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Original article

Multiplex PCR for the detection of *Salmonella* spp. in Indonesian traditional shrimp paste (Terasi)

Affan Gaffar [1], Yoga Dwi Jatmiko [2]*, Asep Awaludin Prihanto [3]

[1]Master Program of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia
[3]Department of Fishery Product Technology, Faculty of Fisheries and Marine Science Universitas Brawijaya, Malang, Indonesia

Abstract

Terasi is a food condiment originating from Indonesia which is processed by fermenting shrimp, fish, or a mixture of both. The processing of terasi in Indonesia is still found traditionally so that it will affect the low quality of terasi which is characterized by being contaminated by pathogenic bacteria such as *Salmonella*. *Salmonella* is a bacterium that causes foodborne diseases. The purpose of this study was to evaluate the presence of *Salmonella* in shrimp paste using conventional method and then confirmed with molecular approach. This study used 10 samples consisting modern terasi and five traditional terasi. The isolation of *Salmonella* spp. carried out by using selective media, and then the isolates were characterized further using biochemical test (TSIA and LIA). The species identification of *Salmonella* spp. was carried out by multiplex PCR (mPCR). The isolates that were not detected by mPCR were further identified using 16s rDNA sequencing. The isolation results showed that *Salmonella* isolates were only detected in traditional terasi with a density range of 2.4 x 10^5-2.9 x 10^8 CFU/g. A total of 20 isolates were characterized biochemically as *Salmonella*, and out of 17 isolates consistently identified as *Salmonella enterica* serovar Typhimurium by using mPCR. The rest three isolates were further identified as *Citrobacter freundii* based on 16s rDNA sequencing with similarity level of 99%. The presence of Salmonella in the shrimp paste indicates that the processing of traditional shrimp paste (terasi) should be evaluated in accordance with good manufacturing process.

Keywords: multiplex PCR, *Salmonella* Typhimurium, terasi, 16s rDNA

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Introduction

Terasi is a fermented product of shrimp, fish, or a mixture of both in the form of shrimp paste. Terasi products are widely liked and spread in several regions in Indonesia. In addition, this product is also spread in several countries, especially Southeast Asia but with a different name. The processing of shrimp paste in each region is the same, but the difference is the treatment and the concentration of added salt. In Indonesia, two types of terasi are found based on processing, namely modern and traditional terasi. Modern terasi processing has been produced on a large scale by factories utilizing technology such as grinding machines, dryers, and packaging to produce good quality shrimp paste. Traditional terasi processing is identical to unhygienic processing. The use of wood as a shrimp paste processing tool is known not to meet the requirements because the wood has pores that allow microorganisms to multiply due to a poor cleaning process. In addition, in the shrimp paste processing process, there are stages of drying, which are generally carried out in the open using a tarpaulin mat placed on a wooden floor. This drying process is susceptible to the entry of foreign objects such as microorganisms that can contaminate and contaminate raw materials and can pose a food safety hazard to consumers (Humairani et al., 2019). According to Ismail and Putra (2016), geographical conditions such as temperature and humidity also affect the length of the shrimp paste drying and fermentation process. The quality of the shrimp paste produced is low.

The Low-quality terasi will be easily contaminated by some pathogenic bacteria, one of which is *Salmonella* bacteria. Arumugaswamy et al. (1995) found Salmonella in belacan (terasi) research results. *Salmonella* is a major cause of food-borne disease. The disease caused by *Salmonella* bacteria is called Salmonellosis. In humans, salmonellosis causes gastroenteritis, bacteremia, and more serious diseases such as typhoid and typhoid fever (Bibi et al., 2015). According to the World Health Organization (WHO), about 153 million cases of non-typhoid enteric *Salmonella* infections worldwide in 2010, 56,969 of them were fatal and about 50% of them were foodborne (Kirk et al., 2015). Data collected by the foodborne disease surveillance system in China from 2006 to 2010, show that *Salmonella* is the second most common bacterium that causes foodborne disease (Pang et al., 2011).

The presence of *Salmonella* spp. in food, especially in terasi, is thought to come from the main ingredient, namely shrimp. According to Mohammed Hatha and Lakshmanaperumalsamy, (1997), that shrimp body parts are contaminated by *Salmonella* spp. namely the surface of the shrimp body, gills, and digestive tract. cephalic parts tend to have more *Salmonella* than other parts of

* Corresponding Author:
Yoga Dwi Jatmiko
Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia
Phone: +62 341-575840 Fax: +62 341-554403
E-mail: jatmiko_yd@ub.ac.id

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the body (Wan Norhana et al., 2010). In addition, according to Carvalho et al. (2013), *Salmonella* spp. can be sourced from shrimp ponds contaminated with sewage. Several previous research results have not identified the *Salmonella* species found in terasi.

Multiplex PCR (mPCR) is a variation of the PCR method that can be used to amplify two or more target sequences simultaneously. The advantages of using the mPCR method are that it is cheaper and saves processing time because it can amplify two or more target sequences simultaneously with one PCR reaction to save on tools and reagents used. (Adikara et al., 2017). Several researchers have succeeded in identifying *Salmonella* using the mPCR method (Alvarez et al., 2004; de Freitas et al., 2010; Lestari et al., 2015; Liu et al., 2012; Malkawi & Gharaibeh, 2003; Park et al., 2009). In this study, the multiplex PCR (mPCR) method was used to identify the genus and serotype of Salmonella isolated from shrimp paste. Therefore, in this study, the identification of *Salmonella* species found in terasi was carried out using the multiplex PCR method.

Methods

Isolation of *Salmonella*

A total of 10 samples of terasi consisted of 2 types of terasi, namely modern made and traditional made. Modern terasi were collected from supermarkets produced by some terasi making companies, while traditional terasi were taken from local markets in Malang City, East Java, Indonesia. The pre-enrichment technique was performed first. A total of 25 g of each terasi sample was weighed, then put into an Erlenmeyer flask containing 225 ml of Buffered Peptone Water (BPW) media. The suspension was homogenized using a vortex, then allowed to stand at room temperature for 1 h. The pH of the suspension was adjusted to reach 6.8. The suspension was incubated at 37°C for 24 h. Bacterial growth is characterized by turbidity and a characteristic odor (National Standardization Agency, 2006). The next step was selective enrichment. A total of 0.1 mL of BPW inoculated with the samples was put into 10 mL of Rappaport Vassiliadis (RVS) media, then incubated at 42 °C for 24 h.

Samples that had been incubated in selective enrichment media (RVS) were taken 1 mL and dissolved into 9 ml of saline solution (NaCl 0.85%) to make a serial dilution from 10^{-1} to 10^{-4}. Then 0.1 mL of each dilution result was taken and poured onto Xylose Lysine Desoxycholate Agar (XLD) Agar, then incubated at 37 °C for 24 h. The suspected colonies as *Salmonella* isolates appeared large and pink colonies with or without the black core in the middle (National Standardization Agency, 2006). The suspected colonies were Gram stained and characterized using biochemical tests including Triple Sugar Iron Agar (TSIA) and Lysin Iron Agar (LIA) tests. The characteristics of *Salmonella* bacteria from the TSIA test results are that the upright part changes color to yellow with or without black color (H_2S), and the oblique part is red (no color change). While the positive results of the LIA test were indicated by a change in color to purple in the entire tube. The isolates selected from the results of biochemical tests were then identified further (Nugroho et al., 2016).

Multiplex PCR analysis

The DNA extraction method used was the heat-treatment method. One loop-full of the isolate was suspended in 200 µL of sterile ddH2O. The suspension was homogenized using a vortex for 1 min, then put into a water bath at 95 °C for 20 min. The suspension was centrifuged for 5 min at 4 °C. The supernatant was taken and transferred into a new microtube and then stored at -20 °C (Mulyawati et al., 2019).

The extracted DNA samples were then identified using mPCR with specific primers based on the serotype, namely ompC (ompCF and ompCR), SdfI (EntF and EntR), Spy (TyphF and TyphR), and ViAB (ViABF and ViABR) (Tabel 1). The mPCR reaction was made with the composition of Go Taq Green 12.5 µL, with each primer reverse forward 0.5 µL, DNA template 5 µL, and added with sterile ddH_2O to reach the final volume of 25 µL. The thermocycler mPCR was programmed with 30 cycles, each one with an initial denaturation at 95 °C for 3 min, denaturation at 95°C for 2 min, annealing at 57 °C for 2.5 min, an extension of 72 °C for 2.5 min, and post extension at 72 °C for 2.5 min. The samples were maintained cooled at 4°C until their withdrawal. The program specifications for Typhimurium serotype were the same, except for the annealing temperature, which was established at 55 °C. All mPCR reactions were visualized at 1.5% (w/v) agarose gel in a UV transilluminator (de Freitas et al., 2010). A positive control used was *Salmonella* Typhimurium. The 16s rDNA sequencing using universal primers (27f and 1492r) was also performed when the isolates were not identified by mPCR.

Table 1. *Salmonella* sp. serotypes, the target gene, sequence and the size of fragments to be amplified adapted from

	Bacteria	Target Gen	Primer	Sequence 5′–3′	Size (bp)	Access Number (GenBank)
Genus	*Salmonella* spp.	ompC	OMPCF	atc gct gac tta tgc aat cg	204	AY341077
			OMPCR	cgg gtt gcg tta tag gtc tg		
Serotipe *Salmonella enterica*	Enteritidis	Sdf I	ENTF	tgt gtt tta tct gat gca aga gg	304	AF370707
			ENTR	tga act acg ttc gtt ctt ctg g		
	Typhi	ViaB	ViaBF	cac gca cca tca ttt cac cg	738	D14156

Bacteria	Target Gen	Primer	Sequence 5′–3′	Size (bp)	Access Number (GenBank)
Typhimurium	Spy	ViaBR	aac agg ctg tag cga ttt agg	401	AE008757
		TyphF	ttg ttc act ttt tac ccc tga a		
		TyphR	ccc tga cag ccg tta gat att		

Results

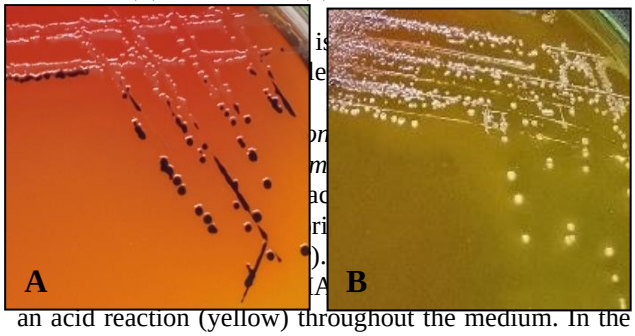
Salmonella in shrimp paste (Terasi)

All samples of traditional terasi contained suspected *Salmonella*, but not in modern made. The shape of the colonies obtained was small round which was marked by a black color in the center of the colony and black in the whole colony, that some colonies were found to be small round yellow in colour without any black color in the center of the colony or the whole colony (Figure 1). The *Salmonella* cell count of each sample was presented in Table 2.

No	Terasi Samples	Isolate Code	Cell number (cfu/gram)
1	Modern A	TMA	Not detected
2	Modern B	TMB	Not detected
3	Modern C	TMC	Not detected
4	Modern D	TMD	Not detected
5	Modern E	TME	Not detected
6	Traditional A	TRA	3.4×10^5
7	Traditional B	TRB	2.4×10^5
8	Traditional C	TRC	2.9×10^5
9	Traditional D	TRD	2.9×10^8
10	Traditional E	TRE	2.7×10^8

Table 2. The cell density of *Salmonella* spp. (CFU/ml) in terasi

Figure 1. The morphological characteristics of *Salmonella* spp. on XLD media: (A) Black colonies, (B) Yellow colonies



an acid reaction (yellow) throughout the medium. In the LIA test, all isolates showed positive results which were indicated by the formation of an alkaline reaction (purple) in the entire tube, but some isolates formed H_2S in the LIA test. The isolates of TRB1, TRB3, TRB4, TRB6, and TRC2 (Table 3) showed negative *Salmonella* results in both TSIA and LIA tests. It is suspected that the isolate was *Escherichia coli* with the criteria for the TSIA slant/depth (yellow/yellow) test. The isolates showing positive results of TSIA and LIA were then further characterized using multiplex PCR (mPCR) to determine the genus and serotype of isolates.

Table 3. Results of gram staining and biochemical tests

No	Isolates	Gram Stain	TSIA Test	LIA test
1	TRA1	Rod(Gram Negative)	K/A	K/K & H_2S
2	TRB1	Rod (Gram Negative)	A/A	K/K
3	TRB2	Rod (Gram Negative)	K/A	K/K
4	TRB3	Rod (Gram Negative)	A/A	K/K
5	TRB4	Rod (Gram Negative)	A/A	K/K
6	TRB5	Rod (Gram Negative)	K/A	K/K
7	TRB6	Rod (Gram Negative)	A/A	K/K
8	TRC1	Rod (Gram Negative)	K/A	K/K
9	TRC2	Rod (Gram Negative)	A/A	K/K
10	TRD1	Rod (Gram Negative)	K/K	K/K & H_2S
11	TRD2	Rod (Gram Negative)	K/K	K/K & H_2S
12	TRD3	Rod (Gram Negative)	K/K	K/K & H_2S
13	TRD4	Rod (Gram Negative)	K/K	K/K & H_2S
14	TRD5	Rod (Gram Negative)	K/K	K/K & H_2S
15	TRD6	Rod (Gram Negative)	K/K	K/K & H_2S
16	TRD7	Rod (Gram Negative)	K/K	K/K & H_2S
17	TRD8	Rod (Gram Negative)	K/K	K/K & H_2S
18	TRD9	Rod (Gram Negative)	K/K	K/K & H_2S
19	TRE1	Rod (Gram Negative)	K/K	K/K & H_2S
20	TRE2	Rod (Gram Negative)	K/K	K/K & H_2S
21	TRE3	Rod (Gram Negative)	K/K	K/K & H_2S
22	TRE4	Rod (Gram Negative)	K/K	K/K & H_2S
23	TRE5	Rod (Gram Negative)	K/K	K/K & H_2S
24	TRE6	Rod (Gram Negative)	K/K	K/K & H_2S
25	TRE7	Rod (Gram Negative)	K/K	K/K & H_2S

Notes: K/A : Alkaline/Acid (red/yellow); K/K : Alkaline (red/red); A/A : Acid/Acid (yellow /yellow); K/K : Alkaline/Alkaline (purple/purple)

Identification of *Salmonella*

Total DNA of 20 isolates that were successfully extracted using the heat treatment method were used as templates in the multiplex PCR (mPCR) reaction. All suspected *Salmonella* isolates succeeded in amplifying the ompC primer by forming a DNA band with a size of 204 bp. The results of DNA bands on the ompC primers showed that all of the isolates belonged to the genus of *Salmonella*. A total of 17 isolates were able to amplify

spy primers by forming a DNA band of 401 bp. The results of DNA bands on the spy primer showed that the isolate was a Typhimurium serotype (Figure 2). In addition, three isolates that could not amplify the specific primer for *Salmonella* serotype were confirmed using 16S rDNA sequences.

PCR results showed that the three isolates could amplify 16S rDNA primers by forming a DNA band length of 1500 bp. The phylogeny tree showed that those three isolates were *Citrobacter* bacteria (Figure 3). TRB2 isolates had similarities with *Citrobacter freundii* 1109-3-F1 (99.9%), while TRB5 and TRC1 isolates had similarities with *Citrobacter freundii*_NR-46 (99.9% and 99.9%).

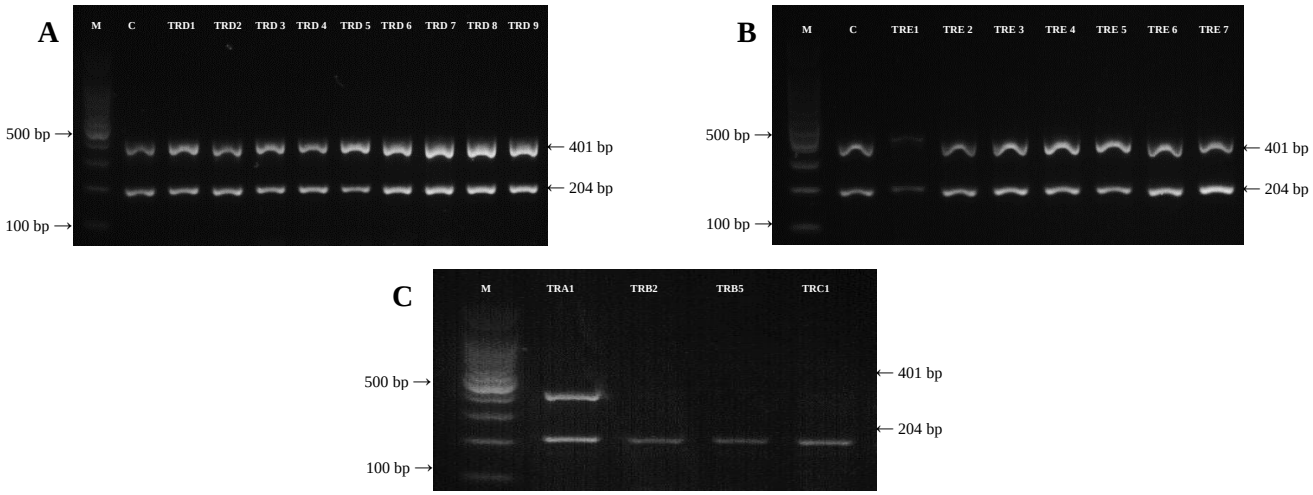


Figure 2. Multiplex PCR for identification of *Salmonella* sp. and Typhimurium serotype from terasi. 2-A:M) DNA Markers; C) Positive Control; TRD1, TRD2, TRD3, TRD4, TRD5, TRD6, TRD7, TRD8, TRD9) mPCR fragment 204 bp is of the genus *Salmonella* sp. and the 401 bp fragment is the Typhimurium serotype. 2-B: :M) DNA Marker; C) Positive Control; TRE1, TRE2, TRE3, TRE4, TRE5, TRE6, TRE7,) mPCR fragment 204 bp of the genus *Salmonella* sp. and the 401 bp fragment is the Typhimurium serotype. 2-C: :M) DNA Marker; TRA1) mPCR fragment 204 bp of the genus *Salmonella* sp. and the 401 bp fragment is the Typhimurium serotype; , TRB2, TRB5, TRC1) 204 bp fragment is the genus *Salmonella* sp.

Discussion

Salmonella is one of the main pathogenic bacteria causing foodborne diseases worldwide. In this study, *Salmonella* was found in traditional terasi products. A total of 10 samples (five traditional and five modern terasi) were examined bacteriologically to isolate *Salmonella* isolates. In this study, all samples of traditional terasi were suspected to be *Salmonella* because colonies growing on XLD media were characteristics of Salmonella colonies. The modern terasi were negative for *Salmonella*, which showed no colonies growing on XLD media. XLD media is a selective medium for *Salmonella* that can suppress the growth of gram-positive bacteria. This medium contains sodium deoxycholate, besides that XLD also contains thiosulfate as an H_2S indicator which shows a typical black *Salmonella* colony. Generally, *Salmonella* cultures form large colonies, shiny black cores or almost all colonies look black, other characteristics are pink colonies with or without black cores. In addition, according to the National Standardization Agency (2006), some *Salmonella* colonies showed unusual morphological characteristics such as forming yellow round colonies with or without a black core. The existence of a positive suspicion of the morphological characteristics of the colony on XLD media was then carried out a confirmatory test consisting of the TSIA and LIA tests.

The positive TSIA test for *Salmonella* was indicated by the formation of yellow color on the upright agar medium and red color on the inclined agar medium with or without H_2S. The yellow color on upright agar media is caused by the reaction of *Salmonella* which can ferment glucose, while the red color on tilted agar media is caused because *Salmonella* is unable to ferment sucrose and lactose in TSIA media (Christanti and Azhar, 2019). The negative results of *Salmonella* in the TSIA test are indicated by the criteria for yellow slant and depth sections, this is because these bacteria can ferment glucose, sucrose, and lactose (Hermono et al., 2017).

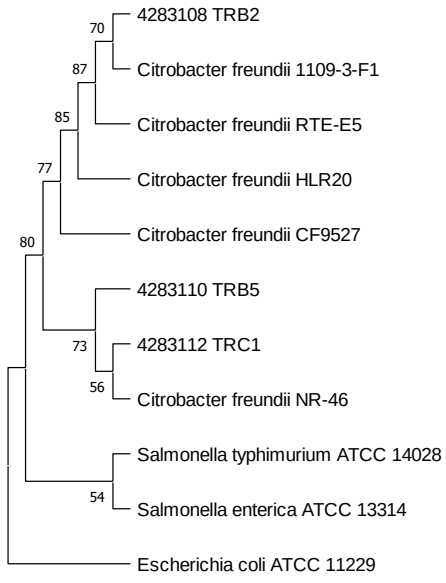


Figure 3. Phylogenetic tree of *Citrobacter* isolates and the reference strains based on 16S rDNA sequences with Neighbor Joining method and bootstrap 1000

The Lysine Iron Agar (LIA) test positive for *Salmonella* was indicated by the absence of color change or remaining purple with or without H_2S on upright and slanted LIA media. This is due to the process of lysine deamination and lysine decarboxylation. The lysine deamination process is an aerobic process that occurs on slanted agar which produces ammonia, the resulting ammonia will react with ferric ammonium citrate which forms a purple color on the slanted agar. While the lysine decarboxylation process is an anaerobic process for upright agar, in this process it will produce an amine end product, which will then react with a pH indicator to produce a purple color on the media to stand upright (Nisa et al., 2018).

DNA extraction methods can affect the results of multiplex PCR. DNA extraction should be fast and simple to reduce the possibility of contamination (Sepp et al., 1994) when many different DNA extraction methods are available (Kumalasari et al., 2020). The heat treatment method is an effective method for obtaining bacterial genomic DNA (Mulyawati et al., 2019); by lysing the cell wall by heating without the addition of buffer (Dashti et al., 2009) and DNA can be easily extracted from Gram-negative bacteria such as Salmonella spp. (Li et al., 2013; Gwida and Al-Ashmawy, 2014). Extraction of DNA from *Salmonella* enterica serovar Brandenburg (Perera and Murray, 2008) and *Salmonella* Enteritidis (Moganedi et al., 2007) by heat treatment method was used.

The results of multiplex PCR in this study showed that all isolates isolated from terasi belonged to the genus *Salmonella* sp. characterized by the formation of a DNA band with a size of 204 bp. The DNA bands formed were caused because the isolate was able to amplify the ompC gene. The omPC gene is involved in protein C, which is responsible for epithelial cell invasion (Lestari, Wibawa & Rohmi 2015). According to Jawad et al. (2016), the OMPC gene is a gene that contains a unique sequence for *Salmonella* isolates and indicates that this gene is a suitable PCR target for the detection of *Salmonella* strains (Jawad & Al-Charrakh 2016). However, in this study, the ompC gene was less accurate in identifying the genus *Salmonella*. The multiplex PCR results for isolates TRB2, TRB5, and TRC1 indicated the genus *Salmonella* because it could amplify the ompC target gene with a length of 204 bp. Still, after confirmation using the 16S rDNA sequence, the three isolates were *Citrobacter freundii* species. Several studies have reported that during the detection of *Salmonella* in food, false-positive results are often found due to the presence of *Citrobacter* (van der Walt and Steyn, 1989; Gaillot et al., 1999). Both genera, such as *Citrobacter* and *Salmonella*, have the same biochemical and antigenic culture characteristics. In addition, the genus *Citrobacter* has a large number of somatic (O) and flagellar (H) antigens, which are usually found in other species of the Enterobacteriaceae family, especially *Salmonella* (Delgado et al., 2013). Other studies have found homologous or identical virulence genes with *Salmonella* and *E.coli* (Samuel et al., 2004).

Most of the *Salmonella* isolates showed the Typhimurium serotype, which was characterized by forming a DNA band measuring 401 bp. This was because the isolate was able to amplify the spy gene. According to de Freitas et al. (2010), the spy gene can code for prismatic proteins. The mPCR method was successfully used by Sahu et al. (2019) to detect the presence of *Salmonella* in seafood. Pui et al. (2011) used the same method to detect and differentiate *Salmonella* sp., *Salmonella* Typhi, and *Salmonella* Typhimurium simultaneously. Several researchers have developed the mPCR method to detect several pathogenic bacteria. Lestari et al. (2015) developed the mPCR method to detect the presence of *Salmonella* in meat preparations by detecting the presence of the ompC gene found in *Salmonella* sp., the sdf gene in *Salmonella* serotype Enteritidis and the spy gene in *Salmonella* serotype Typhimurium. Park et al. (2009) used the same technique to detect *Salmonella* enterica serovar Typhimurium, Enteritidis.

The presence of *Salmonella* Typhimurium and *Citrobacter freundii* bacteria in traditional terasi shows a weakness in the hygienic practices of terasi producers and sellers. In addition, terasi raw material can be the primary source of the presence of *Salmonella*. Pramono et al. (2019) reported that *Salmonella* sp. was mainly found in shrimp products. The high number of *Salmonella* in shrimp products is caused by the polluted environment where shrimp culture is grown. Meanwhile, according to Kumar et al. (2015), shrimp or seafood products are not considered a natural habitat for *Salmonella*. Still, *Salmonella* contamination can be sourced from poor or unhygienic handling processes, significantly contaminated water. Environmental factors such as water play an essential role in the presence of *Salmonella* in food (Araújo et al., 2013). According to Caarasco (2012), *Salmonella* can survive for a long time on food surfaces because these bacteria can form biofilms. In addition, *Citrobacter* is also found in terasi. *C. freundii* is one of the pathogenic bacteria involved in foodborne diseases, and sometimes it can cause opportunistic gastroenteritis. This disease is similar to the disease caused by *Salmonella* (Hidayatullah et al., 2020). In conclusion, traditional terasi is more easily contaminated by pathogenic bacteria, especially *Salmonella* Typhimurium and *Citrobacter freundii*. While the terasi was produced modernly, *Salmonella* Typhimurium and *Citrobacter freundii* were not found because the processing of modern terasi has passed various sensory, chemical, and microbial contamination tests. Modern terasi is produced by several large companies by SNI 2716:2016. Therefore measures to control bacterial growth need to be described to ensure product safety and quality.

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