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Review on Protocol Development for Detection and Identification of *Listeria monocytogenes* in Foods

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Occurrences of the Gram positive pathogenic *Listeria monocytogenes*in foods are often found to be low in level with the possibility of being in an injured state due to the food processes. Various standard protocols have been established to enable the recovery and identification of*L. monocytogenes*cells from foods. Even though *L. monocytogenes* is a comprehensively studied bacterium, due to its high mortality rate in immunocompromised individuals, the numerous studies carried out on recovery methods each proposed a different enrichment medium for different injuries implicated on the cell and that the existing protocols were shown to have insufficient efficiency in recovering the pathogen. The differences in recovery inefficiencies by each protocol may result in underestimation of *L. monocytogenes*in food. This review looks into the development and comparison studies of recovery methods for *L.monocytogenes*which may be useful as a reference on protocol and modification methods to be adopted for recovery as well as standardizing future optimization and developmental studies on culturing and resuscitating sub-lethally injured *L. monocytogenes*from foods.

Key words: *Listeria monocytogenes;* Recovery; Resuscitation; Identification methods; Sub-lethal injury.

*Listeria monocytogenes*is a major foodborne pathogen of public health concern because of the potential to cause a wide range of health complications in the immunocompromised or susceptible individuals.Human listeriosis are the consequence of the infection which are characterized by flu-like symptoms but may be severe, manifested as meningitis, septicaemia, spontaneous abortion or stillbirth and mortality rate as high as 30% [1]. *L. monocytogenes*have adaptive responses when exposed to sublethal food processing interventions which may contributed to the contamination of foods[2-4]. For example, Shabala *et al*.,[5] indicated that the acquired acid tolerance of *L. monocytogenes*have important implications on survival of *Listeria* in the environment and foods. *L. monocytogenes*were found to survive for more than 20 hours at the pH level of 3.5 and 4.0 in the absence of glucose, which pH 6.0 that was initially used in the experiment may have contributed to the adaptive acid tolerance response of *L. monocytogenes* following exposure to mild acid in their study. The work

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presented the high capability of *L. monocytogenes* to tolerate acidic environment and the ability to sustain a large pH change which was initially thought to be between 0.5 to 0.7 pH unit change from the intracellular pH. Shabala *et al*.,[5] showed that the survival of cells at lower pH was dependent on the glucose concentration in the minimal media.

Development and changes to existing protocols have contributed to the improvement of the sensitivity and isolation of *L. monocytogenes*in food. The trend of the media used as well as modifications to the methods showed the more frequent application of USFDA methods as well as incorporation of chromogenic medium have obvious impact on the selection of protocols used. For example:

Publications from the 1980s showed methods using tryptose broth or 2% trisodium citrate aqueous solution in 20°C and 45°C in cold enrichment procedure on food samples such as cheese have enhanced recovery of *L. monocytogenes*in higher temperature of 45°C[6]. In Golden, Beuchat[7], study on six types of media used in series of concentration of injured cells plated on McBride *Listeria* agar with 5% sheep blood (MLA), modified MLA, GBNTSA (gum-base-nalidixic acid-tryptone-soya-agar) and modified Despierres agar MDA[8] showed that for freeze injured cells in pasteurized milk and chocolate ice cream mix, MLA and GBNTSA were the media of choice in the study.

In some publications from the 1990s, Yu and Fung[9] compared the protocols between FDA and USDA procedures using; i) LEB enrichment followed by McBride Agar (MMA) and ii) UVM followed by LPM agar and iii) FB followed by modified Oxford agar (MOX) in ground beef. Findings reported the highest efficiency of recovery was for LEB followed by FB and then UVM, and the best combination was LEB with MOX agar. In Osborne and Bremer[10], enrichment broth for heat injured cells at 54°C for 30 mins also reported highest recovery on LEB, followed by mUVM , UVM and FB. For findings in Jiang, Larkin[11], universal pre-enrichment broths were evaluated for heat treated bacterial strains. Five universal broths (Buffered peptone water, TSB, NB, LEB and Universal Pre-enrichment broth –UPB) were used together to recover induced injury cells in UHT milk and cheese. Report on the unstressed culture showed highest count of colonies in LEB after 24 hours from initial inoculums of 10^1cfu/ml to 10^6cfu/ml, however, for the heat injured cells, recovery was found to be better for UPB after 24 hours in 35°C. The study concluded that growth of *L. monocytogenes*were slower in LEB after 18 hours.

From the year 2000 onwards, multiple studies have addressed the various factors of cell stress and tested the efficiency of enrichment regimes. In Mendonca and Knabel[12], several stress factors such as heat, pH, alcohol, sugar and salt brine as well as frozen storage were tested on the five enrichment media which are LRB, BLEB, LEB, FB and UVM on different food matrix. Heat-treated mussels, alcohol-soy marinade salmon in 4°C storage both showed highest recovery of cells in BLEB; sugar and salt brimmed salmon with highest recovery effiency in LRB and BLEB; and frozen Hoki fish fillet recovered in LRB. BLEB/LEB was found to have recovered different types injured cells more effectively than other broths in this study. In addition to that, in Bull, Hayman[13], effect of hydrostatic pressure on recovery of cells were studied, comparing Listeria enrichment broth (LEB), BLEB, mBLEB, oPSUB (optimized Penn State University broth) from skim and whole raw milk that was subjected to pressure processing. Reported in Vlaemynck, Lafarge[14], in 2006, publication on utility of chromogenic agar for detection of listeria was reported. Findings showed that when compared OCLA, Oxford agar and ALOA, sensitivity of agar in reculturing listeria cells from milk, pate, salmon and lettuce using the ISO 11290 1:1996 method showed that Oxford agar has the highest recovery at 24 and 48 h of enrichment procedure.

Further studies were conducted to evaluate the increased efficiency of dual primary enrichment of environmental samples[15]. In this study, mUSDA-PCR method showed the highest number of recovery. This method used the primary enrichment of two broths, UVM and LRB with second enrichment MOPS-BLEB, followed by selective plating on MOX agar.

Contamination in foods as sublethally injured cells or VBNC

It has been reported that a wide variety of foods have been known to be contaminated with *L. monocytogenes*such as meat, dairy products such as cheese[16], fresh produce[17] and even

processed ready-to-eat foods[18, 19]. The sources of contamination may be from manure used as fertilizers and asymptomatic carriers in animals which are the consumed by the population. Although processing stages such as pasteurization and cooking may decrease or kill off *L. monocytogenes,* contamination can still occur after cooking and before packaging[20]. The occurrences of *L. monocytogenes*in low number may be an unavoidable due to its ubiquitous nature however; the pathogen is able to persist in harsh environment for an extended period of time therefore rendering it resilient in food matrix. Studies has shown that *L. monocytogenes*are able to grow at in low oxygen levels or anaerobic conditions (refer to review by Lungu *et al*.,[21] as well as refridgeration temperatures[18]. Other studies conducted to profile the tolerance and persistence of *L. monocytogenes*to the various environmental factors such as temperature tolerance ranging from 0°C to 45°C[22, 23] with "change temperature" which indicated by the increased growth rate between 10°C to 15°C[24], pH range tolerance between 4.1 to 9.6 [5,22, 25], oxidative stress[26] and pressure[27] and the persistence was due to the phenomenon of adaptive response by the response prior exposure to a form of injury (refer review by Sergedilis and Abrahim[28].

*L. monocytogenes*can enter viable but non-culturable (VBNC) state when subjected to adverse environment such as starvation, temperature change, salinity, oxygen saturation as well as solar illumination[29]. The few factors studied in inducing VBNC state were pH, which was found to be a neglected factor, temperature and NaCl concentration which was identified as important factors inducing the loss of colonies formation. The findings also showed that 'strain effect' which means different susceptibility to form VBNC state based on strain types; such as Scott A strain requiring low temperature at 4°C compared to the other strains at 20°C and may remain in the state for up to 100 days.The major concern of sublethally injured cells and VBNC cells is the ability to recover its virulence when passing through the gut (similar to Campylobacter) therefore indicating the risk involved when resuscitation occurs after ingestion of contaminated foods[30]. Persistance of these listeria cells are detected via molecular method but nonculturable as shown in many prevalence studies detecting the presence of *L. monocytogenes* in food but the failure in isolating the bacteria[17, 31].It may be noteworthy to define the difference between sublethally injured cells and VBNC are not entirely similar as VBNC does not necessarily indicate sublethally injured cells.

Recovery media and methods of resuscitation and culturing

There are a variety of broths developed for the recovery of injured *Listeria* cells which have been incorporated into different protocols. Particularly for *L. monocytogenes,* it has to be noted that this bacteria have a low infectious dose of 100 cells in foods and that foods that contain higher concentration should be properly investigated. Regulatory bodies have set the limit of tolerance to 100cfu/g in ready-to-eat foods[32] and in certain regulation, zero tolerance is imposed[33]. Due to the low concentration of *L. monocytogenes*in foods, it is necessary to develop broth that will not only recover the cells, but increase the number to a detectable limit to prevent the underestimation of the pathogens. Composition of broth plays an important role in aiding the recovery of *L. monocytogenes*cells. Besse[29] identified some factors affecting the resuscitation of injured *L. monocytogenes*cells used in broth compositions such as sugar content, divalent cations content, yeast extracts, salt and osmotic pressure, pH and usage of liquid rather than solid media has been identified as the few factors that plays an important role in cell recovery.

Several important compositions of broths played a role to aid the recovery of *L. monocytogenes* cells

Sugar content

Glucose is known to be an energy source for repair mechanism and helps in the re-concentration of the amino acids to enable the repair of the cell membrane[34]. However, in several studies has shown that this may not be applicable to *L. monocytogenes*recovery. Studies by Busch and Donelly[35], heat injured *L. monocytogenes*at 56°C in Tryptone Phosphate broth for 50 mins with 0.5% glucose, lactose, sucrose, mannose, fructose, galactose and esculine shown to enhance the repair of *L. monocytogenes*within 5 hours whereby concentration of glucose 0.5% showed 51.4% of log10 CFU increase. Their results also showed that higher concentration of sugar did not correspond

to improvement of cellular repair.Similarly in Teo and Knabel[25], heat injured *L. monocytogenes*at 62.8°C, 20 mins in milk with 0.25% glucose content in Penn State University Broth reported that the removal of the glucose from PSU broth did not inhibit the resuscitation of heat-injured *L. monocytogenes*. Osborne and Bremer[10] did a comparison on recovery efficiency of 5 broths, (LEB, BLEB, FB, UVM and LRB) using *L. monocytogenes*cells injured with alcohol, pH,heat, and freeze treatment, and found, amongst the five broths, LRB with the highest glucose concentration (0.75%) has the least recovered heat injured cells for *L. monocytogenes.* Since multiple studies have shown that contradicting results of recovery of injured cells, this may indicate the preference of availability of glucose as recovery broth may not be the priority.

Divalent Cations Content and Oxygen Level

Ions such as magnesium ions (Mg^{2+}) has been known to aid the recovery of injured bacteria in the role of stabilizing the ribosomes and cell membranes through activation of Mg^{2+}dependent enzymes. Injured cells contain decreased catalase and superoxide dismutase activities, therefore; they are more susceptible to hydrogen peroxide and superoxide radicals. Superoxide dismutase plays an important role as an enzyme in most aerobic organism to convert the toxic O_2- radicals to H_2O_2, which is toxic to other species. Catalase functions as a detoxification of the hydrogen peroxide (H_2O_2) to H_2O and O_2 [36]. The catalase and superoxide dismutase activities are known to be activated upon heating at 60°C[37]. Findings of Busch *et al*.,[35] with addition of 1% sodium pyruvate or 0.04% catalase showed that the cells increase from 2.9 10^2 to 1.5 × 10^4cfu/ml in pyruvate and 1.2 × 10^2 to 7.4 × 10^3cfu/ml in catalase. In studies by Knabel *et al*.,[38] heat injured cells were tested on TSB with additional magnesium, iron, calcium and manganese ions, against a TSB treated for removal of ions as control. Findings showed that the control study had no repair in injured cells while the ion that contributed to the highest repair is Fe^{2+} while manganese killed the cells. Addition of MOPS-buffer to replace the phosphate salt in Penn State University broth also showed the enhancement of the percentage of detection for the heat injured cells of 62.8°C in 20 mins of filter sterilized milk. Teo and Knabel[25] showed that the excess of magnesium ions may disrupt the divalent metals among anionic groups in teichoic acids.Therefore, when the salts were replaced with MOPS buffer, it enabled the detection in anaerobic condition. Studies by Mendoca and Knabel[12] reported that addition of LiCl to the Penn State University broth decreased the growth of *E. faecium* in the co-culture of heat injured *L. monocytogenes.* The mechanism of LiCl was reported as a involvement in the competition of divalent cations Ca^{2+} and Mg^{2+}. The heat injured *L. monocytogenes*in their studies recovered and grew under concentration of 7g/L of LiCl and allowed the detection of injured *L. monocytogenes.*

Salt and osmotic pressure

Salt concentration and osmotic pressure of cultures can cause osmotic downshock in hypertonic salt solution and damage the cytoplasmic membrane as shown by the level of fluorescence of ethidium bromide in a study by Robinson *et al*.,[39]. In their study, cultures were subjected to NaCl stress (2.2 mmol/L) based on the uptake of ethidium bromide in the membrane permealization. It was found that recovery ability was inversely related to uptake of ethidium bromide in cells. Robinson *et al.*,[39] also noted that further injury to the cultures can occur if membrane has not been repaired due to the changes in the dilution protocol prior a viable count procedure. This will prevent the growth of the cultures on TSA.Busch and Donelly[35], also highlighted that the optimal condition of osmotic pressure is at 0.5% NaCl concentration.

Current protocols for culturing L. monocytogenes

A review by Churchill et al. (40) comparing the five most common culture-based methods are: Food and Drug Administration (FDA) protocol which uses FDA enrichment broth(FDA-EB), ISO-11290-1, NGFIS method USDA-FSIS method and lastly, cold enrichment method. It was noted by Churchill *et al.,*[40] that several other studies concluded the media used in the recovery has efficiency even with combined two methods between USDA-FSIS, NGFIS and FDA protocols, the efficiencies of *L. monocytogenes*detection would be increased to 87% to 91% which may not be sufficient particularly when it involves possible fatal consequences. This justifies the need to evaluate the efficiencies of culturing methods as it may provide underestimation of the pathogens in foods.

Most of the protocols available adopted the dual-step enrichment procedure whereby food samples are homogenized in non-selective media or half-strength, followed by the selective agents being added into the samples subsequently plated on selective agar. In the event of initiating recovery of cells, *L. monocytogenes*cells are still susceptible to no growth when subjected to selective agar for culturing purposes. This is because certain selective agar may contain inhibitory agents such as acriflavine hydrochloride, nalidixic acid and cycloheximide which serve to reduce the number of competing flora but may not be lethal to the target organism[41] particularly when the cells are not properly resuscitated. Considerations should be placed in the lag phase which depends on the magnitude of injury on the cell, subsequently to apply the appropriate broth with the right composition to aid the resuscitation phase because injured cells have increased lag phase therefore requiring a longer time to repair[34].

Efficiencies of recovery methods are highly dependent on the ability of the broth to initiate resuscitation via the lag phase of pre-enrichment in various non-selective media. Jasson *et al.*,[30] focused their study on resuscitation kinetics of the growth during enrichment of several broths in healthy and sublethally injured cells. In the study, three selective enrichment broths (One Enrichment broth, *Listeria* Xpress Broth and *Listeria* Special broth) and eight basal broths were selected for the recovery procedures of *L. monocytogenes.* The study reported that *Listeria* Special Broth as the best growth supporting selective medium that is comparable to nonselective medium whereas LX broth and demi-Fraser broth increases the lag phase of injured *L. monocytogenes.* Recovery of these injured cells may require different optimum temperature to repair, for example; Mackey *et al.*,[42] indicated in their study that recovery of heat-injured cells was found to be at the limit of 25°C subjected to the composition of the recovery medium.

Solid media such as agar is used for culturing which indicates the growth of *L. monocytogenes*from the sample. With reference to the extensive studies on the development of broths, many studies inflicted heat stress for the injury of cells. One of the most comprehensive studied forms of injury is the heat injury on *L. monocytogenes.* McKellar *et al.*,[33] studied on the effect of recovery temperature on the heat stressed cells at 58°C, the cells showed significant injury with prolonged exposure to heat and therefore a prolonged repair time. The study reports on a lag time in repair whereby at the end of the lag phase duration, only 10% of the injured cells had recovered salt tolerance. Although the repair for salt tolerance maybe necessary, it is not a sufficient condition for growth[33]. For cells that are exposed to mild heating, a more sensitive site is affected by the injury that study had indicated a potential in expanding the model into multiple sites injury to develop methods for quantitative repair.

In Osborne and Bremer[10], comparison of different cellular stresses on *L. monocytogenes*and the broth used for the best recovery of the cells was carried out. The findings concluded that the effectiveness of enrichment regimes in recovering cells under alcohol- and soy-marinade (alcohol and pH stress), whereby their results showed the broth with lowest recovery was LRB as opposing to the findings by Busch and Donelly[35]. There were no significant differences between the efficiencies of broth. When compared to naturally contaminated products, LEB and FB showed positive results but none in MPN micro-well plate and the amount in the dilution was significantly smaller than the estimation limit compared to the BLEB. However, in this naturally contaminated sample, LRB manage to recover a significantly higher number of *L. monocytogenes*compared to the other media used.

Perspectives in improvement the recovery methods

Although *L. monocytogenes*is a well-studied bacterium, the various findings indicated that there are no suitable universal broths to be adopted for all types of food analyses. The inefficiency of pre-enrichment and enrichment broths in recovering the cells may be a cause of the underestimation of *L. monocytogenes*in foods and studies for recovery and resuscitation particular from food matrix requires a better system or recommendations. The previous studies reported the efficiencies of broth based on a single treatment to induce injury in cells, however, cells existing in the food matrix are often subjected to more processes and more than one injury which may affect the lag phase for resuscitation. Multi-method approach[19] which reported the mild

activation using heat for 20 mins at 60°C subsequently exposed to mild preservation conditions as applied by the food industry showed no difference in the MPN number of cells recovered between heat-treated and non-treated *L. monocytogenes*. In fact, the study also reported that *L. monocytogenes*reaches the detection limit faster at acidified media. Findings reported the low pH imposed more stress on the cells by increasing the time-to-detection limit to three times longer compared to the recovery on 9% NaCl. This stress, however, did not inhibit the recovery of the cells. On the other hand, high concentration of NaCl was found to be a selective environment for the most resistance survivor cells[19] and when such scenario is applied to the food industry, preservation of food after a mild heat treatment may have be a selective nature for the enhanced growth characteristics of *L. monocytogenes*. This multi-method provided a deeper insight to the recovery abilities of *L. monocytogenes*whereby cells subjected to mild heat are able to resist the following stresses, except for acid stress[43]. Therefore, establishment of procedures for provoking sub-lethal injury[41] is necessary to ensure that future recovery or improvements to the culturing method will be able to induce sub-lethal injury. The recommended stress for *L. monocytogenes*were oxidative stress after comparing to heat, cold, freezing, acid and 'food' stress.Lee *et al*.,[44] conducted a multiple stress on *L. monocytogenes*cells and compared the recovery effect of three protocols using PALCAM and TSAye. In their study, temperature induced injury was carried on listeria cells and found that, dual enrichment protocols can further support the recovery of cells better than the other protocols. Findings also highlighted that despite the increased efficiency, the cost involved was also much higher.

It is a debate in choosing the best protocol for comparisons on the types of cell injury and the application of the appropriate broths to resuscitate the cells and improve the microbiological methods for detection and isolation of *L. monocytogenes* from foods. The chronology of method improvements are increasingly advocating the use of ALOA or chromogenic agar which has superior results in, not only recovery, but also in discriminating among the *Listeria* spp.[45,46].For future studies of developing protocols of culturing *L. monocytogenes,* the collective studies have showed that few key ingredients that play a major role in recovery of cells would be divalent cations and oxygen levels.

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