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Changes in Thermal Resistance of Three *Salmonella* Serovars in Response to Osmotic Shock and Adaptation at Water Activities Reduced by Different Humectants

MARILIA PEÑA-MELÉNDEZ, JENNIFER J. PERRY, AND AHMED E. YOUSEF[1]*

[1]*Department of Food Science and Technology, The Ohio State University, Parker Food Science Building, 2015 Fyffe Road, Columbus, Ohio 43210, USA*

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ABSTRACT

The purpose of this study was to investigate the effect of osmotic shock and adaptation at low water activity (a_w) and the type of humectant used to lower the a_w, on heat resistance of three *Salmonella enterica* serovars (Saintpaul 02-109, Tennessee 2053H, and Elmsbuettel 1236H). The serovars were grown (adapted) or transferred (osmotic shocked) in low-a_w broths and subjected to heat treatment at 55°C for up to 45 min; samples were removed at 5-min intervals and immediately placed in an ice-water bath until plating. The a_w of tryptic soy broth (TSB) was lowered by the addition of 20% (wt/wt) glycerol (a_w 0.94), 4% (wt/wt) sodium chloride (NaCl; a_w 0.97), or 35% sucrose (wt/wt) (a_w 0.95). The type of humectant and cell adaptation significantly affected the $D_{55°C}$-value. Cells merely suspended in 20% glycerol broth (i.e., nonadapted) prior to heat treatment showed a larger $D_{55°C}$-value (3.0 to 3.9 min), when compared with that of cells adapted in the same medium ($D_{55°C}$-values of 0.86 to 0.98 min). Interestingly, cells adapted to TSB plus glycerol were not more resistant to heat than were the controls. NaCl and sucrose showed a net protective effect for all serovars under both the adapted and nonadapted conditions, with sucrose providing the most protection. Highest $D_{55°C}$-values were obtained for cultures adapted to TSB plus sucrose. Based on these results, the effect of reduced a_w on thermal resistance of *Salmonella* serovars varies greatly, depending on medium constituents and adaptation of the pathogen in these media.

Foods may contain different added components that bind water and lower the water activity (a_w) of the product. Lowering a_w has been used as a protective measure against pathogens and spoilage microbiota in food *(11)*. Recently, the validity of this approach has been questioned as disease outbreaks linked to low-a_w foods are on the rise. Disease outbreaks linked to the consumption of peanut butter, pistachios, and cereals have occurred in the past few years *(4–6)*.

Low-moisture foods can be defined as foods with an a_w of 0.85 or below *(19)*. Most bacteria cannot grow at $a_w \leq 0.85$, although some fungi may be able to overcome this limitation *(11)*. Historically, foods with low a_w were assumed to be safe because the prevailing environment would not support growth of microorganisms, especially foodborne pathogens. This assumption of safety is now questionable because strains of some pathogens (e.g., *Salmonella* serovars) have been demonstrated to survive well under low-moisture and dry conditions. Although the infectious dose of a pathogen is serotype and host dependent, relatively small populations of viable *Salmonella* in low-moisture foods are sufficient to cause disease *(2, 12)*. Results derived from volunteer studies suggest that the infectious dose of several *Salmonella* serotypes is 10^5 to 10^{10} organisms *(13)*. However, Kapperud et al. *(12)* reported that the infectious dose decreases (to 2 to 3 CFU/g) when *Salmonella* is present in low-moisture foods, in this case chocolate. Examples of other low-moisture foods that may harbor dryness-resistant pathogens include nuts (peanuts, almonds, pistachios) and cereals.

Salmonella enterica is a small gram-negative rod and facultative anaerobe *(11, 18)*. Although the main reservoir of salmonellae is the intestinal tract of animals (particularly poultry) and humans, this bacterium can also be transmitted by soil, water, insects, and anything that comes in contact with a contaminated source *(11, 18)*. The minimum a_w for growth of *Salmonella* spp. has been determined to be 0.94, but cells can remain dormant at a_w below this value *(11, 14)*.

In addition to tolerance of low a_w, some *Salmonella* serovars are relatively resistant to heat. The resistance or sensitivity of a particular *Salmonella* serovar to heat may depend on the conditions to which the microorganism has been exposed prior to heat treatment, as well as on the food matrix or medium in which it is present at the time of the treatment *(1)*. Heat resistance is also dependent on the serovar; some serovars (e.g., *Salmonella* Senftenberg 775W) are more resistant to heat than others *(16)*. A cross-protection effect may occur when some *Salmonella* strains are successively exposed to various stress conditions *(20)*. Goepfert et al. *(8)* evaluated the effect of lowering

* Author for correspondence. Tel: 614-292-7814; Fax: 614-292-0218; E-mail: yousef.1@osu.edu.

a_w by the addition of different humectants, to 0.01 M phosphate buffer, on heat resistance of eight *Salmonella* strains. According to these researchers, sucrose provided more protection to cells than the other solutes tested (fructose, glycerol, and sorbitol), and heat resistance was not only dependent on a_w but also on the type of solute used and pathogen strain. Another research team *(15)* investigated whether habituation of *Salmonella* at low a_w prior to a heat treatment increased heat tolerance. The solutes used to lower the a_w included glycerol, sodium chloride (NaCl), and a mixture of glucose and fructose. Their findings show that habituation at a_w 0.95 increased heat tolerance at a treatment temperature of 54°C with the tested solutes. In addition to habituation at low a_w, subjecting *Salmonella* spp. to sublethal temperatures also confers heat tolerance. A study by Bunning et al. *(3)* demonstrated that heat shocking *S. enterica* serovar Typhimurium at various temperatures (42, 48, or 52°C) increased the pathogen's heat tolerance.

Despite these published reports, it is unclear whether resistance of *Salmonella* to heat at low a_w is caused by the cells' immediate osmotic shock or that resistance develops gradually as cells adapt to conditions of low-moisture availability. A detailed investigation of contributing food ingredients that lower a_w of the medium (i.e., humectants) to the heat resistance of *Salmonella* is necessary, and these considerations were addressed in this study. Additionally, the contribution of problematic *Salmonella* serovars to thermal resistance at reduced a_w was investigated.

MATERIALS AND METHODS

Preparation of low-a_w broths. Tryptic soy broth (TSB; Difco, BD, Sparks, MD) was used to grow and heat treat *Salmonella* serovars. The medium was prepared following manufacturer's instructions. Three different humectants were then added at different concentrations to lower the a_w of the medium (TSB, a_w 0.99); the modified media contained 20% (wt/wt) glycerol (a_w 0.94), 4% (wt/wt) NaCl (a_w 0.97), or 35% (wt/wt) sucrose (a_w 0.95). The a_w was measured using a meter (Aqualab, Decagon Devices, Inc., Pullman, WA). Levels of humectants were chosen after preliminary experimentation; these represent the highest concentrations that could be used without impairing growth of the tested *Salmonella* serovars.

Culture preparation. *Salmonella enterica* serovars tested in this study were Saintpaul 02-109, Tennessee 2053H, and Elmsbuettel 1236H. These are outbreak strains provided by the U.S. Food and Drug Administration and were originally isolated from cantaloupe rind, thyme, and peanut butter, respectively. Stock cultures were grown in 9 ml of TSB and incubated at 37°C for 24 h prior to use in further experiments (stock culture).

Osmotic shock. These experiments were designed to assess the effect of culture transfer to low-a_w media (i.e., without adaptation) and individual humectants on thermal resistance of *Salmonella* serovars. Cultures of each serovar were grown in TSB incubated for 24 h at 37°C. Sterile, thin-walled 0.2-ml PCR tubes (Midwest Scientific, St. Louis, MO) were used to hold cell suspensions during heat treatment. Aliquots (100 μl) of low-a_w broth (for shocked cells experiments) or unaltered TSB (for unshocked controls) were dispensed into PCR tubes. Following broth addition, 20 μl of the corresponding *Salmonella* cultures were added to their respective tubes, and cell suspensions were tested for heat resistance immediately.

Adaptation to reduced a_w. To determine the effect of adaptation on heat tolerance, 10 μl of stock cultures of each serovar was placed in 9 ml of broths with different a_w values; inoculated broths were incubated at 37°C for 24 h. The resulting cultures were transferred to the respective fresh a_w modified broths, followed by incubation. Culture transfer and incubation was repeated to achieve four 24-h cycles of incubation (three in a_w-modified media). Cells subjected to these transfers will be referred to as ''adapted.'' Growth of serovars in the a_w-modified media is displayed in Figure. 1. The adapted cells were transferred to fresh a_w-modified media and tested immediately for thermal resistance.

Heat treatment. A circulating water bath (Precision 2864, Thermo Fisher Scientific Inc., Marietta, OH) was set to a temperature of 55°C; PCR tubes were submerged in water and removed from the water bath at 5-min intervals over the course of 45 min. Individual tubes were used for different heating time intervals. Immediately after heating, tubes were placed in an ice-water bath until analysis. Determination of survivors was accomplished by dilution of treated cell suspensions in 0.1% peptone water and spread plating onto tryptic soy agar (Difco; BD). Plates were incubated for 24 h at 37°C.

***D*-value calculation.** *D*-value was calculated as (−1/slope) of survivor plots and can be expressed by the following formula

$$D_T = \frac{-t}{\log N - \log N_0} \tag{1}$$

where N_0 is the initial population count at time 0, N is the population count after heating at a steady temperature (T) for a specific period of time (t), and D_T is the decimal reduction time at the specific T *(21)*. When the survival plot is curve linear, the *D*-value was calculated from the linear portion on the plot.

Statistical analysis. Data analysis of triplicate experiments was performed using the general linear models procedure of SAS, version 9.2 (SAS Institute Inc., Cary, NC). The following model was used with the variables serovar, physiological state (shocked or adapted), and humectants classified as class variables:

$$\begin{aligned} D\text{-value} = {} & \mu + \text{serovar} + \text{physiological state} + \text{humectant} \\ & + (\text{serovar} \times \text{physiological state}) \\ & + (\text{serovar} \times \text{humectant}) \\ & + (\text{humectant} \times \text{physiological state}) \\ & + (\text{serovar} \times \text{humectant} \times \text{physiological state}) \\ & + \text{error} \end{aligned} \tag{2}$$

Significant differences were determined by using difference of least-square means comparisons. A probability value of <0.05 was considered significant.

RESULTS

Adaptation to reduced a_w. Preliminary experimentation showed that the selected *Salmonella* serovars can tolerate up to 20% glycerol, 4% NaCl, and 35% sucrose, without impairing culture growth considerably (data not shown). Although maximum concentrations of glycerol and sucrose with minimal effect on growth were very different, these decreased medium a_w to only slightly different values (0.94 and 0.95, respectively). Compared with other

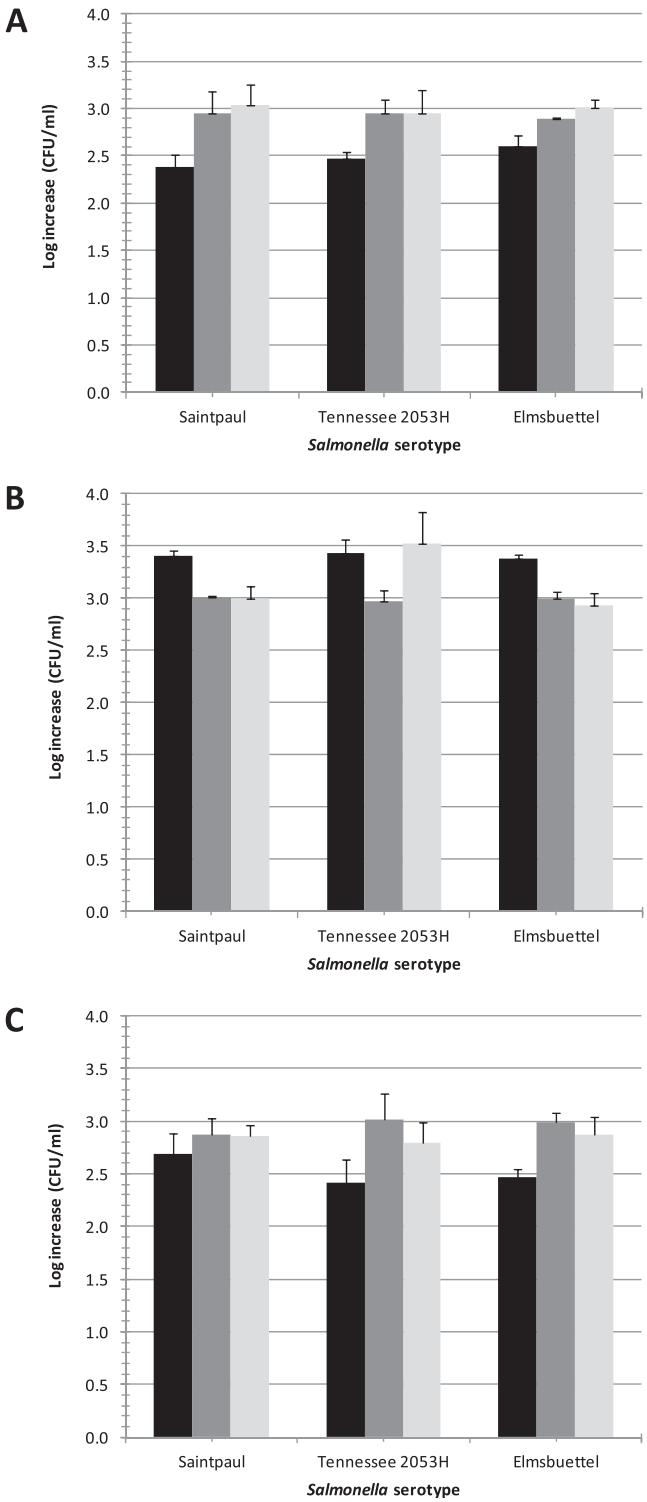

FIGURE 1. *Growth of* Salmonella enterica *serovars Saintpaul 02-109, Tennessee 2053H, and Elmsbuettel 1236H during three transfers in low-a_w broths containing (A) 20% glycerol, (B) 4% NaCl, and (C) 35% sucrose. Log increase represents the change in population during the 24-hour incubation period. Error bars denote standard deviations,* n = *3. Symbols are first transfer,* ■*; second transfer,* ■*; and third transfer,* ■.

humectants, NaCl at a much smaller concentration lowered a_w to only 0.97 but affected the growth in a manner similar to that seen with other two humectants. When *Salmonella* serovars underwent three growth cycles in these low-a_w media, maximum populations generally increased when glycerol and sucrose served as humectants (Fig. 1); this shows that *Salmonella* adapted successfully to low-a_w conditions achieved by these humectants. NaCl, on the contrary, progressively decreased maximum achievable populations (Fig. 1), indicating a general lack of *Salmonella* adaptation to this humectant; an exception was noted during the third transfer of *Salmonella enterica* serovar Tennessee 2053H in this medium.

Increase in thermal resistance after osmotic shock. Survival plots constructed were generally linear, allowing computation of *D*-values. On the occasions when the plot was nonlinear, the *D*-value was calculated using the initial linear portion of the curve. *Salmonella* cells shocked in low-a_w broths became resistant to heat treatment as indicated by significant increases in $D_{55^\circ C}$-values (Tables 1 and 2). The increase in *D*-value varied, depending on the humectant but not on the serovar. Survival of *Salmonella enterica* serovar Tennessee 2053H during heat treatment in the presence of the three humectants is displayed in Figure 2, and the survival of other serovars, which was similar, is not shown.

Changes in heat resistance in response to adaptation. Resistance of *Salmonella* to heat, measured as $D_{55^\circ C}$-values, varied considerably, depending on the suspending medium and physiological state of the cells during the heat treatment, but the difference among serovars was minimal (Tables 1 and 2). When *Salmonella* serovars were adapted in low-a_w broths, resistance to heat depended greatly on the humectant used (Table 2). Although adaptation in sucrose-containing broths and incubation in NaCl-containing medium increased $D_{55^\circ C}$-values significantly ($P < 0.0001$), adaptation in broth containing glycerol did not change heat resistance of *Salmonella* or even decrease it slightly. Compared with NaCl, sucrose resulted in a greater degree of heat resistance in adapted *Salmonella*. Differences among serovars in heat resistance were not significant ($P = 0.94$), regardless of the physiological state or the humectant used (data not shown).

DISCUSSION

The cross-protective effect that occurs when a microorganism is sequentially exposed to various stress conditions has been studied extensively *(11, 20).* The results of this study suggest that the type of humectant (solute) and physiological state of the *Salmonella* serovars tested influence their heat resistance. A study by Corry *(7)* reported an increase in the heat resistance at 65°C of three *Salmonella* serovars (Senftenberg 775W, Typhimurium 7M 4987, and Typhimurium 39H) as the concentrations of sugar or polyols increased. The researcher found no linear relationship between a_w and heat resistance *(7),* which is consistent with our results, as the lowest $D_{55^\circ C}$-value was observed when the serovars were adapted in 20% glycerol (a_w of 0.94, lowest a_w tested). The type of humectant used to lower the a_w of a food or medium can significantly affect the heat resistance of a microorganism, e.g., an ionic humectant

TABLE 1. $D_{55°C}$-*value of* Salmonella *serovars Saintpaul 02-109, Tennessee 2053H, and Elmsbuettel 1236H measured during heat treatment at 55°C for up to 45 min in low–water activity (a_w) broths*

	$D_{55°C}$-value (min)[a]								
	20% glycerol			4% NaCl			35% sucrose		
Serovar	TSB[b]	Adapted	Shocked	TSB	Adapted	Shocked	TSB	Adapted	Shocked
Saintpaul 02-109	1.0 ± 0.04	0.86 ± 0.07	3.0 ± 0.39	1.1 ± 0.10	5.5 ± 1.15	2.6 ± 0.21	1.0 ± 0.23	8.0 ± 4.26	3.9 ± 1.14
Tennessee 2053H	1.1 ± 0.13	0.86 ± 0.22	3.9 ± 1.92	1.2 ± 0.15	2.3 ± 0.45	4.0 ± 0.76	1.6 ± 0.31	6.5 ± 2.37	4.0 ± 2.1
Elmsbuettel 1236H	1.4 ± 0.22	0.98 ± 0.57	3.8 ± 0.90	1.4 ± 0.25	4.1 ± 0.46	4.0 ± 0.06	1.2 ± 0.15	7.6 ± 2.50	5.4 ± 0.98

[a] Average ± standard deviation ($n = 3$).
[b] TSB, tryptic soy broth, without a_w modification; $a_w = 0.99$.

may reduce the heat resistance when added in low concentrations as opposed to nonionic humectants that may have different effects, depending on their molecular weight *(17)*. In the current study, the ionic humectant added (NaCl) provided a protective effect at two physiological states (adapted and shocked; Table 2 and Fig. 2B). The nonionic humectants (sucrose and glycerol) differed in the protection they provided to the serovars (Table 1 and Fig. 2A and 2C). Glycerol did not change significantly the heat resistance of all three serovars studied when they were adapted in the low-a_w broth, but the osmotic-shocked cultures showed an increase in resistance, possibly because glycerol protects the cell when it is not incorporated intracellularly. Cells adapted in TSB with 20% glycerol seemed to become most sensitive to the heat treatment, resulting in the lowest $D_{55°C}$-values observed. Sucrose showed a more protective effect than glycerol or NaCl for the three serovars under both adaptation and osmotic shock conditions, with the highest $D_{55°C}$-values observed for cultures adapted in TSB plus sucrose. Overall, adaptation in sucrose offered the most heat protection of any condition tested (Tables 1 and 2).

It has been suggested that a microorganism may have greater heat resistance under low-a_w conditions because the reduced amount of water present decreases the water molecule vibrations as the cells are heated *(17)*. When there is a high water content in the medium, water molecule vibrations increase during heating, and this may cause protein denaturation; i.e., disruption of disulfide and hydrogen bonds.

During adaptation of the cells in the low-a_w broth, it is possible that solutes were taken up by cells and differences in the cellular utilization of these solutes resulted in the observed variability in $D_{55°C}$-value after the heat challenge. Microbial cells posses an internal osmotic pressure higher than that of the environment, causing an outward turgor pressure exerted on the cell wall *(10)*. Microorganisms lose turgor when they are placed in a low a_w environment because the water in the cytoplasm of the cell migrates to the extracellular environment; as a consequence, cells are unable to replicate *(17)*. To reestablish the turgor, microorganisms will accumulate solutes in the cytoplasm, and this may provide an adaptive response to the osmotic stress to which they are subjected *(10)*. A particular class of solutes, called compatible solutes, does not affect the metabolic and reproductive functions of the cell even at high concentrations *(10)*. These include glycerol and sucrose. Glycerol is known to easily permeate the cell and cause little or no plasmolysis. Sucrose and NaCl, on the other hand, do not permeate as easily and can cause a more severe plasmolysis; osmoregulatory responses are different, depending on how permeable are the solutes *(9, 17)*.

TABLE 2. *Average of* $D_{55°C}$-*value for the three* Salmonella *serovars, at different physiological states, during heat treatment in different low-a_w broths*

	$D_{55°C}$-value (min)[a]		
Physiological state	20% glycerol	4% NaCl	35% sucrose
TSB[b]	1.2 ± 0.2 A	1.2 ± 0.15 A	1.3 ± 0.31 A
Adapted	0.9 ± 0.07 A	4.0 ± 1.60 B	7.4 ± 0.78 D
Shocked	3.6 ± 0.49 BC	3.5 ± 0.8 C	4.4 ± 0.84 BC

[a] Average ± standard deviation. Letters represent significant differences among *D*-values across rows and columns ($P < 0.05$).
[b] TSB, tryptic soy broth, without a_w modification; $a_w = 0.99$.

Although not investigated in this study, researchers have documented the expression of different genes when microbial cells are placed under stress conditions. This stress response increases the cell's ability to survive the applied stress by allowing it to more rapidly respond to damage induced by the external conditions. An example is the gene *rpoS,* which encodes for the stationary-phase sigma factor known as RpoS *(14)*. RpoS regulates the expression of genes that play an important role in the survival of microorganisms when they are exposed to stress conditions like low a_w and high temperature *(17)*.

As observed in this study, it is evident that type of solute present in the heating medium greatly influences the heat resistance of bacterial cells, but it is unclear exactly what is happening in the cell during exposure to the different solutes. More research is needed to understand how the cell is behaving in the low-a_w broth and how it is responding to the heat challenge at the molecular level. Despite the fact that low a_w plays an important role in limiting the growth of microorganisms in food, some microorganisms are able to survive these conditions, and depending on the solutes present, they may be able to resist the lethal effects of subsequent treatments, e.g., heat, more effectively.

In conclusion, adaptation of *Salmonella* to media with low a_w is expected to increase serovars resistance to heat.

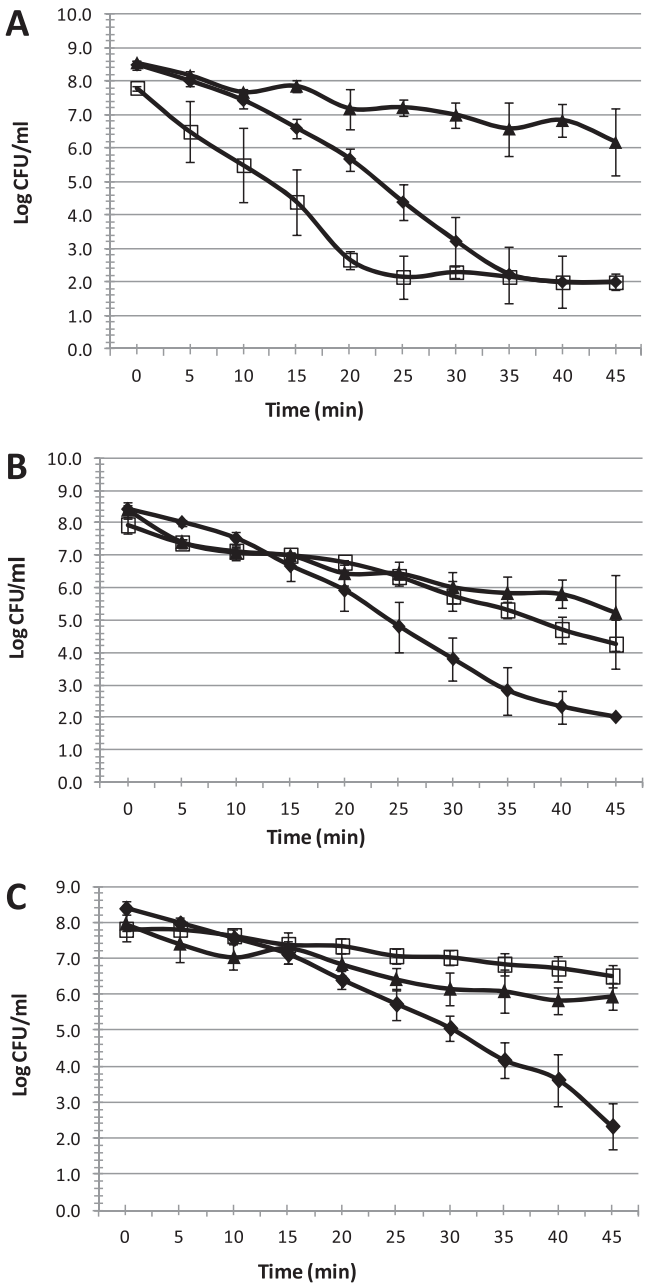

FIGURE 2. *Inactivation of* Salmonella *Tennessee 2053H, adapted and nonadapted to (A) 20% glycerol, (B) 4% NaCl, and (C) 35% sucrose, during heat treatment at 55°C. Error bars indicate standard deviations,* n = 3. *Symbols are TSB,* ◆*; adapted,* □*; osmotic shocked,* ▲.

This hypothesis was confirmed when salt or sugar was used as humectant. On the contrary, when glycerol was used to lower a_w, adaption of serovars to this medium did not increase their heat resistance. Interestingly, when the serovars were merely shocked in this glycerol-containing medium, the short treatment increased bacterial resistance to heat.

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