

Detection and quantification of *Salmonella* in fresh vegetables in Perak, Malaysia

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Abstract

The eating of fresh and minimally processed vegetables is getting popular among Malaysians. This trend poses an increased risk of food poisoning associated with the consumption of fresh produce contaminated with pathogenic bacteria. Salmonellosis is a foodborne disease caused by several non-typhoidal *Salmonella enterica* serovars, predominantly serovars Enteritidis and Typhimurium. The present study aimed to determine the prevalence of *Salmonella* spp., *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium in fresh leafy vegetables such as cabbages (n = 40), lettuces (n = 20), and fruit vegetables such as tomatoes (n = 40), carrots (n = 40) and cucumbers (n = 40), which were sold by three different hypermarkets and a wet market in Kampar, Perak, Malaysia. The study was performed over a period of 13 months (January 2018 to January 2019). A combination of most probable number-multiplex polymerase chain reaction (MPN-mPCR) method was used to quantify the concentrations of *Salmonella* spp., *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium in the examined samples. The results of this study demonstrated that of the vegetables tested, tomatoes, carrots and lettuces were not contaminated by *Salmonella* spp., *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium. However, the presence of *Salmonella* spp. was detected in 3.3% of cabbages from the hypermarket, with estimated microbial loads ranging from <3.0 MPN/g to 15.0 MPN/g. On the other hand, *S. enterica* serovar Typhimurium was detected in 10.0% of the cucumbers from hypermarkets and 20% of them from the wet market. Their microbial loads were ranging from <3.0 MPN/g to >1,100 MPN/g. This indicated that cabbages and cucumbers could be the potential sources of salmonellosis. Therefore, the monitoring of food safety and hygienic practices should be strictly enforced by relevant government agencies to avoid potential poisoning by foodborne pathogens.

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1. Introduction

Salmonella is a Gram-negative rod-shaped bacterium which is responsible for causing salmonellosis in humans. In the United States, 1.2 million illnesses, 23,000 hospitalizations and 450 deaths cases caused by

Salmonella were reported every year (CDC, 2019b). Most of the individuals infected with *Salmonella* develop diarrhoea, fever and abdominal cramps within 12 to 72 hrs. Vulnerable groups such as children, older people and immune-compromised individuals may experience

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severe dehydration which could be life-threatening when infected with *Salmonella* (WHO, 2018).

Among the 2,659 serovars of *Salmonella*, *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium is the most common foodborne pathogens in the developed countries (Hendriksen et al., 2011). Generally, *Salmonella* poisonings are mostly found to be associated with the consumption of contaminated bovine or chicken products, as these pathogens have been reported to reside in the intestine of these animals (Najwa et al., 2015). Nevertheless, *Salmonella* poisonings had also been reported after consuming ready-to-eat (RTE) vegetables such as bean sprouts, lettuces, tomatoes, carrots, alfalfa sprouts, Asiatic pennyworts, water dropworts, cabbages and cucumbers (Bordini et al., 2007; Centre For Food Safety, 2014; Najwa et al., 2015; Kuan et al., 2017). In 2004, salmonellosis outbreak had occurred in the UK, involving 350 people who had consumed lettuces in fast-food restaurants; following an outbreak of *S. Typhimurium* in the US two years after the incident, resulting in 183 individuals fallen sick after eating the contaminated tomatoes (Von Haaren, 2004; CDC, 2006). *Salmonella* food poisoning was also reported in Australia involving 92 individuals who had consumed contaminated pre-packed lettuces (Suzanne, 2016). Thus, the presence of *Salmonella* in RTE vegetables should not be taken lightly globally.

In Malaysia, the exact number of incidences of foodborne illnesses associated with the consumption of fresh produce remains unknown as there has been little attempt to address this problem (Kuan et al., 2017). However, a salmonellosis outbreak involving 171 individuals and mortality after the consumption of RTE vegetables was reported in Kelantan in 2005 (Tunung et al., 2007). Malaysians pose a higher risk in salmonellosis as they have become more health-conscious and consume more vegetables especially cabbage (*Brassica oleracea* var. *capitata*), celery (*Apium graveolens*), spinach (*Spinacia oleracea*), carrot (*Daucus carota*), cauliflower (*Brassica oleracea* var. *cauliflora*), tomato (*Lycopersicon esculentum*), cucumber (*Cucumis sativus*) and long bean (*Vigna sesquipedalis*) (Nurul Izzah et al., 2012). Hence, more prevalence studies on *Salmonella* in vegetables are needed to create awareness about the safety and quality of the vegetables.

This study aimed to detect the presence of *Salmonella* species and two of its serovars (*S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium) in five different vegetables (cabbages, lettuces, tomatoes, carrots and cucumbers) collected from three hypermarkets and a wet market in Kampar, Perak. The microbial level of *Salmonella* in these vegetables was

compared using the most probable number-multiplex polymerase chain reaction (MPN-mPCR) method.

2. Materials and methods

2.1 Sample collection

A total of 40 cabbages (*Brassica oleracea* var. *capitata*), 20 lettuces such as iceberg lettuce (*L. sativa* var. *capitata*), leafy lettuce (*L. sativa* var. *crispa*), butter-head lettuce (*L. sativa* var. *capitata*) and romaine lettuce (*L. sativa* var. *longifolia*), 40 tomatoes (*Solanum lycopersicum*), 40 carrots (*Daucus carota*), and 40 cucumbers (*Cucumis sativus*) were randomly purchased from the hypermarket A, B and C and a wet market in Kampar Perak, Malaysia over a period of 13 months (January 2018 to January 2019). The samples were collected and kept in a sterile polyethylene bag before delivered to the laboratory for further analysis on the same day.

2.2 Detection and quantification of *Salmonella* by MPN-mPCR method

A 10 g of freshly cut vegetables were weighed aseptically and transferred into a sterile stomacher bag (Interscience, Singapore). A 90 mL of sterile Buffered Peptone Water (BPW) (Merck, Germany) was added to the bag and pummelled for 60 s using BagMixer[®] 400 stomacher machine (Interscience, France). The homogenized suspension was diluted to 100 and 1000 folds. Then, the 3-tube MPN method was carried out by transferring 1 mL from each dilution into three replicate tubes and incubated at 37°C for 4 hrs. After pre-enrichment, each of the suspensions was added to 10 mL of Rappaport-Vassiliadis-Soya (RVS) broth (Merck, Germany), followed by incubation at 37°C for 20 hrs under aerobic condition (Pui et al., 2011; Najwa et al., 2015; Thung et al., 2018). The turbid MPN tubes were plated on Xylose Lysine Deoxycholate (XLD) agar (Oxoid Ltd, UK) and DNA was extracted before multiplex PCR (mPCR) was performed. The microbial load in each sample was quantified by referring to the MPN Table of Bacterial Analytical Manual (BAM) from the US FDA (Sutton, 2010).

2.2.1 DNA extraction

Boiled-cell DNA extraction method (Kuan et al., 2017) was used to extract the bacterial DNA. Briefly, 1 mL of broth culture from MPN tube was centrifuged at 15,000 × g for 3 mins. The pelleted cells were re-suspended in 200 µL of Tris-EDTA buffer. The mixture was vortex and boiled for 10 mins. The solution was cooled immediately at -20°C for 10 mins before it was centrifuged at 15,000 × g for 3 mins. The supernatant was used as the DNA template for multiplex polymerase

chain reaction (mPCR).

2.2.2 Multiplex PCR (mPCR)

The first primer pairs of ST11 (5'-GCC AAC CAT TGC TAA ATT GGC GCA-3') and ST15 (5'-GGT AGA AAT TCC CAG CGG GTA CTG G-3') was used to amplify the endonuclease gene at the size of 429 bp for *Salmonella* spp. and the second primer pairs of ENTF (5'-TGT GTT TTA TCT GAT GCA AGA GG-3') and ENTR (5'-TGA ACT ACG TTC GTT CTT CTG G-3') was used to amplify the *sdfl* gene which encodes for the transcriptional regulator on *S. enterica* serovar Enteritidis at 304 bp. The final set of primer pairs, Fli15 (5'-CGG TGT TGC CCA GGT TGG TAA T-3') and Typ04 (5'-ACT GGT AAA GAT GGC T-3') was used to detect the presence of *S. enterica* serovar Typhimurium by targeting the *fliC* gene which encodes for the flagellin protein at the amplicon size of 620 bp (Kuan et al., 2017).

3. Results

3.1 PCR amplification of *Salmonella* spp., *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium in vegetables

The positive amplification of the three target genes specific to *Salmonella* spp., *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium produced products of 429 bp, 304 bp and 620 bp, respectively (Figure 1).

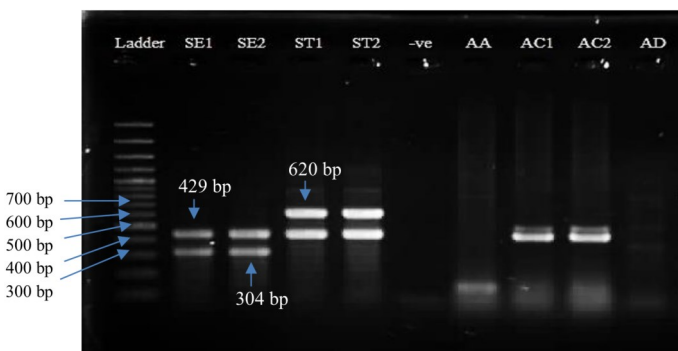


Figure 1. Representative amplification of 429 bp segment of random sequence, 304 bp segment of *Sdfl* gene and 620 bp segment of *fliC* gene for the identification of *Salmonella* spp., *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium, respectively. Lane Ladder: 100 bp DNA ladder (Vivantis Technologies, USA). Lane SE1 and SE2: positive controls of *S. enterica* serovar Enteritidis with PCR amplicons specific to *Salmonella* spp. at 429 bp and *S. enterica* serovar Enteritidis at 304 bp. Lane ST1 and ST2: positive controls of *S. enterica* serovar Typhimurium with PCR amplicons specific to *Salmonella* spp. at 429 bp and *S. enterica* serovar Typhimurium at 620 bp. Lane -ve: negative control. Lane AA and AD: negative samples. Lane AC1 and AC2: positive samples with PCR amplicon specific to *Salmonella* spp. at 429 bp.

3.2 Prevalence of *Salmonella* spp., *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium in vegetables

The prevalence of *Salmonella* spp., *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium in vegetables from the hypermarkets and wet market are tabulated in Table 1. Out of the five different types of vegetable samples tested, only cabbages and cucumbers were found to be positive to *Salmonella* spp. with the prevalence of 2.5% (one positive sample out of 40 samples) and 12.5% (five positive samples out of 40 samples), respectively. Besides, the total prevalence of *S. enterica* serovar Typhimurium in cucumbers purchased from the wet market and the hypermarkets was 10.0% (four positive samples out of 40 samples). The contamination rates of *Salmonella* spp. and *S. enterica* serovar Typhimurium was found to be slightly higher in the samples collected from the wet market than those from the hypermarkets. However, *S. enterica* serovar Enteritidis was absent in all the vegetable samples analysed.

3.3 Microbiological load of *Salmonella* in vegetables

In this study, *Salmonella* spp. was found to be the most prevalent in cucumber with the maximum concentration of >1,100 MPN/g, followed by cabbage with the maximum concentration of 15.0 MPN/g (Table 2). Among five different types of vegetables, cucumber was found to be an important reservoir for *Salmonella*, particularly *S. enterica* serovar Typhimurium with the highest concentration of 23.0 MPN/g and >1,100 MPN/g were detected in cucumbers that were purchased from wet market and hypermarkets, respectively.

4. Discussion

In this study, the prevalence of *Salmonella* spp. and *S. enterica* serovar Typhimurium was found to be higher in the wet market than the hypermarkets. Nevertheless, both retail markets possess the risk of selling *Salmonella* contaminated vegetables. Hypermarkets are one-stop grocery stores, selling a variety of goods. The goods are organized systematically, and similar goods are placed together in different zones to minimize cross-contamination. It is always perceived to be clean and tidy. The centralised cooling facilities at the hypermarkets are built to maintain their inner temperature and humidity so that the quality of the goods, especially food items can be maintained (Castillo, 2010). However, the quality of vegetables and fruits may sometimes be affected due to late delivery by suppliers. Besides, Najwa et al. (2015) found that vegetables in hypermarkets are displayed for up to a week and hence have a longer holding time compared to wet markets.

Table 1. Prevalence of *Salmonella* spp., *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium in fresh vegetables purchased from the hypermarkets and the wet market in Kampar, Perak

Vegetables	Hypermarkets				Wet market			
	No. of positive sample, n (%)				No. of positive sample, n (%)			
	Number of samples (n)	<i>Salmonella</i> spp.	<i>S. enterica</i> serovar Enteritidis	<i>S. enterica</i> serovar Typhimurium	Number of samples (n)	<i>Salmonella</i> spp.	<i>S. enterica</i> serovar Enteritidis	<i>S. enterica</i> serovar Typhimurium
Cabbage	30	1 (3.3%)	0 (0.0%)	0 (0.0%)	10	0 (0.0%)	0 (0.0%)	0 (0.0%)
Tomato	30	0 (0.0%)	0 (0.0%)	0 (0.0%)	10	0 (0.0%)	0 (0.0%)	0 (0.0%)
Lettuce	14	0 (0.0%)	0 (0.0%)	0 (0.0%)	6	0 (0.0%)	0 (0.0%)	0 (0.0%)
Carrot	30	0 (0.0%)	0 (0.0%)	0 (0.0%)	10	0 (0.0%)	0 (0.0%)	0 (0.0%)
Cucumber	30	3 (10.0%)	0 (0.0%)	2 (6.7%)	10	2 (20.0%)	0 (0.0%)	2 (20.0%)
Total	134	4 (3.0%)	0 (0.0%)	2 (1.5%)	46	2 (4.4%)	0 (0.0%)	2 (4.4%)

Table 2. Microbial load of *Salmonella* spp., *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium (MPN/g) in fresh vegetables purchased from hypermarkets and wet market in Kampar, Perak

Vegetables	Hypermarkets									Wet market								
	<i>Salmonella</i> spp.			<i>S. enterica</i> serovar Enteritidis			<i>S. enterica</i> serovar Typhimurium			<i>Salmonella</i> spp.			<i>S. enterica</i> serovar Enteritidis			<i>S. enterica</i> serovar Typhimurium		
	Min	Med	Max	Min	Med	Max	Min	Med	Max	Min	Med	Max	Min	Med	Max	Min	Med	Max
Cabbage	<3.0	<3.0	15	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
Tomato	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
Lettuce	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
Carrot	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
Cucumber	<3.0	<3.0	>1100	<3.0	<3.0	<3.0	<3.0	<3.0	>1100	<3.0	<3.0	23	<3.0	<3.0	<3.0	<3.0	<3.0	23

Min, minimum MPN/g value; Med, median MPN/g value; Max, maximum MPN/g value

This scenario has compromised the freshness of vegetables which leads to the growth of foodborne pathogens. This is supported by the findings in the present study that the microbial load of *Salmonella* spp. (>1,100 MPN/g) was higher in hypermarket. On the other hand, wet markets are often perceived as wet, filthy and smelly. They are not equipped with any cooling facilities. Consequently, this will also promote the growth of *Salmonella*.

On the contrary, Vital *et al.* (2014) found that the incidence of *Salmonella* contamination in the wet markets and the hypermarkets were similar (Vital *et al.*, 2014). However, Najwa *et al.* (2015) reported that the prevalence of *Salmonella* spp., *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium in raw vegetables from hypermarkets were higher than those from the wet markets. Similarly, Loo *et al.* (2013) found that the prevalence and microbial load of other foodborne pathogen, Shiga toxin-producing *Escherichia coli* (STEC) in raw vegetables from hypermarkets was higher as compared to the wet markets.

The number of reported cases of *Salmonella* contamination in raw vegetables is very rare. This is supported by the observation of very low or the absence *Salmonella* in tomatoes, lettuces and carrots in this study. Food with no or low concentration of foodborne pathogens does not imply that they are safe for

consumption, as other microbes can grow to a certain level to cause food poisoning under favourable conditions. Susceptible groups such as immune-compromised patients or pregnant women are easily infected by a sub dosage of foodborne pathogens. Other foodborne pathogens such as *E. coli* O104:H4, *E. coli* O157:H7, *Cyclospora cayetanensis*, *Norovirus*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Klebsiella pneumoniae* had also been reported in raw vegetables (Puspanadan *et al.*, 2012; Chang *et al.*, 2013; Loo *et al.*, 2013; Kuan *et al.*, 2017; CDC, 2019a). Therefore, active surveillance must be conducted routinely to ensure that these raw vegetables are safe for consumption.

In the present study, the prevalence of *Salmonella* in cabbages was found to be much lower compared to the results of Nillian *et al.* (2011), where the contamination rates of *Salmonella* spp., *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium were 28.0%, 24.0% and 16.0%, respectively. However, in an earlier report by Kuan *et al.* (2017), *Salmonella* spp., *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium were not detected in cabbages from Kuala Lumpur, Selangor and Putrajaya. Such differences in the detection of foodborne pathogens are not uncommon as differences arise during pre- and post-harvest handling, especially in hygienic and agricultural practices (Jung *et al.*, 2014; Kuan *et al.*, 2017).

Throughout the sampling period, it was observed that cucumbers in the retail markets were poorly sanitized as dirt and soil could be seen adhering on the cucumbers. The presence of dirt or soil might pose an increased risk of contamination by *Salmonella*. This might be the reason for the highest prevalence of *Salmonella* (12.5%) in cucumbers among the other vegetables in this study. A study has been reported that *Salmonella* can survive in the soil for as long as 231 days (Islam et al., 2004). Bacteria present in the non-hygienic or contaminated display sites which initiated its biofilms formation on the vegetable surfaces.

Interestingly, *Salmonella* was not found in tomatoes, lettuces and carrots in the present study. These findings suggested that tomato, lettuce and carrot might not be a good vehicle for the *Salmonella* contamination. However, contrary results were reported by Kuan et al. (2017), with 7.7% of carrot sample was contaminated with *S. enterica* serovar Enteritidis. Similarly, Sabbithi et al. (2014) reported that 58.5% of the carrots used in the salads sold as street food in Hyderabad, India, were contaminated with *Salmonella*. In Dhanbad, India, 3.3% of the carrots collected from the local market were contaminated with *Salmonella* (Mritunjay and Kumar, 2017).

Apart from *Salmonella*, other microbial pathogens such as *E. coli*, *L. monocytogenes*, *S. aureus*, may grow in cabbages (Mritunjay and Kumar, 2017). According to the studies done by Prazak et al. (2002) and Kuan et al. (2017), 4.7% and 18.2% of cabbages were contaminated by *L. monocytogenes* in Texas, United States and Selangor, Malaysia respectively. Outbreaks of food poisoning associated with contaminated cabbage had been reported (Solomon et al., 1990; Dunn et al., 1995; CDC, 2018). *C. botulinum*, *Shigella flexneri*, *E. coli* O157:H7, *E. coli* O169:H49, *E. coli* O111 and *S. aureus* were detected in these outbreaks.

Although the prevalence of *Salmonella* was low in this study, several sources that may cause raw vegetables being contaminated with *Salmonella* or other foodborne pathogens along the food supply chain including pre-harvesting, harvesting, post-harvesting, transportation and processing. The environment, animal and human may serve as the diverse risk factors of fresh produce contamination (Antwi-Agyei et al., 2015). Soil is one of the sources that may cause cross-contamination during pre-harvesting as foodborne pathogens may transfer to vegetables from contaminated soil. Therefore, vegetables that are close contact with the soil during planting have higher contamination risk (Nillian et al., 2011; Loo et al., 2013). The contaminated irrigation water, animal manure, untreated sewage sludge, insects, pests and the

access of domestic or wild animals in the farm will cause the soil and the crops being contaminated with the pathogens. Besides that, uneducated and untrained workers with poor handling practices during harvesting and post-harvesting could lead to cross-contamination on the raw vegetables. Apart from that, the non-hygienic workplaces and surrounding environments with poor sanitation play a major role in microbial contamination. This is especially true for raw vegetable display areas which are seldom or improperly sanitized and cleaned at the retail markets (Loo et al., 2013; Najwa et al., 2015).

Therefore, good agricultural practices (GAP), good manufacturing practices (GMP) as well as Hazard Analysis and Critical Control Points (HACCP) should be incorporated in the food chain in order to reduce and prevent the risks of contamination of fresh produce from farm to plate (Antwi-Agyei et al., 2015). Nina (2019) has recommended consumers to select non-bruised vegetables; vegetables separated from animal origin foods such as meat, poultry and seafood; properly washed vegetables; and properly stored vegetables at an appropriate temperature. Susceptible groups such as pregnant women, elderly or individual who have weakened immune system should avoid consuming raw vegetables and lightly cooked food (Nina, 2019).

According to the guidelines of the Institute of Medicine and National Research Council Committee (2003) as well as the Centre for Food Safety (2014) in Ireland and Hong Kong, respectively, *Salmonella* spp. shall not be detected at 25 g of food that is ready to be eaten because as few as one organism of *Salmonella* spp. could result in non-typhoidal salmonellosis and less than 10^3 organisms could cause typhoid fever. However, this standard varies in different countries (Antwi-Agyei et al., 2015). In Malaysia, there is no clear statement about the acceptable level in the Malaysia Food Act 1983 and Food Regulations 1985.

In this study, MPN-mPCR was chosen for its rapid and high accuracy in the detection of species-specific genes of foodborne pathogens. The multiplex PCR was used to detect the presence of *Salmonella* by amplifying a specific short target sequence of *Salmonella* DNA. The target sequences chosen are endonuclease gene of *Salmonella* spp., *SdfI* gene of *S. enterica* serovar Enteritidis and *fliC* gene of *S. enterica* serovar Typhimurium. However, PCR-based methods are limited to qualitative determination. It amplifies DNAs of targeted sequence present in the samples including the viable but non-culturable cells (VBNC) and dead pathogens in food samples (Saroj et al., 2008). This might create false-positive results. According to Borowsky, Schmidt and Cardoso (2007), the quantity of

microorganisms present in the food sample is critical for the microbiological risk assessment (Borowsky *et al.*, 2007). Therefore, to overcome the limitation of mPCR, MPN method is often applied to accurately quantify the pathogens. Over the past 10 years, there were numerous studies utilising MPN-mPCR assay to detect and enumerate the foodborne pathogens in food samples (Nillian *et al.*, 2011; Pui *et al.*, 2011; Puspanadan *et al.*, 2012; Chang *et al.*, 2013; Kuan *et al.*, 2013; Loo *et al.*, 2013; Najwa *et al.*, 2015; Kuan *et al.*, 2017; Othman *et al.*, 2018; Thung *et al.*, 2018). MPN-mPCR is able to produce results within two days compares to the conventional biochemical method which takes up from 7 -10 days (Pui *et al.*, 2011).

5. Conclusion

Overall, *Salmonella* spp., *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium were not detected in tomatoes, carrots and lettuces. However, low prevalence of *Salmonella* spp. was detected in cabbage and cucumber. The detection of *Salmonella* spp. in cabbage and cucumber raises the concern on the hygienic and safety of the consumption of not only cabbages and cucumbers, but in other raw vegetables. Various types of foodborne pathogens may grow and cause a threat to the consumer's health. As such, susceptible groups such as elderly, immunocompromised patients and pregnant women should avoid consuming raw vegetables to reduce the risk of bacterial infection. The results obtained from this study might be useful as baseline information for the risk assessment on the microbiological quality of vegetables since there is an increasing number of Malaysians eating raw or minimally processed vegetables daily.

Conflict of Interest

The authors declare no conflict of interest.

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