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The Effect of Composition of Parenteral Solution on the Thermal Resistance of *Bacillus stearothermophilus* and *Bacillus subtilis* Spores

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Abstract

Large-volume parenteral solutions were submitted to heat treatments after being inoculated with *Bacillus stearothermophilus* ATCC 7953 (T_r = 121°C) and *Bacillus subtilis* ATCC 9372 (T_r = 104.5°C) spores. The average decimal reduction time for *B. stearothermophilus* ranged from a $D_{121°C}$ value of 1.31 to 3.14 min, in glucophysiologic and Ringer's solutions respectively. For *B. subtilis*, $D_{104.5°C}$ value increased from 0.69 to 1.37 min, in Ringer's (pH=5.91) and 50% glucose (pH 3.05) solutions respectively. The *z* value ranged from 7.95°C (20% mannitol solution) to 13.14°C (50% glucose solution), corresponding to an activation energy (*Ea*) of 81.48 and 49.30 kcal/mol, respectively.

Index Entries: Parenteral solutions; moist heat sterilization; *Bacillus stearothermophilus*; *Bacillus subtilis*; decimal reduction time.

Introduction

Parenteral solutions, a group of single-use or hypodermic infusions, are destined to replace water, electrolytes, or energy losses in patients of all ages. They are used as vehicles for the administration of auxiliary medications and do not include in their formulations products of biologic origin.

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In accordance with Directive 500/1997 issued by the Brazilian Health Vigilance Office (ANVISA) *(1)* of the Ministry of Health, "large volume parenteral solutions (LVPS) are water based, sterile, apyrogenic solutions, packed in volumes greater than 100 mL in single vessels, which are terminally sterilized by moist heat. Included in this definition are solutions for iv administration, for irrigation, for peritoneal dialysis solutions and glycerinates."

The LVPS are the most widely applied medications in health care units with about 150 million units used in Brazil annually. They have required special attention by the Ministry of Health authorities in view of the occurrence of outbreaks of hospital infections. In 1996, 60 patients who were undergoing hemodialyses in a clinic died in Caruaru (Pernambuco) following the application of a contaminated dialysis solution, and 35 newborn babies died in a 26 bed nursery of a 200 bed hospital in Roraima (Acre) *(2)*. At that time, with the collaboration of the Hospital Infection Program of CDC *(3)* in the investigation of the outbreaks, the water for injection (WFI) (distilled water) used to dilute the iv medications was found to have high levels of endotoxins. The contaminated distilled water was distributed to other locations in Brazil, where similar outbreaks occurred. Recently, in 2001, six people died in São Paulo from intrinsic contamination of the iv glucophysiologic solution with three kinds of bacteria, including staphylococci, which have not yet been associated with the deaths. Other reports cited LVPS as being responsible for the spreading of emerging infections in hospital care units.

Contamination of LVPS has been shown to occur mainly during manufacture, followed by transportation, storage, and handling in the iv administration of these solutions. Sterilizing operations, as well as environmental control, personal hygiene, and sterile packages, determine the quality of the product before the terminal autoclaving process, ensuring a final product with a preestablished, reliable, safe, sterility level. For the terminal autoclaving procedure, it is absolutely necessary to establish binomial time and temperature parameters for each kind of product and packaging. The choice of bioindicator and the treatment temperature depend on the type of package. The material used to manufacture the vessels for parenteral solutions are plastic polymers, the bottles being made of polyethylene (thermal labile) and the bags polypropylene (thermal stable). Despite their high cost, the superior structural quality of polypropylene bags makes them resistant to autoclaving at 121°C. The type of packaging most used is made of low-density polyethylene; the bottles are manufactured in LVPS industries at 250°C, free of microorganisms, apyrogenic, and ready for use in the product packaging area.

Bacillus subtilis spores are chosen as bioindicator of LVPS packed in polyethylene bottles, which are autoclaved at a maximum of 110°C. *Bacillus stearothermophilus* spores are used as bioindicator to monitor LVPS packed in polyethylene bags, which are submitted to higher temperatures, generally 121°C.

The aim of the present study was to investigate the kinetic parameters of thermal inactivation of *B. stearothermophilus* and *B. subtilis* spores in different formulations of LVPS, industrially manufactured in order to optimize their use in autoclaving processes.

Materials and Methods

Biological Indicators

B. subtilis var. globigii ATCC 9372 and *B.stearothermophilus* ATCC 7953 were developed at 37°C for 3 d and at 65°C for 6 d, respectively, on plate count agar (Difco, Detroit, MI) supplemented with 0.10% soluble starch, 0.05% magnesium sulfate, 0.05% manganese sulfate, and 0.05% calcium chloride at pH 7.0. The growth was harvested in 0.02 *M* calcium acetate solution, adjusted to a final pH of 9.7 with 0.14% (w/v) calcium hydroxide ($Ca[OH]_2$) solution and centrifuged (2000 *g* for 30 min) four times. The pelleted spores were suspended in chilling 0.02 *M* calcium acetate solution, the suspension adjusted to pH 9.7 with 0.14% $Ca(OH)_2$ solution and stored at 4°C, and used as required *(4)* . The viability of 10^7–10^8 CFU/mL of heat-shocked spores in suspension (80°C for 10 min for *B. subtilis* and at 98.7°C for 30 min for *B. stearothermophilus*) was estimated through pour plate on trypticase soy agar (TSA) at 37°C for 48 h for *B. subtilis* and at 65°C for 48 h for *B. stearothermophilus*.

Large Volume Parenteral Solutions

LVPS industrially manufactured and supplied by producers named A–I presented the following compositions (% w/v): WFI with conductivity ≤1 µS; physiologic (0.9% NaCl) solution; glucose (5, 10, 50%) solutions, glucophysiologic (0.9% NaCl plus glucose 5%) solution; Ringer's (0.86% NaCl, 0.033% CaC_2, 0.030% KCl) solution and Ringer's with lactate (0.30% sodium lactate, 0.6% NaCl, 0.020% $CaCl_2$, 0.030% KCl) solution; peritoneal dialyses (0.56% NaCl, 0.5% sodium lactate, 0.026% $CaCl_2$, 0.015% $MgCl_2$) solutions with 1.5, 5.0 and 7.0% glucose; mannitol (20%) solution; glycerin (6, 12, 25%) solutions.

Buffer solutions were made up of sodium citrate/citric acid at acid molar concentration and respective pH value at (25°C): 0.0330 mol/L, pH 4.0; 0.0205 mol/L, pH 5.0; 0.0095 mol/L, pH 6.0; and sodium phosphate/citric acid at 0.0065 mol/L and pH 7.0.

LVPS Inoculation with Bioindicator

A volume of 100 mL of each of the LVPS was inoculated with 1.0 mL of the bioindicator suspension to perform an initial spore count of about $1–5 \times 10^6$/mL. After homogenization, 3.0-mL samples were transferred to sterile-ampoule type glass bottles (60 mm high × 25 mm bottom diameter) with a total capacity of 10 mL. The bottles were closed with a sterile flange rubber stopper, which was covered with plastic tape to avoid leakage of the

solution through the top, and then closed with an aluminum seal. The air was removed (5 min) by suction (vacuum pump, 1725 rpm, 1/6 hp; General Electric), using a 22-gage stainless steel needle (30 × 7 mm) through the rubber stopper of the sealed bottle. A type J thermocouple (2x32AWG), with a sensor end inside a stainless 316 steel needle (1.5 mm diameter × 100 mm length), was introduced into the solution through the hole in the rubber stopper left by the pump needle. The bottles were immersed in a thermostatically controlled oil bath and continuously agitated to keep the oil (Dow Corning® silicone 200/220 CS, $\rho = 0.948$ g/cm^3) circulating at the set temperature. The temperature of the oil bath was measured simultaneously with a PT 100 thermocouple (IOPE therm 48, calibrated to 1.0 ± 0.1°C; Incoterm) and monitored visually with reference calibrated thermometers (nitrogen filled, ASM 67C, external Hg scale, +95 to +155°C, subdivided and calibrated to 1.0 ± 0.2°C). For bioindicator spore activation in solution, the experimentally determined lag correction adopted ranged from 5 to 7 min, equivalent to the come-up-time required by the system to achieve the process temperature from 94 to 121°C. The time intervals of 2 and 1 min were selected for temperatures at 121 and below 110°C, respectively. In buffer solutions with a pH between 4.0 and 5.0, shorter time intervals were chosen. For each time interval, a triple set of bottles was treated between 94 and 104.5°C for *B. subtilis* and at 121° C for *B. stearothermophilus*, cooled, and held in an ice-water bath until spore recovery. Quantification of survivors was made by pour plate on TSA with incubation under optimum conditions for *B. subtilis* (37° C for 48 h) and *B. stearothermophilus* (65°C for 48 h), and was expressed by decimal logarithms of the average colony-forming units per milliliter of solution from at least 10 plates for each time intervals heating condition and system used.

Decimal Reduction Times

Decimal reduction times (D_{Tr} value), the interval of time required to reduce one decimal logarithm of the initial spore population, at reference temperature, were determined from the negative reciprocal of the slopes (b) of the regression lines using the linear portions of the survivor curves ($\log_{10}$ population vs time of exposure at a constant temperature). The slope SE (b) and the multiple determination coefficient (R^2) were also calculated. The estimated value of the D value SE(D) was obtained from the root of the variance $(V[D])^{1/2}$, which was calculated as follows:

$$V(D) = ([1/b^2]^2 \times [SE\{b\}]^2)$$

The upper (D – mean + SE [D]) and lower (D – mean – SE[D]) D values were also expressed.

Z-value (°C) and Activation Energy (Ea, kcal/mol)

The resistance coefficient z value, expressed as the number of centigrade required for one log change in the D value, may be given by:

$$z = ([T_1 - T_2]/[\log D_2 - \log D_1]),$$

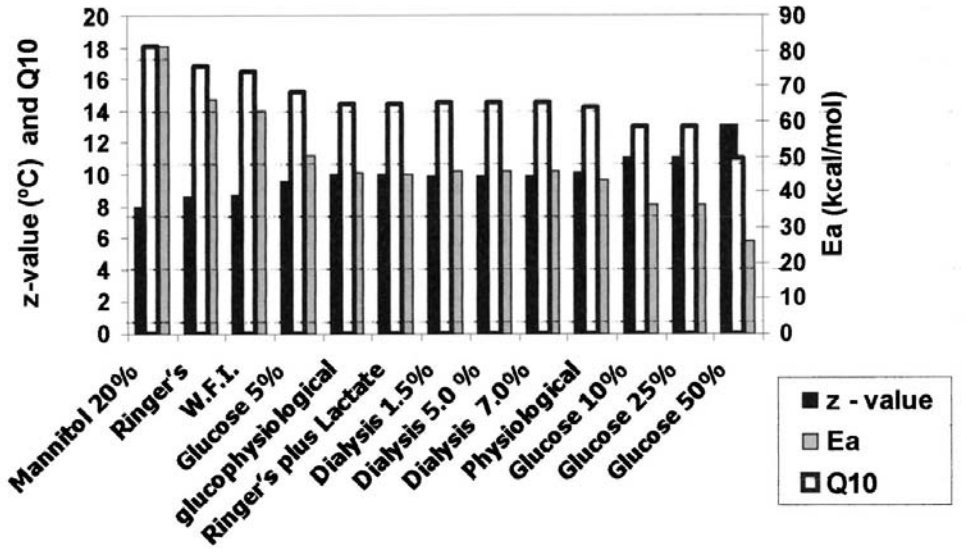

Fig.1. Thermal resistance parameters in LVPS, expressed in z value (°C), Ea (kcal/mol), and Q_{10} coefficient.

in which T_1 and T_2 are the heating temperatures in centigrade. The z -value may be related to the Ea using the Arrhenius equation:

$$Ea = ([2.303 \times Rx \{T_1 \times T_2\}/z]),$$

where T_1 and T_2 are the heating temperatures in degrees Kelvin (°K), and R is the universal gas constant (1.987 calories/ [mol·°K]). The temperature coefficient Q_{10} value of the process may be related to z value by the relation: $Q_{10} = (10^{10/z})$. The z, Ea and Q_{10} values are shown in Fig. 1, for the LVPS investigated in relation to the bioindicator used.

Results and Discussion

The kinetic parameters of inactivation of the bioindicator should be determined for each kind of LVPS before autoclaving. Nine national manufacturers associated with the Brazilian Association of Parenteral Solution Producers (Associação Brasileira dos Produtores de Soluções Parenterais, [ABRASP]) supplied samples of their products and were identified as companies A–I. The WFI obtained in our laboratory was identified by the letter J. The influence of various compositions of LVPS on thermal resistance of bioindicator spores, expressed by decimal reduction time (D_{Tr}, the time in minutes to kill 90% of the initial spore population) at a reference temperature, is shown in Tables 1 and 2 for *B. stearothermophilus* and *B. subtilis* spores, respectively.

The principal physical and chemical characteristics of LVPS formulations, which interfere with the bioindicator's thermal resistance, are as follows: the conductivity value of WFI, the concentration and degree of purity

Table 1
Kinetic Parameters of Inactivation of
B. stearothermophilus ATCC 7953 Spores in LVPS, expressed in D_{Tr} (min)

LVPS	Producer[a]	pH at 25°C[b]	D121°C-value (min)[c]
WFI	A	5.70	3.34 ± 0.32
WFI	B	6.15	3.37 ± 0.27
WFI	C	5.72	3.45 ± 0.33
WFI	F	5.80	3.88 ± 0.24
WFI	J	5.92	3.26 ± 0.32
	A–C, F, J	5.86 ± 0.23	3.46 ± 0.30
Physiologic	F	6.08	3.47 ± 0.77
Physiologic	E	5.60	2.82± 0.17
Physiologic	A	5.55	3.59 ± 0.42
Physiologic	B	5.67	2.78 ± 0.25
Physiologic	D	5.62	3.08 ± 0.14
Physiologic	C	5.95	2.81 ± 0.18
	A–F	5.75 ± 0.23	3.09 ± 0.38
Glucophysiolog	H	4.93	1.11 ± 0.10
Glucophysiolog	A	4.69	1.17 ± 0.03
Glucophysiolog	D	4.53	1.59 ± 0.22
Glucophysiolog	B	4.47	1.35 ± 0.14
		4.66 ± 0.33	1.31 ± 0.17
5% Glucose	A	4.75	1.42 ± 0.28
5% Glucose	F	5.13	1.85 ± 0.28
5% Glucose	B	4.64	1.97 ± 0.08
5% Glucose	D	4.52	1.61 ± 0.17
5% Glucose	C	4.87	1.83 ± 0.18
5% Glucose	E	4.62	1.86 ± 0.29
		4.76 ± 0.23	1.75 ± 0.21
10% Glucose	A	4.52	1.77 ± 0.04
10% Glucose	D	4.43	1.71 ± 0.09
10% Glucose	B	4.42	1.49 ± 0.12
		4.46 ± 0.14	1.66 ± 0.36
50% Glucose	F	3.50	2.03 ± 0.11
50% Glucose	A	2.60	1.87 ± 0.27
	A, F	3.05 ± 0.45	1.95 ± 0.11
Ringer's	B	5.95	2.82 ± 0.22
Ringer's	D	5.55	2.65 ± 0.14
		5.75 ± 0.20	2.74 ± 0.12
Ringer's plus lactate	H	6.55	2.87 ± 0.22
Ringer's plus lactate	A	5.76	3.70 ± 0.41
Ringer's plus lactate	C	6.40	3.18 ± 0.38
Ringer's plus lactate	D	5.65	2.81 ± 0.21
Ringer's plus lactate	B	6.54	2.90 ± 0.21
Ringer's plus lactate	E	6.20	3.40 ± 0.08
		6.18 ± 0.41	3.14 ± 0.37
1.5% Dialysis	A	5.60	2.65 ± 0.15
1.5% Dialysis	B	6.02	2.19 ± 0.17
		5.81 ± 0.21	2.42 ± 0.23
7.0% Dialysis	B	5.44	2.26 ± 0.37
20% Mannitol	A	5.56	2.92 ± 0.18
20% Mannitol	D	5.80	3.23 ± 0.24
20% Mannitol	B	5.24	3.24 ± 0.16
		5.53 ± 0.28	3.13 ± 0.45
25% Glycerin	A	4.22	3.79 ± 0.18
12% Glycerin	G	4.20	2.78 ± 0.06
6% Glycerin	G	4.40	3.40 ± 0.26
Buffer	J	4.00	0.49 ± 0.02
Buffer	J	5.00	0.56 ± 0.08
Buffer	J	6.00	1.32 ± 0.07
Buffer	J	7.00	2.26 ± 0.22

[a] J = our laboratory [b] Mean ± SD = mean ± SE ($p < 0.05$) [c] Mean ± CI = mean ± CI at 95%.

of components diluted in WFI, the pH and water activity of the final product, the viscosity values of those in which glucose is diluted,and the formation of 5-hydroxymethylfurfural (5-HMF) base compounds by hexose transformation in an acid medium. These variables should be checked since they may either increase or decrease thermal resistance of the bioindicator, altering the total autoclaving time.

WFI, the most important solvent for the preparation of parenteral formulations, is water purified by distillation (less used) or by the reverse osmosis process, ensuring conductivity lower than 1 µS/cm, a pH between 4.5 and 7.0, and freedom from microorganisms and mainly from bacterial endotoxins (not more than 0.25 µg of bacterial endotoxin units/mL) *(5)*.

For *B. stearothermophilus* ATCC 7953 spores, the average $D_{121°C}$ value of 3.46 ± 0.30 min was attained in ABRASP WFI (pH 5.86 ± 0.23), confirming similar results obtained by Lopez et al. *(6)*, $D_{121°C}$ = 3.83 ± 0.09 min, and by Pflug *(7)*, from 2.86 to 3.43 min. The WFI (sample J) exhibited the lowest $D_{121°C}$ of 3.26 ± 0.32 min, with a conductivity of 0.80 µS and pH of 5.92. Fernández et al. *(8)* found a $D_{121°C}$ value of 3.1 min and a *z* value of 7.1°C for *B. stearothermophilus* ATCC 12980 spores inoculated in bidistilled water (pH 7.0 at 25°C), and Feeherry et al. *(9)* found a $D_{121°C}$ value of 3.33 min.

For *B. subtilis* spores in WFI (pH 5.84 ± 0.32), the average *D* value of 0.66 ± 0.14 min at 104.5°C ranged from 0.53 ± 0.08 min (pH 5.70) to 0.83 ± 0.17 min (pH 6.15), for water purified by reverse osmosis (producer A) and by distillation (producer B), respectively. The influence of the pH range from 5.45 to 6.18 on moist thermal resistance was not significant (Table 1). Sala et al. *(10)* verified for *B. subtilis* spores suspended in citrate phosphate buffer corresponding to pH 4.0, 5.0, 6.0, and 7.0 that the nonlinear influence of the pH range of 4.0 to 5.0 showed greater significance on heat resistance.

The addition of 0.9% (w/v) NaCl to the WFI in preparing the physiologic solution caused a slight change in the pH value and in the thermal resistance of the bioindicator. For *B. stearothermophilus*, the $D_{121°C}$ value dropped to an average $D_{121°C}$ value of 3.09 ± 0.38 min (pH 5.75) in relation to that found in WFI. For manufacturers A and F, we found that the $D_{121°C}$ values of 3.59 ± 0.42 and 3.47 ± 0.77 min, respectively, in physiologic solution were similar to those obtained in WFI ($D_{121°C}$ = 3.34 ± 0.32 and 3.88 ± 0.24 min, respectively). A decrease of 1 logarithmic cycle in relation to the results obtained by sample B (D121°C = 2.78 ± 0.25 min) was probably owing to the drop in the pH value from 6.15 to 5.67. This result is equivalent to the $D_{121°C}$ value obtained in sample E, which reached a $D_{121°C}$ value of 2.82 ± 0.17 min at pH 5.60. Lopez et al. *(6)* found a $D_{121°C}$ = 2.60 minutes for *B. stearothermophilus* ATCC 7953 in a 6% NaCl solution.

For *B. subtilis* the average $D_{104.5°C}$ value of 0.93 ± 0.20 min increased 41% in the physiologic solution over that attained by WFI, with a slight variation in pH (5.55–6.60). The influence of the addition of electrolyte on the thermal resistance of the bioindicator was described by Berger and Nelson *(11)*, who observed for *Clostridium sporogenes*, that the 0.9% NaCl solution afforded the highest average $D_{121°C}$ of 1.1 minutes and a *z* value of 12.9°C,

Table 2
Kinetic Parameters of Inactivation of *Bacillus subtilis* ATCC 9372 Spores in LVPS, Expressed in D_{Tr} (min), z Values (°C), Ea (kcal/mol)

LVPS	Producer	pH at 25°C[a]	$D_{104.5°C}$ value (min)[b]
WFI	H	6.18	0.66 ± 0.03
WFI	A	5.70	0.53 ± 0.08
WFI	B	6.15	0.83 ± 0.17
WFI	C	5.72	0.58 ± 0.08
WFI	E	5.45	0.69 ± 0.09
WFI		5.84 ± 0.32	0.66 ± 0.14
Physiologic	H	6.06	0.88 ± 0.13
Physiologic	I	6.60	0.85 ± 0.09
Physiologic	E	5.60	1.07 ± 0.16
Physiologic	A	5.55	0.99 ± 0.21
Physiologic	B	5.67	0.67 ± 0.16
Physiologic	D	5.62	1.32 ± 0.11
Physiologic	C	5.95	0.75 ± 0.12
Physiologic		5.86 ± 0.38	0.93 ± 0.20
Glucophysiologic	H	4.93	0.71 ± 0.10
Glucophysiologic	A	4.69	0.88 ± 0.09
Glucophysiologic	D	4.53	0.57 ± 0.07
Glucophysiologic	B	4.47	0.90 ± 0.08
Glucophysiologic	A, B, D, H	4.66 ± 0.33	0.77 ± 0.25
5% Glucose	G	5.20	0.79 ± 0.09
5% Glucose	I	5.18	0.62 ± 0.04
5% Glucose	F	5.13	0.61 ± 0.08
5% Glucose	B	4.64	0.91 ± 0.08
5% Glucose	D	4.52	0.70 ± 0.07
5% Glucose	C	4.87	0.77 ± 0.09
5% Glucose	A	4.75	0.55 ± 0.08
5% Glucose	E	4.62	0.88 ± 0.17
5% Glucose	A-G, I	4.86 ± 0.23	0.73 ± 0.11
10% Glucose	H	4.86	0.83 ± 0.03
10% Glucose	A	4.52	0.93 ± 0.10
10% Glucose	D	4.43	0.85 ± 0.04
10% Glucose	B	4.42	0.86 ± 0.04
10% Glucose	A, B, D, H	4.56 ± 0.33	0.87 ± 0.07
25% Glucose	A	4.22	1.05
50% Glucose	F– [a]105°C	3.50	1.45 ± 0.31
50%Glucose	A	2.60	1.29 ± 0.34
50%Glucose	A, B, D, H	3.05 ± 0.50	1.37± 0.33
Ringer's	H	6.25	0.71 ± 0.07
Ringer's	B	5.95	0.63 ± 0.05
Ringer's	D	5.54	0.74 ± 0.11
Ringer's	B, D, H	5.91 ± 0.88	0.69 ± 0.14
Ringer's plus lactate	H	6.55	0.72 ± 0.08
Ringer's plus lactate	A	5.76	0.66 ± 0.03
Ringer's plus lactate	C	6.40	0.68 ± 0.08
Ringer's plus lactate	D	5.65	0.77 ± 0.11
Ringer's plus lactate	B	6.54	1.14 ± 0.09
Ringer's plus lactate	E	6.20	0.85 ± 0.09
	A-E, H	6.18 ± 0.41	0.75 ± 0.20
1.5% Dialysis	A	5.60	0.71 ± 0.09
1.5% Dialysis	B	6.02	0.88 ± 0.14
1.5% Dialysis	A, B	5.81 ± 0.21	0.80 ± 0.12
5.0 % Dialysis	B	5.81	0.82 ± 0.06
7.0% Dialysis	B	5.44	0.72 ± 0.10
20% Mannitol	H	6.05	0.53 ± 0.13
20% Mannitol	A	5.56	0.69 ± 0.18
20% Mannitol	G	6.20	0.94 ± 0.08
20% Mannitol	D	5.80	0.76 ± 0.10
20% Mannitol	B	5.24	0.82 ± 0.02
20% Mannitol	A, B, D, G, H	5.77 ± 0.38	0.75 ± 0.19

[a] Mean ± SD = mean ± SE ($p < 0.05$). [b] Mean ± CI = mean ± CI at 95%.

among NaCl concentrations (0.45–23.4 %) in solutions. A similar increase in the resistance of *B. stearothermophilus* spores was not noted.

The pH values fell sharply, from 5.86 ± 0.38 to 4.66 ± 0.33, on addition of 5% (w/v) glucose to the physiologic solution, on preparation of the glucophysiologic solution. For *B. stearothermophilus*, the $D_{121°C}$ values dropped approx 2.5 times when compared with the results obtained in WFI, and 2.4 times when compared with the results obtained in the physiologic solution; the average value obtained for $D_{121°C}$ being 1.31 ± 0.17 minutes. Lopez et al. *(12)* observed a reduction of three times the thermal resistance of *B. stearothermophilus* spores when the heating menstruum was acidified from pH 7.0 to pH 5.0.

For *B. subtilis* spores, the average $D_{104.5°C}$ value of 0.77 ± 0.25 min in glucophysiologic solution was an intermediate value between that for WFI and the physiologic solution (Table 2). Berger and Nelson *(11)* emphasized that the addition of glucose to electrolyte solutions significantly reduced the $D_{121°C}$ value of *C. sporogenes* spores, although KCl provides greater thermal resistance in comparison with the formulation with NaCl.

On preparing 5% glucose solution, a significant drop in the average pH value (4.86 ± 0.23) occurred, although sample F showed a higher pH of 5.13. For *B. stearothermophilus* spores, the average $D_{121°C}$ value obtained by 5% glucose solution was 1.75 ± 0.21 min, two times lower than that for WFI and approx 34% higher than that attained by the glucophysiologic solution (Table 1). Comparison of the results revelaed that the addition of 0.9% NaCl to the 5% glucose solution caused the sharpest reduction in the $D_{121°C}$ value. However, for *Bacillus subtilis* spores the average $D_{104.5°C}$ value of 0.73 ± 0.11 min attained was similar to that obtained for the glucophysiologic solution (Table 2). For samples A, I, and F, $D_{104.5°C}$ values were obtained in the range of 0.60 min, very close to those reached by WFI. Theoretically, a lower average $D_{104.5°C}$ value was expected, but the maintenance of the result was perhaps dependent on the conductivity of the WFI used in the dilution of chemical solutes.

The pH value of 4.46 for 10% glucose solution was very close to the pH of 4.62 for 5% glucose solution. This slight reduction in the pH did not cause a significant variation in the $D_{121°C}$ value for *B. stearothermophilus* spores, since for manufacturers A and D the $D_{121°C}$ values rose from 1.42 ± 0.28 to 1.77 ± 0.04 min, and from 1.61 ± 0.17n to 1.71 ± 0.99 min, in 5% glucose and 10% glucose solutions, respectively (Table 1). There was only a reduction in the $D_{121°C}$ value in sample B, from 1.97 ± 0.08 to 1.49 ± 0.12 min, corresponding to an increase of glucose from 5 to 10%, respectively.

The $D_{121°C}$ values of the 50% glucose (w/v) solution for samples A and F were very similar to those obtained by the 10% solution, with an average value of $D_{121°C}$ = 1.95 ± 0.11 min, even though the solution showed a sharp drop in the pH value of 3.05 ± 0.45. The effect of the increase in glucose from 10 to 50% may possibly be explained by the alteration in the rheologic and chemical properties of the product. A sharp drop in the average pH value can be provoked by degradation of the glucose caused by the heat,

converting it into other products, such as 5-HMF, without a simultaneous alteration in $D_{121°C}$ values.

For *B. subtilis* spores in 10% glucose (average pH of 4.56), the average $D_{104.5°C}$ value of 0.85 min for producers B, D, and H was lower than that obtained by producer A (0.93 min). The 25% glucose solution afforded a close $D_{104.5°C}$ value of 1.05 min at pH 4.22. The increase in glucose concentration from 10 to 25 % did not alter either the thermal resistance of *B. subtilis* spores nor the average pH value of the LVPS. An increase in glucose to 50% reduced the pH 3.05; however, it increased the average $D_{104.5°C}$ value to 1.37 ± 0.33 min and the *z* value from 11.03 to 13.14°C, the highest obtained for LVPS.

In Ringer's solution, in which the concentration of NaCl is approx 0.9% (w/v), the average $D_{121°C}$ value of 2.74 ± 0.12 min was slightly lower than that obtained in the physiological solution (Table 1), even though its formulation contains $CaCl_2$ and KCl in concentrations close to 0.03% (w/v), which apparently did not appear to cause any significant alteration in the thermal resistance of the bioindicator. For producer B, the $D_{121°C}$ values of 3.37, 2.78 and 2.82 min, attained by WFI, physiologic and Ringer's solutions, respectively, showed that the addition of electrolytes to WFI dropped the thermal resistance of *B. stearothermophilus* spores to the same extent, with a slight decrease in pH from 6.15 to 5.95. For producer D, the $D_{121°C}$ value varied from 3.08 ± 0.14 (physiologic solution) to $D_{121°C}$ = 2.65 ± 0.14 min (Ringer's solution), for a pH range from 5.62 to 5.55, respectively. Similar results for *B. subtilis* spores were obtained; the average $D_{104.5°C}$ of 0.69 ± 0.14 min in Ringer's solution was very close to that obtained in WFI (Table 2), suggesting that the addition of electrolytes was insufficient to cause any significant alteration in the thermal resistance of this bioindicator.

The addition of 0.5% sodium lactate to Ringer's solution caused an increase in the $D_{121°C}$ values from 2.74 ± 0.12 min (pH 5.75) in Ringer's solution, to $D_{121°C}$ = 3.14 ± 0.37 min (pH 6.18) in Ringer's plus lactate solution (Table 1), magnifying the thermal resistance of the *B. stearothermophilus* spores by approx 15%.

For the Ringer's solution with sodium lactate that presented the highest LVPS pH of 6.18 ± 0.41, the average $D_{104.5°C}$ value of 0.75 ± 0.20 min found for *B. subtilis* spores was similar to that obtained for glucose 5% and glucophysiologic solutions, an intermediate value between those attained for WFI and physiologic solution, and higher than that obtained for Ringer's solution (Table 2). Sodium lactate added to Ringer's solution may be the cause of the increase in thermoresistance, independent of the pH values of the different solutions considered. Producer B had the highest $D_{104.5°C}$ value of 1.14 min (pH 5.69) in Ringer's solution with lactate, which was very close to that obtained in the physiologic solution ($D_{104.5°C}$ =1.07 min) by producer E, and in 25 % glucose ($D_{104.5°C}$ =1.05 min) by producer A, for similar *z* values at about 10°C, although the pH dropped from 6.18 in Ringer's solution with lactate to 4.22 in 25 % glucose.

Ringer's solution and Ringer's solution with lactate provided $D_{104.5°C}$ values of 0.69 and 0.75 min, respectively, both lower than $D_{104.5°C}$ value of 0.93 min for physiologic solution, even though NaCl was a common component, about 0.86, 0.60 and 0.90%, respectively.

The preparation of solutions for peritoneal dialysis with 1.5, 5.0, and 7.0% of glucose, despite not being for parenteral administration, follows the same standards (Directive 500/1997) *(1)* required for parenteral solutions and was also studied. Independent of the concentration of glucose, the other components have a fixed concentration—0.56% NaCl, 0.026% $CaCl_2$, 0.015% $MgCl_2$, and 0.500% sodium lactate, the last component common to Ringer's solution.

The thermal resistance of *B. stearothermophilus* spores was shown to be dependent on glucose ranging from 1.5 to 7.0%, considering producers A and B (Table 1). The average $D_{121°C}$ values for the solutions with 1.5 and 7% glucose were 2.42 ± 0.32 (pH 5.81) and 2.26 ± 0.37 min (pH 5.44), respectively, both results lower than that attained for Ringer's solution ($D_{121°C}$ = 2.74 min at pH 5.75), but higher than that for 5% glucose solution ($D_{121°C}$= 1.75 min), whose pH of 4.76 decreased sharply. The addition of electrolytes and sodium lactate to dialysis solutions helped to maintain the pH values similar to those for WFI (pH 5.86), despite the decrease of approx 50% in the $D_{121°C}$ value from 3.46 (WFI) to 2.26 min (dialysis plus 7% glucose).

The average $D_{104.5°C}$ value of 0.79 min (pH 5.69) was common for the three kinds of peritoneal dialysis solutions, independent of the concentration of glucose added: 1.5, 5.0 or 7.0%. Ringer's solution with sodium lactate provided similar thermal resistance (average $D_{104.5°C}$ = 0.75 min) for spores of *B. subtilis* to those in peritoneal dialysis solutions (Table 2). The addition of glucose to dialysis solutions did not interfere in the thermal resistance of *B. subtilis* spores as much as sodium lactate did in the different kinds of solutions which were studied.

The $D_{121°C}$ value of 3.13 ± 0.45 min (pH 5.53) for *B. stearothermophilus* in 20% mannitol solution was approx 10% below that obtained for WFI and similar to that for physiologic solution (Table 1). The thermal resistance was confirmed to be independent of the chemical composition of the three LVPS considered. The 20% (w/v) mannitol solution reached average $D_{104.5°C}$ values of 0.75 ± 0.19 min at pH 5.77 ± 0.38, similar to those obtained in the 5% glucose, glucophysiologic, Ringer's with lactate, and peritoneal dialysis solutions (Table 2), demonstrating the independence of thermal resistance of *B. subtilis* spores from solution formulations and pH values, the average ranging from 4.86 to 6.18.

Glycerin solutions are not used parenterally; nevertheless, they were studied because they also are submitted to the same terminal autoclaving procedure as LVPS. Independent of glycerin (6, 12, 25%) concentrations, the acidification of the solutions to pH values between 4.20 and 4.40 did not interfere with the $D_{121°C}$ values for *B. stearothermophilus* spores, the average for the three solutions being $D_{121°C}$ = 3.32 min, in comparison with that attained for WFI (Table 1).

Germination of spores did not occur after having been submitted to moist heat treatments in sodium carbonate solution. This phenomenon was also reported by Cheung et al. *(13)* when they attempted to germinate spores of *B. stearothermophilus* NTCTC 10003 in solutions of sodium bicarbonate up to 1%.

For citric acid buffers at pH of 4.0, 5.0 and 6.0, corresponding to molar concentrations of 0.0339, 0.0205, and 0.0095 mol/L, respectively, the $D_{121°C}$ values for *B. stearothermophilus* were 0.49, 0.56 and 1.32 min, respectively. For the bioindicator suspended in citric acid plus phosphate buffer at pH 7.0 and 0.0065 mol/L, the $D_{121°C}$ was 2.26 min. This last result was lower than that obtained for WFI and close to that for dialysis solutions, the pH values of which were between 5.0 and 6.0. Lopez et al. *(12)*, using Mcllvaine buffer solutions, obtained at 120°C similar $D_{120°C}$ values for *B. stearothermophilus* of 0.43 min at pH 4.0, 0.59 min at pH 5.0, 1.25 min at pH 6.0, and 2.10 min at pH 7.0.

On inoculating *B. stearothermophilus* ATCC 12980 spores in an acidified yeast extract medium up to pH 6.0 and in a yeast extract medium to pH 7.0, Fernández et al. *(8)*, attained $D_{120°C}$ values of 1.70 and 2.20 min, respectively.

Wang *(14)* reported that citric acid, disodium phosphate, and glycol solutions enhanced the thermal resistance of *B. stearothermophilus* spores.

The composition of LVPS had a remarkable influence on the *z* value: $z = 8.73°C$ for WFI, the conductivity of which was about 1.0 µS/ cm, and $z = 13.4$ °C for 50 % glucose, corresponding to $D_{104.5°C}$ values of 0.66 and 0.69 min, respectively. Sala et al. *(10)* observed an increase in *z* values for *B. subtilis* with acidification of the heating menstruum.

The *Ea* for *B.stearothermophilus* spores varied from 80 kcal/mol for 20% mannitol solution to 49.36 kcal/mol for 50% glucose solution to 74.20 kcal/mol for WFI, as shown in Fig. 1. According to Nash *(15)*, an $Ea = 71$ kcal/mol corresponds to an acceptable value. The 50% glucose solution showed high stability to maintain spore thermal resistance over moist heat treatments.

The Q_{10} values reported for spores in solutions ranged from 8.07 (10 and 25% glucose) to 18.11 (20% mannitol) and decreased to 5.77 for 50% glucose solution, reinforcing the respective *Ea* results obtained (Fig. 1). According to Gauthier et al. *(16)*, the Q_{10} value for microorganism destruction may range from 8 to 20 for moist heat. Fifty percent glucose and 20% mannitol were the most stable and unstable solutions, respectively, among all LVPS studied, adopting *B. subtilis* as bioindicator and low moist heat temperatures and long term intervals of exposure. *B. subtilis* spores in WFI and Ringer's systems were also vulnerable to heat treatments.

In summary, a producer of LVPS should keep strict control of the quality (physical, chemical, and biologic) of the parenteral solution, preventing significant modifications in the characteristics of the final solution, in order not to interfere with the specific *D* value previously attained for bioindicator, consequently exposing the LVPS to terminal autoclaving processes for longer or shorter periods of time than necessary, and preju-

dicing the safety assurance sterility level of the final product. Despite the heterogeneity of the solution, the thermal stability is greater in autoclaving at 121 than at 94 or 104.5°C, a phenomenon that was evident in the 20% mannitol solution. The validation of terminal autoclaving at 121°C in thermal stable packaging inoculated with bioindicator of *B. stearothermophilus*, for variable volumes, establishes appropriate autoclaving time for the product in question.

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