# Inoculum Size of *Clostridium botulinum* 56A Spores Influences Time-to-Detection and Percent Growth-Positive Samples

L. ZHAO, T.J. MONTVILLE, AND D.W. SCHAFFNER

**ABSTRACT: The influence of inoculum size on the growth kinetics of *Clostridium botulinum* 56A and percentage of growth-positive samples was studied in a complete factorial design with factors of inoculum size (1, 100, or 10,000 spores), pH, and sodium-chloride concentration. Growth was followed hourly as change in $A_{620}$. Polynomial regression was used to analyze the data. The time-to-detection and percent growth-positive samples were significantly affected by inoculum size and its quadratic term. When inoculum size increased from 1 to 100 spores/sample, the percent growth-positive samples increased, and the time-to-detection decreased. When the inoculum was 1000 spores/sample or higher, there was little additional effect. Inoculum size might influence results through simple probability or quorum sensing. The maximum growth rate was independent of inoculum levels.**

**Key Words: *C. botulinum*, spores, inoculum size, Gompertz model, quorum sensing**

## Introduction

THE ASSUMPTION OF STATISTICAL EQUIVALENCE (THAT IS, RESULTS from $10^3$ spores in 1 sample are equivalent to results from $10^3$ samples each with 1 spore) is used in predictive modeling, inoculated pack studies, and thermal processing calculations. Microbiologists make this assumption to simplify studies, which would otherwise require large numbers of samples containing low spore concentrations. Statistical equivalence is valid only for independent events that do not influence the outcome of other events. However, if cells interact with nearby cells through "quorum sensing," the "independence" requirement for statistical equivalence is violated. Quorum sensing is used by many Gram-negative bacteria to regulate specific sets of genes in a cell density-dependent manner (Pearson and others 1995; Crockford and others 1995). It plays an essential role in regulating bioluminescence (Engebrecht and others 1983), antibiotic biosynthesis (Bainton and others 1992), virulence determination (Passador and others 1993), catalase activity (Crockford and others 1995), and initiation of chromosomal replication (Withers and Nordstrom 1998). Lytic germination-inducing enzymes and small molecules, such as lactate and bicarbonate, have been identified as potential initiators for *Clostridium botulinum* spore germination (Gould and Dring 1972). When high spore densities (100 to 300 per plate) are plated, and a germinated spore gives rise to a colony, this colony is often surrounded by "satellite" colonies. It appears that spores that successfully form colonies send a "signal" to other spores, indicating that conditions are favorable and that they should germinate. Satellitism is widely reported in many bacteria (Cohen and others 1989; Ruoff 1991; Dot and others 1993).

Although predictive food microbiology has made great strides in developing comprehensive mathematical models to describe microbial growth as a function of food conditions (McMeekin and others 1993; Whiting and Buchanan 1994; Whiting 1995), fundamental assumptions such as that of statistical equivalence remain unexamined. Early reports that considered inoculum size as a variable (Lindroth and Genigeorgis 1986; Jensen and others 1987; Graham and Lund 1993) all occurred before the statistical importance of heteroscedasticity and changing confidence intervals were recognized (Ratkowsky and others 1991; Alber and Schaffner 1992; Ratkowsky 1992).

Some research on inoculum size has been done with *Clostridium botulinum*. Cann and others (1965) found that inoculum size has a contradictory effect on time to 1st toxic sample of *C. botulinum* type E spores and concluded that initial spore inoculum had little effect on toxigenesis. In a study to model the toxigenesis of *C. botulinum* in fresh fish under modified atmospheres, Baker and Genigeorgis (1990) found that inoculum size accounted for 7.4% of the experimental variation, second only to temperature. The difference in lag phase of toxigenesis caused by inoculum was more apparent at low temperatures. In a later study (Meng and Genigeorgis 1993), they found increasing levels of spore inoculum allowed toxigenesis at lower storage temperature and higher salt levels. The time for a median number of the positive tubes to show turbidity and its variance was shown to increase with decreasing spore numbers (Whiting and Oriente 1997; Whiting and Strobaugh 1998). Although these studies shed light on the effect of inoculum size, they did not examine its influence on true kinetic parameters, and continuous spectrophotometer measurements of growth were not made.

This research examines the hypothesis that inoculum size can be an important variable in predicting the germination of *C. botulinum* spores, that is, the assumption of statistical equivalence is not always valid. In this paper, we modeled the growth of *C. botulinum* at different inoculum, pH, and salt levels. The time-to-turbidity data were fitted to a form of the Gompertz Equation, and the influence of each variable on the percent growth-positive samples, maximum growth rate, and time-to-detection, were examined in the polynomial models.



## Materials and Methods

### Experimental design

The inoculum size dependence hypothesis was tested in a complete 3-×-3 factorial design where the putative variable, inoculum size, was examined with the known variables of pH and salt. Each of the 3 variables was fixed at 3 levels: inoculum size of 1, 100, and 10,000 spores per sample; pH values of 5.5, 6.0, and

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6.5; and sodium-chloride concentrations of 0.5, 2 and 4% (w/v). The experiments were conducted using Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, Mich., U.S.A.) in 96-well micro-titer plates (Corning Costar Co., Cambridge, Mass., U.S.A.) where each well represented a separate experimental sample. Forty-four wells were used for each pH-salt-inoculum combination, and 4 wells contained uninoculated BHI medium as controls. The 9 pH-salt combinations were prepared separately in BHI, autoclaved, and dispensed into the 96-well plates after being inoculated with the appropriate concentration of spores (see below).

### Spore crop preparation and inoculation

*Clostridium botulinum* 56A spores were prepared according to the biphasic method (Anellis and others 1972) and purified using an aqueous polymer 2-phase system (Sacks and Alderton 1961). Spores were enumerated by the Petroff-Hausser chamber and confirmed by plating on Brain Heart Infusion (BHI) Agar plates. Spores were then allocated to 1.5-mL siliconized microcentrifuge tubes, fast frozen with liquid nitrogen, and stored at -70 °C. Three spore stock solutions were prepared for the 3 inoculum sizes studied. One mL of the original spore stock at $4 \times 10^9$ spores/mL was thawed on ice and then diluted to $5 \times 10^6$ spores/mL, $5 \times 10^4$ spores/mL, and $5 \times 10^2$ spores/mL using sterile water. These 3 spore stocks were stored at 4 °C. All culture manipulations and incubations were conducted in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, Mich., U.S.A.) with an atmosphere of 10% $H_2$, 5% $CO_2$, and 85% $N_2$.

For $10^4$ spores/sample experiments, 250 mL $5 \times 10^6$ spores/mL spore stock was transferred to a 1.5-mL siliconized microcentrifuge tube and heat shocked at 80 °C for 10 min. Two hundred mL of spore stock solution was mixed with 20mL BHI medium to get a 100-fold dilution. Two hundred mL of this $5 \times 10^4$ spores/mL BHI solution was allocated to each well of a 96-well microplate, which resulted in a final concentration of $10^4$ spores/well. The same spore-medium mixture allocated into the microplate wells was diluted and plated on 50 BHI agar plates to estimate the actual number of spores contained in 1 well. We conducted 5 enumeration experiments with an inoculum of $10^4$ spores/well, which resulted in a total of 250 plates being counted. The average plate count was then used in the modeling. Similar procedures were used for experiments at 100 spores/sample and 1 spore/sample, except that the spore stocks used were $5 \times 10^4$ spores/mL and $5 \times 10^2$ spores/mL, respectively. To achieve the target of 1 spore/well, the dilution was adjusted to a target of 2 or 3 spores per sample to decrease the chance of a sample receiving an inoculum size of 0.

### Kinetic studies

Each well of the 96-well microtiter plate was covered with 50 µL sterile mineral oil (Sigma Chemical Co., St. Louis, Mo., U.S.A.) prior to sealing the plate with a sterile adhesive film (Rainin Instrument Co. Inc., Woburn, Mass., U.S.A.) to prevent evaporation and contamination. The samples were incubated in a microplate reader (Multiskan MCC/340, MTX Labsystems Inc., Mclean, Va., U.S.A.) maintained at 30 °C inside the anaerobic chamber. The $A_{620}$ of each sample (well) was read hourly for 14 d using autoreading software (Spectrosoft, MTX Labsystems Inc.). If after 14 d, not all the wells on a plate showed growth ($A_{620} > 0.015$ above the blank), the plate was kept in the incubator, and a reading was taken every 3 d until the maximum percent growth-positive samples (that is, maximum number of growth-positive wells/number of wells inoculated × 100) was reached.

### Modeling

After preliminary analysis of several primary models that could be used to analyze the absorbance data, the 3-parameter Gompertz model was selected. Parameters derived from fitting were used to calculate maximum growth rate and lag time. The Gompertz Equation can be expressed as:

$$A_{620} = A_0 + Ce^{-e^{-B(t-M)}} \qquad (1)$$

where:

$A_{620}$ is the absorbance at 620 nm at time t.

$A_o$ is the absorbance at 620 nm at time 0 (which is always 0, so this term drops out of the equation, reducing the 4-parameter model to a 3-parameter model).

C is the change of $A_{620}$ between inoculation and stationary phase.

B is maximum relative growth rate.

M is the time to reach B.

t is time (in hours).

After fitting this equation, the parameters were used to calculate the maximum growth rate and time-to-detection (Gibson and others 1987; Zwietering and others 1990).

$$\text{Maximum growth rate} = \frac{B \times C}{e} \quad (A_{620}/\text{hour}) \qquad (2)$$

$$\text{Time-to-detection} = M - \frac{1}{B} \qquad (3)$$

Fitting of the primary model was done using regression tools in Sigmaplot Version 3 (SPSS Inc., Chicago, Ill., U.S.A.)

Two polynomial equations, 1 containing interaction terms and 1 not, were used to describe the influence of the 3 variables on maximum growth rate. Log transformation was used for inoculum size and time-to-detection. Equation 4 shows the polynomial equation containing interaction terms.

$$\begin{aligned}\text{Maximum growth rate} &= \text{intercept} + A_1 \mathit{Linoc} + A_2 \mathit{pH} + A_3 \mathit{NaCl} \\ &+ A_4 \mathit{Linoc}^2 + A_5 \mathit{pH}^2 + A_6 \mathit{NaCl}^2 \\ &+ A_7 \text{Linoc} \cdot \text{ph} + A_8 \text{Linoc} \cdot \text{NaCl} + A_9 \text{pH} \cdot \text{NaCl}\end{aligned} \qquad (4)$$

where:

Linoc = log(inoculum size).

NaCl = percent of sodium-chloride concentration.

pH = initial pH of the medium.

The time-to-detection and percent growth-positive were analyzed using the same model format.

Eight of the 27 conditions studied generally had less than 10% of the samples reach stationary phase within 14 d, so maximum growth rate and time-to-detection could not be calculated for these conditions. These data were omitted rather than using 0 maximum growth rate and a 14-d time-to-detection, which were found to introduce a statistical bias into the model.

SAS (SAS Institute Inc., Cary, N.C., U.S.A.) backward regression was used to eliminate the insignificant terms in the model. The simplified versions of these models were compared for simplicity (number of variables in the model), collinearity, and $R^2$. A condition index number of 40 or less was considered to have no or a low level of collinearity. The choice of model was made based on a better $R^2$, no or small collinearity, and parsimony.

## Results and Discussion

### Time-to-detection

Both models with and without interaction terms had an $R^2$ of 0.94, and both models had 5 terms, no collinearity. Predictions from the 2 models matched very well (not shown). For ease of interpretation, the model without interaction terms was chosen:

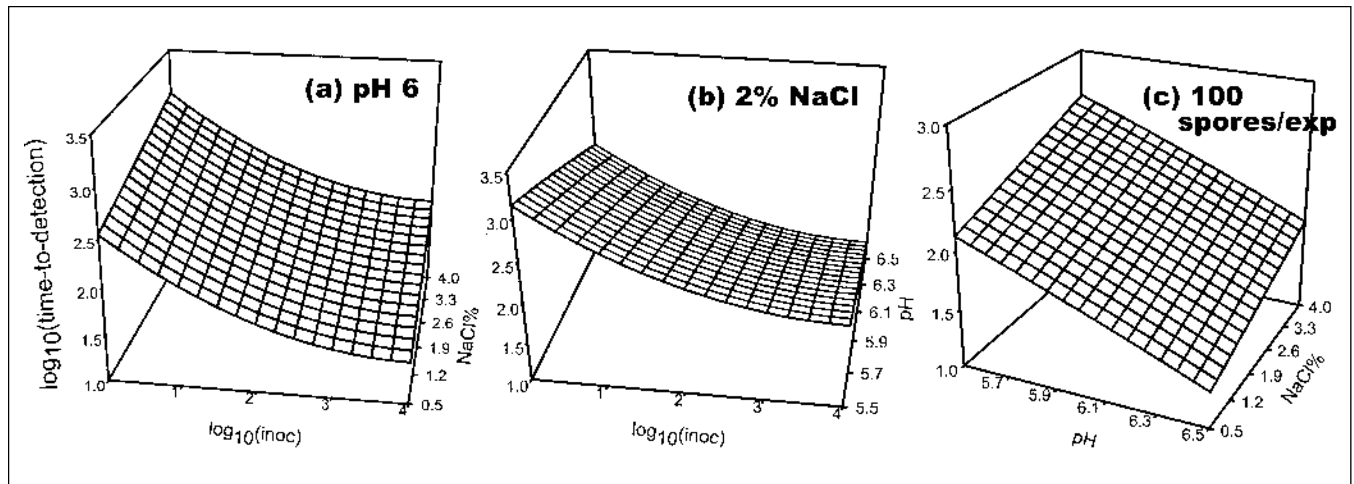


**Figure 1—Influence of inoculum size, pH, and sodium chloride on log, o(time-to-detection) (h). A. Combined effect of inoculum and sodium chloride at pH 6. B. Combined effect of inoculum and pH at 2% sodium chloride. C. Combined effect of sodium chloride and pH at 100 spores/sample.**

$$\log_{10}(\text{time-to-detection}) = 4.910 - 0.533Linoc + 0.139NaCl + 0.055Linoc^2 - 0.068pH^2 \quad (5)$$

The model is plotted in Figure 1 A to C. One variable was kept constant for each panel: pH at 6 in panel A, sodium chloride at 2% in panel B, and inoculum at 100 spores/sample in panel C. In both panels A and B, the response surface curvature demonstrates the quadratic nature of inoculum size as a variable. The decrease in time-to-detection is very apparent when inoculum increases from 1 spore/sample to 100 spores/sample, while the time-to-detection decrease when inoculum size was 1000 spores/sample or higher was very small, if any (panels A and B). The predicted response of time-to-detection corresponding to sodium chloride and pH change was approximately linear as shown in panels A through C. This linearity is visibly apparent despite the presence of a quadratic pH term.

The observed time-to-detection, predicted time-to-detection, and 95% prediction interval for each observation is shown in Table 1. The prediction interval at very low inoculum increased slightly when compared to that at higher levels. The agreement between predicted and observed time-to-detection was very good (Figure 5A). All time-to-detection values calculated from data fell into the 95% prediction interval.

There are 3 possible explanations for the observed inoculum size effect. First, lower numbers of spores may need more time to reach the detection limit. This effect can be accounted for by subtracting the amount of time required for a specific inoculum to reach 10,000 cells. As an example, the original and adjusted log(time-to-detection) for pH 6.0, 2.0% sodium chloride were plotted in Figure 2. The adjusted log(time-to-detection) showed very little difference from the original values. Secondly, the spore populations may contain some portion of spores that are more "active" than others (that is, fast germinators). If the population is large, the chances of it containing 1 or more of these spores would be higher. This could explain why, when inoculum size increased from 1 spore/sample to 100 spores/sample, time-to-detection decreased substantially. If this was the only factor, however, the decrease should extend to the higher inoculum levels. This is clearly not the case. Although our experiment neither proved nor refuted this hypothesis, the quadratic character of inoculum size cannot be explained by this hypothesis alone. The final explanation for the inoculum size effect is spore interaction or "quorum sensing." It has long been proposed that cells or spores use signaling molecules to exchange information on population density. When the population is large, this molecule accumulates to the critical amount quickly, and the whole population responds faster and more uniformly to the environment. When the population is small, it can take much longer for the substance to reach the threshold amount to be active, which would be reflected by an extended time-to-detection and larger variance. This hypothesis is supported by the data. When inoculum size increased from 1 to 100 spores/sample, the concentration of the hypothetical substance increased faster because more cells/spores were producing it, and the time-to-detection for the whole population was shortened. At spore levels of 1000 or higher, however, the net effect of a faster accumulation rate of the substance

**Table 1—Observed and predicted log (time-to-detection) at 30 °C under different inoculum size, pH, and sodium chloride concentration**

| Factors | | | Log(time-to-detection) (h) | | | |
|---|---|---|---|---|---|---|
| Log(spores /well) | pH | NaCl% | Observed | Predicted | Lower 95% of predicted | Upper 95% of predicted |
| 4.0 | 6.5 | 0.5 | 1.090 | 1.0288 | 0.7422 | 1.3154 |
| 4.0 | 6.5 | 2.0 | 1.228 | 1.2369 | 0.9605 | 1.5133 |
| 4.0 | 6.5 | 4.0 | 1.600 | 1.5144 | 1.2284 | 1.8004 |
| 4.0 | 6.0 | 0.5 | 1.212 | 1.4520 | 1.1782 | 1.7259 |
| 4.0 | 6.0 | 2.0 | 1.843 | 1.6601 | 1.3930 | 1.9273 |
| 4.0 | 6.0 | 4.0 | 1.842 | 1.9376 | 1.6554 | 2.2199 |
| 4.0 | 5.5 | 0.5 | 1.866 | 1.8414 | 1.5566 | 2.1262 |
| 4.0 | 5.5 | 2.0 | 2.039 | 2.0495 | 1.7676 | 2.3314 |
| 4.0 | 5.5 | 4.0 | .[a] | 2.3270 | 2.0263 | 2.6276 |
| 1.7 | 6.5 | 0.5 | 1.370 | 1.4044 | 1.1198 | 1.6890 |
| 1.8 | 6.5 | 2.0 | 1.469 | 1.5708 | 1.2935 | 1.8481 |
| 1.8 | 6.5 | 4.0 | 1.858 | 1.8573 | 1.5697 | 2.1449 |
| 1.8 | 6.0 | 0.5 | 1.962 | 1.7998 | 1.5265 | 2.0730 |
| 1.7 | 6.0 | 2.0 | 2.166 | 2.0370 | 1.7702 | 2.3038 |
| 1.7 | 6.0 | 4.0 | 2.188 | 2.3101 | 2.0269 | 2.5932 |
| 1.7 | 5.5 | 0.5 | 2.13 | 2.2029 | 1.9186 | 2.4872 |
| 1.7 | 5.5 | 2.0 | 2.465 | 2.4147 | 2.1324 | 2.6971 |
| 1.8 | 5.5 | 4.0 | .[a] | 2.6543 | 2.3523 | 2.9564 |
| 0.5 | 6.5 | 0.5 | 1.824 | 1.8744 | 1.5819 | 2.1670 |
| 0.4 | 6.5 | 2.0 | 2.147 | 2.1257 | 1.8382 | 2.4132 |
| 0.2 | 6.5 | 4.0 | 2.525 | 2.5060 | 2.1945 | 2.8175 |
| 0.4 | 6.0 | 0.5 | .[a] | 2.3554 | 2.0490 | 2.6619 |
| 0.4 | 6.0 | 2.0 | .[a] | 2.5547 | 2.2563 | 2.8532 |
| 0.2 | 6.0 | 4.0 | .[a] | 2.9068 | 2.5825 | 3.2311 |
| 0.4 | 5.5 | 0.5 | .[a] | 2.7060 | 2.3811 | 3.0310 |
| 0.3 | 5.5 | 2.0 | .[a] | 2.9657 | 2.6341 | 3.2972 |
| 0.4 | 5.5 | 4.0 | .[a] | 3.2304 | 2.8857 | 3.5750 |

*Insufficient data to calculate time-to-detection, generally lass than 10% growth-positive sample was observed within 14 days.

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might be very small.

The effect of initial inoculum on lag time has been reported in many studies (Jason 1983; Meng and Genigeorgis 1993; Gay and others 1996; Vescovo and others 1997). Most of these studies, however, limited their conclusions to the observation that lag time was longer at very low inoculum levels. The effect of inoculum, pH, NaCl, and temperature on the time to turbidity of nonproteolytic (Whiting and Oriente 1997) and proteolytic *C. botulinum* (Whiting and Strobaugh 1998) was also recently studied. Change in percent positive samples over time was modeled by a Logistic equation. A parameter comparable to lag time, t, the time to reach the median number of positive tubes, was derived from these experiments. The model for $\log_{10}$(t) was the full model with 14 parameters in both studies. Despite all the differences in experimental design (turbidity kinetic for each sample over time in this study in contrast to probability estimate over time in the above 2 studies), the results from the above 2 studies were similar to our observations. Whiting and Strobaugh (1998) showed that at 30 °C-pH 5.5-3.5% NaCl, t decreased when inoculum increased from 1 spore to 100 spores, and above this inoculum no further effect was apparent. In Whiting and Oriente (1997), the boundary was approximately 10,000 spores at 21 °C-pH 6-3% NaCl. A review of data from Genigeorgis and others (1991) shows the same trend of curvature in the effect of inoculum size on lag. No hypothesis for the inoculum size effect has been proposed in any of the above referenced literature. No studies have shown that quorum sensing exists in *C. botulinum*, although many studies have pointed to the possibility that cell-to-cell communication is needed for bacterial growth (Kaprelyants and Kell 1996). More studies are needed to verify the hypothesis presented here.

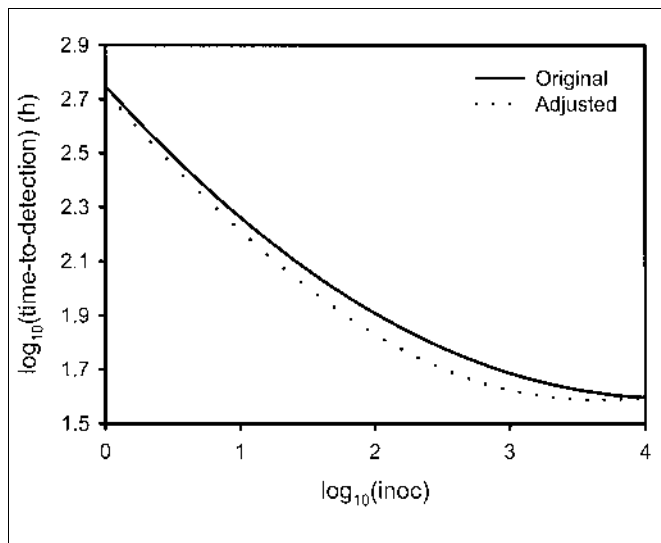


**Figure 2—Comparison of log, 0(time-to-detectio n) (pH 6.0 and 2.0% NaCl) predicted directly by the secondary model, and adjusted for initial spore number.**

### Percentage of growth-positive samples

The inoculum size was also a significant variable for the model of the percentage of growth positive samples, which has $R^2$ = 0.85 and no collinearity:

$$\begin{aligned}\text{Percent growth - positive} = 65.235 + 24.737 Linoc - 107.702 NaCl \\ - 3.811 Linoc^2 - 4.120 NaCl^2 \\ + 19.098 pH \cdot NaCl \qquad (6)\end{aligned}$$

The model with interaction terms was chosen because the model without interaction terms had a much lower $R^2$ (0.65) and very large prediction intervals (not shown).

The model predictions are shown graphically in Figure 3 A to C. The pH value was kept at 6 for panel A, sodium chloride at 2% for panel B, and inoculum at 100 spores/sample for panel C. Sodium chloride and inoculum size both had a nonlinear effect on percent positive samples, with the decrease of percent positive more pronounced at high salt (more than 2%) and lower inoculum size (less than 100 spores/sample). These results verified what we had observed in the model of $\log_{10}$(time-to-detection): There might exist a critical quorum size for spore inocula, such that spores above and below that critical concentration behave differently in terms of germination possibility, outgrowth, and growth.

It is interesting to point out that at higher pH (6.5), sodium chloride had almost no effect on the percent positive sample (Figure 3, panel C). Only when pH dropped to 6 and lower is the



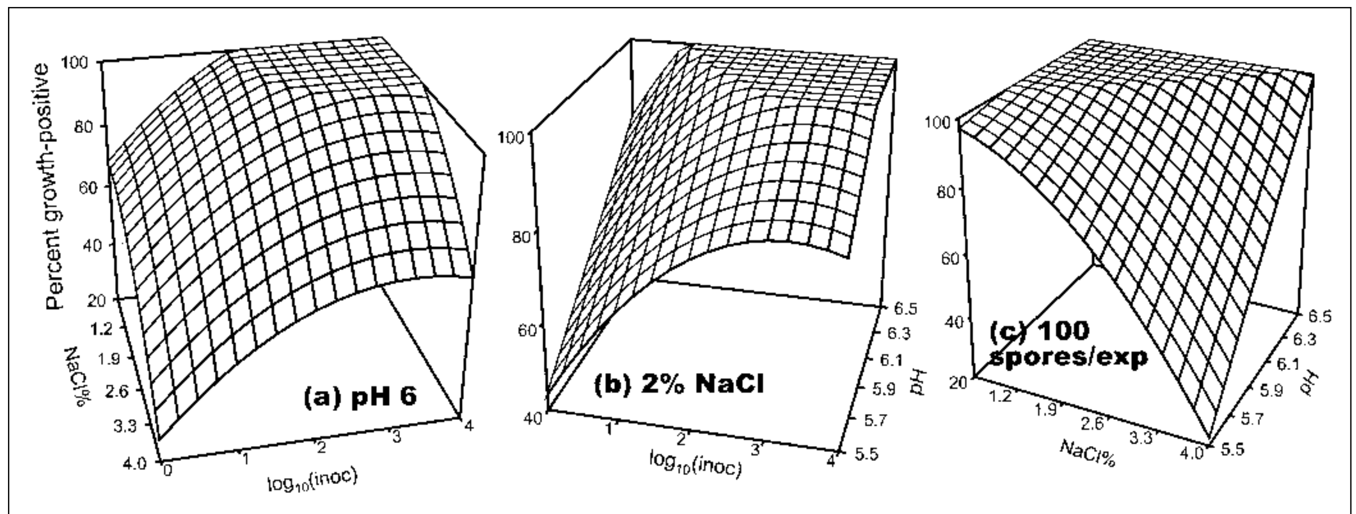


**Figure 3--Influence of inoculum size, pH and sodium chloride on percent growth-positive. A. Combined effect of inoculum and sodium chloride at pH 6. B. Combined effect of inoculum and pH at 2% sodium chloride. C. Combined effect of sodium chloride and pH at 100 spores/sample.**

**Table 2—Observed and predicted percent growth-positive sample at 30 °C under different inoculum size, pH, and sodium-chloride concentration**

| Factors | | | Percent growth-positive sample | | | |
|---|---|---|---|---|---|---|
| Log(spores /well) | pH | NaCl% | Observed | Predicted | Lower 95% of predicted | Upper 95% of predicted |
| 4.0 | 6.5 | 0.5 | 100 | 100[b] | 76 | 100[b] |
| 4.0 | 6.5 | 2.0 | 100 | 100[b] | 85 | 100[b] |
| 4.0 | 6.5 | 4.0 | 100 | 100[b] | 67 | 100[b] |
| 4.0 | 6.0 | 0.5 | 100 | 100[b] | 71 | 100[b] |
| 4.0 | 6.0 | 2.0 | 100 | 100 | 66 | 100[b] |
| 4.0 | 6.0 | 4.0 | 100 | 65 | 31 | 99 |
| 4.0 | 5.5 | 0.5 | 100 | 100[b] | 67 | 100[b] |
| 4.0 | 5.5 | 2.0 | 100 | 81 | 47 | 100[b] |
| 4.0 | 5.5 | 4.0 | 14 | 27 | 0[c] | 63 |
| 1.7 | 6.5 | 0.5 | 100 | 100[b] | 69 | 100[b] |
| 1.8 | 6.5 | 2.0 | 100 | 100[b] | 78 | 100[b] |
| 1.8 | 6.5 | 4.0 | 100 | 97 | 60 | 100[b] |
| 1.8 | 6.0 | 0.5 | 100 | 99 | 65 | 100[b] |
| 1.7 | 6.0 | 2.0 | .[a] | 93 | 58 | 100[b] |
| 1.7 | 6.0 | 4.0 | .[a] | 58 | 23 | 93 |
| 1.7 | 5.5 | 0.5 | 100 | 94 | 60 | 100[b] |
| 1.7 | 5.5 | 2.0 | 100 | 74 | 39 | 100[b] |
| 1.8 | 5.5 | 4.0 | 0 | 21 | 0[c] | 58 |
| 0.5 | 6.5 | 0.5 | 93 | 84 | 50 | 100[b] |
| 0.4 | 6.5 | 2.0 | 93 | 91 | 56 | 100[b] |
| 0.2 | 6.5 | 4.0 | 68 | 70 | 33 | 100[b] |
| 0.4 | 6.0 | 0.5 | 77 | 76 | 42 | 100[b] |
| 0.4 | 6.0 | 2.0 | 80 | 71 | 37 | 100[b] |
| 0.2 | 6.0 | 4.0 | 30 | 33 | 0[c] | 67 |
| 0.4 | 5.5 | 0.5 | 77 | 73 | 39 | 100[b] |
| 0.3 | 5.5 | 2.0 | 30 | 51 | 17 | 86 |
| 0.4 | 5.5 | 4.0 | 0 | 0 | 0[c] | 33 |

[a]This condition was followed only up to 14 d, maximum percent growth-positive sample may not be reached.
[b]Actual prediction greater than 100%
[c]Actual prediction lower than 0%

**Table 3—Observed and predicted maximum growth rate at 30 °C under different inoculum size, pH, and sodium-chloride concentration**

| Factors | | | Maximum growth rate ($A_{620}$/h) | | | |
|---|---|---|---|---|---|---|
| Log(spores /well) | pH | NaCl% | Observed | Predicted | Lower 95% of predicted | Upper 95% of predicted |
| 4.0 | 6.5 | 0.5 | 0.150 | 0.147 | 0.125 | 0.168 |
| 4.0 | 6.5 | 2.0 | 0.118 | 0.123 | 0.102 | 0.144 |
| 4.0 | 6.5 | 4.0 | 0.047 | 0.047 | 0.025 | 0.069 |
| 4.0 | 6.0 | 0.5 | 0.105 | 0.127 | 0.106 | 0.148 |
| 4.0 | 6.0 | 2.0 | 0.108 | 0.103 | 0.083 | 0.124 |
| 4.0 | 6.0 | 4.0 | 0.040 | 0.027 | 0.005 | 0.049 |
| 4.0 | 5.5 | 0.5 | 0.109 | 0.107 | 0.085 | 0.129 |
| 4.0 | 5.5 | 2.0 | 0.077 | 0.083 | 0.062 | 0.105 |
| 4.0 | 5.5 | 4.0 | .[a] | 0.007 | -0.017 | 0.031 |
| 1.7 | 6.5 | 0.5 | 0.142 | 0.147 | 0.125 | 0.168 |
| 1.8 | 6.5 | 2.0 | 0.121 | 0.123 | 0.102 | 0.144 |
| 1.8 | 6.5 | 4.0 | 0.045 | 0.047 | 0.025 | 0.069 |
| 1.8 | 6.0 | 0.5 | 0.139 | 0.127 | 0.106 | 0.148 |
| 1.7 | 6.0 | 2.0 | 0.100 | 0.103 | 0.083 | 0.124 |
| 1.7 | 6.0 | 4.0 | 0.031 | 0.027 | 0.005 | 0.049 |
| 1.7 | 5.5 | 0.5 | 0.109 | 0.107 | 0.085 | 0.129 |
| 1.7 | 5.5 | 2.0 | 0.082 | 0.083 | 0.062 | 0.105 |
| 1.8 | 5.5 | 4.0 | .[a] | 0.007 | -0.017 | 0.031 |
| 0.5 | 6.5 | 0.5 | 0.163 | 0.147 | 0.125 | 0.168 |
| 0.4 | 6.5 | 2.0 | 0.125 | 0.123 | 0.102 | 0.144 |
| 0.2 | 6.5 | 4.0 | 0.033 | 0.047 | 0.025 | 0.069 |
| 0.4 | 6.0 | 0.5 | .[a] | 0.127 | 0.106 | 0.148 |
| 0.4 | 6.0 | 2.0 | .[a] | 0.103 | 0.083 | 0.124 |
| 0.2 | 6.0 | 4.0 | .[a] | 0.027 | 0.005 | 0.049 |
| 0.4 | 5.5 | 0.5 | .[a] | 0.107 | 0.085 | 0.129 |
| 0.3 | 5.5 | 2.0 | .[a] | 0.083 | 0.062 | 0.105 |
| 0.4 | 5.5 | 4.0 | .[a] | 0.007 | -0.017 | 0.031 |

[a]Insufficient data to calculate maximum growth rate, generally less than 10% growth-positive sample was observed within 14 d.

nonlinear effect of sodium chloride apparent. This interaction between pH and sodium chloride was very significant. When this term was deleted from the model, the $R^2$ dropped dramatically. This finding supports the hurdle theory: When spores were exposed to acid stress alone, even at pH 5.5, the probability of germination and growth is not affected much. Similarly, 4% salt alone does not show much effect at high pH. When spores were exposed to moderate levels of both stresses, the effect was much greater than 1 stress factor alone at high levels.

The observed percent growth-positive samples, the predicted values, and the 95% prediction intervals are shown in Table 2. The prediction was considerably lower than thet observed value in 3 conditions: $10^4$ spores/sample-pH6-4%NaCl, $10^4$ spores/sample-pH5.5-2%NaCl, $10^2$ spores/sample-pH5.5-2%NaCl. All observations fell into the range of the 95% prediction interval. The predicted value was plotted against the observed value in Figure 5B. The model was not bounded, so predictions lower than 0 or higher than 100 were possible. When the prediction was presented in Table 3 and all the plots, negative values were forced to be 0, and values higher than 100% were forced to 100%.

## Maximum growth rate

The model for maximum growth rate had $R^2 = 0.95$, no collinearity, and was expressed as:

$$\text{Maximum growth rate} = -0.108 + 0.039 \times pH - 0.006 \times NaCl^2 \quad (7)$$

For maximum growth rate, a model without interaction terms was chosen because this model had only 2 variables as compared to 5 variables in the model with interaction terms, in which the $R^2$ was only 0.02 higher than this model. The model with interaction terms also showed collinearity while the chosen model did not.

The inoculum size variable was not significant in the growth-rate model. In Table 3, the predicted value at different inoculum sizes was the same for each pH and sodium-chloride combination. This was expected because the maximum growth rate was observed after the cell population had already passed the detection limit, which was the case for all inoculum sizes. Lower inoculum conditions took longer to get to this point, which was reflected by a longer time-to-detection, but once this threshold was reached, whether it was from 1 spore or from 10,000 spores made no difference. This agrees with the results from previous studies

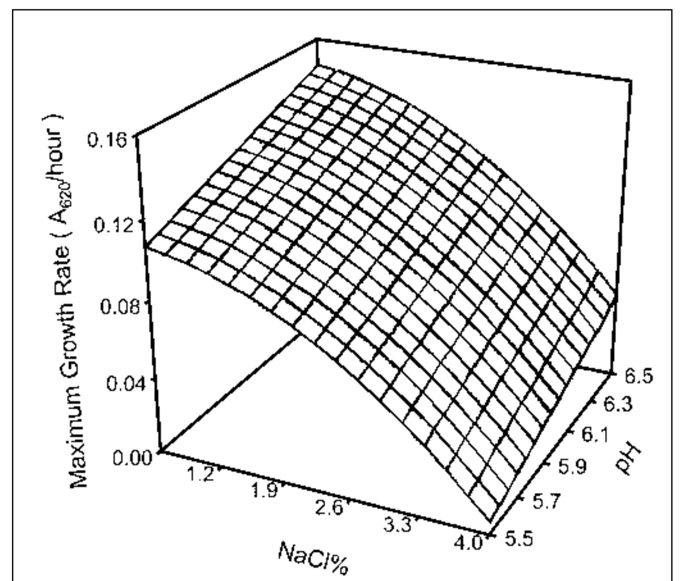


**Figure 4—Influence of pH and salt on the maximum growth rate. ( A620 / h).**

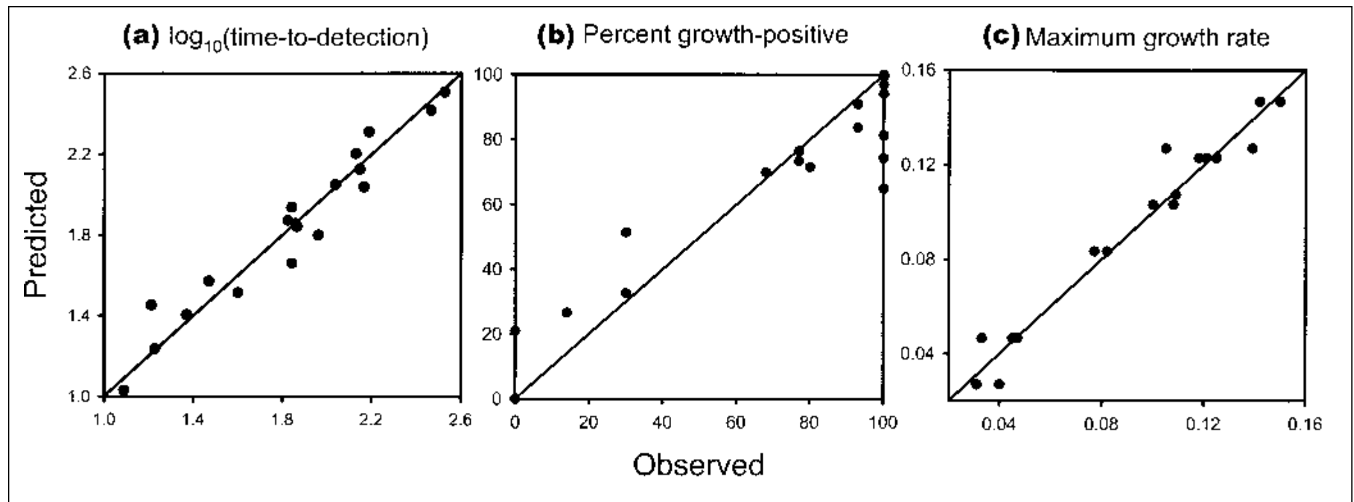


**Figure 5—Goodness-of-fit of the secondary models. (a) log, o(time-to-detection) (h). (b) Percent growth-positive. (C) Maximum growth rate (A620/h).**

in *Salmonella* (Mackey and Kerridge 1988) and *Listeria* (Duffy and others 1994).

The effect of pH on growth rate was positive and linear. Salt affected growth rate negatively and appeared only in a quadratic form, which indicated that only at high salt concentrations would the decline of maximum growth rate be very obvious. The equation is shown graphically in Figure 4. The optimum condition was at pH 6.5 and 0.5% sodium chloride, while at pH 5.5 and 4% sodium chloride, maximum growth rate declined to 0. Curvature of the surface at 0.5% to 1.5% salt was much smaller compared to that when salt increased from 2% to 4%.

A plot of the predicted value against the observed maximum growth rate (Figure 5C) demonstrates good agreement between predicted and observed values. All but 1 of the observed values fell into the 95% prediction interval, and the 1 that did not ($10^4$ spores/sample-pH6-0.5%NaCl) was only slightly lower than the lower boundary (Table 3). A bar plot of the prediction interval (not shown) showed that the 3 extreme combinations of salt and pH ($10^4$ spores/sample –pH 5.5-4%NaCl, 100 spores/sample -pH5.5-4%NaCl, 1 spore/sample -pH5.5-4%NaCl) had slightly larger prediction intervals than that of the rest, and their prediction intervals included 0. (These 3 conditions did not show growth within 14 d.) This indicates that even if these samples could have been observed for longer intervals, the expected growth rate would be extremely slow or nonexistent. For the other 5 conditions that did not show growth within 14 d (all at 1 spore/sample, pH **6-0**.5, 2% and 4% salt, and pH 5.**5-0**.5 and 2% salt), the predicted growth rate was significantly greater than 0. This indicates that 1 would expect these conditions to eventually show growth rates comparable to those that did grow within 14 d. This postulation was confirmed by the fact that 4 of the 5 conditions had predicted time-to-detection values longer than 15 d (Table 1).

## Conclusions

THIS STUDY DEMONSTRATES THAT INOCULUM SIZE IS A SIGNIFICANT variable for time-to-detection and percent growth-positive of *C. botulinum* spores. Low and high inoculum sizes behave differently with a possible differentiating point of inoculum around 1000 spores/mL in this system. Clearly, the assumption of statistical equivalence does not hold in this system, possibly due to quorum sensing. Further studies are needed to verify this hypothesis.

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*Authors are with the Dept. of Food Science, Cook College, the New Jersey Agricultural Experiment Station, Rutgers, The State University of New Jersey, 65 Dudley Road, New Brunswick, NJ 08901-8520. Direct correspondence to D.W. Schaffner (E-mail: schaffner@aesop.rutgers.edu).*