Occurrence of *Listeria monocytogenes* and *Salmonella* Typhimurium in Fruit Juices from Local Stalls and Restaurant in Kuching, Sarawak

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ABSTRACT

Listeria spp. and *Salmonella* spp. are capable of causing food-borne outbreaks and diseases in humans. This study aimed to quantify and detect the occurrence of *Listeria monocytogenes* and *Salmonella* Typhimurium in fruit juices by utilizing Most Probable Number (MPN) in combination with Polymerase Chain Reaction (PCR). In this study, a total of 50 fruit juice samples, consisting of orange, papaya, watermelon, honeydew and apple were collected from Kota Samarahan and Kuching. Specific Polymerase Chain Reaction (PCR) assay targeting the virulence gene, *hlyA* gene in *L. monocytogenes* and *fliC* gene in *S.* Typhimurium was performed, with the expected size of 730 bp and 559 bp, respectively. MPN analysis showed that the estimated microbial loads of *Listeria* spp. and *Salmonella* spp. in all samples were more than 1100 MPN/g. However, based on the PCR analysis, none of the samples (0%) were positive for *L. monocytogenes* or *S.* Typhimurium. This study presented as a preliminary food safety screening for the occurrence of *Listeria* spp. and *Salmonella* spp. from retailed fruit juices. Hygienic practices and food safety measures should be adhered by all food vendors and restaurants in order to avoid foodborne disease outbreaks in the future.

Keywords: *fliC*, *hlyA*, *Listeria*, PCR, *Salmonella*

INTRODUCTION

Fruits are rich in vitamins, minerals and fibers which are essential component in a healthy diet. They are becoming a popular choice of dietary supplement in the modern days society as healthy eating is part of a dietary routine. Despite their health benefits, fruits are also capable of harboring pathogenic microorganisms. As fresh fruits are minimally-processed with no proper disinfection techniques, the probability of acquiring food-borne infections are high. The disease threat is exacerbated when the fruits are eaten raw (Nillian *et al.*, 2011). Normally, fresh fruit juices are prepared by extraction or mechanical means, and have no or little steps taken to reduce contamination and pathogen level (Victorian Government Department of Human Services, 2005). Contamination sourced from raw fruits, equipment and food handlers involved in fruit juices preparation can transmit bacteria to the final products. With the rise in these ready-to-eat foods, attention has been drawn towards the foodborne disease associated with fresh fruits such as salmonellosis and listeriosis.

Listeria monocytogenes (*L. monocytogenes*) is a Gram-positive, facultatively anaerobic, non-spore forming, rod-shaped bacteria that is known to be the causative agent of listeriosis (Jeyaletchumi *et al*., 2010). Listeriosis can cause abortions and premature deliveries, septicemia and meningitis among neonates. In immunocompromised individuals, it can cause meningitis, encephalitis, meningoencephalitis (Jadhav, Bhave & Palombo, 2012). A study that was conducted in Malaysia by Jeyaletchumi *et al*. (2010) found that *L. monocytogenes* was present in the fresh juice samples. *HlyA* gene is a virulent gene which encodes for Listeriolysin O (LLO) toxin, that is responsible to promote haemolytic activity. It can be used to detect the presence of *L. monocytogenes*. In Malaysia, *L. monocytogenes* has been detected in various food samples including raw and ready-to-eat foods such as vegetarian burger patties, salad, vegetables, chicken and egg products (Jamali, Chai & Thong, 2012; Marian *et al.*, 2012; Wong *et al.*, 2011).

Salmonellosis is a foodborne disease caused by the *Salmonella* bacteria. Among *Salmonella* serovars, *S.* Typhimurium and *S.* Enteritidis are the most common causes of salmonellosis. The genus *Salmonella* is within the family Enterobacteriaceae, that is morphologically and biochemically homogenous group of facultatively anaerobic, non-spore forming, Gram-negative rod-shaped bacteria. A local study by Diana, Pui and Son (2012) reported that the highest prevalence of *Salmonella* spp. was in carrot juice while *S.* Typhimurium was the highest

in apple juice. The study concluded that the level of ascorbic acid in the fruit juices was the main factor that affected the pH needed for growth of the *Salmonella*.

However, there is a scarcity of information on the distribution of *L. monocytogenes* and *S.* Typhimurium in fruit juices retailed in East Malaysia (Sarawak). Therefore, the present study aimed to enumerate and detect *L. monocytogenes* and *S.* Typhimurium in fruit juices retailed in Kota Samarahan and Kuching by MPN method and PCR analysis.

MATERIALS & METHODS

Sample collection and processing

Fifty fresh fruit juices were purchased from fruit juice stalls and restaurants located in Kota Samarahan and Kuching, Sarawak. The fruit types and the number of samples are shown in Table 1 and 2. The fruit juice samples were placed in an ice box immediately after collection and they were processed at the Molecular Microbiology Laboratory, Faculty Resource Science and Technology, Universiti Malaysia Sarawak (UNIMAS).

Table 1. Fruit types and the number of fruit juices examined for the detection and concentration of *Listeria* spp. and *L. monocytogenes.*

Types of Fruit	Scientific name	Number of samples
Orange	*Citrus sinensis*	10
Papaya	*Carica papaya*	10
Watermelon	*Citrullus lanatus*	10
Honeydew	*Cucumis melo*	10
Apple	*Malus domestica*	10
Total:		50

Table 2. Fruit types and the number of fruit juices examined for the detection and concentration of *Salmonella* spp. and *S.* Typhimurium.

Types of Fruit	Scientific name	Number of samples
Orange	*Citrus sinensis*	10
Guava	*Psidium*	10
Watermelon	*Citrullus lanatus*	10
Honeydew	*Cucumis melo*	10
Apple	*Malus domestica*	10
Total:		50

Pre-enrichment for *Listeria* spp. and *Salmonella* spp.

Sample processing for *Listeria* spp. and *Salmonella* spp. was performed as described by Wong *et al.* (2011) and Malorny and Helmuth (2003), respectively. *Listeria* spp. were enriched by transferring 10 ml of the samples into 90 ml of Tryptic Soy Broth (Becton, Dickinson & Company, France). While, *Salmonella* spp. were enriched by transferring 10 ml of the samples into 90 ml of Buffered Peptone Water (Merck, Germany). Afterwards, the broths were incubated at 37 °C for 24 hr.

Enumeration by Most Probable Number (MPN) Method

The pre-enriched samples were subjected to the three-tube MPN analysis as described in Sutton (2010). The samples were diluted with three-fold dilution series. Briefly, the homogenised fluid from each of the enriched fruit juice samples was serially diluted into 100-fold and 1000-fold dilutions with nutrient broth (Merck, Germany) and incubated at 37˚C for 24 hours. After incubation, the turbid tubes were chosen for DNA extraction.

DNA Extraction

Boil cell method as described by Diana *et al.* (2011) was adapted in this study. Briefly, 1 ml of the enriched bacterial cultures in the broth was centrifuged (Hettich EBA 21 Zentrifugen, Germany) at 12,000 rpm for 3 min. The supernatant was discarded, and the cell pellet was re-suspended in 200 µl of sterile distilled water and was vortexed (Labnet International, USA). The cell suspension was boiled for 15 min and immediately cooled at -20 °C for 15 min before being centrifuged again (Hettich EBA 21 Zentrifugen, Germany) for 3 min at 12,000 rpm.

PCR Assay for the Detection of *Listeria monocytogenes*

PCR assay was performed as described by Kargar and Ghasemi (2009) with minor modifications in the concentration of reagents and primer used. One set of primers as summarized in Table 3 was used to detect *hlyA* gene in *L. monocytogenes* isolates.

Table 3. Primers used for the detection of *Listeria monocytogenes.*

Targeted gene	Primer nucleotide sequences (5' – 3')	Amplicon Size (bp)	Reference
hlyA	*hly*A-F CAT TAG TGG AAA GAT GGA ATG	730	(Gouws & Liedemann, 2005)
	*hly*A-R GTA TCC TCC AGA GTG ATC GA		

Prior to the PCR assay, positive and negative controls were prepared. The positive control contained 5 µl of DNA template of *L. monocytogenes* ATCC 15313 (American Type Culture Collection, USA), whereas 5 µl of sterile distilled water was used as the negative control. The amplification was performed in the Eppendorf Mastercycler® Personal (Hamburg, Germany). An initial denaturation was started at 95 °C for 5 min, followed by 35 cycles each of denaturation at 95 °C for 1 min, primer annealing at 62 °C for 45 sec and extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. The presence of amplified *hly*A gene fragment was detected by using gel electrophoresis with 1.2% of agarose gel, 0.5X TBE buffer at 80 V for 1 h before visualization under UV transilluminator.

PCR Assay for the Detection of *Salmonella* Typhimurium

PCR was carried out as described by Jamshidi, Bassami and Afshari-Nic (2009) and Soumet *et al*. (1999). *Fli15*-F and *Tym*-R primers were used specific for *Salmonella* Typhimurium with *fliC* gene (Table 4). The PCR reactions were carried out with 25 µl amplification mixture.

Table 4. Primers used for the detection of *Salmonella* Typhimurium.

Target Gene	Primer sets	Nucleotide sequences (5' to 3')	Amplicon size (bp)	Reference
fliC gene	*Fli15*-F	CGG TGT TGC CCA GGT TGG TAA T	559	Jamshidi *et al*. (2009)
	Tym-R	ACT CTT GCT GGC GGT GCG ACT T		

Positive and negative controls were included in each PCR assay. The positive control contained 5 µl of DNA template of *S.* Typhimurium ATCC 14028 (American Type Culture Collection, USA), whereas 5 µl of sterile distilled water was used as the negative control. The amplification was performed in the Eppendorf Mastercycler® Personal (Hamburg, Germany). An initial denaturation was started at 95 °C for 5 min, followed by 35 cycles each of denaturation at 94 °C for 1 min, primer annealing at 56 °C for 30 sec and extension at 72 °C for 30 sec and a final extension at 72 °C for 10 min. The presence of amplified *fliC* gene fragment was detected by using gel electrophoresis with 1.2% of agarose gel, 0.5X TBE buffer at 85 V for one hour before visualization under UV transilluminator.

RESULTS & DISCUSSIONS

The findings from pre-enrichments indicated that all the fruit juices (*n*=100) showed turbidity in the Tryptic Soy Broth and Buffered Peptone Water. Furthermore, the MPN analysis revealed that all samples were contaminated with more than 1100 MPN/g of *Listeria* spp. and *Salmonella* spp. Tables 5 and 6 summarize the results for the detection of *Listeria* spp. and *L. monocytogenes*, and *Salmonella* spp. and *S.* Typhimurium, respectively in five different fruit juices collected from local stalls and restaurants.

The agarose gel images for the detection of *hlyA* (*L. monocytogenes*) and *fliC* (*S.* Typhimurium) genes in fruit juices are shown in Figures 1 and 2, respectively. Nevertheless, no visible bands were detected at 730 bp (for *hlyA* gene) and 559 bp (for *fliC* gene), respectively.

Table 5. Detection of *Listeria* spp. and *L. monocytogenes* in five different fruit juices collected from local stalls and restaurants.

Types of Fruit Juice (Total isolates)	Preliminary detection of ***Listeria*** spp. by MPN/g (No. positive Isolates)	PCR Detection of *Listeria monocytogenes* (Percentage of positive Isolates)
Apple (*n*=10) Orange (*n*=10) Watermelon (*n*=10) Honeydew (*n*=10) Papaya (*n*=10)	>1100 (*n*=50)	0/50 (0%)
	Total: 50/50 (100%)	Total: 0/50 (0%)

Table 6. Detection of *Salmonella* spp. and *S.* Typhimurium in five different fruit juices collected from local stalls and restaurants.

Types of Fruit Juice (Total isolates)	Preliminary Detection of *Salmonella* spp. MPN/g (No. positive Isolates)	PCR Detection of *Salmonella* Typhimurium (Percentage of positive Isolates)
Apple (***n***=10) Orange (*n*=10) Watermelon (*n*=10) Honeydew (*n*=10) Guava (*n*=10)	>1100 (*n*=50)	0/50 (0%)
	Total: 50/50 (100%)	Total: 0/50 (0%)

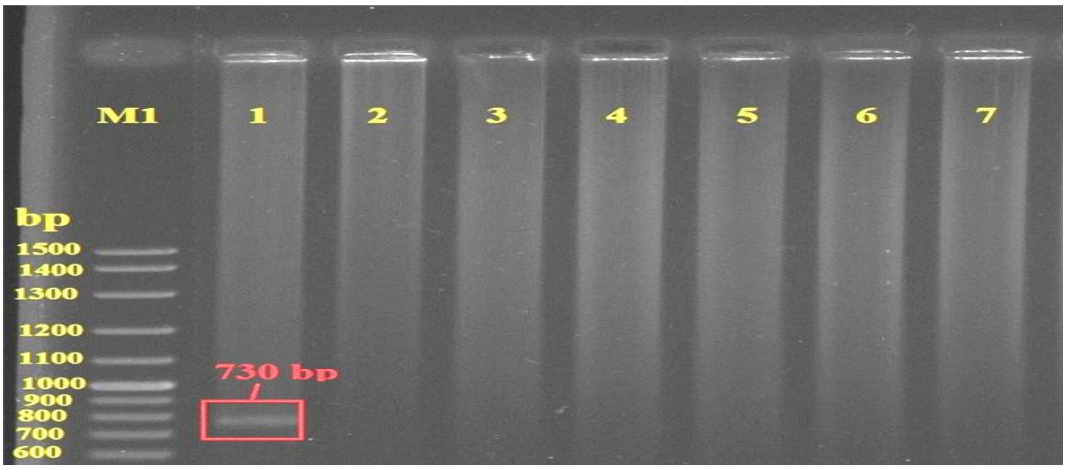

Figure 1. Representative gel image of PCR amplification of *hlyA* gene detection from five different fruit juice samples. M1: 100-bp DNA ladder; Lane 1: *L. monocytogenes* positive control (ATCC 15313), lane 2: negative control, lane 3 – 7: apple, orange, watermelon, honeydew and papaya juice samples.

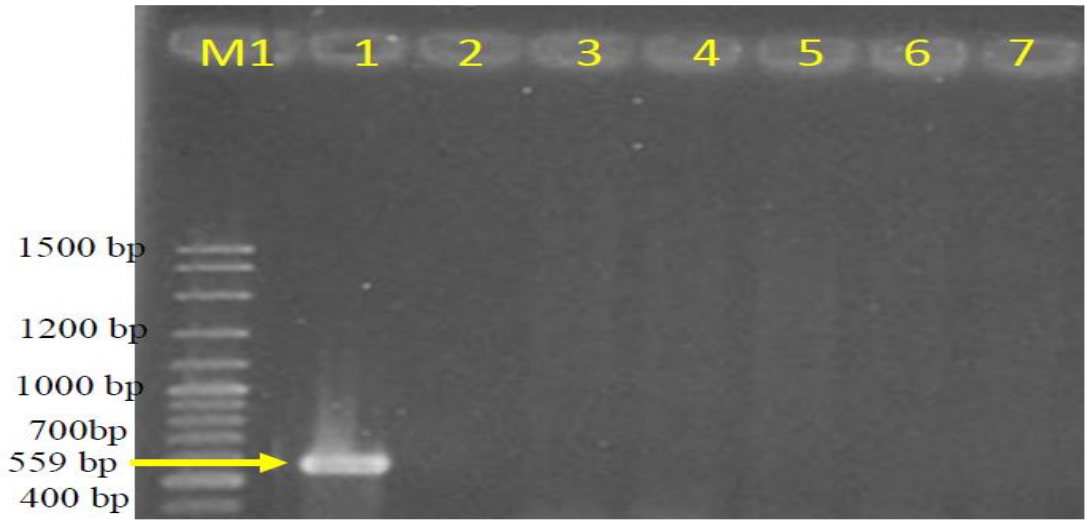

Figure 2. Representative gel image of PCR amplification of *fliC* gene detection from five different fruit juice samples. M1: 100-bp DNA ladder; Lane 1: Positive control for *S.* Typhimurium; Lane 2: Negative control; Lane 3-7: apple, orange, watermelon, honeydew and guava juice samples.

In this study, no sample of fruit juices (n = 0/50) was positive with *L. monocytogenes* and *S.* Typhimurium from the PCR analysis. The absence of *Listeria* spp. and *Salmonella* spp. in this study is due to the vertical growing mechanism of the fruit from the soil which indirectly reduce the potential of Listeria or Salmonella contamination. (Brackett, 2007). It has been frequently reported that *Salmonella* was detected from other foods such as meat, vegetables and seafood. A study by Titarmare, Dabholkar and Godbole (2009) reported that almost 50% of vegetable juice samples tested in the study, showed the presence of *Salmonella*.

Contamination of fruit juices can result from cross-contamination or mishandling during harvesting, processing, transporting and during juices preparation (Diana *et al.*, 2012). Pathogen reduction depends largely on the hygienic and sanitary production and processing practices used to reduce colonization, transmission and cross-contamination among foods and environment (Huda & Adzitey, 2010). In this study, the fruit juice samples were mostly collected from fruit juice stalls at malls and restaurants. It was noteworthy that the environments of fruit juice stalls and restaurants were tidier, cleaner and had more favourable hygienic status compared to hawker stalls. This includes proper sanitary practices presented by the stall workers (malls and restaurants) such as wearing gloves, proper hand washing practices, and washing fruits and utensils at every preparation. The fruits were washed to remove any visible debris and dirt on the fruits. According to Park, Alexander, Taylor, Costa and Kang (2008), chlorinated or electrolyzed water can be used to decontaminate foodborne pathogen on fruits. In this recent study, pasteurized milk was added to the papaya juice during the preparation. Addition of pasteurized milk to fruit juices can be a source of *Listeria* contamination particularly *L. monocytogenes* that is commonly associated with tainted milk products. However, PCR detection assay revealed the absence of *L. monocytogenes* in the papaya juice samples and it is suggested that the milk added was well pasteurized. This is supported by Lado and Yousef (2007), which highlighted that high temperature during pasteurization process can eliminate bacteria.

As opposed to the similar study conducted by Diana *et al*. (2012) in West Malaysia, this study in East Malaysia showed no presence of *S.* Typhimurium in fruit juices. The utilization of purified and distilled water during the preparation of fruit juices is a contributing factor to the absence of *S.* Typhimurium in the fruit juice samples. Purified water normally contains fewer microorganisms than non-purified water. Fresh-cut fruits were further subjected to storage in refrigerator. Chilling process (< 4 °C) helps to inhibit the growth of *Salmonella*. Through our observation, the hawkers used purified water from a purified filter system and reverse osmosis drinking water in the making of fruit juice. This further minimizes the contamination of *Salmonella* as the water had been treated.

The nature of apple, orange and guava growing on trees minimise the chances for the fruits to harbour contaminants from soil. A study by Ukuku and Sapers (2006) stated that the smooth surface of honeydew and watermelon provides less microbial attachment sites and thus the microorganisms can be easily washed off during washing treatment. Besides, adequate amount of sugar in food and drinks can absorb water content which leads to the deterioration of bacterial growth (Trickett, 2001).

CONCLUSION

This study highlighted the absence of *L. monocytogenes* and *S.* Typhimurium in the fruit juices samples tested. Overall, this study serves as a preliminary study on the current safety level of fruit juices consumption (towards *Listeria* spp. and *Salmonella* spp.) purchased from local fruit stalls and restaurants. The absence of *L. monocytogenes* and *S.* Typhimurium in this study may be due to hygienic and sanitary production and processing practises at the sampling premises assist in reducing the possibilities of foodborne bacterial contamination. Proper temperature and shorter storage period of fruits are also encouraged to further reduce microbial contamination on ready to eat fruits.

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