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ORIGINAL ARTICLE

Optimization of broth recovery for repair of heat-injured *Salmonella enterica* serovar Typhimurium and *Escherichia coli* O157:H7

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Keywords
broth recovery, foodborne pathogen, heat injury, injured cell, overlay.

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Abstract

Aims: The purpose of this research was to determine optimum conditions for broth recovery of heat-injured *Salmonella* Typhimurium and *Escherichia coli* O157:H7.

Methods and Results: Exposure to 55°C for 15 and 25 min, respectively, induced cellular injury to those pathogens. Comparison was made with the commonly used overlay method using selective medium for recovering sublethally injured cells of *S.* Typhimurium. For *E. coli* O157:H7, phenol red agar base with 1% sorbitol was used. After cell suspensions were heated at 55°C for selected time intervals, microbes were 10-fold diluted with brain heart infusion (BHI), tryptic soy broth (TSB) and TSB with 0·6% yeast extract (TSBYE) and incubated at 37°C for up to 3 h. At hourly intervals, diluents were plated onto selective medium for recovery. Simultaneously, diluents were plated onto tryptic soy agar (TSA) for recovery of sublethally injured cells. For overlays, diluents were plated onto TSA and overlaid with selective agar after a resuscitation interval. Broth recovery conditions for *S.* Typhimurium and *E. coli* O157:H7 were determined to be 1 h in any of the following broth media: BHI, TSB or TSBYE. When liquid resuscitation was applied to sublethally injured cells in food samples (milk), 1 h was also sufficient time for recovery.

Conclusions: The broth recovery method is a convenient alternative to conventional recovery methods.

Significance and Impact of the Study: Cells sublethally injured by control interventions might not grow on selective medium because they have no resistance to several selective compounds. However, injured cells can recuperate and multiply under conditions sufficient for recovery. To repair and detect heat-injured cells, the overlay method is commonly used but this method has some limitations. This study confirms the effectiveness of liquid resuscitation method on recovery of injured cells. The broth recovery can replace the overlay method due to greater convenience and timesaving.

Introduction

Heat treatment helps ensure food safety by destroying micro-organisms (Song *et al.* 2016). To control contamination of foodstuffs involving foodborne pathogens, thermal-based treatment is considered to be one of the most effective methods of inactivation (Silva and Gibbs 2012). Novel heating methods have been developed and investigated such as microwave, ohmic and radio frequency heating (Jeong and Kang 2017; Kim *et al.* 2017; Ryang *et al.* 2016; Song and Kang 2016a,b). These techniques result in effective pathogen inactivation of various food categories. However, some populations of bacteria inactivated by various control methods can still survive as

injured cells. Although healthy cells can multiply on selective agar containing selective agents, such as salts, acids, dyes and antimicrobials, cells sublethally injured by control interventions might not grow on selective medium because they have no resistance to several selective compounds. However, sublethally injured cells can recuperate and multiply under conditions sufficient for recovery. For this reason, the inactivation efficacy of various interventions could be overestimated (Foegeding and Ray 1992). Therefore, verifying the exact populations of not only healthy but also injured cells is becoming increasingly important.

To repair and detect heat-injured cells, the overlay (OV) method (Hartman *et al.* 1975; Speck *et al.* 1975) is commonly used. After spread plating heat-injured cells onto a nonselective medium such as tryptic soy agar (TSA), plates are incubated for 2 h at the optimum growth temperature of the target micro-organism. In this process, the nutritive, nonselective agar helps sublethally injured cells to repair themselves and resuscitate. Subsequently, 10–12 ml of melted selective agar is overlaid (poured) onto the nonselective agar. After solidification, an additional 22 h of incubation is typically performed for a total of 24 h. However, this method has some limitations, for example, sensitive injured cells can be adversely affected by the mild heat of melted agar (Kang and Fung 1999) and a possible change in oxygenation due to layering on top of inoculated agar may influence microbial growth (Baird-Parker and Davenport 1965; Harries and Russell 1966). Furthermore, additional investigations may be more difficult because colonies are trapped between two layers of agar making isolation difficult (Kang and Fung 2000). Also, there are some organisms for which the overlay recovery method is not suitable. When applying the overlay method to *Escherichia coli* O157:H7 which produces cream-coloured colonies on sorbitol MacConkey agar (SMAC), detecting these colourless colonies between two opaque agar layers is considered to be problematic. Because of this reason, many researchers have used phenol red agar base with 1% sorbitol (SPRAB) for recovery of *E. coli* O157:H7. But the main problem with SPRAB is that it is not a selective medium which means that further confirmation tests are needed.

In general, injured cell recovery methods can be divided into liquid or solid media repair methods. Liquid repair methods use nonselective broth to facilitate repair (Wu 2008). One of the most popular liquid repair systems is the twofold dilution (2FD) method. The 2FD method was developed by Kang and Siragusa (2001). Samples are twofold serially diluted using buffered peptone water (BPW) as a nonselective broth. The diluents are then incubated at 37°C for 3 h for recovery of injured cells. After incubation, equal volumes of double strength selective broth are added and incubated at 37°C for 13 h in the dark. The 2FD method has benefits such as saving time and space. But additional investigations are difficult because it is not possible to isolate micro-organisms with the 2FD method.

Therefore, there is a need to overcome these limitations of the overlay and 2FD methods. In this study, a broth recovery method was optimized for heat-injured *Salmonella* Typhimurium and *E. coli* O157:H7.

Materials and methods

Bacterial strains

Three strains of each *S.* Typhimurium (ATCC 19585, ATCC 43971, DT 104) and *E. coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890) were obtained from the bacterial culture collection of Seoul National University (Seoul, Republic of Korea). Culture stocks consisting of 0·7 ml of 24 h cultures in tryptic soy broth (TSB; MB Cell) and 0·3 ml of 50% glycerol were stored at −80°C. One hundred microlitres of each strain were incubated in 5 ml TSB at 37°C for 24 h and one loopful of growth was streaked onto TSA (MB Cell), incubated at 37°C for 24 h and maintained at 4°C before use. Each strain of *S.* Typhimurium and *E. coli* O157:H7 was incubated in 5 ml TSB at 37°C for 24 h, harvested by centrifugation at 4000 ***g*** for 20 min at 4°C and washed three times with 0·2% peptone water (PW; Becton, Dickinson and Company, Franklin Lakes, NJ). Separate culture cocktails of each pathogen species were prepared by combining equal volumes of the three corresponding pathogen species strains.

Determination of heat injury

Heat treatment was performed in a constant temperature water bath (BW-10G; Jeio Tech, Seoul, Republic of Korea) maintained at 55°C. One millilitre of each pathogen species cocktail was mixed with 9 ml of preheated (55°C) 0·2% PW in a 50-ml conical tube and the tube cap was closed. Tubes were completely immersed in the water bath. To measure levels of sublethally injured cells of each pathogen, heat treatment was performed at 55°C for 0, 5, 15, 25 and 35 min for both pathogens. Heated sample tubes were removed at each time interval and cooled in crushed ice-water, then immediately 10-fold diluted with 0·2% PW at 4°C. After further 10-fold serial dilution with 0·2% PW, 100 μl of appropriate diluents were spread plated onto TSA and also onto selective agar appropriate for each pathogen species. xylose lysine desoxycholate agar (XLD; Difco, Detroit, MI) and SMAC (Oxoid, Basingstoke, Hampshire, UK) were used as selective agar for *S.*

Typhimurium and *E. coli* O157:H7 respectively. Plates were incubated at 37°C for 24 h. Log population differences between TSA and selective agars were calculated to determine levels of sublethally injured cells.

Broth for cell recovery

Brain heart infusion (BHI; Bacto), BPW (Oxoid), nutrient broth (NB; Difco), TSB and TSB with 0·6% yeast extract (Bacto) (TSBYE) were used as recovery broths. These broths are nonselective and their preparation methods are considered to be simple.

Growth curve of heat-injured cells

Growth curves of heat-injured pathogens were plotted after optical density (OD) was measured with a UV-visible spectrophotometer (Molecular Devices, San Jose, CA) in order to identify the most suitable broths for each pathogen. *Salmonella* Typhimurium and *E. coli* O157:H7 were 10-fold diluted in 0·2% PW and heated for 15 and 25 min, respectively, at 55°C. After thermal treatment, inoculated tubes were cooled in crushed ice-water and immediately 10-fold diluted with the five broths (BHI, BPW, NB, TSB and TSBYE). Two hundred microlitres of heat-treated cells were respectively dispensed onto a 96-well plate (Corning, Corning, NY). Inside temperature of the spectrophotometer was set at 37°C before measurement. As a growth index, OD at 600 nm was measured for 10 h at intervals of 30 min.

Overlay and SPRAB for recovery of heat-injured cells

The common overlay method for recovering sublethally injured cells was used with XLD for *S.* Typhimurium. Conversely, phenol red agar base (MB cell) with 1% sorbitol (SPRAB) has been used for direct-plating recovery of heat-injured *E. coli* O157:H7 (Rhee *et al.* 2003). *Salmonella* Typhimurium and *E. coli* O157:H7 were treated with conventional heating at 55°C for 15 and 25 min, respectively, to generate sublethally injured cells. Treated cell suspensions were cooled in crushed ice-water and immediately 10-fold serially diluted with 0·2% PW. One hundred microlitres of aliquots were plated onto TSA for *S.* Typhimurium and onto SPRAB for *E. coli* O157:H7. *Salmonella* Typhimurium plates were incubated at 37°C for 2 h followed by layering with 10 ml of XLD. Plates were further incubated at 37°C for 22 h. In the case of *E. coli* O157:H7, SPRAB plates were incubated at 37°C for 24 h and the latex agglutination confirmation test was performed with presumptive white colonies using the *E. coli* O157:H7 latex agglutination assay (RIM; Remel, Lenexa, KS) (Ha and Kang 2015).

Recovery with broth

Cell suspensions of *S.* Typhimurium and *E. coli* O157:H7 were treated with conventional heat (water bath) at 55°C for 15 and 25 min respectively. Heat-treated dispersions were cooled in crushed ice-water and 10-fold diluted with BHI, TSB and TSBYE. Diluents were incubated at 37°C for 0, 1, 2 and 3 h. After incubation, diluents were serially diluted with 0·2% PW and 100-μl aliquots were plated onto XLD and SMAC for enumeration of micro-organisms. Plates were further incubated at the same temperature for 21–24 h.

Confirmation of no growth of heat-injured cells during liquid recovery

Heat-treated cell suspensions of *S.* Typhimurium and *E. coli* O157:H7 were cooled in crushed ice-water and immediately 10-fold diluted with three broths (BHI, TSB and TSBYE). Diluents were incubated at 37°C for 0, 1, 2, and 3 h. After incubation, diluents were 10-fold serially diluted with 0·2% PW and 100 μl aliquots were plated onto TSA for enumeration of micro-organisms. Plates were incubated at 37°C for 24 h.

Application of liquid resuscitation for recovery of heat-injured cells in milk

We choose milk as a model for applying the liquid recovery method to food. Low fat milk (<2% milk fat) samples which were pasteurized by ultra-high–temperature treatment were purchased from a local market (Seoul, Republic of Korea) and stored at 4°C before use. We did not store milk for more than 1 week. Nine millilitres of commercial milk in 50-ml conical centrifuge tubes were preheated to 55°C. One millilitre of each pathogen species cocktail was mixed with 9 ml of preheated (55°C) milk in a 50-ml conical tube which was then securely closed. Tubes were completely immersed in the water bath and thermally treated (55°C for 15 min). Heat-treated milk samples were cooled in crushed ice-water and immediately 10-fold diluted with BHI, TSB and TSBYE. Diluents were incubated at 37°C for 0, 1 or 2 h. After incubation, diluents were 10-fold serially diluted with 0·2% PW and 100-μl aliquots were plated onto XLD and SMAC for enumeration of micro-organisms. Plates were further incubated at the same temperature for 22–24 h. For the OV method, heat-treated milk was 10-fold diluted with 0·2% PW blanks. One hundred microlitres of 0·2% PW diluents were plated onto TSA for *S.* Typhimurium and onto SPRAB for *E. coli* O157:H7. *Salmonella* Typhimurium plates were incubated at 37°C for 2 h followed by layering with 10 ml of molten XLD tempered to 48°C.

Plates were further incubated at 37°C for 22 h. In the case of *E. coli* O157:H7, SPRAB plates were incubated at 37°C for 24 h and presumptive white colonies were confirmed using the *E.coli* O157:H7 latex agglutination assay (Ha and Kang 2015).

Statistical analysis

Micro-organism populations (CFU per ml) were converted to $\log_{10}$ values. Means and standard deviations were based on three experimental replicates. Analysis of variance was performed using the Statistical Analysis System (SAS Institute, Cary, NC). Significant differences among treatments were evaluated using Duncan's multiple range test. Differences were considered statistically significant at the 95% confidence level ($P < 0{\cdot}05$).

Results

Heat-injured populations were calculated by subtracting surviving cell numbers enumerated on selective agars from those plated on TSA (Fig. 1). For *S.* Typhimurium, differences in log populations were greatest at 15 min of treatment, and for *E. coli* O157:H7 this occurred at 25 min. The maximum log differences between selective and nonselective agar for these pathogens were about 1·67 and 1·52 log CFU per ml for *S.* Typhimurium and *E. coli* O157:H7 respectively. Further recovery tests used these time intervals (15 min for *S.* Typhimurium and 25 min for *E. coli* O157:H7) as they were determined to be optimal for inducing heat injury.

Figure 2 shows the growth curves of *S.* Typhimurium and *E. coli* O157:H7 in different recovery broths. Growth curves of heat-injured *S.* Typhimurium cultured in five different broths are shown in Fig. 2a. Injured *S.* Typhimurium started to grow after 5 h. The growth rate of injured *S.* Typhimurium differed depending on the resuscitation broth. Injured cells in BHI, TSB and TSBYE showed faster growth rates than those in BPW and NB. The maximum OD values at 10 h were 0·6152 (BHI) >0·5347 (TSBYE) >0·4982 (TSB) >0·2858 (BPW) >0·1915 (NB). This same trend was also observed for *E. coli* O157:H7 (Fig. 2b). The maximum OD values of injured *E. coli* O157:H7 were 0·6786 (BHI), 0·5876 (TSBYE), 0·5282 (TSB), 0·3071 (BPW) and 0·1879 (NB). Based on these growth curves, further tests were performed with BHI, TSB and TSBYE which appeared to yield better growth rates than for the other broth media.

Figures 3 and 4 show recovered populations of heat-injured pathogens using the overlay for *S.* Typhimurium and SPRAB for *E. coli* O157:H7 and the broth repair methods. For heat-injured *S.* Typhimurium, 5·32 log

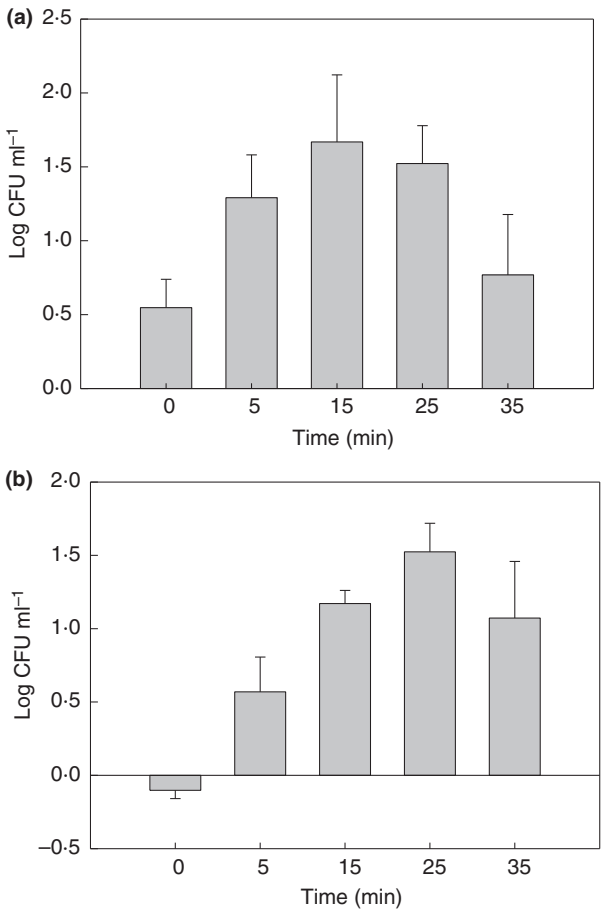

Figure 1 Population differences in pathogens treated by conventional heat treatment (55°C) and plated on tryptic soy agar and selective agars (xylose lysine desoxycholate agar for *Salmonella* Typhimurium or SMAC for *Escherichia coli* O157:H7). (a) *Salmonella* Typhimurium and (b) *E. coli* O157:H7.

CFU per ml was recovered using the overlay method. The number of nonrecovered (0 h incubation) *S.* Typhimurium cells resuscitated in BHI (4·83 log CFU per ml) was significantly lower than those enumerated on OV-XLD (5·32 log CFU per ml). After 1 h of incubation, none of the broths yielded results significantly different from that of the OV-XLD procedure. And there were no significant differences relative to recovery time (1, 2 and 3 h). In the case of *E. coli* O157:H7, 6·44 log CFU per ml was recovered using SPRAB. The populations of *E. coli* O157:H7 cells resuscitated in the three broths were significantly different from those of cells enumerated on SPRAB. But, populations of *E. coli* O157:H7 plated onto selective agar appeared to significantly increase following 1 h recovery in all broths, but there was no significant additional increase when incubation exceeded 1 h. However, 1 h of broth recovery was not significantly different from populations recovered on SPRAB (Fig. 4). For the

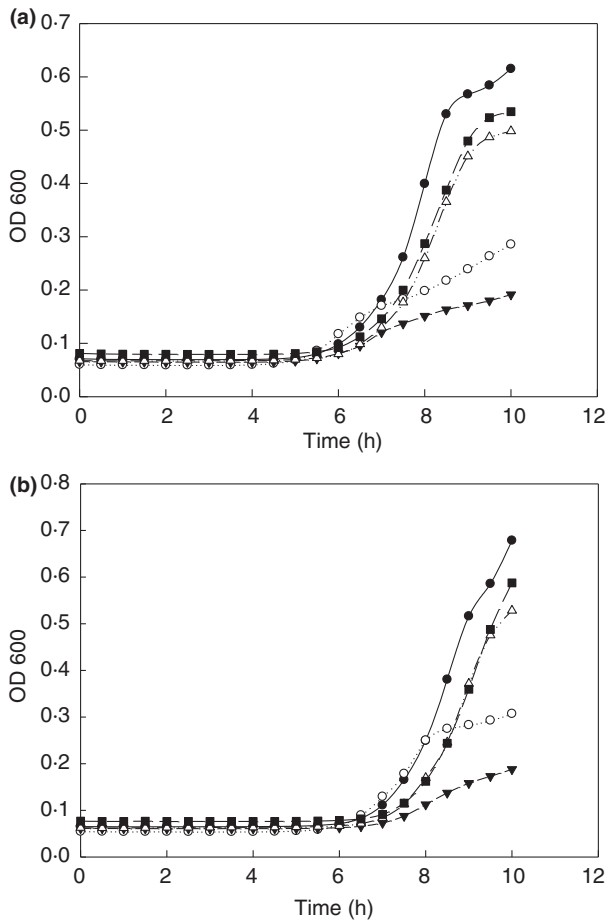

Figure 2 Growth curves of heat-injured pathogens inoculated into BHI (●), BPW (○), NB (▼), TSB (△) and TSBYE (■) at 37°C. (a) *Salmonella* Typhimurium and (b) *Escherichia coli* O157:H7. BHI: brain heart infusion, BPW: buffered peptone water, NB: nutrient broth, TSB: tryptic soy broth, TSBYE: tryptic soy broth with 0·6% yeast extract.

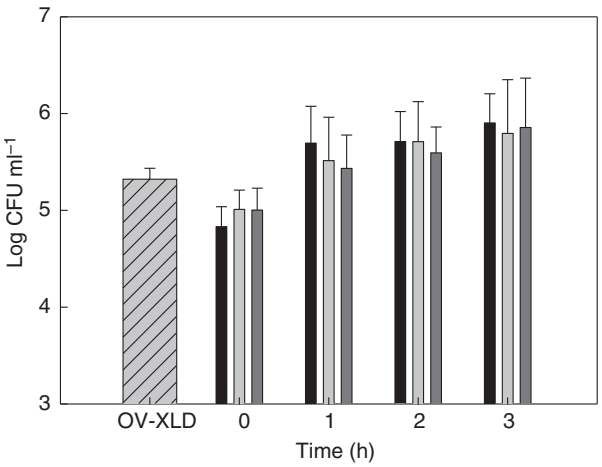

Figure 3 Recovery of heat-injured *Salmonella* Typhimurium populations in 0·2% PW using Overlay-XLD (▨), BHI (■), TSB (▩) and TSBYE (▩). For broth recovery, heat-treated samples were 10-fold diluted with each broth and incubated at 37°C followed by spreadplating onto XLD for further incubation.

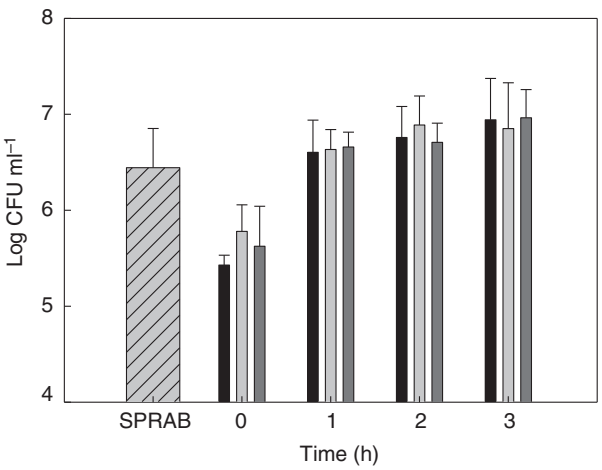

Figure 4 Recovery of heat-injured *Escherichia coli* O157:H7 populations in 0·2% PW using SPRAB (▨), BHI (■), TSB (▩) and TSBYE (▩). For broth recovery, heat-treated samples were 10-fold diluted with each broth and incubated at 37°C followed by spread plating onto SMAC for further incubation.

same recovery time interval, there were no significant differences between the three broths.

Table 1 shows the total populations of heat-treated pathogens recovered in the three broths and plated on TSA. The number of total pathogens recovered at 0 h was slightly lower than those recovered after 1, 2 and 3 h in all three broths, but these differences were not significant ($P > 0·05$). For all tested pathogens diluted with the three broths and plated on TSA, the total number of pathogens did not increase significantly during 3 h of recovery ($P > 0·05$). Figure 2 also shows that pathogens did not increase during 3 h of broth recovery based on plotted growth curves.

Figures 5 and 6 show the effect of the liquid resuscitation method on heat-injured pathogens in milk samples. When *S.* Typhimurium in milk was heated for 15 min at 55°C, populations of this pathogen plated on OV-XLD and XLD were 6·83 and 5·62 log CFU per ml respectively. The number of nonrecovered *S.* Typhimurium (incubated 0 h) diluted in BHI, TSB and TSBYE was 5·79, 5·79 and 5·85 log CFU per ml respectively. But after 1 h of incubation in BHI, TSB and TSBYE at 37°C, populations of *S.* Typhimurium increased to 6·87, 6·69 and 6·67 log CFU per ml respectively. Those were not significantly different from OV-XLD pathogen counts ($P > 0·05$). And there were no significant differences relative to recovery time

Table 1 Populations (log CFU per ml) of injured pathogens in three recovery broths incubated at 37°C

Pathogens	Recovery time (h)	Recovery broths		
		BHI	TSB	TSBYE
Salmonella Typhimurium	0	5·74 ± 0·32*	5·75 ± 0·28	5·66 ± 0·69
	1	6·06 ± 0·47	5·91 ± 0·51	5·87 ± 0·41
	2	6·06 ± 0·36	5·93 ± 0·34	5·72 ± 0·41
	3	6·07 ± 0·53	5·77 ± 0·52	5·74 ± 0·26
Escherichia coli O157:H7	0	6·41 ± 0·73	7·07 ± 0·46	6·42 ± 0·58
	1	7·04 ± 0·20	7·11 ± 0·11	6·77 ± 0·18
	2	7·07 ± 0·43	7·14 ± 0·20	6·71 ± 0·25
	3	7·20 ± 0·15	7·16 ± 0·19	7·02 ± 0·13

BHI: brain heart infusion, TSB: tryptic soy broth, TSBYE: tryptic soy broth with 0·6% yeast extract.
*Mean ± standard deviation. No significant differences were found between time intervals for any of the micro-organisms.

(1 and 2 h). A similar trend was also confirmed in the case of *E. coli* O157:H7. After 1 h of liquid resuscitation, sublethally injured pathogen counts increased by a little <1·0 log CFU per ml which is not significantly different from the number of pathogens plated on SPRAB.

Discussion

In the present study, there were no significant differences in sublethally injured cell recovery between BHI, TSB and TSBYE for each time interval, even though the nutrient composition of each broth was different. These results are congruent with those of Taormina *et al.* (1998) and Jasson *et al.* (2009). Taormina *et al.* (1998) reported that populations of heat-stressed *E. coli* O157:H7 plated on TSA were not significantly different from those plated on BHIA (brain heart infusion agar). Jasson *et al.* (2009) investigated sublethally injured *E. coli* O157:H7 incubated at 37°C in two brands of TSB and in TSBYE. Their results showed that maximum specific growth rates were not significantly different from each other ($P > 0·05$).

Determining the presence of foodborne pathogens is one of the main critical points of food quality and safety. Detection methods which can identify both normal and injured micro-organisms have been developed but other problems have occurred as a consequence. In the case of liquid recovery method, the main disadvantage discussed in previous research studies is that uninjured micro-organisms can multiply during recovery (Wu 2008). Clark and Ordal (1969) recovered heat-injured *S.* Typhimurium in TSB at 37°C. Although injured cells completely recovered during the extended lag phase (4 h), untreated cells appeared to increase during recovery. However, in the present study, broth recovery showed

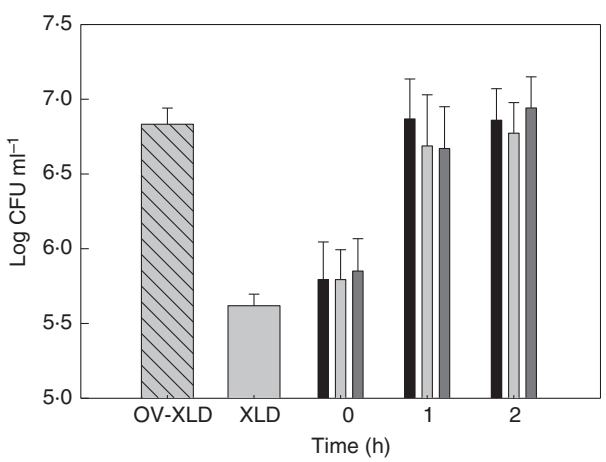

Figure 5 Recovery of heat-injured *Salmonella* Typhimurium populations in milk sample using Overlay-XLD (▨), BHI (■), TSB (□) and TSBYE (▩). For broth recovery, heat-treated samples were 10-fold diluted with each broth and incubated at 37°C followed by spread-plating onto XLD for further incubation.

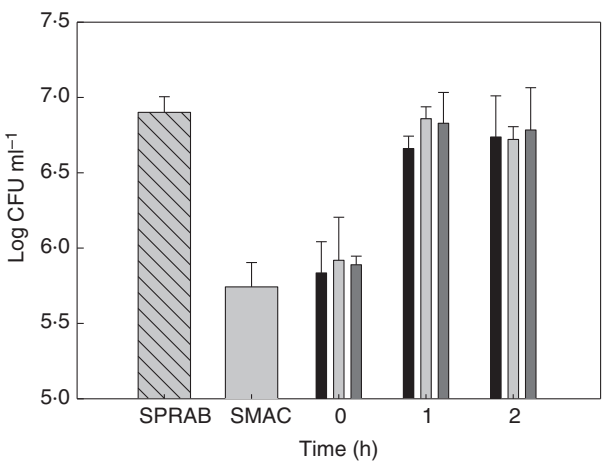

Figure 6 Recovery of heat-injured *Escherichia coli* O157:H7 populations in milk sample using SPRAB (▨), BHI (■), TSB (□) and TSBYE (▩). For broth recovery, heat-treated samples were 10-fold diluted with each broth and incubated at 37°C followed by spread plating onto SMAC for further incubation.

that total populations of *S.* Typhimurium and *E. coli* O157:H7 showed no statistically significant change during 3 h of incubation in nonselective broths (Table 1). These data demonstrate that during incubation in broth media for this portion of lag phase, multiplication of sublethally injured or noninjured cells did not occur to any significant degree. Therefore, when it can be demonstrated that micro-organisms do not grow during a specific recovery period, liquid repair offers greater advantages compared to other methods. Specifically, broth recovery offers the following advantages versus overlay methods: (i) the absence of mild heat inactivation caused by melted agar used for layering, (ii) reduced time and labour costs due to a simpler procedure, (iii) compared with 2 h resuscitation and overlay, resuscitation times for broth recovery were equal or shortened, (iv) isolation of colonies for additional experiments is easier, (v) flexibility in broth selection, and (vi) special equipment or plates for recovery are not needed.

For solid media, thin agar layer (TAL) (Kang and Fung 1999) and agar underlay (Kang and Siragusa 1999) methods have been developed. The TAL method consists of a thin layer of nonselective agar poured onto a layer of selective agar in a Petri dish and, immediately after solidifying, spread-plated with heat-treated cells. Sublethally injured cells recover before selective agents can diffuse into the top nonselective layer. But there are problems with white contaminant colonies appearing on the selective agar. With the agar underlay method, sublethally injured cells are recovered on nonselective agar and after 2 h repair selective agar is underlaid to the bottom chamber of a Lutri plate. Although this method is able to differentiate white colonies, this complicated preparation process is considered cumbersome. Also, other methods for recovery such as addition of compounds that facilitate repair, and recovery utilizing membrane filtration have been investigated (Wu and Fung 2004; Taskila *et al.* 2011). However, these methods are considered to be complicated and costly.

The detection of foodborne pathogens is a major concern for food products having undergone food processing (Wu 2008). For this reason, investigating recovery methods using actual food is important. In this research, we choose milk as a model food. Milk has long been associated with many foodborne illness outbreaks. There were a total of 177 outbreaks involving milk and milk products in France from 1988 to 1997 (De Buyser *et al.* 2001). Also, from 2007 to 2012, there were 81 outbreaks due to unpasteurized milk in the United States. (Mungai *et al.* 2015). A total of 13 outbreaks were caused by Shiga toxin-producing *E. coli* and 2 of them were caused by *S.* Typhimurium. Milk is usually pasteurized by conventional thermal treatment such as high temperature, short time or low temperature and long time which can generate heat-injured foodborne pathogen. Our results with milk were not different from those of 0·2% PW. There was no significant difference between populations of pathogens on OV-XLD or SPRAB and those on XLD or SMAC plated with samples recovered for 1 h in BHI, TSB or TSBYE ($P > 0{\cdot}05$). Similar results were found by Kang and Fung (2000). They reported that numbers of heat-injured *S.* Typhimurium in skim milk recovered by the TAL method were not significantly different from those recovered by OV-XLD ($P > 0{\cdot}05$).

In conclusion, broth recovery can replace the overlay method due to greater convenience and timesaving. Optimum broth recovery conditions for *S.* Typhimurium and *E. coli* O157:H7 were determined to be 1 h in BHI, TSB or TSBYE. No growth of total viable cells ensures no multiplication of healthy cells occurs during recovery time.

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Conflict of Interest

No conflict of interest declared.

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