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Research Article

Detection of the *Yersinia enterocolitica* Bacteria Targeting the *myfA* and *ystA* Genes in Contaminated Vegetable Samples using Real-Time PCR to Develop Rapid Detection of Food Poisoning Bacteria

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ABSTRACT

Yersinia enterocolitica is a pathogenic bacterium with the ability to survive and multiply in food in a low-temperature environment that can cause death in humans. In previous studies, the optimum annealing temperature of *ymoA, ystA*, and ail gene primers with amplicons of 185 bp, 123 bp, and 192 bp, respectively, was successfully found. This study aims to develop a pathogenic bacteria detection kit with confirmation, sensitivity, and specificity of *myfA* and *ystA* primers in detecting *Yersinia enterocolitica* bacteria quickly and accurately using the real-time Polymerase Chain Reaction method. The results showed that *myfA* and *ystA* primers have optimum annealing temperatures at 60°C with amplicon lengths of 181 bp and 123 bp, respectively. Primer *myfA* was able to amplify the target with real-time PCR at Ct 12.07±1 and Tm 81±1°C, while the *ystA* primer at Ct 12.38±1 and Tm 83±1°C. *myfA* and *ystA* primers were also able to distinguish target and non-target bacteria based on Ct or Tm. The designed primers successfully detected *Yersinia enterocolitica* bacteria with the smallest concentration of 0.000439 ng/µL equivalent to 7.024×10^2 CFU. The detection limit obtained is smaller than the contamination threshold set by the Food and Drug Administration (BPOM). Primer *myfA* and *ystA Yersinia enterocolitica* also successfully detected the target bacteria in cabbage and lettuce samples artificially. Based on these results, *myfA* and *ystA* primers successfully detected *Yersinia enterocolitica* in vegetable samples using real-time PCR quickly, sensitively, specifically, and accurately.

1. Introduction

Vegetables have such good properties for human health that vegetables are widely consumed by the public. The standard consumption of vegetables, according to the World Health Organization (WHO) is 250 grams or equal to 62.5 kcal per day. The large consumption of vegetables has led to the emergence of new problems. Food that is processed improperly can be a good medium for the spread of pathogenic bacteria. Therefore, food quality and safety must be well maintained. Diseases

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caused by consuming food contaminated with microorganisms are called foodborne diseases or food poisoning. WHO data states that from one case related to food poisoning in a developing country, there are at least 99 other unreported cases. In addition, WHO also states that 1 in 10 people in the world fall ill as a result of consuming contaminated food, causing more than 420.000 deaths every year (WHO 2015).

One of the microbes that cause food poisoning is *Yersinia enterocolitica. Yersinia enterocolitica* is a gram-negative bacterium in the Enterobacteriaceae family that does not form spores is facultatively anaerobic, and is able to survive and reproduce in food at low temperatures, such as refrigerators(Annamalai & Venkitanarayanan 2005). This bacterium is one of the pathogens that cause food poisoning and infections in humans, known as yersiniosis disease, with clinical manifestations in the form of fever, diarrhea, and abdominal pain (Saraka *et al.* 2017). Yersiniosis due to *Y. enterocolitica* infection is the third most frequently reported zoonosis in Europe, with 7,202 confirmed cases in 2015, after salmonellosis and campylobacteriosis. The highest total specific cases were reported in several countries, including Germany with 2,752 cases, the Czech Republic with 678 cases, France with 624 cases, and Finland with 582 cases(EFSA & ECDC 2017).

This results in the need for a rapid, sensitive, specific, and accurate detection method for detecting *Y. enterocolitica* pathogen contamination in vegetables. Existing methods for detecting pathogen contamination are bacteriological culture (Hassanzadeh *et al.* 2022), conventional Polymerase Chain Reaction (PCR) (Bonardi *et al.* 2016), multiplex Polymerase Chain Reaction (mPCR) (Piras *et al.* 2021). However, these three methods sometimes still have some weaknesses in terms of sensitivity, specificity, and speed. Therefore, real-time PCR, which is a development of conventional PCR methods that can monitor products by measuring fluorescence signals continuously, is often used as a fast and reliable detection tool due to its high sensitivity and specificity.

Previous research has successfully designed primers for the *ymoA* gene as a detection target for *Yersinia enterocolitica* (Nurjayadi *et al.* 2024). This study aims to develop a pathogenic bacteria detection kit with confirmation, sensitivity, and specificity of *myfA* and *ystA* primers in detecting *Yersinia enterocolitica* bacteria quickly and accurately using the real-time Polymerase Chain Reaction method. The *myfA* gene is one of the virulent genes found in *Y. enterocolitica*. Yersinia mucoid factor (*myfA*) is expressed at 37°C under acidic conditions. The 21-kDa *myfA* is a basic component of the fibrillar structure, and the *myfA* gene is grouped into an operon with *myfB*, encoding a putative chaperone, and *myfC* which is a membrane usher protein (Iriarte *et al.* 1993). The *ystA* gene functions as a toxin that activates the particulate form of guanylate cyclase and increases cyclic GMP levels in host intestinal epithelial cells. The novelty reported in this study is the design of primers in a region that is different from previously found genes and the formula used for the PCR process. Therefore, it is necessary to test the ability of primers on vegetable samples artificially contaminated by *Yersinia enterocolitica* bacteria.

2. Materials and Methods

2.1. Design and Synthesis Primer

Primer pairs for the *myfA* gene of *Yersinia enterocolitica* were designed using the National Center for Biotechnology Information or NCBI website (https://www.ncbi.nlm.nih.gov/) by entering the nucleotide sequence based on the database of the ATCC website (https://www.atcc.org/) and also with the Primer-BLAST (Primer-Basic Local Alignment Search Tool) program to design specific primers. The *myfA* primer pair produced an amplicon length of 181 bp. The *ystA* primer pair in this study is an NCBI Primer Blast design that has been done in previous studies with an amplicons length of 123 bp (icreams). The test bacteria used in this study were *Yersinia enterocolitica* from KWIK-STICKTM (Microbiologist, Minnesota) with strain ATCC 23715. Primer pairs were synthesized at Macrogen Laboratory, inc-Korea.

2.2. Preparation of *Yersinia enterocolitica* Culture

Yersinia enterocolitica ATCC 23715 designed in the form of KWIK-STICKTM (Microbiologist, Minnesota) was resuspended with hydrating liquid and cultured on Blood Agar Plate (BAP), then incubated overnight at 37°C. The gray round colonies obtained were then grown in Tryptic Soy Broth (TSB) and incubated for 24 hours at 37°C using an Orbital Shaking Incubator (YIHDER LM-400D) at 150 rpm. Bacterial culture results were then measured by Optical Dencity (OD_{600}) after the incubation period using a UV/VIS Spectrophotometer. Turbidity that occurs in TSB media indicates that the bacteria have grown well.

2.3. Artificial Contaminated Vegetables Sample with *Yersinia enterocolitica*

The vegetable samples used were cabbage and lettuce purchased from the traditional market, which were then boiled until the vegetable juices were released. The vegetable juice was then transferred to Erlenmeyer (Pyrex) and contaminated with bacterial suspension with a volume ratio of 1:1. For the test samples, the bacterial suspension was used at a dilution of 10-7, while the positive control used undiluted bacterial suspension to inoculate the vegetable samples and the negative control used vegetable samples without bacterial contamination. Each sample was incubated at 37°C for 18 hours using an Orbital Shaking Incubator (YIHDER LM-400D) at 150 rpm.

2.4. DNA Isolation

1.5 ml of culture suspension from TSB was transferred into a 1.5 ml Eppendorf tube and centrifuged at 14,000-16,000 ×g for 1 minute until a residue (cell pellet) was produced. Furthermore, bacterial DNA was isolated using the GRS Genomic DNA Kit-Bacteria by following the stages of the gramnegative bacteria DNA isolation protocol from the kit. The resulting bacterial DNA isolate was then measured for purity (A_{260}/A_{280}) and concentration using a nanodrop spectrophotometer (Nanovue Plus).

2.5. Optimization of Annealing Temperature

The annealing temperature optimization process used Gradient PCR (TaKaRa PCR Thermal Cycler). The total reaction volume was 25 µL, which consisted of *Y. enterocolitica* DNA isolate as a template, forward and reverse primers of *myfA* and *ystA* genes, NZYTaq II 2x Colorless Master Mix (nyztech), and Nuclease Free Water (NFW) (Qiagen). Amplification of the two target genes was performed under Gradient PCR conditions as follows: initial denaturation step at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing in the temperature range of 53-62°C for 30 seconds, and extension at 72°C for 1 minute. The PCR amplification process ends with the final elongation stage at 72°C for 10 minutes.

The annealing temperature optimization results were then visualized by 2% agarose gel electrophoresis (nyztech) in 1X Tris-EDTA buffer using green safe (nyztech) as fluorescence. An electrophoresis cocktail using 10 µL of the total mixture containing 7 µL of PCR gradient results and 3µL ExcelDyeTM 6X DNA Loading Dye (SMOBIO) was put into the electrophoresis well. Then the electrophoresis process was carried out at a voltage of 70V, a current of 400 A for 70 minutes. The length of amplicons was visualized with UV Transilluminator (Vilber Lourmat) and compared with 100 bp DNA marker (SMOBIO). The annealing temperature optimization results were applied to the annealing temperature for the real-time PCR method.

2.6. Confirmation Test of *Yersinia enterocolitica* Stock Culture

Confirmation test of *myfA* and *ystA Yersinia enterocolitica* primer pairs was carried out to determine the ability of primers to detect the presence of *Yersinia enterocolitica* bacterial DNA using real-time PCR method (BMS MicqPCR Cycler) and the total volume used for one reaction mixture of 20 µL which includes ExcelTaq 2× qPCR Master Mix, forward and reverse primers *myfA* and *ystA*, *Y. enterocolitica* DNA pure isolate template and Nuclease Free Water (NFW). Sample amplification was made twice (duplo) with the use of Non-Template Control (NTC) as a negative control made as much as 20 µL without DNA isolate and also NTC containing only NFW and Master Mix (MM). The real-time PCR program used includes the initial denaturation stage with a temperature of 95°C for 3 minutes, denaturation with a temperature of 95°C for 10 seconds, annealing with a temperature of 60°C for 30 seconds, extension with a temperature of 72°C for 30 seconds, and final extension with a temperature of 72°C for 420 seconds which was repeated as many as 40 PCR cycles. The results of the confirmation test using real-time PCR are in the form of an amplification curve and a melting curve.

2.7. The Sensitivity and Specificity Testing of *myfA* Primer to *Yersinia enterocolitica* from Stock Culture

Specificity tests were carried out using *myfA* and *ystA* primer pairs which will be cross-tested to determine the level of selectivity of these primers against target bacterial DNA isolates or, in other words, to ensure that primers only target *Y. enterocolitica* bacteria or to determine whether primers can show positive results on other bacteria. *myfA* gene primers that should only detect *Yersinia enterocolitica* ATCC 23715 will be reacted with the target DNA template (positive control) and other non-target bacterial DNA, such as *Cronobacter sakazakii, Vibrio parahaemolyticus, Listeria monocytogenes, Klebsiella pneumonia, Staphylococcus aureus, Vibrio Alginolyticus,* and *Bacillus cereus*. The reaction mixtures were made into

20 µL each. *MyfA* and *ystA* gene primers were mixed into the reaction mixture with each different bacterial DNA template. The concentration of each bacterial isolate was equalized. The real-time PCR curve results will be analyzed based on the amplification curve and melting curve produced.

The sensitivity test of *myfA* and *ystA* gene primers was carried out by multistage dilution of *Y. enterocolitica* bacterial DNA template concentration. This test was conducted to determine the lowest detection limit that the myfA primer pair can achieve to amplify *Yersinia enterocolitica* DNA. Concentration dilution was carried out by inserting 1 µL of template DNA isolate of pure culture of *Yersinia enterocolitica* into 9 µL of NFW up to seven times dilution. Then, the test will be done with real-time PCR based on the amplification curve and standard curve.

2.8. Confirmation Test of the *myfA* Primer to Contaminated Vegetable Sample

Primer pairs of *myfA* and *ystA* genes of *Y. enterocolitica* were tested on vegetable samples artificially contaminated with *Yersinia enterocolitica* bacterial DNA as the positive control, vegetable samples with *Y. enterocolitica* bacterial suspension of 10-7th dilution as test samples, and vegetable samples without bacterial contamination as negative control. The total reaction of each sample was 20 µL. The real-time PCR conditions were the same as the previous confirmation test.

3. Results

3.1. Primer Design

Primers are specifically designed to detect only the target bacteria. Evaluation of the *ystA-FR* primer pair was carried out in a previous study (icreams). The primer pair for *Y. enterocolitica* was designed to target the *myfA* gene fragment in the bacteria. The data from the *in silico* analysis in scripto shows that the *myfA* gene is specific for *Y. enterocolitica* bacteria and is present in almost all strains. In this study, primers were designed based on the *myfA* gene sequence of *Yersinia enterocolitica* with a size of 479 base pairs, and the specific region of the gene with a size of 181 base pairs was used. The nucleotide sequence of the *myfA* primer is based on the ATCC database page Figure 1.

Primer design is based on several requirements, namely primer length of 18-24 bp, %GC value is 35-65%, amplicons length is about 100-250 bp, and the maximum Tm difference between forward and reverse primers is 3°C. The size of the whole genome of *Yersinia enterocolitica* ATCC 23715 is 4,509,790 bp. Based on the results of the *in silico* analysis in Table 1, the *myfA* primer has good primer requirements. The length of the designed primer is 20 bp, this is in accordance with the literature which states that a good primer length is 18-24 bp. If the designed primer is too short, it can cause low specificity. The value of %GC is 50%. The Tm value obtained for each *ystA* forward primer is 58.25°C and the reverse is 56.69°C.

3.2. Quantitative Test of DNA Sample

Quantitative analysis was performed to measure the purity and concentration of DNA. The purity and concentration of *Yersinia enterocolitica* pure culture DNA have been carried out in previous studies (icreams). The purity is 2.06 and the concentration of *Yersinia enterocolitica* culture stock is 225.6 ng/µL. So that the concentration value obtained in this study is relatively good and shows success in the DNA isolation process. The results of quantitative analysis testing on vegetable samples contaminated with *Yersinia enterocolitica* bacteria are presented in Table 2.

3.3. Optimization Annealing Temperature of *myfA* Gene Primer Pairs

PCR annealing temperature optimization process with primer pairs *myfA*-FR and *ystA*-FR using genomic DNA template of *Yersinia enterocolitica* bacteria has

CATTCATTATCGTTATTCATCTTCATACTAATCAATTAATGTGAGATATAAAATGAATAT
GAAAAAATTTGTTAAAAAACCACTAGCAATTGCTGTGTTAATGTTGGCTAGTGGCGGGAT
GGTTAATATGGTACATGCAGAACCGACTGTTATTAATAGTAAAGACATCTCTGCAACAAA
AACTGTTAAAGAGGGAGGTTCGTTCTCAGTTGAATTCAAGGCTACTGAAAACGAGATTGT
GTCAGGCAAATTGGATGCAGATACACCTGCCTTCCATCTGGTAATGTCGGACTCAGGGGA
ACATAAAGGTTGGAATGTTCGGCCTACCGGTGCATCTGAGGGAGGACAGATGGTTTCTGC

Figure 1. Nucleotide sequence of *myfA* primer, which is 479 bp at 1.581.233-1.581.712. Primer forward (red), primer reverse (blue), *myfA* primer amplicons (yellow)

Table 1. Primer and paramater of the primer

Primer	Sequence (5'-3')	Primer length	Tm (°C)	%GC	Self-dimer (kcal/mol)	Hairpin (kcal/mol)	Cross dimer	Amplicon length
ystA-F	TTTCAGGGCAGTTCAGTGAT	20 bp	58.25	50	-4.85	-	-	181 bp
ystA-R	AACATACATCGCAGCAATCC	20 bp	56.69	50	-4.05	-		

Table 2. Purity and concentration DNA isolated from vegetable samples contaminated by *Yersinia enterocolitica* bacteria

Sample	Concentration (ng/µL)	Purity (A_{260}/A_{280})
Selada *Non-Dilute* (ND)	16.4	1.81
Selada *Dilute* (D)	14.3	1.84
Kol *Non-Dilute* (ND)	16.7	1.88
Kol *Dilute* (D)	14.6	1.82

successfully obtained amplicons measuring 181 bp and 123 bp, respectively. The amplification process of *Yersinia enterocolitica* was carried out with variations in annealing temperature. This aims to determine the optimum primer annealing temperature. The amplification process was carried out with variations in annealing temperature ranging from 53-62ºC using PCR Gradient, which was then visualized with 2% agarose gel electrophoresis as shown in Figure 2. Based on the results of *myfA* primer electrophoresis, one band was obtained for the entire temperature range from 53 to 62°C. The optimum temperature of *myfA* primer was obtained in the range of 55-62°C based on the brightness of the band, and only one band was formed. Based on the electrophoresis results, it can be seen that there is no non-specific amplification or miss-priming. This shows that the primer is able to anneal to the template well and amplify the targeted DNA region. While the results of electrophoresis of the *ystA* gene at 53-57°C there are two bands formed because there is still non-specific amplification so it is considered not to be the optimal annealing temperature. Non-specific amplification will decrease if the temperature used increases. The optimum temperature of the ystA primer was obtained in the range of 58 to 62°C. The positive control used is the *Salmonella typhi fimC* gene with an amplicons length of 95 bp.

From the results obtained for each of the *myfA* and *ystA* primers, a temperature of 60°C was selected as the optimum annealing temperature based on the brightness of the band, and only one band was formed. The formation of one band indicates that there is no dimer from the primer pair. In addition, 60°C is the standard annealing temperature of real-time PCR instruments, making it easier for commercial use.

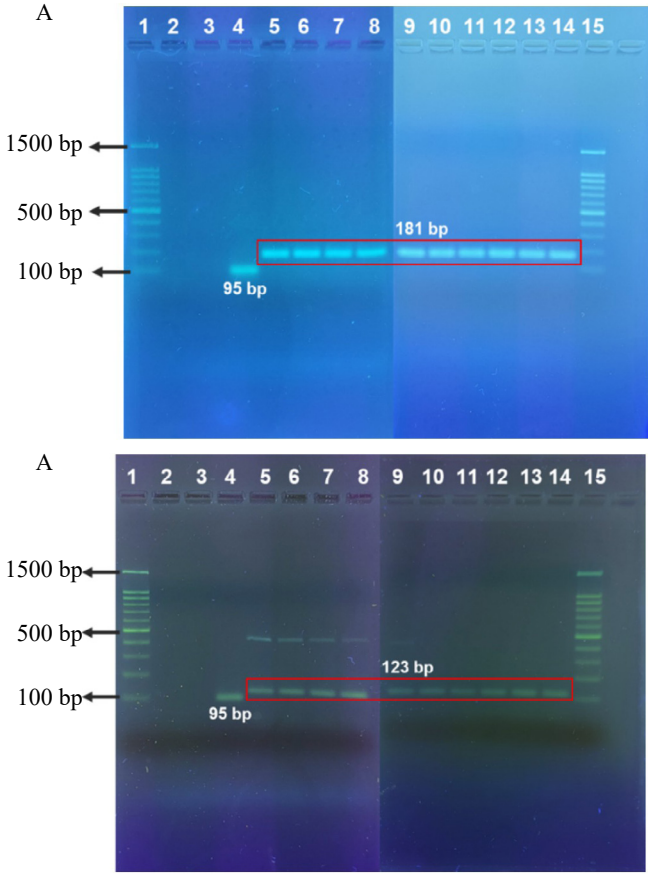


Figure 2. Characterization of *myfA* (A) and *ystA* (B) genes annealing temperature optimization results. (1) and (15) DNA ladder 100 bp; (2) NTC; (3) negative control; (4) positive control fim C *Salmonella typhi* 95 bp; (5)- (14) DNA fragment at 53-62°C

3.4. *Yersinia enterocolitica* DNA Confirmation Test Using Primers *myfA* and *ystA* with Real-Time PCR

The confirmation test aims to determine the ability of primers to amplify and recognize target bacteria by real-time PCR method. In this study, the BMS MicqPCR Cycler instrument was used to amplify *myfA* and *ystA* gene fragments of *Y. enterocolitica* bacteria as targets. In this study, the annealing temperature used was based on the optimization results from the previous stage, namely 60°C. Amplification curve and melting curve will be generated from this test. Figure 3 shows the amplification curve of *myfA* and *ystA*

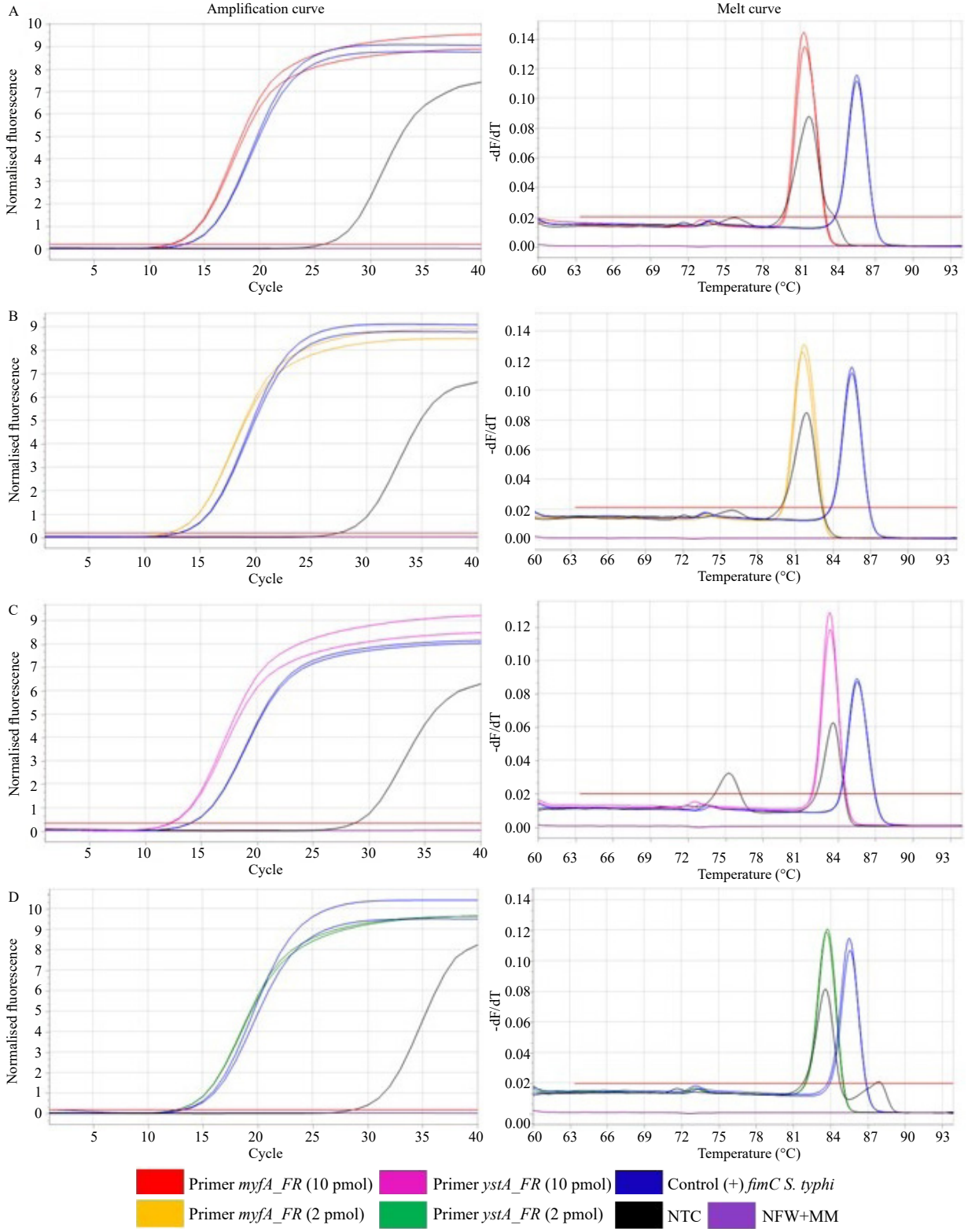


Figure 3. *Yersinia enterocolitica* bacterial stock culture amplification and melt curve with a 50 ng DNA template concentration and negative control. (A) *myfA* 10 pmol, (B) *myfA* 2 pmol, (c) *ystA* 10 pmol, (D) *ystA* 2 pmol

primers, it was found that both primers were able to detect the presence of bacterial target *Y. enterocolitica* with a concentration of 50 ng.

The results of primary confirmation test of *Yersinia enterocolitica myfA* and *ystA* shows at Table 3. The ability of primers to detect the *myfA* gene with a concentration of 10 pmol (red) is indicated by the appearance of Ct values at cycle 11.90 and 12.02 (duplo), for *myfA* gene concentration of 2 pmol (yellow) resulted in Ct values of 12.18 and 12.21 (duplo). While *ystA* primer concentration of 10 pmol (pink) produced Ct values of 12.27 and 12.18 (duplo), and *ystA* primer concentration of 2 pmol (green) produced Ct values of 12.54 and 12.55 (duplo). The appearance of Ct indicates that there is target DNA in the sample. This indicates the success of primers in amplifying *myfA* and *ystA* fragments from *Y. enterocolitica*. Meanwhile, based on the melting curve, each primer produced one peak with Tm (melting temperature) values of *myfA* 10 pmol primer 81.29°C and 81.38°C (duplo), *myfA* 2 pmol primer 81.59°C and 81.73°C (duplo), *ystA* 10 pmol primer 83.39°C and 83.35°C (duplo) and *ystA* 2 pmol primer 83.68°C and 83.76°C (duplo).

Positive control of *Salmonella typhi fimC* (blue) on myfA 10 pmol primer resulted in Ct values of 13.19; 13.15 (duplo), *myfA* 2 pmol primer resulted in Ct values of 13.15; 13.11 (duplo) and Tm 85.53°C; 85.52°C (duplo), the ystA 10 pmol primer produced Ct 14.05; 14.03 (duplo) and Tm 85.57°C and 85.54°C (duplo), the *ystA* 2 pmol primer produced Ct 13.09; 13.26 (duplo) and Tm Tm 85.52°C and 85.59°C. In this confirmation test, the appearance of Ct in the positive control shows that the reaction is going well, and the temperature obtained in the positive control is in accordance with the previous experiment. In this experiment, there is also still a negative control that appears in the form of NTC, which is indicated as the formation of dimers and is not a contamination.

This was reinforced in the MM+NFW negative control where no Ct value appeared, so it can be indicated that the appearance of Ct on NTC is due to the presence of dimers. In addition, the non-appearance of the sigmoid curve in the NFW+MM negative control (purple) also states that during the real-time PCR process there is no disturbing impurity. Negative control NTC (black) on *myfA* primers 10 and 2 pmol produced Ct values of 25.75 and 27.49 with Tm of 81.69°C and 81.88°C, respectively. The ystA 10 and 2 pmol primers were 28.67 with Tm 83.60°C; 87.86°C. The Ct value on NTC is more than 10 cycles then NTC can be tolerated. The appearance of Ct in NTC and not in NFW+MM indicates that the curve occurs due to dimers between primers, but differences of more than ten cycles can be interpreted as negative and are non-targets so they can be ignored. With a difference of ±15 cycles between the sample and NTC, the appearance of Ct on NTC in this study can be ignored.

3.5. Assay Sensitivity and Specificity of *myfA* and *ystA* Primer Using Real-Time PCR

The specificity test aims to determine that the primers designed only amplify the target bacteria and can distinguish them from non-target bacteria. The specificity test compared the results of real-time PCR amplification of *Yersinia enterocolitica* DNA with several non-target bacterial DNA templates using *myfA* and *ystA* primers. Seven non-target bacteria used in this study were *Cronobacter sakazakii, Vibrio parahaemolyticus, Listeria monocytogenes, Klebsiella pneumonia, Staphylococcus aureus, Vibrio alginolyticus,* and *Bacillus cereus*. The amplification curve and melting curve of the primer specificity of the *Yersinia enterocolitica* shows at Figure 4. In the specificity test, a target and non-target DNA template concentration of 50 ng was used with primers *myfA* and *ystA Yersinia enterocolitica* ATTC 23715. More than 22 cycles of Ct values were generated by these seven bacteria, and more than one peak and different Tm values were generated by the melting curves. The results of primary specificity test of *Yersinia enterocolitica myfA* and *ystA* shows at Table 4.

In this sensitivity test, dilutions of *Y. enterocolitica* DNA isolates were carried out. Dilutions were carried out seven times with an initial concentration of 34.3

Table 3. Results of primary confirmation test of *Yersinia enterocolitica myfA* and *ystA*

Primer / Sample	*myfA* 10 pmol		*myfA* 2 pmol		*ystA* 10 pmol		*ystA* 2 pmol	
	Ct	Tm (°C)	Ct	Tm (°C)	Ct	Tm (°C)	Ct	Tm (°C)
NTC	25.75	81.69	27.49	81.88	28.67	75.28; 83.61	28.67	83.60; 87.86
MM+NFW	-	-	-	-	-	-	-	-
fimC S. typhi	13.19; 13.15	85.53	13.15; 13.11	85.52	14.05; 14.03	85.57; 85.54	13.09; 13.26	85.52; 85.59
Sample	11.90; 12.02	81.29; 81.38	12.18; 12.21	81.59; 81.73	12.27; 12.18	83.39;83.35	12.54; 12.55	83.68;76

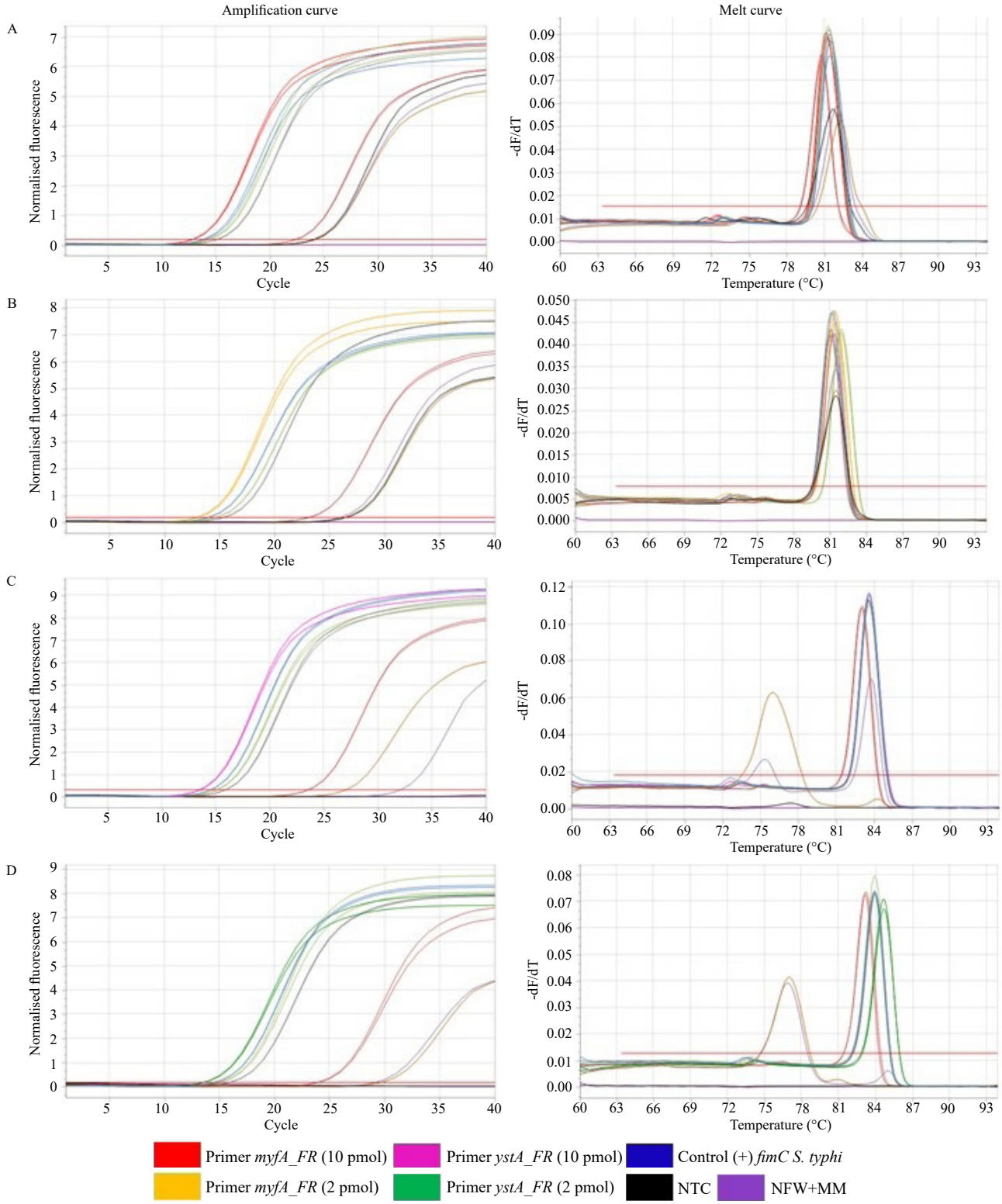


Figure 4. Amplification curve and melting curve of the primer specificity of the *Yersinia enterocolitica*. (A) *myfA* 10 pmol, (B) *myfA* 2 pmol, (c) *ystA* 10 pmol, (D) *ystA* 2 pmol

Table 4. Results of primary specificity test of *Yersinia enterocolitica myfA* and *ystA*

Sample	Color	*myfA* 10 pmol		*myfA* 2 pmol		*ystA* 10 pmol		*ystA* 2 pmol	
		Ct	Tm (°C)	Ct	Tm (°C)	Ct	Tm (°C)	Ct	Tm (°C)
NTC		25.58	80.87	27.69	82.49	31.33	76.86; 85.35	33.57	75.81
MM+NFW		-	-	-	-	-	-	-	-
C. sakazakii		25.03	81.49	22.58	81.38	29.36	75.96; 84.41	31.16	76.23;84.68
V. parahaemolyticus		24.80	81.43	26.14	82.41	29.28	84.11	25.70	76.58
L. monocytogenes		24.93	81.52	27.05	82.29	29.80	76.33	30.18	76.96
K. pneumonia		24.99	81.40	27.29	82.43	29.76	75.85; 84.28	32.42	77.02
Staphylococcus A.		24.83	81.32	26.22	82.21	28.40	84.08	28.49	84.36
V. alginolyticus		24.72	81.18	26.59	82.18	30.02	76.43	32.36	77.08
Bacillus cereus		24.39	81.15	25.85	82.18	29.45	75.59; 84.16	29.70	76.16;84.59

Table 5. Results of primary sensitivity test of *Yersinia enterocolitica myfA* and *ystA*

Sample	Color	Ct				Concentration (ng/µL)
		myfA 10 pmol	*myfA* 2 pmol	*ystA* 10 pmol	*ystA* 2 pmol	
D0		11.77	12.28	12.32	12.46	34.3
D1		14.21	14.95	15.38	15.25	6.86
D2		16.63	17.40	17.60	17.39	1.372
D3		18.81	19.86	20.13	20.05	0.2744
D4		21.14	22.28	22.77	22.63	0.05488
D5		23.63	25.11	25.05	23.71	0.01098
D6		26.49	27.08	27.59	26.61	0.002195
D7		28.61	29.66	29.33	29.57	0.000439
NTC		33.06	34.68	-	33.29	-
NFW+MM		-	-	-	-	-

ng/µL to a concentration of 4.39×10^{-4} ng/µL with Ct values on primers *myfA* 10 pmol, *myfA* 2 pmol, ystA 10 pmol, and *ystA* 2 pmol respectively, namely 28.71; 29.66; 29.33; and 29.57. Equation y = -3.45x + 16.72 with efficiency of 0.95 and R^2 value of 0.9990 for myfA 10 pmol primer; y = -3.98 x + 17.49 with efficiency of 0.98 and R^2 value of 0.9994 for *myfA* 2 pmol primer; y = -3.49x + 17.31 with efficiency of 0.93 and R^2 value of 0.9975 for ystA 10 pmol primer; y = -3.36x + 17.52 for *ystA* 2 pmol primer with efficiency of 0.98 and R^2 value of 0.9946. The results of primary sensitivity test of *Yersinia enterocolitica myfA* and *ystA* shows at Table 5 and the amplification curve and sensitivity test curve for *Yersinia enterocolitica* shows at Figure 5.

3.6. Confirmation Test of the *myfA* Primer to Contaminated Vegetable Sample

Pure culture of *Yersinia enterocolitica* was used as positive control, artificially and nonartificially contaminated cabbage and lettuce samples as negative control, analyzed using real time PCR. Based on the amplification curves of food sample confirmation test results at Figure 6, pure culture *Y. enterocolitica myfA* and *ystA* primers successfully produced Ct values. In the *myfA* primer, both cabbage and lettuce samples that were diluted 10-7 and non-diluted had a Tm value of 81±0.5°C, while the *ystA* primer in cabbage and lettuce samples had a Tm value of 83±0.5°C and a similar peak position, so that the melting curve data indicated that the cabbage and lettuce samples were successfully detected using the primers that had been designed because they had typical Tm values of *Yersinia enterocolitica* according to their respective gene targets. In the negative control of food samples, both primers also formed a melting curve, but with lower intensity. This indicates in both negative controls that the Ct that appears is not the target amplicons but the presence of mutually amplified dimers. The oncentration of *Y. enterocolitica* in food samples shows at Table 6.

4. Discussion

Foodborne disease is a disease caused by consuming food that contains microbial agents such as pathogenic bacteria, viruses, and parasites or food contaminated with toxic chemicals or biotoxins(Mirriam *et al.* 2013). In the case of food poisoning contaminated with microbial agents, a detection method that is very fast, sensitive, specific, and accurate is needed so that the handling of victims becomes more effective and efficient(Widyastuti & Nurdyansyah 2017; Federici *et al.* 2018; Luo *et al.* 2021). real-time PCR has several advantages, namely the

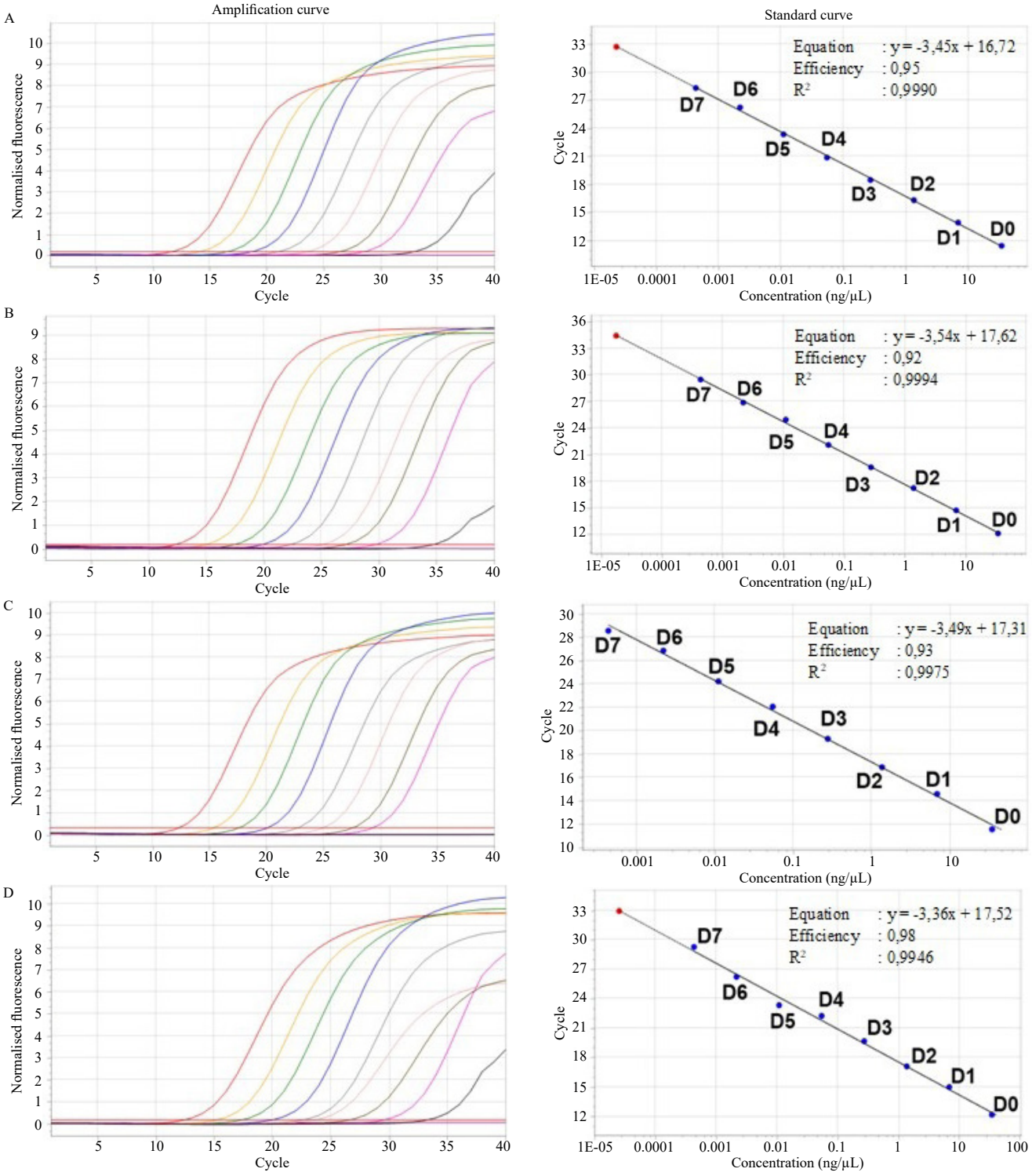
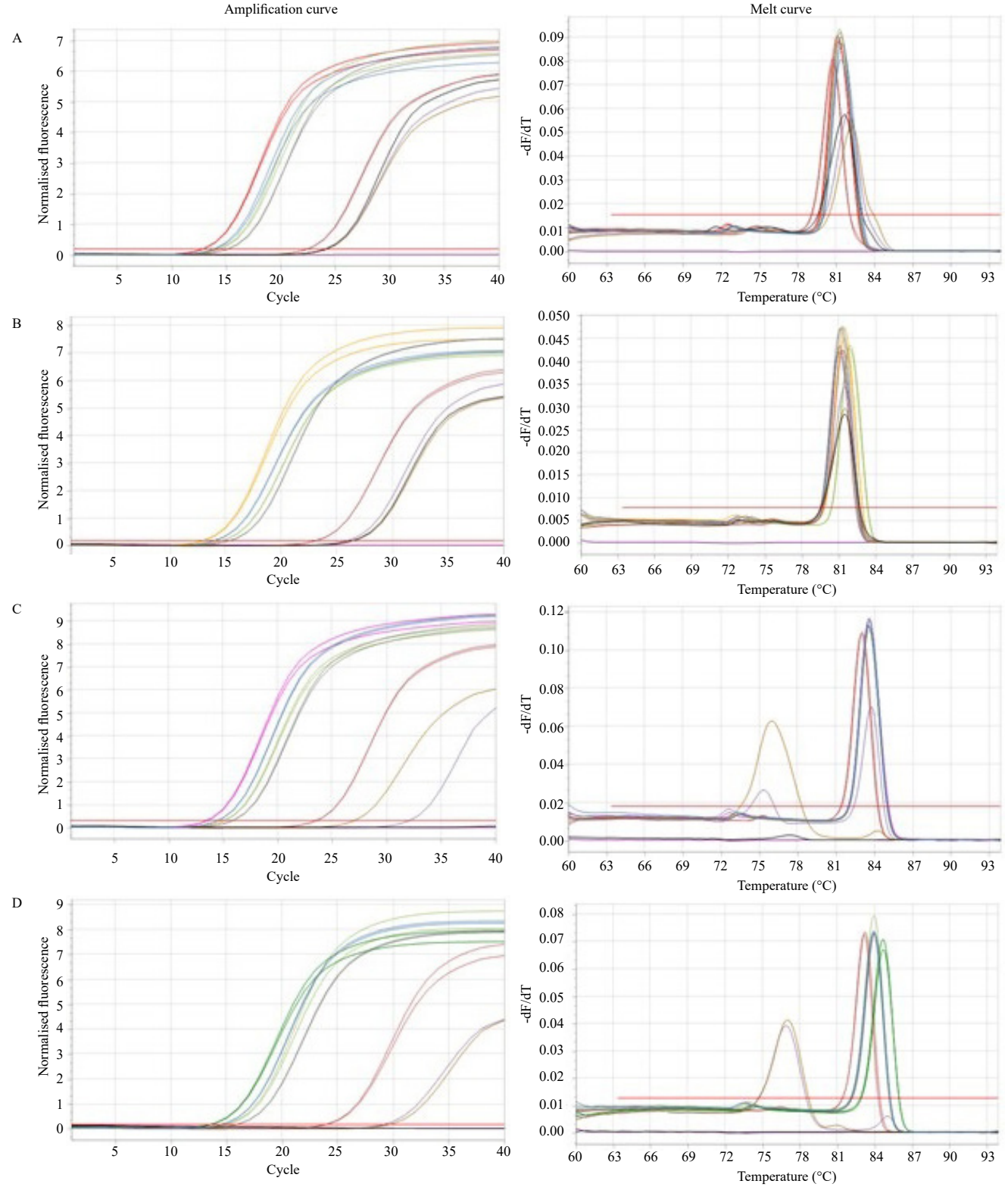


Figure 5. The amplification curve and sensitivity test curve for *Yersinia enterocolitica*. (A) *myfA* 10 pmol, (B) *myfA* 2 pmol, (c) *ystA* 10 pmol, (D) *ystA* 2 pmol



Sample	Color	Ct	Tm (°C)
myfA 10 pmol		12.82; 12.87	81.14; 81.20
myfA 2 pmol		13.06; 12.99	81.47
ystA 10 pmol		13.55; 13.50	83.57; 83.60
ystA 2 pmol		13.51; 13.56	84.67;84.69

Figure 6. Amplification and melt curve for *Yersinia enterocolitica*. (A) *myfA* 10 pmol, (B) *myfA* 2 pmol, (c) *ystA* 10 pmol, (D) *ystA* 2 pmol

Table 6. Concentration of *Y. enterocolitica* in food samples

Sample	Primary DNA concentration (colony/g)			
	myfA 10 pmol	*myfA* 2 pmol	*ystA* 10 pmol	*ystA* 2 pmol
Selada Non-Dilusi	2.6×10^{-1}	3.5×10^{-1}	2.1×10^{-1}	2.9×10^{-1}
Selada Dilusi	1.2×10^{-3}	1.4×10^{-3}	7.9×10^{-4}	6.15×10^{-4}
Kol Non-Dilusi	3.01×10^{-1}	5.2×10^{-1}	3.1×10^{-1}	3.9×10^{-1}
Kol Dilusi	1.3×10^{-1}	2.2×10^{-1}	1.2×10^{-1}	1.5×10^{-1}

ability of sensitive, specific, and reproducible analysis in detecting the target bacteria *Yersinia enterocolitica* (Thoerner *et al.* 2003) so the study used real-time PCR method to detect *Yersinia enterocolitica* bacteria in vegetable samples.

This study used primers *myfA* and *ystA Yersinia enterocolitica*, which have amplicons length of 181 bp and 123 bp, respectively. Based on Figure 2, the amplification results with PCR Gradient annealing temperature at 53-62°C showed good results. This shows the presence of Yersinia enterocolitica DNA bands with a size of 181 bp for *myfA* primers and 123 bp for *ystA* primers. If the designed primer is too short, it can cause low specificity, because the primer sequence can attach to the wrong DNA fragment. Too long primers are feared to form dimer primers (Wu *et al.* 2007). The value of %GC is 50%, this value is in accordance with the literature of Wu *et al.* (2007) which states that the percentage for good G and C bases used for general rules as primer design is 40 to 60%. A %GC value that is too high can cause an unfavorable denaturation process and can also cause mispriming of the primer with the DNA template. Conversely, if the %GC value is too low, the AT will be more so that it allows the bonding process between the primer and the DNA fragment to be less good. The Tm value obtained for each ystA forward primer is 58.25°C and the reverse is 56.69°C, this is in accordance with the literature where the difference between forward and reverse primers is a maximum of 3°C (Rahayu *et al.* 2018). One of the influential parameters is the presence or absence of secondary structures in the form of hairpins and dimer primers (self-dimer and cross-dimer). The presence of secondary structure that exceeds the tolerance limit can result in the designed primer not attaching to the target bacterial DNA template. The maximum limit of ΔG value on self-dimer is -6 kcal/mol, cross dimer is -5 kcal/mol, and hairpin is -3 kcal/mol.

The optimal temperature chosen is 60°C, which is characterized by the thickest and brightest single band, indicating that the annealing temperature produces optimal amplification so that it can be used in the real-time PCR process. The temperature of 60°C was chosen based on previous research, which showed that this temperature is ideal for a number of primers in test bacteria (Nurjayadi *et al.* 2019). The selection of this gene is based on the function of the gene, the *myfA* gene shows high homology with the Psa protein, thought to play an important role in enhancing thermoinduction and hemagglutination in *Y. enterocolitica* (Rakin *et al.* 2014).

In the confirmation test of *Yersinia enterocolitica* bacteria using *myfA* and *ystA* primers, the concentration used was 50 ng. In this test, *myfA* and *ystA* primers successfully amplified *Yersinia enterocolitica* DNA because they produced a small Ct value, and the resulting melt curve was one peak. The formation of one peak on the melting curve indicates that only one specific amplicon product is formed. The primers *myfA* and ystA successfully discriminated between target and nontarget bacteria in the specificity test, where the non-target microorganisms included *Yersinia enterocolitica*, which is responsible for food poisoning. This is due to the fact that pure *Yersinia enterocolitica* DNA differs by more than 10 cycles. If the difference is ten cycles, it is considered negative (Jia 2012).

The sensitivity test results show that the lowest concentration of bacterial DNA isolates that can still be detected by myfA and ystA primers is 4.39×10^{-4} ng/µL of DNA with a LOD of 7.024×10^{2} CFU/ml. Based on extrapolation from the standard curve results of each primer, the 10 pmol *myfA* primer with a cut-off value was taken based on the Ct at the lowest concentration, namely at a concentration of 8.78×10^{5} ng/µL with a Ct of 30.71±1 and 1.756×10^{5} ng/µL with a Ct value of 34.45±1 for 2 pmol *myfA*. The *ystA* 10 pmol primer had a cut-off value of 38.77±1 with the lowest concentration of 7.024×10^{7} ng/µL. In the *ystA* 2 pmol primer, the cut-off value was taken as 31.15±1 with the lowest concentration of 8.78×10^{5} ng/ml. The final step involved artificially sampling food and using *myfA* and *ystA* primers to test for the presence of *Yersinia enterocolitica*. In this test, cabbage and lettuce were found to contain *Yersinia enterocolitica* bacteria with *myfA* and *ystA* primers.

The results of the bacterial concentration of food samples (Table 7) in this study are good because they are below the maximum limit standard for microbial contamination in vegetable samples issued by BPOM,

Table 7. Confirmation test results for primary food samples *myfA* and *ystA* of *Yersinia enterocolitica*

Sample	Color	*myfA* 10 pmol		*myfA* 2 pmol		*ystA* 10 pmol		*ystA* 2 pmol	
		Ct	Tm (°C)	Ct	Tm (°C)	Ct	Tm (°C)	Ct	Tm (°C)
NTC		24.09	81.66	26.42	81.50	-	-	-	-
MM+NFW		-	-	-	-	-	-	-	-
Selada ND		14.27	81.31	14.65	81.91	15.11	83.58	14.92	83.94
		14.40	81.30	14.65	81.97	15.17	83.62	15.06	83.89
Selada D		22.37	80.80	23.16	81.35	23.59	83.07	23.93	83.23
		22.42	80.74	23.19	81.11	23.55	83.00	24.04	83.14
Kol ND		14.03	81.43	14.03	81.15	14.53	83.57	14.51	83.91
		13.79	81.5	14.02	81.03	14.50	83.62	14.48	83.94
Kol D		15.10	81.44	15.36	81.29	15.91	83.65	15.92	84.00
		15.13	81.35	15.38	81.15	15.97	83.61	15.94	83.96
(-) Selada		24.18	82.00	26.01	81.50	32.12	75.33; 83.77	29.17	76.89
(-) Kol		24.20	82.24	26.53	81.51	27.09	75.98	29.68	77.01

which is 1×10^4 colonies/g (BPOM 2019). So, it can be said that the artificially contaminated cabbage and lettuce samples are positive for *Y. enterocolitica* bacteria with concentration values below the BPOM standard. Thus, by using real-time PCR technique, this study was able to identify Yersinia enterocolitica bacteria in cabbage and lettuce samples with *myfA* and *ystA* primers quickly, specifically, and sensitively.

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References

Annamalai, T., Venkitanarayanan, K., 2005. Expression of major cold shock proteins and genes by *Yersinia enterocolitica* in synthetic medium and foods. *Journal of Food Protection*. 68, 2454-2458. https://doi.org/10.4315/0362-028X-68.11.2454

Bonardi, S., Bruini, I., D'Incau, M., Van Damme, I., Carniel, E., Brémont, S., Cavallini, P., Tagliabue, S., Brindani, F., 2016. Detection, seroprevalence and antimicrobial resistance of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in pig tonsils in Northern Italy. *International Journal of Food Microbiology*. 235, 125–132. https://doi.org/10.1016/j.ijfoodmicro.2016.07.033

BPOM, 2019. Laporan tahunan pusat data dan informasi obat dan makanan tahun 2019. *Journal of Chemical Information and Modeling*. 53, 1689–1699.

EFSA, ECDC, 2017. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. *EFSA Journal*. 15, e05077. https://doi.org/10.2903/j.efsa.2017.5077

Federici, S., Serrazanetti, D. I., Guerzoni, M. E., Campana, R., Ciandrini, E., Baffone, W., Gianotti, A., 2018. Development of a rapid PCR protocol to detect *Vibrio parahaemolyticus* in clams. *Journal of Food Science and Technology*. 55, 749–759. https://doi.org/10.1007/s13197-017-2986-9

Hassanzadeh, P., Ghasemzadeh Limoee, E., Nouri Gharajalar, S., 2022. Molecular detection, biotyping and serotyping of *Yersinia enterocolitica* isolated from chicken livers in Tabriz. *Comparative Immunology, Microbiology and Infectious Diseases*, 83, 101777. https://doi.org/10.1016/j.cimid.2022.101777

Iriarte, M., Vanooteghem, J.C., Delor, I., Diaz, R., Knutton, S., Cornelis, G.R., 1993. The Myf fibrillae of *Yersinia enterocolitica*. *Molecular Microbiology*. 9, 507–520. https://doi.org/10.1111/j.1365-2958.1993.tb01712.x

Jia, Y., 2012a. Real-Time PCR. *Methods in Cell Biology*. 112, 55-68. https://doi.org/10.1016/B978-0-12-405914-6.00003-2

Luo, G., Zhang, J., Zhang, S., Hu, B., Hu, L., & Huang, Z., 2021. High-quality RT-PCR with chemically modified RNA controls. *Talanta*. 224, 121850. https://doi.org/10.1016/j.talanta.2020.121850

Mirriam, E.N., Rol, N, N., 2013. The challenges of foodborne pathogens and antimicrobial chemotherapy: a global perspective. *African Journal of Microbiology Research*. 7, 1158–1172. https://doi.org/10.5897/ajmrx12.014

Nurjayadi, M., Pertiwi, Y. P., Islami, N., Azizah, N., Efrianti, U. R., Saamia, V., Wiranatha, I. M., Nastassya, L., El-Enshasye, H.A., 2019. Detection of the *Salmonella typhi* bacteria in contaminated egg using real-time PCR to develop rapid detection of food poisoning bacteria. *Biocatalysis and Agricultural Biotechnology*. 20, 101214. https://doi.org/10.1016/j.bcab.2019.101214

Nurjayadi, M., Putri, G.I., Declan, J.L., Krisdawati, I., Juliansyah, D.A., Azzahra, M., Maulana, I., Kartika, I.R., Kurniadewi, F., Liman, N.K., Fahriza, T., Sukmawati, D., Rahayu, S., Saamia, V., Saputro, D.A.O., Wiranatha, I.M., Enshasy, H.E., 2024. Optimal annealing temperature of *Yersinia enterocolitica ymoA* gene primers using the polymerase chain reaction method. *AIP Conf. Proc.* 2982, 040018. https://doi.org/10.1063/5.0183856

Nybo, K., 2011. qPCR: avoiding signals in the no-template control. *BioTechniques*. 50, 213–215. https://doi.org/10.2144/000113648

Piras, F., Spanu, C., Sanna, R., Siddi, G., Mocci, A.M., Demontis, M., Meloni, M.P., Spanu, V., De Santis, E.P.L., Scarano, C., 2021. Detection, virulence genes and antimicrobial resistance of *Yersinia enterocolitica* in sheep and goat raw milk. *International Dairy Journal*. 117, 105011. https://doi.org/10.1016/j.idairyj.2021.105011

Rahayu, W.P., Nurjanah, S., Komalasari, E., 2018. *Escherichia coli*: patogenitas, analisis, dan kajian risiko. *Journal of Chemical Information and ModelingInformation and Modeling*. 53, 5.

Rakin, A., Garzetti, D., Bouabe, H., Sprague, L.D., 2014. *Yersinia enterocolitica*. In: Tang YW, Sussman M, Liu D, Poxton I, Schwartzman J. *Molecular Medical Microbiology, Second Edition*. Academic Press: Boston. pp. 1319-1344. https://doi.org/10.1016/B978-0-12-397169-2.00073-1

Saraka, D., Savin, C., Kouassi, S., Cissé, B., Koffi, E., Cabanel, N., Brémont, S., Faye-Kette, H., Dosso, M., Carniel, E., 2017. *Yersinia enterocolitica*, a neglected cause of human enteric infections in côte d'Ivoire. *PLOS Neglected Tropical Diseases*. 11, e0005216. https://doi.org/10.1371/journal.pntd.0005216

Thoerner, P., Bin Kingombe, C.I., Bögli-Stuber, K., Bissig-Choisat, B., Wassenaar, T.M., Frey, J., Jemmi, T., 2003. PCR detection of virulence genes in *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* and investigation of *Virulence* gene distribution. *Applied and Environmental Microbiology*. 69, 1810–1816. https://doi.org/10.1128/AEM.69.3.1810-1816.2003

[WHO] World Health Statistic Report 2015. World Health Organization. World Health Organization. Available at: https://www.who.int/news-room/fact-sheets/detail/food-safety. [Date accessed: 24 October 2023]

Widyastuti, D.A., Nurdyansyah, F., 2017. Deteksi molekuler mikroorganisme patogen pada bahan pangan dengan metode RT-PCR. *Jurnal Ilmu Pangan Dan Hasil Pertanian*. 1, 54–63. https://doi.org/10.26877/jiphp.v1i1.1356

Wu, L.C., Horng, J.T., Huang, H.Y., Lin, F.M., Huang, H.Da, Tsai, M.F., 2007. Primer design for multiplex PCR using a genetic algorithm. *Soft Computing*. 11, 855–863. https://doi.org/10.1007/s00500-006-0137-8