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Growth Simulation and Risk Assessment of *Listeria monocytogenes* in Chicken Liver at Refrigeration Condition

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ABSTRACT

Listeria monocytogenes is an important emerging foodborne pathogen with infections causing high mortality rates (20-30%). It can cause sporadic but serious cases of listeriosis especially among pregnant women and the elderly. Emergence of multiple antibiotic resistant strains further complicate treatment of listeriosis cases. The goals of present study include to assess the risk of listeriosis among general population, pregnant women, elderly and immunocompromised from contaminated chicken liver consumption in Malaysia. *L. monocytogenes* growth data at temperature 4°C were used to determine the probability of listeriosis from chicken liver consumption using exponential model. The data obtained was used to calculate probability of infection among vulnerable groups (pregnant women, elderly and immunocompromised). Monte Carlo simulation with 10 000 iteration was used to determine the distribution of expected cases and rate of infection. Monte Carlo simulation showed *L. monocytogenes* in chicken liver at refrigerated increased by 20.50 fold. Immunocompromised patient was estimated to show the highest risk of listeriosis followed by pregnant women, elderly and general population. This study concluded that *L. monocytogenes* presence in chicken liver and inadequate safe food practices that cause cross contamination pose significant risk of listeriosis among the consumers.

Keywords: Chicken liver, *Listeria monocytogenes*, Monte Carlo simulation, high risk population, risk characterization.

INTRODUCTION

Recently, the amount of accessible edible meat offal has expanded significantly from slaughterhouses, meat processors and wholesalers (Darine et al., 2010). Chicken gizzard, chicken heart and chicken liver are the common poultry offal consumed by Malaysians as a side dish for rice which is also a cheap source of protein (Kuan et al., 2013). After World War II, an alternative to beef was needed to address the issue of food shortage and poultry meat become one of the alternatives, the liver which is also part of the poultry products is regarded

as the important economic and nutritional resource that rich in protein, iron and vitamin A (Rüegg & Dimenstein, 2018). Offal is deemed as a good source of protein and now considered as delicacies because it is able to resolve the issue of protein malnutrition and food insecurity in many countries (Alao et al., 2018). In Malaysia, there are many Japanese Yakitori restaurants operating in the city of Kuala Lumpur and based on Japanese eating culture, all of the edible chicken offal are made as Japanese traditional dish or chicken offal skewers (Seong et al., 2015). The demand of chicken by-products which includes chicken offal is increasing because its low-cost, low-fat content as well as the short preparation time needed (Alvarez-Astorga et al., 2002). In Malay food, satay or skewered marinated meat or offal grilled over charcoal is very popular in Malaysia, Indonesia, Thailand and Singapore (Noorman, 2020).

As chicken liver is one of the by-products of the poultry sector, *L. monocytogenes* is possible to be isolated from chicken offal because poultry flocks are deemed as one of the main vehicles of *Listeria*. *L. monocytogenes* is widely present in the environment and commonly referred as a saprophyte that is mostly adapted to soil, water and plants (Schoder et al., 2014; Linke et al., 2014). Therefore, the widespread nature of *L. monocytogenes* can lead to the contamination of poultry carcasses and the processing facilities (Chiarini et al., 2009). The two main sources of *L. monocytogenes* contamination of poultry by *L. monocytogenes*, first, environmentally; secondly, unhygienic conditions during production or mishandling (Fallah et al., 2012). *L. monocytogenes* is a causative agent of listeriosis which means it can lead to self-limiting or non-invasive gastroenteritis to general population, however, it can lead to adverse effects such as sepsis, meningitis, neonatal infections and miscarriage to the high-risk groups such as elderly, immunocompromised patients and pregnant women (Halbedel et al., 2020). The incidence rate of listeriosis among pregnant women (3.42 cases per 100,000 population) in the USA was higher than in adults aged more than 65 years (1.21 cases per 100,000 population) (Xu et al., 2017). The vulnerability among these groups is different as the World Health Organization reported that the elderly has 2.6 times higher susceptibility compared to the general population while pregnant women has 17 times vulnerability as compared to the general population (Dumitrascu et al., 2020).

Potential outbreak of listeriosis in Malaysia is still poorly understood because most cases are sporadic (Hernandez-Millian et al., 2014). Besides, the intake of contaminated poultry is rarely associated with high incidence of listeriosis and the data on the probability of occurrence of *L. monocytogenes* on poultry products or chicken offal is very scarce (Chlebicz et al., 2018). This is because for a foodborne disease case to be recognized by health authorities, it requires a long process which is initiated when patient seek medical care, followed by appropriate clinical test by physicians, detection of the relevant pathogens by the laboratory, and the test result must be reported in a timely manner (Haas et al., 2014).

When managing the risks linked to *L. monocytogenes* in food, the understanding about the probability of acquiring listeriosis after consumption of a given amount of *L. monocytogenes* cells is necessary (Hoelzer et al., 2013). Pathogens are commonly known to grow in food stored at danger zone temperature ranging from 5 to 60^{o}C (Ricci et al., 2020) and growth inhibit below 4^{o}C (Yip, 2024). However, *L. monocytogenes* is psychrophilic bacteria that capable of growing at refrigeration temperature (0 - 4^{o}C). Its ability to grow at these temperatures has defeated the goal of refrigeration to inhibit the growth of bacteria and pose significant risk of food contamination which will result in foodborne illness. Risk assessment of *L. monocytogenes* based on its growth characteristics at refrigeration temperature will provide insight on the potential risk of foodborne illness from consuming contaminated chicken liver. This study aimed to simulate the growth of *L. monocytogenes* in chicken liver stored at refrigeration temperature and estimate the risk of listeriosis among different population group (general population and high risk population) from contaminated chicken liver consumption.

MATERIALS AND METHODS

L. monocytogenes Growth in Chicken Liver

Inoculum preparation

L. monocytogenes ATCC 7944 and isolate AMPG1 from our previous study (Wai et al., 2020a) was revived in 10 ml of Tryptic Soy Broth (TSB) (Merck, Germany) for 24 h at 37°C. The isolate was purified using selective PALCAM agar (Merck, Germany). Pure *L. monocytogenes* culture was grown in TSB (Merck) for inoculum preparation as described in previous study with slight modification (Tang et al., 2017). The density of bacteria was determined by light spectrophotometer (600 nm wavelength) and the inoculum was approximately 9.20 log CFU/ml. The inoculum of *L. monocytogenes* was determined using light spectrophotometer at 600 nm wavelength and at OD 0.99, it corresponds to 9.20 log CFU/ml.

Survivability at refrigeration condition

L. monocytogenes isolate AMPG1 was used to determine the growth pattern in chicken liver in comparison to *L. monocytogenes* ATCC 7455. *L. monocytogenes* in saline served as a control. Three grams of chicken liver was placed in universal bottles. A total of 20 μL of inoculum was inoculated onto the chicken liver and control (saline). The experiment was tested in a chiller (4°C) over a period of 7 days. Numbers of *L. monocytogenes* was enumerated using a spread plate method on PALCAM agar (Merck, Germany) on day 0, 1, 3, 5 and 7.

Statistical analysis and Simulation

Results for *L. monocytogenes* survivability on chicken offal were analysed using ANOVA test on SPSS 17.0 software. Findings with a p-value < 0.05 were considered to be statistically significant. Simulation of probability of infection based on the survival experiments were performed using Microsoft Excel with Argo add-in function.

Risk Assessment

Statement of purpose

This study aimed to determine the risk of listeriosis from consumption of chicken liver stored at 4°C. The calculation of risk estimate was based on data from this study, previous study (Goh *et al.,* 2014), and other studies and government data. The likelihood of listeria infection from chicken liver consumption was calculated with consideration of all the assumptions and uncertainties which were clearly stated.

Hazard identification

L. monocytogenes is ubiquitous in the environment and contaminate various food products (Buchanan *et al.,* 2017). Studies have found *L. monocytogenes* present in chicken liver from 2 to 25% (Kuan *et al.,* 2013; Wai et al., 2020a). Studies have shown food stored at danger zone temperature (between 5°C to 60°C) led to of listeriosis incidences among high risk population such as young children, pregnant woman, elderly and immunocompromised (FDA/FSIS, 2003, Kuan *et al.,* 2015). However, *L. monocytogenes* is psychrophilic bacteria that grows at refrigeration temperature which pose significant risk of pathogen growth in numbers during storage.

Hazard Characterization

L. monocytogenes generally cause mild and febrile illness in healthy adult with symptoms include fever and diarrhea (Aureli *et al.,* 2000). However, it is known to cause serious illness in susceptible groups such as meningitis and sepsis (Buchanan *et al.* 2017). In healthy individuals, around 10 to 100 million cells are needed to cause illness but only between 0.1 to 10 million cells can cause disease in susceptible groups. Invasive listeriosis which resulted in severe symptoms and fatality is more common in susceptible groups (Buchanan *et al.,* 2017). This study investigates the potential risk from chicken liver that are stored at recommended storage temperature (4°C).

Dose response model FAO/WHO (2004) used for estimating the probability of listeriosis in this study is shown below:

$P_{illness} = 1- exp^{-r*N}$ Equation (1)

where

r = a variable that describes the dose/response relationship

N = amount of microbes ingested.

The probability of illness among the high risk groups is assumed from ingestion of single *L. monocytogenes* cell (Buchanan et al., 2000).

However, if the likelihood of a single organism to cause infection is relatively small, assumption is made based on the value of the parameter, "r" (Chen et al., 2003) as shown in Table 1.

Table 1. Dose-response relationship for *L. monocytogenes* for different high-risk groups.

High-risk Groups	Dose- response relationship (r- value)	Reference
General population	8.5×10^{-16}	FAO/ WHO (2004)
Immunocompromised	5.6×10^{-10}	Lindqvist and Westwoo (2000)
Pregnant women	2.6×10^{-11}	FDA/FSIS (2003)
Elderly (> 60 years old)	8.4×10^{-15}	FDA/FSIS (2003)

The probability of illness per year was calculated based on the formula described in Haas et al., (2014):

Probability of illness per year $= 1 – [1 – P_{illness}]^{fA}$ Equation (2)

where fA = the frequency per year

Exposure Assessment

The exposure assessment conducted in this study was based on the survivability of *L. monocytogenes* (log cfu/g) in chicken liver with reference to a previous study on the transmission of the bacteria from raw to cooked samples after contact with food contact surfaces (Goh et al., 2014). An average of 44% transmission percentage was used in this study to cover all the possible scenarios. The framework of exposure assessment was shown in Figure 1.

Probability of illness from consumption of contaminated chicken liver was calculated based on the growth pattern during storage at refrigeration and room temperature.

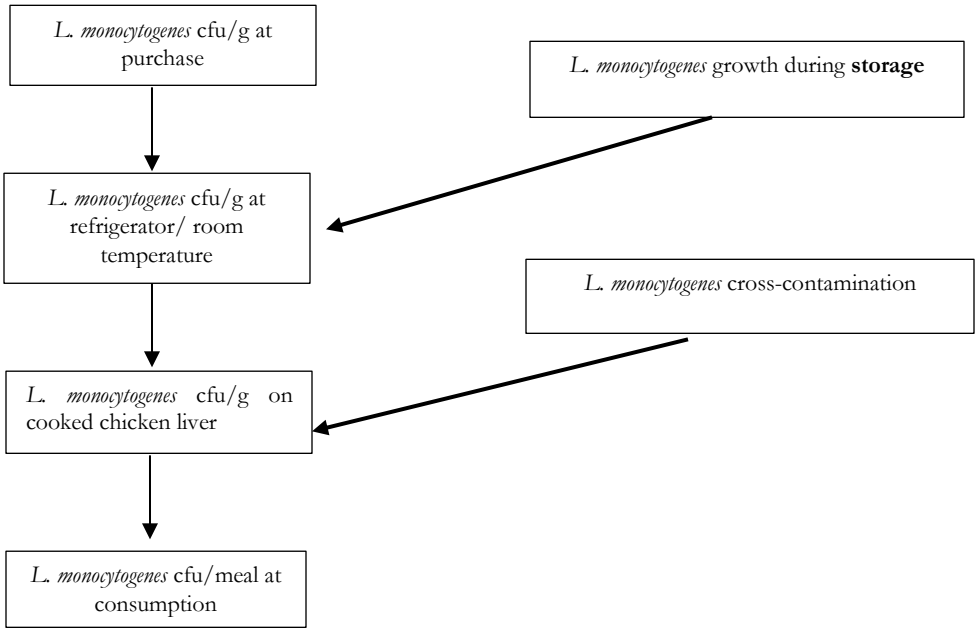

Figure 1. Post-purchase exposure assessment of *L. monocytogenes* in chicken liver stored at refrigerator temperature.

Risk characterization of L. monocytogenes

The dose-response model was used to estimate the risk based on different storage temperature and storage time which was calculated from the data generated in this study and assumptions based on data from other studies. The model simulation was calculated with Argo software as Microsoft Excel add-in (Booz Allen). All of the calculations were performed by the Monte Carlo method of sampling from specified input distributions in order to generate the output distributions and 10,000 iterations were undertaken.

RESULTS AND DISCUSSION

Survivability of L. monocytogenes in Chicken Offal

The mean values from the survivability data of chicken offal at cold temperature (4°C) were required for the risk estimates for acquiring listeriosis from chicken offal consumption. The mean values were obtained from the survivability study of *L. monocytogenes* on the chicken liver. The experiments were performed in triplicates. Based on the statistical analysis result, the mean value obtained for cold temperature (4°C) was approximately 0.40 log CFU/g.

The survivability study of the control set, *L. monocytogenes* ATCC 7644 and AMPG1 at 4°C for 7 days period was used to simulate the condition of chicken offal that was stored at prolong storage. From Table 2, *L. monocytogenes* isolates ATCC 7644 and AMPG1 were found to grow significantly ($p<0.05$) after day 1. Based on the simulated distribution result, the number of *L. monocytogenes* (CFU) cells of isolate AMPG1 were able to increase by 20.50 folds after 3 days incubation at 4°C which was showed in Figure 2.

Table 2. Survivability of *L. monocytogenes* ATCC 7644 and isolate AMPG1 on liver at 4°C for 7 days.

Day	Control (Saline) (log CFU/g)	*L. monocytogenes* ATCC 7644 (log CFU/g)	*L. monocytogenes* Isolate AMPG1 (log CFU/g)
0	6.14 ± 0.08^{Aa}	6.08 ± 0.11^{Aa}	6.36 ± 0.13^{Aa}
1	6.25 ± 0.10^{Aa}	7.27 ± 0.11^{Bab}	7.28 ± 0.13^{Bab}
3	6.48 ± 0.15^{Aab}	8.20 ± 0.25^{Bbc}	8.33 ± 0.25^{Bbc}
5	6.71 ± 0.02^{Abc}	8.60 ± 0.35^{Bc}	8.49 ± 0.21^{Bc}
7	7.01 ± 0.08^{Ac}	9.14 ± 0.43^{Bc}	9.17 ± 0.38^{Bc}

[A, B] Data in the same row with different letter is different significantly ($p < 0.05$).
[a, b] Data in the same column with different letter is different significantly ($p < 0.05$).

Prolonged refrigeration that frequently occurs in the food industry, will select *L. monocytogenes* variants with enhanced cold tolerance. *L. monocytogenes* may also acquire such mutations during cold exposure in the natural environment, therefore, *L. monocytogenes* isolates from this study showed growth in the survivability study (Hingston et al., 2019). A study by Sheng et al. (2017) showed that food industry should not rely on cold storage alone in controlling this pathogen as they proved that *L. monocytogenes* was able to survive on apples at 4°C with no significant reduction for 12 weeks in refrigerated storage. Besides, another study reported that at 4°C, *L. monocytogenes* can grow by 2.0 log cycles during extended storage (30 days) on frankfurters while during the recommended shelf-life of 7 days for frankfurters, *L. monocytogenes* population still can increase by 1.5 log cycles at 4°C (USDA-FSIS, 2006). Based on the observation, the chicken offal in the supermarket were kept for three days and stored in cold temperature. At lower temperature, the growth of *L. monocytogenes* might slow down, but once low level of *L. monocytogenes* contaminated the food products, the microorganism can increase to a dangerous level that pose risk to consumers (Lakicevic et al., 2015).

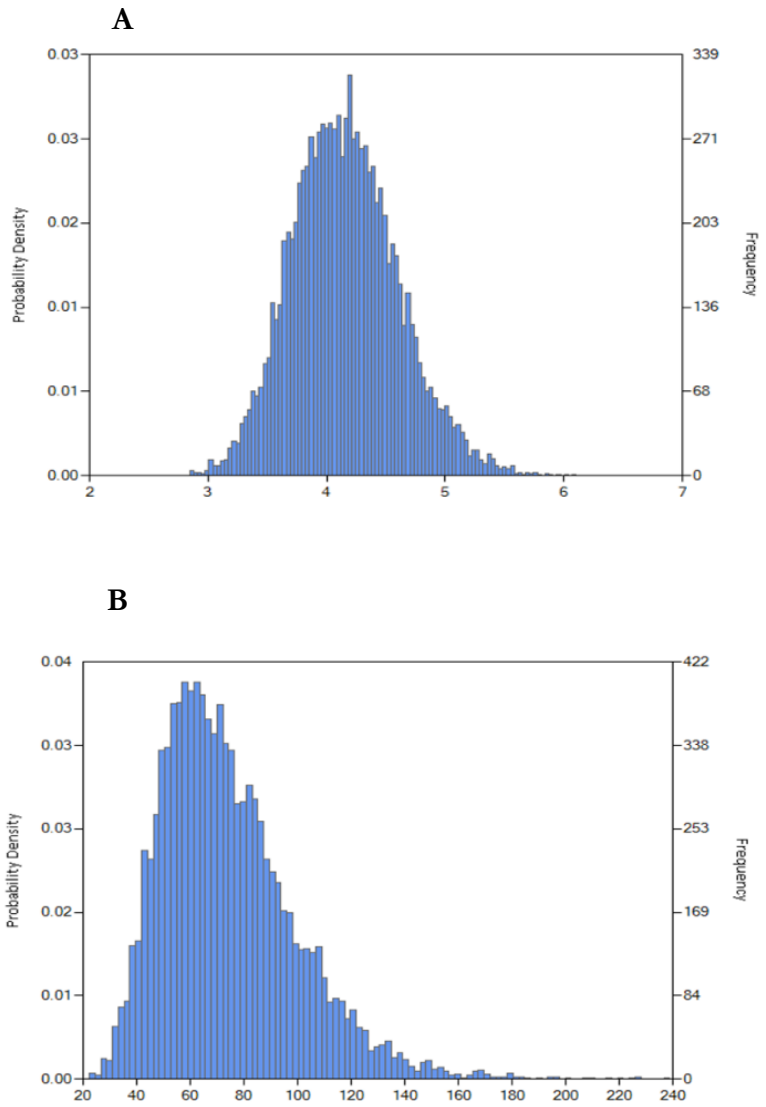

Figure 2. Simulated distribution of *L. monocytogenes* (AMPG1) on liver at 4°C for 3 days. A) Day 1 at 4°C, Mean = 4.16 CFU; B) Day 2 at 4°C, Mean = 85.30 CFU

The exponential risk assessment model was used to estimate risk of prolonged storage and temperature abused condition. The details of the risk analysis framework based on the exponential model was summarized in Table 3. The differences in the population, population that consume liver and r-value (dose-response) among general population, pregnant women, elderly and immunocompromised patient indicate the susceptibility of the host toward listeriosis.

Table 3. Risk analysis framework based on exponential model.

Description	Formula/model/value	Units	Reference
Initial concentration (In_{Conc})	10 (PCR) or 100 (Plating)	CFU	This study
Growth at 4°C (G_4)	0.40±0.11	Log CFU/day	This study
Growth at 28°C (G_{28})	0.18±0.04	Log CFU/h	This study
Percentage of *L. mono* transfer from raw to cook samples ($\%_{Tr}$)	44.0	%	Goh et al., 2014
Dose (d)	anti-Log(Log(In_{Conc}) x (G_4 or G_{28}) x incubation duration x $\%_{Tr}$/100)	CFU	This study
frequency per month (f_M)	3	--	NMHS 2014
frequency per year (f_A)	f_M x 12	--	NMHS 2014
Percentage population consuming offal ($\%_{ConOff}$)	29.8	%	NMHS 2014
Population KL & Selangor	7 250 000	--	DOSM 2020
Population that consume offal (N_{offal})	$\%_{ConOff}$/100 x 7 250 000	--	This study
$r_{general}$	8.5 x 10^{-16}	--	FAO/WHO (2004)
$r_{pregnant}$	2.6 x 10^{-11}	--	FAO/WHO (2004)
$r_{elderly}$	8.4 x 10^{-15}	--	FAO/WHO (2004)
Probability of illness ($P_{illness}$)	1- exp^{-r*d}	--	FAO/WHO (2004)
$P_{illness}$ per year	1 - [1 - $P_{illness}$]^{fA}	--	Haas, Rose and Gerba, 2014
Expected cases per year (E_{Case})	$P_{illness}$ per year x N_{offal}	--	Haas, Rose and Gerba, 2014
Rate illness per 100 000 population	E_{Case} x 100 000/7 250 000	--	Haas, Rose and Gerba, 2014

Based on the result for the refrigerated chicken offal (4°C) stored for 3 days (Table 4), the rate of illness per 100,000 was the highest for immunocompromised group (2.92× 10^{-2}), followed by pregnant women (9.78 × 10^{-4}), elderly (4.02 × 10^{-7}) and general population (5.22 × 10^{-8}).

Table 4. Probability of *L. monocytogenes* illness among Kuala Lumpur and Selangor population from refrigerated chicken offal stored for 3 days.

Description	General population	Pregnant woman	Elderly	Immunocompromised patient
Concentration				
Initial *L. monocytogenes* number	10	10	10	10
Growth (4^{o}C)	0.40	0.40	0.40	0.40
Percent transfer	44%	44%	44%	44%
Probability of transfer	0.44	0.44	0.44	0.44
Dose	85.30	85.30	85.30	85.30
Population				
Population in Kuala Lumpur and Selangor	7,250,000	28,444	522,000	261,000
Population consume offal	2,161,950	8,476	155,556	77,778
Probability				
r-value	8.5×10^{-16}	2.6×10^{-11}	8.4×10^{-15}	5.6×10^{-10}
Probability of illness single consumption	6.74×10^{-14}	1.26×10^{-09}	5.19×10^{-13}	3.77×10^{-08}
Probability of illness per year	1.75×10^{-12}	3.28×10^{-08}	1.35×10^{-11}	9.80×10^{-07}
Expected cases per year	3.78×10^{-06}	2.78×10^{-04}	2.10×10^{-06}	7.62×10^{-02}
Rate of illness per 100 000 population	5.22×10^{-08}	9.78×10^{-04}	4.02×10^{-07}	2.92×10^{-02}

In Australia, a quantitative risk assessment study reported that the risk of listeriosis from consumption of processed meat was 1.00×10^{-8}, 2.28×10^{-9} for pâtés and 7.06×10^{-9} for cooked sausage, while pâtés and cooked sausage presented a lower risk of listeriosis when compared with that of present study (Ross et al., 2009). Besides, study by Sun et al. (2008) reported that the average risk from consuming cooked meat in bulk products for children population was 8.24×10^{-7}, young people was 2.58×10^{-8}, elderly was 8.24×10^{-7} and pregnant women was 1.05×10^{-6} per meal and the result was in agreement with the present study that showed high risk population (pregnant women, elderly and infant) had a higher risk of listeriosis than low risk population (young adult). In China, the estimated number of cases of listeriosis each year per 100 000 people by consuming meats was 5.53×10^{-3} for children (0-4 years old), intermediate age (5-64 years old) was 1.7×10^{-4} and for elderly was the highest (7.57×10^{-3}). The sensitivity analysis showed that contamination level at retail ($r=0.607$) was the highest positive risk factors of listeriosis compared with storage time at home ($r=0.339$) and storage temperature ($r=0.257$) (Tian et al., 2011). Recently, a study reported that based on Monte Carlo simulation, the median number of listeriosis per year associated with consumption of chicken salad was 8.73×10^{-5} and the risk assessment showed that *L. monocytogenes* was able to grow in chicken salad at ambient temperature and refrigerated temperature (Bernardo et al., 2020). The result was in agreement with the findings of present study showing that chicken liver supported the growth of *L. monocytogenes* at ambient temperature and refrigerated temperature.

The relationship between strain virulence and host susceptibility was reflected by the average probability of a single *L. monocytogenes* CFU to cause illness in a targeted host (r- value) and the value varies from the least (general population without any underlying sickness) to the most susceptible subpopulation (i.e. haematological cancer), as a result, the probability of illness depending on the variability in host susceptibility and *L. monocytogenes* virulence (Ricci et al., 2018). The susceptible population (pregnant women, elderly and individuals with compromised immune system) were involved in present study because they have higher risk of getting invasive listeriosis (Buchanan et al., 2017). A study reported that 90% of the cases for bacteraemia of *L. monocytogenes* in Spain was associated with immunosuppressed patients including pregnant women (Hernandez-Milian et al., 2014). Besides, Lamont et al. (2011) described that listeriosis associated with pregnant women could be 18 times higher than general population, which was in agreement with the present study that showed the estimated risk of listeriosis from pregnant women was higher than from general population. Immunocompromised individuals such as patients on haemodialysis, patients undergo organ transplants, diabetes patients and HIV carriers have higher risk of getting invasive listeriosis likely due to impaired cell mediated immunity (Hernandez- Milian et al., 2014). From 1992 to 2013, most of the sporadic non-clustered cases of hospital associated listeriosis (91.4%) happened on immunocompromised patients and they had several diseases associated with immune deficiency (Lee et al., 2014). Elderly consumers are at risk of listeriosis due to improper food handling practices in their household kitchen, besides, they do not aware that domestic food practices are actually associated with the risk of listeriosis (Maia et al., 2018).

CONCLUSION

The study concludes that *Listeria monocytogenes* contamination of chicken liver stored at refrigerated temperatures poses serious health hazards. This pathogen's presence in chicken liver can cause major health problems especially for susceptible populations like the elderly, pregnant women, and people with weakened immune systems. The growth patterns shown under refrigeration highlight *L. monocytogenes'* adaptability and capacity to flourish in extended refrigeration storage settings, underscoring the drawbacks of using cold storage alone as a preventive strategy. Consumer protection depends on efficient methods for lowering contamination risks, such as careful monitoring and safe food handling procedures.

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