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# A rapid twofold dilution method for microbial enumeration and resuscitation of uninjured and sublethally injured bacteria

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# A rapid twofold dilution method for microbial enumeration and resuscitation of uninjured and sublethally injured bacteria

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**Aims:** A rapid and simple method for enumerating uninjured and sublethally injured bacterial cells, the twofold dilution method (2FD), was developed and evaluated.

**Methods and Results:** Following twofold serial dilution of samples in a 96 well microtiter plate, double strength selective broth or nonselective broth was added to each well. For resuscitation of heat-injured (55°C for 10 min) coliforms, the selective broth was added to the wells after 3 h preresuscitation time in buffered peptone water. The results of the 2FD were compared to plating methods for total and coliform plate counts from mixed cultures and beef carcass surface tissue samples.

**Conclusions:** The 2FD method results were not significantly different for uninjured cells ($P > 0{\cdot}05$) from those obtained using Petrifilm and standard plating. Correlation of the scatterplot of spread plating and 2FD indicated a high level of agreement between these two methods ($R^2 = 0{\cdot}98$ for total counts and $R^2 = 0{\cdot}96$ for coliforms from mixed cultures; $R^2 = 0{\cdot}98$ for total cell counts and $R^2 = 0{\cdot}94$ for coliforms from faeces inoculated beef carcasses).

**Significance and Impact of the Study:** The twofold dilution method recovered significantly higher numbers of heat-injured coliforms compared to conventional plating methods ($P < 0{\cdot}05$).

## INTRODUCTION

Several methods and variations for conducting bacterial plate counts have been developed (Swanson *et al*. 1992; Koch 1994; Jett *et al*. 1997). Sublethally injured bacterial cells are generally not resuscitated on selective agar and several methods have been developed to recover these populations (Hartman *et al*. 1975; Speck *et al*. 1975; Cole *et al*. 1993; Clavero and Beuchat 1995; McCleer and Rowe 1995; Sage and Ingham 1998; Kang and Siragusa 1999). Cole *et al*. (1993) reported that injured cells are easily resuscitated on nonselective complete broth or liquid medium in less than 2 h.

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Although the liquid medium resuscitation method is simpler and faster than solid agar resuscitation methods, resuscitation in liquid broth systems generally precludes any potential for bacterial enumeration (Cole *et al*. 1993). To date, no rapid and simple viable cell counting method has been reported with the potential for resuscitation of sublethally injured cells coupled with quantification in liquid culture. Our laboratory has developed a method for the accurate enumeration of total culturable bacterial cells and coliform counts from samples based on a liquid medium format. This paper presents a comparison of the twofold dilution (2FD) method for the enumeration of micro-organisms, as well as its application for the enumeration of sublethally injured coliform bacteria.

## MATERIALS AND METHODS

### Bacterial cultures

*Escherichia coli* (ATCC 25922), *E. coli* O157:H7 (ATCC 35150), *Salmonella typhimurium* (ATCC 19855 and UNL

10636–97), *Listeria monocytogenes* (LCDC 81–861 and ATCC 49593), *Lactobacillus acidophilus* (PA3), and *Staphylococcus aureus* (ATCC 12598) were maintained as part of the culture collection of the Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, NE (MARC), as glycerol suspensions at −70°C. Cultures were prepared by subculturing in tryptic soy broth (TSB; Difco-BBL, Cockeysville, MD, USA) at 37°C for 24 h under static conditions. Following incubation, 1 ml of each individual culture was mixed together in a test tube. Part of the mixture (0·1 to1 ml) was serially diluted with buffered peptone water (BPW, Difco-BBL) to obtain a total of 25 different dilutions and cell concentrations. This set of assorted dilutions was used for the evaluation of the twofold dilution method.

### The 2FD method for nonselective bacterial enumeration

All of the 2FD growth assays were performed in standard, clear polyethylene flat bottomed microtitre plates (Becton Dickinson and Company, Franklin Lake, NJ, USA). To each well, 100 $\mu$l of BPW was aliquotted and samples were serially diluted twofold through the entire row of wells (1 : 2–1 : 4096). For nonselective growth, 100 $\mu$l of double strength Purple Broth base (Difco) with 1% (w/v) glucose (PBG) was then added and the plate incubated for 16 h at 37°C. For enumerating coliform bacteria, double strength modified Violet Red Bile broth (mVRBB; substituted brom cresol purple for neutral red indicator) was used. The reciprocal of the dilution of the endpoint or the last well with positive growth (defined by conversion of the indicator dye) was used to calculate the minimum cell number possible for that dilution normalized per unit volume and transformed to the $\log_{10}$ cfu ml$^{-1}$.

### Standard culture methodology for nonselective and coliform bacterial enumeration

At the time of inoculating the 2FD plates, samples were also plated on Petrifilm aerobic count and *E. coli* Petrifilm (3M, Minneapolis, MN, USA) or spread plated 100 $\mu$l per plate on tryptic soy agar (TSA, Difco-BBL) and incubated under the same conditions as for the 2FD plates. Following incubation at 37°C for 24 h under aerobic conditions, colony counts were normalized per unit volume and transformed to the $\log_{10}$ cfu ml$^{-1}$.

### The 2FD method for enumerating bacterial counts from beef carcass surface tissues inoculated with fecal suspensions

Beef carcass tissues were spoon-inoculated as described previously (Cutter and Siragusa 1994; Dorsa *et al*. 1997) with a bovine faecal mixture made from mixing equal masses of fresh bovine faeces obtained from three different animals located on the laboratory site's research feedlot pens. After inoculation, the tissues were incubated for 15 min at room temperature (Cutter and Siragusa 1994; Dorsa *et al*. 1997). Using cotton swabs (Hardwood Products Co., Guilford, ME, USA), a 10 cm × 10 cm area was sampled and mixed with 5 ml of sterile BPW. Samples were subjected to analysis by the 2FD and standard culture methods for total mesophilic bacteria and presumptive coliforms described above.

### Enumeration of sublethally heat-injured coliforms using 2FD method

The bovine tissue inoculated with the 1 : 2 diluted bovine faeces was sampled by the swab method as described above. One hundred microlitres of the diluted sample suspension was added to 5 ml of BPW in a screw capped tube, which had been preheated and maintained at 55°C. After inoculation, the tube was tightly sealed, immersed in a shaking water bath, and heated at 55°C for 10 min. After heating, tubes were cooled immediately in slush ice. Heat injured cell suspensions were spread-plated on VRB (Difco-BBL) and *E. coli* Petrifilm then incubated for 24 h at 37°C. For the twofold dilution method, another 100 $\mu$l of sample was twofold serially diluted on a 96 well microtitre plate using BPW. The twofold dilution set was incubated at 37°C for 3 h for resuscitation of injured cells, after which an equal volume of double strength mVRBB was added to each well. The set was incubated for a further 13 h at 37°C in the dark. After incubation, the normalized bacterial counts from Petrifilm and the 2FD method were compared. Each experiment was performed three times.

### Statistical analysis

Bacterial populations were converted to $\log_{10}$ cfu ml$^{-1}$ and analysed statistically by ANOVA using the SAS General Linear Models procedure (SAS 1991). Means of three replicates were reported. Differences among treatments were examined for level of significance by Duncan's multiple range test.

## RESULTS AND DISCUSSION

From the mixed culture suspension of eight bacterial strains representing six species, only *E. coli* strains grew and fermented lactose (turning indicator yellow) in the mVRBB selective medium. Agreement between the conventional TSA spread plate method and the 2FD method from the mixed culture system was high for both the total mesophilic count ($R^2 = 0{\cdot}98$) and the coliform bacterial counts

($R^2 = 0{\cdot}96$) (Fig. 1 a,b, respectively). Correlations between the 2FD method and Petrifilm (aerobic or *E. coli* Plate) on total viable cell counts ($R^2 = 0{\cdot}98$) and coliforms ($R^2 = 0{\cdot}94$) from bovine faeces-inoculated carcass tissue samples are presented in Fig. 2(a,b). The data shown in Fig. 3 illustrates is that the 2FD method is a much more efficacious method for enumeration of heat-injured cells than is direct spread plating on selective media VRBA and *E. coli* Petrifilm ($P < 0{\cdot}05$). Significantly higher counts ($P < 0{\cdot}05$) from heat injured samples were obtained with the 2FD method than from either spread plating on VRBA or on *E. coli* Petrifilm. Using the 2FD method, a significantly high number of sublethally heat injured bacteria were recovered from tested carcass surface samples.

Overall, the 2FD method shows high agreement with the conventional agar and Petrifilm agar plate methods for counting viable bacterial cells and coliform bacteria from mixed cultures and bovine faeces-inoculated beef carcass tissues. Moreover, the efficiency of the 2FD method in

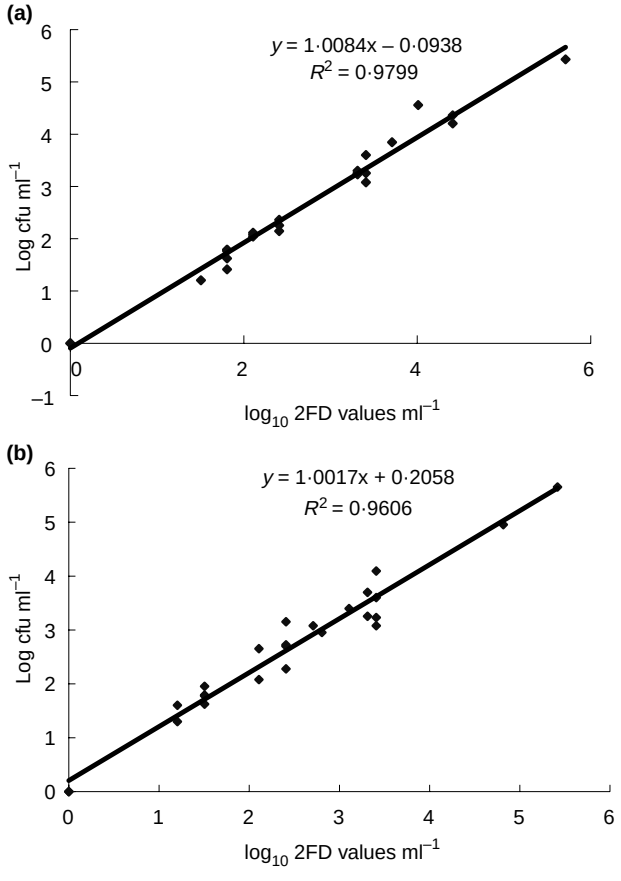


**Fig. 1** The comparison of total viable bacterial counts (a) and coliforms (b) from mixed cultures obtained by TSA spread plating on tryptic soy agar and the twofold dilution (2FD) method

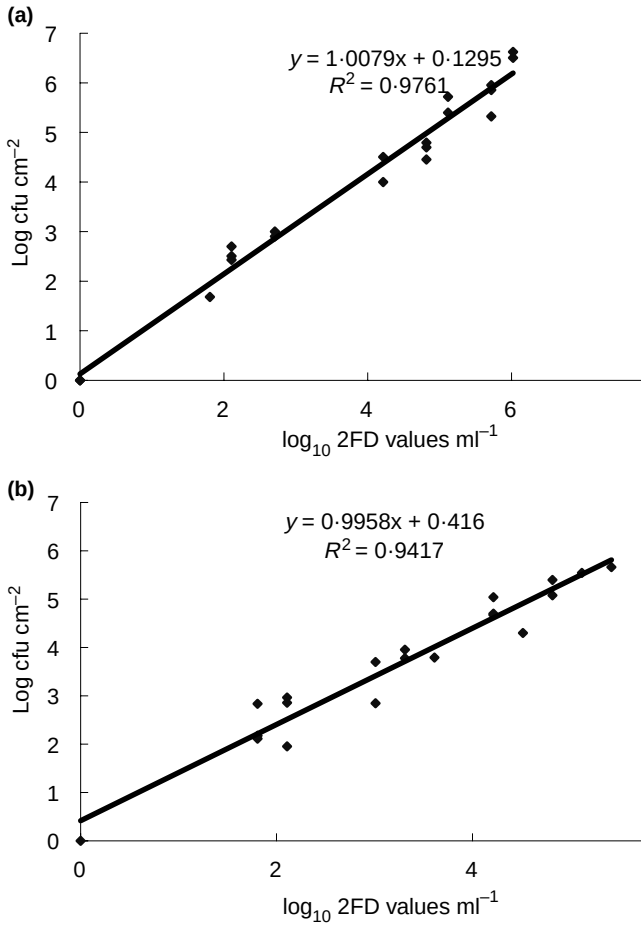


**Fig. 2** The comparison of total viable bacterial counts (a) and coliforms (b) from beef carcass tissues inoculated with bovine faeces obtained by the PetriFilm method and the twofold dilution (2FD) method

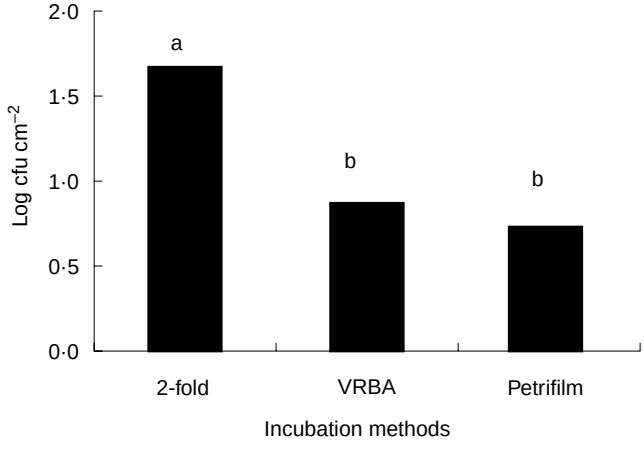


**Fig. 3** Recovery of heat-injured coliforms using plating methods (VRBA: Violet red bile agar; and Petrifilm: *E. coli* Petrifilm) and the twofold dilution (2FD) method with resuscitation and subsequent outgrowth in mVRBB

terms of economy of space, time, and material far exceeded the agar plate method.

In the 2FD method, the final dilution positive well theoretically could contain only one target cell or cfu; therefore, the sensitivity of the 2FD method is as high as that from plating a 1 ml sample with 1 cfu $ml^{-1}$. Other rapid plating techniques are less sensitive. For instance, Siragusa (1999) reported the track-dilution plating method for enumeration of micro-organisms with 10 $\mu$l inoculum occluded samples with less than 100 cfu $ml^{-1}$. Fung and LaGrange (1969) reported a microtitre plate dilution step followed by deposition of 25 $\mu$l of a diluted sample on a Petri dish of agar medium without subsequent spreading of the inoculum, limiting the method to a threshold of more than 40 cfu $ml^{-1}$.

Population counts obtained using the 2FD method preceded by an injury resuscitation step averaged about 80% higher than counts from direct plating or the Petrifilm plating method. In a preliminary experiment (data not shown), we evaluated the resuscitation times of 1 and 3 h in BPW and found 3 h to result in greater injury repair, a result congruent with that reported previously (Cole *et al*. 1993). In the case of the conventional spread plating and Petrifilm, the injured cells are directly exposed to selective agents in selective medium, precluding their recovery and colony formation (Ray and Adams 1988). In the 2FD method, the time of addition of selective broth to the diluted samples of potentially injured bacteria can be adjusted to achieve recovery while retaining selectivity. Post-resuscitation bacterial counts of heat injured cultures of *E. coli* O157:H7 (ATCC 35150) determined by the 2FD method were not significantly different ($P < 0{\cdot}05$) from homologous counts determined on nonselective agar, TSB (data not shown).

As with previously reported end-point dilution methods (Bathelier *et al*. 1996; Palenzuela *et al*. 1997), the 2FD method offers several advantages both practical and technical over the agar plate method. These advantages and potential variations include: (1) utilization of one microtitre plate instead of dilution bottles; (2) utilization of the same microtitre plate instead of several Petri dishes for duplicate plating of several dilutions; (3) only a very small area is needed for operation and storage; (4) ease of waste sterilization and disposal; (5) savings of time and media [in terms of medium volume, the 2FD method requires about 0·05% of media volume compared to conventional methods] for the enumeration of bacteria; (6) anaerobic incubation could be achieved both by using anaerobic jars or chambers or by including oxygen scavenger agents, such as Oxyrase, then sealing the microtitre plate; (7) no melting and tempering of agar in solid medium injury repair procedures; and (8) the means to perform several replicate determinations on each sample if necessary. One disadvantage is the inability to isolate micro-organisms for further study without re-culturing well contents. In the setting of routine testing of products or process monitoring, the 2FD values offer a reasonable estimate of microbial load with a high correlation to plate culture. Once the 2FD is optimized for specific sample types, its advantages should become fully manifested.

In conclusion, the twofold dilution method (2FD) is a simple and economical method for bacterial cell enumeration offering a means for testing a large number of samples as well as recovery of injured cells.

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