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MICROBIOLOGY

Occurrence and molecular characterization of different virulence-associated genes of *Cronobacter sakazakii* isolates from some foods and dust samples

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ABSTRACT: *Among the Cronobacter genus, Cronobacter sakazakii is the most common species posing a severe health risk for newborns, infants and children. Some infant formulas, cereal-based foods, and food production environments may be the potential reservoirs of C. sakazakii. This pathogen possesses different virulence factors encoded by different virulence genes. Therefore, characterizing these genes is important for distinguishing pathogenic strains from nonpathogenic ones. The objective of this study was to characterize some virulence genes [OmpA, OmpX, zpx, and Cpa] by real-time polymerase chain reaction (PCR) in C. sakazakii isolates from a total of 120 samples (20 each of milk powder, starch, rice flour, semolina, infant formula and dust samples from food production environments). Overall, 13 isolates (7 from milk powder, 2 rice flour, 1 semolina, and 3 dust) were cultured, identified by bioMérieux API® 20E test kit, and then subjected to real-time PCR application for screening the target virulence-associated genes. Our results showed that all of 13 isolates were positive for the virulence genes OmpA, OmpX, zpx, and Cpa. In summary, our study revealed that some of the analyzed foods and environmental samples were contaminated with pathogenic C. sakazakii with its virulence-associated markers, far above the allowable limit; and therefore, this level of contamination may pose a severe health threat for newborns, infants, and children.*
Key words: *C. sakazakii, dust, food, health, virulence genes.*

Ocorrência e caracterização molecular de diferentes genes associados à virulência de *Cronobacter sakazakii* detectados em alguns alimentos e amostras de poeira

RESUMO: *Dentre o gênero Cronobacter, Cronobacter sakazakii é a espécie mais comum que representa um grave risco para a saúde dos recém-nascidos, bebês e crianças. Algumas formulas infantis paracrianças, alimentos a base de cereais e locais de produção de alimentos, foram considerados como potenciais locais de contaminação de C. sakazakii. Este patógeno possui diferentes agentes de virulência codificados por diferentes genes de virulência. Portanto, a caracterização dos genes é importante para distinguir as cepas patogênicas das não patogênicas. O objetivo deste estudo foi caracterizar os diferentes genes de virulência [OmpA, OmpX, zpx, and Cpa] em C. sakazakii isolados em um total de 120 amostras (20 de cada uma delas - leite em pó, amido, farinha de arroz, sêmola, comida para bebês e amostras de poeira provenientes dos ambientes de produção de alimentos) por reação em cadeia da polimerase em tempo real (RT-PCR). No total, foram cultivadas 13 estirpes (7 de leite em pó, 2 de farinha de arroz, 1 de sêmola e 3 poeiras) e depois identificadas pelo kit de teste bioMérieux API® 20E. As estirpes identificadas foram submetidas ao PCR em tempo real para caracterizar os genes alvo associados à virulência. Os resultados mostraram que todos os genes C. sakazakii isolados eram patogenicos e positivos às OmpA, OmpX, zpx e Cpa com um padrão de coexistência. Em resumo, o nosso estudo revelou que os alimentos e os ambientes de produção de alimentos analisados constituíam uma ameaça à saúde dos recém-nascidos, bebês e crianças devido à contaminação por C. sakazakii patogênico como marcadores associados à virulência.*
Palavras-chave: *C. sakazakii, Poeira, alimentação, genes de virulência.*

INTRODUCTION

Cronobacter sakazakii is an opportunistic pathogen with high mortality rates of 40–80%. It causes severe infections, including necrotizing enterocolitis, bacteremia and meningitis, in newborns, infants, children up to three years of age, and immunocompromised adults less than 70 years (HEPERKAN et al., 2017).

Globally, very few surveillance data are available for *C. sakazakii*-related diseases because of the lack of available information on surveillance systems, which are rarely sufficient to examine exposure factors. The World Health Organization has identified roughly 120 individually documented cases of *Cronobacter* spp. infections among infants and young children up to three years of age (IVY et al., 2013). The collection of food borne pathogenic isolates is important for developing detection methods, intervention strategies, and an understanding of pathogenesis. Food borne disease surveillance

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and/or outbreak reporting systems encompassing *C. sakazakii* infection have been employed in most countries and the data by these systems suggested that very young infants are at a greater risk of severe disease and death from infection with this organism (FAO & WHO, 2008).

Many studies have reported the presence of *C. sakazakii* in infant formula, follow-up formula, growing-up formula, children's formula, semolina, milk powder, starch, and rice flour (SHAKER et al., 2007; GÖKMEN et al., 2010; XU et al., 2014; YAN et al., 2015; LI et al., 2016; AKINEDEN et al., 2017), milk and whey powder (HEPERKAN et al., 2017), dairy products, sahlab, and dust samples (EL-SHAROUD et al., 2009; MÜLLER et al., 2013), herbs and spices (JARADAT et al., 2009), dried herbs and vegetables (OGIHARA et al., 2014) and frozen food, seafood, spices, and ready-to-eat snacks (MIRANDA et al., 2017).

Infant formulas and other cereal-based foods consumed by infants and young children must be free of this pathogen according to national and international authorities. In addition, *C. sakazakii* contamination has repeatedly been detected in factories processing baby foods and ingredients used for making baby foods (PARRA-FLORES et al., 2015). Dust particles in the air of such a facility may act as a vector of *C. sakazakii* dissemination. The higher levels of *C. sakazakii* are mostly observed in dust filters, vacuum cleaners, bagging, and packaging areas (FEI et al., 2015).

C. sakazakii is the most frequently isolated species of the *Cronobacter* genus. However, its virulence factors remain poorly studied (ALMAJED & FORSYTHE, 2016). Therefore, their characterization is important for distinguishing pathogenic from nonpathogenic strains. An advanced understanding of this bacterium has begun to characterize the virulence factors and pathogenic potential of *C. sakazakii*. These developments have been obtained by improved DNA-based techniques (HUNTER & BEAN, 2013). Recent studies have identified many virulence factors in *C. sakazakii* such as seven O-serogroups and eleven proteolytic enzymes (DU et al., 2015). Among the virulence-related proteins, outer membrane proteins (*OmpA* and *OmpX*) are involved in the colonization of the gastrointestinal tract and may have roles in helping the organism penetrate the blood–brain barrier (KYUMSON et al., 2010; ZIMMERMANN et al., 2014; ALMAJED & FORSYTHE, 2016). The virulence factors Zinc-metalloprotease (*zpx*) causes cell deformation and cells rounding , other virulence factor *Cronobacter* plasminogen activator (*Cpa*) provides resistance against the bactericidal activity of serum, activates plasminogen, and inactivates alpha2-antiplasmin (YE et al., 2016; ESHWAR et al., 2016).

The objective of this study was to characterize the different virulence genes (*OmpA, OmpX, zpx*, and *Cpa*) in *C. sakazakii* isolates by real-time PCR from samples of each milk powder, starch, rice flour, semolina, infant formula and dust samples from food production environments.

MATERIALS AND METHODS

Reference cultures

As standardized cultures, *C. sakazakii* ATCC® 29544 (Liofilchem, Istanbul, Turkey) and *E. coli* ATCC® 25922 (Liofilchem) were used for control testing in the phenotypic and genotypic methods.

Sample collection

During the period from 2015 to 2016, 120 samples (20 each of milk powder, starch, rice flour, semolina, infant formula, and dust from dust collection systems in food production environments) were randomly collected from public bazaars, markets and food production environments in Istanbul, Turkey. Samples were placed in sterile sampling bags and then taken to the laboratory in a thermobox container at 4°C for further examinations.

Sample preparation and microbiological analysis

Samples were prepared in accordance with the Method "ISO 8261:2001 Milk and Milk Products General Guidance for the preparation of the test samples, initial suspensions, and decimal dilutions for microbiological examination". Isolation of the presumptive *C. sakazakii* species was performed according to the Guidelines of the Method "ISO/TS 22964:2006 Milk and Milk Products-Detection of *Enterobacter sakazakii*". Of each sample, 25g was homogenized in 225mL of buffered peptone water (LAB103, UK) for 2min using a stomacher (EasyMix-AES Chemunex, France). The homogenized suspension was then exposed to aerobic incubation at 37°C for 18h. Then, 100µL of the pre-enriched suspension was mixed with 10mL of Modified Laurylsulfate-Tryptose Vancomycin (mLST/vancomycin) broth (Liofilchem). The inoculated plate was incubated at 44°C for 24h under aerobic conditions. Of the incubated suspension, 10µL was streaked on Harlequin CSIM chromogenic selective media (LABM, UK) by using a sterile loop and allowed for incubation at 44°C for 24h. Fifteen blue-green colonies were selected for the

confirmation and subcultured on a Tryptone soya agar plate (CM0131, Oxoid, Turkey), incubated at 37°C for 46h, and checked for yellow coloration. Finally, the subcultured isolates were subjected to the biochemical identification test.

Biochemical identification

The species identification of presumptive *C. sakazakii* isolates was conducted by API® 20E Test Kit (bioMérieux, France) according to the manufacturer's instructions. Readings were evaluated according to the criteria by the API Reading Scale. Finally, the identified isolates were stored in tryptic soy broth (LABM, UK) containing 10% glycerol at –20°C until further workup.

DNA extraction

The plasmid DNA of the identified *C. sakazakii* isolates were extracted from the isolates refreshed on Luria-Bertani broth using the FastLyse Miniprep Kit (MDI Membrane Tech, Turkey). The extracted DNA was stored at –20°C for further molecular analyses.

Real-time PCR *primers for virulence genes and reaction conditions*

The primer pairs of the virulence-related genes, *OmpA*, *OmpX*, *zpx*, and *Cpa*, were prepared according CAI et al. (2013), AMALARADJOU et al. (2014), KOTHARY et al. (2007) and FRANCO et al. (2011), respectively (Table 1). All primers were designed by Exim Ltd. (Istanbul, Turkey) and Integrated DNA Technologies (Istanbul, Turkey). As standardized cultures, *C. sakazakii* ATCC® 29544 (Liofilchem) and *E. coli* ATCC® 25922 (Liofilchem) were used for control testing. All real-time PCR analyses were conducted as a single-plex assay for amplifying *OmpA*, *OmpX*, *zpx*, and *Cpa*. A typical PCR mixture, in a final volume of 18µL (0.6µM of each primer [10µM], 10µL of 2x SYBR Green Master Mix [Analytik Jena, Turkey], and 6.8µL of DNase/RNase-free water, was prepared. A total of 18µL of this prepared master mix solution was pipetted into each well after the addition of 2µL of extracted DNA. Each sample was run in duplicate. Sterile water was placed in the negative control well in place of DNA, and *C. sakazakii* ATCC® 29544 and *E. coli* ATCC® 25922 DNA (in 2 wells) were used for control testing. FAM/SYBR® Green (Excitation: 492nm, Emission: 516nm) was used. Thermal processing conditions were optimized at the laboratory according to the primers used. To verify the specificity of the reactions using SYBR Green I as the fluorescent dye, melting curve analysis was performed. The analysis was performed using the Agilent Stratagene Mx3000P real-time PCR (Waldbronn, Germany). The sensitivity of the real-time PCR assay undergoing Ct≤40 cycles of amplification was accepted to be positive in accordance with the melting point setting because of the interference of undesirable nonspecific amplifications (Table 1).

Table 1 - Primer design, sequences, and amplification conditions for virulence genes.

Primer (f / r*)	Sequence	Amplicon (bp)	PCR	Reference
OmpA-f *OmpA*-r	5'-GGT GAA GGA TTT AAC CGT GAA CTT-3' 5'-GCG CCT CGT TAT CAT CCA AA-3'	70	PCR-1	XIAN-QUAN CAI et al. (2013)
OmpX-f *OmpX*-r	5'-GTC TTT CAG CAC TGG CTT GTG T-3' 5'-GGT GCC AGC AAC AGC AGA A-3'	150	PCR-2	AMALARADJOU et al. (2014)
zpx-f *zpx*-r	5'-GAA AGC GTA TAA GCG CGA TTC-3' 5'- GTT CCA GAA GGC GTT CTG GT-3'	350	PCR-3	KOTHARY et al. (2007)
Cpa-f *Cpa*-r	5'-GCC TGG CGG AAT TCA ATG G-3' 5'-GAT CAA AGC TGC AGT CAG AAA CG-3'	936	PCR-4	FRANCO et al. (2011)

*f: forward and r: reverse.
PCR-1: 1 cycle for 2 min at 50°C, 1 cycle for 10 min at 95°C followed by 40 cycles for 15 s at 95 °C, and finally 1 cycle for 1 min at 60°C.
PCR-2: 1 cycle for 2 min at 50°C, 1 cycle for 10 min at 95°C followed by 40 cycles for 15 s at 95 °C, and finally 1 cycle for 1 min at 60°C.
PCR-3: 1 cycle for 15 min at 95°C, followed by 35 cycles for 1 min at 95°C, 1 min at 62 °C, 1 min at 72°C, and finally 1 cycle for 7 min at 72°C.
PCR-4: 1 cycle for 5 min at 95°C, followed by 40 cycles for 15 s at 95°C, 15 s at 62°C, and 30 s at 72°C, and finally 1 cycle for 1 min at 95°C, 30 s at 55°C, and 30 s at 95°C.

RESULTS

In this study, 13 *C. sakazakii* isolates (7 from milk powder, 2 rice flour, 1 semolina, and 3 dust) were cultured, and then identified by the bioMérieux API® 20E test kit. The identified strains were subjected to real-time PCR for characterizing the target virulence-associated genes. Our results showed that all of 13 isolates were positive for the virulence genes *OmpA, OmpX, zpx*, and *Cpa*, revealing that some of the analyzed foods and environmental samples were contaminated with pathogenic *C. sakazakii* with its virulence-associated markers, far above the allowable limit; and therefore, this level of contamination may pose a severe health threat for newborns, infants, and children (Table 2, Figure 1, 2, 3 and 4).

DISCUSSION AND CONCLUSIONS

The recorded history for *Cronobacter* spp. is short but they have certainly existed for millions of years. *Cronobacter* (Formerly Enterobacter sakazakii) is a newly classified genus including seven species (*C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dubliniensis*, *C. condimenti*, and *C. universalis*) (SINGH et al., 2015). Among them, *C. sakazakii* is the most common and most often isolated species with high mortality rates of 40–80% in infants, children, and adults especially elderly and immunocompromised adults (HEPERKAN et al., 2017).

This opportunistic microorganism is unique in the *Cronobacter* genus in encoding genes enabling the use of some clinically significant exogenous substances such as sialic acid, which is a major evolutionary host adaptation, as the compound present in breast milk and infant foods (JOSEPH et al., 2012). In the last few years, much has been learned about the complexity of *C. sakazakii* since being first described as *Enterobacter sakazakii* in 1980 (SHASHKOV et al., 2015). However, many uncertainties are associated with the assessment of the public health risk posed by this pathogen (HEALY et al., 2010; BAO et al., 2017). Disease surveillance and/or outbreak reporting systems encompassing *C. sakazakii* infection have suggested that infants and children are at a higher risk of severe disease and death from the infection with food borne *C. sakazakii* (FAO & WHO, 2008). Our study proved that the foods and food production environments analyzed posed a health threat for newborns, infants, and children because of contamination by *C. sakazakii*.

International studies conducted between 2008 and 2014 have indicated that 5.7% of the food samples of animal origin and 19% of plant origin harbored *Cronobacter* spp., including *C. sakazakii* (SANI & ODEYEMI, 2015). Another study in the Czech Republic showed that the foods of plant origin were most frequently contaminated with *C. sakazakii* (54.7%) (HOCHEL et al., 2012). However, in our study, only 35% of *C. sakazakii* strains were isolated from milk powder samples, followed by dust, rice flour, and semolina samples. Our findings suggest that infants and children are at a risk of disease and death from infection with the analyzed foods and environmental sources harboring *C. sakazakii*.

In Jordan, SHAKER et al. (2007) and JARADAT et al. (2009) detected *C. sakazakii* in infant formulas (1.4% to 17.4%) and semolina samples, while another study conducted in Egypt reported the organism in milk powder and infant formula samples (EL-SHAROUD et al., 2009). XU et al. (2014) and

Table 2 - Occurrence of *C. sakazakii* isolates and their virulence genes in the sampling groups.

Sample	No. of sample (n)	No of *C. sakazakii* + sample (n,%)	No of virulent + isolate (n, %)	*OmpA*	*OmpX*	*zpx*	*Cpa*
Starch	20	0 (0%)	0 (0%)	-	-	-	-
Infant formula	20	0 (0%)	0 (0%)	-	-	-	-
Semolina	20	1 (5%)	1 (100%)	+	+	+	+
Rice flour	20	2 (10%)	2 (100%)	+	+	+	+
Dust	20	3 (15%)	3 (100%)	+	+	+	+
Milk powder	20	7 (35%)	7 (100%)	+	+	+	+
Total	120	13 (11%)	13 (100%)	+	+	+	+

The numbers in parentheses represent percentage values.

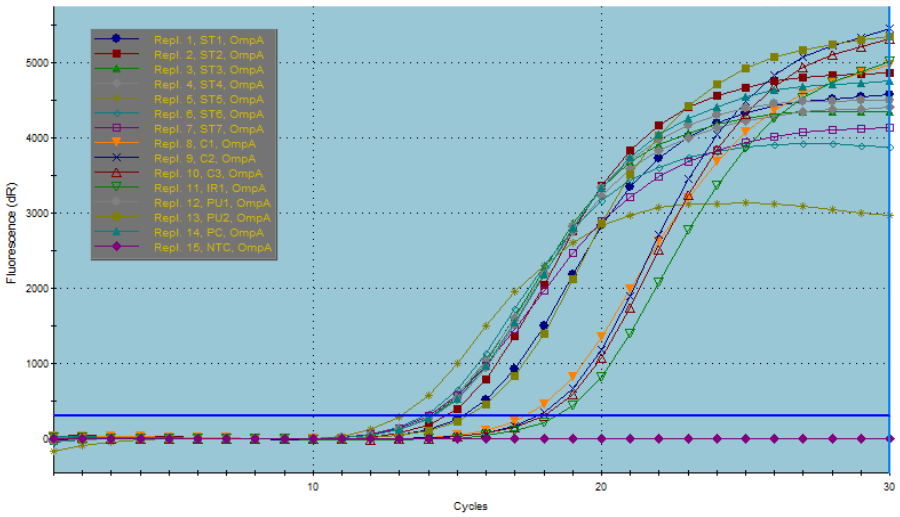

Figure 1 - Amplification curves of the isolates for *OmpA*.

HUANG et al. (2015) found this opportunistic organism in general formula (6.25%), infant formula (1.82% to 16.9%), follow-up formula (3.64%), growing-up formula (5.45%), children's formula (2.5%), and rice flour (28.8%) consumed in China between 2010 and 2012. Milk powder, rice flour, and semolina are widely used as ingredients for producing various infant and children foods. Level of contamination with *C. sakazakii* was higher in the analyzed milk powder samples in this study than in international studies but was lower in semolina and rice flour samples compared with results obtained by JARADAT et al.

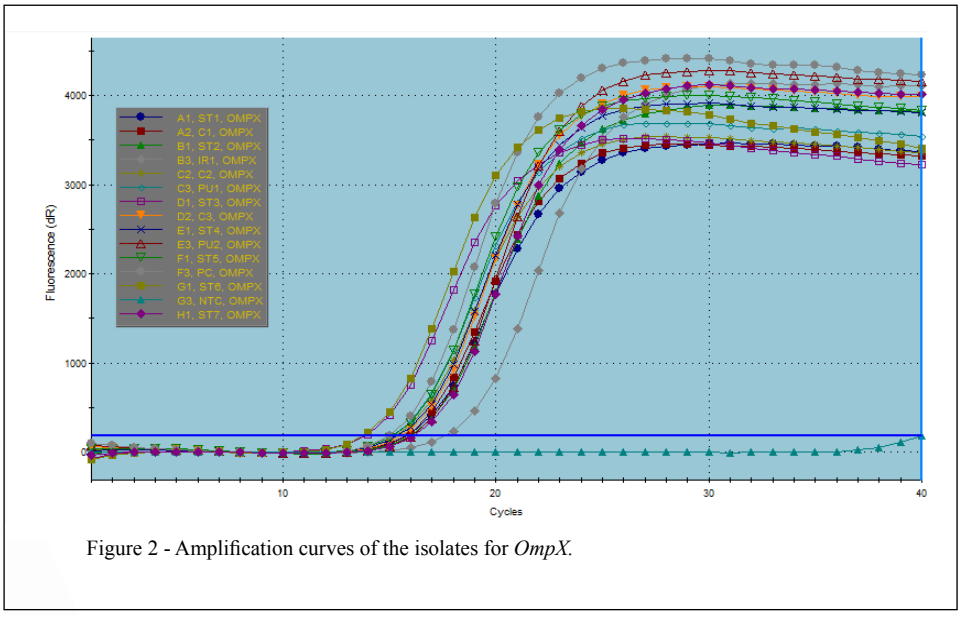

Figure 2 - Amplification curves of the isolates for *OmpX*.

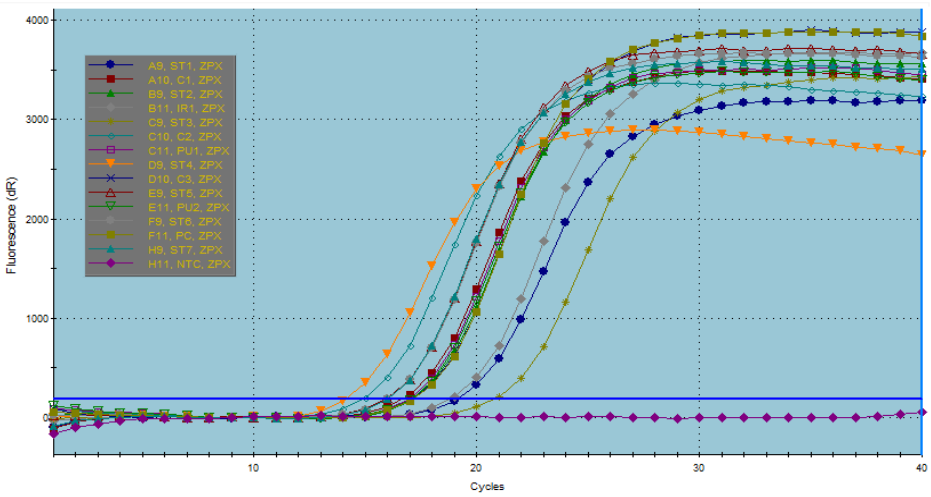

Figure 3 - Amplification curves of the isolates for *zpx*.

(2009) and XU et al. (2014). By contrast, *C sakazakii* was not detected in starch and infant formula samples in this study. The results presented in this assay were similar to those obtained by MIRANDA et al. (2017) in the United States of America and by HOCHEL et al. (2012) in the Czech Republic.

In Turkey, some studies have provided significant data for the frequency of *Cronobacter* spp., especially *C. sakazakii*, in different sources. *C. sakazakii* was reported in milk powder (5% to 7.5%), starch (5%), rice flour (5%), semolina (5%), and whey powder samples (7.5%) (GÖKMEN et al.,

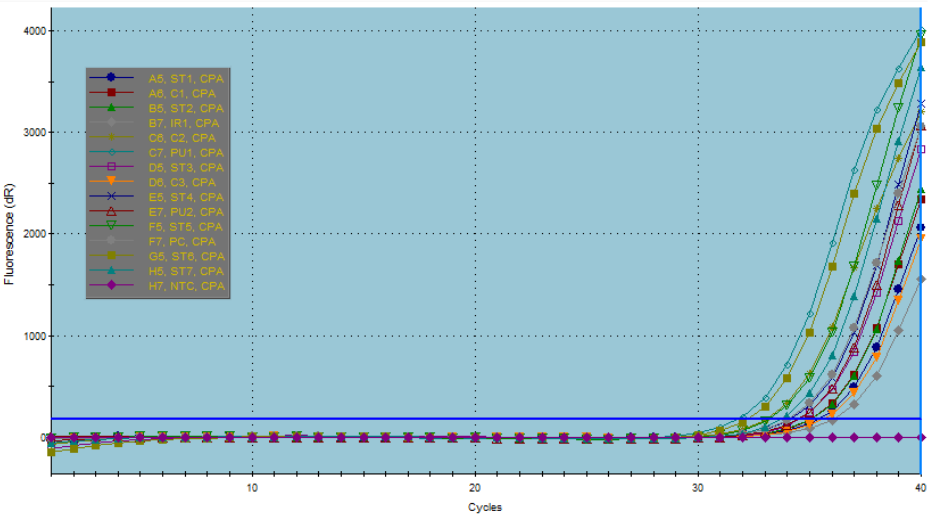

Figure 4 - Amplification curves of the isolates for *Cpa*.

2010; CAVA GÜMÜŞ et al., 2017; HEPERKAN et al., 2017); but whey powder and powdered infant formulas from three retail brands did not contain the organism (GÖKMEN et al., 2010; GÜNER et al., 2011). Results obtained in this study showed that milk powder, rice flour, and semolina were important sources of *C. sakazakii* posing a health risk for infants and children.

In Jordan, the frequency of *C. sakazakii* reported in vacuum dust of a food production facility was 18%, *C. sakazakii* has also been isolated from food production areas (SHAKER et al. 2007; JANINE, 2015). MÜLLER et al. (2013) showed that *C. sakazakii* was the most prevalent species identified (93.6%) in a facility processing powdered infant formula in Switzerland. Similarly, MOZROVÁ et al. (2014) reported the organism in samples from a dairy farm and dust from vacuum cleaners in the Czech Republic. For instance, textile filters for exhaust air of spray-drying towers in a milk powder-producing plant were found to be internal reservoirs of *C. sakazakii*. This situation occurs because of economic reasons, the waste powder from the filters is reintroduced into the product flow for optimization and cost-reduction goals in the production cycle (JACOBS et al., 2011). To lower the contamination risk by *C. sakazakii* in the production area, air humidity and the number of dust particles in the air should be kept minimal and production equipment should be frequently cleaned and waste powder should be effectively treated (FEI et al., 2015). In this study, 25% of the analyzed dust samples were positive for *C. sakazakii*. Future studies would discover the impact of the production area and equipment as risk factors on the dissemination of *C. sakazakii* and create methods to better control this pathogen and reduce its infections.

The virulence factors indicate the pathogenic potential of *C. sakazakii* with their plausible connection with clinical manifestations, including meningitis and necrotizing enterocolitis in infants, and septicemia and catheter-associated infections in elderly and immunocompromised people (SINGH et al., 2017). However, the virulence factors and the pathogenesis of *C. sakazakii* infection are poorly understood. Therefore, toxicological experiments, *C. sakazakii* subtyping, molecular and proteomics analyses comprehensively evaluate the virulence-related characteristics of *C. sakazakii*. The detection of *Cronobacter* spp. according to ISO/TS 22964 takes up to a week, and traditional methods do not provide information about the virulence potential of a strain. Because of this reason, fast and sensitive methods as mentioned above are required (YAN et al., 2012). For instance, the outer membrane protein *OmpA*, a potential virulence factor involved in the crossing of the blood-brain barrier before the onset of meningitis, has been used for identification purposes (FEI et al., 2015). *OmpA* works synergistically with some other virulence genes *in vitro* and *in vivo* in the pathogenesis of *C. sakazakii* infection (CHANDRAPALA et al., 2014). Recent studies also have revealed that there are also other proteins having virulence-related potential. This situation simply indicates that *Cronobacter* virulence is dependent on multiple factors (JARADAT et al., 2009).

In last few years, many studies have examined the virulence characteristics of *C. sakazakii* isolated from a wide range of sources. A study showed that 13 isolates from a powdered infant formula factory harbored the virulence gene *zpx*, while no isolate contained *OmpA* (JARADAT et al., 2009) Another study revealed that *OmpA* was reported in 64.7% of *Cronobacter* strains tested in low-moisture food products, including powdered infant formulas (YAN et al., 2011). Similarly, *OmpA* and *OmpX* were reported in all *Cronobacter* spp., whereas 98% of *Cronobacter* strains possessed *Cpa* (JARADAT et al., 2009). Studies in Germany have revealed that 11% of *C. sakazakii* isolates from infant and baby foods were highly virulent (ZIMMERMANN et al., 2014; AKINEDEN et al., 2017). In a milk powder factory in China, the prevalance of virulence genotypes carrying *Cpa-OmpX* was 79.3% (WANG et al., 2015). Similarly, *C. sakazakii* isolates isolated from milk-based infant and baby food samples between 2010 and 2012 in China harbored mostly *OmpA* (LI et al., 2016). Another study showed that *C. sakazakii* isolates derived from plant-based materials and environmental samples harbored mainly *OmpA*, followed by *Cpa* (60%) (SINGH et al., 2017). In this study, the isolated strains harbored all four virulence genes, *OmpA*, *OmpX*, *zpx*, and *Cpa*, simultaneously. Studies are needed to determine the conditions that influence survival and growth or cause death of *C. sakazakii* in a broad range of locations. Differences in the hygiene and storage conditions are major key factors on the variations of the results. Also, culture-based methods are time-consuming as well as having insufficient effectiveness of virulence factors in bacteria.

This is the first comprehensive report in Turkey with characterization of some virulence genes in *C. sakazakii* isolates, and this led to develop a better understanding of its virulence characteristics mainly from infant and baby foods, and production areas of these foods. The literature showed that there is not adequate data in this field in Turkey. Overall,

our study revealed that some of the analyzed foods and environmental samples were contaminated with pathogenic *C. sakazakii* with its virulence-associated markers, far above the allowable limit; and therefore, this level of contamination may potentially pose a severe health threat for newborns, infants, and children.

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DECLARATION OF CONFLICTING OF INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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