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ORIGINAL ARTICLE

A novel primer targeted *gyrB* gene for the identification of *Cronobacter sakazakii* in powdered infant formulas (PIF) and baby foods in Iran

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Abstract

The aim of this study was to perform a molecular identification and design a polymerase chain reaction (PCR) method based on a specific gene (*gyrase subunit B [gyrB])* for rapid detection of *Cronobacter sakazakii*. In this study, from February 2017 to January 2018, 100 powdered infant formula milks (PIF1-6) and 100 baby food items (BF1-8) (total number = 200 samples) were purchased from different commercial brands from different pharmacies of the Mashhad city, Iran from different pharmacies of the Mashhad city, Iran.

After isolation of *Cronobacter*, DNA extraction and PCR assay were performed to detect and confirm genus and species of isolated bacteria with *16S ribosomal RNA (16S rRNA)* and designed *gyrB* primers, respectively. The abundance of *C. sakazakii* in PIFs and baby foods by culture method were 5/100 (5%) and 8/100 (8%), respectively; also, the final analysis based on *gyrB* primer pairs in PIF and baby food showed contamination rates of 0/100 (0%) and 3/100 (3%), respectively. These findings indicated that Iranian baby foods were a real threat for children's health. It is also recommended that this designed primer could be used for detection of *Cronobacter sakazakii* in these products.

Practical application

Cronobacter sakazakii is an opportunistic bacteria that is known worldwide as a foodborne pathogen especially in Infant Formula and baby foods, therefore the quality of these products has to be evaluated by a rapid, sensitive and specific method. Our findings indicated that Iranian baby foods were a threat for children's health so implementation of adequate sanitary measures to prevent *Cronobacter sakazakii* illness is necessary. It was also recommended that the designed primer based on *gyr B* gene could be used for specific detection of *Cronobacter sakazakii* in these products.

1 | INTRODUCTION

Food safety considered as one of the most important worldwide health issues; many microbes cause foodborne diseases, and are major cause of morbidity and mortality throughout the world. According to the reports, 1.8 million people died from diarrheal diseases in 2005, but the global incidence of foodborne disease remains unknown.

Cronobacteria spp. are considered as emerging opportunistic pathogens; and its infection can be life threatening for humans (Zhao et al., 2010b). According to the molecular identification, *Cronobacteria* classifies into seven species: *Cronobacter sakazakii*, *Cronobacter muytjensii*, *Cronobacter malonaticus*, *Cronobacter universalis*, *Cronobacter condiment*, *Cronobacter dublinensis*, & *Cronobacter turicensis* (Joseph, Hariri, & Forsythe, 2013). Among them, *C. malonaticus*, *C. turicensis* and *C. sakazakii* are very common species that have been separated from human infections (Jackson, Sonbol, Masood, & Forsythe, 2014; Zimmermann, Schmidt, Loessner, & Weiss, 2014).

C. sakazakii is an opportunistic pathogen that is known worldwide as a foodborne pathogen; also, it has the highest prevalence among

the mentioned species (Ye et al., 2018). This bacterium is gram-negative, motile by peritrichous flagella, rod-shape, facultative anaerobe, and nonspore-forming (Feeney et al., 2014; Heperkan, Dalkilic-Kaya, & Juneja, 2017). It causes infections among all age groups, including infants, children, and adults, especially the elderly and immunocompromised persons; also, it is the most severe cause of infections among newborns as well. According to these reports, this bacterium with a mortality rate of 40–80%, has caused meningitis, septicemia, and enterocolitis (Heperkan et al., 2017). Therefore, the International Commission for Microbiological Specifications for Foods (ICMSF) classifies *Cronobacter* spp. as a serious threat to the health of individuals which can have significant effects over the long term (Shaker, Osaili, Al-Omary, Jaradat, & Al-Zuby, 2007).

This bacterium has been identified in various food sources, although its relevance to powdered infant formula (PIF) and baby food are major sources of concern (Biering et al., 1989; Teramoto et al., 2010; Yan et al., 2012). Raw materials of different origin or heat-sensitive materials that are added to the PIF heating, extrinsic contamination of opened cans and carrier humans can be potential sources of bacteria (Abdel-Galil, Abdel-Latif, Ammar, & Serry, 2015; Jaradat, Al Mousa, Elbetieha, Al Nabulsi, & Tall, 2014; Mardaneh & Dallal, 2017).

In recent years, current detection methodologies on major food borne pathogens like *E. coli* (Zhao et al., 2010), *Salmonella* (Zhao, Wang, et al., 2010b), *Vibrio parahaemolyticus* (Zhao et al., 2010c), *Pseudomonas aeruginosa* (Zhao et al., 2011), *Staphylococcus aureus* (Xu et al., 2012), *Listeria monocytogenes* (Wang, Li, Chu, Xu, & Zhong, 2012) have been developed.

Of the diagnostic methods for the *C. sakazakii* is the gold standard based on the FDA-microbial culture but since this method is very time-consuming and has low sensitivity and accuracy, molecular methods are used based on the nucleotide sequence such as PCR (Chen et al., 2015). The enzyme-linked immunosorbent assay (ELISA) and PCR-based methods are efficient, rapid, and sensitive; however, they often give false results either due to primer nonspecificity or residual target DNA in food samples. To overcome these problems, LAMP method and real-time PCR-based methods have been used for rapid identification of this bacteria. Generally, real-time PCR assays are not recommended for routine analysis of contaminated food samples because they require TaqMan, SYBR Green, probe and a thermal cycler with an expensive fluorescence detector. Also, LAMP has high risk of potential contamination during work (Cai et al., 2013; Shukla, Lee, Song, Park, & Kim, 2016).

Another method for bacterial identification is PEGE; but its major disadvantage is its low stability, and the time taken (2–4 days). MLST is another identification methd, but its high costs and time-consuming protocols associated with sequencing for MLST method have limited its use. It was shown that by by nonspecific primers, PCR-RFLP has also been impeded by difficulties, such as the minor differences of band sizes between some species. However, diagnostic sequencing methods requires special equipments and are very time-consuming. As a result, comparing these methods; simple PCR by choosing a suitable part of a gene, could be a simple, quick and cost effective tool for bacterial identification (Cai et al., 2013).

One of the molecular methods which is used to identify *Cronobacteria* species is analyzing *16S rRNA* genomic sequences, this gene is not able to differentiate some species such as *C. sakazakii* and *C. malanticus* due to their genetic similarity (Kucerova et al., 2010; Ye, Ling, Han, Cao, & Wu, 2015). Also, it has been observed that *16S rRNA* gene sequences among the *C. sakazakii* group strains have high degrees of similarity (reaching 97.8–99.7%) (Iversen et al., 2008). Therefore, it is essential to study other genes and primer for the detection of *C. sakazakii* by molecular methods. There are many previous studies on various genes of *C. sakazakii* such as *wzx* (Jarvis et al., 2011), *rpoB* (Stoop, Lehner, Iversen, Fanning, & Stephan, 2009), *cgcA* (Carter et al., 2013), *gyrB* (Huang, Chang, & Huang, 2013), *dnaG* (Seo & Brackett, 2005), *wehI*, *wehC* (Mullane et al., 2008).

In the same way, some studies have also been done on *gluA*, *SG*, *zpx*, and *OmpA*, but the results of *Cronobacter* spp. detection was not satisfactory (Chen et al., 2015). Based on *rpoB* sequences studies, different primer pairs were designed. But using this gene (*rpoB*) has a flaw; the PCR should be repeated to distinguish *C. sakazakii* from other species. (Stoop et al., 2009). Studies targeting *cgcA* gene showed the problem of nonspecific bands appearance on the agarose gels (Carter et al., 2013). The *gyrB* gene has also been used as a molecular marker for *C. sakazakii* and *C. dublinensis* and the designed primer pairs showed acceptable results (Huang et al., 2013).

The aim of this study was to design a PCR method based on the *gyrB* gene for the rapid detection of *C. sakazakii* in a variety of PIFs and baby foods. Also, the quality of PIFs and baby foods regarding the presence of *C. sakazakii* with culture and molecular methods were evaluated.

2 | MATERIALS AND METHODS

This descriptive cross-sectional study was performed on 100 PIF samples from six different commercial brands (PIF1-6) and 100 baby food samples from eight different baby food types (BF1-8) (brands names were coded for their privacy protection). Samples were purchased from different pharmacies of the Mashhad city, Iran, based on different brands and different production dates from February 2017 to January 2018. Control positive strain (*C. sakazakii* PTCC 1550) was purchased from Iranian Research Organization for Science and Technology (IROST) and four species of *Entrobacteriacea* including *Serratia marcescens*)PTCC 1187(, *Shigella dysenteriae* (PTCC 1188), *Escherichia coli* (PTCC 1399) and *Salmonella enteritidis* (PTCC 1709) were prepared from Microbiology Department, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran, as negative control in order to evaluate the designed primer.

2.1 | Isolation of *C. sakazakii* by culture method

Based on the Institute of Standards and Industrial Research of Iran (ISIRI) method for "Identification of *Cronobacter sakazakii* in milk and its products" ISIRI: NO.9430 protocol, *C. sakazakii* was isolated from PIFs and baby foods (Abbasi, Goli, & Momtaz, 2017; ISIRI, 2007). Isolation and identification of *C. sakazakii* by PCR assay.

2.2 | DNA extraction of bacteria

All strains of bacteria) positive control, negative control and possible *C. sakazakii*) were subcultured on EMB (Eosin Methylene Blue) agar and were incubated at 37 °C for 24 hr. Genomic DNA from these bacteria was extracted by DNA extraction kit (Qiagen, Valencia, CA) based on manufacturer's instructions. Finally, the extracted DNA was kept at −20 °C for PCR assay.

2.3 | DNA quantification and purity

The quality and quantity of the extracted DNA were determined by spectrophotometry, using a NanoDrop 2000/2000c spectrophotometer (Thermo Scientific). The concentration and purity of the DNA was determined under UV absorbance at 260 nm and by the ratio of absorbance at 260 and 280 nm, respectively.

2.4 | Identification of *C. sakazakii* with *16S rRNA* primer pair

According to the Abbasi et al. (2017) study, *16S rRNA* primer pairs were used to confirm C. *sakazakii* at the genus level. The final volume (25 μL) of PCR solution was comprised of 12.5 μL master-mix (American Applied Biosystem), 1 μL of the forward and 1 μL of the reverse *16S rRNA* primers (Table 1), 5 μL of sterile distilled water and 5 μL of extracted DNA. The thermal and time cycling conditions, included an initial incubation at 94 °C for 5 min, followed by 30 cycles at 95 °C for 60 s, 55 °C for 60 s, 72 °C for 90 s, and the final extension at 72 °C for 8 min.

2.5 | Designing a primer pair based on *gyrB* gene

Complete data from several different genomic sequences related to the *C. sakazakii* and other *Cronobacter* species were taken from the nucleotide database section of the GenBanK (http://www.ncbi.nlm.nih.gov/genbank), and were stored in the FASTA format. Then, all genomic sequences related to *C. sakazakii* and other species were compared by Bio Edit software and MEGA 7. Finally, the genomic sequence related to the *gyrB* gene of *C. sakazakii*, which was different from other species, was selected for primer designing. The primer pair *gyrB*-F (5′-GCC GCG AAG GTA AAA TCC AC-3′) and *gyrB*-R (5′-AGG CGC TTC GCC AGA ATA TC-3′) was designed for the identification of *C. sakazakii* based on the 90 *gyrB* reference sequences. Finally, the BLAST service of the NCBI was used to ensure the specificity of the final primer pairs.

TABLE 1 Characteristics of primer pairs used in the PCR of *C. sakazakii*

Target	Primer name	Primer sequence (5′–3′)	Product size (base pair)
16S rRNA	Esak2	CCC GCA TCT CTG CAG GAT TCT C	832
	Esak3	CTA ATA CCG CAT AAC GTC TAC G	
gyrB	F	GCC GCG AAG GTA AAA TCC AC	169
	R	AGG CGC TTC GCC AGA ATA TC	

2.6 | Determination of the limit of detection (LOD) for the *gyrB* primer pair

Extracted DNA from a positive control strain (PTCC 1550) with concentrations of 3.43 pg/μL was used to determine its sensitivity. For this purpose, 1 μL of the extracted DNA was sequentially diluted sevenfold in 9 μL of sterile distilled water. Finally, 5 μL of diluted DNA was used in a 25 μL PCR reaction, and the minimum dilution with a distinct band on the agarose gel was assumed as the limit of detection.

2.7 | Determination of the specificity of *gyrB* primer

For the specificity measurement of the designed primers, the same PCR reaction was performed with the extracted DNA of *Serratia marcescens*)PTCC 1187(, *Shigella dysenteriae* (PTCC 1188), *Escherichia coli* (PTCC 1399), and *Salmonella enteritidis* (PTCC 1709) by the designed *gyrB* primers.

2.8 | Identification of *C. sakazakii* with *gyrB* primer pair

PCR with the *gyrB* primer pair in the final volume of 25 μL was used for the confirmation and specific identification of *C. sakazakii*. Each microtube contained 12.5 μL of master mix, 1 μL of each forward and reversed primer of *gyrB* (Table 1), 5 μL sterile distilled water and 5 μL of the extracted DNA. The thermal cycles were as followed: 94 °C for 5 min, 30 cycles at 95 °C for 60 s, 60.5 °C for 1 min (the gradient PCR program was adjusted for various temperatures and finally the temperature of 60.5 °C was considered as the main and appropriate reaction temperature), 72° for 90 s, and a final cycle at 72 °C for 8 min. The PCR products were loaded on to %1 (w/v) agarose gel in a TBE 0.5xbuffer (0.5 mM EDTA, pH 8.0, 44.5 mM Tris/Borate). The gel was stained with green viewer (Parstous Co., Iran). Then, the gel electrophoresis was run at a constant voltage of 90 V for 45 min, and the gels were visualized *via* gel image system (Uvi tech., UK(.

2.9 | Sequencing the isolates

Each sample along with the corresponding primer pairs, were sent to the Macrogen, Inc. (Rockville, MD) for sequence reading.

2.10 | Molecular phylogeny of the isolates

Sequence readings were performed by the BioEdit Sequence Alignment Editor software. Then Blast software was used to identify the sequences at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Finally, the phylogenetic tree of the registered isolates in the NCBI was drawn by MEGA7 software. The evolutionary analysis for the genomes of bacteria was performed by the Neighbor-Joining method, and evolutionary distances were calculated by the Likelihood Maximum method.

TABLE 2 Prevalence of *C. sakazakii*, in various types of powdered infant formula and baby food

Sample	Total number	No.(%) of contaminated samples by culture method	No.(%) of contaminated samples based on *16S rRNA* gene	No.(%) of contaminated samples based on *gyrB* gene
Powdered infant formula.	100	5 (5%)	1 (1%)	0 (0%)
Baby food	100	8 (8%)	4 (4%)	3 (3%)
Total	200	13 (13%)	5 (5%)	3 (3%)

3 | RESULTS AND DISCUSSION

3.1 | Isolation of *C. sakazakii* with culture and molecular methods

In this study, two different methods were used to identify *C. sakazakii* in PIFs and baby foods; method of ISIRI: NO.9430 and *16S rRNA* and *gyrB* molecular methods. Because of the differences in the accuracy and sensitivity of these two methods, the results of culture and molecular methods showed different levels of contamination. In this study, the results of microbiological culture on the ESIA following the biochemical tests (Microgen GN ID analytical profile showed 84% similarly with the *C. sakazakii*, this might be due to the lack of some biochemical tests in this kit) revealed that 8 out of 100 samples (8%) and 5 out of 100 samples (5%) were contaminated in baby foods and PIFs samples, respectively (Table 2).

Baby food samples had the highest contamination rate (especially foods for 6 and 12 months babies) which were based on cereal-fruit and grain-date. Confirmation of the results by *16S rRNA* primer showed that among the five probable *C. sakazakii* detected in PIFs by culture method, only 1 (1%), including PIF1, and among the eight potential bacteria detected in the baby foods, only 4 (4%) were positive. The baby foods included BF1 (at the end of 6 months of age), BF3 (at the end of 8 months of age), BF5 (at the end of 12 months of age) and BF8 (at the end of 6 months of age). Indicating the contamination of cereals and fruits based food samples (Table 2). Since studies have shown that *16S rRNA* PCR could not distinguish different species, especially *C. sakazakii* from *C. malonaticus* (Iversen et al., 2007), so, positive samples were further analyzed by the designed *gyrB* primers.

The *gyrB* gene (encodes the subunit B protein of a type II DNA topoisomerase) has a crucial role in DNA replication, and is distributed universally among bacterial species. It has been suggested that this gene is an excellent molecular marker for bacterial species identification (Huang et al., 2013). The results of the present study showed that *gyrB* gene had high specificity in the identification of *C. sakazakii*. These findings were consistent with the results obtained by Huang et al. (2013). In the present study, using the *gyrB* gene indicated a contamination rate of 0% and 3% in PIFs and baby food (BF3, BF5, BF8) samples, respectively (Table 2). The *gyrB* and *16S rRNA* gene sequences were submitted to the GenBank with the accession numbers of MG637049.1 and MG637050.1.

The contamination rate of *C. sakazakii* in Heperkan et al. (2017) and Abdel-Galil et al. (2015) studies were reported as 1 (2.5%) (Heperkan et al., 2017) and 9 (5.2%), respectively (Abdel-Galil et al., 2015), which were higher than our findings. Comparing the results with other studies in Iran, our findings showed a decrease in contamination rate of PIFs. According to Mardaneh and Dallal study in 2017 on 125 samples of PIFs, the contamination rate was reported 7.2% (Mardaneh & Dallal, 2017). On the other hand, contamination of baby foods in our study showed a higher contamination rate than some other studies, such as Abdel-Galil et al., 2015 who reported only one contaminated sample (1.6%) (Abdel-Galil et al., 2015) and lower contamination rate than (Aigbekaen & Oshoma, 2010; Iversen & Forsythe, 2004) studies who reported 27.1% (Aigbekaen & Oshoma, 2010) and 24%, respectively (Iversen & Forsythe, 2004).

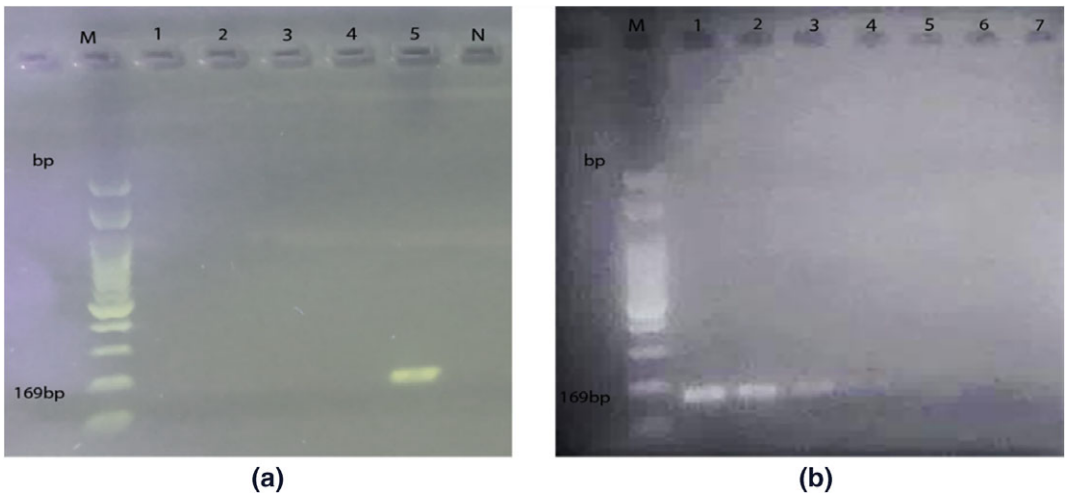

FIGURE 1 (a) PCR results of different bacteria using gyrB pairs. Lane M = 100bpDNA ladder; Lanes 1-5:*Serratia marcescenes* (PTCC 1187), *Shigella dysenteriae* (PTCC 1188), *Escherichia coli* (PTCC 1399), *salmonella enteritidis* (PTCC 1709) and *C. sakazakil* (PTCC 1550); Lane N = negative control. (b) The sensitivity of PCR using gyrB primer pairs for the detection of *C. sakazakil* strain PTCC1550. Lane M = 100bpDNA ladder; Lanes1-7: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}

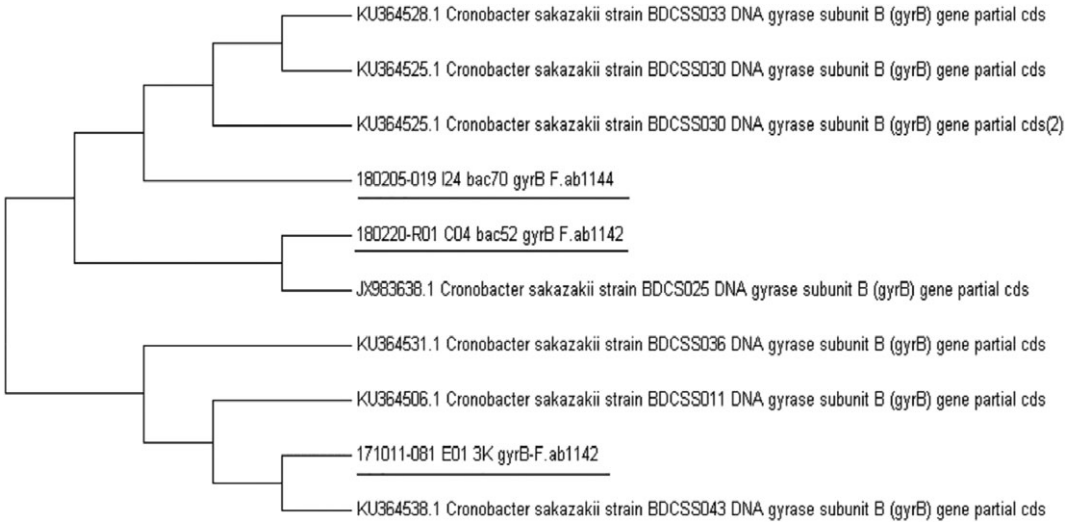

FIGURE 2 The evolutionary history was inferred using neighbor-joining method. The optimal tree with the sum of branch length = 0.12017073 is shown. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 95 positions in the final dataset. Evolutionary analyses were conducted in MEGA7

Generally, the highest and lowest levels of contaminations were related to cereals and fruits-based baby foodsat the end of six months, respectively. Therefore fruits and grains may be the main sources of contamination in these products. These findings were consistent with the results of former studies (Al-Timimi, 2007; Shaker et al., 2007). In this study, one sample of PIF (PIF1) and one sample of baby food (BF1) were not approved as *C. sakazakii* by *gyrB* primer pairs, indicating possible contamination with other species of this genus. Our results showed that PIFs were not contaminated with *C. sakazakii* in Iran, but improving surveillance and hygiene level in producing baby foods is necessary.

3.2 | Study the specificity and sensitivity of *gyrB* primer

The designed primer showed high specificity for *C. sakazakii* (Figure 1a). Evaluating the sensitivity of the primers, four bands (dilution of 10^{-1} to 10^{-4}) with the size of 169 bp were observed. In this study, with an initial concentration of 34.3 ng/μL of the DNA, the detection limit was calculated as 3.43 pg/μL, which is equivalent to 762 copy/μL (Figure 1b).

3.3 | Results of phylogenetic tree

Sequencing results of the isolated bacteria and comparing them with the NCBI Blast database showed 100% similarity of these bacteria with *C. sakazakii* (Figure 2) results also showed the phylogeny relationships between the isolates and the existed bacteria in the Gene Bank (NCBI/Gene Bank). In the plotted dendrogram, two major clusters were defined based on *gyrB* gene, bac70 *gyrB* F.ab1144 sample was classified in a separate branch, but bac52 *gyrB* F.ab1142 and 3 K *gyrB*-F.ab1142 were adjacent to *C. sakazakii* strain BDCS025 and strain BDCSS043, respectively. The dendrogram shows, the genetic similarity of the studied bacterium with these strains.

4 | CONCLUSION

According to the obtained results, it can be concluded that the hygienic state of the PIFs in Iran is acceptable, however, there was some degree of contamination in the baby foods. In this study, a pair of primers were designed based on *gyrB* gene; which could detect *C. sakazakii* to the genus and species level with high specificity. Therefore, using this primer is recommended for the detection of *C. skazakii* in PIFs and baby foods.

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