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Prevalence and molecular characterization of *Cronobacter* species in Egyptian table eggs and egg-based desserts; special insight on *Cronobacter sakazakii*

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Abstract

The occurrence of *Cronobacter* spp. was investigated in table eggs and egg-based desserts obtained from retail stores. *Cronobacter* was isolated from 57 out of 180 (31.7%) (95% Confidence Interval [CI]: 26–43%) examined samples of eggs and egg-based desserts. The prevalence of *Cronobacter* spp. was significantly higher in farm eggs (67%) ($P < 0.05$) than in Balady eggs (23%), cream cake (27%) and small-scale ice cream (27%). *Cronobacter (C.) sakazakii* and *C. muytjensii* were the predominant isolates obtained in the present study. All *C. sakazakii* isolates were molecularly confirmed with higher incidence in small-scale ice cream (62.5%).

Keywords: *Cronobacter* spp.; *C. sakazakii*; desserts; eggs; PCR; *16S* rRNA sequencing

Introduction

For decades, eggs and egg-based products have been a main ingredient of recipe in human diet all over the world. Eggs are catalogued as convenient foods, readily available, with a high organoleptical quality and nutritional value, providing humans with proteins, fats, minerals and vitamins. They can be used in manufacturing of some desserts, such as cream cake and ice cream. Egg yolks are used in ice cream as an emulsifier (binding fat and water together in a creamy emulsion) or stabilizer agents (reduce its tendency to melt rapidly) to extend the shelf-life of ice cream during freezing. However, microbial contamination of eggs at primary production or during processing of egg could be associated with the presence of food-borne pathogens, *Salmonella* spp. being the most prevalent one, although other pathogenic microorganisms have been identified, such as *Cronobacter (C.) sakazakii* or *Staphylococcus aureus* (Galiş *et al.*, 2013; Hochel *et al.*, 2012). This means that contaminated raw eggs can pose a public health risk if proper cooking procedures and/or inactivation treatments at industry are not followed. Despite preventive measures being implemented during the egg-processing chain, risks associated with the consumption of table eggs and egg-based products are not negligible, and are being classified by the European Scientific Committee on Veterinary Measures as a food group posing public health hazards (European Food Safety Authority [EFSA], 2014).

In Egypt, egg-based desserts are very popular and consumed by all age groups. Small-scale ice cream, for instance, is a frozen product prepared by traditional methods through freezing a mixture of milk, cream, milk solids, emulsifiers, stabilizers, and flavoring and coloring agents. It is processed in small-scale production units that usually have certain doubts regarding the hygienic measures of the produced ice cream (Warke *et al.*, 2000). Additionally, cream cake as a ready-to-eat food is widely consumed worldwide and prepared easily by mixing of

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different ingredients, such as flour, milk, butter cream, fruit, chocolate, and mainly eggs. Since both ice cream and cream cake require uncooked/raw eggs during their preparation, they constitute a favorable medium for growth of different pathogens because of the availability of nutrients and moisture content as well as neutral pH (Siriken *et al.*, 2009). Hence, such products could transmit public health hazards to consumers.

Cronobacter spp. is considered an opportunistic foodborne pathogen-causing life-threatening infection in all age groups, particularly in neonates and immunocompromised adults (Mullane *et al.*, 2008). The *Cronobacter* genus was first defined by Iversen *et al.* (2007), including different species, such as *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis*, *C. genomospecies 1, C. universalis*, and *C. condiment* (Joseph *et al.*, 2012). It is a Gram-negative, motile by Peritrichous flagella, rod-shaped and non-spore-forming bacteria. Moreover, it is an exemplary facultative, oxidase-negative and catalase-positive anaerobe (Iversen *et al.*, 2007). *C. sakazakii* could resist osmotic stress and dryness for a long period, and could be isolated from powdered infant formula even after 2½ years of storage (Bai *et al.*, 2019).

The virulence factors of *Cronobacter* spp. have been reported, including the presence of endotoxins, invasion and adherence in cellular lines, flagella and presence of a capsule that may facilitate its attachment to surfaces, biofilms formation, and persistence under desiccated conditions (Holý and Forsythe, 2014). Notably, 77% of these organisms produce biofilms, which are removed with difficulty using the disinfectants commonly applied in hospitals, daycare centers, and food industries (e.g., T.B.Q.® *disinfectant*, Zep DZ-7 infectant, and Zep FS Formula 386L acid cleaner sanitizer) (Kim *et al.*, 2007; Oh *et al.*, 2007).

In 2008, Food and Agriculture Organization/World Health Organization (FAO/WHO, 2008) classified *Cronobacter* as a pathogenic microorganism associated with sporadic infections and disease outbreaks. *Cronobacter* spp. was previously recovered from different food matrices, such as plant foods, including vegetables, herbs, spices and cereal products, as well as foods of animal origin, such as powdered infant formula (PIF), milk, milk products, fish, meat and meat products (Das *et al.*, 2021; Hayman *et al.*, 2020; Saad and Ewida, 2018).

The composition of dry foods together with their low water activity (a_w) could significantly affect the survival of *Cronobacter* spp. in such foods (Beuchat *et al.*, 2009). Gurtler and Beuchat (2007) reported that reduction level in the count of such a pathogen in powdered food was significantly higher with a_w between 0.43 and 0.50, compared to other items with a_w between 0.25 and 0.30. The authors found that the pathogen was more persistent in formulas with lower a_w for long time of storage. Thus, the presence of *Cronobacter* spp. in food items is often associated with contaminated PIF and may be presented in dry egg products.

Although several studies have covered the incidence of *Cronobacter* spp. in foodstuffs, little is known about its occurrence in table eggs and egg products. Some previous works reported its detection on the eggshell surface, cloacal swabs, and fertilized eggs (Amer and Mekky, 2019). In this context, the present study was designed to evaluate farm and Balady hens' eggs (laid by the Egyptian native breeds of hens) together with the most common Egyptian desserts (small-scale ice cream and cream cake) for the presence of *Cronobacter* spp., with special reference to *C. sakazakii*, by conventional culture-based methods followed by molecular characterization of isolates.

Materials and Methods

Samples collection and preparation for microbiological analysis

From November 2020 to June 2021, a total of 300 eggs, including Balady hens' eggs and farm hen's eggs (150 eggs, each represented by 30 samples, with every 5 eggs constituting one sample) and 60 samples of egg-based desserts (small-scale ice cream and cream cake) comprising 30 samples each, were collected on a random basis in Assuit province (Egypt). Farm eggs and egg-based desserts were acquired, respectively, from different food stores and sweets outlets of Assiut city. Balady eggs were obtained from farmers residing in different villages of Assiut governorate. The frozen or refrigerated products (egg-based desserts) were transported to the laboratory in approved insulated containers containing ice (4–5°C) within 1 h of collection, stored at refrigeration temperature, and tested on the same day.

Each sample of eggs was washed in sterile plastic bags with 100 mL of sterile saline solution (NaCl 0.9%). The bags were held at an angle with eggs and saline held in one corner. The washing of eggs was done twice for 1 min at an interval of 5 min by rubbing each egg shell through the bag (Pienaar *et al.*, 1995). On the other hand, the egg content was prepared according to method demonstrated by Vanderzant and Splittstoesser (1992). In brief, each egg was washed with warm water (32°C) using a brush and soap. Then, the egg was drained and immersed in 70% ethanol (BP 82011; Thermo Fisher Scientific, MA, USA) for 10 min, and then flamed. A hole was made at the wider end of the egg by using a sterile scalpel, and the contents of each sample was collected aseptically in a sterile mixer until the sample became homogeneous.

Isolation and biochemical identification of *Cronobacter* spp.

The isolation technique was performed using Cronobacter Screening Broth (CSB) (CM1121; Thermo Fisher Scientific) method according to Iversen *et al.* (2008). First, 11 mL of the prepared samples (egg contents, small-scale ice cream and cream cake) were pre-enriched in 99 mL of sterile 0.1% peptone water (CM0009B; Thermo Fisher Scientific) to obtain a dilution of 1:10; then, incubation was done at 37±1°C for 18±2 h. Afterwards, the enrichment was carried out with 0.1-mL egg shell or pre-enriched samples inoculated aseptically into 10 mL of CSB supplemented with vancomycin 10 µg/mL (Oxoid, Basingstoke, Hampshire, UK) separately and incubated at 42°C for 24 h.

Samples with carbohydrate fermentation, resulting in a color change from purple to yellow, were used for further examination. Then, a loopful from purple tubes was sub-cultured onto *Cronobacter* chromogenic isolation agar (CM1122; Oxoid) and incubated at 44±1°C for 24±2 h. One typical colony from the most abundant morphologically distinct colonies was selected, sub-cultured, and grown in the same condition for further identification. The suspected colonies (blue/green colonies) were picked onto Tryptose Soya agar (TSA) (CM0131; Oxoid) slants and incubated at 37°C for 48 h before being subjected to biochemical identification. The conventional biochemical tests for identification of *Cronobacter*, included sugar fermentation (sucrose, dulcitol, and sorbitol), indole production, and malonate utilization were performed (Iversen *et al.*, 2008).

Molecular characterization of *Cronobacter sakazakii*

From the overnight incubated TSB (QingDao Hope Bio-technology, Qingdao, China) sub-cultured with the obtained isolates, 1 mL was taken into 1.5-microcentrifuge tube and centrifuged at 10,000 ×g for 1 min. The supernatant was discarded and the content was resuspended in 500-µL nuclease-free water. Then the tube was heated in a thermomixer at 100°C for 10 min, re-centrifuged at 10,000 ×g for 1 min., and the supernatant was stored at -20°C for further use.

A 929-bp fragment of *16S* rRNA gene was amplified using the primer pairs of Esakf (5' GCT YTGCTG ACG AGTGGCGG 3') and Esakr (5' ATC TCT GCA GGATTCTCT GG 3') (Applied Biosystems, MA, USA) according to the method demonstrated by Lehner *et al.* (2004). Briefly, a volume of 15 µL of reaction mixture was used and consisted of 2 µL genomic DNA (150 ng), 7.5 µL Cosmo Master Mix (Promega®, USA), 1 µL of each primer (0.5 µM) and final volume was adjusted to 15 µL by adding nuclease free water.

The amplification was performed in a programmable heating block (Gradient Thermal Cycler; Veriti Applied Biosystems, CA, USA) at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1.30 min and then kept for 5 min at 72°C for final extension. The amplified polymerase chain reaction (PCR) products were revealed by electrophoresis in 1% agarose gel containing ethidium bromide (1-µL/mL electrophoresis buffer) at 100 V for 30 min and finally visualized and documented under ultraviolet (UV) trans-illuminator (UVsolo TS® Imaging System, Biometra®, Jena, Germany). The bands of PCR products containing the positive DNA sequence of 929-bp *16S* rRNA gene were analyzed using Doc-It®LS image acquisition software (Biodoc Analyzer, Biometra).

PCR products were sequenced by Applied Biosystems 3130 genetic analyzer (HITACHI, Japan) as described below. PCR products were purified using QIA quick PCR product extraction kit (Qiagen, Hilden, Germany). In brief, the DNA fragments of *C. sakazakii* were cut from agarose gel using clean and sharp scalpel and placed in a colorless tube with buffer QG in a ratio of 1:3. The tubes were incubated at 50°C for 10 min and vortexed every 2–3 min to dissolve the gel. Isopropanol was added to the sample and mixed; then, the QIAquick spin column were placed in the provided 2-mL collection tube. The mixture was centrifuged twice at 5,000 rpm for 1 min; 500 µL of buffer QG was added and centrifuged for 1 min, then discard the flow-through and placed the QIAquick spin column again into the same tube. The content was washed with 750 µL of buffer PE and centrifuged for 1 min. After waiting for 5 min, the content was centrifuged and placed in another 1.5-mL microcentrifuge tube. Finally to elute DNA, 50 µL of buffer BE or water was added to the center of the QIAquick membrane and centrifuged for 1 min. The obtained purified DNA was sequenced at the Colors Medical Laboratories, Cairo, Egypt. Bigdye Terminator V3.1 cycle sequencing kit (PerkinElmer MA, USA) was used for sequence reaction and the purification was done using the Centrisep spin column. DNA sequences were obtained by Applied Biosystems 3130 genetic analyzer (HITACHI). BLAST® analysis (a basic local alignment search tool) (Altschul *et al.* 1990) was initially performed to establish sequence identity to the GenBank entries. The sequence of strain ON197907 was manually aligned against sequences obtained from the GenBank database using Molecular Evolutionary Genetics Analysis (MEGA).

Statistical analysis

Descriptive statistics, such as mean values, standard deviation, and 95% CI, were calculated from the obtained data with MS Excel (Microsoft Corporation).

The observed data were statistically analyzed using SPSS v21 for Windows (IBM SPSS, Amonk, NY, USA). The statistical analysis performed consisted of mean comparison tests, Univariate Analysis of Variance (ANOVA), followed by Tukey's post-hoc test ($p < 0.05$) to evaluate significant differences between incidence of *Cronobacter* spp. in eggs and egg-based desserts.

Results

Prevalence of *Cronobacter* spp. in the examined samples of table eggs

A total of 60 egg samples (farm and Balady eggs, 30 samples each) were subjected to bacteriological examination. *Cronobacter* spp. were isolated from 27 of the 60 (45%) examined egg shells and 14 (23.3%) from egg contents.

For farm eggs, 67% (95% CI: 49–85%) of the examined egg shells and 30% (95% CI: 13–47%) of egg contents were positive for *Cronobacter* (Table 1). In shell samples, the distribution of *Cronobacter* spp. was higher for *C. turicensis* (26.67%), followed by *C. muytjensii* (16.67%). Moreover, the prevalence of different *Cronobacter* spp. in farm egg contents was 6.67% except for *C. genomospecies 1* strain that was not detected in such samples (Table 2). *C. turicensis* had the highest frequency rate (40%) among all species of *Cronobacter* in farm eggs.

On the other hand, only 7 (23%) of the examined shell and 5 (17%) of the content samples of Balady eggs were contaminated with this pathogen (Table 1). *C. muytjensii* had the highest prevalence and frequency distribution among the isolates either on the shell (13.33%) or in content (6.67%) of eggs. Both *C. malonaticus* and *C. dublinensis* were not detected in these examined samples (Table 2).

Table 1. Prevalence of *Cronobacter* spp. in the examined samples of farm and Balady eggs and egg-based desserts (cream cake and small-scale ice cream).

Examined samples			No. of examined samples	Positive samples		
				No.	Proportion	95% Confidence Interval (95% CI)
Eggs	Farm	Shell	30	20	0.67[a]	0.49–0.85
		Content	30	9	0.30[b]	0.13–0.47
	Balady	Shell	30	7	0.23[b]	0.07–0.39
		Content	30	5	0.17[b]	0.03–0.31
Egg-based desserts	Cream cake		30	8	0.27[b]	0.10–0.43
	Small-scale ice cream		30	8	0.27[b]	0.10–0.43
Total			180	57	0.32	0.26–0.43

[a,b]Different letters indicate significant difference ($p < 0.05$).

Table 2. Prevalence of different isolated *Cronobacter* spp. in the examined samples of farm and Balady eggs and egg-based desserts (cream cake and small-scale ice cream).

Isolated *Cronobacter* spp.	Positive samples											
	Eggs								Egg-based desserts			
	Farm				Balady				Cream cake		Small-scale ice cream	
	Shell		Content		Shell		Content					
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
C. sakazakii	2	6.67	2	6.67	2	6.67	1	3.33	1	3.33	5	16.67
C. malonaticus	2	6.67	2	6.67	*	*	*	*	2	6.67	2	6.67
C. muytjensii	5	16.67	2	6.67	4	13.33	2	6.67	*	*	1	3.33
C. dublinensis	*	*	2	6.67	*	*	*	*	4	13.33	*	*
C. turicensis	8	26.67	1	3.33	1	3.33	1	3.33	*	*	*	*
C. genomospecies 1	3	10.00	*	*	*	*	1	3.33	1	3.33	*	*
Total	20	66.67	9	30.00	7	23.33	5	16.67	8	26.67	8	26.67

*Not detected.

Prevalence of *Cronobacter* spp. in the examined samples of egg-based desserts

Of the 30 examined samples of cream cake or small-scale ice cream, 8 (27%) (95% CI: 0.10–0.43%) samples each of these were contaminated with *Cronobacter* spp. (Table 1). The incidence of *C. dublinensis* was higher (13.33%) in the examined samples of cream cake; however, *C. sakazakii* was determined in the five samples of ice cream with a frequency rate of 62.5% (Table 2).

Molecular characterization of *C. sakazakii* recovered from the examined samples of eggs and egg-based desserts

Herein, identification of isolates was confirmed by molecular methods (*16S* rRNA) for *C. sakazakii* only. In total, 13 (22.8%) (95% CI: 12–34%) out of 57 *Cronobacter* strains recovered from the examined samples of eggs and egg-based desserts were identified for *C. sakazakii* using conventional methods that included several biochemical tests (e.g., sugar fermentation of sucrose, dulcitol, and sorbitol; indole production; and malonate utilization). Further, all the tested *C. sakazakii* isolates (100%) were confirmed by PCR technique using *16S* rRNA gene sequencing. The obtained *16S* rRNA gene sequence (strain ON197907) revealed a 99.8% identity to other *C. sakazakii* sequences deposited in GenBank (accession numbers KY971635, KX056903, KC818156, and HQ880382). The *C. sakazakii* sequence generated in the present study and its annotation data are available in the GenBank database under accession number ON197907.

Discussion

Cronobacter spp. could be present in various commonly consumed food substrates as well as in the environment (Ling *et al.*, 2018). To date, limited data are available regarding *Cronobacter* spp. present in eggs and egg-based desserts in Egypt. To fill this gap, the present study was conducted to evaluate table eggs and some of egg-based desserts for the presence of *Cronobacter* spp. and to characterize molecularly the obtained *C. sakazakii*. The obtained results reported that the examined samples of table eggs and egg-based desserts were highly contaminated with *Cronobacter* spp., particularly eggs. In addition, *C. sakazakii* was the most prevalent isolates in small-scale ice cream in comparison to other food categories. Therefore, these food matrices could be a potential source of *Cronobacter* infection in children and rest of the population. Consequently, considerable attention must be paid to the safety issues of egg products related to *Cronobacter* spp.

In the present study, occurrence of *Cronobacter* in the examined samples of table eggs (Balady and farm) was high (45%) (95% CI: 32–58%), compared to egg-based desserts (cream cake and small-scale ice cream) (27%). The high level of *Cronobacter* contamination in eggs was expected considering the ubiquitous nature of such pathogen in foods, beverages, and the environment (Jaradat *et al.*, 2009). Contrary to our results, lower incidence was reported by Hochel *et al.* (2012), who isolated *C. sakazakii* from 10% of the examined egg samples. This variability observed in results was probably due to the number of samples analyzed, difference in chicken breeds, the applied hygienic measurements, and isolation methods. Importantly, it is obvious from the obtained results that the incidence of *Cronobacter* spp. was lower in Balady eggs than farm eggs in case of either shells or content samples. This could be due to the higher resistance of the Egyptian Balady breed chicken to infectious diseases than other breeds (Hassan *et al.*, 2004).

Of particular note, different isolates of *Cronobacter* spp. were detected in table egg samples, including *C. sakazakii*, *C. malonaticus*, *C. muytjensii*, *C. dublinensis*, *C. turicensis*, and *C. genomospecies 1*. World Health Organization/Food and Agriculture Organization (WHO/FAO 2008) reported that all *Cronobacter* species were retrospectively linked to clinical infection in infants or adults, and therefore all species must be considered as pathogenic. Hence, our results revealed that eggs could be a source of all *Cronobacter* spp., because they are widely consumed in original condition or used as a food ingredient in cooking. Strikingly, a significant increase ($p < 0.05$) in the prevalence of *Cronobacter* spp. was observed between eggs and other food samples in the present study. This could be due to the increased contamination rate in farms and farmers' houses. Besides, the examined egg samples in the present study were raw and not priorly exposed to any stress factors (e.g., heating, cooling, or freezing) that could destroy *Cronobacter*.

Cream cake and small-scale ice cream in Egypt are traditional products often produced in small and not well-qualified establishments, lacking preventive measures for production and distribution of such products. The occurrence of *Cronobacter* spp. in the examined samples of egg-based desserts (27%) was high and in concurrence with the findings observed by Abou Elkhair (2014) and Yörük (2019). Although lower incidences of *Cronobacter* spp. were reported previously by El-Gamal *et al.* (2013) and Mathews *et al.* (2013), Saad and Ewida (2018) isolated *C. sakazakii* from 3.33% of ice cream samples in Egypt. On the other hand, Baumgartner *et al.* (2009) and Kandhai *et al.* (2010) failed to isolate bacterium from 27 and 89 tested ice cream samples in Switzerland and the Netherlands, respectively. Importantly, in the present study, the high level of contamination with *Cronobacter* spp. in the examined samples of egg-based desserts could be attributed to

cross-contamination of raw materials, contaminated equipment, and improper food handling practices during processing of these products. Notably, ice cream produced in small-scale industries presented the highest contamination rate (5 out of 8 samples were infected, 62.5%) with *C. sakazakii*, compared to other examined samples in this study. This finding proved that small-scale ice cream could potentially disperse *C. sakazakii* and implicated in human infections. In Egypt, small-scale ice cream is produced under poor hygienic conditions using low-quality ingredients. Additionally, lack of heat treatment during processing of such products could be the main cause of its contamination. Contrary to small-scale ice cream, the majority of cream cake samples (13.33%) were contaminated with *C. dublinensis.* Similarly, Ling *et al*. (2018) found in China that 21.05% of vegetable samples were positive to *C. dublinensis*. However, a limitation in studies was observed concerning the occurrence of *Cronobacter* spp. in cream cake samples; hence, it is worth to pay more attention toward such products to avoid post-consumption *Cronobacter* infection.

Overall, the present findings showed that the biochemical identification of the obtained isolates could efficiently discriminate between different species of *Cronobacter*. Similarly, Lu *et al*. (2013) reported that biochemical identification of *Cronobacter* spp. showed better identification accuracy, compared to other methods. On the other hand, several alternative molecular methods for identification of *C. sakazakii* have been investigated, including PCR assay. The *16S* rRNA gene-based PCR identification system has been a reliable tool to correctly identify *C. sakazakii* isolates (Lehner *et al.*, 2004).

Importantly, in the present study, all suspected isolates subjected to PCR technique were confirmed as *C. sakazakii*. Our results were in agreement with the findings reported by Berhilevych and Kasianchuk (2017) and Saad and Ewida (2018), while lower incidence was obtained by Moustafa (2021), who using PCR technique confirmed 15 out of 20 (75%) tested samples as positive for *C. sakazakii*. In ready-to-eat foods, Aksu *et al.* (2019) reported that 8 (66%) of the obtained isolates were identified as *C. sakazakii*. In the present study, it is remarkable that the results obtained by conventional method and PCR followed by *16s* rRNA gene sequencing were similar. This indicated that using selective media (*Cronobacter* chromogenic isolation agar), followed by biochemical tests, could be effectively applied for the isolation and identification of *C. sakazakii* in foods. All in all, the obtained findings highlighted the need for regular monitoring of eggs and egg-based desserts for the presence of *Cronobacter* spp. to determine the risk of such food matrices in Egyptian consumers. Additionally, as the present investigation was performed using a limited sample size, further studies are required on *Cronobacter* spp. in Egyptian egg products for reliable determination of contamination level.

Conclusions

The data obtained in the present study illustrated that *Cronobacter* spp. was widely detected in the examined samples of eggs and egg-based desserts. The higher incidence of *C. sakazakii* was determined in small-scale ice cream samples, rather than in cream cake samples. The confirmed proportion of *C. sakazakii* was 22.8% (95% CI: 12–34%) using *16s* rRNA gene sequencing. Altogether, our results established that eggs and their products could be a transmission vehicle of *Cronobacter* spp. to consumers if good hygienic practices and/or improper storage conditions are not followed. Hence, preventive measures must be taken by food operators to prevent contamination of eggs and egg-based products with *Cronobacter* spp.

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