The Risk of Radio-Resistance Development in Wild-Type *Salmonella enterica* serotype Typhimurium Isolated from Chicken Carcass Towards Gamma Irradiation Treatment

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ABSTRACT

Introduction: *Salmonella enterica serotype* Typhimurium *(S.* Typhimurium*) is a foodborne pathogen whose presence in foods should be avoided. Food preservation methods such as gamma irradiation can eliminate S.* Typhimurium *without compromising food quality. However, as S.* Typhimurium*'s resistance to irradiation increases, there is a possibility of treatment failure, which coincides with a public health problem. The objective of this study was to characterize the sensitivity of wild-type S.* Typhimurium *isolated from chicken carcasses in Surabaya's wet market toward gamma irradiation treatment, as part of a radio-resistant S.* Typhimurium *monitoring.*

Method: *Using a conventional Salmonella isolation methodology, S.* Typhimurium *was recovered from chicken carcasses sold at Surabaya's wet market. The suspected Salmonella isolates were then subjected to a biochemical test, followed by serological and PCR confirmation. The S.* Typhimurium *isolate suspension was irradiated with a 0 to 0.4 kGy dose using a cobalt-60 panoramic batch irradiator (1.1 kGy/h dosage rate). The viable colonies of an irradiated isolate were counted, and the D_{10} value was calculated by plotting the number of irradiated isolates per initial isolate number (0 kGy radiation) versus the dose.*

Results: *Four S.* Typhimurium *isolates from chicken carcasses sold in Surabaya's wet market showed varying slopes of a linear regression derived from viable cell enumeration results. As a result, the computed D_{10} value differed for each isolate. KS-1 had the lowest D_{10} value (0.09 kGy), followed by PK-1 (0.14 kGy) and PK-2 (0.15 kGy), while KS-2 had the greatest value (0.19 kGy).*

Conclusion: *The radio-sensitivity of the isolate varied according to the D_{10} value, which ranged from 0.09 to 0.19 kGy. Simply stated, it shows that radio-resistance development has occurred in wild-type S.* Typhimurium*. Despite being unclear, varied stress exposure to wild-type S.* Typhimurium *was identified as the most potentially impacting factors; nonetheless, further research is required for this purpose.*

Keywords: *Irradiation, Resistance, Salmonella,* Typhimurium

INTRODUCTION

Salmonella enterica serotype Typhimurium (*S.* Typhimurium) is a non-typhoidal serotype that is commonly found in human and animal gastrointestinal tracts. Although *S.* Typhimurium does not cause typhoid fever, it is well-known as a foodborne pathogen (1). Cross-contamination risks in food processing increase the risk of *S.* Typhimurium-related foodborne diseases. The World Health Organization (WHO) recently reported that *S.* Typhimurium was involved in multiple outbreaks in the United States as well as numerous countries in Europe as of 25 April 2022, emphasizing the necessity of controlling this pathogen (2).

Several treatments can prevent the presence of *S.* Typhimurium in food

products. However, the majority involve heat, which may cause undesired changes and damage the quality of food products. Due to its capacity to destroy bacteria while maintaining food quality, gamma irradiation has the potential to address this issue (3). Numerous studies concerning the use of gamma irradiation in food preservation technologies and its efficacy against *S.* Typhimurium have been well documented (3,4). Some studies, recently also provide information regarding the D_{10} value, a required dose to reduce the viable bacterial population by 90%, of *S.* Typhimurium in various food products (5, 6). Nevertheless, limited information on standalone *S.* Typhimurium sensitivity and/or resistance to gamma irradiation treatment was published, and it was generally outdated since it was published back in 1990(7).

As the growth of antibiotic-resistant bacteria increases, their general resistance to threats such as irradiation may evolve as well. *S.* Typhimurium is able to adapt to environmental stress, including irradiation. The radioresistant strain Typhimurium LT2, for example, provides clear evidence of evolution due to a gradual rise in resistance to gamma irradiation (8). The lack of regular monitoring of radioresistant *S.* Typhimurium raises the possibility of food preservation failure, which thus escalates the risk to public health.

The insufficient data of recent studies regarding the standalone radio-sensitivity and/or radio-resistance of *S.* Typhimurium against gamma irradiation encourages us to initiate the monitoring of radioresistant *S.* Typhimurium. The objective of this study was to characterize the sensitivity of wild-type *S.* Typhi-murium on chicken carcasses sold in Surabaya's wet market toward gamma irradiation. Since *S.* Typhimurium is prevalent in chicken carcasses, there is a considerable hazard of cross-contamination between fresh chicken carcasses and food products. The findings will be useful in designing future studies to monitor radioresistant strains of *S.* Typhimurium in foods.

MATERIAL AND METHODS

Samples

Four chicken carcasses from the wet market in Surabaya, Indonesia, were used as a sample. Two samples, each chicken leg and skull, were collected from the Pacar Keling (PK) market in September 23rd, 2013. While in September 24th 2013, Kendangsari (KS) market provided two more samples, a skull and a wing each. All samples were transported to laboratory by using an ice box. Microbial analysis was directly conducted after samples arrived in the laboratory.

Isolation and Identification of *S.* Typhimurium

S. Typhimurium was isolated from chicken carcass samples following the standard isolation for *Salmonella*. Isolation was consisted of pre-enrichment, selective enrichment and selective plating step. In pre-enrichment, 25-gram samples were added with 225 ml of Buffered Peptone Water (BPW), followed by an incubation at 37°C for 24 h. Later, each 1 ml of bacterial suspension from pre-enrichment step was transferred into 9 ml of both Selenite Cystine (SC) Broth and Tetrathionate (TT) Broth for the selective enrichment. After being incubated at 37°C for 24 h, the bacterial suspension of both SC and TT were plated on *Salmonella-Shigella* (SS) Agar as a selective plating step. Bacterial colonies growth on the surface of SS Agar after the 24 h of incubation at 37°C were observed.

The suspected *Salmonella* colony, which appeared as a black center-colorless

colony, was selected for further analysis. A biochemical test was performed as an initial screening for the selected colony, comprising a triple sugar iron (TSI) along with an indole, methyl-red, Voges-Proskauer, citrate (IMViC) test. TSI test was conducted on TSI Agar, whilst IMViC test was performed on various media, SIM Agar was used for the hydrogen-sulphide, indole and motility test; MR-VP Broth for the methyl red and Voges-Proskauer test and Simmon's Citrate Agar for the citrate test. A specific identification by *Salmonella* latex agglutination test kit and detecting the *invA* gene with polymerase chain reaction (PCR) method were performed to ensure isolate was a *Salmonella* sp. Besides, *S.* Typhimurium isolate was further identified by detecting the *fimA* gene as well as using the multiple antisera kit. Verified *S.* Typhimurium isolates were chosen for further analysis of gamma irradiation susceptibility. All *S.* Typhimurium isolate were stored at -80°C before being treated by gamma irradiation.

Isolates Preparation

Each isolate was subcultured on a Nutrient Agar (NA) followed by 24 h of incubation at 37 °C. *S.* Typhimurium isolate suspension was made by diluting the subcultured isolates into 10 ml of sterile distilled water until its turbidity was equal to the McFarland standard no. 1 (approximately 3 x 10^8 CFU/ml). Isolate suspension further distributed into 5 tubes, 1 ml each.

Gamma Irradiation Treatment

Irradiation was performed at Panoramic Batch Irradiator with 1.1 kGy/h of dose rate by using a cobalt-60 (^{60}Co) as the irradiation source. Five irradiation doses (0, 0.1, 0.2, 0.3, 0.4 kGy) were applied to each *S.* Typhimurium isolate suspension at the same time to ensure the irradiation uniformity between isolates. To minimize bias of the results, three replication was made in irradiation treatment.

Enumeration of *S.* Typhimurium

S. Typhimurium viable cells were enumerated by using a total plate count (TPC) method. They were homogenized and serially diluted in sterile dH_2O, then plated on a Plate Count Agar (PCA). After a 37°C incubation for 24 h, growth colonies at each dilution were counted. The total number of each irradiated isolate was obtained from the average number of three dilutions. Result was expressed in colony forming unit per milliliter (CFU/ml).

Calculation of D_{10} Value

A survival curve was obtained by plotting the survival fraction (S), which was acquired by dividing Na with No, against the irradiation dose (kGy) on a logarithmic scale. Further determination of D_{10} value, the dose to reduce 90% of microbial population, was calculated by using the following equation: D_{10} value = D/(log No – log Na). D is the irradiation dose, No is considered the number of initial or untreated isolates, and Na is the number of irradiated isolates. However, D_{10} values could also be taken as the negative reciprocal of the slope of the regression line from the survival curve.

RESULT

_S._ Typhimurium Isolates

Each *S.* Typhimurium isolate was obtained from a different chicken carcass sample. Two PK market isolates were labeled as PK-1 and PK-2, suggesting that they were both enriched with SC broth and originated from the leg and skull, respectively. The KS-1 and KS-2, both from the KS market, were isolated from

the skull and wing through enrichment with SC broth.

Total Number of Irradiated Isolates

The total number of irradiated isolates varied due to differences in the given irradiation dosage and initial isolate suspension. Table 1 showed the average number of isolates based on irradiation dosages applied. The number of viable cells irradiated with 0 kGy dose was changed from $1.20x10^8$ CFU/ml (PK-1) to $1.57x10^7$ CFU/ml (KS-2). PK-1 isolate had the greatest starting number and also had the highest number for isolates irradiated with 0.1 kGy (1.19×10^7 CFU/ml), 0.2 kGy (2.34×10^6 CFU/ml), 0.3 kGy (6.82×10^5 CFU/ml), and 0.4 kGy (1.14×10^5 CFU/ml).

Regardless of its low initial number, KS-2 did not always show the lowest number on each irradiation dose This isolate, however, showed a greater amount (2.34×10^6 CFU/ml) after 0.3 kGy irradiation compared to PK-2 and KS-1 (3.08×10^5 CFU/ml and $2.30 x10^4$ CFU/ml, respectively). Despite being lower, the total number of KS-2 (3.90×10^6 CFU/ml) did not differ significantly from PK-2 (3.95×10^6 CFU/ml) at 0.1 kGy dose. At 0.4 kGy of irradiation, no significant difference was noted between KS-2 (1.07×10^5 CFU/ml) and PK-1 (1.14×10^5 CFU/ml).

Table 1. The total number of irradiated *S.* Typhimurium isolate (CFU/ml)

Isolate Code	Irradiation Dose (kGy)				
	0	0.1	0.2	0.3	0.4
PK-1	1.20×10^8	1.19×10^7	2.34×10^6	6.82×10^5	1.14×10^5
PK-2	4.00×10^7	3.95×10^6	8.30×10^5	3.08×10^5	8.50×10^4
KS-1	5.30×10^7	4.80×10^6	3.85×10^5	2.30×10^4	-
KS-2	1.57×10^7	3.90×10^6	1.20×10^6	4.25×10^5	1.07×10^5

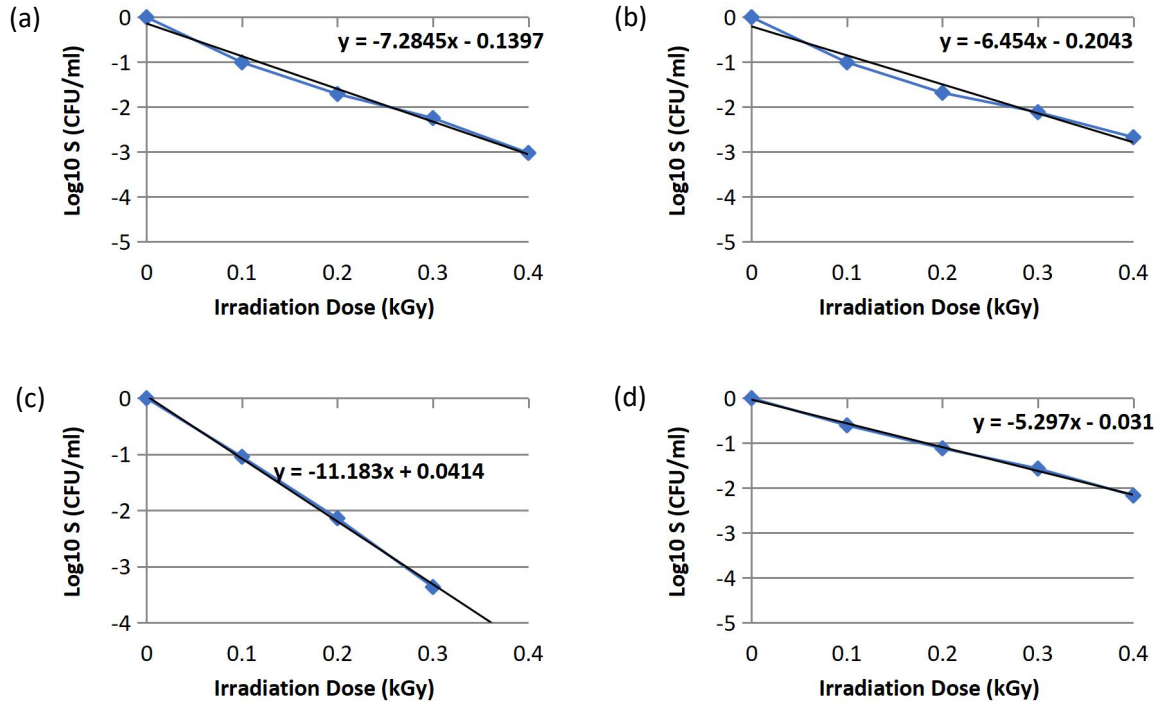

Figure 1. Survival curve of (a) PK-1 , (b) PK-2 , (c) KS-1 , (d) KS-2

PK-2, which had a lower isolate number (4.00×10^7 CFU/ml) than KS-1 at 0 kGy irradiation (5.30×10^7 CFU/ml), interestingly showed a larger number at 0.2 (8.30×10^5 CFU/ml) and 0.3 kGy (3.08×10^5 CFU/ml) compared to KS-1 (3.85×10^5 CFU/ml at 0.2 kGy and 2.30×10^4 CFU/ml at 0.3 kGy). Unfortunately, due to a technical error, the enumeration data of KS-1 at 0.4 kGy dose was missing. Because all irradiation isolate suspensions were produced from a single homogeneous initial isolate suspension, any concern about non-uniformity was eliminated.

Decrease Rate of Isolate

An additional investigation was required to ascertain the decrease rate of the bacterial isolate by obtaining the D_{10} value. Figure 1 presented the survival curves of isolates, together with the regression line. The generated regression line for four types of isolates, nevertheless, had different slopes. The maximum slope of the regression line was seen on KS-1 (11.183), followed by PK-1 (7.2845), PK-2 (6.454), and KS-2 had the least slope (5.297). Furthermore, because D_{10} is calculated from the reciprocal of the slope, the slope of each isolate also reflected its sensitivity to gamma irradiation.

Table 2 displayed the D_{10} value for each *S.* Typhimurium isolate. KS-1 was

found to have the lowest D_{10} value as the highest slope (0.09 kGy). PK-1 and PK-2 had almost similar D_{10} values of 0.14 and 0.15 kGy, respectively. Meanwhile, the KS-2 isolation had the highest D_{10} value (0.19 kGy).

Table 2. D_{10} value of irradiated *S.* Typhimurium isolate

Isolate Code	D_{10} value (kGy)
PK-1	0.14
PK-2	0.15
KS-1	0.09
KS-2	0.19

DISCUSSION

Enumeration of four *S.* Typhimurium isolates used in this study revealed a significant decrease in number as the irradiation dose increased. Gamma irradiation reduced the number of *S.* Typhimurium isolates; however, the number of isolates reduced varied according to radio-sensitivity. Figure 1 depicted the variation in radio-sensitivity of *S.* Typhimurium isolates in this study by the difference in the slope of the survival curve generated by each isolate. Furthermore, the D_{10} value obtained from the isolates in Table 2 determined their sensitivity to gamma irradiation.

KS-1 was determined to be the most radio-sensitive isolate among all irradiated samples due to its lowest D10 value (0.09 kGy). KS-2, on the other hand, was the most resistant isolate to gamma irradiation, as demonstrated by its high D_{10} value (0.19 kGy). Despite coming from the same market, the KS market, the radio-sensitivity of these isolates differed significantly. This suggests that the sensitivity of *S.* Typhimurium isolates to gamma irradiation may vary depending on their sampling origin. PK-1 and PK-2, on the contrary, were identical due to the similarity of their D_{10} values. It is reasonable to presume that they came from the same source and that there was contamination between the origin samples. However, environmental stress in the PK market may be more widespread than in the KS market, resulting in greater development of radio-resistance of S. Typhimurium in the PK market compared to the KS market.

Overall, only KS-1 had the D_{10} value under 0.10 kGy, while the rest showed above 0.10 kGy. Unfortunately, the simplicity of this study brings a limitation to explain the phenomena of varied radio-sensitivity of *S.* Typhimurium isolates. Various factors were involved in the kinetics of microbial destruction by gamma irradiation, comprises extrinsic and intrinsic factors (9). The extrinsic factors could be ignored in this study since all isolates were in the same conditions of growth either in the growth phase, medium, temperature as well as water content and water activity. Thus, the difference in the D_{10} value among isolates was driven mainly by intrinsic factors.

Since the serotypes and origins of the four isolates employed in this investigation were identical, their impact can indeed be neglected; yet, the stress condition of each isolate might differ. Despite the fact that it was isolated from a similar sample, *S.* Typhimurium isolates could be exposed to a variety of stresses. Bacterial stress adaptation leads to increased radio-resistance. Furthermore, Gaougaou et al. (10) discovered a link between the acquisition of antibiotic resistance genes and the growth in radio-resistance of pathogenic bacteria.

CONCLUSION

D_{10} values for *S.* Typhimurium isolates ranged from 0.09 to 0.19 kGy, indicating the emergence of different radio-sensitivity among isolates. KS-1 demonstrated the lowest D_{10} value (0.09 kGy), which was less than 0.10 kGy,

suggesting it was the most radio-sensitive isolate among the four *S.* Typhimurium isolates tested. The different D_{10} values show the different levels of radio-resistance for each isolate, which was primarily affected by various stresses. The fact that three of the four isolates used in this study had D_{10} values more than 0.10 kGy warns of an increase in radio-resistance in wild-type *S.* Typhimurium, particularly those isolated from a chicken carcass. These findings call for further investigation into the wider *S.* Typhimurium population, as well as the stress involved in the development of increased radio-resistance.

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