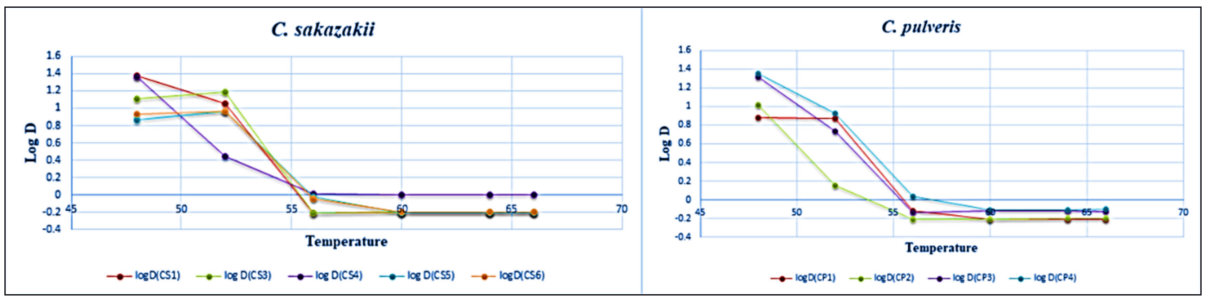

Fig. 3. Thermal death time curve of strains of *C. sakazakii* (left) and *C. pulveris* (right) in RIMF

factors including physiological conditions, the growing temperature of the inoculum, and heating menstruum (fat, solids, and sugar concentration) besides bacterial recovery method (Nazarowec-White and Farber, 1997). Moreover, these variations in *D*-values may be due to differences in the environmental condition of the inspected isolates (Chauhan *et al.*, 2020). The *z*-value was also calculated in this study, it ranged from 2.50°C to 4.28°C, which was lower than previously reported for *C. sakazakii*. Both studies by Edelson-Mammel and Buchanan (2004) and Iversen *et al.* (2004) found that the *z*-values were ranging from 5.6°C to 5.8°C. Nevertheless, Al-Holy *et al.* (2009) reported much higher *z*-values, 3.76°C and 10.11°C. Furthermore, the highest *z*- value (17.42°C) was reported for *C. sakazakii* by (Awadallah *et al.*, 2018). With regard to *C. pulveris*, the *D*-value and *z*-value have not been reported to date. As shown in Table 2, the *z*-value ranged from 3.33°Cto 4.89°C which was relatively similar to *C. sakazakii* results. In comparison, D_{48} ranged from 7.60 to 22.32 minutes while D_{52} ranged from 1.42 to 8.45 minutes which were lower than that reported for the same *D*-values of *C. sakazakii.* On the other hand, the findings of $D_{56,}$ $D_{60,}$ D_{64}, and D_{66} values for *C. pulveris* strains were correspondingly equivalent to that reported for *C. sakazakii*. All of these results were in agreement with the WHO which recommends using hot water at more than 70°C to prepare infant formula to reduce the risk of viable *Cronobacter* cells (Al-Nabulsi *et al.*, 2009).

Conclusion

In conclusion, IMF has been recognized as the major vehicle of transmission of *C. sakazakii* especially when prepared under unhygienic conditions. *C. sakazakii* was previously isolated from infant products sold in the Libyan market. The thermal resistance of *Cronobacter* spp. is considered a vital parameter assisting infection of infant formula. In this work, the extent of thermos-tolerance amongst dissimilar *Cronobacter* isolates was evaluated by calculating *D*-value and *z*-value for each isolate. The results of this study indicate that a significant disparity in thermal resistance between *C. sakazakii* isolates is noted. Some *C. sakazakii* isolates can grow and proliferate at cooling temperatures with time. With regard to *C. pulveris*, the *z*- value was relatively similar to *C. sakazakii* results. It is likely that the usage of hot water at more than 70°C is essential to prepare infant formula as WHO recommends. To date, only a few studies considered the growth variation in heat resistance amongst *C. sakazakii* strains in infant products. Moreover, this is the first study in Libya investigating the thermos-tolerance of *Cronobacte*r species in RIMF. This study may contribute to reducing the risk of *C. sakazakii* survival during the preparation of infant food.

Authors' contributions

A.M.G, S.M.A, and S.A.F. planned and designed the study. S.M.A, S.A.F, J.A.S, A.F.L, and F.T.G carried out the laboratory work. All authors A.M.G, S.A.F, S.M.A, J.A.S, A.F.L, H.L.E, F.T.G, H.T.N, A.A.E.S. and I.M.D contributed equally to the analysis and presentation of data in addition to the preparation and revision of the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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