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SINGLE AND PARALLEL DYE-BASED REAL-TIME PCR DETECTION OF FOODBORNE PATHOGENS *SALMONELLA ENTERICA* AND *CAMPYLOBACTER JEJUNI*

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ABSTRACT

Salmonella enterica and *Campylobacter jejuni* are some of the common foodborne pathogens causing gastrointestinal illnesses worldwide. The development of sensitive and specific detection methods is essential to ensure food safety. Dye-based real-time PCR assay using SYBR™ GreenER™ dye was developed for the detection of *Salmonella enterica* and *Campylobacter jejuni*. Designed primer sets specifically targeting the genes *ompF* and *omp50* in *Salmonella* and *Campylobacter*, respectively, were utilised in the study. The assay was able to detect *Salmonella* and *Campylobacter* at as low as 50 fg/µl and 10 fg/µl, respectively. Specificity analysis performed using 16 different bacterial strains to check for cross-reactivity with the respective bacteria found the assay to be specific to *Salmonella* and *Campylobacter*. The assay successfully detected *Salmonella enterica* in inoculated food at as low as 5 fg/reaction for some food samples. Meanwhile, the detection limit for *Campylobacter jejuni* in all inoculated food samples was 2000 fg/reaction. The coefficient variations (CV%) of the assays for both pathogens indicated that the assays were highly reproducible. Therefore, the developed real-time PCR assays for both *Salmonella enterica* and *Campylobacter jejuni* detections were specific and sensitive and can be used for rapid screening to detect these foodborne pathogens.

1.Introduction

Foodborne pathogens, such as bacteria, viruses and parasites, can cause gastrointestinal illnesses in humans. Factors such as globalization, population movement, and supply chain can introduce pathogens to different regions causing emerging infections. Approximately 600 million people, which is 1 in 10 people worldwide, have foodborne illnesses due to the consumption of contaminated food, and 420,000 people die from it annually (World Health Organization, 2022). Most foodborne illnesses are acute, with an infected person typically showing symptoms lasting four to seven days. The most common symptoms include fever, diarrhoea, headache, abdominal cramps, nausea, and vomiting. The disease is generally self-limiting, and most do not require medical treatment to recover. However, some foodborne diseases, such as gastroenteritis, may

lead to severe complications, thus, requiring medical attention, and untreated foodborne infections can lead to death, especially in immunocompromised patients and older adults. Hence, the disease can still burden public health in developed and developing countries.

The typical route for most foodborne illnesses in humans is through the consumption of contaminated food and water, also known as faecal-oral transmission. Other transmission routes include contact with an infected person or animals through zoonosis and via contaminated surfaces (North Dakota Department of Health, 2020). Fresh produce, such as fruits and vegetables, has a higher risk of contamination by foodborne pathogens due to the many access points for contamination from pre-harvesting, harvesting, packaging, and food preparation processes. Poor quality of irrigated water can easily transmit pathogens to fresh produce. Moreover, the transmission of foodborne pathogens can occur during the production, distribution, handling, and cooking of raw meat, such as poultry and beef (Bosch *et al.*, 2011).

Some of the most common foodborne pathogens include *Salmonella* and *Campylobacter* (North Dakota Department of Health, 2020). *Salmonella* is a Gram-negative and flagellated anaerobic bacterium. It causes gastroenteritis called salmonellosis, whereby the gastrointestinal tract is infected with *Salmonella* bacteria. Symptoms caused by *Salmonella* infection begin six hours to six days after the infection and can last up to four days to one week (Centers for Disease Control and Prevention, 2013). These symptoms include diarrhoea, fever, abdominal cramps, and nausea. *Salmonella enterica* (*S. enterica*) is the prominent cause of foodborne illness around the world (Silbergleit *et al.*, 2020). Approximately 93.8 million cases of *Salmonella* were reported every year worldwide, with 155,000 mortalities, of which 80.3 million cases were foodborne (Ao *et al.*, 2015; Majowicz *et al.*, 2010). Among those, 17 to 33 million cases are *Salmonella*-caused typhoid fever and diarrhoeal diseases, with 600,000 mortalities annually (Ranjbar *et al.*, 2014). The detection of *Salmonella* species is crucial, as the method must be able to detect the specific serovars, to prevent infection by the pathogen in food which could lead to illnesses and, if not controlled, an outbreak.

Campylobacter is Gram-negative, spiral-shaped, and can move in a corkscrew-like motion (Chon *et al.*, 2020; Frasao *et al.*, 2017). Gastroenteritis caused by *Campylobacter* bacteria is known as campylobacteriosis, which causes diarrhoea that can turn severe, fever, abdominal cramps and vomiting (Centers for Disease Control and Prevention, 2019; Same *et al.*, 2018). These symptoms typically begin two to five days after infection and can last up to one week (Centers for Disease Control and Prevention, 2019). *Campylobacter* infection is additionally a common antecedent of Guillain-Barré syndrome, an autoimmune disorder of the peripheral nervous system and is known to mainly cause acute flaccid paralysis (Centers for Disease Control and Prevention, 2022; Rees *et al.*, 1995; Sejvar *et al.*, 2011). *Campylobacter* infections that lead to Guillain-Barré syndrome have shown a slower recovery rate, degeneration of axons, and severe residual disability (Rees *et al.*, 1995). *Campylobacter* has also been reported as one of the etiological factors of Crohn's disease and ulcerative colitis in humans (Moore *et al.*, 2005). Moreover, other clinical manifestations of the infection include Bell's palsy, Miller Fisher syndrome, reactive arthritis and acute febrile disease. *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*) are the most common strains to cause campylobacteriosis in humans (Frasao *et al.*, 2017). The Centers for Disease Control and Prevention (CDC) has reported that *C. jejuni* is the second biggest cause of foodborne illnesses, with more than 1.5 million cases reported in the United States every year (Osaili and Alaboudi, 2016; Tack *et al.*, 2019). Approximately 9% of gastroenteritis was caused by *Campylobacter* spp., with 15% of the cases leading to hospitalisations (Scallan *et al.*, 2011).

The growing concern around outbreaks of foodborne illnesses demanded better control of food safety, including food handling, processing, storage and packaging. This highlighted the need to analyse food to ensure its safety and quality (Salihah *et al.*, 2016).

Molecular-based techniques such as polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) have made it easier for microbiologists to identify and detect foodborne pathogens (Saravanan *et al*., 2021). ELISA is a highly specific immunological approach for detecting foodborne pathogens (Saravanan *et al*., 2021); however, ELISA has low sensitivity, and cross-reaction between the antibodies and closely related antigens may occur, leading to false results (Law *et al*., 2015). PCR is a widely used method mediated by specific primers and requires thermostable DNA polymerase to amplify the target DNA sequence *in vitro.* Conventional PCR is a qualitative approach requiring gel electrophoresis to detect the presence of the PCR products (Kadri, 2019). Real-time PCR is the advanced variation of PCR whereby the target DNA sequence is amplified in real-time at the end of every amplification cycle. Among the molecular detection methods, real-time PCR is rapid and has higher sensitivity due to its ability to detect very low concentrations of the target sample. Thus, it has become the new "gold standard" for detecting and quantifying foodborne pathogens in food and microbial population studies with absolute and relative quantification (Malorny *et al*., 2008).

In comparison to conventional PCR, real-time PCR can be used as a one-step method without needing post-PCR analysis to determine the presence of the PCR products. Thus, real-time PCR is less labour-intensive and less prone to contamination due to the reduced number of steps. Unsurprisingly, the use of real-time PCR for *Salmonella* detection is favoured due to its accuracy, rapidity and sensitivity (Rodríguez-Lázaro *et al*., 2003) and has been utilised to detect and quantify *Salmonella* spp. from sheep faeces and tissue samples with 91% sensitivity and 100% specificity (Parker *et al*., 2020). Similarly, the detection of *Campylobacter* by real-time PCR has been reported to produce rapid and sensitive results compared to conventional culture methods (Sails *et al*., 2003; Vencia *et al*., 2014; Yang *et al*., 2003). Methods employing biosensors have facilitated the speedy detection of foodborne pathogens. However, the biosensor's practicality is dependent on the samples' condition, and the non-interaction of the target with the bioreceptor may contribute to inaccurate data and findings (Saravanan *et al*., 2021). Being broadly applied for pathogen detection, real-time PCR has been chosen as the detection method in this study. Furthermore, real-time PCR is relatively more straightforward than other detection methods, and it serves a central role in DNA amplification, making it a crucial application in pathogen detection.

The purpose of this study was to develop rapid and sensitive real-time PCR assay methods for the detection of the two most common foodborne pathogens found in Brunei Darussalam, *S. enterica* and *C. jejuni*, using novel designed primers that specifically identify targeted genes of the pathogens. The majority of the developed *Salmonella* PCR assays amplify virulence genes like invasion gene (*inv*), fimbriae Y protein gene (*fimY*), and type-1 fimbrial protein subunit A gene (*fimA*) (Azinheiro *et al*., 2020; Wang *et al*., 2018; Zhai *et al*., 2014). Target genes for *Campylobacter* PCR assays include 16S ribosomal RNA (*16S* rRNA), hippuricase (*hipO*), flagellin (*flaA*), and elongation factor G (*fusA*) (Hong *et al*., 2007; Perelle *et al*., 2004; Reis *et al*., 2018). To the best of found knowledge, only a number of studies have investigated the use of outer membrane protein F gene (*ompF*) and 50kDa outer membrane protein gene (*omp50*), which are the target genes in this study, for the detection of *S. enterica* and *C. jejuni*, respectively. The assays were developed in such a way that both assays employed the same temperature protocol; hence, the detection of the two bacterial species could be conducted simultaneously in a single PCR run or it can be performed separately. Assessment of assays' performance demonstrated better sensitivity and applicability for *Salmonella* and *Campylobacter* detection in foods.

2. Materials and methods

2.1. Bacterial strains and bacterial culture

The genomic DNA (gDNA) of *S. enterica*, *C. jejuni* and 15 other bacterial strains were

obtained from American Type Culture Collection (ATCC, USA) and are listed in Table 1. The strains were stored at -80 °C and aliquoted to tubes to be used as working stock and kept at -30 °C. All strains were tested with gel electrophoresis to check the quality of the gDNA using 0.8% agarose gel and electrophoresed at 90 V for 45 min. Quick-load 2-log DNA ladder (New Englands Biolabs, USA) was used to determine the molecular weight of the gDNA. NanoPhotometer™ P-Class (Implen, Germany) was used to measure the concentration and purity of the gDNA of all bacterial strains. To prepare artificially contaminated food samples, *S. enterica* subsp. enterica serovar Paratyphi ATCC strain number 9150D-5 bacterial cultures in blood agar (BA) plates and *C. jejuni* AS-84-79 ATCC strain number 33292D-5 bacterial cultures in Campylobacter Selective agar (CAMPY) plates were received from Microbiology Lab, Department of Laboratory Services, Ministry of Health, Brunei. The bacteria colony was cultured in buffered peptone water (BPW) and incubated at 37 °C for 24 hours.

Table 1. List of bacterial strains used in this study

Bacterial Strains	ATCC strain number
Salmonella enterica subsp. enterica serovar Paratyphi	ATCC 9150D-5
Campylobacter jejuni AS-84-79	ATCC 33292D-5
Streptococcus pyogenes group A	ATCC 19615D-5
Yersinia enterocolitica subsp. enterocolitica	ATCC 27739D-5
Escherichia coli H10407	ATCC 35401D-5
Bacillus subtilis 168	ATCC 23857D-5
Shigella flexneri type 2 24570	ATCC 29903D-5
Clostridium perfringens	ATCC 13124D-5
Aeromonas hydrophila CDC 359-60	ATCC 7966D-5
Plesiomonas shigelloides	ATCC 51903D
Mycobacteria avium K-10	ATCC BAA-968D-5
Cronobacter sakazakii 2001-10-01	ATCC BAA-894D-5
Escherichia coli FDA Seattle 1946	ATCC 25922D-5
Staphylococcus epidermidis	ATCC 12228D-5
Pseudomonas aeruginosa Boston 41501	ATCC 27853D-5
Legionella pneumophila Philadelphia-1	ATCC 33152D-5
Bacillus subtilis subsp. spizizenii	ATCC 6633D-5

Table 2. List of primer pairs designed for the detection of *S. enterica* and *C. jejuni*

Bacteria species	Target gene	Primer Sequences (5' – 3')	Location	Amplicon length (bp)
S. enterica	*ompF*	F: CAACGACCGGCGATAGTAAA R: ATTCCCACTGACCGAAACC	137 – 157 223 – 242	105
C. jejuni	*omp50*	F: GTAGGCGGACGCTATGATTT R: GTTGATACTTGGACGGCTCATA	1143 – 1163 1220 – 1242	99

2.2. Primer design of *S. enterica* and *C. jejuni*

The *ompF* and *omp50* genes in *S. enterica* (Accession number CP035301.1) and *C. jejuni* (Accession number AJ582064.1), respectively, were targeted, and primer pairs were designed accordingly (Tatavarthy and Cannons, 2010; Dedieu *et al*., 2004). DNA sequences of the target genes were derived from Standard Nucleotide Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The

primer pairs were designed using PrimerQuest Tool at Integrated DNA Technologies (IDT PTE, Singapore) website : (https://www.idtdna.com/Primerquest/Home/Index). *In silico* PCR amplification was performed to confirm the specificity and compatibility of the designed primers for *Salmonella* and *Campylobacter* detection : (http://insilico.ehu.es/PCR/).

Designed primer sets for *S. enterica* and *C. jejuni* presented in Table 2 were purchased from SBS Genetech Co., Ltd. (Beijing, China) and Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China), respectively. Lyophilised primers were then suspended in Tris-EDTA (TE) buffer (10 mM, pH 8, 1 mM) at their respective amounts.

2.3. Real-time PCR protocol

For each real-time PCR reaction, a total volume of 20 µl containing 10 µl of 2× SYBR™ Select Master Mix (Applied Biosystems, Thermo Fisher Scientific), 0.5 µl of 10 µM forward and reverse primers, 5 µl nuclease-free water and 4 µl DNA template was set up. The real-time PCR assay was performed on Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, USA), with the following thermal cycling conditions: 50 °C for 5 min, followed by 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The melt curve was set after amplification at 95 °C for 5 s, 60 °C for 1 min, followed by 95 °C for 30 s and finally 60 °C for 15 s. All real-time PCR runs include nuclease-free Ultrapure MilliQ water as negative control and *S. enterica* subsp. enterica serovar Paratyphi ATCC 9150D-5 and *C. jejuni* AS-84-79 ATCC 33292D-5 as the positive control for *S. enterica* and *C. jejuni* detection, respectively.

2.4. Specificity analysis

The specificity analysis of the real-time PCR assay was performed against 1×10^2 pg/µl of the bacterial strains (Table 1). The PCR products were validated and confirmed by electrophoresis in 2% agarose gel stained with 1.5 µl FloroSafe DNA stain (1st BASE, Singapore). The gel was electrophoresed in TBE buffer at 90 V for 45 to 50 min and subsequently visualised under UV light to obtain the gel image.

2.5. Sensitivity analysis

The gDNA of *S. enterica* subsp. enterica serovar Paratyphi ATCC 9150D-5 and *C. jejuni* AS-84-79 were diluted to 10-fold serial dilutions from 1×10^6 fg/µl to 1 fg/µl with TE buffer. Serial dilutions were amplified in triplicates in an assay following the real-time PCR protocol for subsequent determination of the limit of detection (LOD). Data obtained from the amplification plot was subsequently used to plot the standard curve, which will be used to further validate the real-time PCR assay.

2.6. Validation of real-time PCR assay

Standard curves were plotted, and the R^2 value and PCR efficiency were determined to validate the real-time PCR assays. The standard curves were plotted using the 10-fold serial dilutions of respective gDNA strains, *S. enterica* and *C. jejuni*, ranging from 1×10^6 fg/µl to 1 fg/µl. PCR efficiency (E) was calculated using the following equation:

$$E = \left(10^{(-1/gradient)} - 1\right) \times 100\% \quad (1)$$

where the gradient is the slope of the curve.

To determine the reproducibility of the assay, the intra- and inter-assay coefficient variations (CV%) were calculated by dividing the analysed sample's standard deviation by its average.

2.7. Preparation of artificially contaminated food samples

Bacterial cell cultures of *S. enterica* subsp. enterica serovar Paratyphi on BA agar plate and *C. jejuni* on CAMPY agar plate were obtained from Microbiology Laboratory, Department of Laboratory Services, Ministry of Health, Brunei. The serial dilutions of *Salmonella* and *Campylobacter* culture were performed by pre-enrichment to obtain the 10-fold serial dilution suspensions, which were then incubated at 37 °C for 24 h. The concentration range of serial dilutions was made 1 to 1×10^6 cells/ml in which one cell of *S. enterica* and *C. jejuni* was equivalent to approximately 5 fg and 2 fg of DNA, respectively. Food samples were diced and autoclaved at 121 ℃ for 15 min. 22.5 ml

BPW was added to 2.5g of the autoclaved food sample to yield a dilution of 1:10 (w/v), and 500 µl of the diluted culture was inoculated to the sample. After incubation for 24 h at 37 °C, 1 ml of the sample was heat-treated at 100 °C for 10 min, followed by DNA extraction using Purelink™ Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher Scientific, USA).

2.8. Detection of *S. enterica* and *C. jejuni* in artificially contaminated food

Following DNA extraction, 4 µl of the extracted DNA was used as the template for the real-time PCR assay. Autoclaved sterile food samples without artificially inoculated bacterial culture and nuclease-free Ultrapure MilliQ water were used as negative controls. The detection of pathogens in artificially contaminated food was performed in triplicates using the real-time PCR protocol.

3. Results and discussions

3.1. Specificity analysis and primer designing

Primer sets that target the *ompF* and *omp50* genes of *Salmonella* and *Campylobacter*, respectively, were confirmed to be homologous to *S. enterica* and *C. jejuni* via *in silico* PCR amplification. As shown in Table 3 and the amplification plot in Figure 1, *in vitro* analysis of the primer sets specificity revealed no cross-

Table 3. Specificity analysis of *S. enterica* and *C. jejuni*

Bacterial strains	**Cross-reactivity analysis[a]**	**Cross-reactivity analysis[b]**
Salmonella enterica subsp. enterica serovar Paratyphi	+	-
Campylobacter jejuni AS-84-79	-	+
Streptococcus pyogenes Group A	-	-
Yersinia enterocolitica subsp. enterocolitica	-	-
Escherichia coli H10407	-	-
Bacillus subtilis 168	-	-
Shigella flexneri type 2 24570	-	-
Clostridium perfringens	-	-
Aeromonas hydrophila CDC 359-60	-	-
Plesiomonas shigelloides	-	-
Mycobacterium avium K-10	-	-
Enterobacter sakazakii 2001-10-01	-	-
Escherichia coli FDA Seattle 1946	-	-
Staphylococcus epidermidis	-	-
Pseudomonas aeruginosa Boston 41501	-	-
Legionella pneumophila philadelphia-1	-	-
Bacillus subtilis subsp. Spizizenii	-	-

[a] – Cross-reactivity for *S. enterica* detection, [b] – Cross-reactivity for *C. jejuni* detection, + showing positive amplification, - showing negative amplification

reactivity with non-target bacterial species. The specificity assay was performed in triplicates and further confirmed using 2% agarose gel electrophoresis, which shows positive bands for the target bacterial strains and no bands for the non-target strains. By allowing substrates to cross the membrane, *ompF* contributes to *Salmonella*'s physiology (Abd El Tawab *et al*., 2016; Elkenany *et al*., 2019). Studies on the *ompF* gene found that the gene is not identified in other prevalent foodborne pathogens such as *Vibrio* spp., *Listeria* spp., *Staphylococcus* spp., *Bacillus* spp., *Shigella* spp., *Escherichia coli*, and *Campylobacter* spp. (Abd El Tawab *et al*., 2016; Tatavarthy and Cannons, 2010). Similarly, the *omp50* gene was discovered to be

Campylobacter-specific (Dedieu *et al*., 2004; Xia *et al*., 2013). As the genes are specific to the targeted pathogen species, one might argue that the specificity of the P2ompF and Pomp50 assays to detect *S. enterica* and *C. jejuni*, respectively, is expected to be high.

3.2. Sensitivity analysis and limit of detection

Sensitivity analysis using different concentrations of the pathogens' gDNA from 1×10^6 fg/µl to 1 fg/µl was performed in triplicate, with three replicate measurements taken for each serial dilution (Table 4). LOD is

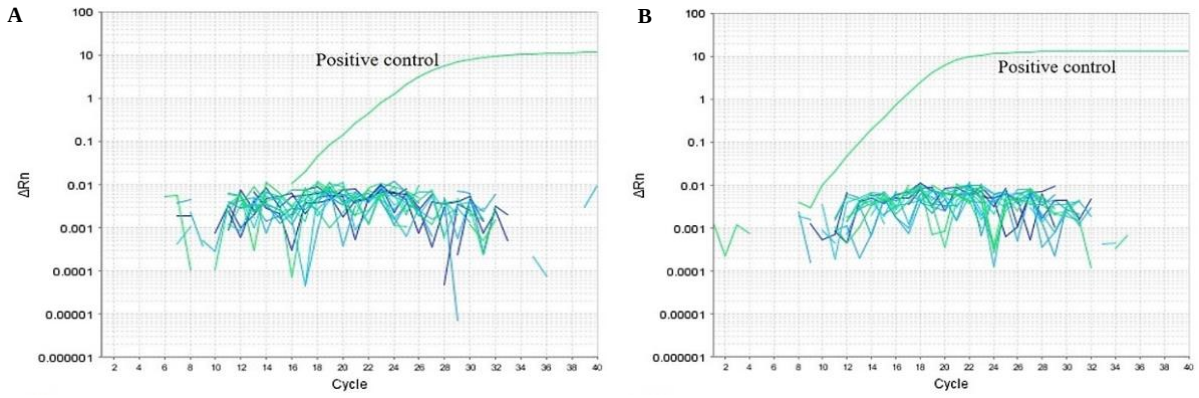

Figure 1. Amplification plot for specificity analysis. A – P2ompF assay. B – Pomp50 assay

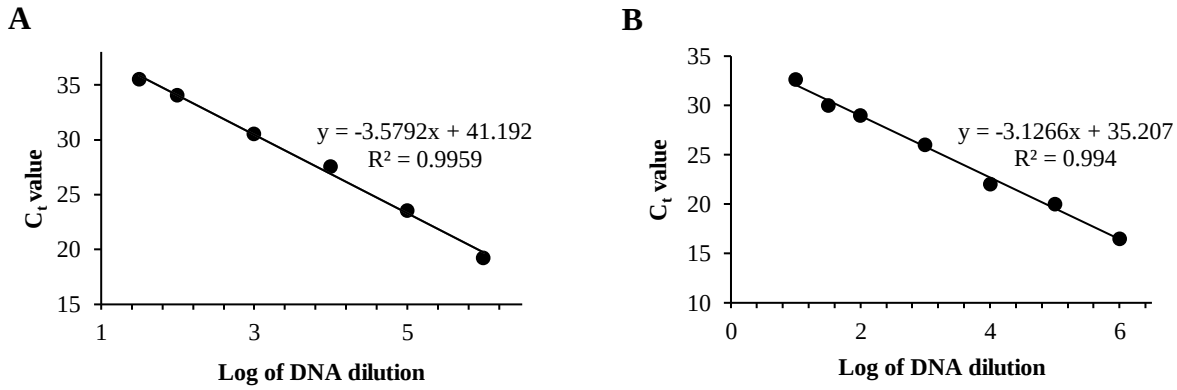

Figure 2. Standard curves of the real-time PCR assays. A – P2ompF assay. B – Pomp50 assay

the lowest analyte concentration at which positive amplification can be obtained with at least a 95% confidence level (US FDA, 2020). The LOD of the P2ompF assay for *S. enterica* was 50 fg/µl. Compared to previous research that targets the virulence genes, the limit of the P2ompF assay is lower. Azinheiro *et al*. (2020), Wang *et al*. (2018), and Zhai *et al*. (2014), reported a limit of 1.6 pg/µl, 10^{-3} ng/µl, and 1.87 pg/µl *Salmonella* gDNA, respectively. The Pomp50 primer set used in this study successfully detected as little as 10 fg/µl of *C. jejuni*, whereas the primers utilised by Reis *et al*. (2018) to target the *fusA* gene of *C. jejuni* achieved an analytical sensitivity of 200 fg.

3.3. Validation of real-time PCR assay

The performance of the assay was validated by evaluating the amplification plot and the standard curve (Figure 2). The standard curve was generated by plotting the 10-fold serial dilutions of the gDNA of each bacterial strain, *S. enterica* and *C. jejuni*. The PCR assay for *S. enterica* detection achieved a PCR efficiency of 91% and an R^2 of 0.9959 (Table 5). The intra- and inter-assay coefficient variations (CV%) were calculated as 0.784 ± 0.194 and 0.988 ±

0.243, respectively. On the other hand, the PCR assay for *C. jejuni* showed an efficiency of 104% and an R^2 of 0.994 with intra- and inter-assay coefficient variations of 0.639 ± 0.139 and 0.658 ± 0.139, respectively. The assays' efficiency and R^2 value were within the recommended range of E = 90 to 110% and $R^2 > 0.99$ (Johnson *et al*., 2013), demonstrating that the designed primer sets were of high quality. Furthermore, as the intra- and inter-assay coefficient variations (CV%) were less than 1%, the developed PCR assays were indicated to be highly reproducible and repeatable (Pfaffl, 2004).

Table 4. Sensitivity analysis of *Salmonella* and *Campylobacter* real-time PCR assay, ratio of positive amplification, copy number and LOD

Bacteria	Assay	Concentration[a] [fg/µl]	Ratio of positive amplification[b]	Copy number[c]	LOD[d] [fg/µl]
S. enterica	P2ompF	1×10^6	6/6	4.41×10^5	50
		1×10^5	9/9		
		1×10^4	9/9		
		1×10^3	9/9		
		1×10^2	9/9		
		10	4/9		
		1	0/9		
C. jejuni	Pomp50	1×10^6	6/6	9.36×10^4	10
		1×10^5	9/9		
		1×10^4	9/9		
		1×10^3	9/9		
		1×10^2	9/9		
		10	9/9		
		1	4/9		

[a] – serial dilutions of respective *S. enterica* and *C. jejuni* in fg/µl, [b] – ratio of positive amplification per 3 individual reactions, [c] – copy number per bacterial cell, [d] – limit of detection of assay

Table 5. PCR efficiency, R^2 value and intra- and inter-assay coefficient variations (CV%) for P2ompF and Pomp50 assays

Bacteria	Assay	Concentration[a] [fg/µl]	R^2 value	PCR Efficiency (E)	Mean CV% ± SD[b]	
					Intra-assay	Inter-assay
S. enterica	P2ompF	1×10^6	0.9959	91%	0.784 ± 0.194	0.988 ± 0.243
		1×10^5				
		1×10^4				
		1×10^3				
		1×10^2				
		10				
		1				
C. jejuni	Pomp50	1×10^6	0.994	104 %	0.639 ± 0.139	0.658 ± 0.139
		1×10^5				
		1×10^4				
		1×10^3				
		1×10^2				
		10				
		1				

[a] – serial dilutions of *S. enterica* and *C. jejuni* in fg/µl, [b] – standard deviation

Table 6. The LOD of *Salmonella enterica* in artificially contaminated food samples

Food sample	Concentration[a] [fg/reaction]	Ratio of positive amplification[b]	LOD[c] [fg/reaction]
Lamb	5×10^6	3/3	5
	5×10^5	3/3	
	5×10^4	3/3	
	5×10^3	3/3	
	5×10^2	3/3	
	50	3/3	
	5	3/3	
Fish	5×10^6	3/3	5
	5×10^5	3/3	
	5×10^4	3/3	
	5×10^3	3/3	
	5×10^2	3/3	
	50	3/3	
	5	3/3	
Chicken	5×10^6	3/3	5
	5×10^5	3/3	
	5×10^4	3/3	
	5×10^3	3/3	
	5×10^2	3/3	
	50	3/3	
	5	3/3	
Beef	5×10^6	3/3	5
	5×10^5	3/3	
	5×10^4	3/3	
	5×10^3	3/3	
	5×10^2	3/3	
	50	3/3	
	5	3/3	
Pork	5×10^6	3/3	50
	5×10^5	3/3	
	5×10^4	3/3	
	5×10^3	3/3	
	5×10^2	3/3	
	50	3/3	
	5	0/3	
Processed Fish	5×10^6	3/3	500
	5×10^5	3/3	
	5×10^4	3/3	
	5×10^3	3/3	
	5×10^2	3/3	
	50	1/3	
	5	0/3	

[a] – approximate quantity of gDNA of *S. enterica* subsp. Enterica serovar Paratyphi ATCC 9150D-5 in fg/reaction, [b] – ratio of positive amplification per 3 individual reactions, [c] – limit of detection of the assay

Table 7. The LOD of *Campylobacter jejuni* in artificially contaminated food samples

Food sample	Concentration[a] [fg/reaction]	Ratio of positive amplification[b]	LOD[c] [fg/reaction]
Lamb	2×10^6	3/3	2000
	2×10^5	3/3	
	2×10^4	3/3	
	2×10^3	3/3	
	2×10^2	1/3	
	20	0/3	
	2	0/3	
Fish	2×10^6	3/3	2000
	2×10^5	3/3	
	2×10^4	3/3	
	2×10^3	3/3	
	2×10^2	0/3	
	20	0/3	
	2	0/3	
Chicken	2×10^6	3/3	2000
	2×10^5	3/3	
	2×10^4	3/3	
	2×10^3	3/3	
	2×10^2	1/3	
	20	0/3	
	2	0/3	
Beef	2×10^6	3/3	2000
	2×10^5	3/3	
	2×10^4	3/3	
	2×10^3	3/3	
	2×10^2	1/3	
	20	0/3	
	2	0/3	
Pork	2×10^6	3/3	2000
	2×10^5	3/3	
	2×10^4	3/3	
	2×10^3	3/3	
	2×10^2	0/3	
	20	0/3	
	2	0/3	
Processed Fish	2×10^6	3/3	2000
	2×10^5	3/3	
	2×10^4	3/3	
	2×10^3	3/3	
	2×10^2	1/3	
	20	0/3	
	2	0/3	

[a] – approximate quantity of gDNA of *C. jejuni* AS-84-79 ATCC 33292D-5 in fg/reaction, [b] – ratio of positive amplification per 3 individual reactions, [c] – limit of detection of assay

3.4. Detection of *S. enterica* and *C. jejuni* in artificially contaminated food

To determine the practicality of the real-time PCR assay to detect *S. enterica* and *C. jejuni* in foods, food samples artificially inoculated with the bacteria were prepared and analysed using the proposed techniques. Detection of *Salmonella* and *Campylobacter* in artificially contaminated lamb, fish, chicken, beef, pork, and processed fish was performed with 4 µl of extracted DNA using respective primer sets. Autoclaved sterile food samples without artificially inoculated bacterial culture and nuclease-free Ultrapure MilliQ water were used as negative controls in which no amplification was observed for both samples. The real-time PCR assay for *S. enterica* reached a detection limit of as low as 5 fg/reaction for lamb, fish, chicken, and beef. However, the LOD for pork and processed fish was 50 fg/reaction and 500 fg/reaction, respectively (Table 6). Meanwhile, the real-time PCR assay for *Campylobacter* detected as low as 2000 fg/reaction in all the inoculated food samples (Table 7).

Taking into account that 5 fg of *S. enterica* gDNA corresponds to one cell, the P2ompF assay could detect 1 cell/ml of *S. enterica* in lamb, fish, chicken, and beef. In contrast, a minimum of 10 cells/ml and 100 cells/ml of *S. enterica* was successfully detected in pork and processed fish, respectively. Considering that one cell of *C. jejuni* is equivalent to approximately 2 fg gDNA, the Pomp50 assay detected 1000 cells/ml of *C. jejuni* in all the inoculated food samples. The detection limits in foods achieved by the assays are comparable to published works. A real-time PCR assay developed by Alves *et al*. (2016) detected 1 CFU/ml of *Salmonella* spp. and *Campylobacter* spp. in spiked chicken meat. Toplak *et al*. (2012) were able to detect 10 to 10000 CFU/ml of *C. jejuni* in spiked tap water and chicken juice. A study on *Salmonella* has reported a limit of detection of 8.5 CFU/ml in inoculated poultry meat (Siala *et al*., 2017). The differences in the detection limits may be attributed to the varying food matrices and inhibitory features of the food type under investigation (Siala *et al*., 2017). According to the findings of this study, the novel primer sets could contribute to enhancing the sensitivity of real-time PCR approaches for the detection of *Salmonella* and *Campylobacter* spp. in food samples.

4. Conclusions

The proposed SYBR™ GreenER™ dye-based real-time PCR assays successfully detected *S. enterica* and *C. jejuni* with 100% specificity and high sensitivity, reproducibility and repeatability. The primer sets target the *ompF* and *omp50* genes, which are known to be inclusive for *Salmonella* and *Campylobacter*, respectively. Application of the assays to various artificially contaminated food samples showed the potential and usefulness of the assay to be implemented in food microbiology laboratories and for food monitoring.

5.References

Abd El Tawab, A. A., Amaar, A. M., El-bialy, A. I., Elsayed, M. M. (2016). A study of outer membrane protein (OMPs) genes for the detection of *salmonella* organisms in poultry farms. *Benha Veterinary Medical Journal*, 30(1), 231–237.

Alves, J., Hirooka, E. Y., de Oliveira, T. C. R. M. (2016). Development of a multiplex real-time PCR assay with an internal amplification control for the detection of *Campylobacter* spp. and *Salmonella* spp. in chicken meat. *LWT - Food Science and Technology*, 72, 175–181.

Ao, T. T., Feasey, N. A., Gordon, M. A., Keddy, K. H., Angulo, F. J., Crump, J. A. (2015). Global burden of invasive nontyphoidal *salmonella* disease, 2010. *Emerging Infectious Diseases*, 21(6), 941-949.

Azinheiro, S., Carvalho, J., Prado, M., Garrido-Maestu, A. (2020). Multiplex detection of *salmonella* spp., *E. coli* O157 and *L. monocytogenes* by qPCR melt curve analysis in spiked infant formula. *Microorganisms*, 8(9), 1359.

Bosch, A., Bidawid, S., Le Guyader, F. S., Lees, D. N., Jaykus, L.-A. (2011). Norovirus and Hepatitis A virus in shellfish, soft fruits and water. In J. Hoorfar (Ed.), Rapid detection, identification, and quantification of

foodborne pathogens. (pp. 466), Washington, D.C.: ASM Press.

Centers for Disease Control and Prevention. (2013). National Enteric Disease Surveillance: *Salmonella* Annual Report, 2011. Retrieved from https://www.cdc.gov/ncezid/dfwed/pdfs/salmonella-annual-report-2011-508c.pdf

Centers for Disease Control and Prevention. (2019). *Campylobacter* (Campylobacteriosis): Questions and Answers. Retrieved from https://www.cdc.gov/campylobacter/faq.html

Centers for Disease Control and Prevention. (2022). *Campylobacter* (Campylobacteriosis): Guillain-Barré syndrome. Retrieved from https://www.cdc.gov/campylobacter/guillain-barre.html

Chon, J.-W., Seo, K.-H., Kim, B., Jeong, D., Song, K.-Y. (2020). Advanced Methods for Isolating from and Confirming *Campylobacter* spp. in Milk and Dairy Products: Review. *Journal of Dairy Science and Biotechnology*, 38(3), 121–133.

Dedieu, L., Pagès, J. M., Bolla, J. M. (2004). Use of the *omp50* Gene for Identification of *Campylobacter* Species by PCR. *Journal of Clinical Microbiology*, 42(5), 2301–2305.

Elkenany, R., Elsayed, M. M., Zakaria, A. I., El-sayed, S. A., Rizk, M. A. (2019). Antimicrobial resistance profiles and virulence genotyping of *Salmonella enterica* serovars recovered from broiler chickens and chicken carcasses in Egypt. *BMC Veterinary Research*, 15, 124.

Frasao, B. da S., Marin, V. A., Conte-Junior, C. A. (2017). Molecular Detection, Typing, and Quantification of *Campylobacter* spp. in Foods of Animal Origin. *Comprehensive Reviews in Food Science and Food Safety*, 16(4), 721–734.

Hong, J., Jung, W. K., Kim, J. M., Kim, S. H., Koo, H. C., Ser, J., Park, Y. H. (2007). Quantification and Differentiation of *Campylobacter jejuni* and *Campylobacter coli* in Raw Chicken Meats Using a Real-Time PCR Method. *Journal of Food Protection*, 70(9), 2015–2022.

Johnson, G., Nolan, T., Bustin, S. A. (2013). Real-time quantitative PCR, pathogen detection and MIQE. *Methods in Molecular Biology*, 943, 1–16.

Kadri, K. (2019). Polymerase Chain Reaction (PCR): Principle and Applications. In M. L. Nagpal, O. Boldura, C. Baltă, S. Enany (Eds.), Synthetic Biology - New Interdisciplinary Science. London, IntechOpen Limited.

Law, J. W.-F., Ab Mutalib, N.-S., Chan, K.-G., Lee, L.-H. (2015). Rapid methods for the detection of foodborne bacterial pathogens: principles, applications, advantages and limitations. *Frontiers in Microbiology*, 5, 770.

Majowicz, S. E., Musto, J., Scallan, E., Angulo, F. J., Kirk, M., O'Brien, S. J., Jones, T. F., Fazil, A., Hoekstra, R. M. (2010). The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clinical Infectious Diseases*, 50(6), 882–889.

Malorny, B., Lofstrom, C., Wagner, M., Kramer, N., Hoorfar, J. (2008). Enumeration of *Salmonella* Bacteria in Food and Feed Samples by Real-Time PCR for Quantitative Microbial Risk Assessment. *Applied and Environmental Microbiology*. 74(5), 1299–1304.

Moore, J. E., Corcoran, D., Dooley, J. S. G., Fanning, S., Lucey, B., Matsuda, M., McDowell, D. A., Mégraud, F., Millar, B. C., O'Mahony, R., O'Riordan, L., O'Rourke, M., Rao, J. R., Rooney, P. J., Sails, A., Whyte, P. (2005). *Campylobacter*. *Veterinary Research*, 36(3), 351–382.

North Dakota Department of Health. (2020). Foodborne and Gastrointestinal Illness. Retrieved from https://www.health.nd.gov/diseases-conditions/foodborne-and-gastrointestinal-illness

Osaili, T. M., & Alaboudi, A. R. (2016). Antimicrobial resistance of *campylobacter* sp. In: O. V. Singh (Ed.), Foodborne Pathogens and Antibiotic Resistance. (pp

417–429), Hoboken, New Jersey: Wiley-Blackwell.

Parker, A. M., Mohler, V. L., Gunn, A. A., House, J. K. (2020). Development of a qPCR for the detection and quantification of *Salmonella* spp. in sheep feces and tissues. *Journal of Veterinary Diagnostic Investigation*, 32(6), 835–843.

Perelle, S., Josefsen, M., Hoorfar, J., Dilasser, F., Grout, J., Fach, P. (2004). A LightCycler real-time PCR hybridization probe assay for detecting food-borne thermophilic *Campylobacter*. *Molecular and Cellular Probes*, 18(5), 321–327.

Pfaffl, M. W. (2004). Quantification strategies in real-time PCR. In S. A. Butin (Ed.), A-Z of quantitative PCR. (pp. 87-112), La Jolla: International University Line. https://www.gene-quantification.de/chapter-3-pfaffl.pdf

Ranjbar, R., Naghoni, A., Farshad, S., Lashini, H., Najafi, A., Sadeghifard, N., Mammina, C. (2014). Use of TaqMan® real-time PCR for rapid detection of *Salmonella enterica* serovar Typhi. *Acta Microbiologica et Immunologica Hungarica*, 61(2), 121–130.

Rees, J. H., Soudain, S. E., Gregson, N. A., Hughes, R. A. (1995). *Campylobacter jejuni* infections and Guillain-Barré syndrome. *The New England Journal of Medicine*, 333(21), 1374–1379.

Reis, L. P., Menezes, L. D. M., Lima, G. K., Santos, E. L. de S., Dorneles, E. M. S., de Assis, D. C. S., Lage, A. P., Cançado, S. de V., de Figueiredo, T. C. (2018). Detection of *Campylobacter* spp. in chilled and frozen broiler carcasses comparing immunoassay, PCR and real time PCR methods. *Ciência Rural*, 48(2), 1–7.

Rodríguez-Lázaro, D., Hernández, M., Esteve, T., Hoorfar, J., Pla, M. (2003). A rapid and direct real time PCR-based method for identification of *Salmonella* spp. *Journal of Microbiological Methods*, 54(3), 381–390.

Sails, A. D., Fox, A. J., Bolton, F. J., Wareing, D. R. A., Greenway, D. L. A. (2003). A real-time PCR assay for the detection of *Campylobacter jejuni* in foods after enrichment culture. *Applied and Environmental Microbiology*, 69(3), 1383–1390.

Salihah, N. T., Hossain, M. M., Lubis, H., Ahmed, M. U. (2016). Trends and advances in food analysis by real-time polymerase chain reaction. *Journal of Food Science and Technology*, 53(5), 2196–2209.

Same, R. G., & Tamma, P. D. (2018). *Campylobacter* Infections in Children. *Pediatrics in Review*, 39(11), 533–541.

Saravanan, A., Kumar, P. S., Hemavathy, R. V., Jeevanantham, S., Kamalesh, R., Sneha, S., Yaashikaa, P. R. (2021). Methods of detection of food-borne pathogens: a review. *Environmental Chemistry Letters*, 19, 189–207.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., Jones, J. L., Griffin, P. M. (2011). Foodborne illness acquired in the United States-Major pathogens. *Emerging Infectious Diseases*, 17(1) 7–15.

Sejvar, J. J., Baughman, A. L., Wise, M., Morgan, O. W. (2011). Population Incidence of Guillain-Barré Syndrome: A Systematic Review and Meta-Analysis. *Neuroepidemiology*, 36(2), 123–133.

Siala, M., Barbana, A., Smaoui, S., Hachicha, S., Marouane, C., Kammoun, S., Gdoura, R., Messadi-Akrout, F. (2017). Screening and Detecting *Salmonella* in Different Food Matrices in Southern Tunisia Using a Combined Enrichment/Real-Time PCR Method: Correlation with Conventional Culture Method. Frontiers in Microbiology, 8, 2416.

Silbergleit, M., Vasquez, A. A., Miller, C. J., Sun, J., Kato, I. (2020). Oral and intestinal bacterial exotoxins: Potential linked to carcinogenesis. In: J. Sun (Ed.), Progress in Molecular Biology and Translational Science. (pp. 131-193), Amsterdam, Netherlands: Elsevier Science.

Tack, D. M., Marder, E. P., Griffin, P. M., Cieslak, P. R., Dunn, J., Hurd, S., Scallan, E., Lathrop, S., Muse, A., Ryan, P., Smith, K., Tobin-D'Angelo, M., Vugia, D. J., Holt, K. G., Wolpert, B. J., Tauxe, R., Geissler, A.

L. (2019). Preliminary incidence and trends of infections with pathogens transmitted commonly through food — Foodborne Diseases Active Surveillance Network, 10 U.S. sites, 2015–2018. *American Journal of Transplantation*, 19(6), 1859–1863.

Tatavarthy, A., & Cannons, A. (2010). Real-time PCR detection of *Salmonella* species using a novel target: The outer membrane porin F gene (*ompF*). *Letters in Applied Microbiology*, 50(6), 645–652.

Toplak, N., Kovač, M., Piskernik, S., Možina, S. S., Jeršek, B. (2012). Detection and quantification of *Campylobacter jejuni* and *Campylobacter coli* using real-time multiplex PCR. *Journal of Appied. Microbiology*, 112(4), 752–764.

US FDA. (2020). Guidelines for the Validation of Analytical Methods for Nucleic Acid Sequence-Based Analysis of Food, Feed, Cosmetics and Veterinary Products.

Vencia, W., Nogarol, C., Bianchi, D. M., Gallina, S., Zuccon, F., Adriano, D., Gramaglia, M., Decastelli, L. (2014). Validation according to ISO 16140:2003 of a commercial real-time PCR-based method for detecting *Campylobacter jejuni*, *C. coli*, and *C. lari* in foods. *International Journal of Food Microbiol*ogy, 177, 78–80.

Wang, M., Yang, J., Gai, Z., Huo, S., Zhu, J., Li, J., Wang, R., Xing, S., Shi, G., Shi, F., Zhang, L. (2018). Comparison between digital PCR and real-time PCR in detection of *Salmonella typhimurium* in milk. International Journal of Food Microbiology, 266, 251–256.

World Health Organization. (2022). Food safety. Retrieved from https://www.who.int/news-room/fact-sheets/detail/food-safety

Xia, Q., Muraoka, W. T., Shen, Z., Sahin, O., Wang, H., Wu, Z., Liu, P., Zhang, Q. (2013). Adaptive mechanisms of *Campylobacter jejuni* to erythromycin treatment. *BMC Microbiology*, 13, 133.

Yang, C., Jiang, Y., Huang, K., Zhu, C., Yin, Y. (2003). Application of real-time PCR for quantitative detection of *Campylobacter jejuni* in poultry, milk and environmental water. *FEMS Immunology and Medical Microbiology*, 38(3), 265–271.

Zhai, L., Kong, X., Lu, Z., Lv, F., Zhang, C., Bie, X. (2014) Detection of *Salmonella enterica* serovar Dublin by polymerase chain reaction in multiplex format. *Journal of Microbiological Methods*, 100, 52–57.

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