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Comparison of supplements* to enhance recovery of heat-injured *Salmonella* from egg albumen

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Abstract

Aims: The purpose of this study was to determine the proficiency of supplements to enhance the recovery of *Salmonella* from heat-treated liquid egg albumen on solid agar media.
Methods and Results: *Salmonella*-inoculated albumen, heated at 53·3°C for 4 min, was plated on 39 combinations of solid media with or without the addition of 12 supplements. Greater numbers of *Salmonella* ($P < 0·05$) recovered with the addition of 1·0 g l^{-1} ferrous sulfate ($FeSO_4$) than with any other supplements, except for 0·5 or 1·0 g l^{-1} 3′3′-thiodipropionic acid (TDP), which recovered equivalent populations. Addition of 1·0 g l^{-1} sodium pyruvate or 6·0 g l^{-1} yeast extract plus 1·0 g l^{-1} sodium pyruvate supported greater resuscitation than unsupplemented tryptic soy agar (TSA) or supplementing with 0·01 or 0·1 g l^{-1} N-propyl gallate, 10 g l^{-1} activated charcoal, 0·1 g l^{-1} $KMnO_4$ or 50 mg l^{-1} ethoxyquin. The remaining supplements supported recovery of equivalent numbers of *Salmonella*, which were fewer cells than recovered with 1·0 g l^{-1} $FeSO_4$, yet greater populations than recovered with 50 mg l^{-1} ethoxyquin.
Conclusion: Supplementation of plating media with $FeSO_4$, TDP or sodium pyruvate enhanced recovery of sublethally injured *Salmonella* from albumen.
Significance and Impact of the Study: Pasteurizing albumen impedes recovery of pathogens. These results suggest that the addition of supplements to plating media may assist resuscitation and colony development of heat-injured salmonellae.

Introduction

Pasteurized liquid egg products have not been implicated in known foodborne illnesses in the United States since 1970, although the UK Food Standards Agency recently reported two outbreaks of salmonellosis linked to the consumption of commercially pasteurized liquid egg imported from France (UK FSA 2007a,b). Two foodborne outbreaks from pasteurized liquid egg were also reported to have occurred in Japan between 1998 and 2002 (Hara-Kudo and Takatori 2009). The US Department of Agriculture (USDA), FSIS Pathogen Reduction–Hazard Analysis and Critical Control Point Program found that 48 of 10 146 (0·47%) pasteurized egg samples analysed between 1999 and 2003 were positive for the genus *Salmonella* (White *et al.* 2007). Recovering viable salmonellae from liquid egg white (albumen), however, is challenging (Thammasuvimol *et al.* 2006) because of the inherent antimicrobial make-up of the milieu (Kang *et al.* 2006). This could lead to underestimates of salmonellae in pasteurized egg albumen that may be responsible for *c.* 2600 foodborne illnesses per year, according to a recent USDA risk analysis (Latimer *et al.* 2007).

Thermal sterilization of bacteriological media stimulates autoxidation of compounds such as sugars and phosphates, which form reactive oxygen species (ROS) leading

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to substrate-accelerated bacterial inactivation and reduced recovery of sublethally injured cells (Postgate and Hunter 1963; Calcott and Postgate 1972; Mackey and Derrick 1982, 1986; Kell *et al.* 1998; Baylis *et al.* 2000; Wesche *et al.* 2009). Various supplements have been reported to support the recovery of sublethally injured cells (Wu 2008). This study evaluates amending solid plating media with compounds that may reduce ROS or in other ways assist in the recovery of injured salmonellae from egg albumen.

Materials and methods

Bacterial strains

Five strains of *Salmonella enterica* (Enteriditis PT 4 NVSL 94-13062, Enteriditis PT 13 NVSL 96-15835, Typhimurium TM-1 NVSL 96-6904, Blockley NVSL 95-3698 and Heidelberg 3347-1 Sheldon), which were reported in the International Egg Pasteurization manual (Froning *et al.* 2002), were provided by Dr Jayne Stratton (University of Nebraska). In preparation for thermal challenge studies in liquid egg albumen, isolates were selected for spontaneously occurring mutants resistant to 200 ppm nalidixic acid to minimize chances for contamination, as described elsewhere (Muriana *et al.* 1996; Muriana 1997). The *Salmonella* strains used in the present study were grown in 10 ml of tryptic soy broth with 200 ppm nalidixic acid (TSBN; Difco, Becton Dickinson, Sparks, MD, USA) at 37°C for 24 h, 10 μl of suspension was transferred to 10 ml TSBN after 24 h and incubated for another 24 h. Cells were pelleted by centrifugation at 10°C and 8700 *g* for 15 min, washed in sterile deionized water, centrifuged a second time and resuspended in 1 ml of sterile deionized water to concentrate the population ten-fold. Suspensions were serially diluted in 0·1% bacteriological peptone (Difco) diluent (pH 7·4) and plated on tryptic soy agar (Difco) with 200 ppm nalidixic acid (TSAN) and incubated for 24 h at 37°C before the number of colonies was counted.

Preparation of egg white

Large grade A shell eggs were purchased from a local grocer, shell-sanitized with 70% ethanol, aseptically opened, and the albumen and yolk were separated. Albumen was surface-plated (0·25 ml on each of four plates) on TSA and incubated for 24 h at 37°C. Albumen (95 ml) was placed into a sterile Waring model CAC32 glass blending jar (Waring Products, Torrington, CT, USA.) and blended with a Waring model LB10G variable speed blender for 1 min at the lowest setting. One ml of each of the five strains of concentrated *Salmonella* suspensions were added to the albumen and blended on low speed for 1 min. One millilitre of the uninoculated and inoculated albumen were serially diluted, spiral-plated on TSA and TSAN, respectively, and incubated at 37°C for 48 h.

Media preparation

Solid agar media were purchased in dehydrated form, prepared according to manufacturers' directions and are listed in Table 1. Supplements were added to media before or after autoclave sterilization as specified in Table 1. Media were poured (*c.* 12 ml) into sterile petri dishes and held at 21°C for 24 h before plating.

Albumen heat treatment

Albumen was heat-treated in thermal-death-time (TDT) discs (Jin *et al.* 2008). Sterile TDT discs were filled with 0·9 ml of inoculated egg white, sealed and heated in a Pharmacia Biotech Multitemp III recirculating water bath (Pharmacia Biotech AB, Uppsala, Sweden) at 53·3°C for 4 min. This time and temperature (although lower than USDA pasteurization requirements of 56·7°C for 3·5 min) was determined in preliminary studies so as to effect *c.* 2-log inactivation of salmonellae and impart sublethal injury to a proportion of the surviving population. Type K thermocouples connected to a model 94461-35 multi-input thermocouple thermometer and datalogger (Sper Scientific, Ltd, Scottsdale, AZ, USA) were placed at the centre of the water bath as well as affixed inside a control TDT disc through a hole to monitor the come-up time and treatment temperature. Heating times began when the disc was within 0·5°C of the target temperature and ended when discs were immersed in a slush ice bath.

Spiral plating, enumeration and pH measurement

Cooled TDT discs were sanitized by a 70% ethanol immersion followed by two sterile deionized water immersions, and 0·5 ml of the albumen was transferred to 4·5 ml of 0·1% peptone solution. A second serial dilution was made and plated in duplicate on media with or without supplements using a DW Whitley WASP II spiral plater (Microbiology International, Frederick, MD, USA). Plates were incubated at 37°C for 48 h and counted with a Synbiosis åCOLyte Supercount automated colony counting system (Microbiology International). An Accumet single junction, gelled Ag/AgCl, flat surface electrode (Fisher Scientific, Pittsburgh, PA, USA) connected to a model UB-5 bench top pH meter (Denver Instruments, Denver, CO, USA) was used to measure the pH of duplicate samples of each medium as well as albumen.

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Table 1 Formulations of media with supplements used to recover a five-strain composite of *Salmonella* from liquid egg white heated at 53·3°C for 4·0 min

Medium or supplement added to medium	pH	Source	Concentration of supplement per litre of medium	Descriptor for compounds or media
Tryptic soy agar (TSA)	7·50	Difco*	—†	TSA
TSA + yeast extract	7·41	Bacto‡	6·0 g	Yeast extract
TSA + sodium pyruvate	7·44, 7·37, 7·34§	Sigma¶	1, 3·5, 6·0 g	Na pyruvate added before or after sterilization
TSA + yeast extract + sodium pyruvate	7·37	Bacto and Sigma, respectively	1 and 6 g, respectively	Yeast/Pyruvate
Fluid thyoglycollate medium + granulated agar**	7·37	BBL†† and Difco, resp.	15 g	FTM
TSA + N-propyl gallate	7·50, 7·52, 7·12	MP‡‡	0·01, 0·1, 1·0 g	NPG
TSA + ethoxyquin	7·54, 7·54, 7·54	MP	0·5, 5·0, 50·0 mg	Ethoxyquin
TSA + ferric ammonium citrate ('Brown')	7·51, 7·51, 7·38	Spectrum§§	0·01, 0·1, 1·0 g	Ferric ammonium citrate
TSA + ferrous sulfate	7·50, 7·49, 6·93	Spectrum	0·01, 0·1, 1·0 g	$FeSO_4$
TSA + activated charcoal	7·55, 7·63, 7·69	Fisher¶¶	1·0, 5·0, 10·0 g	Activated charcoal
TSA + 3′3′-thiodipropionate	6·81, 6·01, 4·36	Acros***	0·5, 1·0, 5·0 g	3′3′-thiodipropionic acid
TSA + ethylenediaminetetraacetic acid	7·40, 7·42, 6·95	Sigma	0·05, 0·1, 1·0 g	EDTA
TSA + potassium permanganate	7·50, 7·53, 7·77	Acros	1·0, 10, 100 mg	$KMnO_4$
Plate count agar	7·06	Difco	—	PCA
TSA + sodium chloride	7·47	MP	15 g	NaCl
TSA + sodium thiosulfate	7·48, 7·54, 7·58		0·05, 0·5, 5·0 g	Sodium thiosulfate

*Difco, Beckton Dickinson, Sparks, MD.
†—, prepared according to manufacturer's directions with no added supplements.
‡Bacto, Beckton Dickinson, Sparks, MD.
§Values represent average pH measurements for each of three supplement concentrations added, respectively.
¶Sigma-Aldrich Corp., St Louis, MO.
**Granulated agar was combined with FTM, boiled, and sterilized at 121°C and 15 psi for 15 min.
††BBL, Beckton Dickinson, Sparks, MD.
‡‡MP Biomedicals, Santa Ana, CA.
§§Spectrum Chemicals and Laboratory Products, Gardena, CA.
¶¶Fisher Scientific, Pittsburg, PA.
***Acros Organics, Geel, Belgium.

Statistical analysis

Three replicate experiments were conducted, and duplicate samples from each trial were averaged. Transformed $\log_{10}$ values were analysed by the GLM using Statistical Analysis Software ver. 9·1 (Statistical Analysis Systems Institute, Cary, NC, USA). Significant differences ($P < 0·05$) between media in the recovery of *Salmonella* were determined by the least significant differences test.

Results

Heating and cooling times and pH measurement

Albumen samples in TDT discs required 45 s to come up to within 0·5°C of the target temperature (53·3°C) and cooling to 15°C required 12 s. Albumen pH averaged 7·84. The pH levels of media are reported in Table 1.

Population of *Salmonella* in inocula and inoculated albumen

Population averages of five strains of *Salmonella* after a ten-fold concentration was 9·46 log CFU ml^{-1}. Uninoculated control samples of albumen did not contain aerobic bacterial contaminants, as determined by plating on TSA and incubation at 37°C for 48 h. Inoculated albumen samples (1 ml of each of five strains of *Salmonella* added to 95 ml of albumen) contained an average of 7·72 log CFU ml^{-1} of *Salmonella* before heating. Identical numbers of *Salmonella* were recovered on TSA as well as TSA + 1·5% NaCl, which was used to assess injury.

Recovery of *Salmonella* from heat-treated egg white on nonselective agar with supplements

Populations of salmonellae that recovered on nonselective media with supplements are represented in Fig. 1 and

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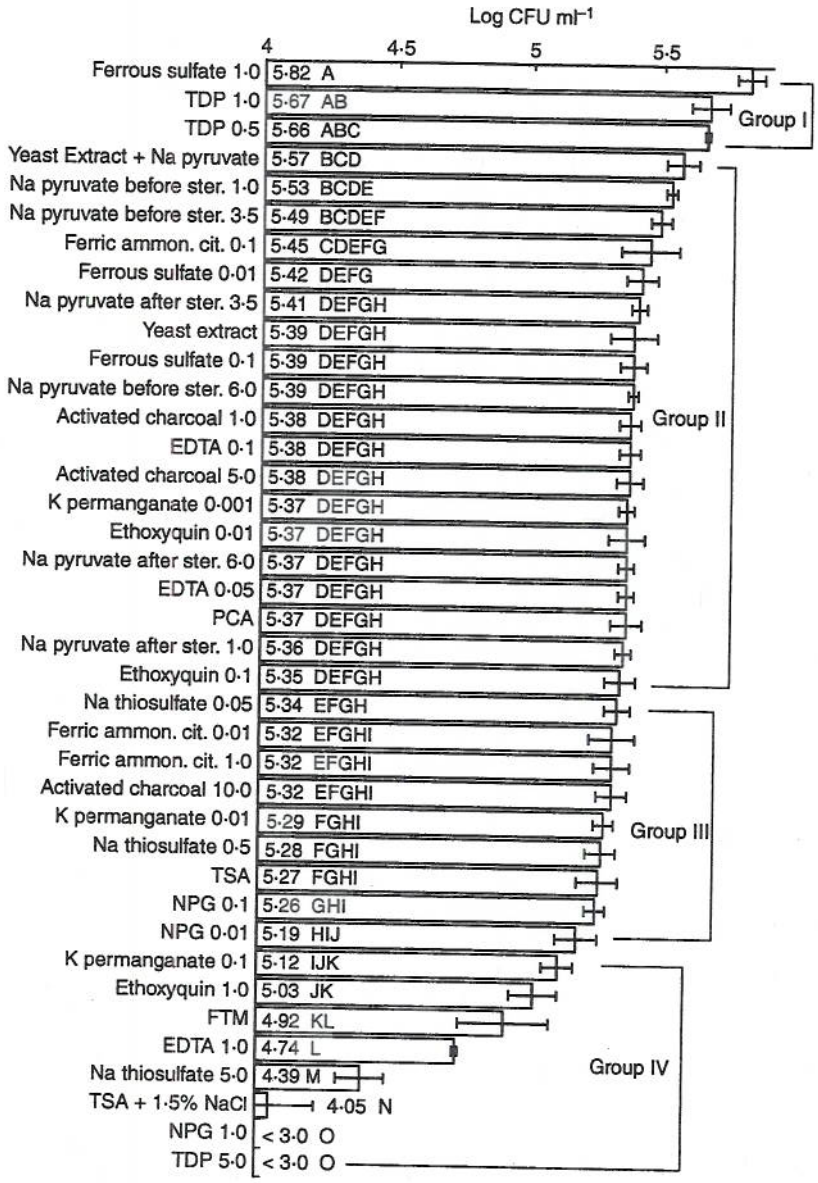

Figure 1 Populations of a five-strain composite of *Salmonella* inoculated into egg albumen, thermally treated at 53·3°C for 4 min and recovered on 39 combinations of plating agar and supplements. Values represent mean $\log_{10}$ CFU ml^{-1} of three replicate experiments. Mean values that are not followed by the same letter are significantly different ($P \leq 0{\cdot}05$). Error bars represent standard deviations. The detection limit was 3 log CFU ml^{-1}.

subdivided into groups for discussion. Significantly higher populations of injured cells were recovered on TSA + 1·0 g l^{-1} $FeSO_4$ than on all other media tested except for those recovered on TSA + 0·5 or 1·0 g l^{-1} TDP. Mean inactivation of salmonellae (1·90 log CFU ml^{-1}) was determined by comparing recovery of cells from untreated albumen (7·72 log CFU ml^{-1}) with the recovery from treated samples on TSA + 1·0 g l^{-1} $FeSO_4$ (5·82 log CFU ml^{-1}). Log injury of salmonellae is reported as the difference in log CFU ml^{-1} recovered on TSA + 1·0 g l^{-1} $FeSO_4$ and TSA + 1·5% NaCl (4·05 log CFU ml^{-1}). The proportion of salmonellae injured because of thermal treatment and exposure to innate antimicrobial constituents in albumen was 1·77 log CFU ml^{-1}, or 98·3% injury as calculated by the equation (1·0 − [CFU ml^{-1} of injured cells ÷ CFU ml^{-1} of healthy cells]) × 100%. Logarithmically fewer (0·55 $\log_{10}$ CFU ml^{-1}) and proportionally fewer (72%) cells were recovered on TSA in comparison with TSA + 1·0 g $FeSO_4$, and logarithmically greater inactivation of salmonellae occurred as assessed by TSA (2·45 log CFU ml^{-1}) when compared with TSA + 1·0 g $FeSO_4$ (1·90 log CFU ml^{-1}). The individual and separate addition of the four reducing agents, 0·1 g l^{-1} $FeSO_4$, ethoxyquin, sodium thiosulfate and NPG or supplementation with yeast extract, activated charcoal, ferric ammonium

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citrate, EDTA or $KMnO_4$ to TSA resulted in no greater recovery of cells than samples plated on TSA alone. The addition of 1·0 g l^{-1} $FeSO_4$ or 0·5 or 1·0 g l^{-1} TDP to TSA supported greater recovery of injured salmonellae than did TSA or PCA alone. All media in group II supported resuscitation of equivalent populations of cells. Recovery on TSA + 1·0 g l^{-1} NPG or 5·0 g l^{-1} TDP fell below the detection threshold of 3 log CFU ml^{-1}, as determined by spiral plating 50 μl samples in duplicate from 100-fold serial dilutions of heated albumen.

Discussion

Although TSA without resuscitation amendments is often used as a nonselective medium for resuscitating and supporting colony development of injured salmonella (Dykes and Moorhead 2001; Wuytack *et al.* 2003; Yuste *et al.* 2004; Perni *et al.* 2007), the present results report five combinations of supplements, which when added to TSA, support the recovery of statistically greater numbers of cells than TSA alone. Ferrous sulfate, TDP and sodium pyruvate act as antioxidants or reducing agents, which neutralize or prevent the formation of ROS known to injure and prevent resuscitation of bacterial cells. Media in group I, in addition to TSA + 0·1% sodium pyruvate and 0·6% yeast extract, or 0·1% sodium pyruvate added before autoclaving, supported recovery of significantly higher numbers of salmonellae than were recovered on TSA. Supplementing nonselective media with reducing agents such as 1·0 g l^{-1} $FeSO_4$, which recovered 72% more injured cells than TSA, should be considered when the resuscitation of a maximum number of stressed bacteria is desired.

The proficiency of TDP to enhance the repair of stressed bacteria has been sparingly reported in the literature, and has never, to the authors' knowledge, been reported to increase recovery of injured salmonellae. Calabrese and Bissonnette (1990) reported that the addition of TDP between 0·5 and 20 g l^{-1} to tryptone glucose yeast extract agar did not enhance the recovery of coliforms from acid mine water. McDonald *et al.* (1983), however, found that tryptone glucose extract agar or violet red bile agar amended with either sodium pyruvate (3·3–6·6 g l^{-1}) or TDP (2·5–10 g l^{-1}) significantly increased ($P < 0·01$) the recovery of heat- or freeze-injured *Escherichia coli* proportionate with the amount of supplement added; although synergism did not occur when supplements were added simultaneously. Mizunoe *et al.* (1999) determined that the addition of TDP between 0·1 and 1·0 g l^{-1} to LB agar restored the culturability of *E. coli* following cold (4°C) low osmotic/starvation stress commensurate with increasing concentrations of the supplement. While our results indicate that 5 g l^{-1} of TDP inhibited the growth of thermally injured salmonellae in comparison with TSA, lower concentrations (0·5 and 1·0 g l^{-1}) were effective in enhancing resuscitation of injured cells in comparison with TSA alone.

As with TDP, the use of $FeSO_4$-ammended TSA to promote the resuscitation of injured bacteria is infrequently described in the literature. Ferrous sulfate has been used as an iron source to improve detection of *Salmonella* in pre-enrichments and enrichments of egg albumen (Gast and Holt 1995; Valentín-Bon *et al.* 2003). Rayman *et al.* (1978) reported that adding 0·5 g l^{-1} filter-sterilized $FeSO_4$ to TSA after autoclaving resulted in the recovery of 10% and 60% more healthy and heat-injured cells of *Salmonella* Senftenberg, respectively, which was attributed to the neutralization of H_2O_2 that is spontaneously formed during media preparation. In the same study, 6% fewer salmonellae recovered on TSA + 0·5 g l^{-1} $FeSO_4$ in comparison with TSA + 2·5 g l^{-1} sodium pyruvate. In the present study, however, the 72% greater numbers of salmonellae that recovered with 1·0 g l^{-1} $FeSO_4$ *vs* unamended TSA may be a result of adding $FeSO_4$ prior to autoclaving, which may be the optimal time to incorporate ROS-reducing compounds (Stephens *et al.* 2000). Supplementating TSA with 0·1 or 0·01 g l^{-1} ferrous sulfate prior to autoclaving also resulted in the recovery of equivalent numbers of sublethally injured *Salmonella* as that of TSA supplemented with 1 or 3·5 g l^{-1} sodium pyruvate. However, TSA + 1·0 g l^{-1} of ferrous sulfate recovered significantly greater numbers (49%) ($P < 0·05$) of salmonellae than TSA with 1 g l^{-1} sodium pyruvate added before autoclaving and 65% more salmonellae when 1 g l^{-1} sodium pyruvate was added to TSA after autoclaving. Another iron source (ferric ammonium citrate) does not have the ROS-reducing power of $FeSO_4$ and, consequently, supported the recovery of between 63% and 68% fewer cells than TSA + 1·0 g l^{-1} $FeSO_4$.

It has been suggested that because sodium pyruvate is a key substrate in the Krebs cycle and electron transport system, its inclusion in media could aid in the cellular production of ATP, necessary for bacterial resuscitation (Kobayashi *et al.* 2005). While this may be true, the present results indicate that $FeSO_4$ and TDP, possibly playing the role of ROS-reducing compounds, enhanced the recovery of an equivalent number of injured cells as did sodium pyruvate. Furthermore, the H_2O_2-quenching ability of sodium pyruvate has been previously reported (Martin *et al.* 1976; McDonald *et al.* 1983; Wu 2008). The inability of many injured cells to recover on media in group IV may indicate the utility of these compounds added singly or in combination as selective supplements for assessing bacterial injury; although the proficiency of such media to support colony development of a maximum population of healthy cells would need to be validated.

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Although the present study examined only solid agar media, results as to the efficacy of resuscitating compounds in this article may be useful in research on the recovery of heat-injured *Salmonella* from albumen in liquid pre-enrichments. Ferrioxamine E, which was not tested in this study, is known to be a selective source of iron for *Salmonella* but not *E. coli* in egg albumen enrichments when used at concentrations less than 50 $\mu g\ ml^{-1}$ (Reissbrodt *et al.* 1996; Kingsley *et al.* 1999; Thammasuvimol *et al.* 2006). Future studies may explore the addition of this compound to solid agar media.

The five strains of *Salmonella* used in this study were all selected for resistance to nalidixic acid, which should not significantly alter thermal resistance. Stopforth *et al.* (2008) demonstrated that there was no significant difference between the heat resistance of nondrug-resistant *Salmonella* isolates and multidrug-resistant isolates of *Salmonella*. Future studies may examine the thermal resistance of wild-type *Salmonella* in egg albumen in place of nalidixic acid-resistant strains, to more closely reflect natural contamination. The addition of $FeSO_4$, TDP, yeast extract and sodium pyruvate may also be further evaluated to optimize combined concentrations in solid media or selective and nonselective enrichment broths for resuscitating injured salmonellae from pasteurized liquid egg white.

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