

Incidence and Interrelation of *Cronobacter sakazakii* and Other Foodborne Bacteria in Some Milk Products and Infant Formula Milks in Cairo and Giza Area

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Abstract: A total of 140 samples including different milk products, 20 Domiati cheese, 25 ice cream, 25 yoghurt, as well as 20 milk powder and 50 powdered infant formula milk samples (PIFM) were randomly collected from Cairo and Giza area and examined for the incidence of *Cronobacter* (formerly *Enterobacter*) *sakazakii* and its interrelation with the other foodborne pathogenic bacteria. Detection of *C. sakazakii* was performed using both of FDA procedure as a traditional method and chromogenic medium as a rapid technique. To identify and confirm the detection of the pathogen, both biochemical reactions according to FDA and biochemical identification kits (HiBio-ID™, Hi25™ Enterobacteriaceae identification kits KB003) were comparatively applied. *Cronobacter sakazakii* was successfully isolated from 14.3% (20/140) using chromogenic medium (HiCrome *Enterobacter sakazakii* agar) compared to 11.4% (16/140) by traditional media (violet red bile glucose agar, VRBGA and tryptone soy agar, TSA). The pathogen could be detected in Domiati cheese samples in percentages of 30% (6/20) on the chromogenic medium and 20% by the traditional media. Furthermore, *C. sakazakii* was isolated from one sample (4%) of each of ice cream and yoghurt samples using both of the two methods, equally. *C. sakazakii* was detected in (PIFM) samples at percentages of 24 and 20% using chromogenic medium and VRBGA & TSA media, respectively. However, *C. sakazakii* could not be isolated from any of powdered milk samples using both of the isolation media. Generally, conventional biochemical reactions and biochemical identification test kits (HiBio-ID™, Hi25™ Enterobacteriaceae identification kits KB003) showed similar efficiency for identification of *C. sakazakii* in positive samples. Furthermore, the positive samples for *C. sakazakii* in the tested products were determined for the frequency of other bacteriological criteria involving total aerobic colony count (TACC), mold and yeast count, Gram- positive foodborne pathogenic bacteria including *Staphylococcus aureus*, *Bacillus cereus* and *Listeria monocytogenes* and Gram- negative foodborne pathogens comprising coliform bacteria, *Yersinia enterocolitica*, *Escherichia coli* O157: H7 and *Salmonella* spp. In positive samples of Domiati cheese, yoghurt and ice cream, coliform group showed the highest frequency, whereas *B. cereus* and *L. monocytogenes* were the ones in the positive samples of PIFM (25% each). Both of *Y. enterocolitica* and *E. coli* O157: H7 were detected in one positive cheese sample for each. *Salmonella* spp. could not be isolated from any positive samples for *C. sakazakii*. The results emphasize the relationship between *C. sakazakii* and PIFM and indicate that Domiati cheese, yogurt, ice cream and PIFM distributed in Cairo and Giza area represent a risk for human health as many of these products did not comply with microbiological criteria of international or Egyptian standards. The necessary precautions have to be taken to carry out effective sanitary practices during the production in the plants, handling and distribution in the markets.

Key words: *C. sakazakii* • Food-borne pathogens • Incidence • Interrelation • Milk products • Milk powder • Powdered infant formula milk

INTRODUCTION

Food-borne illnesses rates remain largely unchanged and continue to be a serious public health problem as reported by The Centres for Disease Control and Prevention [1]. These food-borne diseases are associated with 31 known pathogens and with an assemblage of unspecified agents, including microbes, toxins and other substances [2]. Therefore, considerable attention has been recently directed at the microbiological safety of food particularly dairy products and powdered infant formula milks (PIFM) [3]. Most of the attention has focused on PIFM as a response to the warning regarding the presence of *Cronobacter sakazakii* in baby formula [4], as its presence in powdered infant formula for use with newborn babies is of particular concern [5] due to infants lacking a developed immune system, or a competing intestinal flora [6]. *Cronobacter* is an emerging genus of opportunistic Gram-negative pathogens associated with potentially fatal neonatal infections, including meningitis, sepsis and necrotizing enterocolitis (NEC), [7]. It has been confirmed as the cause of mortality in premature newborns with underlying medical conditions [8], however, it rarely affects adults, causing less severe infections [9]. Historically, *Cronobacter spp.* were thought to be a single species known as *Enterobacter sakazakii* [10]. With improved culture media and identification techniques, including partial 16S ribosomal DNA, hsp 60 sequencing and polyphasic analysis, *Cronobacter* was recognized as its own genus within the family of Enterobacteriaceae. *Cronobacter* contains seven species including *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis*, *C. condiment* and *C. universalis* [11]. Despite natural habitat and primary reservoir still remain unknown, *C. sakazakii* have been isolated from many sources including milk powder, formula constituents and from environments within manufacturing plants [12-15] and household utensils such as blenders, infant bottle cleaning brushes and spoons [16-18]. Furthermore, the organism has been isolated from different kinds of foods including cheese, meat, vegetables, grains, herbs and spices [19-24].

In addition to food, *C. sakazakii* was detected in human clinical samples comprising cerebrospinal fluid, blood, skin wounds, breast abscess, urine, respiratory secretions and digestive tract samples [25, 26]. The pathogen was also isolated from various insect's intestinal tracts of insects such as the Mexican fruit fly *Anastrepha ludens* and the stable fly *Stomoxys calcitrans*.

Farthermost, the organism has been isolated from rats, soil sediment, wetland and even crude oil [27-31]. While, a reservoir for *C. sakazakii* is unknown, a growing number of reports suggest a role for powdered infant formula as a vehicle for infection [8]. PIF is not manufactured as a sterile preparation and some strains of *Cronobacter spp.* have been repeatedly reported as remarkably resistant to osmotic stress and dryness and moderately thermotolerant as some encapsulated *Cronobacter spp.* were still recoverable from desiccated infant formula after storage for up to 2-5 years [32, 33, 34]. This may explain why the pathogen was isolated from this product by numerous investigators in a number of countries throughout the world [23, 24, 35]. Milk and other dairy products are healthy foods that provide us with high-quality protein, minerals, vitamins and omega-3 fatty acids [36]. The nutritional value of these foods is such that, as part of a healthy diet, it is recommended that three servings of dairy products such as milk, yogurt and cheese as well as PIF consumed by millions of infants throughout the world be eaten every day and are considered to be safe products [37]. Although the micro-organisms in infant milk cannot grow due to its low moisture content and do not play any direct role in their spoilage, their occurrence in powdered infant formula milk is of great significance and serves as an index of hygienic standards maintained during production, processing and handling. The infant milk and other dairy products provide highly nutritious substrates that can support the wide variety of bacteria as well as yeast and molds for their growth and reproduction [38, 39]. Among foods in which *C. sakazakii* has been found are milk and other dairy products, including cheese, milk powder and ultra-high temperature processed milk [15, 25]. Because of its potential virulent effects, the possibility of *Cronobacter* in foods that make up an important part of our daily diet raises important concerns regarding food safety and the need to control the presence of this organism and other harmful food pathogens [40]. Although many studies have been done by numerous researchers throughout the world to determine the presence of *C. sakazakii* in PIFM, there is paucity of information and there are no sufficient works in Egypt.

Therefore, this work aimed to investigate the incidence of *C. sakazakii* and its interrelation with the associating other foodborne pathogenic bacteria in Domiati cheese, yoghurt, ice cream, milk powder and powdered infant formula milk (PIFM) samples collected from Cairo and Giza markets and pharmacies under Egyptian conditions of production and/or distribution.

MATERIALS AND METHODS

Samples Collection: A total number of 140 samples of different milk products and powdered infant formula milk (PIFM) were randomly collected from Cairo and Giza local markets and pharmacies. These samples comprised powdered infant formula (50 samples), Domiati cheese (20 samples), ice-cream (25 samples), yogurt (25 samples) and milk powder (20 samples). Cheese, yogurt and ice cream samples were collected into clean, dry and sterile containers in an ice-box and transferred to the laboratory as soon as possible to be microbiologically examined.

Detection, Isolation and Identification of *C. sakazakii*:

The methods described by FDA [41] were applied for preparation of all samples for *C. sakazakii* detection and isolation. All samples were screened for the pathogen using the traditional procedure and a rapid technique. The conventional one depended on three successive steps including pre-enrichment in buffered peptone water (BPW) broth (Oxoid), enrichment in selective Enterobacteriaceae Enrichment Broth (EEB) (Himedia, Mumbai, M287) and plating on selective violet red bile glucose agar (VRBGA) (Himedia, Mumbai and M043) and tryptone soy agar (TSA) (Oxoid, CMO 131). However, in the rapid method, chromogenic media (HiCrome *Enterobacter sakazakii* agar) was used instead of (VRBGA) and (TSA) for the third step of selection reducing the overall time procedure of FDA [41] by up to 3 days. Milk powder and PIFM were pre-enriched by reconstitution in sterilized, distilled water (10g sample/90 ml water), whereas Domiati cheese and yoghurt samples were pre-enriched by mixing 25g sample with 225ml BPW broth. Ice cream samples were pre-enriched by adding 10ml homogenized sample to 90 ml sterile saline. After incubation at 36°C for 24 h, 10 ml of the pre-enrichment culture was inoculated into 90 ml of the EEB, which was then incubated for 24h at 36°C. The selection step was carried out by aseptically streaking a loop full (10µl) of the EEB culture onto a duplicate plates of VRBGA followed by incubation at 36°C for 24h. Suspect purple colonies surrounded by purple halo were picked up and sub-cultured onto TSA plates in duplicates by streaking. The plates were observed for yellow pigmented colonies after incubation at 25°C for 48-72h. But in the rapid technique, VRBGA and TSA were replaced by HiCrome *Enterobacter sakazakii* agar, which was inoculated by streaking with a loop full (10µl) of the EEB culture in duplicates. Suspect blue-green colonies were observed after incubation at 36°C for 24h. Both of yellow pigmented

colonies and blue-green ones were morphologically examined under microscope and Gram-staining reaction. Typical colonies that were short rods and Gram negative were further identified using both biochemical reactions according to FDA [41] and Biochemical identification test kits (HiBio-ID™, Hi25™ Enterobacteriaceae identification kits KB003).

Interrelation Between Incidence of *Cronobacter sakazakii* and Other Foodborne Pathogenic Microorganisms:

The incidence of *Cronobacter sakazakii* in the previously different kinds of milk and milk products was studied in relation to the other bacteriological criteria such as: total aerobic colony count (TACC), mold and yeast counts and foodborne illness, Gram- positive bacteria comprising *Staphylococcus aureus*, *Bacillus cereus* and *Listeria monocytogenes* and Gram- negative bacteria including coliform bacteria, *Yersinia enterocolitica*, *E. coli O157: H7* and *Salmonella spp* as detailed below.

Total Aerobic Colony Count (TACC): Total aerobic colony count (TACC) was carried out due to the conventional method [42] using plate count agar (Oxoid).

Molds and Yeasts Count: Enumeration of molds and yeasts was carried out in the samples using the medium of acidified potato dextrose agar (Mu96, Himedia, Mumbai). The method recommended by FDA [42] was followed up.

Detection of *Listeria monocytogenes*: Tryptose soy broth (Fluka, Switzerland) supplemented with 0.5% yeast extract and listeria selective enrichment supplement (CoodeSR140, Oxoid) was used. After incubation of the flasks at 30°C for 7 day [43], suspected colonies, black with halo on esculent containing media, were picked up and propagated for further specifically morphological and biochemical tests as recommended by FDA [42].

Enumeration of *Staphylococcus aureus*: Enumeration of *S. aureus* in the samples was carried out using the Baird Parker agar medium [44] supplemented with egg yolk and potassium telurite solution as described by APHA [45] and FDA [42].

Enumeration of *Bacillus cereus*: *Bacillus cereus* was determined by the surface plating technique onto *Bacillus cereus* selective agar (Oxoid) supplemented with egg yolk and polymyxin solution (SR99, Oxoid) according to Holbrook and Anderson [46]. Further specific identification was followed up according to FDA [42].

Determination of Coliform Bacteria: Coliform group was determined using solid medium method onto plates of violet red bile agar (VRBA) according to the method reported by FDA [42].

Detection of *Escherichia coli* O157: H7: *Escherichia coli* O157: H7 was detected by using Sorbitol MacConkey (SMAC) agar medium (Oxoid, England). Biochemical-serological identification was applied according to FDA [42].

Isolation and Identification of *Salmonella*: Pre-enrichment, selective enrichment and selective plating on *Salmonella* & *Shigella* agar (SS agar) (Oxoid) plates were done. Lactose negative suspected *Salmonella* or *Shigella* spp. was biochemically identified according to FDA [42] and APHA [45].

Detection of *Yersinia enterocolitica*: Detection of *Y. enterocolitica* was carried out by using *Yersinia* selective agar medium (Oxoid code CM653) supplemented with *Yersinia* selective supplement (SR 109), (1.25mg Novobiocin, 7.5 mg cefsulodin and 2.0 mg Irgasan). The method and the media were recommended by FDA [42].

Statistical Analysis: Statistical analyses were performed using the GLM procedure with SAS [47] software. Duncan's multiple comparison procedure was used to compare the means. A probability to $P \leq 0.5$ was used to establish the statistical significance.

RESULTS AND DISCUSSION

Incidence and Frequency Distribution of *Cronobacter sakazakii* in Domiati Sheese: Twenty Domiati cheese samples, collected from Cairo and Giza markets, were tested for the incidence of *C. sakazakii* and other foodborne pathogenic bacteria, total aerobic colony count and mold and yeast. Results in Table 1 revealed that *C. sakazakii* was isolated from Domiati cheese samples in different percentages according to the used methods or media. The higher the incidence was shown in Domiati cheese samples using the chromogenic media (HiCrome *Enterobacter sakazakii* agar), as *C. sakazakii* was isolated from six out of twenty samples (30%) against four out of twenty samples (20%) using the traditional method (VRBGA and TSA). These results are in parallel with the findings of Gökmen *et al.* [48] who pointed out the presence of *E. sakazakii* in 4% (2/50) of white cheese samples. The results were also consolidated by El-Sharoud *et al.* [49] who examined 40 cheese samples

including stored Domiati cheese, fresh Domiati cheese, Kariesh cheese and Ras cheese, obtained from the Nile-Delta region of Egypt, for the incidence of *C. sakazakii*. They isolated *C. sakazakii* from 40% (4/10) fresh Domiati cheese samples, but they could not isolate the pathogen from the other tested cheese samples. However, El-Sharoud *et al.* [50] did not detect *C. sakazakii* in 37 cheese samples including the same above. The matter may be attributable to the methods of isolation and identification as, in 2009; they identified and characterized the isolates using pheno- and genotyping experiments before confirming the detection of *Cronobacter* using real-time PCR. Also, De Haast and Britz [51] stated that *C. sakazakii* has been isolated from a cheese whey substrate. On the other hand, Meshref and Hassan [52] reported that none of 100 examined soft cheese samples comprising kariesh and tallaga cheeses contained *C. sakazakii*. Baumgartner *et al.* [22] found that *C. sakazakii* was not isolated from 39 samples of soft cheese from milk by selective enrichment followed by plating on chromogenic agar. The findings of Wahyuni and Budiarto [53] also showed that *E. sakazakii* was not isolated from both district Sleman and Boyolali dairy cow's milk farms in Indonesia. Lehner *et al.* [54] could not find *Cronobacter* spp. either in 100 raw milk samples or in 91 milk concentrate samples. These findings were supported by Baumgartner and Niederhauser [55] who reported that all the tested 875 bulk milk samples were negative for *Cronobacter* spp. concluding that milk cannot be a relevant source for *Cronobacter* spp. Thus, if cheese samples were positive for *C. sakazakii*, they would be certainly postcontaminated.

Identification of *C. sakazakii*: Results in Table 2 show the difference between *C. sakazakii* isolates identified by the traditionally biochemical method and the rapid method (Hi25™ Enterobacteriaceae identification kits) in positive samples. Results revealed that the higher the efficiency of the traditionally biochemical method, for identifying the microbe in cheese samples (30%), compared to the rapid identification kits (20%). So, it seems that there are differences between the two identification methods for testing the microbe in Domiati cheese samples to make the use of traditional method an essential procedure for confirmation. For ice cream and yoghurt samples, results illustrated in Table 2 revealed the similar efficiency of the traditionally biochemical method, for identifying the microbe in ice cream and yoghurt samples (4% each), with the rapid method kits (Hi25™ Enterobacteriaceae identification kits) (4% each). So, it sounds no differences between the two identification methods for testing the

Table 1: Incidence of *C. sakazakii* in some milk products and milk powders collected from Cairo and Giza markets.

Type of samples	Number of analyzed samples	Positive Samples			
		HiCrome <i>E. Sakazakii</i> Agar (Chromogenic medium)		Traditional method (violet red bile glucose agar and tryptone soy agar)	
		Number	%	Number	%
Domiaty Cheese	20	6	30	4	20
Ice cream	25	1	4	1	4
Yoghurt	25	1	4	1	4
Milk powder	20	0	0	0	0
Powdered infant milk	50	12	24	10	20
Total samples	140	20	14.3	16	11.4

Table 2: Identification of *Cronobacter sakazakii* by the traditionally biochemical method and the rapid biochemical kits

Source of isolates	Identification of <i>Cronobacter sakazakii</i>			
	No. (%) of the isolates identified by traditionally biochemical test		No. (%) of the isolates identified by Hi25™ Enterobacteriaceae identification kits	
Domiaty Cheese (PIFM)	6/20	30%	4/20	20%
Ice cream	10/50	20%	12/50	24%
Yoghurt	1/25	4%	1/25	4%
Total	1/25	4%	1/25	4%
Total	18/140	12.9%	18/140	12.9%

microbe in ice cream and yoghurt samples rather than in cheese samples. In PIFM samples, Hi25™ Enterobacteriaceae identification kits were more slightly successful in identification of *C. sakazakii* than the ordinary biochemical tests, 24% against 20%, respectively. Although the rapid kits were faster and showed similar accuracy in comparison to traditionally biochemical tests, genotype analysis should be applied to further characterize *C. sakazakii* in food products.

Interrelation Between *Cronobacter sakazakii* and Other Foodborne Pathogenic Microorganisms in Domiaty Cheese:

Also, the microbiological analysis of Domiaty cheese samples (Table 3) revealed that this variety of cheese having high frequency of *C. sakazakii* was inferior quality since out of twenty Domiaty cheese samples, 6 samples (30%) which were positive for *C. sakazakii* contained total aerobic colony count ranging from 6.69 to 10.47 log cfu/g. Whilst molds and yeasts counts ranged from 2.00 to 3.47 log cfu/g in 5 samples (83%) out of the those 6 samples. In regard to Gram- negative bacteria, coliform bacteria were found in 4/6 samples (66%) in counts ranged from 3.0 to 5.30 log cfu/g. One of these positive samples for coliform group was found to contain *E. coli O157: H7* and *Y. enterocolitica* (16.6% each). For Gram- positive bacteria, *L. monocytogenes* was isolated from three samples (50%)

in counts ranged from 2.0 to 3.2 log cfu/g. Meanwhile, *B. cereus* was isolated from two samples (33%) and yielded counts of 2.9 and 3.1 log cfu/g. Moreover, *S. aureus* was also isolated from two samples (33%) and yielded counts of 2.1 and 3.1 log cfu/g. However, the current results revealed that the high the contamination of Domiaty cheese samples with *C. sakazakii* (30%), the high the count of TACC count (10.47 log cfu/g) and the worse the mycological quality. The obtained results were similar to that found by El Kholy *et al.* [56], since they rejected 80% of the vended Domiaty cheese samples that did not meet the Egyptian standards (ES no.1008-2000) because of high TACC and mycological counts. Ahmed [57] detected the coliform group in Domiaty cheese samples collected from Assiut and Beni Swef governorates with similar count rates as obtained in the current study. El Kholy *et al.* [56] also found high incidence of coliform, *E. coli O157:H7*, *Salmonella* spp and the other Gram- negative enteric bacilli in Domiaty cheese, consolidating the current result in concern to *E. coli O157:H7*. In besides, the current results showed that the pathogenic *E. coli O157:H7* has been isolated from 16.6% of the total 6 positive Domiaty cheese samples for *C. sakazakii* as one of the most popular hazardous enteric food borne bacteria in Egypt (personal communication with Central laboratories of Ministry of Health). In respect of *Y. enterocolitica*, Mostafa [58] did not find *Y. enterocolitica* in any of the Domiaty cheese samples collected from different retail

outlets in Assiut city, contradicting the obtained results that revealed the presence of this organism in Domiati cheese sample containing *C. sakazakii*.

Regarding to Gram-positive foodborne pathogenic bacteria, the obtained results (Table 3) were of similarity to findings of El Kholly *et al.* [56] who isolated *S. aureus*, *B. cereus* and *L. monocytogenes* from Talaga and Domiati cheese samples collected from Cairo and Giza areas. Also, Kaldes [59] reported the presence of *S. aureus* in Domiati cheese samples collected from different sites in Egypt. In besides, Fathi and Saad [60] isolated *B. cereus* from the Domiati cheese samples collected from Assiut city. Therefore, they paid similar attention to the probable intoxication due to enterotoxins that might be produced at the optimal level of contamination and conditions in cheese containing *C. sakazakii*.

Contrarily, El-Zayat [61] reported that *B. cereus* was not isolated from any of 50 Domiati cheese samples collected from Ismailia governorate. According to our findings, the results indicate how Egyptian white soft cheeses are inferior quality and hazardous food as they might be an etiology for food borne illness in Cairo and Giza locals. According to the Egyptian Standard ES 1008-2000, there were 66 % of the samples would not be accepted due to the high counts of coliform, 16.6% due the presence of *E.coli* and *Y. enterocolitica*, 33% due to the presence of *S. aureus* and *B. cereus* and 50% due to the presence of *Listeria monocytogenes*, in samples containing *C. sakazakii* in the same time. This may pay much attention to that variety of cheese in particular to the side of hygienic quality. Furthermore, specifications and legislation must be taken to the presence of these pathogens in white soft cheese samples.

Incidence of *Cronobacter sakazakii* in Ice Cream and Yoghurt and its Interrelation with Other Foodborne Pathogenic Microorganisms: Twenty five samples of each of ice cream and yoghurt, collected from Cairo and Giza markets, were tested for the presence of *C. sakazakii* and other foodborne bacteria, total bacterial count and mold and yeast. Results in Tables 1 and 4 show only one sample (4%) of ice cream was found to contain *C. sakazakii* out of 25 samples, either using chromogenic or traditional media and yielded TACC 7.69 log cfu/g and molds & yeasts counts 2.0 log cfu/g. The sample was positive for coliform group which yielded 2.30 log cfu/ gm. On the other hand, Gram-positive foodborne pathogenic bacteria were not detected in the sample. Similarly, only one sample of yoghurt was also positive for *C. sakazakii* out of 25 samples and yielded 8.71 log cfu/g of TACC and

2.60 log cfu/g of mold and yeast as shown in Tables 1 and 4. The sample was positive for coliform group which yielded 3.27 log cfu /g. Meanwhile, Gram- positive foodborne pathogenic bacteria were not detected in the sample. The obtained results contradict the findings by El-Sharoud *et al.* [49] who reported *C. sakazakii* was not detected in any sample of dried ice-cream, dried artificial cream and dried whey samples. On the same track, Baumgartner *et al.* [22] found that *C. sakazakii* was not isolated from 27 samples of ice-cream using selective enrichment followed by plating on chromogenic agar. For yoghurt, the current results are inconsistent with that done by El-Sharoud *et al.* [50] who reported that *C. sakazakii* was not detectable in the examined yoghurt samples. From the current study and literature, it seems, in general, *C. sakazakii* could not be found in yoghurt and ice cream because of its sensitivity to low pH or freezing temperature, respectively. However, the presence of the pathogen in the current work reflects the badly hygienic quality and postcontamination of these products.

Incidence and Frequency Distribution of *Cronobacter sakazakii* in Milk Powder and Powdered Infant Formula Milks:

A total of 50 powdered infant formula milk samples (PIFM) and 20 powdered milk samples collected from Cairo and Giza pharmacies and markets were tested for the presence of *C. sakazakii* and other foodborne pathogenic bacteria. Regarding to powdered infant formula milks, the obtained results in Table 1 revealed that *C. sakazakii* was isolated from 12 out of 50 PIFM samples in percentages of 24 % onto chromogenic media (HiCrome *Enterobacter sakazakii* agar) and from 10 out of 50 samples (20%) by the traditional methods and media (VRBGA and TSA) reflecting the excel of chromogenic media and Hi25™ *Enterobacteriaceae* kits for the detection and identification of *C. sakazakii* in PIFM samples, respectively in comparison to traditional methods. The obtained results were closely similar to and advocated by Shaker *et al.* [15] who found the pathogen was detected in 25% (2/8) of infant formula milk samples using the FDA enrichment procedure and a chromogenic medium. The current results were higher than the previous studies by Iversen and Forsythe [5] and Muytjens *et al.* [13] who isolated *C. sakazakii* from 2.4% of 82 and from 14.8% of 141 samples of powdered infant formula milk analyzed, respectively. Nazarowec-White and Farber [62] isolated *C. sakazakii* from 6.7% of 120 dried PIFM. Oonaka *et al.* [63], Shetty *et al.* [64] and Fu *et al.* [65] could detect *C. sakazakii* in 6.6% (9/149), 5.4% (11/202) and 3.9% (3/77) PIFM samples,

Table 3: Relationship between incidence of *C. sakazakii* and other pathogenic bacteria in Domiati cheese samples collected from Cairo and Giza markets.

Sample type	No. of positive samples/ total samples	Gram-positive bacteria			Gram-negative bacteria				Total aerobic colony count (TACC)	
		<i>L. monocytogenes</i> Log cfu/g	<i>B. cereus</i> Log cfu/g	<i>Staph. aureus</i> Log cfu/g	Coliform log cfu/g	<i>E. coli O157:H7</i> Log cfu/g	<i>Salmonella spp.</i>	<i>Y. enterocolitica</i>	Log cfu/ g	Mold and yeast Log cfu/ g
Domiati	D3/20	2.1 b ±0.1	2.9 b±0.13	0.0d	5.30 ±0.06a	0.0b	ND	ND	7.47 bc ± 0.3	2.47d ±.02
Domiati	D6/20	0.0c	0.0c	2.1 c ±0.06	0.0e	0.0b	ND	ND	7.47 bc± 0.22	2.0e ± 0.0
Domiati	D9/20	0.0c	3.1a ±0.06	0.0d	3.00 d± 0.06	2.00 a ± 0.0	ND	+	10.47 a± 0.3	0.0f
Domiati	D13/20	0.0c	0.0c	3.1 a ± 0.1	3.84 ± 0.03c	0.0b	ND	ND	7.00 cd± 0.12	3.47b ± 0.06
Domiati	D15/20	3.2 a±0.15	0.0c	0.0 ^e	4.00 b± 0.22	0.0b	ND	ND	6.69d ± 0.15	2.77c ± 0.12
Domiati	D20/20	2.0 b±0.0	0.0c	0.0d	0.0e	0.0b	ND	ND	7.69 b± 0.06	3.47b ± 0.13
Total samples	6/20	3/6	2/6	2/6	4/6	1/6	0/6	1/6	6/6	5/6
(%) of positive samples	30	50	33.3	33.3	66	16.6	0	16.6	100	83

Values are means ± slandered error, ND not detected, Means with the same letters are not significantly different ($p \leq 0.05$).

Table 4: Relationship between the the incidence of *C. sakazakii* and other pathogenic bacteria in ice cream and yoghurt samples collected from Cairo and Giza markets

Sample type	No. of positive samples/ total samples	Gram-positive bacteria			Gram-negative bacteria				Total aerobic colony count (TACC)	
		<i>L. monocytogenes</i> Log cfu/g	<i>B. cereus</i> Log cfu/g	<i>Staph. aureus</i> Log cfu/g	Coliform log cfu/g	<i>E. coli O157:H7</i> Log cfu/g	<i>Salmonella spp.</i>	<i>Y. enterocolitica</i>	Log cfu/ g	Mold and yeast Log cfu/ g
Ice cream	Ice 6/25	0.0a	0.0a	0.0a	2.30b ± 0.17	0.0a	ND	ND	7.69a ± 0.41	2.00b ± 0.057
Yoghurt	Y17/25	0.0a	0.0a	0.0a	3.27a± 0.46	0.0a	ND	ND	8.71a ± 0.46	2.60a ± 0.17
Total samples	2/50	0/2	0/2	0/2	2/2	0/2	0/2	0/2	2/2	2/2
(%) of positive samples	4	0	0	0	100	0	0	0	100	100

ND (Not detected), + (Positive). Means with the same letters are not significantly different ($p \leq 0.05$), Values are means±standard error.

respectively. However, Seo and Brackett [66], O'Brien *et al.* [67], Sani and Yi [68] and Putthana *et al.* [69] did not detect any positive samples in 50, 390, 30 and 7 PIFM evaluated, respectively. On the other hand, the contamination of powdered infant formula milk samples with *C. sakazakii* in this work was lower than that of Aigbekaen and Oshoma [70] who reported that 38 (27.1%) of total 140 powdered foods were positive for *C. sakazakii*, with powdered infant foods having the highest frequency of 33.9%. Also, David *et al.* [71] found the pathogen to be detectable in 40% (4/10) of the PIFM samples analyzed to be higher than our findings.

According to the current and previous studies, it could be clearly stated that there is a direct relationship between powdered infant formula and *C. sakazakii*. Many studies have focused on the infant formula as the main source of this serious pathogen, despite the fact that formulas are exposed to heat treatment during processing. Post-processing contamination of the infant formula from food production environments may be responsible for the presence of this pathogen in infant formula since standard pasteurization practices are effective for the inactivation of the organism [8, 17, 70, 72]. Contamination of PIF, powdered infant drinks or other infant foods with *Cronobacter spp.* can occur during post-pasteurization processing, via the addition of dry ingredients (such as vitamins and minerals) or during packaging. Once it has become established as part of the in-house flora, there is an ongoing need to control the levels of *Cronobacter* strains in the environment of manufacturing

facilities. However, there is evidence to suggest that the organism may continually enter the processing chain in certain raw ingredients or from the environment [73]. There is therefore a need for data on the prevalence of *Cronobacter sakazakii* in the raw materials used in the production of PIF and other baby foods in order to determine the most prevalent primary sources of contamination and enable effective control strategies to be put in place [19, 67] reported that the presence of *C. sakazakii* in powdered infant milk formula depends on the process conditions and nature of the product. However, the prevalence of the organism following the drying stage and survival in powdered foods for a long time may be partially due to the organism's ability to resist desiccation and osmotic stress [74]. Furthermore, powdered infant formulas including dried bovine milk and milk products are not sterile products [4]. So, Powdered infant formula has been known to be contaminated with bacterial pathogens, including *Bacillus* species, *Clostridium* species, *Staphylococcus* species and *Enterobacteriaceae*, notably *Cronobacter* [75]. Therefore, hygienic measures and practices must be used during the manufacture of formula to minimize entry of contaminants into the process.

In respect to the incidence of *C. sakazakii* in powdered milk, the pathogen was not detectable in any of the 20 powder milk samples analyzed either using chromogenic or violet red bile glucose agar & tryptone soy agar media, reflecting the same efficiency of the two used methods for the isolation of the pathogen in milk powder samples. The obtained results were agreeable with

Shaker *et al.* [15], Jaradat *et al.* [76] and Wang *et al.* [77] who could not isolate *C. sakazakii* from any samples of milk powder analyzed. The current findings were also consolidated by El-Sharoud *et al.* [49] as they reported *C. sakazakii* was not detected in any of full-fat milk powder samples marketed in the Nile-Delta region of Egypt. The presence of *C. sakazakii* in these products is unlikely since the liquid milk is normally pasteurized before manufacturing the milk powders, as stated by Shaker *et al.* [15]. Iversen *et al.* [74] has been reported that pasteurization treatment is effective in the elimination of this pathogen. Moreover, *C. sakazakii* was found to be more heat-sensitive than other pathogenic organisms like *Listeria monocytogenes* [32]. Although it is not detected in milk powder samples, *C. sakazakii* is not ruled out from being a possible pathogen present in this product, putting in consideration that each sample represents itself only [15]. On the other hand and contrarily to our findings, Iversen and Forsythe [5] could isolate *C. sakazakii* from 4.2% (3/72) milk powder samples. Also, Gökmen *et al.* [48] and Aigbekaen and Oshoma [70] found the pathogen to be detected in 5% (3/60) and 6% (3/50) milk powder samples, respectively. The variations on the findings may be attributable to the fact that milk to be processed may contain different levels of *C. sakazakii* according to different processing techniques and analysis methods. Moreover, differences in the hygiene and storage conditions at the dairies and retail points are other key factors on the variations of the results [15, 48, 49]. Also, it could be that the organism is unequally distributed in the sample or its presence escaped detection [13].

Therefore, the current study recommends that good hygiene practices for production of infant formula and milk powder should take this organism in consideration so as to monitor *C. sakazakii* in these products routinely to comply the standard specifications. Several reports have implicated rehydrated powdered infant formula as a source of *C. sakazakii* in neonatal infections, so it is highly recommended to include this organism specifically in PIFM and milk powder standards by the Egyptian organization of standard specifications.

Interrelation Between *C. sakazakii* and Other Foodborne Pathogens in Powdered Infant Formula Milk (PIFM) Samples: Results in Table 5 show the relationship between the presence of *C. sakazakii* in powdered infant formula milk (PIFM) samples and simultaneous association of other food borne pathogenic microorganisms in the same samples.

Out of fifty PIFM samples, twelve were positive for *C. sakazakii* and were examined for their microbial quality. While the total aerobic colony counts ranged from 4.07 to 6.39 log cfu/g, mold and yeast were isolated from only three samples yielding 2.0, 1.0 and 2.6 log cfu/g, respectively. The results show wide differences between TACC and mold & yeast in PIFM samples. The twelve PIFM samples did not comply with of E.S.S No. 2082-1992 due to total aerobic colony counts that exceed 10^3 cfu/g, whilst mold and yeast counts were in acceptable quality range not exceed 10^2 cfu/g. The obtained results agree with El-Mossalami and Noseir [78] who examined 25 PIFM samples from different localities of Cairo and Giza cities. They found that TACC varied from 2.0 to 3.3 log cfu/g, while mold and yeast counts varied between 1.0 and 2.0 log cfu/g. Also, Oonaka *et al.* [63] and David *et al.* [71] reported that the aerobic bacterial load in PIFM samples analysed was much higher than the microbiological specification for powdered infant formula. In regard to Gram- negative bacteria, one sample was positive for *Y. enterocolitica*. Coliform group was also isolated from one sample giving 2.30 log cfu/g. *Salmonella* spp and *E. coli* O157:H7 were not detected in PIFM samples. The results were similar to findings of Magalhaes *et al.* [79] who examined 30 PIFM samples and found Enterobacteriaceae, Coliforms were $< 1 \times 10$ cfu/g. *Salmonella* spp. and *C. sakazakii* were not detected in 25g and 10g, respectively. Deeb *et al.* [80] could not detect *Salmonella* spp. in any of 50 PIFM samples tested. Al Timimi [81] could identify some G (-) bacteria as *Klebsiella pneumoniae*, *Citrobacter freundii*, *C. cloacae* and *C. sakazakii* in some samples of powdered infant formula (PIF) and dried infant cereals collected from different specialties that are available in Hilla city, Iraq. Zhou *et al.* [3] reported that 7 *C. sakazakii* and 6 *Klebsiella pneumoniae* isolates were obtained from 8 samples (26.7%) out of 30 PIFM samples randomly purchased in Chinese market. It is noteworthy that even though *C. sakazakii* was not detected in 132 PIFM samples collected from different brands retailed in Turkey, important pathogens in Enterobacteriaceae such as *Escherichia coli* (3 samples), *Citrobacter freundii* (2 samples), *Klebsiella pneumoniae* ssp. *pneumoniae* (2 samples), *Salmonella* (1 sample) and *Enterobacter cloacae* (12 samples) were detected [35]. Contradicting to the current results, El-Mossalami and Noseir [78] could not detect coliform bacteria in PIFM samples.

For Gram-positive bacteria, three samples were positive for *L. monocytogenes* with count of 2.0, 2.3 and 2.0 log cfu/g, respectively. While *B. cereus* was detected in three samples yielding 2.41, 2.19 and 2.24 log cfu/g,

Table 5: Relationship between the incidence of *C. sakazakii* and other pathogens in powdered infant formula milk (PIFM) samples collected from Cairo and Giza area.

Sample type	No. of positive samples/ total samples	Gram-positive bacteria			Gram-negative bacteria				Total aerobic colony count (TACC)	
		<i>L. monocytogenes</i> Log cfu/g	<i>B. cereus</i> Log cfu/g	<i>Staph. aureus</i> Log cfu/g	Coliform log cfu/g	<i>E. coli O157:H7</i> Log cfu/g	<i>Salmonella spp.</i>	<i>Y. enterocolitica</i>	Log cfu/ g	Mold and yeast Log cfu/ g
PIFM	3/50	2.00 b± 0.05	0.0c	0.0b	0.0b	0.0a	ND	ND	6.30 a ± 0.33	0.0 d
PIFM	5/50	0.0c	0.0c	0.0b	0.0b	0.0a	ND	ND	4.47d ± 0.06	0.0 d
PIFM	6/50	2.30 a± 0.17	2.41a ± 0.15	0.0b	0.0b	0.0a	ND	ND	6.39 a ± 0.35	2.00 b ± 0.0
PIFM	8/50	2.00 b± 0.0	2.19 b ± 0.057	0.0b	0.0b	0.0a	ND	ND	5.17 c± 0.017	0.0d
PIFM	9/50	0.0c	2.24 b± 0.23	0.0b	0.0b	0.0a	ND	ND	5.17 c ± 0.09	0.0d
PIFM	15/50	0.0c	0.0c	0.0b	0.0b	0.0a	ND	ND	4.30 d ± 0.17	0.0d
PIFM	17/50	0.0c	0.0c	0.0b	0.0b	0.0a	ND	ND	4.17 e± 0.066	1.00 c ± 0.0
PIFM	23/50	0.0c	0.0c	2.89 a ± 0.17	2.30 a ± 0.33	0.0a	ND	ND	5.69 b ± 0.35	0.0d
PIFM	29/50	0.0c	0.0c	0.0b	0.0b	0.0a	ND	+	5.91 b ± 0.52	0.0 d
PIFM	38/50	0.0c	0.0c	0.0b	0.0b	0.0a	ND	ND	4.11e ± 0.09	2.60 a ± 0.55
PIFM	39/50	0.0c	0.0c	0.0b	0.0b	0.0a	ND	ND	5.17c ± 0.024	0.0 d
PIFM	41/50	0.0c	0.0c	0.0b	0.0b	0.0a	ND	ND	4.07e ± 0.066	0.0 d
Total samples	12/50	3/12	3/12	1/12	1/12	0/12	0/12	1/12	12/12	3/12
(%) of positive samples	24	25	25	8.3	8.3	0	0	8.3	100	25

ND: not detected, +: positive, Means with the same letters are not significant ($P \leq 0.05$), Values are means ± standard error.

respectively, *S. aureus* in one sample with count of 2.89 log cfu/g. These results were compatible with that obtained by Tunio *et al.* [82] who reported that out of the 21 powdered food products including infant formula milks and powdered protein-based shakes, 7 samples were found to have been contaminated with different combinations of the three bacterial pathogens including *S. aureus*, *B. cereus* and *C. Sakazakii* and by Al Timimi [81] who found that *Bacillus subtilis* and *Staphylococcus aureus* were the most commonly identified isolates in PIFM samples analyses. Similar results were reported by El-Mossalami and Noseir [78] who found *Bacillus cereus* in 24% of PIFM samples. While, Helmy *et al.* [83] found that 70% of dried infant formula milk were contain *Bacillus cereus*. In this concern, Becker and Terplan [84] reported that counts of *Bacillus cereus* in dried milk products especially in infant formulas, must be remain below 100 cfu/g in order to minimize the risk of growth to critical level, through incorrect handling by consumers and free from *C. sakazakii* as well. Contrarily, the current results contradict those reported by Mohamed [85] and Sleem [86] who could not find *S. aureus* or *L. monocytogenes* in any of the examined infant formula milk samples.

Looking through the current and previously related results, it could be reported that infant formula could support the growth of bacterial pathogen despite their low water activity, hence could be a good vehicle for the transmission of pathogens. Presence and high counts of the foodborne pathogen in infant formula powder foods indicating unacceptable quality that did not meet the international acceptable standards may be due to methods of processing, preparation, handling and storage of formulas and/or may come from recontamination by environments, workers or equipments during the ultimate

phases of the process as dried cereals blended with skim milk powder, vitamins and some other ingredients or finally during packaging [54, 71, 78, 86].

CONCLUSION

The study revealed that *C. sakazakii* was found in Domiati cheese, yoghurt, ice cream and powdered infant formula milk samples representing Cairo and Giza area. The matter is highly important for these products to be potential transmission vehicles of the pathogen. Thus, the highest hygienic requirements must be satisfied to avoid contamination and the Critical Control Points should be carefully applied to minimise the microorganisms entry into the hygiene zones and prevent the proliferation of those that are already present. The Egyptian Standard Specification (E.S, No.2082-1992) for infants and children foods did not state the absence of *C. sakazakii* within the microbiological standards which is highly recommended and appreciated as an outcome of this study. Data presented in tables statistically analyzed and results with the similar letters show no significantly difference.

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