Research Note

Efficacy of the Thin Agar Layer Method for the Recovery of Stressed *Cronobacter* spp. (*Enterobacter sakazakii*)

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ABSTRACT

Cronobacter spp. (*Enterobacter sakazakii*) are emerging opportunistic pathogens for all age groups, and are of particular concern when it comes to infants. Prior to contaminating food, the organism may be exposed to a variety of stresses, leading to a generation of sublethally injured cells that may not be detected by selective media unless a protracted recovery period is included in the isolation procedure. This study evaluated the efficacy of the thin agar layer (TAL) method for the recovery of *Cronobacter* cells that had been exposed to various stress conditions. Five strains of *C. sakazakii* and *C. muytjensii* were exposed to starvation, heat, cold, acid, alkaline, chlorine, or ethanol, with or without further exposure to desiccation stress. The recovery of the stressed cells was determined on tryptone soy agar (TSA; nonselective control medium), violet red bile glucose agar (VRBGA; selective agar), Druggan-Forsythe-Iversen (DFI; selective agar), and TAL media (viz., VRBGA overlaid with TSA, and DFI overlaid with TSA). Regardless of stress type, there were no significant differences among the recoveries of stressed desiccated *Cronobacter* spp. cultures on TSA, DFI+TSA, and VRBGA+TSA, but there was significantly less recovery on VRBGA. The recovery of prestressed desiccated *Cronobacter* spp. on DFI+TSA was similar to that on TSA, whereas the recovery on VRBGA+TSA was lower. DFI+TSA performed better than VRBGA+TSA did in differentiating *Cronobacter* spp. within mixed bacterial cultures. The results of this study suggest the use of the TAL method DFI+TSA as an improved method for the direct recovery of stressed *Cronobacter* spp.

Cronobacter (*Enterobacter sakazakii*) is considered an emerging opportunistic pathogen, causing infections in all age groups, and is a serious concern due to rare but severe forms of neonatal infections such as meningitis, necrotizing enterocolitis, and sepsis *(3, 6, 12, 24, 35)*.

The natural habitat of *Cronobacter* may be plant material, as the organism has been isolated from various plant-related products and ingredients *(8, 13, 18, 20, 31, 32)*. Consequently, the organism possesses physiological characteristics that aid survival under different environmental stresses *(27)*. These characteristics include the ability of most (92%) *Cronobacter* spp. to produce a yellow pigment that protects the cell against UV rays in sunlight, to form exopolysaccharide capsular material to aid in adherence to surfaces, to form a biofilm to enhance resistance against harsh environmental conditions, and to resist desiccation during dry periods *(4, 5, 25, 27)*. *Cronobacter* spp. are exposed to similar environmental stresses such as starvation, desiccation, and temperature extremes, as well as detergents and sanitizers, during food production *(25, 26, 30, 33)*.

Cronobacter spp. are widely distributed in nature and have been isolated from various food ingredients, foods, food production environments, and households *(7, 10, 13, 18, 21, 22, 32)*. However, to date there are no official procedures for detecting *Cronobacter* spp. from environmental samples, food ingredients, or foods other than infant formula. U.S. Food and Drug Administration *(34)* and International Organization for Standardization *(16)* methods for the isolation of the organism from powdered infant formula (PIF) involve preenrichment and enrichment steps, followed by plating on selective agar such as violet red bile glucose agar (VRBGA) or a chromogenic agar. As VRBGA is not specific for *Cronobacter* spp., suspect colonies are streaked on tryptone soy agar (TSA), and any yellow-pigmented colonies are selected for identification by biochemical tests. It should be noted that this method will miss a significant portion (8%) of *Cronobacter* spp., which are nonpigmented, and phenotyping databases are not as accurate as are DNA sequencing techniques for the identification of *Cronobacter* spp. *(2, 19)*.

Several media, based on the capability of *Cronobacter* spp. to produce α-glucosidase, have been developed to improve detection of *Cronobacter* spp. in PIF *(11)*.

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Druggan-Forsythe-Iversen (DFI) agar is one of these media, and it has high specificity and sensitivity to detect *Cronobacter* spp. *(17)*. It contains the chromogen 5-bromo-4-chloro-3-indolyl-α,D-glucopyranoside, which serves as an indicator of α-glucosidase and results in blue-green *Cronobacter* colony formation. DFI agar was developed to replace the steps involving VRBGA and TSA in the isolation of *Cronobacter* spp., and it decreases the detection period by 2 days. However, stressed *Cronobacter* spp. cells are sensitive to sodium deoxycholate in DFI and to crystal violet and bile salts in VRBGA *(14, 19)*, which limits the use of these media in the direct detection of *Cronobacter* from foods and environmental samples.

Gurtler and Beuchat *(14)* compared the recovery of stressed *Cronobacter* spp. cells on various selective media (fecal coliform agar, DFI, Oh and Kang agar, VRBGA, and *Enterobacteriaceae* enrichment agar) and nonselective media (TSA plus sodium pyruvate). They found that recovery on the selective media was significantly lower than on nonselective media. Al-Holey et al. *(1)* recovered stressed *Cronobacter* cells by overlaying inoculated nonselective agar with selective agars. However, this overlay method has some limitations, as colonies that grow between the two agar layers are small and difficult to isolate for further characterization. In addition, the temperature of the molten selective overlay agar (45 to 48°C) can further stress injured cells that are otherwise resuscitated on the nonselective agar *(23, 38)*.

An alternative is the thin agar layer (TAL) method. In this procedure, the selective medium is overlaid with a thin layer of nonselective agar before inoculation. The nonselective agar layer allows injured microorganisms to revive in a favorable environment before exposure to the selective agents diffusing from the lower layer. Kang and Fung *(23)* developed a TAL method for recovery of heat-injured *Listeria monocytogenes* by overlaying prepoured modified Oxford medium with the nonselective medium TSA. Hajmeer et al. *(15)* showed that a TAL media of MacConkey sorbitol plus TSA was more effective than MacConkey sorbitol agar alone in recovering starved *Escherichia coli* O157:H7. Duan et al. *(9)* used the TAL method to recover heat- and cold-injured *Vibrio parahaemolyticus*. Wu et al. *(36, 37)* showed that the TAL method was as effective as TSA in recovering heat- and acid-injured *E. coli* O157:H7, *L. monocytogenes, Salmonella* Typhimurium, *Staphylococcus aureus,* and *Yersinia enterocolitica,* and it recovered higher numbers of organisms than did selective media alone. The evaluation of the TAL method, based on VRBGA and DFI selective agars with a TSA overlay, is reported here for the recovery of stressed *Cronobacter* cells as would occur in environmental and food samples.

MATERIALS AND METHODS

***Cronobacter* strains and culture preparation.** The *Cronobacter* strains used were four *C. sakazakii* strains isolated from infant milk formula, cereal formula, and semolina, as described by Shaker et al. *(32),* and *C. muytjensii* ATCC 51329 (source unknown). The latter strain was used, as it was the ATCC Perceptrol (quality control) strain for *E. sakazakii*. Cultures were maintained at −40°C, and cultures were grown overnight in brain heart infusion broth (Oxoid, Ltd., Basingstoke, UK) at 37°C for 24 h.

Preparation of stressed *Cronobacter* cells. As previously described *(28–30, 33),* equal volumes of stationary-phase *Cronobacter* cells were combined to form a five-strain composite suspension (ca. 10^9 CFU/ml). The cocktail culture was exposed to starvation, heat, cold, acid, alkaline, chlorine, or ethanol stresses, as previously described by Osaili et al. *(26)*. Control cells were unstressed cocktail cultures.

Desiccation of *Cronobacter* spp. in PIF. Stressed and unstressed (control) *Cronobacter* cells at ca. 10^2 CFU/g of PIF were inoculated in the formulas as described by Osaili et al. *(26)*. Inoculated formulas were transferred to sterile 500-ml screw-cap bottles and stored at 21°C for 30 days. The viability of desiccated *Cronobacter* spp. in PIF was determined immediately after inoculation (day 0) and after 30 days of storage. The PIF was purchased locally and had an intrinsic bacterial flora of <300 CFU/g, as determined by aerobic mesophilic plate count on TSA incubated at 37°C for 48 h.

Water activity measurement. The water activity of inoculated PIF was measured with a water activity meter (Hygrolab, Rotronic Instrument Corp., Huntington, NY) on day 0 and after 30 days of storage.

Media. TSA (CM0131, Oxoid, Ltd.), a nonselective medium, was used as a control medium to enumerate both stressed and unstressed *Cronobacter* spp. The selective media were VRBGA (selective medium for *Enterobacteriaceae*; CM0978, Oxoid, Ltd.) and DFI (selective medium for *Cronobacter* spp.; CM1055, Oxoid, Ltd.). Media were prepared according to the manufacturer's instructions. The number of sublethally injured cells was defined as the difference between the viable counts on TSA and the selective agars.

Preparation of TAL media. The TAL method was developed for both VRBGA and DFI agar media. Twenty-milliliter quantities of sterilized VRBGA and DFI were poured into petri dishes (8.5 cm in diameter) and left at room temperature for 2 h. After solidification, 10 ml of molten TSA (45°C) was poured onto the selective agar layer.

Enumeration of *Cronobacter* spp. Ten-milliliter samples of stressed and unstressed cultures or 10-g samples of inoculated PIF were thoroughly mixed with 90 ml of sterile 0.1% peptone water (Difco, Becton Dickinson, Sparks, MD) and further diluted as necessary. Sample aliquots of 0.1 ml or 1 ml were plated in duplicate onto one plate or three plates, respectively, of TSA, VRBGA+TSA, VRBGA, DFI+TSA, or DFI. The plates were incubated for 24 h at 37°C before enumeration of *Cronobacter* colonies.

Evaluation of TAL method. The TAL method for the detection of *Cronobacter* spp. was further evaluated with a mixed bacterial culture of *E. coli* O157:H7, *Salmonella* Typhimurium, *Listeria innocua, Bacillus cereus, Bacillus subtilis, S. aureus, Pseudomonas,* and the five strains of *Cronobacter*. Each microorganism was grown separately in brain heart infusion broth at 37°C for 24 h. After incubation, equal volumes (0.1 ml) of each culture were combined to form the mixed culture (ca. 10^9 CFU/ml). Decimal dilutions of the mixed culture were spread (0.1 ml) on
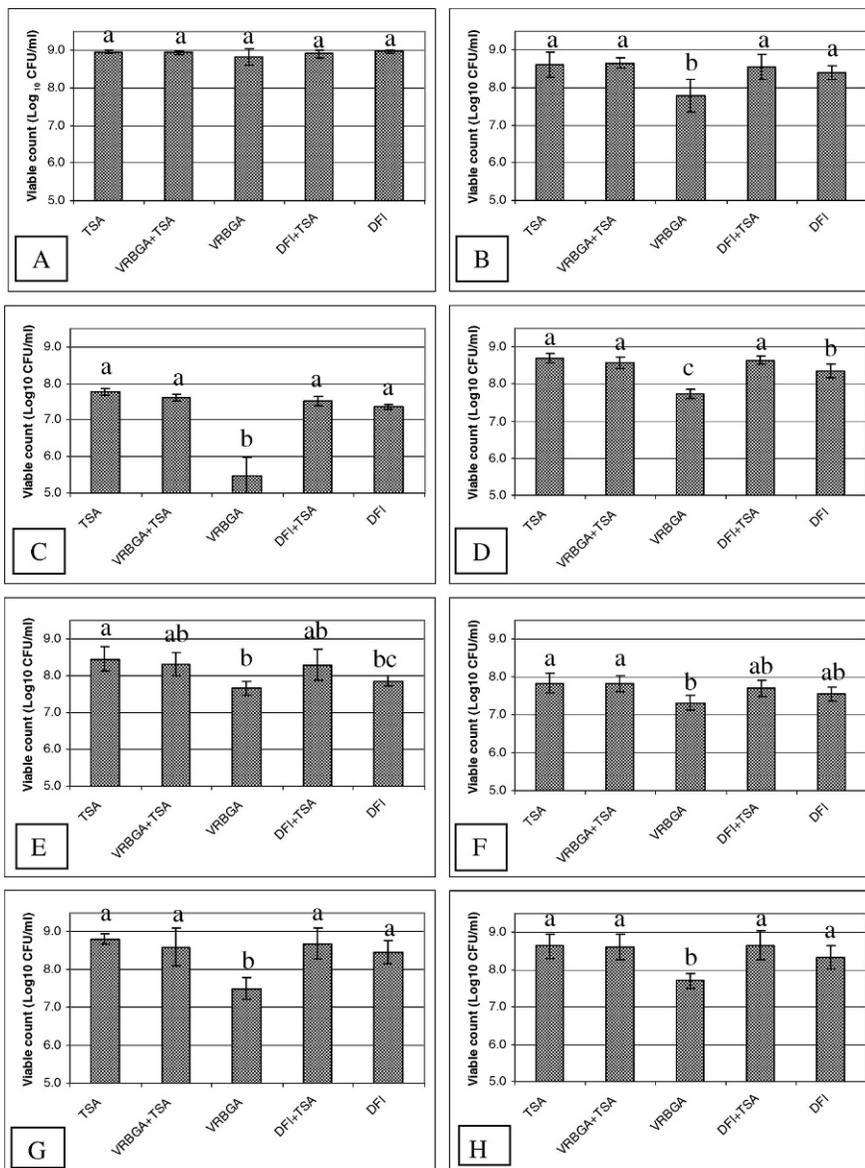
FIGURE 1. *Recovery of stressed* Cronobacter *cultures on TSA, VRBGA, TAL of VRBGA+TSA, DFI, and TAL of DFI+TSA. (A) Control (unstressed) cells, (B) starvation-stressed cells, (C) heat-stressed cells, (D) cold-stressed cells, (E) acid-stressed cells, (F) alkaline-stressed cells, (G) chlorine-stressed cells, (H) ethanol-stressed cells. Within the same stress type, means with the same letter are not significantly different (*P* ≥ 0.05). Error bars represent standard error. Each value represents the mean of three replications.*

VRBGA+TSA and DFI+TSA in duplicate and their viable count determined after 24 h at 37°C.

Statistical analysis. The differences in the recovery of stressed *Cronobacter* spp. by the various media was analyzed with the least-squares means method at a 5% level of significance by using the Statistical Analysis System (SAS) program, version 8.1 (SAS Institute Inc., Cary, NC).

RESULTS

Recovery of stressed undesiccated *Cronobacter* spp. cultures. Figure 1 shows the recovery of unstressed, starvation-, heat-, cold-, acid-, alkaline-, chlorine-, and ethanol-stressed *Cronobacter* cells, without desiccation on the various media. There were no significant (*P* ≥ 0.05) differences between the recovery of the unstressed cells of *Cronobacter* cells on VRBGA or DFI as compared with the TAL media (VRBGA+TSA and DFI+TSA) (Fig. 1A). There were no significant differences between the recovery of stressed *Cronobacter* cells on TSA, and the TAL media VRBGA+TSA and DFI+TSA demonstrated that the TAL

media recovered sublethally injured cells that did not grow on VRBGA or DFI alone.

There was significantly (*P* < 0.05) less recovery of sublethally injured cells from all stress treatments on VRBGA. This was especially apparent after heat treatment, which resulted in a greater than 2-log CFU/ml difference with the viable count on TSA (Fig. 1C). DFI agar did recover sublethally injured cells better than VRBGA did for all treatments but, in general, was not as efficient as was the DFI+TSA (TAL) combination (*P* ≥ 0.05).

The difference between viable counts on the selective agars and the TAL combinations varied according to the stress-inducing treatment. Heat-treated *Cronobacter* cells were recovered by 2 log orders greater on VRBGA+TSA than on VRBGA alone (Fig. 1C), compared with 0.2 log orders for DFI and DFI+TSA. Cold-stressed cells were significantly (*P* < 0.05) less recovered on DFI and VRBGA as compared with the TAL media (VRBGA+TSA and DFI+TSA), by 0.85 and 0.30 log CFU/ml, respectively. Both TAL media recovered sublethally acid-injured cells better than did VRBGA and DFI alone by 0.66 and 0.4 log

FIGURE 2. *Recovery of stressed* Crono-
bacter *from powdered infant formula on
day 0 (dotted bars) and after 30 days of
storage at 21°C (striped bars) on TSA,
VRBGA, TAL of VRBGA+TSA, DFI, and
TAL of DFI+TSA. (A) Control (unstressed)
cells, (B) starvation-stressed cells, (C)
heat-stressed cells, (D) cold-stressed cells,
(E) acid-stressed cells, (F) alkaline-
stressed cells, (G) chlorine-stressed cells,
(H) ethanol-stressed cells. Within the same
stress type, means with the same letter are
not significantly different (P ≥ 0.05).
Error bars represent standard error. Each
value represents the mean of three repli-
cations.*
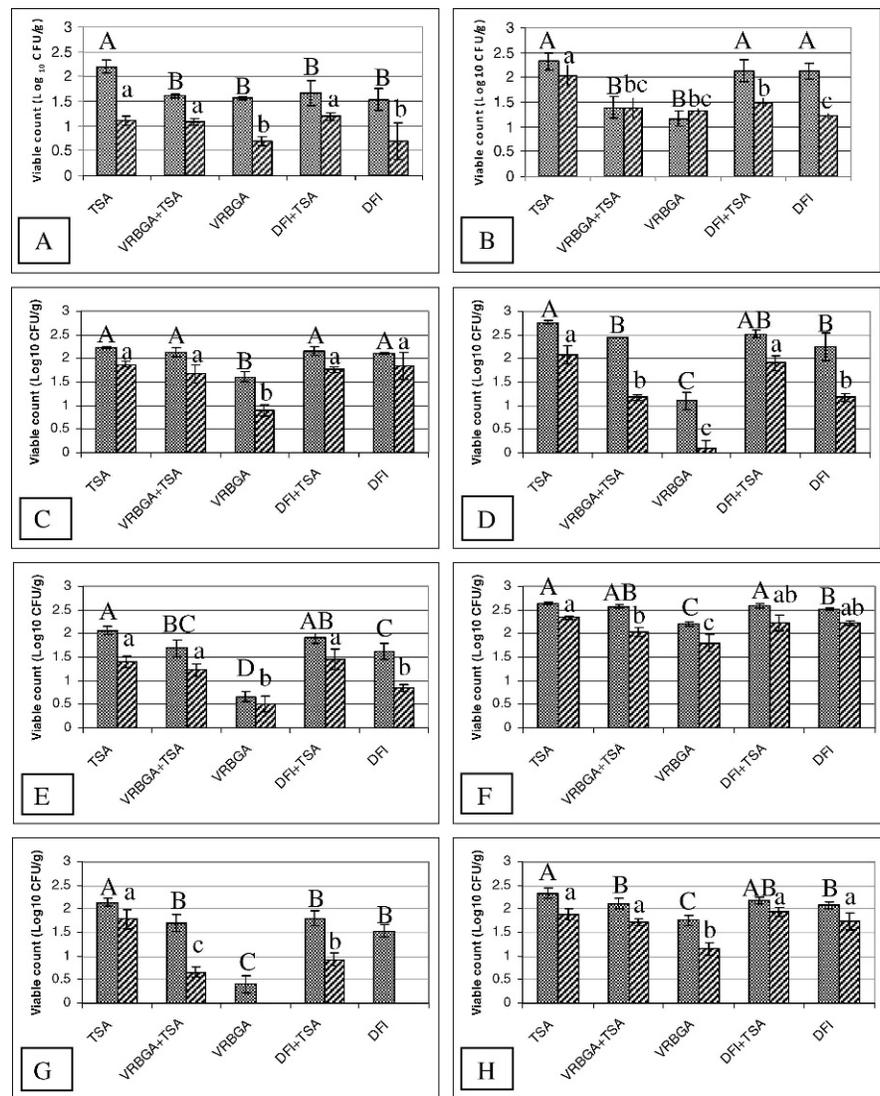
CFU/ml, respectively (Fig. 1E). VRBGA+TSA recovered
alkaline-stressed cells at higher levels ($P ≥ 0.05$) than
VRBGA, 0.52 log CFU/ml. All sublethally injured cells,
after chlorine and ethanol exposure, were recovered on the
TAL media at levels not significantly different from the
viable count on TSA.

Recovery of desiccated *Cronobacter* spp. The perfor-
mance of the TAL media was evaluated for the recovery of
prestressed *Cronobacter* cells after further desiccation stress
by storage in PIF for 30 days (Fig. 2). The water activity of
the inoculated PIF was $0.21 ± 0.01$ on day 0 and had
increased to $0.28 ± 0.01$ after 30 days. During this period,
there was a significant decrease in the viability of desiccated
Cronobacter (Fig. 2A).

The media performances were similar to those for cells
without desiccation. The recovery on VRBGA was
significantly lower for most prestressed cells than on TSA,
VRBGA+TSA, and DFI+TSA (Fig. 2). There were no
significant differences between the recoveries of prestressed
(heat, cold, acid, alkaline, and ethanol) desiccated cells on
TSA and DFI+TSA, but there was significantly less
recovery of prestressed (starvation, cold, alkaline, and

chlorine) desiccated cells on VRBGA+TSA as compared
with TSA. The VRBGA and DFI media did not recover
chlorine-desiccated stressed cells from PIF after 30 days
storage, whereas VRBGA+TSA and DFI+TSA recovered
Cronobacter at levels of 0.5 and 1.0 log CFU/g,
respectively. Similarly, DFI+TSA and VRBGA+TSA were
able to recover heat-, cold-, acid-, and ethanol-stressed cells,
and desiccated cells from PIF at levels 1- to 2-log CFU/g
greater than what VRBGA was able to recover.

The TAL method did not hinder typical color formation
by *Cronobacter* on VRBGA (pink-red colonies) or DFI
(blue-green colonies). However, as other *Enterobacteriace-
ae* are also pink-red on VRBGA, *Cronobacter* spp. were
only distinguishable within mixed cultures of *E. coli*
O157:H7, *Salmonella* Typhimurium, *L. innocua, B. cereus,
B. subtilis, S. aureus,* and *Pseudomonas* on DFI+TSA
plates. Additionally H$_2$S-producing *Salmonella* are distin-
guishable by their black colony formation on DFI+TSA.

Exposure of *Cronobacter* spp. to various stresses
(starvation, heat, cold, acid, alkaline, chlorine, ethanol, or
desiccation) occurs in a variety of situations that could have
implications on food safety monitoring, for instance,
exposure of *Cronobacter* spp. to nutritional stress (starva-

tion) or physical stresses (heat and cold) in a persistent low-nutrient environment or by using high heat treatments and cold storage in processing facilities, respectively. *Cronobacter* spp. will be exposed to chemical stresses frequently in food processing facilities using detergents to remove soil residues from equipment and floors and by using sanitizers after cleaning. Additionally, *Cronobacter* cells will be desiccated in low-water-activity environments such as powdered foods and ingredients.

In this study, the TAL method used VRBGA and DFI agar plates overlaid with TSA to recover stressed *Cronobacter* cells. The TSA top layer provided a suitable environment for stressed cells to revive in the first few hours of incubation. Thereafter, resuscitated cells were exposed to the selective agents that diffused into the upper agar layer from the VRBGA or DFI. Figures 1 and 2 show that the recovery of stressed *Cronobacter* cells was higher on the TAL media DFI+TSA and VRBGA+TSA than on DFI or VRBGA. This was probably because stressed (injured) cells were sensitive to the selective agents, i.e., crystal violet, bile salts, and sodium deoxycholate, when plated directly on VRBGA and DFI (14, 19).

The study indicates that the TAL medium DFI+TSA is more appropriate than VRBGA+TSA in detecting and enumerating stressed *Cronobacter* cells from mixed cultures. Additionally, the black pigmentation of *Salmonella* on DFI is advantageous as a dual detection system for detecting this pathogen as well as *Cronobacter* spp.

In summary, combining TSA with other selective differential media in a TAL method is a suitable procedure to recover stressed *Cronobacter* cells from mixed cultures from environmental samples, food ingredients, or foods. In particular, this study shows the greater recovery of such stressed cells by the direct-plate TAL method of TSA+DFI as compared with conventional selective and differential media.

ACKNOWLEDGMENT

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