

The Effect of Temperature and Low pH on Survival of *Shigella flexneri* in Broth^{†‡}

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ABSTRACT

The survival characteristics of *Shigella flexneri* strain 5348 were determined in brain heart infusion broth as a function of low pH (2 to 5) and temperature (4 to 37°C). Stationary-phase cells were inoculated into sterile media to give initial populations of 6 to 7 log₁₀ CFU/ml. Bacterial populations were determined periodically by aerobic plate counts. Survivor curves were fitted from plate count data using a two-phase linear model to derive lag times and slopes of the curves, from which *D*-values and times to a 4-D (99.99%) inactivation (*T*_{4D}) were calculated. In general, survival increased as temperature decreased and as pH increased. Bacterial populations reached undetectable levels (<1.3 log₁₀ CFU/ml) at 37, 28, 19, 12, and 4°C in media adjusted to pH 4 after 5, 15, 23, 85, and 85 days, respectively, and in media adjusted to pH 3 after 1, 7, 9, 16, and 29 days, respectively. In media adjusted to pH 2, bacterial populations were stable for 2 to 12 h at temperatures of 19°C or lower and reached undetectable levels after 1 to 3 days, while at 28 and 37°C, the bacteria were undetectable after 8 and 2 h, respectively. In media adjusted to pH 5, bacterial levels decreased only 0.5 to 1.5 log₁₀ CFU/ml after 75 days at 4°C and decreased to undetectable levels after 135 days at 12°C, while growth occurred at higher temperatures. These results indicate that *S. flexneri* is acid resistant and that acidic foods may serve as vehicles for infection.

Shigella is recognized as a major cause of foodborne gastrointestinal illness (15). It is highly host adapted, infecting only humans and certain other primates. Numerous shigellosis outbreaks have been associated with foods that are consumed raw, as well as with multiple-ingredient foods, such as potato salad, and cooked foods that are not reheated before serving, such as cooked frozen shrimp. Based on the Centers for Disease Control and Prevention survey data, the incidence of infection with *Shigella* in the United States has been estimated at 448,000 cases per year, with 20% of the cases due to foodborne transmission of the pathogen (10). Epidemiological studies indicate that infected food handlers with poor personal hygiene and fecally contaminated water are frequently associated with shigellosis outbreaks. The potential for illness due to consumption of contaminated foods is relatively high, since the infective dose of *Shigella* may be as low as 10 to 500 organisms (5).

Shigella spp. may survive for extended periods of time in various foods, even in foods of low pH (12, 14, 16). To develop control strategies for the bacterium in food, it is necessary to evaluate its potential for growth or survival as a function of nutritional factors or environmental conditions. However, little, if any, quantitative data on growth or survival of *Shigella* in foods are available, since many of

the published reports do not provide sufficient experimental details (9). We previously determined the growth kinetics of *Shigella flexneri* strain 5348 in brain heart infusion (BHI) broth as a function of temperature, pH, and NaCl and NaNO₂ concentrations and developed models to predict the growth of the organism under aerobic (18) and anaerobic conditions (17). We also validated these models by comparing growth kinetics parameters observed in food with those predicted by the models (18). The objective of the present work was to systematically study the survival of *S. flexneri* strain 5348 in a microbiological medium under conditions of pH and temperature that do not support growth. The data obtained will be incorporated into the development of a nonthermal inactivation model that can be used to predict inactivation kinetics parameters (lag time, *D*-value, and time to 99.99% inactivation) for the bacterium as a function of temperature and pH, as well as additional factors important to its survival.

MATERIALS AND METHODS

Microorganism. *S. flexneri* strain 5348 (obtained from Dr. David W. Nisael, University of Texas Medical Branch, Galveston, Tex.) was used throughout the study. This strain has been studied extensively in our laboratory and was used for the development of growth models for *S. flexneri* (17, 18). The strain 5348 stock culture was stored at -70°C in cryovials (Nalgene) containing BHI (Difco Laboratories, Detroit, Mich.) broth supplemented with 10% (vol/vol) glycerol. To prepare the inoculum for each trial, a 0.1-ml portion of the thawed stock culture was added to 10 ml of BHI and incubated without shaking for 18 to 24 h at 37°C. The resulting stationary-phase cells were used to inoculate test media.

Medium. The BHI broth was adjusted to pH 2, 3, 4, or 5 with 6 N HCl, distributed in 100-ml portions into 250-ml Erlen-

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TABLE 1. Model to mathematically represent individual survivor curves^a

Two-Phase Linear Inactivation Model	
Phase 1: $N = N_0$	$t < t_L$
Phase 2: $N = N_0 + s(t - t_L)$	$t \geq t_L$
Where	
N = Log ₁₀ count of bacteria at time t	Log ₁₀ (CFU/ml)
N_0 = Log ₁₀ count of bacteria at time $t = 0$	Log ₁₀ (CFU/ml)
s = Slope of survivor curve	Log ₁₀ (CFU/ml)/h
t = Time	h
t_L = Duration of lag period prior to commencement of inactivation	h

^a D -values were calculated by taking the negative reciprocal of the "s" term, and "time to a 4-D (99.99%) inactivation" was calculated using the following equation: $T_{4D} = t_L + (4 \times D)$.

meyer flasks capped with foam plugs, and sterilized by autoclaving. Autoclaving of the media did not result in significant changes in pH (± 0.1 pH unit). A model 501 ionanalyzer (Orion Research, Boston, Mass.), equipped with a sealed combination electrode with silver/silver chloride reference, 0 to 14 pH (VWR Scientific, San Francisco, Calif.), was used for pH determination.

Culture conditions. Portions (1 ml) of inoculum were added to BHI media to give an initial population level of 10^6 to 10^7 CFU/ml. The media were incubated on a rotary shaker (150 revolutions/min) at the desired temperature (4, 12, 19, 28, or 37°C) until bacterial populations reached undetectable levels. A minimum of two trials (i.e., fresh cells) were conducted for each variable combination-treatment, with one to three replicates or flasks per treatment per trial.

Determination of microbial populations. Bacterial populations were determined at appropriate time intervals by surface plating cultures, or dilutions thereof prepared in 0.1% peptone water, onto duplicate plates of tryptose agar (Difco) using a spiral plater (model D; Spiral System Instruments, Bethesda, Md.). The plates were incubated for 48 h at 37°C, and the colonies were counted with the aid of a bacteria colony counter (model 500A; Spiral).

Curve fitting. Survivor curves were generated, using the method of Buchanan et al. (3), by fitting the population versus time data to the linear function that allows for the presence of a lag period before the beginning of the exponential decline in bacterial population density. The model for survival curves is shown in Table 1. The curves were fitted using ABACUS, a nonlinear regression program that employs a Gauss-Newton iterative procedure (4). A population decrease of greater than or equal to 3 log₁₀ CFU/ml was designated as acceptable to generate valid survivor curves.

RESULTS AND DISCUSSION

Survival characteristics of *S. flexneri* strain 5348 at 4 to 37°C were studied using stationary-phase cells inoculated into BHI media adjusted to pH 2 to 5. Survival of *S. flexneri* in BHI broth increased as the pH of the medium increased at all of the temperatures used in this study. A typical trial is shown in Figure 1 (19°C). During incubation at 19°C and pH 2, the bacteria declined to undetectable levels in 30 h but were still present at greater than or equal to 2 log₁₀ CFU/ml at pH 3 after 198 h (8 days) and at pH 4 after 558 h (23 days).

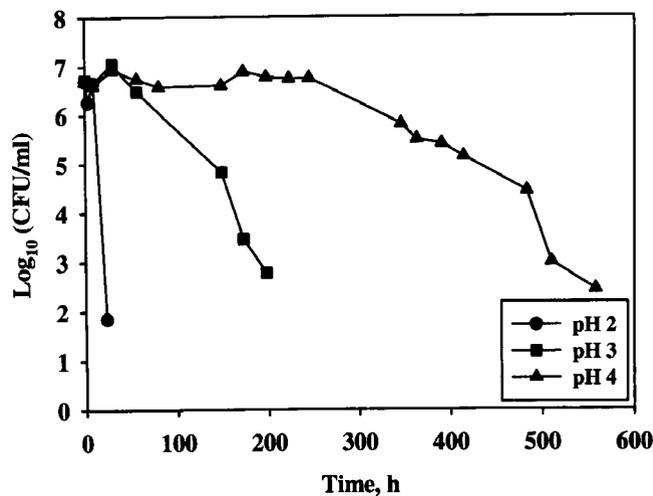


FIGURE 1. Survival of *S. flexneri* strain 5348 at 19°C in BHI adjusted to pH 2, 3, and 4.

In a trial conducted at 12°C, the population level decreased by approximately 5 log₁₀ CFU/ml after 317 h (about 13 days) at pH 3, while at pH 5, the population level decreased by approximately 1.1 log₁₀ CFU/ml after 1,401 h (about 58 days). In other trials, at pH 5, growth occurred at 19, 28, and 37°C, while population levels were stable for more than 1,000 h at 4 and 12°C. In BHI adjusted to pH less than or equal to 5, survival of *S. flexneri* decreased with increasing temperature, yet even at 37°C, the pathogen survived for 1 to 2 h at pH 2 and for at least 10 h at pH 3.

The two-phase linear model (3) used to fit the bacterial population data gave a convenient mathematical representation of *S. flexneri* inactivation under the nongrowth conditions studied. Curves obtained for all variable combinations consisted of a lag period and a declining curve. Examples of experimental data and the generated survivor curves are shown in Figure 2 (pH 3, 37°C) and Figure 3 (pH 4, 28°C).

A summary of the inactivation parameters derived from the individual survivor curves is presented in Table 2. The data shown are mean values obtained from at least two trials containing two to eight replicate survivor curves

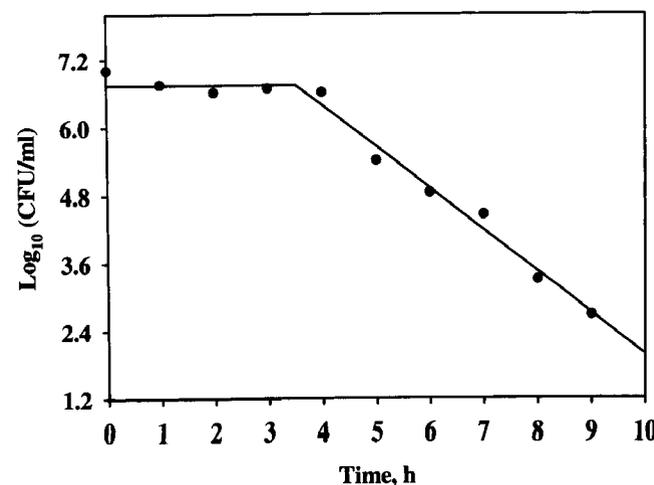


FIGURE 2. Survival curve obtained with a two-phase model for *S. flexneri* strain 5348 at 37°C in BHI adjusted to pH 3.

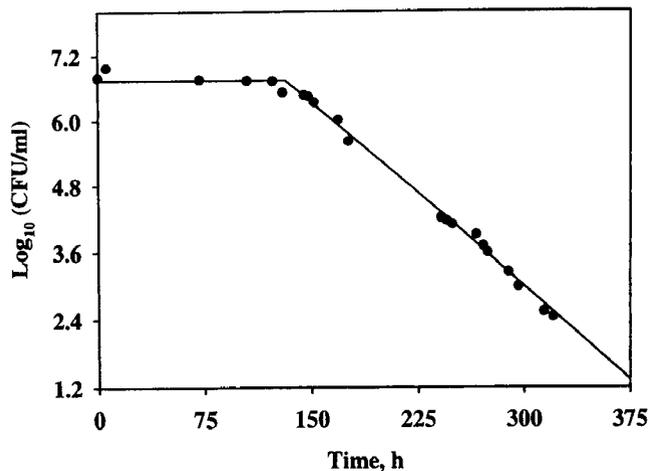


FIGURE 3. Survival curve obtained with a two-phase model for *S. flexneri* strain 5348 at 28°C in BHI adjusted to pH 4.

for each variable combination of pH and temperature. The *D*-values ranged from 0.3 h at pH 2 and 37°C to 470.7 h (about 20 days) at pH 5 and 12°C. The time to a 4-D (99.99%) inactivation (*T*_{4D}) value is an informative way to compare the response of *S. flexneri* under various combinations of temperature and pH, since it includes both the lag time and the *D*-value (Table 1). The *T*_{4D} values increased with increasing pH and decreasing temperature (Table 2). However, one exception to this trend was observed

at pH 2, 4°C, the lowest pH and the lowest temperature tested. In media adjusted to pH 2, the *T*_{4D} value at 4°C (15.4 h) was lower than that at 12°C (63.1 h). We have no explanation for this discrepancy at this time.

In previous studies, the survival of 21 strains each of *S. flexneri* and *Shigella sonnei* at room temperature in nutrient broth adjusted to pH 3 to 4.5 was studied by Fehlhäber (6). Fifteen of the *S. flexneri* strains survived at pH 3 after 30 min, but none survived after 4 h. All strains survived for 4 h, and two strains survived for 2 days at pH 4.5, but none survived after 4 days. Similar results were obtained for *S. sonnei*. These results suggest that *S. flexneri* and *S. sonnei* have little resistance to low pH. However, Fehlhäber (6) employed cells that were in the midexponential phase of growth (i.e., cultures were grown for 5 h at 37°C in nutrient broth). It is generally recognized that bacterial resistance to acids is greater for cells in the stationary phase, as used in the present study, than for cells in the exponential phase of growth (2). In this regard, Gordon and Small (7) determined that acid resistance in *S. flexneri* was highly dependent on growth phase and that acid resistance is not fully expressed until the stationary phase. Also, these workers reported that 9 of 12 isolates of *Shigella* spp. tested were acid resistant, characterized by the ability of 10% or more of the inoculum to survive exposure to pH 2.5 for 2 h (7).

S. flexneri and *S. sonnei* can survive for long periods

TABLE 2. Survival of *S. flexneri* at 4 to 37°C in BHI media adjusted to pH 2 to 5^a

Temp.	pH	<i>n</i> ^b	<i>N</i> ₀ (log ₁₀ [CFU/ml])	<i>t</i> _L (h)	<i>s</i> (log ₁₀ [CFU/ml]/h)	<i>D</i> -value (h)	<i>T</i> _{4D} (h) ^c
4°C	2	4	6.87	2.6	-0.3254	3.2	15.4 (2.8)
	3	4	6.77	234.3	-0.0149	74.5	532.4 (50.1)
	4	3	6.80	918.4	-0.0051	208.6	1752.8 (67.0)
	5	4	7.09	— ^d			
12°C	2	2	7.22	11.9	-0.0781	12.8	63.1 (0.6)
	3	8	7.06	174.1	-0.0163	62.0	421.6 (116.1)
	4	3	6.34	455.5	-0.0036	276.5	1562.0 (60.0)
	5	3	6.21	1449.6	-0.0022	470.7	3332.3 (338.9)
19°C	2	3	7.26	1.7	-0.2256	5.3	23.0 (12.8)
	3	3	6.70	76.1	-0.0342	30.2	196.9 (1.2)
	4	7	6.95	224.0	-0.0264	40.8	387.4 (75.3)
	5	6	7.07	— ^e			
28°C	2	2	6.82	0.5	-0.8923	1.1	5.0 (0.3)
	3	4	6.88	13.8	-0.0518	20.2	94.7 (18.3)
	4	7	6.87	114.7	-0.0248	41.4	280.2 (39.9)
	5	2	6.66	— ^f			
37°C	2	2	6.98	0.2	-4.0000	0.3	1.2 (0.0)
	3	5	6.77	3.9	-0.6504	1.6	10.2 (2.6)
	4	3	7.30	21.5	-0.0976	10.5	63.6 (3.8)
	5	3	7.06	— ^g			

^a Abbreviations are defined in Table 1.

^b Number of replicate cultures.

^c Values in parentheses are standard deviations.

^d Population decreased 2 log₁₀ CFU/ml in 85 days.

^e The bacteria grew to 9 log₁₀ CFU/ml in 79 h.

^f The bacteria grew to 9 log₁₀ CFU/ml in 28 h.

^g The bacteria grew to 9 log₁₀ CFU/ml in 21 h.

of time, even in acidic foods, when stationary-phase cells are artificially inoculated into the foods (14, 16). Using stationary-phase cells, Sheth et al. (13) studied the survival of *S. sonnei* ATCC 25931 in low pH beverages. Although the bacterium was not detectable after 48 h of incubation at 36°C in cola, beer, sour mix, and diet cola (pH 2.4, 3.8, 3.1, and 3.2, respectively), populations declined only 1.5 to 2 log₁₀ CFU/ml after 24 h from an initial population of 6 to 7 log₁₀ CFU/ml (13).

Little information is available on the incidence of *Shigella* spp. in foods. Also, in foodborne shigellosis outbreaks, *Shigella* is rarely, if ever, isolated from epidemiologically implicated food. This may be due to the failure of the organism to survive in the food until it is analyzed or to inadequate isolation or identification methodologies. Gorman (8) reported on a shigellosis outbreak due to *S. sonnei* at a school involving very young children. The only factor that could be linked to the outbreak was consumption of stewed apples, which were stored overnight at room temperature. Subsequent experiments established that *S. sonnei* inoculated into stewed apples (pH 3.2) survived for 1 week at 20°C but was undetectable within 24 h at 37°C.

Although few systematic studies have been reported, it is recognized that temperature is a major factor in the survival of *Shigella* spp. at low pH. *S. flexneri* and *Shigella dysenteriae* were inactivated within 24 h at 37°C in nutrient broth adjusted to pH 3.5, while *S. dysenteriae* survived for 170 h at -4°C in orange juice at pH 3.5 (1). Mossell and de Bruin (11) reported the T_{4D} for a number of *Enterobacteriaceae* in tomato juice (pH 3.9 to 4.1), orange juice (pH 3.1 to 3.5), apple juice (pH 3.0 to 3.1), and lemon juice (pH 2.1 to 2.6). At 24°C, the T_{4D} for *S. sonnei* in tomato, orange, apple, and lemon juice was 7 to 10, 4 to 7, 2 to 10, and 1 day, respectively, while at 5°C, the T_{4D} was 38 to 49, 10 to 35, 5 to 35, and 1 day, respectively.

Comparison of the survival of *Shigella* in foods with that in microbiological media requires further study. However, the results of the present study indicate that *S. flexneri* has acid resistance and suggest that foods of pH 5 or lower stored at or below room temperature may permit survival of the organism over long periods of time in sufficient numbers to cause illness. The data obtained in this study will be incorporated into the development of a nonthermal inactivation model that can be used to predict inactivation kinetics parameters such as lag time, *D*-value, T_{4D} for *S. flexneri* as a function of temperature, pH, and additional factors important to its survival in foods.

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