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Real-time PCR-based Detection of Foodborne Pathogen *Cronobacter sakazakii* DNA in Infant Formula Milk with Specific Targeting on the *hfq* Gene

Muktiningsih Nurjayadi[1,2*], Dandy Akbar Juliansyah[1,2], Jefferson Lynford Declan[1,2], Gladys Indira Putri[1,2], Ismaya Krisdawati[1,2], Atikah Nur Rahmawati[1,2], Maharanianska Azzahra[1,2], Irvan Maulana[1,2], Gusti Angieta[1,2], Fera Kurniadewi[1,2], Irma Ratna Kartika[1,2], Vira Saamia[3], I Made Wiranatha[3], Bassam Abomoelak[4], Hesham Ali El-Enshasy[5,6,7]

[1]*Department of Chemistry, Faculty of Mathematics and Natural Science, Universitas Negeri Jakarta, Gedung KH. Hasjim Asj'ari, 6th Floor, Jakarta Timur 13220, Indonesia*
[2]*Research Center for Detection of Pathogenic Bacteria, Lembaga Penelitian dan Pengabdian Kepada Masyarakat, Universitas Negeri Jakarta, Jakarta Timur 13220, Indonesia*
[3]*Center Forensic Laboratory of the Criminal Investigation, Police of the Republic of Indonesia, Cipambuan Babakan Madang, Bogor 16869, Indonesia*
[4]*Arnold Palmer Hospital Pediatric Specialty Diagnostic Laboratory, Orlando, FL 32806, USA*
[5]*Innovation Center in Agritechnology for Advanced Bioprocessing (ICA), Universiti Teknologi Malaysia (UTM), Pagoh, Johor, Malaysia*
[6]*School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia (UTM), Skudai, Johor Bahru, Malaysia*
[7]*City of Scientific Research and Technology Applications, New Burg Al Arab, Alexandria, Egypt*

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ABSTRACT

Cronobacter sakazakii has been linked to cause meningitis, necrotizing enterocolitis, and sepsis in infants and newborns, with case fatality rates ranging from 40 to 80%. The most common source of infection has been identified as *Cronobacter sakazakii*-contaminated infant formula. With a relatively specific target *hfq* gene, this study aims to develop a real-time PCR method to identify *Cronobacter sakazakii* in infant formula milk. Real-time PCR is used as a detection method because rt- PCR has higher specificity and sensitivity compared to conventional PCR methods. The real-time PCR method also has a higher level of effectiveness and time efficiency compared to conventional PCR. *Cronobacter sakazakii* ATCC 29544 genomic DNA was isolated and used in a real-time PCR assay. *Cronobacter sakazakii* DNA was amplified using a primer targeting the *hfq* gene, yielding a 145 bp amplicon. The results of the real-time PCR test showed that *Cronobacter sakazakii* DNA with a concentration of 53 ng/µL could be amplified by the primer pairs of *hfq* gene with Ct values of 11 respectively then had Tm values of 81.7°C±0.5. The specificity test showed that the *hfq* primer pairs could differentiate between the target and some non-target bacteria. The sensitivity test showed the ability of the primer to detect the smallest concentration of 3.392 pg/µL with a Ct of 26.16. Based on the results obtained, it can be concluded that the *hfq* primer has the potential to be used as a fast detection method for *Cronobacter sakazakii* bacteria in infant formula using real-time PCR.

1. Introduction

Foodborne disease is a disease caused by consuming food or drinks contaminated with microorganisms or toxic substances (Haskito *et al.* 2019). Foodborne disease can be caused by contamination with foodborne pathogens, such as bacteria, viruses, or fungi, as well as chemical contaminants, including heavy metals, chemicals, and pesticides. Pathogenic bacteria are the most common cause of foodborne disease (Pigott 2008). In 2020, the number of people affected by food poisoning in

* Corresponding Author
E-mail Address: muktiningsih@unj.ac.id

Indonesia was 3276, with 1528 experiencing symptoms of illness (attack rate of 46.62%) while six experienced death (0.18% case fatality rate) (BPOM 2021).

Cronobacter sakazakii is one of the bacteria that causes a disease and infections like necrotizing enterocolitis, bacteremia, meningitis, and septicemia, with reported case-fatality rates ranging from 40 to 80% in infected infants (Yan *et al.* 2012). Infant formula contaminated with *Cronobacter sakazakii* has been reported to be the most common cause of infection (Bowen & Braden 2006; Elkhawaga *et al.* 2020). In several studies, it has been reported that *Cronobacter sakazakii* bacteria are able to survive in dry conditions (Nurjanah *et al.* 2017). Due to this ability, the bacterium *Cronobacter sakazakii* is thought to be contaminated, particularly in dried food products such as infant formula powder. Therefore, there is an urgent need to develop rapid and accurate methods for detecting foodborne pathogens, particularly for identifying *Cronobacter sakazakii* in food, to ensure food safety.

The culturing method is one of the conventional methods used to detect pathogenic bacteria in food products. However, using this method requires a relatively long time and is not specific for detecting pathogenic bacteria. Real-time PCR is a molecular-based method that offers high sensitivity and specificity, enabling the detection of pathogenic bacteria in food, including *Cronobacter sakazakii* (Liu *et al.* 2006, 2019). The *hfq* gene is one of the genes found in *Cronobacter sakazakii*. The detection of *Cronobacter sakazakii* pathogenic bacteria with the *hfq* gene was carried out because the *hfq* gene is pathogenic and is involved in the expression, including regulation of motility and outer membrane protein expression (OmpD), as well as the secretion of virulence factors in facultative intracellular pathogens (Sittka *et al.* 2007).

In previous studies conducted by the UNJ Salmonella Team, specific primers have been designed and synthesized as target genes for the detection of nine foodborne pathogenic bacteria in food. One of them is a primer pair for the fast and accurate detection of the pathogenic bacterium *Salmonella typhi*, which has successfully amplified a portion of the 95 bp fragment of the fim-C gene using the real-time PCR method (Nurjayadi *et al.* 2019). In this study, the development of rapid detection using the real-time PCR method will be carried out to detect *Cronobacter sakazakii* bacteria in infant formula milk.

2. Materials and Methods

2.1. Design of Specific Primers

The *hfq* gene sequences were retrieved from GenBank with accession number NZ_CP011047.1. The *hfq* gene is located at the sequence 1542202 to 1542510 and measures 309 bp in *Cronobacter sakazakii* strain ATCC 29544 chromosome, complete genome (Accession number: NZ_CP011047.1). The *hfq* gene was designed in silico using the Primer-Blast software, which is available on the NCBI website (https://www.ncbi.nlm.nih.gov/). The designed pairs of primers for the *hfq* gene were synthesized at Macrogen Synthesis, Inc.-Korea commercial laboratory. The primer pair produced in this research are shown in Table 1.

2.2. Culture Media and Bacterial Strains

Cronobacter sakazakii ATCC 29544 in KWIK-STIKTM (Microbiologist, Minnesota) was cultured in Tryptic Soya Agar (TSA) media (Merck) at 37ºC for 18 h (overnight culture). Following incubation, a single colony was inoculated into Tryptic Soy Broth (TSB) and incubated in an incubation shaker (YIHODER LM-400D) at 37°C for 18 hours and 150 rpm. TSA was used to grow bacteria at a dilution of 10^{-5}-10^{-7} using the spread plate method and incubated at 37°C for 18 hours. A sample dilution yielding 30-300 colonies, based on FDA BAM plate count standards, was used due to artificial contamination (Food and Drug Administration Bacteriology Analytical Manual). Then, CFU/mL was calculated based on the number of colonies.

2.3. Artificial Contamination of an Infant Formula Milk Sample

Food samples were prepared by brewing the infant formula according to the amount indicated in the product instructions. After that, 5 mL of bacteria inoculum in the media to be artificially contaminated was added to the formula milk sample in the Erlenmeyer flask. After that, it was put into a Thermal Shaker (YIHODER LM-400D) and incubated at 37°C for 24 hours. The uncontaminated

Table 1. PCR primers and genes for PCR assays

Target gene	Primers	Sequence	Amplification size
hfq	*hfq-F*	5'–GCA AGA TCC GTT CCT CAA C-3'	145 bp
	hfq-R	5'–ATA AAC CAT CTG GCT GAC CG-3'	

food sample served as the negative control, while the undiluted bacteria suspension used to inoculate the food sample served as the positive control.

2.4. Isolation of Bacterial DNA

Microtube was filled with one milliliter of *Cronobacter sakazakii* culture stock from TSB and centrifuged (Sorval Legend Micro 17R) at 5000 x g for five minutes. Following that, gram-negative bacteria DNA from *Cronobacter sakazakii* bacteria was isolated from pellets using Geno Plus Genomic DNA Extraction Miniprep System (Viogene), in accordance with the manufacturer's instructions. Using a nanodrop spectrophotometer (Nanovue Plus), the A_{260}/A_{280} ratio and DNA concentration were measured to determine the quality of the isolated DNA. Then, agarose gel DNA electrophoresis was carried out to confirm that the DNA isolation was appropriate. To see isolated DNA, 0.7% agarose gel electrophoresis and a UV Trans-illuminator (Vilber Lourmat) were utilized. *Cronobacter sakazakii* isolated DNA is kept in a freezer between -20°C and -80°C.

2.5. Optimization of Annealing Temperature of *hfq* Primer Pairs

Gradient PCR (Takara PCR Thermal Cycler) was used to identify the ideal temperature for the annealing stage to which the primer *hfq* is attached. Primer with synthesis has been examined across a specific temperature range of 53-62°C. The PCR assay was performed using a final volume of 25 µL containing 1 µL of pure *Cronobacter sakazakii* DNA isolate, 1 µL of forward and reverse primers, 12.5 µL of master mix (NZYTaq), and 9.5 µL of Nuclease Free Water (NFW), for a total volume of 25 µL in the tube. The gradient PCR assay protocols were as follows: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at a temperature between 53°C and 62°C for 30 s, and an elongation step at 72°C for 1 minute. Through final extension at 72°C for 10 minutes, the PCR amplification process was completed.

2.6. Confirmation Assay of Primer *hfq* using Real-Time PCR

The test was carried out using Magnetic Induction Cycler qPCR (Bio molecular system), and the total volume used for one reaction mixture was 20 µL. The composition used included 10 µL Master Mix SYBR green (Smobio), 1 µL each forward and reverse primer, 1 µL pure *Cronobacter sakazakii* DNA isolate template, and 7 µL NFW. In addition, one reaction was also used as a Non-Template Control (NTC) to serve as a negative control, containing NFW and Master Mix SYBR green. *Salmonella typhi* (as used in a previous study) with fimC primers serves as a positive control (Nurjayadi *et al.* 2019). The amplification process was carried out for 40 cycles under pre-denaturation conditions at 95°C for 3 minutes, followed by denaturation at 95°C for 30 seconds, annealing at 5°C Tm for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 10 minutes.

2.7. Specificity and Sensitivity Assay

The specificity test aims to ensure that the designed *hfq* gene primer pair can specifically amplify *Cronobacter sakazakii* bacteria. Primer specificity test was conducted to determine the specificity of primers in their ability to distinguish between target and non-target bacteria. The target bacteria used were *Cronobacter sakazakii* as a positive control and several non-target bacteria, which are bacteria that cause food poisoning, namely *Vibrio parahaemolyticus, Vibrio alginolyticus, Listeria monocytogenes, Salmonella typhi, Yersinia enterocolitica, Staphylococcus aureus,* and *Klebsiella pneumoniae*. A total of 20 µL of the reaction mixture was utilized in this test. Each reaction mixture containing the target and non-target bacteria DNA templates received a pair of primers. Each isolated bacterial concentration was equalized. Based on the amplification curve and the corresponding melting curve, the real-time PCR curve findings were examined. Multilevel dilution sensitivity testing was done on DNA template isolates of the bacteria. *Cronobacter sakazakii* DNA isolate template was diluted by mixing 2 µL of pure culture into 8 µL of NFW up to seven dilutions. For the real-time PCR process, 2 µL of each dilution result was used. The amplification curves and standard curves represent the outcomes of this sensitivity test.

2.8. Application in Artificially Contaminated Infant Formula Milk

This confirmation test was carried out using DNA isolates from the test sample (formula milk that had been artificially contaminated), positive controls (pure *Cronobacter sakazakii* DNA isolates), and negative controls (NTC without DNA isolates and only containing NFW + Master Mix). Each reaction mixture was made as much as 20 µL. The results of the real-time PCR are in the form of an amplification curve and a melting curve.

3. Results

3.1. Qualitative and Quantitative DNA Genome Analysis

The isolation results were characterized by agarose gel electrophoresis using a 10 Kb ladder as shown in Figure 1. The DNA band on the leftmost lane is a 10,000 bp DNA ladder, which serves as a marker for the size of the DNA bands on the other lanes. Qualitative test results of DNA isolates show that *Cronobacter sakazakii* bacteria have been successfully isolated because the bands formed in lanes 2 to 5 appear above 10,000 bp based on the marker. Based on in-silico tests, the size of *Cronobacter sakazakii* bacteria DNA is around 4.5 Mbp. In the quantitative test, the purity and concentration of DNA were measured using a NanoDrop Spectrophotometer at a wavelength of A_{260}/A_{280}. The results of the qualitative test can be seen in Table 2.

Based on the information in Figure 1, the bright DNA bands from *Cronobacter sakazakii* pure culture DNA isolates and DNA isolates from artificial food

Table 2. The purity and concentration of C. sakazakii DNA

Sample	Purity A_{260}/A_{280}	Concentration (ng/µL)
Pure culture of *C. sakazakii*	1.853	53
Non-dilute bacteria + infant formula milk	2.07	21.9
10-6 dilution bacteria + infant formula milk	2.04	23.1
Infant formula milk without artificial contamination	1.58	12.7

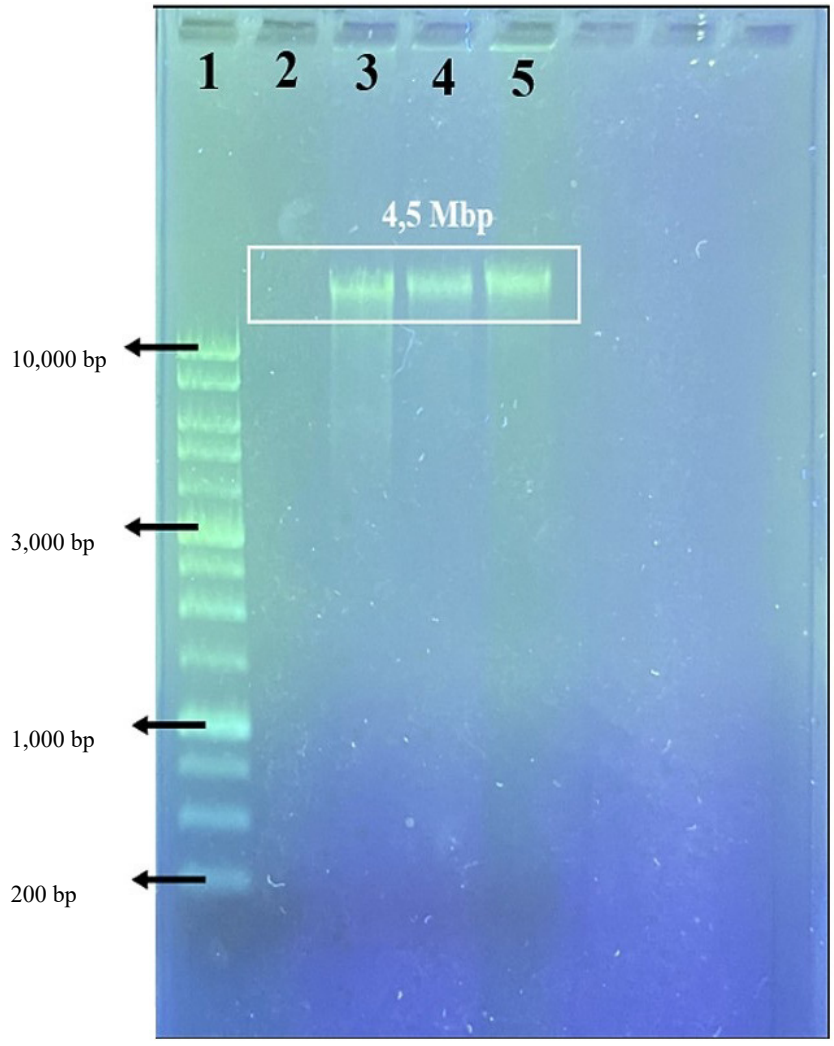

Figure 1. Characterization of *C. sakazakii* DNA on an electrophoresis gel. (M) DNA Ladder 10 kb; (1) negative control; (2-4) *C. sakazakii* DNA

contamination (lanes 2-5) appear at a higher position than the 10,000 bp marker size.

3.2. Optimization of Primer Pairs Annealing Temperature

The results of DNA isolation from a pure culture of *Cronobacter sakazakii*, with a concentration of 53 ng/µL, were used as templates in the gradient PCR process. The results of gradient PCR were then visualized using 2% Agarose Gel Electrophoresis. This optimization is shown in Figure 2, whereas the positive control is toxR *V. parahaemolyticus*, which produced 137 base pairs. Based on the information in Figure 2, the sample with the primer *hfq* produced a *Cronobacter sakazakii* specific DNA band with an amplicon size of 145 bp at all annealing temperature conditions of 53-62°C. And at temperatures ranging from 57-62°C produce the thickest and brightest DNA fragments and these temperatures will be used in the real-time PCR process.

3.3. Confirmation Assay with Real-Time PCR

Confirmation assay resulting from *hfq* primer pairs could amplify *Cronobacter sakazakii* DNA at Ct 11. The melting curve of *Cronobacter sakazakii* was 81.9°C±0.5. Non-template control amplified with the differences >20 cycles and different melt curves with the low peak as shown in (Table 3; Figures 3 and 4).

Table 3. The results of the confirmation assay primer pairs with real-time PCR

Line	Sample	*hfq* Primer	
		Ct	Tm (°C)
	Cronobacter sakazakii	11.01	81.92
		10.83	82.00
	Salmonella typhi (positive control)	12,47	85.33
	NTC	34.15	82.13
	NFW + MM	-	-

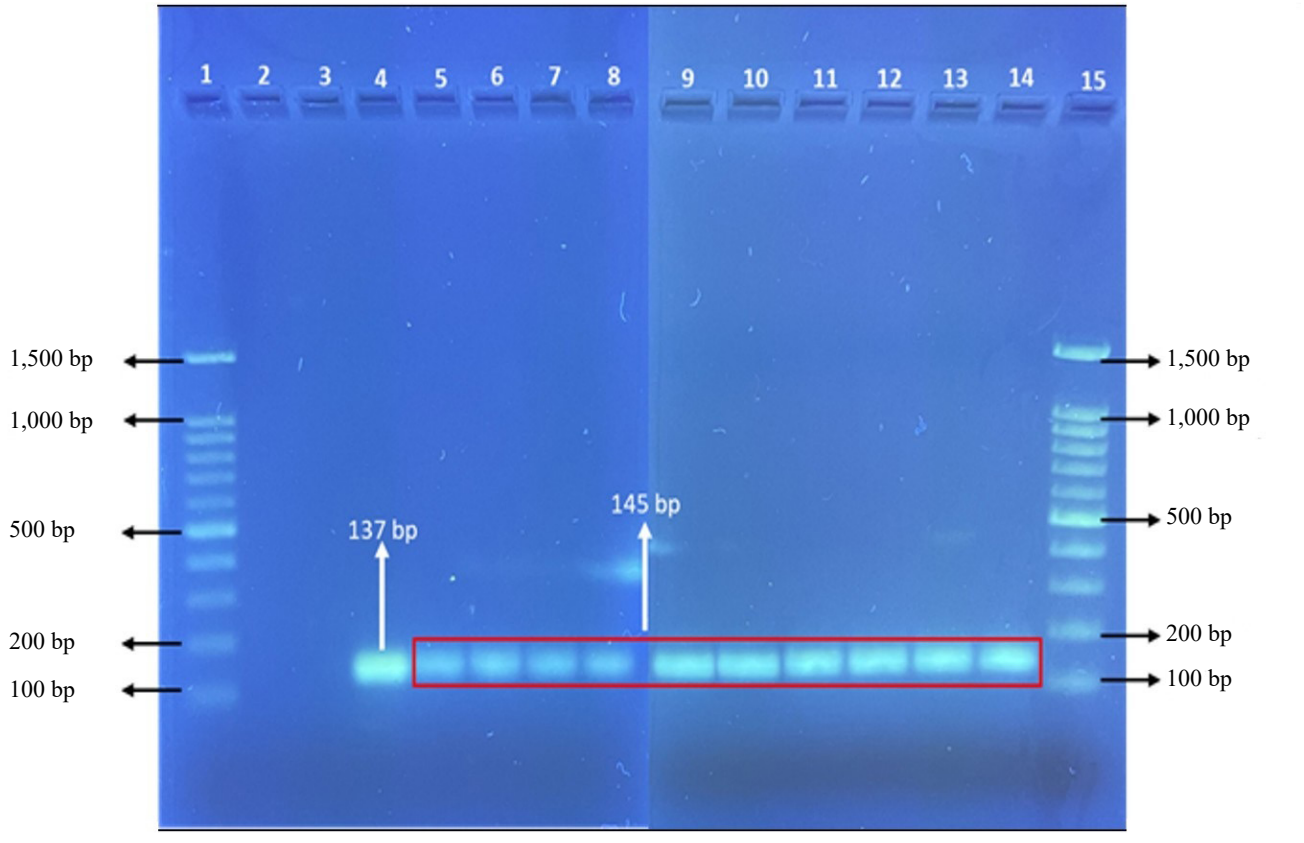

Figure 2. Characterization of *hfq* gene annealing temperature optimization results with agarose gel electrophoresis. (1) DNA Ladder 1,500 bp; (2) NTC; (3) Negative control; (4) Positive control *tox*R Vibrio parahaemolyticus 137 bp; The annealing temperature of PCR was (5) 53°C; (6) 54°C; (7) 55°C; (8) 56°C; (9) 57°C; (10) 58°C; (11) 59°C; (12) 60°C; (13) 61°C; (14) 62°C; (15) DNA Ladder 1,500 bp

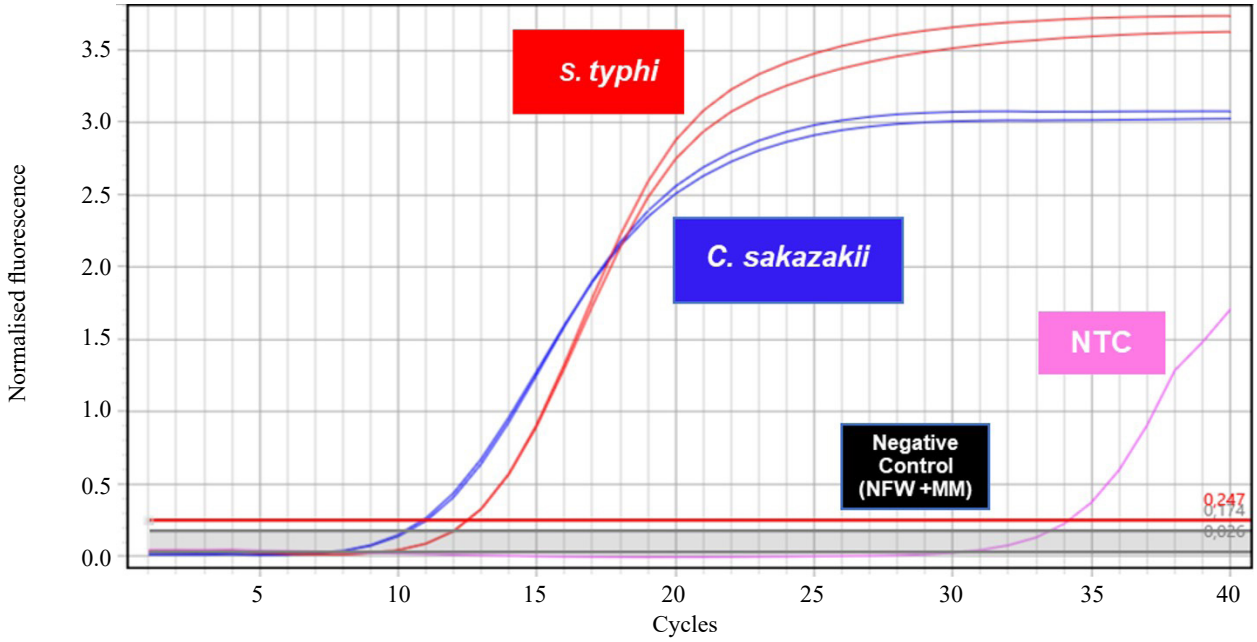

Figure 3. Amplification curve of *hfq* of a concentration 53 ng/µL DNA templated *C. sakazakii* bacteria stock culture, Positive control *fimC Salmonella typhi*, and negative control (NTC & NFW+MM)

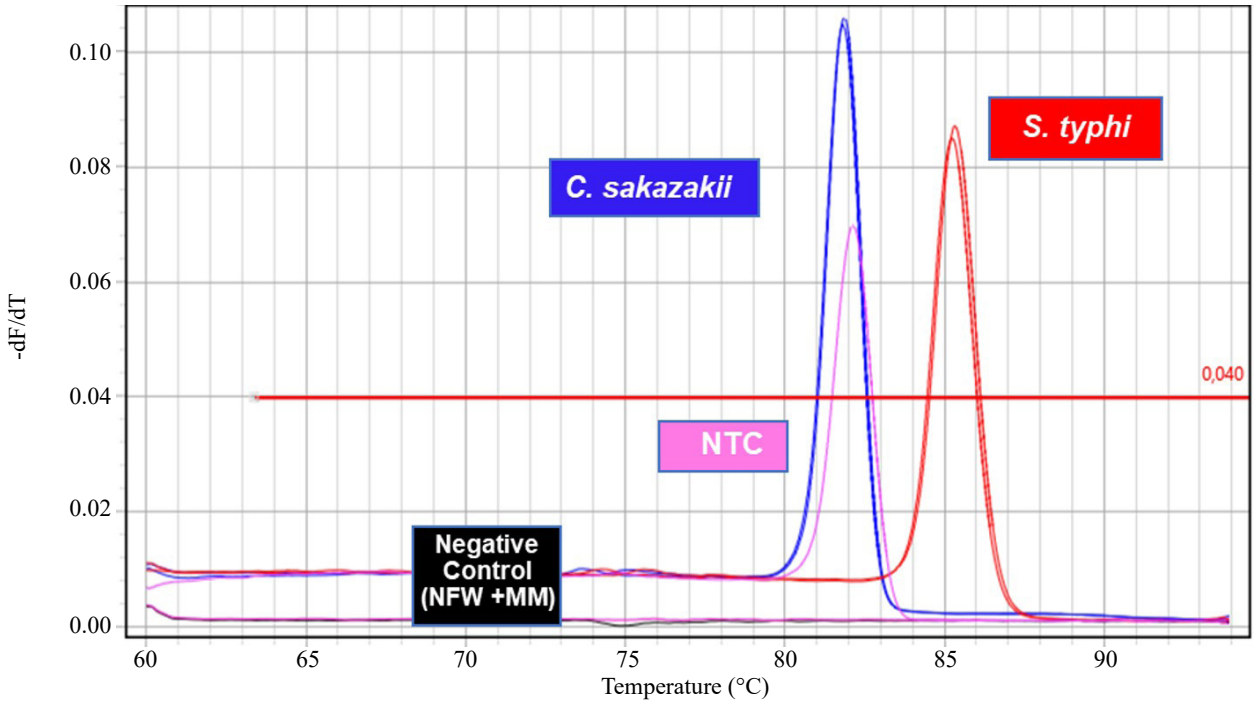

Figure 3. Amplification curve of *hfq* of a concentration 53 ng/µL DNA templated *C. sakazakii* bacteria stock culture, Positive control *fimC Salmonella typhi*, and negative control (NTC & NFW+MM)

3.4. Specificity and Sensitivity Assay of real-time PCR

The *hfq* primer pairs were tested with non-target bacteria as mentioned before and showed a good result, as shown in (Figure 5). Primer pairs can amplify to non-target bacteria with a different range, a few of 10 cycles. DNA and non-target bacteria exhibit different melting curves (Figure 6; Table 4).

The *Cronobacter sakazakii* isolate was diluted up to five times. This assay yielded results indicating

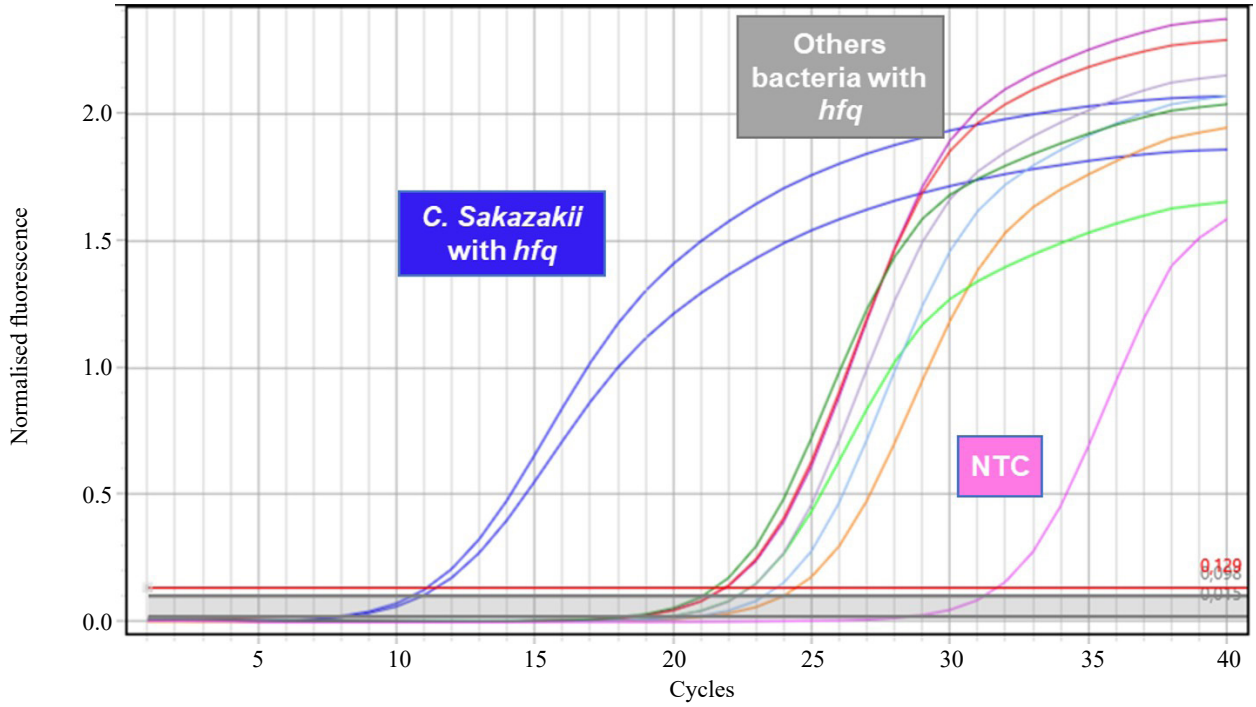

Figure 5. Amplification curve of *C. sakazakii* the *hfq* primer specificity test by real-time PCR

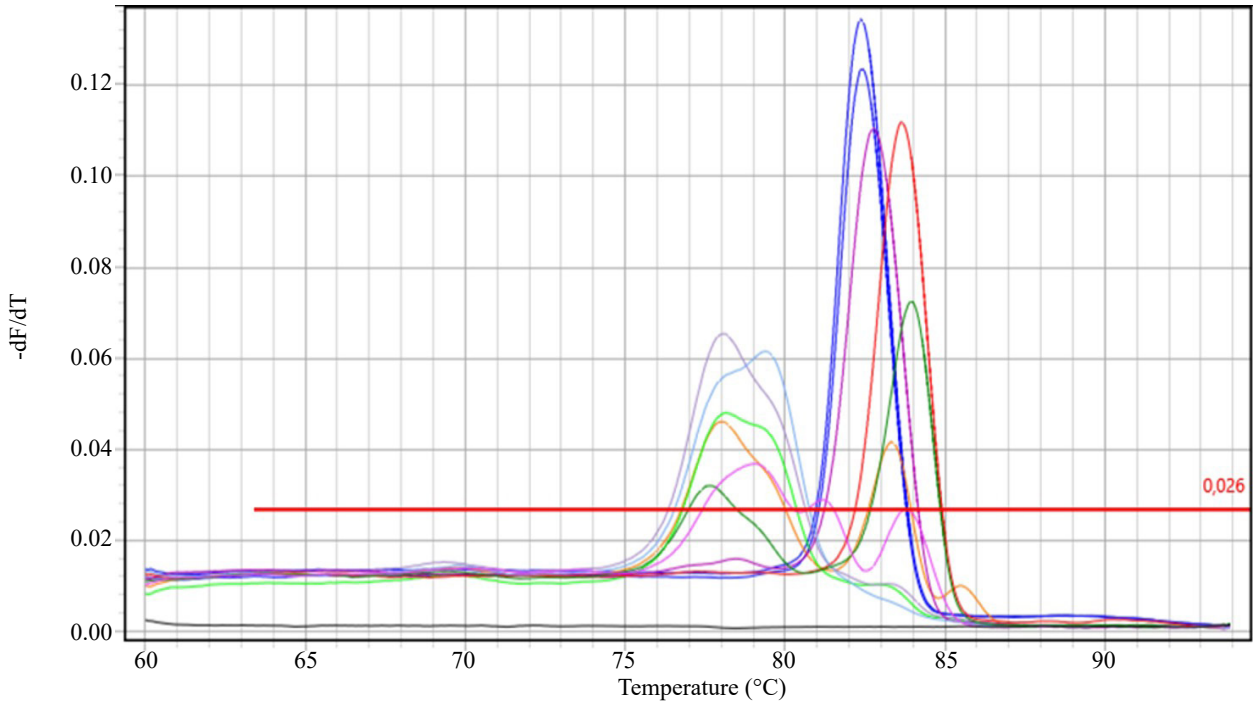

Figure 6. Amplification curve of *hfq* of a concentration 53 ng/µL DNA templated *C. sakazakii* bacteria stock culture, Positive control *fimC Salmonella typhi*, and negative control (NTC & NFW+MM)

that *Cronobacter sakazakii* DNA template could still be detected at a lower concentration of 3,392 pg/µL, with a Ct value of 26.16, using *hfq* primers (Figure 7). The standard curve is obtained by plotting the Ct value (y-axis) against the transformation concentration of the target nucleic acid dilution (x-axis). The Standard curve and R2 value *hfq* are shown in Table 5.

Table 4. The results of a specificity test with real-time PCR

Line	Sample	*hfq* Primer	
		Ct	Tm (°C)
	C. sakazakii	11.36	82.38
	V. parahaemolyticus	24.39	78.03; 83.32
	V. alginolyticus	22.71	78.20
	L. monocytogenes	22.72	78.08
	S. typhi	21.83	82.77
	Y. enterocolitica	21.79	83.65
	S. aureus	23.68	79.35
	K. pneumoniae	21.45	77.66; 83.94
	NTC	31.63	82.15

Table 5. The results of a sensitivity test using real-time PCR

Line	Sample	Concentration (ng/µL)	Ct
			hfq Primer
	Non-Dilute (ND)	53	11.03
	Dilute 1 (D1)	10.6	13.01
	Dilute 2 (D2)	2.12	15.43
	Dilute 3 (D3)	0.424	18.20
	Dilute 4 (D4)	0.0848	20.91
	Dilute 5 (D5)	0.01696	23.09
	Dilute 6 (D6)	0.003392	26,16
	NTC	-	35
Equation			y = -3.543x + 14.99
R^2 Value			0.9977

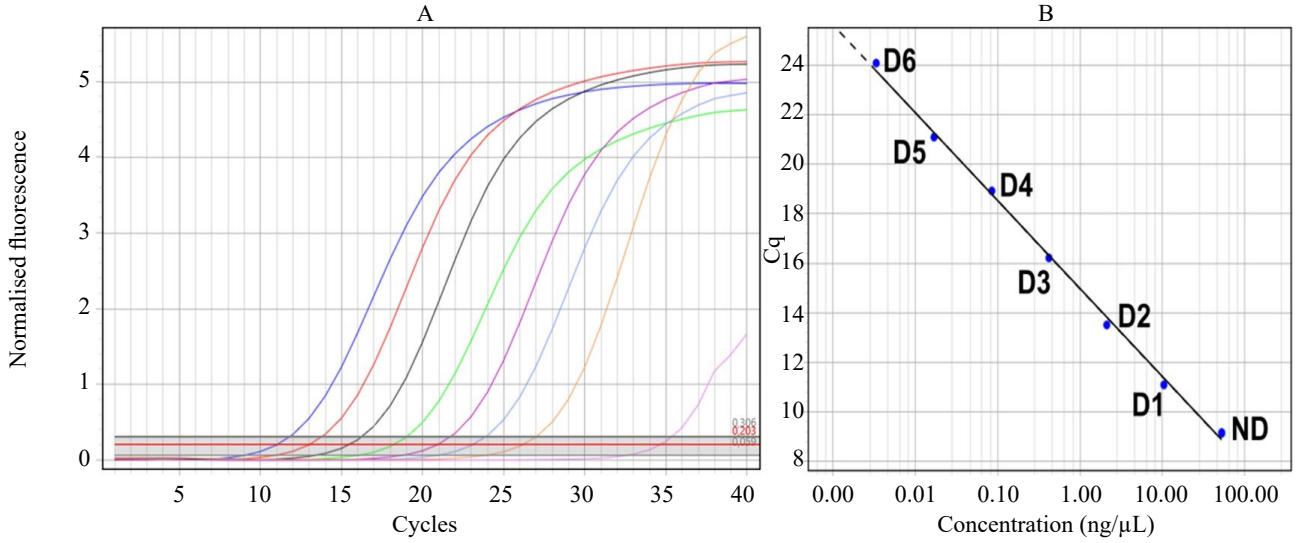

Figure 7. Amplification curve (A) and standard curve (B) test the sensitivity of the *hfq* primer

3.5. Detection of *Cronobacter sakazakii* in Artificially Contaminated Infant Formula Milk

The artificially contaminated infant formula sample and the uncontaminated infant formula sample, serving as the negative control, were examined and then compared with pure cultures of *Cronobacter sakazakii*, the positive control (Figures 8 and 9). The *hfq* primer pair can amplify contaminated infant formula samples at Ct 15.06 (not diluted) and Ct 15.36 (bacteria dilution 10−6) (Figure 8A). The melting curve shows that the two samples have the same melting temperature, which is ±82.2°C (Figure 8B). Uncontaminated samples were seen at Ct ± 29 with a difference of 10-15 cycles with food samples.

4. Discussion

The success of DNA isolation is determined from the results of electrophoresis and the $A_{260nm}/_{A280nm}$ wavelength ratio. Based on the electrophoresis results in Figure 1, the appropriate isolate was obtained based on the genome size of *Cronobacter sakazakii* ATCC 29544, which is 4,511,265 bp (Kim *et al.* 2017). Maximum absorbance of UV light by DNA molecules can be measured at a wavelength of 260 nm, while a maximum absorbance of the spectrum of UV light by protein contaminants is measured at 280 nm. The purity requirement for a good DNA isolate is 1.8-2.0. There are impurities in the genetic material if the purity is less than 1.8 or greater than 2.0 (Dikshit *et al.* 2019). The annealing step in the PCR process is a crucial factor in optimizing PCR amplification results, as the process of attaching a pair of primers to open double-stranded DNA requires an optimal annealing temperature. Intact DNA bands, not smeared, have good thickness and brightness, and indicate high-concentration PCR amplification results (Anggisti & Roslim 2018).

Confirmation tests were carried out to find out that the primers that had been designed could recognize the target bacteria DNA by amplifying *Cronobacter sakazakii* bacteria DNA from pure cultures. The X-axis

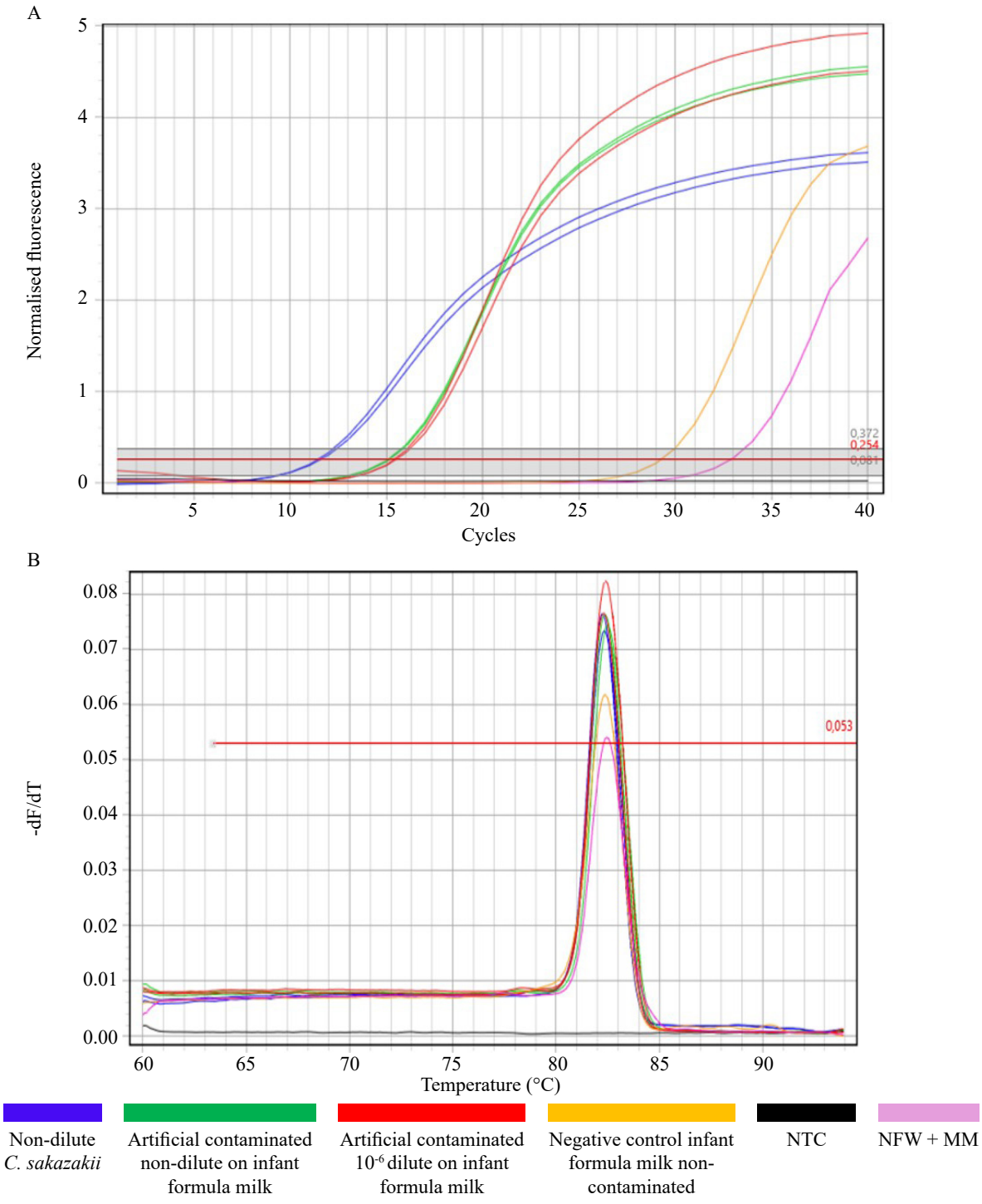

Figure 8. Amplification curve (A) and melting curve (B) confirmation assay in infant formula milk of *hfq* primer

in the real-time PCR amplification curve represents Ct, and the Y-axis represents the accumulated intensity of the fluorescent reporter signal from the total amplicon product produced in each cycle. The lower the Ct value, the more genetic material of the target microorganism in the sample (Wijayanti *et al.* 2022). The results of NTC amplification were considered non-targeted, as evidenced by the difference in Ct distance between target bacteria *Cronobacter sakazakii* and NTC >10 cycles (Nurjayadi *et al.* 2019). Furthermore, the results of the melting curve show that only the target DNA is amplified with the formation of one peak.

The specification test was carried out to ensure that *hfq* primers were specifically designed to detect *Cronobacter sakazakii* bacteria or not. The pair of *hfq* primers amplified the non-target bacteria with a difference in the range of 10-24 cycles when compared to the target bacteria. The Ct of a positive sample is lower than 10 cycles or more than the Ct of the negative control or non-target bacteria; then the sample is considered negative (Nurjayadi *et al.* 2018). Thus, it can be concluded that the *hfq* primer is specific against *Cronobacter sakazakii* bacteria. The sensitivity test aims to obtain a standard curve so that the Limit of Detection (LoD) of *hfq* primer can be identified in detecting *Cronobacter sakazakii* DNA. LoD is a parameter used to describe the sensitivity of a detection method (Rohman 2014). In this research, the *Cronobacter sakazakii* DNA template can still be detected at a lower concentration of 3.392 pg/µL at a Ct value of 26.16 with LoD 2.6×10^{-4} CFU/mL with *hfq* primer (Figure 7 and Table 3).

The pair of *hfq* primers can amplify *Cronobacter sakazakii* DNA in samples of infant formula at Ct 11 to 15. The closeness of the sigmoid curve of pure *Cronobacter sakazakii* culture (positive control) and the resulting sigmoid curve in food contamination samples indicates that *Cronobacter sakazakii* is able to live and reproduce in samples of infant formula, so that bacterial DNA can be detected in real-time PCR. The difference of 2-3 cycles between the amplification results of pure culture samples (positive control) and artificially contaminated formula milk samples may be due to the presence of impurities, which become an obstacle to the PCR process. The presence of inhibitors in the form of impurities affects the efficiency of the real-time PCR amplification process. It raises concerns about the concentration of DNA in doubt, which can affect the resulting fluorescence detection (Hedman & Rådström 2013). The melting curve in Figure 8 shows that pure culture samples and artificially contaminated infant formula have nearly the same melting temperature. These results suggest that the method can be developed into a detection kit model to quickly, sensitively, and specifically determine pathogenic bacteria, yielding accurate results.

In conclusion, in this study, the real-time Polymerase Chain Reaction method used for the detection of pathogenic bacteria *Cronobacter sakazakii* was successfully used to amplify DNA fragments from *Cronobacter sakazakii* using the *hfq* primer at an annealing temperature of 60°C. The optimal temperature chosen was 57-62°C, as it yielded clear results with the formation of a single amplified band. As a result, gene amplification from the *hfq* primer was obtained with a fragment length of 145 bp. The specific test results show that the *hfq* primer specifically detects *Cronobacter sakazakii* target DNA and can distinguish it from other non-target bacteria DNA. The sensitivity test results showed that the lowest concentration of bacterial DNA isolates that could still be detected by the *hfq* primer was 3.392 pg/µL, with a limit of detection (LOD) of 2.6×10^{-4} CFU/mL. Furthermore, this method can be applied in the development of detection kits for the identification of *Cronobacter sakazakii* in food samples by real-time Polymerase Chain Reaction method.

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