Modeling the combined effect of NaCl and pH against *Cronobacter* spp. using response surface methodology

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Abstract
The growth response of Cronobacter spp. to different levels of NaCl and pH was investigated using response surface methodology. Brain heart infusion (BHI) broth containing 0 to 10% (w/v) NaCl at pH values of 4.5 to 8.0 was inoculated with a cocktail of 5 Cronobacter spp. The mixtures were incubated at 37°C and were sampled at intervals of 0, 2, 4, 6, 8, and 24 hr. Cronobacter spp. were recovered on tryptose soy agar and response surface methodology was employed to investigate the effect of NaCl, pH or their combination on growth and viability. At pH ≤ 5.0, the viability of Cronobacter spp. was reduced below the detection limit after 4 h. In addition, ≥6% NaCl significantly affected Cronobacter spp. growth. However, the interactive effects of pH at 5.5 and 2 to 4% NaCl reduced Cronobacter growth. The response surface analysis indicated that combining NaCl with pH would cause a significantly greater reduction in Cronobacter viability than would be caused by each factor alone. These results showed that Cronobacter may be controlled in food products by concentrations of NaCl ≤ 4% at pH values ≤ 5.5.

Practical applications
Cronobacter spp. have been isolated from a wide range of foods including cheese, meat, grains, herbs, spices, fermented bread, tofu, infant foods, and fermented beverages. Cronobacter spp. have a remarkable ability to survive under a variety of environmental stresses including those involving low water activity (a₀), acidic pH, osmotic challenge, and mild heating. In the current study, the results showed that the growth of Cronobacter in food products could be controlled by the combined effect of NaCl and pH at concentrations of NaCl ≤ 4% at pH values ≤ 5.5.

1 | INTRODUCTION

The genus *Cronobacter* consists of 7 species including *C. condimenti*, *C. sakazakii*, *C. malonaticus*, *C. muytjensii*, *C. turicensis*, *C. dublinensis*, and *C. universalis*. *Cronobacter* species (formerly included in the genus *Enterobacter*) are Gram-negative rod-shaped, motile facultative anaerobic bacteria belonging to the family *Enterobacteriaceae* (Iversen et al., 2008; Stephan et al., 2014).

*Cronobacter* species cause severe forms of necrotizing infection and meningitis, especially in neonates, with mortality rates varying from 40 to 80% (Bar-Oz, Preminger, Peleg, Block, & Arad, 2001; Healy et al., 2010; Van Acker et al., 2001). The infective dose is estimated to range from 10³ to ≥ 10⁸ cells (Iversen & Forsythe, 2003; Pagotto, Nazarowec-White, Bidawid, & Farber, 2003). *Cronobacter* spp. have been isolated from a wide range of foods including cheese, meat, grains, herbs, spices, fermented bread, tofu, infant foods, and fermented beverages (Chap et al., 2009; Gassem, 1999; Gassem, 2002; Iversen, Druggan, & Forsythe, 2004; Iversen and Forsythe, 2003; Muytjens, Muytjens, Roelofs-Willemse, & Jaspar, 1988; Skladal, Mascini, Skladal, Salvadori, & Zannoni, 1993). However, powdered infant formula has been epidemiologically linked to *Cronobacter* infections in infants (Jaradat, Al Mousa, Elbetieha, Al Nabulsi, & Tall, 2014). Furthermore, *Cronobacter* spp. have been detected in food production environments such as milk powder facilities and chocolate factories (Al-Nabulsi et al., 2011; Kandhai, Reij, Gorris, Guillaume-Gentil, & Van Schothorst, 2004).

*Cronobacter* spp. have a remarkable ability to survive under a variety of environmental stresses including those involving low water activity (a₀), acidic pH, osmotic challenge and mild heating (Breeuwer, Lardeau, Peterz, & Joosten, 2003; Kim & Beuchat, 2005; Osaili &
Sodium chloride is Generally Recognized As Safe (GRAS) in the USA, and is used as an additive and preservative in many food products (Al-Nabulsi et al., 2009; Ayash, Sherkat, & Shah, 2013). Its ability to inhibit microorganisms is related to changes it causes in aw and ionic strength of food systems (Ha & Ha, 2011). In addition to the antimicrobial effects of NaCl, other properties of a food such as its pH can exert a significant influence on the ability of bacteria to grow (Tienungoon, Ratkowsky, Mcmeekin, & Ross, 2000). It has been shown that acids, including formic, acetic, ascorbic, and lactic can effectively prevent the growth of foodborne pathogens (Calix-Lara et al., 2014; Singh et al., 2012). There are many instances where reduced pH and NaCl were more effectively antimicrobial together than when used separately. In fact, the combination of low pH with NaCl is a classic example of the hurdle approach and is used to preserve a large and diverse range of foods including fermented meats, cheeses, and vegetables. To our knowledge, the combined effect of different NaCl concentrations and pH levels on the growth of C. sakazakii and other Cronobacter spp. has not been reported.

Response surface methodology consists of a set of mathematical and statistical procedures developed for modeling data to identify combinations of experimental variables that will lead to an optimized response (Giovanni, 1983). Modeling has been used successfully for evaluating and predicting the interactions of factors with the growth and survival of Listeria monocytogenes, Shigella spp. as well as other foodborne pathogens (Buchanan & Phillips, 2000; Ribeiro, Manha, & Brito, 2006; Zaika & Phillips, 2005). Therefore, the objective of the current study was to investigate the combined effect of NaCl and pH on the viability of Cronobacter spp. using response surface methodology.

2. MATERIALS AND METHODS

2.1 | Bacterial strains and culture preparation

A mixture of one C. muytjensii ATCC 51329 strain and four C. sakazakii food isolates originally described by Shaker, Osaifi, Al-OMary, Jaradat, and Al-Zuby (2007) was used throughout this study. All bacterial cultures were stored individually at −40°C in 5 ml of 20% (v/v) glycerol and brain heart infusion (BHI) broth (Oxoid, Basingstoke, U.K.). To prepare the inoculum for each trial, a loop from each stock culture was added individually to 15 ml tubes containing 10 ml of BHI broth and incubated for 24 hr at 37°C. Two consecutive transfers were performed before culture use in trials. Before inoculation, equal volumes of the 5 Cronobacter strains were combined to form a cocktail containing 9 log_{10} CFU/ml. The mixed culture was then decimally diluted in peptone water (0.1% w/v) (Becton Dickinson, Sparks, Maryland, U.S.A.) to yield 5 log_{10} CFU/ml.

2.2 | Growth media and culture conditions

The test media were BHI broths containing 1, 2, 3, 4, 5, 6, 8, or 10% NaCl (w/v) (Fluka Chemicals, Switzerland) and the pH of each concentration was adjusted to 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, or 8.0 (Cyberscan 500 pH meter, Eutech Instruments, Singapore) using lactic acid (TEDIA Company, Ohio, U.S.A.) or NaOH (Sigma-Aldrich, St Louis, Missouri, U.S.A.). Control BHI broth without NaCl addition was also prepared. The media were distributed in 100 ml portions into 250 ml flasks. The flasks were then capped with foam plugs and the media were sterilized by autoclaving (121°C, 15 min). The pH of each flask was examined after sterilization and no significant changes were observed. Portions (1 ml) of inoculum were added to the treated BHI broth at an initial population of 10^7 CFU/ml. The media were incubated at 37°C and sampled at 0, 2, 4, 6, and 24 h. A minimum of three trials were conducted for each combination of factors (treatment), with one flask per treatment (N = 3).

2.3 | Enumeration of bacteria

To determine Cronobacter spp. populations at selected intervals, all samples and appropriate decimal dilutions in 0.1% peptone water were spread-plated in duplicate onto tryptose soy agar (TSA) (Oxoid). The plates were incubated aerobically at 37°C for 24 to 48 hr and the colonies were counted.

2.4 | Experimental design, model development, and statistical analysis

Response surface methodology (RSM) with an 8 × 6 (NaCl level × pH of the BHI media) factorial design was applied with JMP 11.1 2013 software (SAS Institute Inc., Cary, North Carolina, U.S.A.). NaCl (1, 2, 3, 4, 5, 6, 8, and 10%) and pH (4.5, 5.0, 5.5, 6.0, 6.5, and 7.0) were studied. The design contained 48 treatments with three replicates (N = 3). A response surface (Y) was obtained for the Cronobacter population, and a second order polynomial model was defined to fit the response:

\[ Y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{1 \leq i < j \leq k} b_{ij} x_i x_j + \epsilon \]  

(1)

Where k is the number of variables, \( b_0 \) is a constant term, \( b_i \) is a coefficient of linear parameters, \( \beta_{ij} \) represents a coefficient of the interaction parameters, \( x_i \) and \( x_j \) represent the variables, and \( \epsilon \) is the experimental residual error with the experiments. In order to determine a critical point (maximum, minimum or saddle), it was necessary for the polynomial function to contain quadratic terms according to the following equation:

\[ Y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ii} x_i^2 + \sum_{1 \leq i < j \leq k} b_{ij} x_i x_j + \epsilon \]  

(2)

The adequacy of the models was determined using a lack of fit test and a coefficient of determination (R^2) was estimated for model analysis. Only terms found statistically significant (p < .05) were
included in the reduced models. It should be noted that some independent variable terms were kept in the reduced model despite non-significance (p > .05). For example, linear terms were kept in the model if a quadratic or interaction term containing the variable was significant (p < .05). The populations of Cronobacter spp. were subjected to an analysis of variance for main effects (NaCl levels and pH values of the growth media). Least-squares means were separated using Fisher’s least significance difference test (LSD) and the General Linear Model. All statistical analyses were performed using JMP 11.1 software.

3. RESULTS AND DISCUSSION

A number of studies have investigated the resistance of Cronobacter spp. to different environmental stress conditions, antibiotics and food processing technologies (Breeuwer et al., 2003; Kim & Beuchat, 2005; Osaili & Forsythe, 2009). The combined effects of pH and NaCl on the growth of Cronobacter spp. For 0, 2, 4, 6, 8, and 24 hrs at 37°C are shown in Fig. 1A–F, respectively, using response surface methodology. The inhibitory effect of NaCl and pH on bacterial growth was evident at 4 h (Fig 1C).
However, after 24 hr, Cronobacter numbers increased by 6 log_{10} CFU/ml at pH 7.0 without NaCl (Fig 1F), while numbers increased by < 2 log_{10} CFU/ml when NaCl was ≥8% and pH was between 6.0 and 8.0.

In control samples (without NaCl) the growth of Cronobacter differed little between pH 6.5 to 8.0, whereas growth was 1-2 log_{10} CFU/ml lower at pH 5.5 and 6.0, and this was significant. Further, Cronobacter cells were not detected at pH values of 5.0 and 4.5 after 4 and 2 hrs, respectively. In contrast to the results of the current study, Dancer et al. (2009) tested 72 clinical and environmental isolates of C. sakazakii from food processing plants for their ability to grow in tryptic soy broth at acidic pH. They found that all 72 isolates were able to grow at pH 4.5. However, some (71, 69, and 57) isolates grew at pH 4.3, 4.1, and 3.9, respectively. The difference in acid resistance found between these two studies was due in part to differences in strains tested and growth media used. In the current study, it seemed that Cronobacter strains tested were sensitive to acidic media with pH ≤ 6.0.

In general, Cronobacter growth increased significantly after 24 hr of incubation at all salt and pH levels except pH 4.5 to 5.5. The addition of ≤4% NaCl was not inhibitory to Cronobacter spp. in BHI broth with pH 6.0 to 8.0, while interactive (additive) effects were observed with pH 5.5 by 24 hr (Cronobacter numbers were reduced 3.0 to 6.0 log CFU/ml compared to the control without NaCl). Remarkably, ≥6% NaCl significantly suppressed Cronobacter growth during the first 8 hr at all pH levels. It is likely that Cronobacter became sensitive to NaCl at levels ≥6%. Moreover, Cronobacter numbers were reduced > 4 log CFU/ml by 6% NaCl at pH 6.0 to 8.0 by 24 hr, while Cronobacter cells were not detected by 24 hr at pH 5.5 and by 2 hr at 4.5 to 5.0. Similarly, Álvarez-Ordóñez et al. (2014) found that all 15 C. sakazakii strains as well as one strain each of C. muytjensii and C. malonaticus were able to grow in LB broth containing 4–8% NaCl. In the current study, it was evident that NaCl was more inhibitory to Cronobacter spp. at acidic (<5.5) than neutral pH.

Statistical analysis of the data, based on variance analysis with two criteria, provided evidence of a significant effect of NaCl levels, pH values and their interaction against Cronobacter growth. Multiple range analysis confirmed significantly higher Cronobacter growth in BHI control broth without salt; however, growth was reduced in acidic broth at higher levels of NaCl. It is clear that there were significant differences (p < .001) in Cronobacter growth between the levels of NaCl or values of pH after 2 hr incubation. However, the combined effect of NaCl with pH on Cronobacter growth was significant (p < .001) at 6 hr to 24 hr (Tables 1 and 2). Different models have been used to predict growth responses following environmental challenge of pathogens including Enterobacter cloacae (Bevilacqua, Gallo, Corbo, & Sinigaglia, 2013), L. monocytogenes (Carrasco et al., 2006; Park et al., 2005), E. coli (García-Gimeno, Barco, Rincón, & Zurera-Cosano, 2005), and Salmonella enterica Typhimurium (Park, Seo, & Ha, 2007).

Ribeiro et al. (2006) investigated effects of salt and pH stress on the growth of dairy isolates of L. monocytogenes using response surface methodology. In contrast to the current study, they found that L. monocytogenes isolates grew optimally at extreme acidic and alkaline pH values. Additionally, they reported that the response surface model distinguished differences between the isolates tested (Ribeiro et al., 2006). In another study, Bevilacqua et al. (2013) examined the growth response of E. cloacae as a function of NaCl, pH, and temperature. As found during the present study, their response surface modeling showed that E. cloacae exhibited better growth at pH 4.5 to 5.5 when the NaCl concentration was low. The authors also reported that pH was more inhibitory than NaCl toward E. cloacae growth as shown in the present work. Further, in the present study, it was obvious that the effect of pH on Cronobacter growth became more apparent as NaCl level increased. At all incubation times, the response surface model indicated that Cronobacter became more sensitive to pH ≤5.5 at all NaCl concentrations (Fig. 1). Moreover, the response surface model predicted that Cronobacter growth at pH 6.0 to 8.0 would be suppressed when the NaCl concentration was increased to 10% at all incubation times. Lee and Kang (2016) found that the addition of NaCl decreased the resistance of L. monocytogenes and S. aureus to acetic

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**TABLE 1** Analysis of variance showing effects of NaCl, pH or their combination against Cronobacter at different sampling times

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Time (hour)</th>
<th>p-value</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>24</th>
</tr>
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<tbody>
<tr>
<td>NaCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl*pH</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

NS: Not significant.

***: p < .001.

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**TABLE 2** Effects (p-value) of pH, NaCl or their combination against Cronobacter incubated at 37°C for ≤24 hr

<table>
<thead>
<tr>
<th>Source</th>
<th>Incubation Time (hour)</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>&lt;.0001</td>
<td></td>
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<td></td>
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<tr>
<td>NaCl</td>
<td>0.0076</td>
<td>&lt;.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>0.8626</td>
<td>&lt;.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl*pH</td>
<td>0.1407</td>
<td>0.2531</td>
<td>0.0023</td>
<td>0.0023</td>
<td>0.7106</td>
<td>0.9108</td>
<td></td>
</tr>
<tr>
<td>pH*pH</td>
<td>0.9637</td>
<td>0.2769</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>R-square</td>
<td>0.0320</td>
<td>0.4920</td>
<td>0.5680</td>
<td>0.7270</td>
<td>0.8213</td>
<td>0.7780</td>
<td></td>
</tr>
<tr>
<td>Lack of Fit (p-value)</td>
<td>0.9990</td>
<td>0.5650</td>
<td>0.6290</td>
<td>0.0826</td>
<td>0.0163</td>
<td>0.0010</td>
<td></td>
</tr>
</tbody>
</table>
acid which is similar to our results, but it increased the acid resistance of \textit{E. coli} O157:H7 and \textit{Shigella}. It seems that antagonistic or synergistic effects between these treatments depends on the bacterial strain, NaCl concentration and possibly the type of acid.

In conclusion, the effects of NaCl, pH and their combination on the growth of \textit{Cronobacter} was studied and a predictive model developed using response surface methodology revealed that pH and NaCl significantly affected \textit{Cronobacter} growth at each incubation time tested. However, a combined inhibitory effect of NaCl and pH against \textit{Cronobacter} was observed after 6 to 24 h. Use of low concentrations of NaCl (<4%) with acidic pH is highly recommended to control \textit{Cronobacter} growth. However, at pH 6.0–8.0 high concentrations of NaCl will be required to reduce its viability.

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**CONFLICT OF INTEREST**

No conflict of interest was declared.

**REFERENCES**


