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Survival and Viability of *Cronobacter sakazakii* and *Cronobacter pulveris* in Reconstituted Infant Milk Formula at Various Storage Temperatures

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Abstract

Background: *Cronobacter sakazakii* is an emerging pathogen shown to be responsible for many neonatal outbreaks with high mortality rate and is remarkably known to resist desiccation and survive in powder infant formula (PIF) for extended period of time. *C. pulveris* is also important as a newly developed foodborne pathogen but there is not yet enough published data on its surviving behavior. Reconstituted infant milk formula (RIMF) usually stated as the main vehicle associated with neonatal *Cronobacter* infections.

Aim: The aim of this study was to investigate the survival of *Cronobacter* spp. (*C. sakazakii* and *C. pulveris*) in RIMF at various storage temperatures.

Methods: The reconstituted formula was inoculated with five *C. sakazakii* isolates and four *C. pulveris* isolates separately and stored at room and refrigeration temperatures for 2, 4, 8, 24, 48, 72 and 96h.

Results: The results showed that *C. sakazakii* and *C. pulveris* were able to grow and multiply in RIMF at room temperature as the storage time increases. At 4ºC, population of CP4, CP2 and CS4 were remained as the initial levels until the end of storage period. Whereas CS1, CS3, CP1 and CP3 were not detected at 4°C after 72, 24, 72 and 8h respectively. However, the viable count of CS5 and CS6 had increased by about 1 log at 4ºC after 8h.

Conclusion: This study demonstrated the significant diverse in behavior between the examined isolates in RIMF at room and refrigeration temperatures as highlighted. Furthermore, these results may improve understanding of *C. sakazakii* and *C. pulveris* surviving strategies which may lead to create an effective control of *Cronobacter* infections.

Keywords: *Cronobacter spp.*; *C. sakazakii*; *C. pulveris*; *Survival*; *RIMF*

Introduction

As consumers become more aware of food-borne diseases, the production of pathogen-free foods has become a fundamental requirement of the food industry, science and public health [1]. Among the huge food products, infant formula remains a major challenge in infant food production. Currently, powder infant formula (PIF) is a basic source of nutrition for infants, consumed as a substitute for breast milk

and can provide essential nutrients for growth, but it is also a major risk factor for transmission of foodborne illness caused by *Cronobacter* spp. [2]. *Cronobacter* spp. especially *C. sakazakii*, is recognized as a new foodborne pathogen and is involved in neonates' outbreak after feeding of contaminated PIF; furthermore, it has been reported to infect people with immunodeficiency, specifically the elderly [3]. In fact, these non-spore forming rod bacteria are found in a variety of environments such as soil, water, waste and dust from households. It is also found in several types of foods, including dried foods, meats, vegetables, infant milk powder, cereal formula and other dairy products [4,5]. Many cases of meningitis, sepsis, or necrotizing enterocolitis have been recorded in the neonatal intensive care unit due to the survival of this microorganism in reconstituted infant milk formula (RIMF) and nutrient preparation equipment [6]. Rarely, *C. sakazakii* has a high mortality rate from the pediatric infections, which can be as high as 80% and the recovered cases may suffer from neuropathy [7,8]. In fact, traditional heat treatments such as pasteurization are an important process for maintaining the quality of milk powder and are a common method for eliminating *C. sakazakii* from baby food. With this in mind, post treatment contamination due to the addition of heat sensitive components and/or the environment may be the cause of *Cronobacter* infections. in addition, poor hygiene during the handling and preparation of baby food may be another route of transmission of this microorganism [9]. According to scientific studies, this pathogen is highly resistant to drought and is capable of surviving in PIF for more than 2 years. It is currently classified as a Class A pathogen by FAO-WHO. Although their infection dose is relatively low (1000 CFU) [2,8,10,11]. This ability of survive may be related to the ability of *C. sakazakii* to produce a yellow pigment that protects cells from UV light. Besides, some of these isolates are capable of forming encapsulating materials and proteases that allow cells to attach to the surface of other cells and survive in food chain and environment [9]. Consequently, these significant characteristic allowing the pathogens to reveal variable survival and virulence phenotype as well as significant alterations in RIMF since mechanisms of pathogenicity is not fully interpreted [12]. Few studies have recently examined the growth variation and surviving of *C. sakazakii* isolates and their effect on reconstituted infant formulas at different storage temperature. This provides the knowledge and the concerns for studying *Cronobacter* spp. behavior diversity and surviving in RIMF in Libya since it had been isolated from Libyan retail dairy and meat products, baby food and RIMF [13]. Therefore, this study focused on the influence of various storage temperatures on survival and viability of each isolate of *C. sakazakii* and *C. pulveris* and their effect on consistency of RIMF in Libya.

Materials and Methods

Bacterial isolates used in this study

The nine isolates of *Cronobacter* spp. have been used in this study (Table 1), were obtained from foodborne Libyan type bacterial collection (FLBC), which were isolated from raw milk, powdered infant formula, some meat and dairy products that were collected from different localities in Libya and stored in beads banking system at -80°C [13]. A single bead was streaked on nutrient agar (NA; CM0003, Oxoid, UK) and incubated at 37°C for 24h then a single colony was streaked on tryptone soya agar slants (TSA, Oxoid, UK) incubated at 37°C for 24h and kept refrigerated. The cultures were transferred from TSA slants individually to sterilized test tubes containing 10 mL BHI and incubated at 37°C for 24h. At this stage, the cells were considered in their early stationary phase.

No.	FLBC Code	Study Code	Isolates of *Cronobacter* spp.	Biofilm	Type of sample
1	10305.2	Cs1	*C. sakazakii*	Capsulated	Raw Camel's milk
2	10456	Cs3	*C. sakazakii*	Non- capsulated	Cereal baby food
3	2204.2	Cs4	*C. sakazakii*	Capsulated	Meat
4	6404.2	Cs5	*C. sakazakii*	Non- capsulated	Fermented milk
5	6208.2	Cs6	*C. sakazakii*	Capsulated	Meat
6	10322.1	Cp1	*C. pulveris*	Capsulated	Raw cow`s milk
7	10324.1	Cp2	*C. pulveris*	Non- capsulated	Raw cow's milk
8	10429	Cp3	*C. pulveris*	Capsulated	Maasora cheese
9	10492	Cp4	*C. pulveris*	Capsulated	Baby milk

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

FLBC: Food-Borne Libyan-Type Bacterial Collection.

Preparation of reconstituted infant milk formula

Infant milk formula was purchased from local supermarkets and pharmacies in Tripoli, Libya. The appropriate amount of powder was added according to the manufacturer's instructions. Reconstitution of infant milk formula in sterile distilled water was carried out according the manufacturer's guidelines (two scoops = 8.6g per 60 mL of warm water). Ten milliliters of RIMF samples were placed into sterile test tubes and sterilized at 121°C for 15 minutes to get rid of the background microorganisms.

Survival of *Cronobacter* spp. in reconstituted infant milk formula

The following procedure was adopted from Awadallah., *et al*. [14] with some modification. The isolate was transferred from TSA slants into (BHI) broth and incubated for 24h at 37°C. Ten-fold serial dilution of the BHI culture was performed in buffered peptone water solutions (BHI; RM 001, HI media, India) by adding 1 mL from cultured brain heart infusion broth onto 99 mL of buffered peptone water dilution. A portion of 100 µL from each dilution was then aseptically plated (0.1 mL in duplicate) onto TSA and incubated at 37°C for 24h (stationary phase). The dilution that had a microbial load of 10^7 - 10^8 CFU/mL was used for the inoculation of the prepared RIMF. Inoculated infant milk formula tubes were then stored at room and refrigeration temperature. Control groups of non-inoculated infant powdered milk were also included. Examination was carried out in triplicate at zero time (15 minutes after inoculation) and at 2, 4, 8, 24, 48, 72 and 96h for both storage temperatures.

The samples were plated on TSA, by adding 0.1 mL from each samples and then cultured on TSA (pouring plate with liquefied agar) and the plates were incubated, for 24h at 37°C and quantification of bacteria at each storage time.

Results

Microbial quantity of each isolate showed relatively similar values and it ranged from 10^7 - 10^8 CFU/ mL. The dilution that had a microbial load of 10^7 - 10^8 CFU/mL was used for the inoculation of the prepared RIMF. Inoculated infant milk formula tubes were then stored at room and refrigeration temperature.

Survival of nine isolates of *Cronobacter* spp. in RIMF at room temperature

The means and standard deviation (SD) of the concentration of *Cronobacter* spp. in RIMF at room temperature after incubation at 0 (15 minutes), 2, 4, 8, 24, 48, 72 and 96h (Table 2). The isolates (CS1, CS3, CS4, CS5 and CS6) of *C. sakazakii*, inoculated in RIMF at a population of 8 log were used as the initial inoculum levels. The survival rate of *C. sakazakii* isolates in RIMF stored at room temperature is shown in figure 1. Concentration of three isolates (CS1, CS4 and CS5) of *C. sakazakii* continued as initial inoculum levels after incubation for 0, 2 and 4h. After 8h at room temperature, their viable count in RIMF had increased by about 1 - 2 log. A higher increase (> 300 colonies) of (CS1, CS4 and CS5) isolates was observed after 24, 48, 72 and 96h of storage. The concentration of (CS3 and CS6) isolates of *C. sakazakii* in RIFM at room temperature remained at the initial inoculum levels after incubation for 0, 2h. There was no change in the number of colonies until 2h post inoculation, the viable count of (CS3 and CS6) isolates had increased by about 1 log after 4h of storage time at room temperature and much more increase by about 2 log was noticed after 8. However, higher increase (> 300 colonies) of (CS3and CS6) isolates were recorded after 24, 48, 72 and 96h of storage time at room temperature in RIMF.

Figure 2 showed that the survival rate of *C. pulveris* isolates in RIFM stored at room temperature. Notably, the concentration of (CP1) isolate of *C. pulveris* stayed at the initial inoculum levels until the end of storage period. Whereas, the concentration of (CP2, CP4) isolates remained at the initial inoculum levels after incubation for 0, 2, 4h, the number of these isolates increased by about 1 log after 8h of storage in RIFM. A drastic increase (> 300 colonies) of (CP2, CP4) isolates was observed after 24, 48, 72 and 96h of storage at room temperature in RIMF. It can be seen that the viable count of (CP3) isolates of persisted as the initial inoculum levels after incubation for 0, 2, 4, 8h,

the number of CP3 isolate increased by about 1.4 log after 24h of storage at room temperature. A higher increase (> 300 colonies) of CP3 isolate was observed after 48, 72 and 96h at room temperature in RIFM. As a result of growth and proliferation of *Cronobacter* isolates a visible viscosity had been developed in RIMF with time after storage at room temperature. As shown in table 3 and 4, both capsulated and non-capsulated isolates of *Cronobacter sakazakii* and *pulveris* can affect the consistency and the viscosity of RIMF which increase as the time of storage increases.

Time (Hours)	Number of Cronobacter spp. (CFU/ mL)								
	CS1	CS3	CS4	CS5	CS6	CP1	CP2	CP3	CP4
0	$1.7x10^8 \pm 1.2$	$1.6x10^8 \pm 0.6$	$1.6x10^8 \pm 1.2$	$3.3x10^8 \pm 0.6$	$2.3x10^8 \pm 0.6$	$0.7x10^7 \pm 1.2$	$0.7x10^8 \pm 1.2$	$0.3x10^8 \pm 0.6$	$0.7x10^8 \pm 0.6$
2	$3.3x10^8 \pm 2.3$	$1.6x10^8 \pm 0.6$	$3.3x10^8 \pm 1.2$	$3.3x10^8 \pm 4.0$	$2.7x10^8 \pm 0.6$	$1x10^7 \pm 1$	$0.7x10^8 \pm 0.6$	$0.6x10^8 \pm 1.2$	$0.7x10^8 \pm 0.6$
4	$0.7x10^8 \pm 0.6$	$5.3x10^8 \pm 1.5$	$310^8 \pm 1$	$3.3x10^8 \pm 2.3$	$27.3x10^8 \pm 2.3$	$0.7x10^7 \pm 0.6$	$2.3x10^8 \pm 0.6$	$1x10^8 \pm 1$	$2.3x10^8 \pm 1.5$
8	$16.7x10^8 \pm 5.7$	$45x10^8 \pm 10.6$	$27.3x10^8 \pm 2.8$	$228.3x10^8 \pm 19.6$	$288x10^8 \pm 10.6$	$0.7x10^7 \pm 0.6$	$14.3x10^8 \pm 3.1$	$0.7x10^8 \pm 0.6$	$8x10^8 \pm 1$
24	$3x10^{10}$	$3x10^{10}$	$3x10^{10}$	$3x10^{10}$	$3x10^{10}$	$2.67x10^7 \pm 1.2$	$3x10^{10}$	$25.7x10^8 \pm 1.5$	$3x10^{10}$
48	$3x10^{10}$	$3x10^{10}$	$3x10^{10}$	$3x10^{10}$	$3x10^{10}$	$0.3x10^7 \pm 0.6$	$3x10^{10}$	$3x10^{10}$	$3x10^{10}$
72	$3x10^{10}$	$3x10^{10}$	$3x10^{10}$	$3x10^{10}$	$3x10^{10}$	$0.3x10^7 \pm 0.6$	$3x10^{10}$	$3x10^{10}$	$3x10^{10}$
96	$3x10^{10}$	$3x10^{10}$	$3x10^{10}$	$3x10^{10}$	$3x10^{10}$	$0.7x10^7 \pm 1.2$	$3x10^{10}$	$3x10^{10}$	$3x10^{10}$

Table 2: *Count of Cronobacter spp. in RIMF at room temperature storage (Mean ± S.D).*

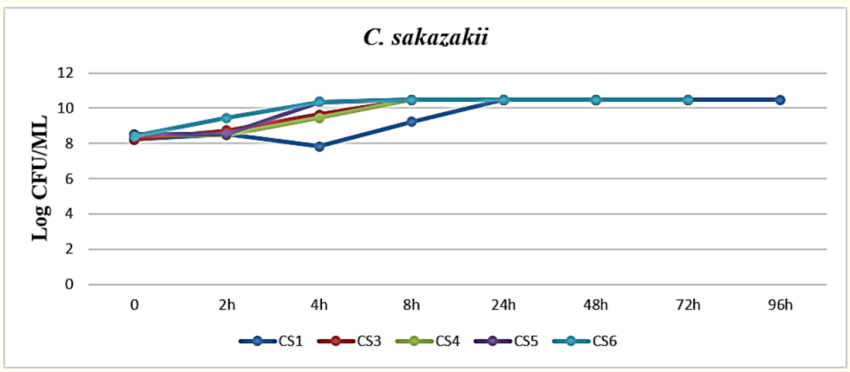

Figure 1: *Survival rates of C. sakazakii isolates in RIMF stored at room temperature (20-25ºC).*

Time/hours	CS1	CS3	CS4	CS5	CS6
0	No Change	No Change	No Change	No Change	No Change
2	No Change	No Change	No Change	No Change	No Change
4	No Change	No Change	No Change	No Change	No Change
8	Viscosity	Viscosity	Viscosity	Viscosity	Viscosity
24	Viscosity	Viscosity	Viscosity	Viscosity	Viscosity
48	Viscosity	Viscosity	Viscosity	Viscosity	Viscosity
72	Viscosity	Viscosity	Viscosity	Viscosity	Viscosity
96	Viscosity	Viscosity	Viscosity	Viscosity	Viscosity

Table 3: *The microbial changes of RIMF by C. sakazakii strains at room temperature.*

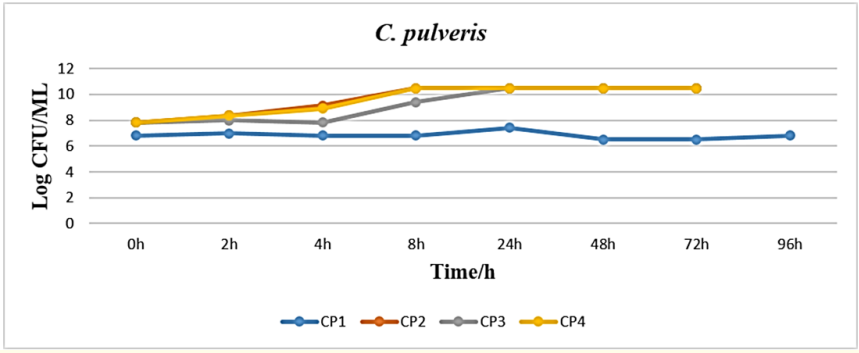

Figure 2: *Survival rates of C. pulveris isolates in RIMF stored at room temperature (20-25ºC).*

Time/Hours	CP1	CP2	CP3	CP4
0	No Change	No Change	No Change	No Change
2	No Change	No Change	No Change	No Change
4	No Change	No Change	No Change	No Change
8	No Change	Viscosity	No Change	No Change
24	No Change	Viscosity	Viscosity	Viscosity
48	No Change	Viscosity	Viscosity	Viscosity
72	No Change	Viscosity	Viscosity	Viscosity
96	No Change	Viscosity	Viscosity	Viscosity

Table 4: *The microbial changes of RIMF by C. pulveris strains at room temperature.*

Survival of nine isolates of *Cronobacter* spp. in RIFM at refrigeration temperature

The means and standard deviation (SD) of *Cronobacter* spp concentration in RIMF at refrigeration temperature after incubation for 0 (15 minutes), 2, 4, 8, 24, 48, 72, and 96h can be seen in table 5. As shown in figure 3, the concentration of (CS1) isolate of *C. sakazakii* in RIMF at refrigeration temperature continued at the initial inoculum levels after storage for 0, 2, 4, 8, 24h. On the other hand, the number of (CS1) increased in RIMF by about 1 log after storage for 48h at 4ºC. After 72h at 4°C, (CS1) was not detected in inoculated RIFM. Until 8h at refrigeration temperature, the concentration of (CS3) isolate of *C. sakazakii* in RIMF remained at the initial inoculum levels. However, after 24h of storage at 4°C, the viable count of CS3 isolate was not been detected in RIMF till the end of storage time. Remarkably, the viable count of (CS4) isolate of *C. sakazakii* in RIMF persisted at the initial inoculum levels until the end of storage period. The cell number of (CS5 and CS6) isolates of *C. sakazakii* remained at the initial inoculum levels after storage in RIMF for 0, 2, 4h at 4°C; though, after 8, 24, 48, 72, 96h their viable count had increased by about 1 log. Figure 4 showed the number of (CP1) isolate of *C. pulveris* remained at the initial inoculum levels after storage for 0, 2, 4, 8, 24 and 48h at refrigeration temperature. However, (CP1) was not detected in RIMF when stored for 72, 96h at 4ºC. Obviously, the population of (CP2 and CP4) isolates of *C. pulveris* continued at the initial inoculum levels until the end of storage period. The number of (CP3) strain of *C. pulveris* remained at the initial inoculum levels after storage for 0, 2, 4h but it was not detected in RIMF when stored for 8, 24, 48, 72, 96h at refrigeration temperature.

Time (Hours)	Number of Cronobacter spp. (CFU/mL)								
	CS1	CS3	CS4	CS5	CS6	CP1	CP2	CP3	CP4
0	$1.66x10^8 \pm 1.52$	$1.66x10^8 \pm 1.52$	$2.33x10^8 \pm 0.57$	$3.33x10^8 \pm 2.31$	$3x10^8 \pm 0$	$0.67x10^7 \pm 1.15$	$0.33x10^8 \pm 0.57$	$0.33x10^8 \pm 0.57$	$0.67x10^8 \pm 0.57$
2	$1x10^8 \pm 1$	$1.33x10^8 \pm 0.57$	$2x10^8 \pm 1.73$	$1.67x10^8 \pm 1.15$	$2.33x10^8 \pm 0.57$	$0.33x10^8 \pm 0.57$	$0.67x10^8 \pm 0.57$	$1.33x10^8 \pm 1.52$	$0.67x10^8 \pm 0.57$
4	$1x10^8 \pm 1$	$1.33x10^8 \pm 0.57$	$2x10^8 \pm 1.73$	$2x10^8 \pm 0$	$3x10^8 \pm 1.73$	$0.67x10^7 \pm 1.15$	$1.67x10^8 \pm 0.57$	$0.33x10^8 \pm 0.57$	$0.67x10^8 \pm 1.15$
8	$1x10^8 \pm 1$	$1x10^8 \pm 0.57$	$1x10^8 \pm 1$	$5.67x10^8 \pm 1.15$	$5x10^8 \pm 1.73$	$0.67x10^7 \pm 0.57$	$1.33x10^8 \pm 0.57$	0 ± 0	$0.67x10^8 \pm 0.57$
24	$0.33x10^8 \pm 0.57$	0 ± 0	$1x10^8 \pm 1$	$4.67x10^8 \pm 1.52$	$6.67x10^8 \pm 3.05$	$0.33x10^7 \pm 0.57$	$0.67x10^8 \pm 0.57$	0 ± 0	$1.33x10^8 \pm 1.15$
48	$11.67x10^8 \pm 11.93$	0 ± 0	$1x10^8 \pm 1$	$16x10^8 \pm 1$	$44x10^8 \pm 1.73$	$0.33x10^7 \pm 0.57$	$0.67x10^8 \pm 0.57$	0 ± 0	$1x10^8 \pm 1$
72	0 ± 0	0 ± 0	$0.33x10^8 \pm 0.57$	$8.33x10^8 \pm 2.88$	$19x10^8 \pm 6.55$	0 ± 0	$0.67x10^8 \pm 0.57$	0 ± 0	$1x10^8 \pm 1$
96	0 ± 0	0 ± 0	$0.33x10^8 \pm 0.57$	$4.33x10^8 \pm 0.57$	$18.33x10^8 \pm 3.05$	0 ± 0	$0.33x10^8 \pm 0.57$	0 ± 0	$1.33x10^8 \pm 0.57$

Table 5: *Count of Cronobacter spp. in RIMF at refrigeration temperature storage (Mean ± SD).*

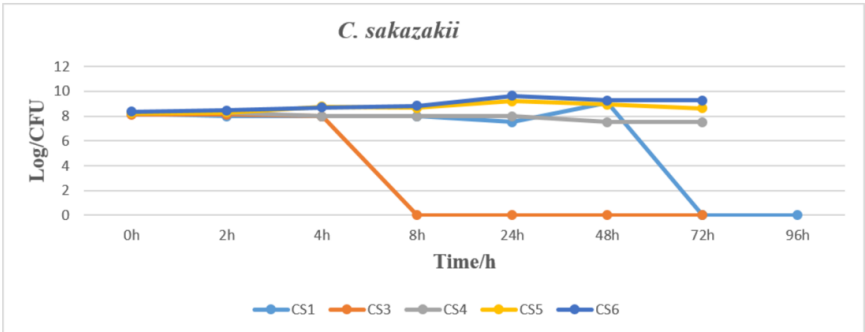

Figure 3: *Survival rates of C. sakazakii isolates in RIMF stored at refrigeration temperature.*

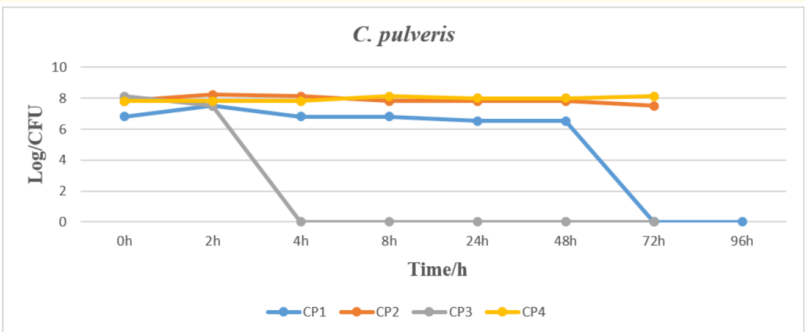

Figure 4: *Survival rates of C. pulveris isolates in RIMF stored at refrigeration temperature (4°C).*

Discussion

RIMF is commonly identified as the primary vehicle associated with neonatal *Cronobacter* infections, increasing consideration of the survival and growth characteristics of this pathogen for neonatal safety [15]. Recently, most previous studies have generally been focused on the ability of *C. sakazakii* to grow in reconstituted powdered infant formula as one single pathogen. However, little attention has been paid on the behavior variation of *C. sakazakii* strains and their influence in reconstituted infant formulas at different storage temperature. Hence, this will be the first study in Libya to investigate the growth pattern of these strains as well as *C. pulveris* as a newly significant foodborne pathogen in rehydrated infant milk powder. In current study, the population count of the nine isolates of *Cronobacter* spp. were determined in RIMF stored at room temperature for 2, 4, 8, 24, 48, 72 and 96h. The differences of surviving ability can be seen among these isolates. The three isolates (CS1, CS4, and CS5) did not grow and remain at the initial level until 4h post inoculation in RIMF. Similarly, both Beuchat., *et al*. [15] and Osaili., *et al*. [16] reported that there is no significant increase in the viable count after 4h of storage at room temperature. This result can indicate that the time held at room temperature should be less than 4h to minimize the potential growth of *C. sakazakii*. However, the microbial number of CS3 and CS6 were clearly increased about 1 log although they were grown at same conditions of other *C. sakazakii* isolates. The possible explanation may be that the behavior disparity between the strains can relatively affect their ability of growing in RIMF. Nonetheless, the findings of the present study were not apparently approved by the recommendation of the Centers for Disease Control and Prevention (CDC), which indicates that prepared formula should not be stored at room temperature for more than 4h [17]. After 8h storage period at room temperature, the growth was obviously increased and amplified in all isolates of interest. In accordance, with previous studies which confirmed that *C. sakazakii* was able to grow and propagate at 8h post inoculation with storage period at the same previous temperature [16,18]. However, these results contradict with a study by Awadallah., *et al*. [14] who demonstrated that the *C. sakazakii* gradually grew in RIMF after 24, 48,72 and 96h at ambient temperature. This may be because of the differences in the origin of examined isolates of *C. sakazakii*. There is far less agreement, however, about the growth time of *C. sakazakii* in RIMF at room temperature. A study which was conducted by Al-Nabulsi., *et al*. [9] who reported that the increase in the number of bacterial cell was slightly after 6h and drastically after 24h. This result is also supported by Al-Holy., *et al*. [19] who studied the growth of *C. sakazakii* in RIMF and storage for long periods of time up to about 14 days at room temperature and found that the strains increase dramatically until the end of the storage period. Furthermore, in another study which concluded that prolonged periods of storage or administration at room temperature might lead to proliferating numbers of *C. sakazakii* that may be present [20]. This finding seems to agree with the present results, which showed extreme increase in microbial count in all isolates of *C. sakazakii* after 24, 48, 72 and 96h at room temperature.

Although, *C. pulveris* has a significant importance as a newly emerged foodborne pathogen, this is by far the first study to report the growth and survival of *C. pulveris* in RIMF. According to the current findings, the concentration of all isolates of *C. pulveris* (CP1, CP2, CP3 and CP4) remained at the initial inoculum levels in RIMF after incubation for 0, 2, 4h at room temperature. In contrast, some isolates of *C. sakazakii* (CS3 and CS6) grew and multiplied at 4h post inoculation at same storage conditions. The viable count of 2 isolates of *C. pulveris* (CP2, and CP4) had gradually increased after 8h. Yet others (CP1 and CP3) did not show any proliferation in RIMF at this time. Similar to *C. sakazakii* finding after 24, 48, 72, 96h all isolates of *C. pulveris* appeared to contain a higher microbial count since the plates showed overgrowth and the number of the colonies was more than 300 CFU (uncountable) with exception of CP1 which stayed as original level. A possible explanation could be that the effect of deep freezing on (CP1) strain that reduced its activity or it could have been injured during storage in the deep freezer at -80°C. Preceding studies have indicated that RIMF is a nonsterile product since provided by an excellent medium for microbial growth in particular *Cronobacter* spp. [8,19]. In this study, the microbial alteration of RIMF caused by *Cronobacter* spp. has been recorded and the changes in the form and consistency of the milk was observed rendering to the isolates type (Table 3). From microbiological point of view, some *Cronobacter* isolates can create capsule material and proteases [12]. Despite of *C. sakazakii* isolates, capsulated (CS1, CS4 and CS6) and/or un-capsulated (CS3, CS5), there was no notable change in RIFM up to 4h post inoculation at room temperature. After 8h at room temperature, the viscosity of RIMF was gradually increased until the end of storage period. Remarkably,

CS6 (capsulated strain) showed increased in viscosity with accumulation of curd at 24h and gas production was noticed after incubation for 48h at room temperature. Hurrell., *et al*. [21] reported that, high density (10^7 CFU/cm) biofilm formation in IMF has been observed after 24h on enteral feeding tubes. This critically increases the risk for neonatal infections, especially in hospitals, as enteral feeding tubes remain *in situ* at 37ºC for several days and nutrients are administered to the infants every 2 - 3h. Additionally, CS3 (un-capsulated) showed the same activity and high viscosity in RIFM after 48 and 72h of the storage period. There were equal results in milk alteration of capsulated and un-capsulated strains. In other words, existence of *C. sakazakii* can clearly affect the consistency of RIMF with prolonged period of storage. On the contrary, both Al-Holy., *et al*. [19] and Iversen., *et al*. [6] observed that the capsulated strains can noticeably increase the viscosity of RIMF with time, owing to the ability of capsulated strain to create the exopolysaccharide that participate in viscous appearance. Interestingly, only CS3 (un-capsulated) and CS6 (capsulated) had developed a viscosity in RIMF at room temperature between 21 to 25°C. According to Iversen., *et al*. [6] the majority of *C. sakazakii* strains are able to ferment lactose and produce gas at 37°C and some strains produce gas at 44°C. This may possible due to the carbohydrate metabolism in a large number of *C. sakazakii* strains that may be sensitive to temperatures of 44°C and above.

The impact of *C. pulveris* on RIMF homogeneity were also observed and reported in the present study. For all isolates (CP1, CP2, CP3 and CP4), there was not any significant alteration in RIMF till 8h at room temperature except CP2, which was un-capsulated strain, showed very slight viscosity in milk. In compare, all *C. sakazakii* isolates able to gradually increase the viscosity of RIMF after 8h at room temperature until the end of storage period. However, the viscosity of the milk inoculated with CP2, CP3, and CP4 was visibly raised after 24h and continued up to the end of the storage period. Only CP2 (un-capsulated) and CP4 (capsulated) had developed viscous materials after 72h at room temperature. Similar results were observed by *C. sakazakii* and approved by previously mentioned work [12]. It is worth stating that RIMF inoculated with CP1 as a capsulated strain did not show any alteration until the end of the storage period at room temperature. This may support the earlier probability of effect of deep freezing as it remained as initial level up to end of storage period.

Despite the fact that *Cronobacter* spp. can grow over a wide temperature range, it is commonly used to keep RIMF at refrigeration during infant feeding. Therefore, it is critical to study the persistence of this pathogen in RIMF at refrigeration. Previous studies have concluded that *Cronobacter* spp. unable to grow and survive at refrigerating temperature but the initial microbial level could be decline with time [14,16,19,20,22-24]. These studies seem to support the recommendation of the Center for Disease Control and Prevention (CDC) which acclaimed that reconstituted infant formula can be stored in the refrigerator for 24h [17]. Additionally, both Gurtler and Beuchat [18] suggested that RIMF should be kept at 4°C to avoid growth of *C. sakazakii*. To some extent, this was not observed in this study as some *C. sakazakii* isolates grew and proliferated at refrigerator temperature with time. For instance, the initial concentration of CS5 and CS6 was gradually raised since 8h post inoculation and sustained until the end of storage period. The discrepancy between the results obtained in the present study with those reported above can be linked to that; these isolates have the ability to resist cold during storage at this temperature. Consequently, it is noteworthy that the refrigerator temperature seems to be insufficient to prevent the microbial propagation of *Cronobacter* spp. and could still be infectious once infant feeding. In agreement, other report confirmed that *C. sakazakii* was able to grow at refrigerator temperature [6]. Oddly, CS1 isolate has increased after 48h and suddenly not detected in RIMF at 72h storage time. This may attribute to that the population reduction is more evident in the capsulated isolates such as CS1 compared to the un-capsulated isolates [19]. Similarly, CP3 and CP1, which are known as capsulated isolates, did not tolerate refrigerating and began to die off during storage, hence, were not detected in milk after 8 and 72h respectively. However, other *C. pulveris* strains remained at initial microbial level as same as CS4 in RIMF when stored at refrigeration conditions.

Conclusion

In conclusion, most previous studies have generally focused on the ability of *C. sakazakii* to grow in reconstituted powdered infant formula as one single pathogen. The results of this study showed that there is a clear behavior variation between *Cronobacter* isolates in RIFM at room and refrigeration temperature. The microbial number of some *C. sakazakii* isolates clearly increased after 4h. Also, some *C.*

sakazakii isolates can grow and proliferate at cooling temperature with time. The behavior disparity between the strains can relatively affect their ability of growing in (RIFM). The refrigerator temperature seems to be insufficient to prevent the microbial propagation of *Cronobacter* and could still be infectious once infant feeding. Nonetheless, the finding of the present study was not apparently approved the recommendation of the Centers for Disease Control and Prevention (CDC), which indicates that prepared formula should not be stored at room temperature for more than 4 hours.

Conflict of Interest

The authors declare that they have no conflict of interest.

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