Evaluation of a New Enrichment Broth for Detection of 
*Cronobacter* spp. in Powdered Infant Formula

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ABSTRACT

The aim of this study was to investigate the potential of using Al-Holy–Rasco (AR) medium, a novel broth for detection and isolation of *Cronobacter* spp. in infant formula milk (IFM). The new medium’s composition is generic brain heart infusion broth with the addition of 1% NaCl, 15% sucrose, and 0.80 g/liter sodium deoxycholate as selective ingredients. AR broth outperformed *Enterobacteriaceae* enrichment broth (EE), *Enterobacter sakazakii* enrichment broth (ESE), modified lauryl sulfate broth, and milk as enrichment media to stimulate the growth of a cocktail of 10 strains of *Cronobacter*. Additionally, AR broth significantly suppressed the growth of competing non-*Cronobacter Enterobacteriaceae* as compared with EE, ESE, modified lauryl sulfate broth, and milk. The recovery of desiccated *Cronobacter* (1 to 5,000 CFU/100 g) from powdered IFM in the presence of competing non-*Cronobacter Enterobacteriaceae* was determined by EE, ESE, and AR broth with 10 and 15% sucrose. AR broth with 15% sucrose outperformed all other examined broths and recovered *Cronobacter* from all samples tested at all *Cronobacter* concentrations. AR broth must be validated before it can be used for rapid detection and isolation of *Cronobacter* from powdered IFM.

*Cronobacter* is an emerging human pathogen of great concern to the baby food industry, because it has been implicated in severe forms of neonatal illness such as meningitis, sepsis, and necrotizing enterocolitis (4, 17, 26). Recently, *Cronobacter* has been proposed as a new genus to replace *Enterobacter sakazakii*. *Cronobacter* currently includes six different species potentially pathogenic to neonates (16). *Cronobacter* is present at very low levels in powdered infant formula milk (IFM), usually ≤1 CFU/g. It was reported that *Cronobacter* was detected at variable levels in 14% of the cans of powdered IFM tested from 35 different countries (21). The occurrence of *Cronobacter* even at low levels is significant, based on its capability to multiply rapidly at room temperature (25). Several outbreaks of *Cronobacter* have resulted from high holding temperatures of reconstituted IFM, poor hygienic practices, or unclean washing brushes or bottles (4, 5, 17). To reduce the occurrence of *Cronobacter* outbreaks, it is very important to reduce the prevalence of the organism in infant formula. This is crucial, as *Cronobacter* exhibits extraordinary capability of surviving in dry environments such as powdered IFM for long periods, relative to other members of *Enterobacteriaceae* (1, 6, 7). Arku et al. (3) were able to detect *Cronobacter* in milk powder after spray drying inoculated milk. The pathogen was still detectable after 12 weeks of storage.

Detection and isolation methods for *Cronobacter* must be robust and reliable to ensure safety of IFM. Several methods are available for isolation of *Cronobacter* from powdered IFM. For example, the current U.S. Food and Drug Administration (FDA) method, which is based on the pioneering work of Muyltjens et al. (20), in essence uses *Enterobacteriaceae* enrichment (EE) broth, then utilizes selective plating on violet red bile glucose agar and tryptic soy agar (TSA) for presumptive identification of *Cronobacter*. However, agar made from EE broth and EE broth itself were proven to be inferior when used in the recovery of heat-, acid-, alkaline-, or desiccation-stressed *Cronobacter* cells (2, 10, 15). Recently, a new method based on a real-time PCR–based assay and chromogenic media to improve isolation and detection of *Cronobacter* from IFM was proposed by the FDA, and it is currently being validated (18).

The recognition of IFM as a primary vehicle of infection prompted the development of culture-based detection methods (8). Development of an enrichment broth to improve the isolation of *Cronobacter* from IFM is needed crucially. Other broths can also be used, such as modified lauryl sulfate tryptose broth, brilliant green bile broth, or *Enterobacter sakazakii* enrichment broth (ESE) broth. However, these broths may contain some selective ingredients that preclude the growth of some strains of *Crono-
bacter, such as lauryl sulfate in lauryl sulfate broth (LSB), or could lack enough selectivity for Cronobacter and at other times promote the growth of non-Cronobacter Enterobacteriaceae (8). Hence, developing a new Cronobacter detection and isolation broth is necessary to promote the growth of Cronobacter, usually present in very low levels in powdered IFM, and to reduce the possibility of positive deviations and negative deviations results due to the presence of other Enterobacteriaceae.

The objective of this study was to investigate the ability of a new Cronobacter enrichment broth, AL-Holy–Rasco (AR broth), based on a modified brain heart infusion (BHI) broth to support the growth of Cronobacter spp. and to suppress the growth of other competing non-Cronobacter Enterobacteriaceae. In addition, the capability of AR broth to promote growth and isolation of Cronobacter was compared with other Cronobacter enrichment broths.

MATERIALS AND METHODS

Bacterial cultures. Thirty strains of Cronobacter (C. sakazakii [ATCC 29004, 12868, and 29544], C. mucyiensi [ATCC 51329], and 26 other Cronobacter isolates [FSM 287, FSM 292, 55, 2.39-1, 2.68, 1, 2, 3, 4, 5, 6, 7, 55, 60, L02-401, 42VS, 501B, E27B, 800DS, FSM 30, FSM 145, FSM 261, FSM 262, FSM 265, FSM 271, and FSM 272]) were used in this study. All the strains were American Type Culture Collection (Manassas, VA) cultures or isolated from dry infant formula or baby food, and baby food processing environments. The Cronobacter strains were transferred individually from stock aqueous glycerol (15%) solutions stored at −20°C, transferred to slants of TSA (Difco, BD, Spark, MD), and kept refrigerated. Prior to the experiment, the cultures were transferred from TSA slants to BHI broth (Difco, BD) and grown for 24 h at 37°C. Then, 10 ml of BHI broth of each strain was compiled to make a 10-strain Cronobacter cocktail. The BHI mixture was centrifuged at 4,000 × g for 15 min, and the resulting pellet was resuspended in 10 ml of 0.9% saline solution. This procedure was repeated two times to remove the BHI residue. The cocktail was 10-fold serially diluted in 0.9% saline solution and inoculated at about 100 CFU/ml into each broth medium.

Enrichment broths. Four different enrichment broths were compared with AR broth for their capabilities to recover and stimulate the growth of Cronobacter. A 10-strain cocktail of Cronobacter (ATCC 29004, 51329, 12868, 29544, and FSM 287, FSM 292, 3, 55, 2.39-1, and 2.68) was inoculated at approximately 100 CFU/ml into each of the five broths. This same microbial cocktail recipe was used in “Optimization of AR broths to improve recovery of Cronobacter;” below.

The Enterobacteriaceae enrichment broth Mossel (Difco, BD) was prepared according to the manufacturer’s instructions.

Modified lauryl sulfate broth (mLSB) was prepared as follows. Lauryl sulfate (Difco, BD) was modified as described by Guillaume-Gentil et al. (9) by incorporating 0.5 M NaCl and 10 mg/ml vancomycin (M.P. Biomedicals, Solon, OH), which was added after sterilizing the broth at 121°C for 15 min, and then cooling the broth to room temperature.

AR broth was composed (in grams per liter) of fluid from 200.0 g of calf brain, fluid from 250.0 g of beef heart, 10.0 g of Proteose Peptone (Difco, BD), 2.0 g of dextrose, 10.0 g of sodium chloride, 2.5 g of disodium phosphate, 0.80 g of sodium deoxycholate (Difco, BD), and 150.0 g of sucrose (Fisher Scientific, Fair Lawn, NJ). The broth was autoclaved at 121°C for 15 min. The final composition of the formula was determined after conducting the optimization experiments as detailed in “Optimization of AR broths to improve recovery of Cronobacter.”

ESE was prepared as described by Iversen and Forsythe (14); 6.5 g of sodium dihydrogen phosphate (Mallinkrodt Baker, Phillipsburg, NJ), 2.0 g of potassium dihydrogen phosphate (Sigma-Aldrich, St. Louis, MO), 1.5 g of yeast extract (Difco, BD), 4.0 g of neutralized peptone (Fisher Scientific), 12.0 g of base tryptone (pancreatic digest of casein; Difco, BD), 4.0 g of sodium chloride (Fisher Scientific), 100.0 g of sucrose (Fisher), and 0.5 g of sodium deoxycholate (Difco, BD) were dissolved in 1 liter of distilled water and autoclaved at 121°C for 15 min.

IFM was reconstituted in sterile distilled water, according to the manufacturer’s instructions and supplemented with 20 mg/liter novobiocin (Oxoid, Ltd., Basingstoke, UK).

Optimization of AR broths to improve recovery of Cronobacter. The capability of AR broth to support growth of Cronobacter was investigated at 37 and 43°C. Cronobacter counts were determined by taking samples from the different broths at 0, 3, 6, and 9 h. Appropriate dilutions were spread plated onto violet red bile lactose agar (VRBL; Difco, BD) and incubated at 37°C for 24 h.

To improve selectivity of AR broth for Cronobacter, the effect of deoxycholate concentration on the growth of Cronobacter was examined. Sodium deoxycholate was added to AR broth at 0.30, 0.50, and 0.80 g/liter. The growth of Cronobacter was monitored by taking samples every 3 h during a 9-h incubation period at 43°C. Appropriate dilutions were spread plated onto VRBL and incubated at 37°C for 24 h.

AR broth was supplemented with either 100 or 150 g/liter sucrose (Fisher Scientific). Cronobacter counts were determined by taking samples from the different broths incubated at 43°C for 0, 3, 6, and 9 h. Appropriate dilutions were spread plated onto VRBL and incubated at 37°C for 24 h.

Growth behavior of Cronobacter strains inoculated individually into AR broth. Thirty strains and isolates of Cronobacter (listed in “Bacterial cultures”) were inoculated individually into AR broth and incubated at 43°C for 8 h. Cronobacter counts were determined by spread plating appropriate dilutions of the broth onto VRBL and incubating them at 37°C for 24 h.

Comparison of growth behavior of competing Enterobacteriaceae in AR and other enrichment broths. A 12-strain mixture of Enterobacteriaceae (Escherichia coli [ATCC 25922, B E4a, DH 5a, DH10B, HB101, K-12 2B], Klebsiella pneumoniae [K1a, Revoco 41, Revoco 55], Enterobacter cloacae [Rev 1210 Case 00-5395, Rev 1343 case 12286], and Enterobacter aerogenes ATCC 13048) was prepared as detailed previously and inoculated at ca. 10 to 100 CFU/ml of each of the following enrichment broths: AR broth, ESE, m-LSB, EE, and reconstituted IFM with novobiocin. The enrichment broths were incubated at 43°C for 9 h. The growth of Enterobacteriaceae was monitored by taking samples every 3 h, spread plating the samples onto VRBL, and then incubating the plates at 37°C for 24 h.

Recovery of desiccation-stressed Cronobacter from dry IFM containing competing Enterobacteriaceae. The ability of the newly developed AR broth and ESE as media specifically formulated to enrich Cronobacter from powdered IFM was examined. EE broth was also tested as a medium used in the reference method of the FDA for selective enrichment of...
Cronobacter. Modified reconstituted IFM and m-LSB were excluded in this component because of their inferiority to support the growth of inoculated cells of Cronobacter as compared with other broths used in the current study. Milk-based infant formula was purchased from a local grocery store. Cronobacter cells were desiccation stressed by inoculating 24-h-old cultures of a cocktail of the five strains of Cronobacter (ATCC 29004, 51329, 12868, 29544, and FSM 287) onto the surface of powdered IFM. Powdered IFM was placed into a sterile 1,000-ml beaker. The inoculum (100 μl) was sprinkled onto the formula in a dropswise manner. After inoculation, the IFM with the inoculum was mixed vigorously with a sterile spatula for 3 min. The initial level of Cronobacter was determined by the overlay method (spread plating on TSA [2-h incubation at 37°C], which was followed by applying another layer of VRBL and then by incubation at 37°C for an additional 22 h) (1). The initial level of Cronobacter was approximately 50 CFU/g. This formula was used to inoculate other quantities of IFM so that the resulting final concentrations of Cronobacter were 1, 10, 1,000, and 5,000 CFU/100 g of powdered IFM. The samples were kept under dry conditions (water activity of the IFM was 0.22) in a desiccator for 50 days. After Cronobacter inoculation, other Enterobacteriaceae (E. coli [ATCC 25922, B E4a, DH 5a, DH10B, HB101, K–12 2B], K. pneumoniae [K1a, Revoco 41, Revoco 55], E. cloacae [Rev 1210 Case 00-5395, Rev 1334 case 12286], and E. aerogenes ATCC 13048) were inoculated into powdered IFM at a level of 100 CFU/100 g. The samples were stored in a desiccator at room temperature for an additional 10 days. The water activity of the powdered IFM was measured at room temperature before and after the inoculation at day 0, and after 30 and 60 days of storage at room temperature by using a water activity meter (model 3TE, Aqualab, Pullman, WA).

Inoculated powdered IFM was reconstituted in sterile buffered peptone water (Difco, BD) at a ratio of 1:9 and incubated at 37°C overnight (14 h). One milliliter of each of the enrichment broths (EE, ESE, AR with 10 or 15% sucrose) and incubated at 37°C for EE and ESE, and at 43°C for AR broth. During incubation, samples were taken after 8 and 16 h, 10-fold serially diluted, and spread plated onto Dreggan-Forsythe-Iversen (DFI) agar, which was prepared in our laboratory, as described by Iversen et al. (12). The plates were incubated at 37°C for 24 h. Typical blue-green colonies on DFI were considered presumptive Cronobacter colonies and were subjected to biochemical confirmation with the API Rapid 20E Biochemical System (bioMérieux, Marcy l’Etoile, France), which is the present reference FDA method, according to the manufacturer’s instructions. (However, the rapid ID 32E is currently recommended.) Other colonies that appeared on the medium were also subjected to biochemical identification with the API rapid 20E system.

Statistical analysis. Three independent replicate trials were conducted for each experiment, and standard deviations were determined. Cronobacter and Enterobacteriaceae counts were log transformed, and data were analyzed with a computer software package (SAS Institute Inc., Cary, NC), with analysis of variance and Fisher’s least-significant difference test for mean separations (P ≤ 0.05).

RESULTS

In this study, a new medium (AR broth) was formulated to enhance growth of Cronobacter and to impede the growth of competing microorganisms. Figure 1 shows the effect of incubation temperature on the growth behavior of Cronobacter in AR broth. After 9 h of incubation, Cronobacter grew to significantly (P ≤ 0.05) higher numbers at 43°C as compared with 37°C. Sodium deoxycholate was incorporated in the AR broth because it serves as a selective agent against gram-positive organisms. No significant differences in the growth of Cronobacter were noticed among the three tested concentrations (0.30, 0.50, and 0.80 g/liter) (Fig. 2); therefore, the concentration of 0.80 g/liter of deoxycholate was chosen as a selective ingredient in the medium. Sucrose was also incorporated in the AR broth because a previous study indicated that all Cronobacter strains could ferment sucrose, whereas the majority of α-glucosidase–positive Enterobacteriaceae cannot utilize sucrose as a carbon source (15). Therefore, high concentrations of sucrose (either 10 or 15%) were tested in the broth (Fig. 3). NaCl was also added to the medium to impart more osmotic stress selectivity for this medium against the growth of competing microflora. Therefore, the effect of different concentrations of NaCl (10.0, 15.0, and 30.0 g/liter) on the growth of Cronobacter was investigated (Fig. 4). The minimum concentration of NaCl (10.0 g/liter) that did not result in suppressing the growth of Cronobacter was selected as the optimum concentration. NaCl concentrations of 30 g/liter or more resulted in complete inhibition of Cronobacter in this medium (data not shown).

Figure 5 compares the growth behavior of Cronobacter in milk with novobiocin, m-LSB with vancomycin, EE, ESE, and AR broth. AR broth generated a significantly
better \( (P \leq 0.05) \) growth performance as compared with all other tested broths, where the numbers of Cronobacter reached up to \( >7 \) log CFU/ml after 9 h of incubation at 43°C.

Figure 6 shows the growth behavior of a 12-strain cocktail of non-Cronobacter–competing Enterobacteriaceae. The growth of inoculated Enterobacteriaceae was significantly lower \( (P \leq 0.05) \) in AR broth as compared with all other tested broths, a crucially important characteristic revealing the selectivity of AR broth for Cronobacter while simultaneously suppressing other competing Enterobacteriaceae.

Table 1 shows the counts of 30 Cronobacter strains grown individually in the novel AR broth. Generally, most of the Cronobacter strains grew to levels of \( \geq 8 \log \text{CFU/ml} \) in the AR broth. A few strains (Cronobacter isolates 55, 60, and E27B) grew, but to a lesser extent in the AR broth. However, it is worthwhile to state that the same strains also experienced a slow growth rate in the general medium, tryptic soy broth (data not shown). Therefore, these strains might need special nutritional supplements added to the medium to stimulate their growth.

The capability of EE, ESE, and AR with 10 or 15% sucrose to recover desiccated cells of Cronobacter from powdered IFM was tested (Table 2). Variable concentrations of Cronobacter cells were incorporated into powdered IFM and stored for a 2-month period, resulting in desiccation-stressed cells. In addition to Cronobacter, other competing Enterobacteriaceae, which are commonly isolated from baby foods and processing environments (13), were also incorporated. This combination of flora makes the model more representative of real food and environmental samples that could be tested from baby food processing plants.

**DISCUSSION**

Cronobacter is a microorganism of growing concern to the infant formula and baby food industry (1). This microorganism was implicated in several cases of fatalities, meningitis, and necrotizing enterocolitis among neonates (4, 5, 10). The diseases were reported to result from consuming contaminated formula or baby foods, or from poor hygienic practices used in the preparation and handling of formula and formula preparation utensils (4, 5). Therefore, developing reliable detection and isolation methods is essential to
TABLE 1. Growth of 30 Cronobacter strains inoculated individually at an initial level of 1 × 10^2 to 1 × 10^4 CFU/ml in Al-Holy–Rasco broth

<table>
<thead>
<tr>
<th>Cronobacter strain or isolate</th>
<th>Mean ± SD (log CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 29544</td>
<td>8.60 ± 0.12</td>
</tr>
<tr>
<td>ATCC 51329</td>
<td>8.48 ± 0.22</td>
</tr>
<tr>
<td>ATCC 12868</td>
<td>8.40 ± 0.16</td>
</tr>
<tr>
<td>ATCC 29004</td>
<td>8.51 ± 0.08</td>
</tr>
<tr>
<td>FSM 30</td>
<td>8.88 ± 0.22</td>
</tr>
<tr>
<td>FSM 145</td>
<td>8.19 ± 0.12</td>
</tr>
<tr>
<td>FSM 261</td>
<td>8.27 ± 0.02</td>
</tr>
<tr>
<td>FSM 262</td>
<td>8.44 ± 0.07</td>
</tr>
<tr>
<td>FSM 265</td>
<td>8.36 ± 0.08</td>
</tr>
<tr>
<td>FSM 270</td>
<td>8.54 ± 0.01</td>
</tr>
<tr>
<td>FSM 271</td>
<td>9.06 ± 0.04</td>
</tr>
<tr>
<td>FSM 272</td>
<td>8.87 ± 0.22</td>
</tr>
<tr>
<td>FSM 287</td>
<td>8.52 ± 0.04</td>
</tr>
<tr>
<td>FSM 292</td>
<td>8.47 ± 0.03</td>
</tr>
<tr>
<td>2.39-1</td>
<td>8.43 ± 0.01</td>
</tr>
<tr>
<td>2.68</td>
<td>8.62 ± 0.01</td>
</tr>
<tr>
<td>1</td>
<td>9.12 ± 0.30</td>
</tr>
<tr>
<td>2</td>
<td>9.20 ± 0.01</td>
</tr>
<tr>
<td>3</td>
<td>8.49 ± 0.03</td>
</tr>
<tr>
<td>4</td>
<td>7.46 ± 0.20</td>
</tr>
<tr>
<td>5</td>
<td>9.16 ± 0.06</td>
</tr>
<tr>
<td>6</td>
<td>7.98 ± 0.71</td>
</tr>
<tr>
<td>7</td>
<td>8.93 ± 0.13</td>
</tr>
<tr>
<td>55</td>
<td>5.74 ± 0.09</td>
</tr>
<tr>
<td>60</td>
<td>4.22 ± 0.25</td>
</tr>
<tr>
<td>L02-401</td>
<td>8.07 ± 0.16</td>
</tr>
<tr>
<td>42VS</td>
<td>7.90 ± 0.59</td>
</tr>
<tr>
<td>501B</td>
<td>9.23 ± 0.12</td>
</tr>
<tr>
<td>E27B</td>
<td>5.31 ± 0.06</td>
</tr>
<tr>
<td>800DS</td>
<td>8.71 ± 0.60</td>
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</tbody>
</table>

*a Strains incubated at 43°C for 8 h (n = 3).

prevent occurrence of disease and to reduce the spread of Cronobacter in infant food and food processing environmental niches, where Cronobacter can be inexpugnably protected into its own biofilm (15, 22). Cronobacter spp. grew more preferably at 43°C as compared with 37°C (Fig. 1). Previous studies reported that Cronobacter is able to grow at temperatures up to 46°C (6, 9, 21). Furthermore, Guillaume-Gentil et al. (9) indicated that Cronobacter exhibits better growth performance at high osmolarity (1 M NaCl) when incubated at a relatively lower incubation temperature of 37°C as compared with 45°C.

Sucrose was incorporated in the AR broth because a previous study indicated that all Cronobacter strains could ferment sucrose, whereas the majority of α-glucosidase–positive Enterobacteriaceae cannot utilize sucrose as a carbon source (14). Because of the extraordinary ability of Cronobacter to tolerate osmotic stress, it proliferated equally at 10 and 15% concentrations of sucrose (Fig. 3). Additionally, Cronobacter exhibits a greater tolerance to desiccation and osmotic stresses as compared with other Enterobacteriaceae (6, 7). Supplementation of the medium with a high sucrose concentration is important, because some α-glucosidase–positive Enterobacteriaceae such as Pantoea spp. and Escherichia vulneris can be easily confused when viewed on newly developed Cronobacter chromogenic media, since the differentiation capability of these media depends on the presence of α-glucosidase (12, 19), and these particular species are able to persist for lengthy periods (over 2 years) in powdered IFM (7). A high sucrose concentration acts as a humectant, hindering the growth of other non-Cronobacter Enterobacteriaceae, especially α-glucosidase–positive Enterobacteriaceae, without affecting the growth of Cronobacter (14).

AR broth supported the growth of Cronobacter considerably, where the count reached up to >7 log CFU/ml after 9 h of incubation at 43°C. In comparison, milk and m-LSB were inferior enrichment broths; the numbers of Cronobacter reached only to about 5 log CFU/ml. EE and ESE resulted in counts of about 6.5 log CFU/ml after 9 h of incubation at 37°C. LSb was modified by adding NaCl and the antibiotic vancomycin (9). A mixture of different Cronobacter strains showed a sluggish growth pattern in this medium (Fig. 5). The growth was more rapid in AR, ESE, and EE broths than it was in m-LSB. Previous reports also indicated that some Cronobacter strains failed to grow

TABLE 2. Comparison of four enrichment broths for recovery of desiccation-stressed cells of Cronobacter from powdered IFM in the presence of other competing non-Cronobacter Enterobacteriaceae after 8 and 16 h of incubation in the broths

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Conc. of Cronobacter (CFU/100 g)</th>
<th>EE</th>
<th>ESE</th>
<th>AR-1</th>
<th>AR-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>5,000</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
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<tr>
<td></td>
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<td>3/3</td>
<td>3/3</td>
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<td></td>
<td>10</td>
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<tr>
<td></td>
<td>1</td>
<td>0/3</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>16</td>
<td>5,000</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
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</tr>
<tr>
<td></td>
<td>1,000</td>
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<td>0/3</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>

*a The average water activity of the powdered IFM before inoculation was 0.212; after inoculation at the first day, 0.261; after 30 days, 0.215; and after 60 days, 0.218. Values are numbers of positive samples/numbers of samples tested.

b EE, Enterobacteriaceae enrichment broth; ESE, Enterobacter sakazakii enrichment broth; AR, Al-Holy–Rasco broth.
Additionally, the growth of other non-
Cronobacter Enterobacteriaceae was also suppressed in
this medium (Fig. 6), suggesting that this broh does not
promote the growth of Cronobacter and other members of
Enterobacteriaceae. Enrichment broths such as buffered
peptone water or tryptic soy broth containing vancomycin as
a selective supplement resulted in a poor growth behavior
of Enterobacteriaceae including Cronobacter and E. coli
O157:H7 (21). Milk containing novobiocin was also inferior
in promoting the growth of Cronobacter. It was reported
that reconstituted IFM without additives supports the
growth of Cronobacter (23). However, reconstituted IFM
with novobiocin apparently did not support the growth of
Cronobacter. Novobiocin exhibits an inhibitory effect
against gram-positive bacteria (23). However, most Enterobact-
eriaceae are resistant to the effect of novobiocin, but
some gram-negative bacteria such as Haemophilus and
Proteus strains are sensitive (24). Notwithstanding, bacte-
riostasis of Cronobacter is possible in enrichment broths
containing 20 mg/liter of novobiocin (23).

Cronobacter was able to thrive in AR broh, possibly
because of its remarkable capability to produce osmoreg-
ulators such as trehalose, rendering it more resistant to osmotic
stress (6). Milk, EE, and ESE supported the growth of
Enterobacteriaceae, in which numbers of about 7 log CFU/
ml were obtained after 9 h. In comparison, less than 6 log
CFU/ml of Enterobacteriaceae was obtained after 9 h of
incubation in m-LSB (Fig. 6), suggesting that the presence of
salt and vancomycin in m-LSB hinders the growth of not only
Cronobacter spp., but also of other Enterobacteriaceae.

AR broh suppressed the growth of non-Cronobacter–
competing Enterobacteriaceae (Fig. 6) while stimulating
the growth of Cronobacter (Fig. 5). This selectivity is
attributable not only to the presence of deoxycholate, but
also to the presence of high sucrose concentrations and
NaCl in the broh, which hinders the growth of other
Enterobacteriaceae.

In this study, DFI medium was used for presumptive
identification of Cronobacter spp. because of the presence
of other competing Enterobacteriaceae that could be
confused with Cronobacter colonies on violet red bile
glucose agar or VRBL. Additionally, some Cronobacter
strains do not produce yellow pigmentation on TSA after
being incubated for 3 days at 25°C, a critical parameter
adopted for presumptive identification of Cronobacter
by the FDA (14). Therefore, DFI was used in the current
study for rapid presumptive identification of Cronobacter, since
Cronobacter can grow rapidly and produce distinctive blue-
green colonies on this medium within 24 h of incubation at
37°C.

At relatively high concentrations of Cronobacter (5,000
or 1,000 CFU/100 g), all of the tested broths were capable
of detecting Cronobacter in all tested samples (Table 2).
The sensitivity of EE broh decreased as the concentration
of Cronobacter in powdered IFM decreased. The sensitivity
was defined as the number of true positives divided by the
sum of true positives plus false negatives (12). However, at
the concentration of 100 CFU/100 g, EE failed to detect
Cronobacter in one of the three samples tested after 8 and
16 h of incubation in the broh. Whereas at the levels of 10
and 1 CFU/100 g, EE broh was inferior in detecting
Cronobacter and completely failed to recover the organism
from any of the samples, with high levels of contamination
taking place by other competing Enterobacteriaceae. This
agrees with our previous finding (2) and other findings (10,
14). ESE was more successful in recovering low levels of
Cronobacter at all concentrations except at 1 CFU/100 g,
where one of three samples tested positive for the presence
of Cronobacter after 8 and 16 h of incubation. However,
low counts of Cronobacter were detected after 8 h of
incubation of the broh at 37°C and high counts of
competing Enterobacteriaceae, especially E. cloacae, were
recovered and produced white colonies on DFI. For AR
broth (10% sucrose), high counts of Cronobacter were
obtained (∼1 × 10⁶ CFU/ml of broth). Nonetheless,
colonies that belong to E. aerogenes were also recovered,
and they produced white colonies on DFI. On the other
hand, AR broh (15% sucrose) had 100% sensitivity at all
the examined concentrations of Cronobacter in powdered
IFM. The presence of competing non-Cronobacter Enterobact-
eriaceae even in higher numbers relative to Cronobac-
ter spp. did not hamper the specificity of the medium for
Cronobacter because of the high exclusivity of this broh
for nontarget microorganisms. AR broh (15% sucrose)
was the most superior among the enrichment broths
examined to promote the growth of Cronobacter spp.
and to preclude the growth of other competing Enterobact-
eriaceae. Cronobacter was recovered from all samples
at all concentrations. Counts of more than 1 × 10⁶ CFU/
ml of the broh were recovered after 8 and 16 h of
incubation in this broh, without detectable growth of
competing Enterobacteriaceae. Generally, the presence of
competing Enterobacteriaceae reduced the sensitivity of
the EE broh and to a lesser extent, the ESE broh, but not
the AR broh with a 15% sucrose concentration. Addition-
ally, AR broh had a low limit of detection (defined as the
smallest number of cultivable microorganisms that can be
detected 50% of the time) as compared with the other
examined broths (Table 2).

Using the AR broh depicted in the current study will
most likely lead to more precise and rapid detection of
Cronobacter spp. in infant formula, as it promotes the growth
of Cronobacter while suppressing the growth of other
Enterobacteriaceae. Additionally, as shown in Table 2, 8 h
of enrichment is sufficient to promote significant growth of
Cronobacter, even if the organism is present at very low
levels in the tested samples, suggesting quicker detection of
Cronobacter with this method as compared with the reference
FDA method. Therefore, AR broh may serve as a potential
medium for rapid detection and isolation of Cronobacter
from powdered IFM after conducting further validation
studies. Further studies are needed to improve the ability of
the AR broh to recover some stressed and poorly growing
Cronobacter strains, this by supplementing AR broh with
ingredients such as sodium pyruvate and 3,3-thiodiopropio-
nic acid (11). Future studies may also focus on testing the
ability of the AR broh to recover Cronobacter spp. from
naturally contaminated powdered IFM.
REFERENCES