A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Optimization of Liquid Resuscitation for Repair of Heat and Acid-injured Foodborne Pathogens

열과 산에 의해 손상된 식중독균의 회복을 위한 액체회복법의 최적화

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Department of Agricultural Biotechnology

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석사학위논문

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지도교수 강동현

이 논문을 석사학위 논문으로 제출함

2017 년 2 월

서울대학교 대학원 농생명공학부

한 지 윤

한지윤의 석사 학위논문을 인준함

2017 년 2 월

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ABSTRACT

Because of the presence of injured cells, the inactivation efficacy of various interventions may be overestimated. Therefore, verifying the exact populations of not only healthy but also injured cells is becoming increasingly important. However, recovery through commonly used method, overlay and SPRAB have several limitations. As there is a need to overcome limitations, liquid resuscitation method was developed and optimized. The purpose of this research was to determine optimum conditions for the liquid resuscitation of heat/acid-injured *Salmonella* Typhimurium and *Escherichia coli* O157:H7. Preliminary experiments showed that exposure to 55℃ in PW for 15 and 25 min respectively as heat-treatment, and to 2% lactic acid for both 60 s as acid-treatment induced cellular injury of *S.* Typhimurium and *E. coli* O157:H7. BHI, TSB, and TSBYE were selected for recovery broth through optical density measurements at 600 nm. After microorganisms were heat and acid-treated at appropriate conditions, microbes were ten-fold diluted with BHI, TSB, and TSBYE and incubated at 37℃ for up to 3 h. At hourly intervals, diluents were plated onto XLD or SMAC for the enumeration of healthy cells. Simultaneously, diluents were plated onto TSA for total viable counts (including recovered injured cells) followed by further incubation at 37℃. Comparison was made with the commonly used overlay

method using XLD for recovering injured cells of *S.* Typhimurium. For *E. coli* O157:H7, SPRAB was used instead of the overlay method. One hour resuscitation were significantly recover heat or acid-injured *S.* Typhimurium and *E. coli* O157:H7, in any of the following broth media: BHI, TSB, and TSBYE. No growth of non-injured cells was observed during incubation. When liquid resuscitation applied to injured cells in food samples (low fat milk, apple juice), also 1 h incubation were enough to recover Thus, the liquid resuscitation method is a convenient alternative to the conventional overlay method.

Keywords: **Injured; Liquid resuscitation; Recovery; Overlay; Foodborne pathogen**

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I. INTRODUCTION

In 2013, over 230 foodborne illness outbreaks occurred in the USA and about 800 people were hospitalized due to foodborne illnesses (Centers for Disease Control and Prevention, 2013). According to the CDC's foodborne disease outbreaks annual report, *Salmonella* (62%), Shiga toxin-producing *Escherichia coli* (14%), were the major pathogens causing hospitalizations.

To control contamination of foodstuffs involving these microorganisms, physical and chemical inactivation techniques have been developed and investigated. Such as microwave, radio frequency heating and organic acids (Rodríguez-Marval et al., 2009, Lagunas-Solar et al., 2005). These techniques result in effective inactivation of various food categories. However, some populations of bacteria inactivated by these techniques can still survive as injured cells. Whereas healthy cells can multiply on selective agar containing selective agents such as salts, acids, dyes and antimicrobials, cells sub-lethally injured by inactivation treatments 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. For this reason, the inactivation efficacy of various interventions may 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 (Speck et al., 1975, Hartman et al., 1975) is commonly used. After spreading heat-injured cells onto non-selective medium like tryptic soy agar (TSA), plates are incubated for 2 h at the optimum growth temperature of target microorganisms. In this process the nutritious, non-selective agar helps injured cells to repair themselves and resuscitate. Subsequently 10~12 ml of melted selective agar is overlaid (poured) onto the non-selective agar. After solidification, an additional 22 h incubation is typically performed for a total of 24 h. This method however has some limitations, e.g., 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 (Harries and Russell, 1966, Baird-Parker and Davenport, 1965). 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 *E. coli* O157:H7 which produces cream-colored colonies on selective agar (SMAC), detecting these colorless colonies between two opaque agar layers is considered to be problematic.

Therefore, there is a need to overcome these limitations of the overlay method. In this research study, a liquid resuscitation method was developed and optimized for the heat and acid-injured *S.* Typhimurium *and E. coli* O157:H7.

II. MATERIALS AND METHODS

2.1. Bacterial strains and preparation of inoculum

Three strains of each *Salmonella enterica* Serovar Typhimurium (ATCC 19585, ATCC 43971, DT 104) and *Escherichia 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 were stored at - 80℃ with 0.7 ml of 24 h cultures in tryptic soy broth (TSB; MBcell, Seoul, Republic of Korea) and 0.3 ml of 50% glycerol. One hundred microliters of each strain were incubated in 5 ml TSB at 37℃ for 24 h and one loopful of growth was streaked onto tryptic soy agar (TSA; MBcell), incubated at 37℃ for 24 h and maintained at 4℃ before use. The nine microorganism strains were incubated separately in 5 ml TSB at 37℃ for 24 h. Three strains of each pathogen species were combined in 50 ml Falcon centrifuge tubes (Corning, USA) and centrifuged at 4000 x g for 20

min at 4℃. Supernatants were removed followed by washing twice with 0.2% peptone water (Bacto, USA). The final pellets were resuspended with 0.2% peptone water.

2.2. Experiments of recovery of heat-injured pathogens

2.2.1. Heat treatment

Heat treatment was performed in a waterbath (JeoiTec Co., Ltd., Republic of Korea) maintained at 55℃. In a preliminary study, temperatures less than 55℃ required longer times to generate >1 log CFU/ml injured cells, and higher temperatures were too sensitive to time, making generation of consistent numbers of injured cells difficult. One milliliter of each pathogen species cocktail was respectively mixed with 9 ml of preheated 0.2% peptone water in a 50 ml Falcon centrifuge tube and the tube cap was closed. Tubes were completely immersed in heated water.

2.2.2. Determination of heat-injury conditions

Conditions for inducing injury varied with the species. To measure levels of injured cells of each pathogen, heat treatment was performed at 55℃ for 0, 5, 15, 25, and 35 min for *S.* Typhimurium and *E. coli* O157:H7. Heated sample tubes were removed at each time interval and cooled in crushed ice-water, then immediately ten-fold diluted with 4℃ 0.2% peptone water. After further ten-fold serial dilution with 0.2% peptone water, 100 μl of appropriate diluents were spread-plated onto TSA (a non-selective medium) and also onto selective agar appropriate for each pathogen species. Xylose Lysine Desoxycholate Agar (XLD; Difco, Maryland, USA) and Sorbitol MacConkey Agar (SMAC; Oxoid, UK) were used as selective agar for *S.* Typhimurium and *E. coli* O157:H7, respectively. Plates were incubated at 37℃ for 24 h. Log population differences between TSA and selective agars were calculated to indicate populations of injured cells. For the control treatment (0 h), differences of non-heated samples between TSA and each selective agar were calculated. Based on experiments, heat treatment of *S.* Typhimurium and *E. coli* O157:H7 for 15, and 25 min, respectively, produced moderate levels of injured cells.

2.2.3. Broth screening

Brain heart infusion (BHI; Bacto), Buffered peptone water (BPW; Oxoid), Nutrient broth (NB; Difco), Tryptic soy broth (TSB; MBcell), and Tryptic soy broth with 0.6% yeast extract (YE; Bacto) (TSBYE) were used as recovery broths. These broths are non-selective and their manufacturing methods are considered to be simple.

2.2.4. Growth curve with general broths

Growth curves of heat-injured pathogens were measured with a UV-visible spectrophotometer (Molecular Devices, California, USA) for select better broths of each pathogens. *S*. Typhimurium and *E. coli* O157:H7 diluted in PW were heated for 15 and 25 min, respectively. Treatment times were determined based on previous experiments as mentioned previously. After thermal treatment, inoculated tubes were cooled in crushed ice-water and immediately ten-fold diluted with the five broths. Two hundred

microliters of heated and unheated treated cells were respectively dispensed onto a 96 well plate (Corning). Inside temperature of the spectrophotometer was set at 37℃ before measurement. As a growth index, OD at 600 nm was measured during 10 h at intervals of 30 min.

2.2.5. Overlay and SPRAB method

The common overlay method for recovering injured cells was used with XLD for *S*. Typhimurium. Conversely, phenol red agar base (MBcell) with 1% sorbitol (MBcell) (SPRAB) has been used for direct-plating recovery of heat-injured *E. coli* O157:H7 (Rhee et al., 2003). *S.* Typhimurium and *E. coli* O157:H7 were treated with conventional heating for 15 and 25 min, respectively, to generate injured cells. Treated dispersions were cooled in crushed ice-water and immediately ten-fold serially diluted with 0.2 % peptone water. One hundred microliters of aliquots were plated onto TSA for *S.* Typhimurium and onto SPRAB for *E. coli* O157:H7. *S.* Typhimurium plates were incubated at 37℃ for 2 h followed by layering with 10 ml of the

XLD agar as a selective agar tempered at 45 ~ 48℃. Plates were further incubated at 37℃ for 22 h. Black colonies characteristic of *S.* Typhimurium were enumerated. In the case of *E. coli* O157:H7, SPRAB plates were incubated at 37℃ for 24 h and the latex agglutination confirmation test was performed with presumptive white colonies using the *E.coli* O157:H7 Latex kit (Oxoid) (Rhee et al., 2003).

2.2.6. Liquid resuscitation method

S. Typhimurium and *E. coli* O157:H7 were treated with conventional heat for 15 and 25 min, respectively. Heat-treated dispersions were cooled in crushed ice-water and immediately ten-fold serially diluted with BHI, TSB, and TSBYE. Appropriate diluents were incubated at 37℃ for 0, 1, 2, and 3 h and 100 μl aliquots were plated onto each selective agar and TSA for enumeration of microorganisms. Plates were further incubated at the same temperature for 21~24 h. The 0 time interval (non-recovered cells) was

examined by spread-plating 100 μl of each broth inoculated with heat-injured microorganisms before incubation.

2.3. Experiments of recovery of acid-injured pathogens

2.3.1. Acid treatment

Acid treatment was performed with 2% lactic acid (Dslab, Seoul, Republic of Korea). One milliliter of each pathogen species cocktail was respectively mixed with 9 ml of 2% lactic acid solution in a test tube.

2.3.2. Determination of acid-injury condition

For both pathogens, cell suspensions were inoculated on lactic acid for 0, 30, 60, and 90 s. Treated sample tubes were neutralized with Dey/Engley (DE) neutralizing broth (Difco, Maryland, USA) at each time interval and immediately ten-fold diluted with 0.2% peptone water. After further ten-fold

serial dilution with 0.2% peptone water, 100 μl of appropriate diluents were spread-plated onto TSA and also onto XLD or SMAC, respectively. Plates were incubated at 37℃ for 24 h. The value of populations on selective agar subtracted from populations on non-selective agar was used as the level of injuries. For the control treatment (0 h), differences of non-heated samples between TSA and each selective agar were calculated. Based on experiments, acid treatment of *S.* Typhimurium and *E. coli* O157:H7 for both 60 second, produced moderated levels of injured cells.

2.3.3. Broth screening

As same with heat-injury experiment, brain heart infusion, buffered peptone water, nutrient broth, tryptic soy broth and tryptic soy broth with 0.6% yeast extract were used as recovery broths.

2.3.4. Growth curve with general broths

Growth curves of acid-injured pathogens were measured with a UV-visible spectrophotometer for select better broths of each pathogens. *S.* Typhimurium and *E. coli* O157:H7 diluted in PW were acid-treated for 60 s both. Treatment times were determined based on previous experiments as mentioned previously. After acidic treatment, inoculated tubes were neutralized with D/E broth and ten-fold diluted with the five broths. Two hundred microliters of acid-treated and untreated cells were respectively dispensed onto a 96 well plate. Inside temperature of the spectrophotometer was set at 37℃ before measurement. As a growth index, OD at 600 nm was measured during 10 h at intervals of 30 min.

2.3.5. Overlay and SPRAB method

S. Typhimurium and *E. coli* O157:H7 were treated with 2% lactic acid for 60 s, to generate injured cells. Treated dispersions were neutralized with D/E broth and ten-fold serially diluted with 0.2 % peptone water. One hundred microliters of aliquots were plated onto TSA for *S.* Typhimurium

and onto SPRAB for *E. coli* O157:H7. *S.* Typhimurium plates were incubated at 37℃ for 2 h followed by layering with 10 ml of the XLD agar tempered at 45 ~ 48℃. Plates were further incubated at 37℃ for 22 h. Black colonies characteristic of *S.* Typhimurium were enumerated. In the case of *E. coli* O157:H7, SPRAB plates were incubated at 37℃ for 24 h and the latex agglutination confirmation test was performed with presumptive white colonies using the *E.coli* O157:H7 Latex kit.

2.3.6. Liquid resuscitation method

S. Typhimurium and *E. coli* O157:H7 were treated with 2% lactic acid for 60 s. Acid-treated dispersions were neutralized with D/E broth and ten-fold serially diluted with BHI, TSB, and TSBYE. Appropriate diluents were incubated at 37°C for 0, 1, 2, and 3 h and 100 μl aliquots were plated onto each selective agar and TSA for enumeration of microorganisms. Plates were further incubated at the same temperature for 21~24 h. The 0 time interval

(non-recovered cells) was examined by spread-plating 100 μl of each broth inoculated with heat-injured microorganisms before incubation.

2.4. Applications of liquid resuscitation for recovery of injured-cells in food samples

S. Typhimurium and *E. coli* O157:H7 inoculated in low fat milk and apple juice (pH 3.1) were thermally treated with waterbath. By the preliminary experiments, for low fat milk, 15 min treatment at 55℃ and for apple juice, 5 min treatment at 55℃ was adequate for the injuries. Heat-treated samples with pathogens were cooled down with crushed ices and ten-fold serially diluted with BHI, TSB, TSBYE and PW. Appropriate diluents of BHI, TSB and TSBYE were incubated at 37°C for 0, 1, and 2 h and 100 μl aliquots were plated onto each selective agar and TSA for enumeration of microorganisms. Plates were further incubated at the same temperature for 21~24 h. The 0 time interval (non-recovered cells) was examined by spread-plating 100 μl of each broth inoculated with heat-injured microorganisms

before incubation. In case of PW diluents, 100 μl of aliquots were plated onto TSA for *S.* Typhimurium and onto SPRAB for *E. coli* O157:H7. *S.* Typhimurium plates were incubated at 37℃ for 2 h followed by layering with 10 ml of the XLD agar tempered at 45 ~ 48℃. Plates were further incubated at 37℃ for 22 h. Black colonies characteristic of *S.* Typhimurium were enumerated. In the case of *E. coli* O157:H7, SPRAB plates were incubated at 37℃ for 24 h and the latex agglutination confirmation test was performed with presumptive white colonies using the *E.coli* O157:H7 Latex kit.

2.5. Statistical analysis

Microorganism populations (CFU/ml) were converted to $\log_{10}$ values. Means and standard deviations were based on three experimental replicates. Analysis of variance was performed using SPSS (SPSS Statistics 23.0; IBM, USA). Significant differences among treatments were evaluated using

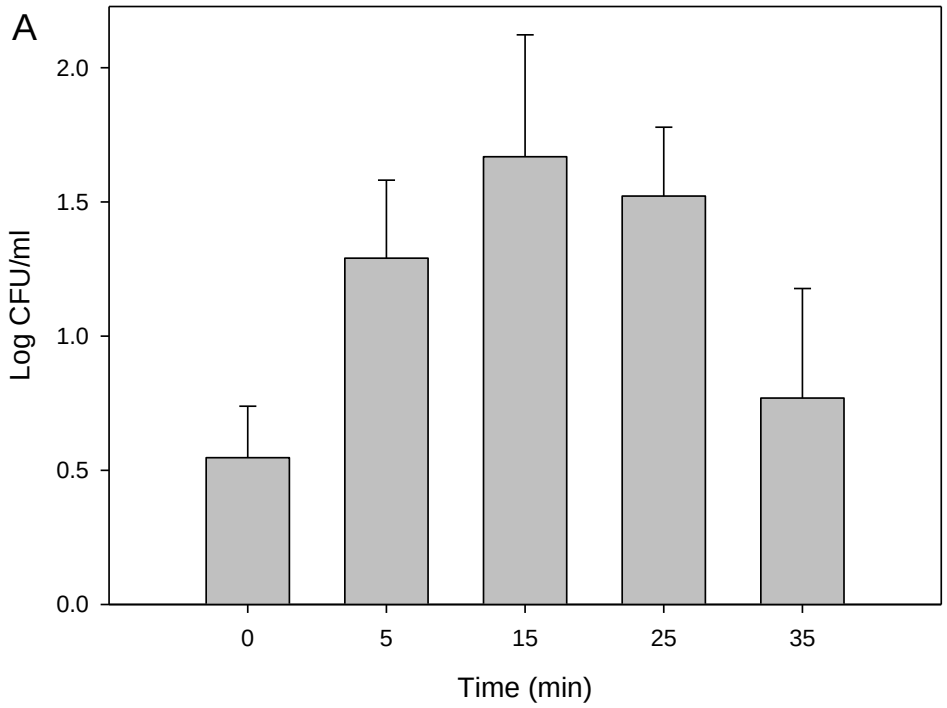
Duncan's multiple range test. Differences were considered statistically significant at the 95% confidence level ($P < 0.05$).

III. RESULTS

3.1. Recovery of heat-injured pathogens

3.1.1. Determination of heat-injury condition

Heat-injured populations were calculated by subtracting surviving cell numbers enumerated on selective agars from those plated on TSA (non-selective agar) (Figure 1). For *S.* Typhimurium, log differences at 15 min treatment were the highest, and for *E. coli* O157:H7 this occurred at 25 min. These time intervals were determined to be optimum for inducing heat-injury. Non-heated (0 h) *S.* Typhimurium showed about a 0.5 log CFU/ml difference due to antimicrobial agents in the selective agars. Longer treatment times led to decreased total microorganism populations (data not shown).


A
2.0
1.5
1.0
0.5
0.0
Log CFU/ml
0
5
15
25
35
Time (min)

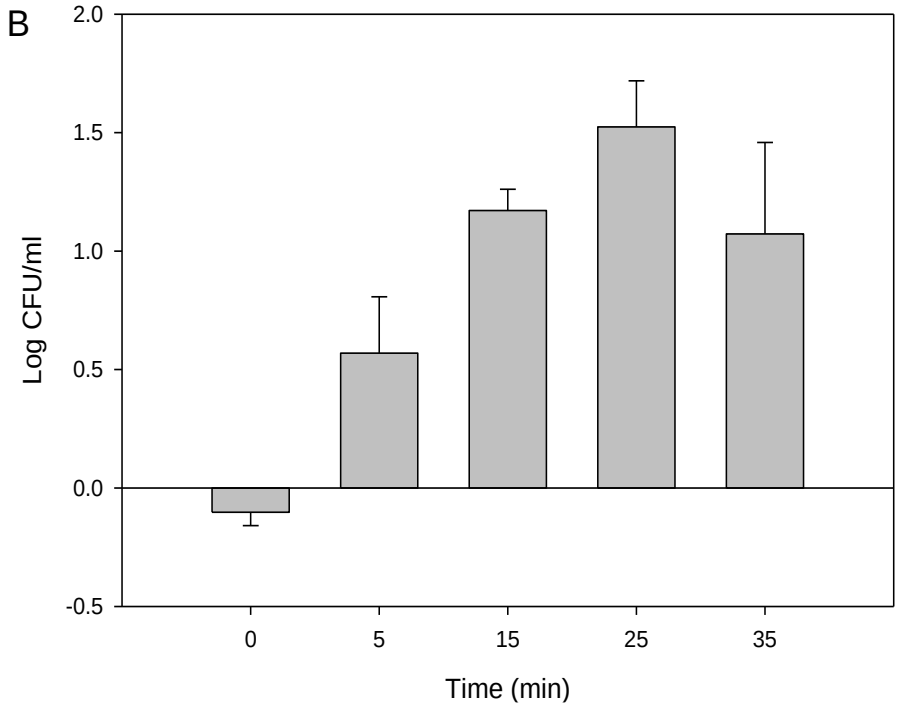
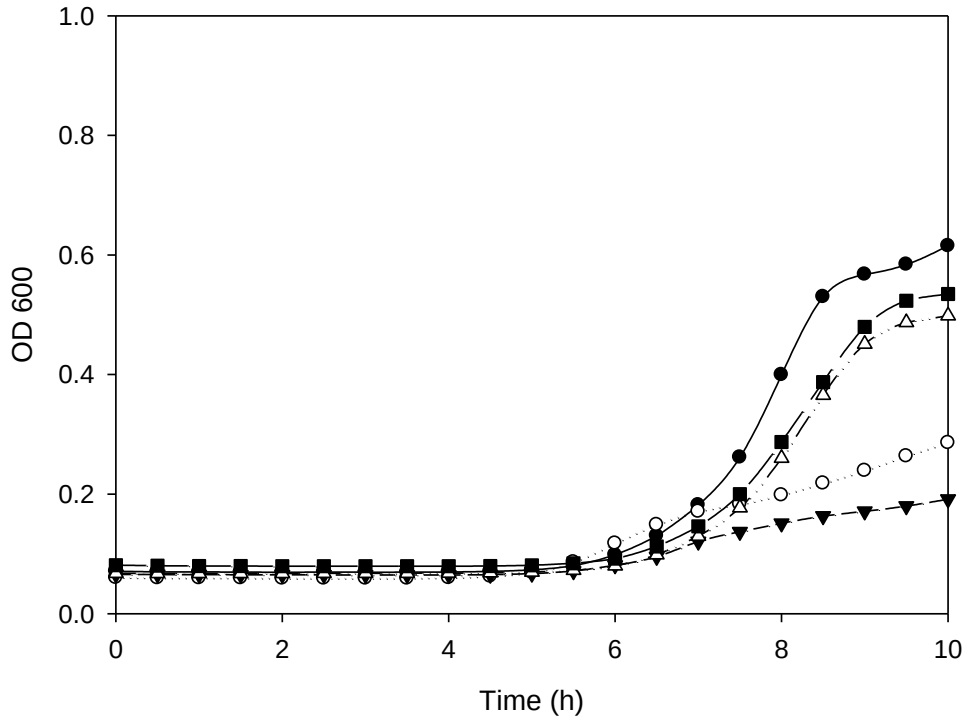


Fig. 1 Log differences of pathogens incubated on TSA and selective agar. Treatments were conducted at 55℃ for *S.* Typhimurium (A) and *E. coli* O157:H7 (B). Heat-treated samples were plated on each selective agar.

3.1.2. Growth curves in common broth media

Growth curves of heat-injured and *S.* Typhimurium cultured in 5 different broths were measured (Figure 2). *S.* Typhimurium diluted with BHI, TSB, and TSBYE yielded better growth rates compared to NB and BPW. The heat-injured *S.* Typhimurium growth curve revealed a prolonged lag phase. Recovery of heat-injured cells involving an extended lag phase has been reported by earlier investigators (Iandolo and Ordal, 1966). These tendencies were also observed for *E. coli* O157:H7. 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.

A



B

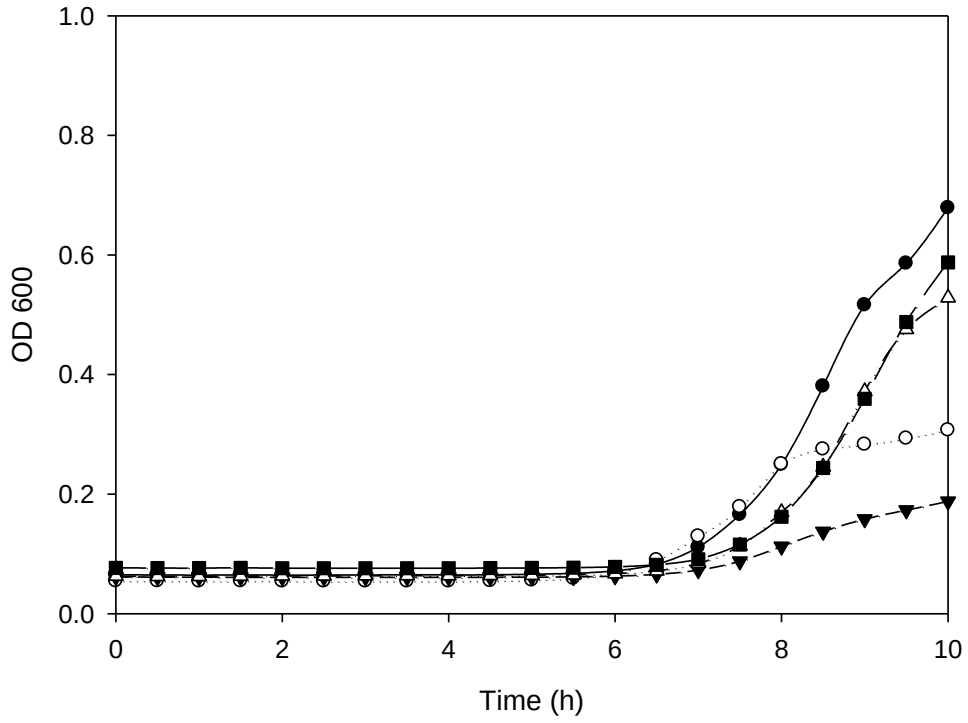
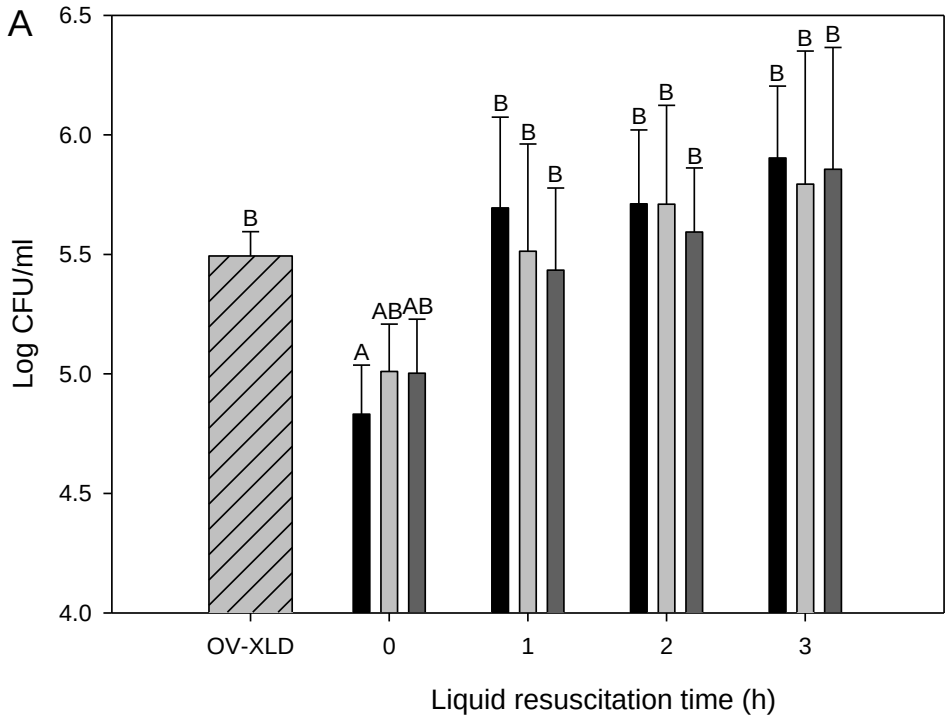


Fig. 2 Growth curve of heat-injured *S.* Typhimurium (A) and *E. coli* O157:H7 (B). Heat-treated samples were inoculated into BHI(—●—), BPW(···○···), NB(-·-▼-·-), TSB(—△—), TSBYE(- —■— -) at 37℃

3.1.3. Recovery of heat-injured pathogens by liquid resuscitation method

Figures 3 show recovered populations of heat-injured pathogens using the overlay (SPRAB for *E. coli* O157:H7) and broth repair methods. For heat-injured *S.* Typhimurium and *E. coli* O157:H7, 5.32 and 6.44 log CFU/ml were enumerated, respectively, using the conventional methods. Numbers of non-recovered (0 time incubation) *S.* Typhimurium cells resuscitated in BHI were significantly different from those enumerated on OV-XLD. After 1 hour incubation, none of the broths yielded results significantly different from that of the OV-XLD procedure. Also, BHI and TSBYE facilitated significant recovery of injured cells during 0 and 1 hour incubation. Populations of *E. coli* O157:H7 plated onto selective agar appeared to significantly increase following 1 hour recovery in all broths, but there was no significant increase when incubation exceeded 1 hour. However, 1 hour broth recovery was not significantly different from populations recovered on SPRAB. For the same recovery time interval, there were no significant differences between the three broths. Also, for all tested

pathogens diluted with the three broths and plated on non-selective agar, means of total viable cells showed no significant growth during 3 hour recovery (Table 1).


A
6.5
6.0
5.5
5.0
4.5
4.0
Log CFU/ml
B
A
AB
AB
B
B
B
B
B
B
B
B
B
B
OV-XLD
0
1
2
3
Liquid resuscitation time (h)

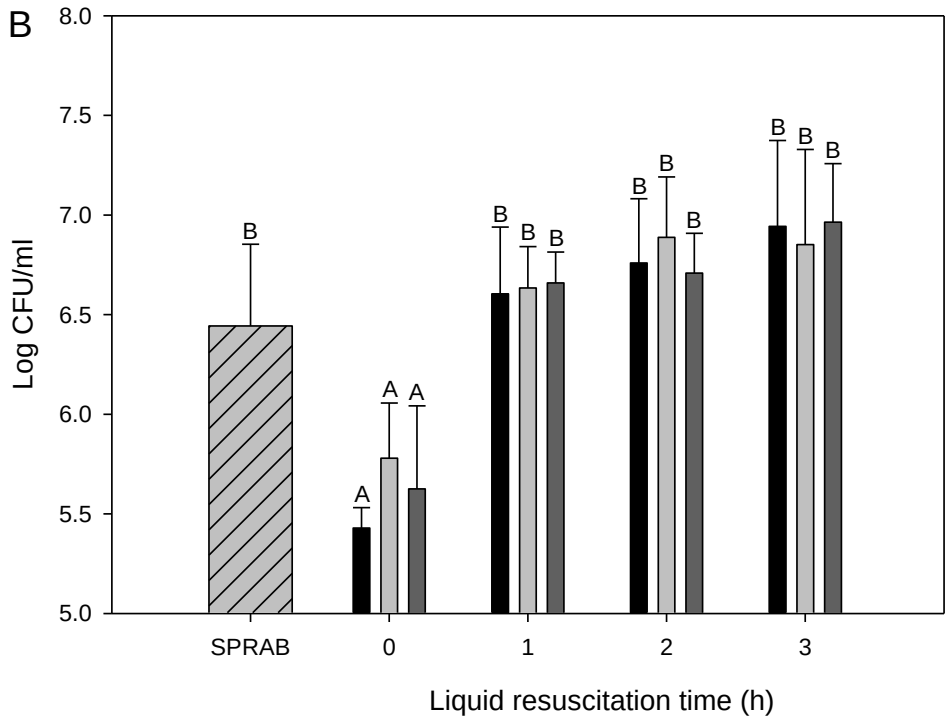


Fig. 3 Overlay-XLD or SPRAB population and recovery of heat-injured *S.* Typhimurium (A) and *E. coli* O157:H7 (B) in BHI(■), TSB(▭) and TSBYE(▬). For liquid resuscitations, heat-treated samples were ten-fold diluted with each broths and incubated at 37℃ followed by on selective agars (XLD, SMAC) for further incubation. Results are explained with means and standard deviations independent triplicate. Bars with different letters are significantly different ($P < 0.05$).

Table 1. Population of pathogens during recovery of heat-injuries in resuscitation broths.

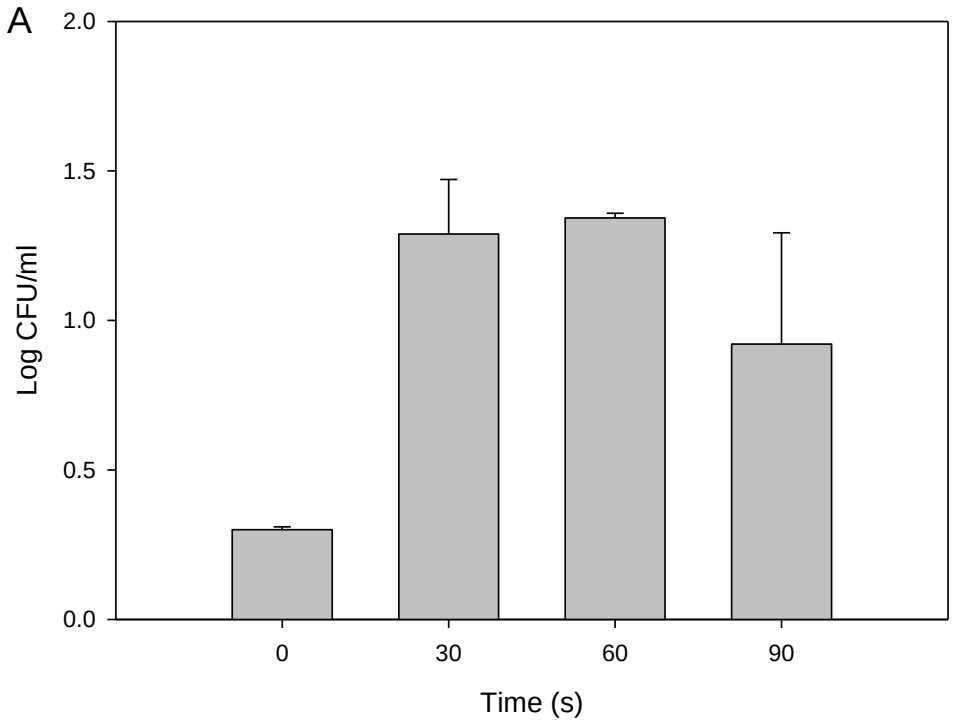
Microorganisms (Heat-treatment time)	Time (h)	Resuscitation broths*		
		BHI	TSB	TSBYE
S. Typhimuirum (15 min)	0	5.74±0.32	5.75±0.28	5.66±0.69
	1	6.06±0.47	5.91±0.51	5.87±0.41
	2	6.06±0.35	5.93±0.34	5.72±0.41
	3	6.07±0.53	5.77±0.52	5.74±0.26
E. coli O157:H7 (25 min)	0	6.88±0.24	7.07±0.46	6.80±0.22
	1	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	7.16±0.10	7.16±0.19	7.02±0.13

* Log CFU/ml. Thermal-treated microorganisms were diluted resuscitation broths and plated on TSA (non-selective agar) followed by incubation at 37℃. The data represent means ± standard deviations from three replications.

3.2. Recovery of acid-injured pathogens

3.2.1. Determination of Injured condition

Acid-injured populations were calculated by subtracting surviving cell numbers enumerated on selective agars from those plated on TSA (Figure 4). For *S.* Typhimurium, log differences at 60 s acidic treatment was the highest, and for *E. coli* O157:H7 also 60 s treatment occurred the highest injury. These time intervals were determined to be optimum for inducing acid-injury.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1.5
1.0
0.5
0.0
Log CFU/ml
0
30
60
90
Time (s)

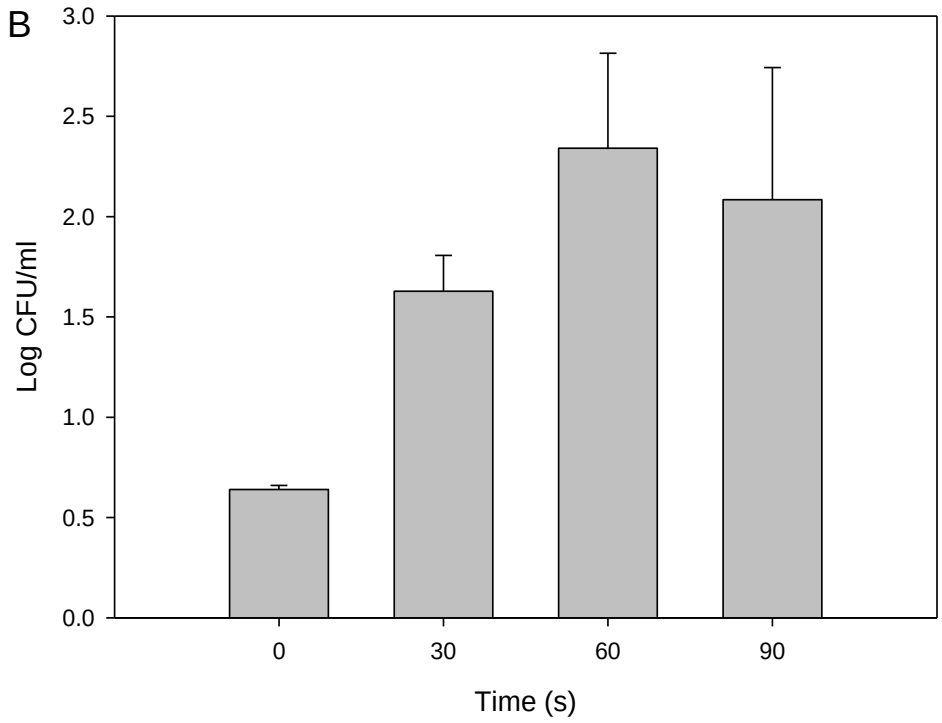
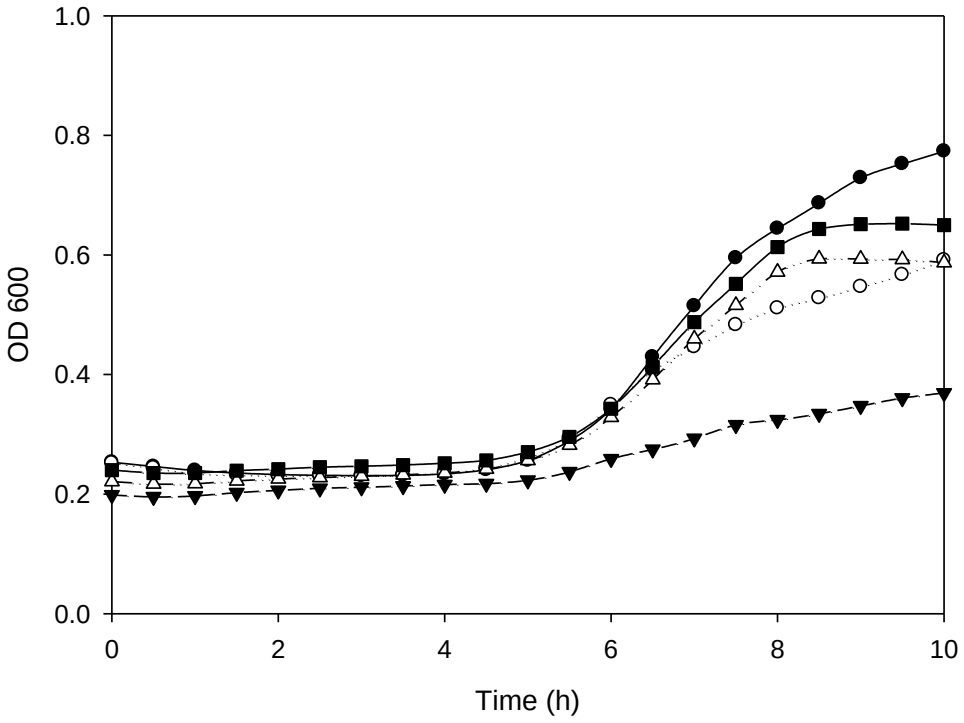


Fig. 4 Log differences of pathogens incubated on TSA and selective agar. Acid treatments were conducted with 2% lactic acid for *S.* Typhimurium (A) and *E. coli* O157:H7 (B). Acid-treated samples were neutralized with D/E broth and plated on each TSA and selective agars.

3.2.2. Growth curves in common broth media

Growth curves of acid-injured and *S.* Typhimurium cultured in 5 different broths were measured (Figure 5). *S.* Typhimurium inoculated in BHI, TSB, and TSBYE yielded better growth rates compared to NB and BPW. The acid-injured *S.* Typhimurium growth curve revealed a prolonged lag phase compared with that of non-treated microbes. Also growth curve of *E. coli* O157:H7 revealed similar tendencies with *S.* Typhimurium. Based on acid-injured pathogens growth curves, further tests were performed with BHI, TSB, and TSBYE.


1.0
0.8
0.6
0.4
0.2
0.0
OD 600
0
2
4
6
8
10
Time (h)

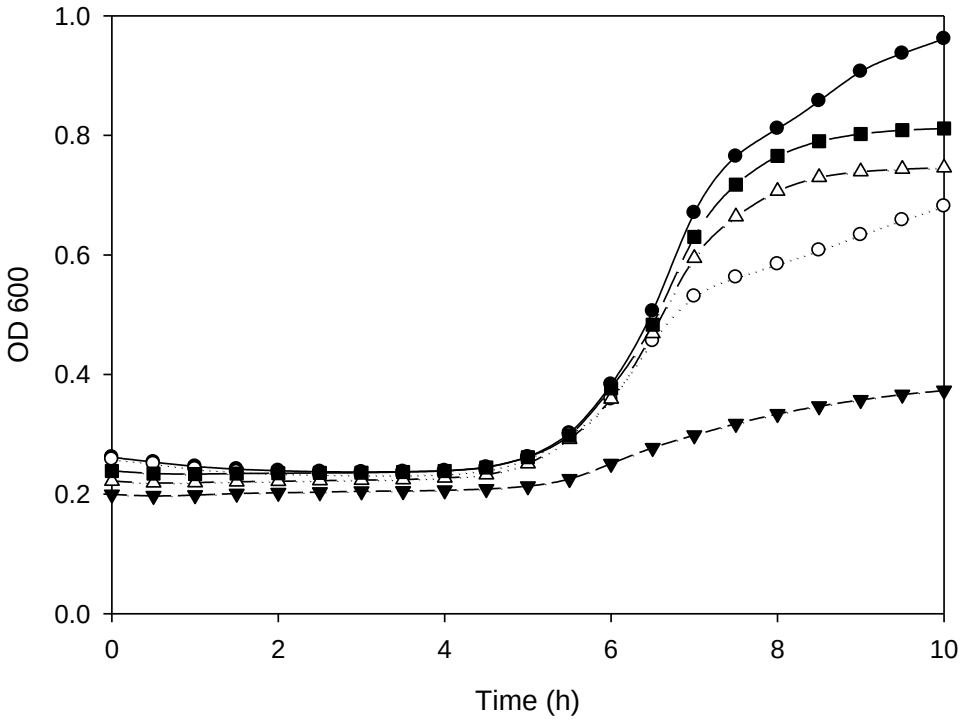
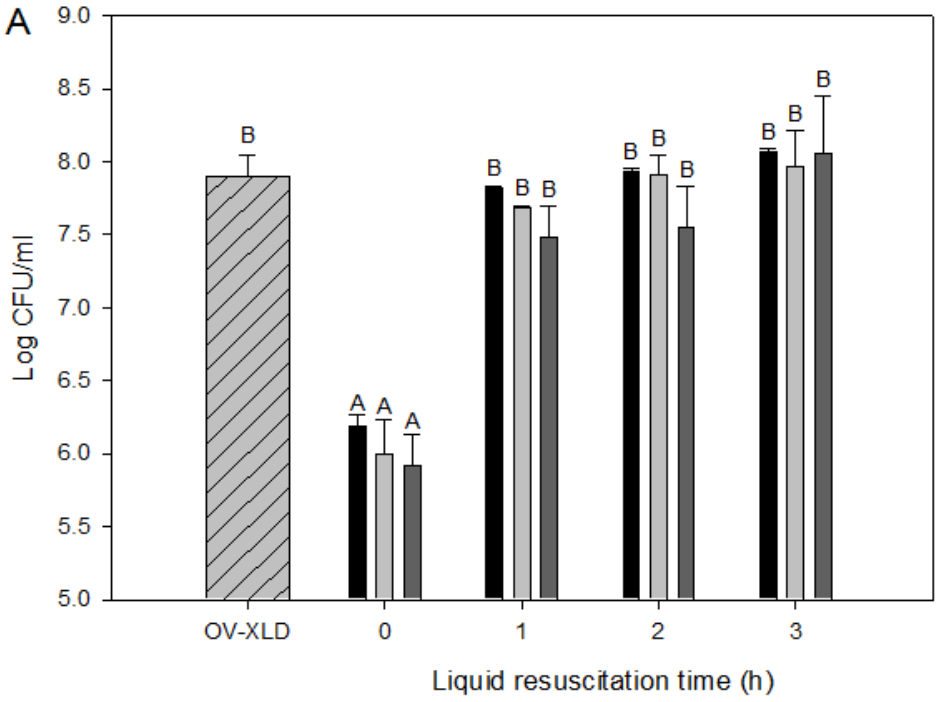


Fig. 5 Growth curve of acid-injured *S.* Typhimurium (A) and *E. coli* O157:H7 (B). Acid-treated samples were inoculated into BHI(—●—), BPW(···○···), NB(-·-▼-·-), TSB(—△—), TSBYE(- —■— -) at 37℃

3.2.3. Recovery of acid-injured cells by liquid resuscitation method

Figures 6 show recovered populations of acid-injured pathogens using the overlay or SPRAB method and liquid resuscitation methods. For acid-injured *S.* Typhimurium and *E. coli* O157:H7, 7.90 and 7.41 log CFU/ml were enumerated, respectively. For *S.* Typhimurium, after 1 hour incubation, none of the broths yielded results significantly different from that of the OV-XLD procedure. Also, there are no significant growth during 1 to 3 hour. Therefore, about 2 log of injured cells were recovered only 1 h incubation by liquid resuscitation method. In case of *E. coli* O157:H7 plated onto selective agar, appeared to significantly increase following 1 hour recovery in all broths, but also there was no significant increase when incubation exceeded 1 hour. However, 1 hour broth recovery was not significantly different from populations recovered on SPRAB. For the same recovery time interval, there were no significant differences between the three broth's populations. Also there are no significant increase of total viable counts during 3 hour recovery (Table 2).


A
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
Log CFU/ml
B
A A A
B B B
B B B
B B B
OV-XLD
0
1
2
3
Liquid resuscitation time (h)

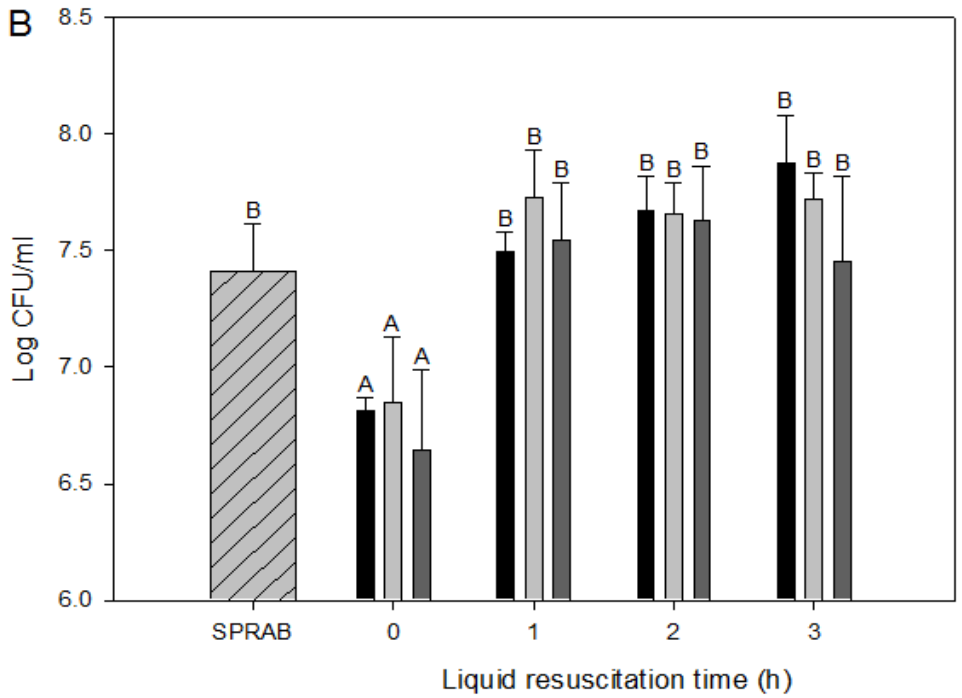


Fig. 6 Overlay-XLD or SPRAB population and recovery of acid-injured *S.* Typhimurium (A) and *E. coli* O157:H7 (B) in BHI(■), TSB(□) and TSBYE(■). For liquid resuscitations, acid-treated samples were ten-fold diluted with each broths and incubated at 37 followed by spreading on selective agars (XLD, SMAC) for further incubation. Results are explained with means and standard deviations independent triplicate. Bars with different letters are significantly different ($P < 0.05$).

Table 2. Population of pathogens during recovery of acid-injuries in resuscitation broths.

Microorganisms (Acid-treatment time)	Time (h)	Recovery broths		
		BHI	TSB	TSBYE
S. Typhimuirum (60 s)	0	8.25±0.30	8.06±0.16	7.83±0.44
	1	8.22±0.11	8.09±0.33	7.84±0.58
	2	8.17±0.32	7.76±0.62	7.79±0.39
	3	8.17±0.10	8.06±0.40	8.20±0.43
E. coli O157:H7 (60 s)	0	7.98±0.04	7.89±0.09	7.66±0.22
	1	7.96±0.05	7.88±0.10	7.65±0.20
	2	7.92±0.06	7.88±0.07	7.76±0.08
	3	8.04±0.04	8.02±0.28	7.86±0.05

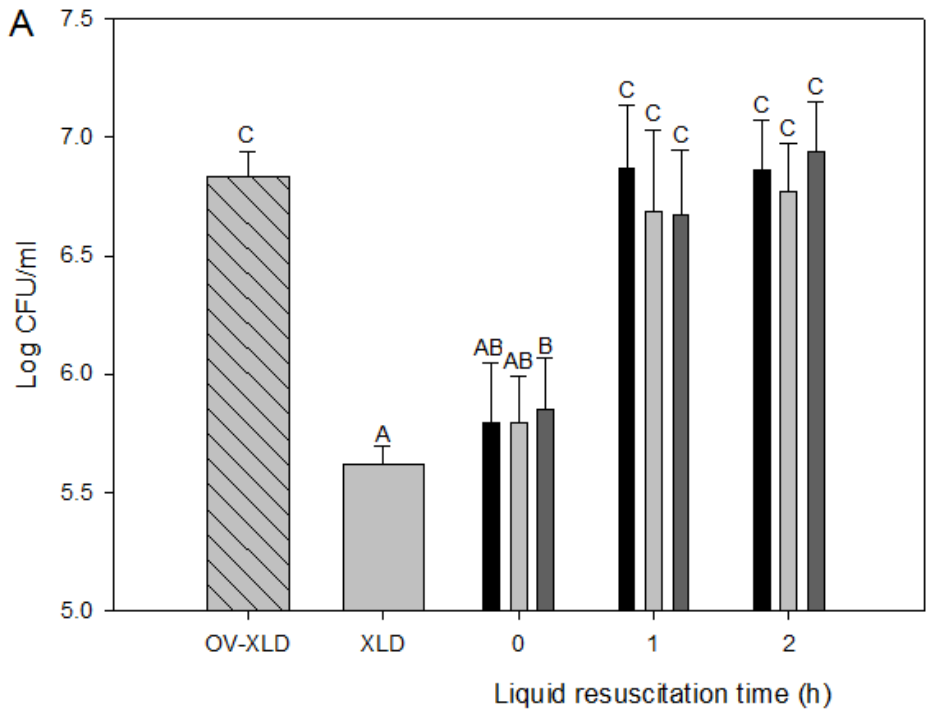
* Log CFU/ml. Acid-treated microorganisms were diluted resuscitation broths and plated on TSA followed by incubation at 37℃. The data represent means ± standard deviations from three replications.

3.3. Food application of liquid resuscitation method

For heat-treated low fat milk, S. Typhimurium was recovered about 1 log CFU/ml and significantly not differentiated with overlay-XLD populations in 1 h incubation in all tested broths (Figure 7). And also recovery of E. coli O157:H7 observed similar tendency.

For thermally treated apple juice, in case of S. Typhimurium, almost injured cells were resuscitated within 1 h incubation in any broths used in this study (Figure 8). Also E. coli O157:H7 revealed similar tendency that 1 h resuscitation was enough to recover injured cells to the level of SPRAB populations.

In both samples, there was no significant increase when incubation exceeded 1 hour. And there are no significant differences between 3 broths at same time intervals.


A
7.5
7.0
6.5
6.0
5.5
5.0
Log CFU/ml
C
A
AB
AB
B
C
C
C
C
C
C
OV-XLD
XLD
0
1
2
Liquid resuscitation time (h)

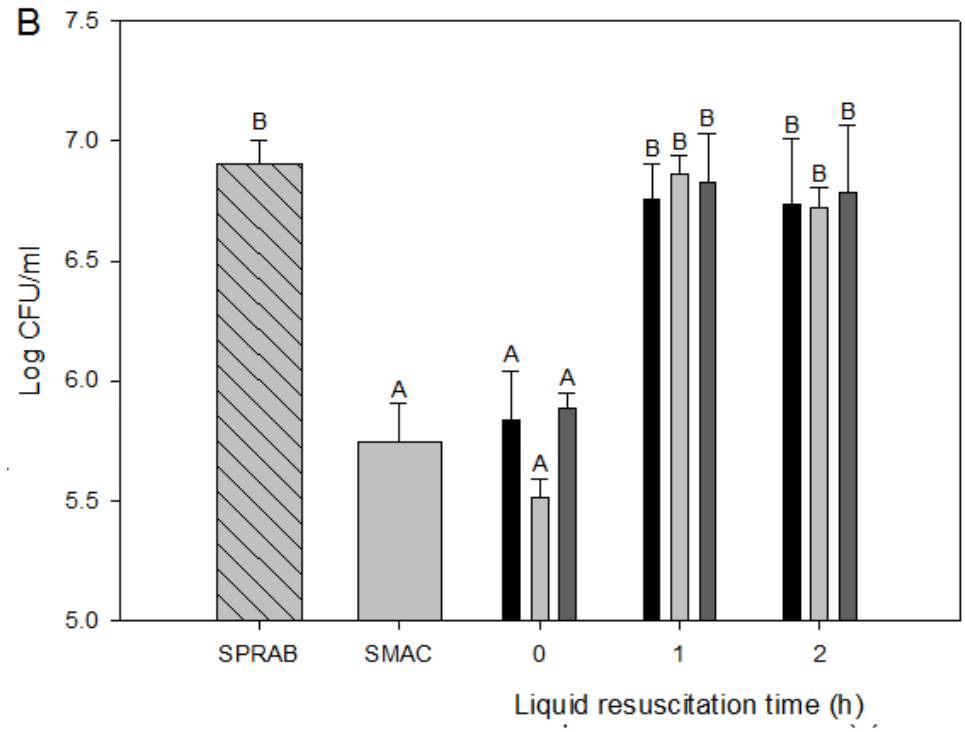
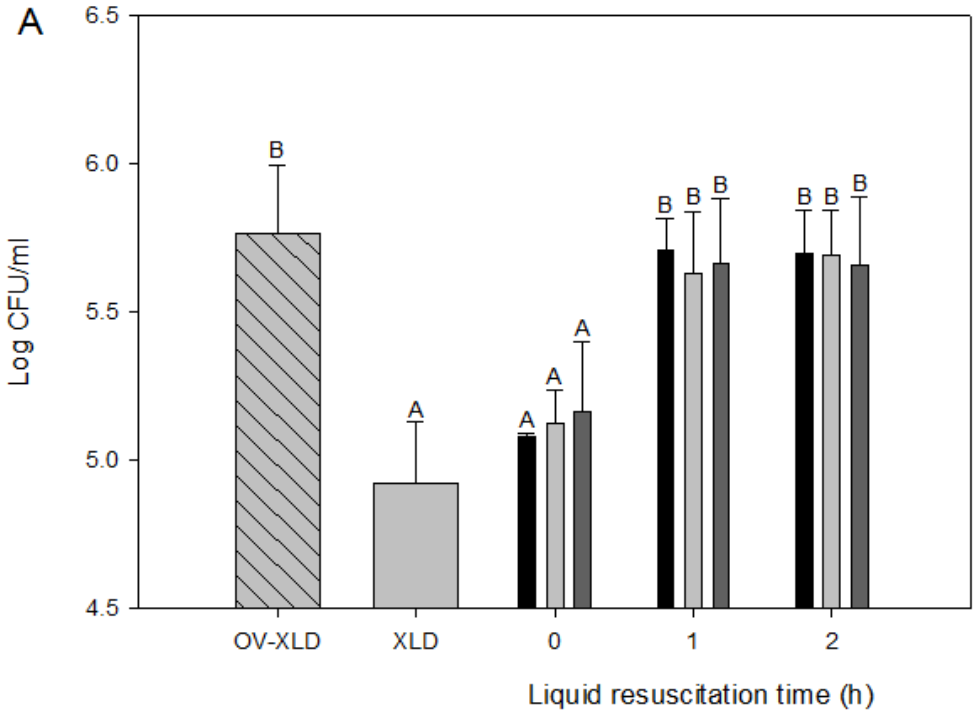


Fig. 7 Recovery of heat-injured *S*. Typhimurium (A) and *E. coli* O157:H7 (B) inoculated at low fat milk. For liquid resuscitations, heat-treated samples were ten-fold diluted with BHI(■), TSB(□) and TSBYE(■). Results are explained with means and standard deviations independent triplicate. Bars with different letters are significantly different ($P < 0.05$).


A
6.5
6.0
5.5
5.0
4.5
Log CFU/ml
B
A
A
A
A
B
B
B
B
B
B
OV-XLD
XLD
0
1
2
Liquid resuscitation time (h)

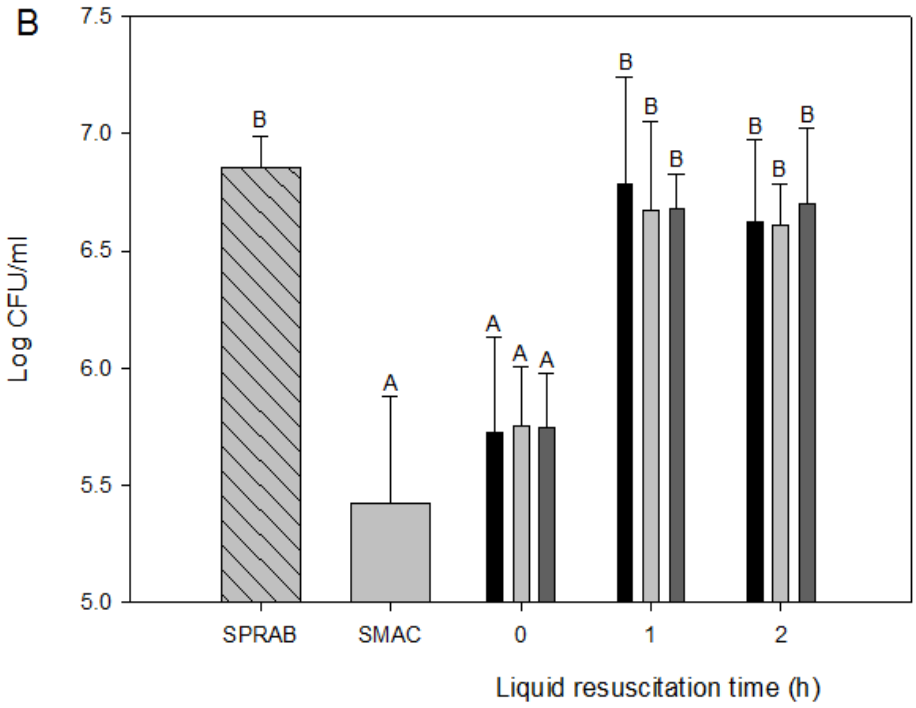


Fig. 8 Recovery of heat-injured *S.* Typhimurium (A) and *E. coli* O157:H7 (B) inoculated at apple juice. For liquid resuscitations, heat-treated samples were ten-fold diluted with BHI(■), TSB(▭) and TSBYE(▬). Results are explained with means and standard deviations independent triplicate. Bars with different letters are significantly different ($P < 0.05$).

IV. DISCUSSION

When bacteria are injured by thermal and acidic stresses, several functional problems occur due to destruction of cell membranes, and damage to ribosomes and ribosomal RNA. Following exposure to those stresses, de novo synthesis of rRNA, protein and ATP occurs during repair (Ray, 1986). Iandolo and Ordal (1966) discovered that to repair heat-injured *S. aureus*, neither cell-wall nor protein synthesis was needed for the recovery of resistance to salt. Furthermore, blocking RNA synthesis by actinomycin D showed that RNA synthesis is necessary for recovery. Since RNA synthesis is dependent on amino acid mixtures (Gale and Folkes, 1953), inadequate concentration and types of amino acids, or absence of specific amino acids in the recovery culture interfere with repair. Based on these research studies, significantly better recovery in some broths at each microbes may be affected by the different amino acid composition and amino acid utilization of each pathogens.

There were no significant differences between TSB and TSBYE recovery for each time interval, even though TSBYE has more nutrients. These results are congruent with those of Jasson et al. (2009). In that investigation, *E. coli* O157:H7 was incubated at 37℃ in two brands of TSB and in TSBYE. Their results showed that maximum specific growth rates were not significantly ($P > 0.05$) different from each other. Also, in application of liquid resuscitation which recover injured cells in food samples, low fat milk and apple juice, TSB and TSBYE were not significantly different in recovery capability. In those two cases, liquid resuscitation weren't affected by several compounds exist in food samples. However as optimization of liquid resuscitation are not concluded by these few experiments. Therefore, there is a need to study that recover the microbes injured by several stresses like freezing, drying or existed at different environments.

To improve on the overlay method, several procedures have been evaluated. For solid media, there are the thin agar layer (TAL) (Kang and Fung, 1999) and agar underlay (Kang and Siragusa, 1999) methods. The TAL method consists of a thin layer of non-selective agar poured onto a

layer of selective agar in a Petri dish and spread-plated with injured cells. Injured cells recover before selective agents can diffuse into the top non-selective layer. With the Agar underlay method, injured cells are recovered on non-selective agar and after 2 h repair selective agar is underlaid to the bottom chamber of a Lutri plate. Although these method are able to avoid effect of melted agar, complicated preparation processes are considered cumbersome. Also, other methods for recovery such as addition of compounds that help repair, and recovery utilizing membrane-filtration have been investigated (Taskila et al., 2011, Wu and Fung, 2004). However, these methods are considered to be complicated and costly.

Because of these disadvantages, liquid-repair has been investigated as an alternative. The two fold dilution (2-FD) method of Kang and Siragusa (2001) alleviates the overlay method's adverse effects on growth attributed to overlaying with melted agar, while obtaining significant recovery compared with non-recovered cells plated directly on selective agar. However, the inability to isolate target organisms for additional study is considered a major problem. Another disadvantage of liquid recovery discussed in previous

research studies is that uninjured microorganisms can multiply during recovery so as to make this method ineffective for regulatory purposes (Wu A.CH, 2008). However, in the present study, liquid resuscitation with broth showed that total populations of *S*. Typhimurium and *E. coli* O157:H7 showed no statistically significant change during 3 hour incubation in non-selective broths. These data demonstrate that during incubation in broth media for this portion of lag phase, multiplication of injured or non-injured cells does not occur to any significant degree. Therefore, when it can be demonstrated that microorganisms do not grow during a specific recovery period, liquid repair offers greater advantages compared to other methods. Also, liquid resuscitation has more advantages versus overlay method such as (1) absence of mild-heat inactivation caused by melted agar used for layering, (2) reduced time and labor costs due to a simpler procedure, (3) compared with 2 h resuscitation and overlay, resuscitation times for broth recovery were equal or shortened, (4) isolation of colonies for additional experiments is easier, (5) flexibility in broth selection, and (7) Special equipment or plates for recovery are not needed.

In conclusion, optimum liquid resuscitation conditions for heat or acid-injured *S*. Typhimurium and *E*. *coli* O157:H7 were determined to 1 h in BHI, TSB, or TSBYE. During recovery intervals, no growth of total viable cells ensures no multiplication of healthy cells. Therefore, liquid resuscitation can replace the overlay method due to greater convenience and timesaving.

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Taskila, S., Osmekhina, E., Tuomola, M., Ruuska, J., and Neubauer, P., 2011. Modification of buffered peptone water for improved recovery of heat-injured *Salmonella* Typhimurium. Journal of Food Science 76, M157-M162.

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VI. 국문초록

식중독균의 살균을 위한 여러 식품기술들의 연구는 활발이 진행되고 있다. 하지만 이러한 연구들의 결과는 손상된 세포들의 회복을 고려하면 과대평가된 것으로 여겨질 수 있다. 따라서 적절한 방법을 통한 손상된 세포의 회복은 살균 실험 설계에 있어 꼭 고려되어야하는 요소이다. 이를 위해 가장 많이 사용되고 있는 방법은 오버레이 방식으로, 비선택한천배지에 미생물을 도말 후 2 시간의 배양을 거쳐 녹아있는 선택배지를 비선택배지 위로 붓는 방법이다. 하지만 이 방법의 여러 단점으로 인해 새로운 회복 방법의 연구가 필요하였다. 따라서 본 실험을 통하여 열과 산에 의해 손상된 식중독균을 액체회복법을 통하여 정상세포로 회복시키기 위한 최적의 조건을 찾고자 하였다. 식중독균의 인위적인 열에 의한 손상을 위하여, *S.* Typhimurium 과 *E. coli*

O157:H7 는 55℃의 펩톤수에서 각각 15 분, 25 분 처리 되었으며, 산에 의한 손상을 위해 2%의 젖산에서 두 균 모두 60 초간 처리되었다. 손상받은 식중독균은 OD 측정을 통하여 선별된 BHI, TSB, TSBYE 에 접종되어 최장 3 시간동안 37℃에서 배양되었으며 각 시간별 회복 정도는 선택배지를 통해 측정되었다. 기존의 회복 방법으로 *S.* Typhimurium 의 경우 오버레이 방법이, *E. coli* O157:H7 의 경우 SPRAB 방법이 대조군으로 설정되었다. 열과 산 처리에 의해 손상된 식중독균 모두 1 시간의 액체회복법을 통해 효과적으로 정상세포로 회복되는 것을 확인할 수 있었으며, 세 액체배지간 유의적인 차이는 관찰되지 않았다. 또한 손상되지 않은 미생물의 유의적인 증가는 관찰되지 않았다 ($P>0.05$). 또한 저지방우유와 사과주스에 접종된 손상세포도 1 시간의 액체회복법에 의해 대부분이 회복되는 것을 확인하였다. 따라서 기존의 회복 방법들을 대신하여, 액체회복법은 더 빠르고, 간편하게 손상세포를 회복시킬 수 있을 것으로 기대된다.

주요어: 미생물 손상, 액체회복법, 회복, 오버레이, 식중독균

학번: 2015-21803