



Cronobacter sakazakii and Microbiological Profile of Infant Formulae and Some Dairy Products Consumed by Infants

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Abstract | *Cronobacter sakazakii* is an opportunistic, food-borne pathogen that causes serious diseases and deaths in children. To the best of our knowledge, This is the first work that evaluated infant formula and other dairy products consumed by infants for the presence of *Cronobacter sakazakii* in Sharkia Governorate, Egypt. Also, we evaluated the possible role of the MALDI-TOF MS system as an accurate and rapid method for *Cronobacter sakazakii* identification by comparing it with the conventional biochemical tests. The results cleared that *Cronobacter sakazakii* was detected in 14.3%, 40%, and 6.25% of the contaminated starting from birth IFMP, follow up IFMP, and rice pudding samples, respectively. On the other hand, microbiological analysis of the samples cleared high contamination levels and the presence of *Aeromonas hydrophila*. The present study enlightens the presence of high danger for infants consuming these contaminated products, thus hygienic precautions and effective control measures should be applied.

Keywords | *Cronobacter sakazakii*, Infant formula, MALDI-TOF MS, *Aeromonas hydrophila*, Baby food

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INTRODUCTION

Cronobacter sakazakii is a rod-shaped gram-negative member of the *Enterobacteriaceae* family. At first, this organism was called “yellow-pigmented *Enterobacter cloacae*” then it was renamed as *C. sakazakii* at 1980 (Farmer *et al.*, 1980). These bacteria are common commensal and can be found in various kinds of foods like meat, vegetables, cheese, grains, and spices. It can be isolated from the rice plant leaves as it was an endophytic bacteria (Yang *et al.*, 1999). By the year 1960, *C. sakazakii* has been frequently detected in various types of infant formula milk powder (IFMP), thus a great attention has been paid for these products. Currently, *C. sakazakii* is classified as a category A organism that is directly associated with occurrence of many infections such as meningitis, meningo-encephalitis, necrotizing enterocolitis, and severe sepsis

especially in babies and children, beside its ability to cause osteomyelitis and bacteremia in immuno-compromised and older individuals (Kucerova *et al.*, 2010). Food safety organizers cleared that, there is a high-risk group of individuals, including infants because of their undeveloped immunity and their little competing intestinal flora. So, microbiological analysis of the products used for infants should be applied regularly for evaluating the quality and confirming the safety of these products.

The conventional identification laboratory methods used for microbe identification may give wrong results, which may not be helpful, especially for medical diagnosis (Cherkaoui *et al.*, 2010). On the other hand, Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) is an accurate, rapid, and cost-effective method for medical diagnosis as it

has been used for the identification of human pathogens and environmental microbes, and it has the advantage of being used successfully for the identification of slow-growing, and fastidious organisms (Biswas and Rolain, 2013).

The aims of this study were to detect and isolate *C. sakazakii* in IFMP, and different types of dairy products consumed by infants, as baby food and rice pudding marketed in Sharkia governorate, Egypt. Also, to evaluate the efficiency of MALDI-TOF MS for *C. sakazakii* identification by comparing its results with conventional culture and biochemical routine tests. In the Present study, microbiological quality of the products were assessed in terms of public health.

MATERIALS AND METHODS

SAMPLING

A total number of eighty samples of different milk products were randomly collected from local markets and pharmacies in Sharkia Governorate, Egypt. These samples were classified into three groups: I- IFMP samples (20) (10 for each of infant formula From birth, and Follow up formula). II- baby food samples (30) (4 for Fruit yoghurt with different flavor, 16 for Blend of milk and grain (powder) with fruit, and 10 for Pudding milk with different flavors). III- Rice pudding (30). Each sample was obtained in its original container without opening and then transferred to the laboratory without delay in a clean, dry, and sterile ice-box at 4°C.

DETECTION, ISOLATION AND IDENTIFICATION OF *C. SAKAZAKII* USING CULTURE AND BIOCHEMICAL TESTING METHOD

The method described by (FDA, 2002) for *C. sakazakii* detection and isolation depending on 3 successive steps, including pre-enrichment in buffered peptone water (BPW) broth (Oxoid LTD, Bagstoke Hampshire, England), enrichment in selective Enterobacteriaceae Enrichment Broth (EEB) (HiMedia, Mumbai, India) and plating on selective violet red bile glucose agar (VRBGA) (Oxoid LTD, Bagstoke Hampshire, England) and tryptone soy agar (TSA) (Oxoid LTD, Bagstoke Hampshire, England). Each suspected colony was picked up and cultured on slope agar for further microscopical and biochemical identification.

IDENTIFICATION OF *C. SAKAZAKII* USING THE MALDI TOF TECHNIQUE (KARAS AND KRÜGER, 2003)

MALDI-TOF MS Ultra Flex system (Brüker Daltonik) for the MALDI MS identification was used, according to the apparatus instructions. To identify unknown bacterial isolates, Spectra were compared to fingerprint database by using the Brüker Biotyper 3.1 software and a library of 5,623 entries.

According to the guidelines of the manufacturer, a score of ≥ 2 depicts identification to the species level and an intermediate log score between $\geq 1.7 < 2$ for identification to the genus level. A low score of < 1.7 was regarded as unreliable for identification.

DETERMINATION OF MICROBIOLOGICAL PROFILE OF SAMPLES

Ten ml of each sample were added aseptically and transferred into a sterile tube containing 90 ml of sterile saline solution. The latter was shaken well to have 1:10 dilution, followed by decimal serial dilution according to APHA (2004). The total aerobic colony count was carried out according to the conventional method (BAM, 2009) Using Standard Plate Count Agar (PCA) (Himedia, Mumbai, India) at $32^{\circ}\text{C} \pm 1$ for 48 ± 2 hours.

Enumeration of molds and yeasts was carried out on the samples using the medium of Sabouraud Dextrose Agar (Oxoid LTD, Bangstoke Hampshire, England). The method recommended by APHA (2003), was followed up.

Isolation and identification of *Aeromonas* spp. were carried out according to Carnahan and Joseph (2005), where IFMP samples were enriched by reconstitution in sterilized, distilled water (10gm Sample / 90ml water), whereas rice pudding and baby food samples were pre-enriched by mixing 10gm sample with 90ml BPW broth. After incubation at 36°C for 24h, 0.1ml of the enrichment culture was evenly spread onto pre-poured plates of *Aeromonas* selective agar medium (Himedia, Mumbai, India) and incubated at 30°C for 24 hours. The presumptive colonies were subjected to Gram staining and biochemical identification.

Statistical analysis was performed using the Statistical Package for Social Sciences version 22.0 (IBM Corp., Armonk, NY, USA).

RESULTS AND DISCUSSION

Infants are the most vulnerable groups of humans to microorganisms because of their lack of intestinal flora and their undeveloped immune system (Townsend and Forsythe, 2008). The Present study focused on different types of dairy products consumed by infants, including IFMP, some types of baby food, and rice pudding. These samples were evaluated for the presence of *C. sakazakii* using the FDA culture based technique, as it is considered as a gold standard technique.

Concerning IFMP, the results cleared that 70% and 50% of starting from birth and follow up samples, respectively were contaminated with *Enterobacteriaceae*

species. The biochemical identification of the strains revealed that *C. sakazakii* was detected in only 42.9% (3/7) of the contaminated samples of starting from birth formula samples, while it was present in 40% (2/5) of the contaminated follow-up samples. On the other hand, other *Enterobacteriaceae* species, as *Pantoea* spp (*Pantoea agglomeranse* (*P. agglomeranse*) and *Pantoea anantis* (*P. anantis*)) and *Enterobacter cloacae* (*E. cloacae*) were isolated in the percentages of 14.3% and 42.9% from the contaminated samples of starting from birth and of 20% and 40% from follow up samples, respectively. Other authors recorded the detection of *C. sakazakii* in infant formula samples with different isolation rates as Jaber et al. (2015) and Saleh (2017) found it in 18%, and 5% of the examined infant formula samples.

Contamination of IFMP with microorganisms occurs during various stages of the manufacture, either from the materials used for its manufacture as bovine milk, which is considered an essential ingredient of IFMP and at the same time it is a potential source of pathogenic bacteria to humans, or from contaminated additional dry ingredients as vitamins and minerals, contaminated equipments or through asymptomatic diseased workers in the plant. Also, it is expected that contamination of these products with *Cronobacter* occurs mainly after pasteurization process, either during drying or packing as these microbes are incapable of surviving pasteurization, but have a great ability to resist osmotic pressure or drying process (Strydom et al., 2012). On the other side, the incorrect storage of contaminated reconstituted IFMP prior to consumption can support rapid growth of *C. sakazakii* during the holding time, even the powder contains low levels and so it is considered to be a risk factor.

Cronobacter sakazakii and other related species were also detected in other examined dairy products consumed by the infants. The results that were presented in the Table 1 showed that only blend powder of milk and grain samples contain *C. sakazakii* strains with percentage of 16.7%. Moreover, we found that the examined yoghurt samples contained *E. Cloacae* with a percentage of 100%, while it was detected in 58.3% and 87.5% of the blend of milk and grain and pudding with different flavor samples, respectively. *Pantoea* species were detected in the blend of milk and grain and pudding with different flavor samples with a percentage of 25% and 12.5% respectively. Similar results were recorded by El-Sharoud et al. (2008) who detected the absence of *C. sakazakii* in the examined yoghurt samples, while (El-Gamal et al., 2013) found *C. sakazakii* in only 4% of their yoghurt samples. Different results were obtained by Iversion and Forsythe (2003) as they found *C. sakazakii* in 10.2% of the examined baby food samples. Low pH may be considered the main cause of *C. sakazakii* absence in the examined yoghurt samples.

The unhygienic quality of the processing environment can explain the presence of this pathogen in other products, as microbiological analysis applied by scientists indicated the presence of correlation between the environment and *Cronobacter* contamination in the final products (Reich et al., 2010).

Concerning rice pudding, *C. sakazakii* was present in 12.5% of rice pudding samples, while *E. cloacae* and *Pantoea* species were detected in the percentage of 75% and 12.5%, respectively. Lower results were reported by (Saad and Ewida, 2018), where *C. sakazakii* was found in only 10% of the contaminated rice pudding samples. The presence of *C. sakazakii* in rice pudding may be explained due to many causes, either contaminated constituents used during manufacturing, or post-pasteurization and processing contamination. Rice of poor quality used during manufacturing is considered as the main cause as *C. sakazakii* could be isolated from many types of cereal foods, including ground rice, starch, and flour (Lee et al., 2012).

C. sakazakii has been considered as a causative agent of many dangerous infections, including septicemia, necrotizing enterocolitis, and meningitis, which may be complicated by brain abscess, cyst formation, and cerebral infarction in newborn infants, which are mostly associated with a bad prognosis specially in premature infants and Low-birth-weight as mortality rates may be recorded up to 80% of infected cases, while few cases have been recorded in adult, where it causes dangerous diseases as malignancies, which is not usually life-threatening (Block et al., 2002).

The correct measures and identification of bacterial pathogens is of great concern, especially in outbreaks for applying effective control measures for these diseases. The conventional culture-dependent methods used in the microbiological laboratories do not supply all the requirements of our fast developed world. As mis-identification can easily occur with these methods, especially in the genera with close biochemical reaction results. Also, different molecular techniques are time and money-consuming. On the other side, MALDI-TOF MS technique is a molecular technique used recently in clinical microbiology for identification of bacterial isolates depending on the protein fingerprint of these isolates using short laser pulses (Abouseada et al., 2016). It has been reported as a potential method for bacterial identification as it gives a solution for the other methods' difficulties as it was a rapid procedure with less cost and greater quality than the conventional methods. It does not need any purification, or concentration of isolates as in other molecular techniques (Prod'hom et al., 2010).

In the current work, the spectra scores were determined

for *C. sakazakii* strains isolated from 9 samples which were identified by biochemical tests, including infant formula, different types of baby food, and rice pudding as 5, 2, and 2 for each respectively. The accurate identification of *C. Sakazakii* were obtained in a range of 1.999 to 2.166, where 7 (77.8%) isolates were identified to the species level, at score >2, while accurate identification to the genus level was present in 2 (22.2%) isolates at score (1.7:2), and no isolates give no reliable identification at a score (<1.7). The data in the Table 2 cleared that 3 strains (out of 9) which were identified as *C. Sakazakii* by biochemical tests were appeared to be *E. cloacae* using MALDI-TOF technique. According to MALDI-TOF technique, the difference in the incidence of isolated *C. sakazakii* and other *Enterobacteriaceae* species presented only in the examined IFMP samples (1/3) and rice pudding samples (1/2), while baby food samples give the same percentages in different species with both of them.

Some authors identified all the examined strains as *Cronobacter* species only to the genus level, but they could not use MALDI-TOF MS to identify them to species level exactly (Wang et al., 2017), while Guo et al. (2014) succeeded to identify many examined strains to both levels with different percentages. On the contrary, others note that this method does not identify the examined isolates with high confidence, and suggested that, it can be used earlier in the identification protocols, then confirm the results using another method (Cherkaoui et al., 2010).

Microbiological examination and detection of pathogens in raw milk and different dairy products are essential parameters for evaluation of the quality of these products to ensure the human safety, as these products are easily contaminated and have many nutrients that support the growth of many microbes efficiently. In the present study, IFMP samples were investigated for the presence of bacterial contaminants, and the results were given in Table 3 cleared that, the mean values for aerobic plate count/ml in the examined IFMP samples were $1.1 \times 10^6 \pm 6.7 \times 10^5$ and $1.2 \times 10^6 \pm 6 \times 10^5$ cfu/g for Infant formula from birth and Follow up formula, respectively. Lower results were obtained by Adebayo-Tayo et al. (2012), Matug et al. (2015) and Tahoun and Abdelfatah (2015).

The manufacturing of IFMP includes many steps that can reduce the microbial content in the end product, however, these microbes might enter to these products after dehydration or before final packaging (Buchanan and Oni, 2012). Also, the total microbial content of this product can increase during the preparation or before consumption. Therefore, it is necessary to be sure of using hygienic measures during the handling and consuming the prepared infant milk as quickly as possible.

The level of bacterial contamination of food samples consumed by infants was $1.2 \times 10^6 \pm 6.8 \times 10^5$, $5.8 \times 10^4 \pm 1.9 \times 10^4$ and $1.4 \times 10^7 \pm 7.2 \times 10^6$ cfu/g for Fruit yoghurt (jar), blend powder of milk and grain with fruit, and pudding with a different flavor, respectively (Table 3). Our findings were lower than those reported by Gun et al. (2008), and higher than those reported by Adebayo-Tayo et al. (2012) and Secim and Ukar (2014). Concerning rice pudding samples, the mean value of aerobic plate count/ml in the examined samples was $1.2 \times 10^6 \pm 3.4 \times 10^5$ cfu/g. Lower results were obtained by Ayok (2002), and Secim and Ukar (2014). While higher results were declared by Abdellatif and Saad (2016). There are many sources for microbial contamination of dairy products, primary sources as raw milk and water, and secondary sources as utensils, and handling steps in production. The differences in results in other works can be explained due to the difference in microbial load of raw materials used in processing, inefficient heat treatment during processing, and the hygienic condition of processing or storing.

Concerning the presence of some pathogenic organisms, the results of the current study revealed that *Aeromonas* species were identified in 70% and 80% of the examined positive infant formula starting from birth and follow up formula, where *A. hydrophila* can be detected in 71.4% and 87.5% of the contaminated samples of both types respectively. While they can be detected in 75%, 75% and 100% of the examined fruit yoghurt, a blend powder of milk and grain with fruit, and pudding with a different flavor, where *A. hydrophila* can be detected in 66.7%, 100% and 100% of the examined positive samples, respectively (Table 4). In the examined rice pudding samples, *Aeromonas* species can be detected in 83.3% of positive samples. *A. hydrophila* can be detected in 92% of the positive samples. Lower results were obtained by Deeb (2005), Awan et al. (2009).

In recent years, *Aeromonas* has received more attention as a reason for foodborne diarrheal diseases in healthy people. Foods of animal origin as dairy products may act as an important vehicle in the transmission of them to humans. Animal feces appear to be the major source of contamination of foods So, the presence of mesophilic *Aeromonas* species in the examined products may be due to contaminated milk or contamination during production or preparation (Igbinsosa et al., 2012). The presence of a high number of *Aeromonas* species, particularly in foods, which are stored under refrigeration as yoghurt and rice pudding, may be explained by the ability of aeromonads to grow at low temperature. Aeromonads have been involved in the severe diarrheal disease of short duration in vulnerable groups, including children or the immune compromised individuals and they are known to cause travelers' diarrhea (Von Graevenitz, 2007).

Table 1: The incidence of isolated *Cronobacter sakazakii* and other *Enterobacteriaceae* species in the examined samples according to the biochemical identification.

Type of samples (N=80)		Contaminated % samples no.		<i>C. sakazakii</i> No %		Other enterobacte- riaceae			
						<i>Pantoea</i> spp.		<i>E. cloacae</i>	
						No	%	No	%
IFMP (N=20)	Infant formula from birth (N=10)	7	70%	3	42.9%	1	14.3%	3	42.9%
	Follow up formula (N=10)	5	50%	2	40.0%	1	20.0%	2	40%
Baby food (n=30)	Fruit yogurt (jar) (N=4)	2	50%	0	0.00%	0	0.00%	2	100%
	Blend of milk and grain with fruit (powder) (N=16)	12	75%	2	16.7%	3	25.0%	7	58.3%
	Pudding milk (with different flavors) (N=10)	8	80%	0	0.00%	1	12.5%	7	87.5%
Rice pudding (n=30)		16	53.3%	2	12.50%	2	12.5%	12	75.0%

Table 2: MALDI Biotyper database for *C.sakazaki* isolated from the examined samples.

NCBI Identifier	Score	Product	Strain	No.
28141	2.032	IFMP	<i>Cronobacter sakazakii</i> LMG 2789 LMG	1
550	1.999	IFMP	<i>Enterobacter cloacae</i> MB-8779-05 THL	2
550	2.097	IFMP	<i>Enterobacter cloacae</i> MB-5277-05 THL	3
28141	2.166	IFMP	<i>Cronobacter sakazakii</i> DSM 4485T DSM	4
28141	2.158	IFMP	<i>Cronobacter sakazakii</i> DSM 4485T DSM	5
550	2.079	Rice pudding	<i>Enterobacter cloacae</i> MB-5277-05 THL	6
28141	2.109	Rice pudding	<i>Cronobacter sakazakii</i> DSM 4485T DSM	7
28141	1.999	Blend of milk and grain	<i>Cronobacter sakazakii</i> LMG 2786 LMG	8
550	2.094	Blend of milk and grain	<i>Cronobacter sakazakii</i> LMG 5740 LMG	9

Table 3: Aerobic plate count (cfu/g) in the examined samples.

Type of samples (N=80)		Aerobic plate count			
		Min.	Max.	Mean	(±) S.E.M
IFMP (N=20)	Infant formula from birth (N=10)	2.0x10 ³	5.7x10 ⁶	1.1x10 ⁶	6.7x10 ⁵
	Follow up formula (N=10)	3.0x10 ⁴	5.0x10 ⁶	1.2x10 ⁶	6.0x10 ⁵
Baby food (N=30)	Fruit yogurt (jar) (N=4)	3.0x10 ⁵	3.2x10 ⁶	1.2x10 ⁶	6.8x10 ⁵
	Blend of milk and grain with fruit (powder) (N=16)	3.8x10 ³	3.0x10 ⁵	5.8x10 ⁴	1.9x10 ⁴
	Pudding (milk with different flavors) (N=10)	3.1x10 ⁵	5.0x10 ⁷	1.4x10 ⁷	7.2x10 ⁶
Rice Pudding (N=30)		3.0x10 ⁴	5.3x10 ⁶	1.2x10 ⁶	3.4x10 ⁵

Table 4: Distribution of *Aeromonas* organisms in the positive examined samples.

Type of samples (N=80)		No. of iso- lates No %		Aeromonas species			
				<i>A. hydrophila</i>		<i>A.scubertii</i>	
				No	%	No	%
IFMP (N=20)	Infant formula from birth (N=10)	7	70%	5	71.4%	2	28.6%
	Follow up formula (N=10)	8	80%	7	87.5%	1	12.5%
Baby food (N=30)	Fruit yogurt (jar) (N=4)	3	75%	2	66.7%	1	33.3%
	Blend of milk and grain with fruit (powder) (N=16)	12	75%	12	100%	0	0.00%
	Pudding (milk with different flavors) (N=10)	10	100%	10	100%	0	0.00%
Rice pudding (N=30)		25	83.3%	23	92.0%	2	8.00%

Table 5: Total yeast and mold count (cfu/g) in the examined samples.

Type of samples (N=80)		Positive samples		Total yeast and mold count		
		No.	%	Min.	Max.	Mean ±S.E
IFMP (N=20)	Infant formula from birth (N=10)	10	100	1.0x10 ³	4.1x10 ⁶	7.4x10 ⁵ ±4.8x10 ⁵
	Follow up formula (N=10)	10	100	1.0x10 ³	2.0x10 ⁵	4.4x10 ⁴ ±1.9x10 ⁴
Baby food (N=30)	Fruit yogurt (jar) (N=4)	4	100	2.0x10 ⁴	2.1x10 ⁵	1.4x10 ⁵ ±4.4x10 ⁴
	Blend of milk and grain (powder) with fruit (N=16)	14	87.5	<1x10 ³	7.0x10 ⁶	9.02x10 ⁵ ±5.1x10 ⁵
	Pudding (milk with different flavor) (N=10)	10	100	2.0x10 ⁴	5.3x10 ⁵	1.7x10 ⁵ ±6.1x10 ⁴
Rice pudding (N=30)		30	100	2.0x10 ⁴	1.6x10 ⁷	8.5x10 ⁵ ±5.6x10 ⁵

The results of the current study revealed that, yeast and mold were present in 100% of all types of the examined samples, except in blend powder of milk and grain with fruit, where they were detected in 87.5% of them, and ranged from 1.4x10⁵ to 9.02x10⁵ cfu/g in the examined samples (Table 5). Various studies had reported the presence of yeast and mold in our examined samples with different isolation rates. In IFMP, lower results were obtained by Sezer et al. (2015), and Tahoun and Abdelfatah (2015). Regarding other samples consumed by infants, our findings were in disagreement with those reported by Secim and Ukar (2014), and Abdel Latif et al. (2016), as they gave lower results. In rice pudding samples, the obtained results were higher than that declared by Secim and Ukar (2014), and Abdel latif et al. (2016), while it was partially different with that obtained by Ayok (2002).

The presence of yeasts and molds in the examined samples in this study highlighted the presence of inadequate sanitary conditions in the equipment, employees or the production area. Some species of yeast as *Candida* species cause human health hazards and they are considered the most common, ubiquitous fungal pathogens that affect humans. Also, many strains of molds are able to produce toxic metabolites called Mycotoxins in milk and other dairy products causing many human diseases ranged from gastroenteritis, kidney failure conditions to cancer (Palumbo et al., 2011).

CONCLUSIONS

The study indicated that the examined products either IFMP or other types of dairy products marketed in Sharkia Governorate have a high contamination level. However, the presence of *C. Sakazakii* in the examined products has a significant public health problem, especially in developing countries because of the lack of hygienic measures and absence of data about this microbe. So, there is an urgent need for the application of strict hygienic guidelines to restrict the hazard of this microbe in hospital neonatal units, factories, and kitchens. Identification of isolates by using MALDI-TOF MS provide more reliable and faster bacteria species identification than conventional

culture and biochemical methods, because of its sensitivity, low cost and short duration.

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AUTHORS' CONTRIBUTIONS

RMYE performed sample collection, and data analysis. IHA, MAHM and ENA designed the study, supervised the experiment, and wrote the manuscript draft. All the authors have read, revised, and approved the final manuscript.

CONFLICT OF INTEREST

All authors declare that there is no conflict of interest.

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