Thermal Resistance of Bacterial Spores in Milk-Based Beverages Supplemented with Nisin†

B. M. BEARD,‡ B. W. SHELDON,* and P. M. FOEGEDING†

1Department of Food Science, Box 7624, North Carolina State University, Raleigh, North Carolina, 27695-7624; and
2Department of Poultry Science, Box 7608, North Carolina State University, Raleigh, North Carolina, 27695-7608, USA

ABSTRACT

The effect of nisin, added in the form of Nisaplin, on the thermal resistance of bacterial spores and the effects of medium composition, exposure time, and pH on nisin enhancement of heat sensitivity were evaluated. Nisin apparently required specific nutrients to sensitize spores to heat. For example, $D_{130}^0$ values of approximately 10 s were observed in sodium phosphate buffer with and without 6% sucrose with no significant ($P \geq 0.05$) differences detected as a result of increased nisin concentration. In a nutrient-rich chocolate milk model system (CMMS), increasing either the time of exposure to nisin (5, 15, or 24 h) before heating or nisin concentration (0, 2,000, or 4,000 IU/ml) increased the sensitivity of Bacillus stearothermophilus spores to heat. In the CMMS with 10 to 12% fat cocoa powder, increasing nisin concentration (at 5 h of exposure) significantly ($P \leq 0.05$) reduced $D_{130}^0$ values; $D_{130}^0$ values were 21.7, 17.2, and 17.8 s, respectively, for the 0-, 2,000-, and 4,000-IU/ml nisin treatments. Fifteen and 24 h of exposure further reduced $D_{130}^0$ values in the nisin-containing treatments compared to the control (0 IU of nisin per ml). A lower-fat CMMS (0 to 1% fat cocoa powder) had lower $D_{130}^0$ values (19.3, 15.8, and 14.7 s for the 0-, 2,000-, and 4,000-IU/ml nisin treatments, respectively). Nisin activity was enhanced by lowering pH in the CMMS (10 to 12% fat cocoa powder), with reductions in $D_{130}^0$ values across all pH values (ranging from 18.0% at pH 6.4 to 41.9% at pH 5.0). $z_D$ values were 9.6, 9.0, and 8.4°C for the 0-, 2,000-, and 4,000-IU/ml nisin treatments, respectively. Spores of B. licheniformis yielded results similar to those obtained with B. stearothermophilus. For example, decreasing CMMS (10 to 12% fat cocoa powder) pH values from 6.4 to 5.0 produced $D_{100}^0$ values of 3.3, 2.8, and 2.8 min (pH 5.0) and 1.0, 0.8, and 0.8 min (pH 5.0) for the 0-, 2,000-, and 4,000-IU/ml nisin treatments. This study clearly verified that the addition of Nisaplin to dairy-based beverages, such as a chocolate milk drink, or other foods intended to be heated reduces the thermal resistance of selected bacterial spores. Increased spore sensitivity to heat may provide food processors with an opportunity to reduce their thermal processes and expenses while maintaining product quality, functionality, and shelf stability.

The marketability of milk-based products with an extended shelf life has been limited by the difficulty in producing a product with acceptable quality attributes. In many cases, these products lack acceptable levels of functionality, viscosity, flavor, or shelf life. To improve product quality, the goal of processors is to reduce the time and/or temperature of thermal processing while destroying even the most persistent microorganisms. In previous studies directed at evaluating sterilization processes of raw milk, the predominant surviving thermophilic organism was Bacillus stearothermophilus (3). B. stearothermophilus spores are widely present in nature and, because of their extreme resistance to heat, are often used to evaluate commercial sterilization. B. licheniformis spores are mesophilic organisms that often occur in raw milk (13, 16, 31) and are difficult to control because they are relatively heat resistant. In more recent studies, this organism was identified as the most common organism isolated at all stages of milk processing (5).

Considerable attention has been directed toward reducing the heat resistance or improving control of bacterial spores through contact with the antimicrobial peptide nisin (4, 12, 17, 29). Nisin has been shown to reduce the thermal process requirements in several food products by enhancing thermal inactivation of bacterial spores and vegetative cells.

Nisin is a bacteriocin or antimicrobial peptide produced by some strains of Lactococcus lactis. This peptide is reported to inhibit the spore germination process at the stage of pre-emergent swelling (6) and to strongly inhibit the growth of a wide range of gram-positive bacteria (30). Nisaplin, a commercial preparation of nisin concentrate produced by Aplin & Barrett (Dorsett, UK), has a high and consistent biological activity (6). Nisaplin is composed of 2.5% nisin, >50% sodium chloride, 23.8% denatured milk solids, and <3% moisture.

By using nisin as an adjunct to thermal processing of selected foods, it may be possible to alter the thermal resistance of contaminating organisms (spores) in such a way that survivors of nisin treatment will be more susceptible...
to thermal processes. By introducing nisin in the form of Nisaplin to milk-based beverages before heat processing, it may be possible to improve product quality and to save time and money through energy savings by reducing thermal processing requirements. Moreover, it may be possible to produce new dairy products, such as carbonated drinks, that cannot withstand current processing requirements.

The objectives of this study were to determine the effect of nisin, in the form of Nisaplin, on the thermal resistance of bacterial spores and to determine the effects of medium composition, exposure time, and pH on nisin enhancement of heat sensitivity. It was also our goal to determine whether nutrients affect the function of nisin. Thermal death kinetics, or $D$ values, and $z_D$ values for spores were determined in pasteurized milk-based model systems containing cocoa and sucrose and in sodium phosphate buffer (SPB), with or without sucrose, supplemented with various concentrations of nisin using an immersion sealed, glass capillary tube heating system.

**MATERIALS AND METHODS**

**Bacterial cultures.** *B. steareothermophilus* strain ATCC 12980 was obtained from the culture collection of Dr. Peggy Foe-geding (Department of Food Science, North Carolina State University, Raleigh, N.C.). *B. licheniformis* ATCC 14580 was obtained from the American Type Culture Collection (Rockville, Md.).

Spores of *B. steareothermophilus* were prepared by the method of Warth (35). Spores were harvested by centrifugation at 3,700 $\times$ g for 15 min at 4°C. The spores were washed five times in sterile, distilled water, suspended in distilled water, and stored frozen at $-20^\circ$C in 0.5-ml aliquots for approximately 4 years before use in this study. Just before use, spores were thawed at room temperature for approximately 20 min and heat-shocked at 100°C for 15 min to obtain a homogeneous spore population. After heat shock, 0.5-ml volumes of the spore crop (approximately $3.5 \times 10^8$ CFU/ml) were added to 10 ml of heating menstrum.

Spores of *B. licheniformis* were prepared by the method of Johnson et al. (20) as used for sporulation of *B. cereus*. Spores were harvested by centrifugation at 1,000 $\times$ g for 20 min at 4°C. Spores were washed, dispensed into 0.5-ml aliquots, frozen, and thawed before use as described above. Spores (approximately $3.8 \times 10^8$ CFU/ml) that were approximately 4 years old were used without heat shock.

**Nisin.** Nisin was added in the form of Nisaplin, a commercial preparation that contains 2.5% active nisin, >50% sodium chloride, 23.8% denatured milk solids, and <3% moisture. Nisaplin has an activity of $1 \times 10^9$ IU/g, whereas pure nisin has an activity of $40 \times 10^8$ IU/g. In 1969, the World Health Organization (37) defined an international unit of nisin as the activity exhibited by 1 g of an international reference preparation that was equivalent to Nisaplin (8).

A nisin stock solution of 800,000 IU/ml was prepared fresh for each trial by dissolving 0.8 g of Nisaplin into 1 ml of 0.02 N HCl. Twenty-five- or 50-µl aliquots of the nisin stock solution were added to the heating menstrum containing spore inoculum to yield nisin concentrations of 2,000 and 4,000 IU/ml, respectively. These two nisin concentrations were determined previously to be effective in laboratory studies involving *B. steareothermophilus* spores (34). For the control treatment, 50 µl of 0.02 N HCl was added per tube to adjust the volume and acidity of the heating menstrum to coincide with those of the experimental treatments.

**Heating menstrum.** Four heating menstrum, each supplemented with three concentrations of nisin (0, 2,000, and 4,000 IU/ml) were evaluated. These four systems included a chocolate milk model system (CMMS) made with either 10 to 12% or 0 to 1% fat cocoa powder and 100 mM SPB (pH 6.4) with or without 6% sucrose. The heating menstrum were chosen to provide baseline data in model dairy beverage formulations of different fat (from cocoa) concentrations at a constant pH. SPB was included to provide a reference nutrient-free (germinant-free), nonfat system and to allow isolation of the effect of sucrose as an ingredient. These systems were evaluated at 130, 135, and 137°C for *B. steareothermophilus*. At 130°C, exposure of spores to nisin for 5, 15, or 24 h before heating was evaluated. Heating at 135 and 137°C was evaluated only for the 10 to 12% fat cocoa CMMS. *B. licheniformis* studies were performed in the 10 to 12% fat cocoa CMMS at 100°C.

The CMMSs were formulated in consultation with an industrial processor of aseptic chocolate milk so that they modeled a realistic commercial formulation. CMMSs contained 33% nonfat dry milk, 6% sucrose, and 1% cocoa powder (10 to 12% or 0 to 1% fat). Nonfat dry milk powder and water initially were mixed and heated to 68°C, followed by the addition of sucrose and cocoa powder. SPB at pH 6.4 simulated pH values found in the CMMSs.

The effect of pH on nisin enhancement of heat sensitivity in *B. steareothermophilus* was evaluated at 130°C using the 10 to 12% fat cocoa CMMS. The CMMS was adjusted from its original pH of 6.4 to 5.7 and 5.0 using 1 or 5 M HCl, respectively. To determine similar effects in *B. licheniformis*, the 10 to 12% fat cocoa CMMS was adjusted from pH 6.4 to 5.0 using 5 M HCl and evaluated at 100°C. Table 1 details the thermal inactivation trials conducted for each heating menstrum, pH, exposure time, and temperature.

**Determination of $D$ values.** Decimal reduction times ($D$ values) were determined using a low-volume, sealed, glass capillary tube method (11). Inoculated CMMS or SPB (50 µl) was dispensed into glass capillary tubes (0.8- to 1.1-mm inner diameter $\times$ 90-mm length; no. 34502, Kimble, Vineland, N.J.) using a syringe that was fitted with a 100-mm blunt-tipped needle. The tubes were sealed using an oxygen torch and placed in ice-water before and after sealing. After sealing, the tubes were refrigerated (4°C) for 5, 15, or 24 h before heating. Tubes were placed in a mesh-covered test tube rack and completely submerged in a circulating oil bath maintained at 100, 130, 135, or 137°C (model W13, Haake, Inc., Karlsruhe, Germany). The time for the capillary tube to reach temperature was previously determined to be approximately 10 s (36). At zero time (no heating) and at seven additional times per trial, a capillary tube was removed from the bath and placed in an ice-water slurry. Each chilled tube was placed in a 200-ppm hypochlorite solution for at least 5 min and then rinsed with sterile, distilled water for at least 5 min. The hypochlorite-treated and rinsed tubes were aseptically transferred to test tubes containing 5 ml of sterile 0.1% peptone water and crushed using a sterile glass rod to release spores.

For *B. steareothermophilus* spores, samples were vortexed, and volumes of 50 to 250 µl were spread, using a spiral plater (model Autoplate 4000, Spiral Biotech, Bethesda, Md.), onto 0.1% tryptic soy agar supplemented with 1% starch (38) to allow for recovery of possibly injured spores. Plates were incubated at 55°C for 120 h to recover injured spores.

*B. licheniformis* samples were vortexed and spread as described above onto 0.1% tryptic soy agar supplemented with 1% starch to allow for recovery of possibly injured spores. Plates were incubated at 35 to 37°C for 72 h to recover injured spores.
TABLE 1. Experimental design of thermal inactivation trials conducted for Bacillus stearothermophilus and B. licheniformis spores tabulated by heating menstruum, pH, exposure time, and temperature

<table>
<thead>
<tr>
<th>Spores</th>
<th>Heating menstruuma</th>
<th>pH</th>
<th>Exposure time (h)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. stearothermophilus</td>
<td>CMMS (10–12% fat cocoa powder)</td>
<td>6.4</td>
<td>5</td>
<td>130, 135, 137</td>
</tr>
<tr>
<td></td>
<td>6.4</td>
<td>5, 15, 24</td>
<td>5</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>6.4, 5.7, 5.0</td>
<td>5</td>
<td></td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>SPB</td>
<td>6.4</td>
<td>5</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>SPB (6% sucrose)</td>
<td>6.4</td>
<td>5</td>
<td>130</td>
</tr>
<tr>
<td>B. licheniformis</td>
<td>CMMS (10–12% fat cocoa powder)</td>
<td>6.4</td>
<td>5</td>
<td>100</td>
</tr>
</tbody>
</table>

a CMMS, chocolate milk model system; SPB, sodium phosphate buffer.

Data analysis. The detection limit of the capillary tube procedure was 100 CFU/ml. Statistical analysis of D values was conducted using the Student’s t test least significant difference with P ≤ 0.05, 0.10, or 0.15. Data were pooled when nonsignificant replicate effects were observed. For B. stearothermophilus and B. licheniformis spores inactivated in the lower-pH CMMS, D values were analyzed by analysis of variance using a randomized, complete-block design with replicates as blocks. Waller-Duncan k ratio t tests were used to separate means (32).

Triplicate thermal inactivation trials (100, 130, 135, or 137°C) were conducted in each case (inoculated CMMS or SPB samples [pH 6.4, 5.7, or 5.0] exposed to nisin [0, 2,000, or 4,000 IU/ml] for 5, 15, or 24 h [4°C before heating]. Survivor curves (log population vs. heating time) were plotted for each trial, and best-fit linear regression lines were determined. D values (time to reduce the population by 90%) were calculated as the negative reciprocal of the survivor curve slope. D value reductions were calculated by percentage differences in D values between nisin treatments (2,000 and 4,000 IU/ml) and controls (0 IU/ml). D values were calculated by the negative reciprocal of the slope of the decimal reduction time curves (mean log D values vs. temperature).

Germination studies. The role of germinants in enhancing the effect of nisin on spore heat sensitivity was evaluated. Sucrose was evaluated as a potential germinant because it is added in CMMSs at a concentration of 6%.

B. stearothermophilus spores (approximately 3.5 × 10⁸ CFU/ml) were suspended in 10 ml of 100 mM SPB (pH 6.4) containing either 0.6 g of germinant (sucrose), 2,000 IU of nisin per ml, or 0.6 g of sucrose plus 2,000 IU of nisin per ml. Spore suspensions were vortexed and placed in a 55°C circulating water bath. A 10-μl sample was taken immediately to represent zero time. Duplicate 10-μl samples were taken every 5 min for a total of 45 min. The samples were placed on coverslips and dried for approximately 10 min by placing the coverslips on a hot plate (Fisher Thermix, Fisher Scientific Co., Dallas, Tex.) set on low temperature (40 to 80°C). Dried samples on coverslips were placed in a dessicator until microscopic examination was performed.

Just before microscopic examination, dried spore samples were rehydrated with a drop of sterile water. Samples were then observed at ×1,000 magnification using a phase-contrast microscope (Olympus BH-2, Tokyo, Japan). A total of 200 organisms per sample were counted. As in the studies performed by Blocher and Busta (2), spores were classified as refractile (phase bright, nongerminated) or nonrefractile (phase dark, germinated). Only spores that were completely phase bright (i.e., no gray spores) were classified as refractile or nongerminated. Percentages of germinated and nongerminated spores were calculated. This experiment was performed in triplicate.

RESULTS

Effect of nisin on thermal resistance of B. stearothermophilus spores. Representative survivor curves for B. stearothermophilus spores in a CMMS supplemented with 0, 2,000, or 4,000 IU of nisin per ml are shown in Figure 1. As depicted, the curves demonstrate a linear order of inactivation with a 3- to 4-log reduction in population and R² values averaging 0.97. Moreover, as the nisin concentration in the CMMS increased, the rate of spore inactivation increased (0.0549 to 0.0796 s⁻¹). Survivor curves followed this same general pattern irrespective of nisin exposure time, heating menstruum, pH, temperature, or spore type.

In preliminary studies, time of spore exposure to nisin (time of contact between spores and nisin in the CMMS before thermal processing) was established. Figure 2 summarizes the mean D₁₃₀°C values for the three exposure times and three nisin concentrations, as well as the percentage of reduction in D₁₃₀°C values for the nisin-supplemented CMMS compared to the control held at 4°C for the same time periods. CMMS (10 to 12% fat cocoa powder) supplemented with nisin and held for 5, 15, and 24 h had significant (P ≤ 0.05) reductions in D₁₃₀°C values when

FIGURE 1. Representative survivor curves for B. stearothermophilus spores heated at 130°C in a CMMS (10 to 12% fat cocoa powder) containing one of three levels of nisin. , 0 IU of nisin per ml; ■, 2,000 IU of nisin per ml; ▲, 4,000 IU of nisin per ml. R² values were 0.96 to 0.98.
FIGURE 2. Thermal inactivation of B. stearothermophilus spores at 130°C in a standard CMMS (10 to 12% fat cocoa powder) with various exposure times to nisin at 4°C. Mean D values with different letters (a through d) are significantly different (P ≤ 0.05). 1Reductions are calculated as the percentage difference in D values between the control (0 IU of nisin per ml) and nisin-containing treatments (D0 nisin − D0 2,000 or 4,000 nisin / D0 nisin × 100). Mean of three trials (n = 3). □ 0 IU of nisin per ml; □, 2,000 IU of nisin per ml; ■, 4,000 IU of nisin per ml.

FIGURE 3. Thermal inactivation of B. stearothermophilus spores at 130°C in various heating menstrua preexposed for 5 hours to one of three levels of nisin. Mean D values with different letters (a through f) are significantly different (P ≤ 0.05). 1Reductions are calculated as the percentage difference in D values between the control (0 IU of nisin per ml) and nisin-containing treatments (D0 nisin − D0 2,000 or 4,000 nisin / D0 nisin × 100). Mean of three trials (n = 3). □ 0 IU of nisin per ml; □, 2,000 IU of nisin per ml; ■, 4,000 IU of nisin per ml.

Compared to controls. As indicated by decreasing D130°C values, spores of B. stearothermophilus were more sensitive to the thermal treatment when preexposed to nisin at 4°C for 15 to 24 h. Moreover, increasing nisin concentration from 2,000 to 4,000 IU/ml did not significantly influence the apparent heat resistance, nor did exposure time beyond 15 h further reduce D values. After 5 h of exposure to nisin, the D130°C values were 21.7, 17.2, and 17.8 s, respectively, for the 0-, 2,000-, and 4,000-IU/ml treatments. After 15 h of exposure, D130°C values significantly (P ≤ 0.05) declined from 18.5 to 14.7 and 13.4 s, respectively. Similar reductions in D130°C values observed between 5 and 15 h of nisin exposure were detected after 24 h of exposure (18.7, 14.0, and 13.9 s, respectively). Regardless of exposure time, nisin-supplemented CMMS reduced the overall heat resistance of B. stearothermophilus. D130°C values declined an average of 20.7 and 18.0%, respectively, for the 2,000- and 4,000-IU/ml treatments after 5 h of exposure. After 15 h of exposure, the average reductions in D values were 20.5 and 27.6%, respectively. Reductions in D values of 25.1 and 25.7%, respectively, were detected for the 24-h exposure treatment. Because the 5-h exposure times produced significant reductions in D values and more closely mimicked industry handling practices during food formulation and mixing, this exposure time was used in subsequent experiments.

Reductions in D130°C values and percentage reductions for nisin-containing treatments versus the control are illustrated in Figure 3. For the CMMSs with 0 to 1% and 10 to 12% fat cocoa powder, there was a significant (P ≤ 0.05) difference between the controls (0 IU/ml nisin) and the nisin-containing treatments. For example, D130°C values for spores in a CMMS with 0 to 1% fat cocoa powder were 19.3, 15.8, and 14.7 s, respectively, for the 0-, 2,000-, and 4,000-IU/ml nisin treatments. However, buffer with or without 6% sucrose resulted in D130°C values of approximately 10 s that did not differ regardless of nisin concentration. Spores in the nutrient-free (i.e., sucrose-free) SPB had D130°C values of 10.3, 10.0, and 10.5 s for the 0-, 2,000-, and 4,000-IU/ml nisin treatments, respectively. Similarly, spores in SPB with 6% sucrose yielded D130°C values of 11.1, 10.3, and 10.4 s for the 0-, 2,000-, and 4,000-IU/ml treatments, respectively. Reductions in D130°C values ranged from 18 to 23.8% in the CMMSs and from 0 to 7.2% in the SPB systems.

D values were calculated for B. stearothermophilus spores by conducting thermal inactivation studies at 130, 135, and 137°C using a CMMS made with 10 to 12% fat cocoa powder (Table 2). zD values were 9.6, 9.0, and 8.4°C for the 0-, 2,000-, and 4,000-IU/ml nisin treatments, respectively. D values for spores at 135 and 137°C were 5.5, 3.9, and 3.3 s (135°C) and 4.0, 3.0, and 2.9 s (137°C) for the 0-, 2,000-, and 4,000-IU/ml treatments, respectively.

Effect of pH on nisin enhancement of heat sensitivity in B. stearothermophilus spores. The effect of pH and nisin on D values for B. stearothermophilus spores heated at 130°C in a CMMS (10 to 12% fat cocoa powder) and the percentage of reduction in D values between nisin treatments are shown in Figure 4. Compared to the reference system (pH 6.4), D130°C values in an acidified CMMS (pH
5.7 or 5.0) are significantly lower (P ≤ 0.05). At a pH of 5.7, $D_{130°C}$ values were 15.5, 11.3, and 10.4 s for the 0-, 2,000-, and 4,000-IU/ml treatments, respectively, whereas $D_{130°C}$ values of spores in the pH 5.0 system were 8.6, 5.6, and 5.0 s for the 0-, 2,000-, and 4,000-IU/ml treatments, respectively. This figure illustrates an increasing effect of nisin with decreasing pH as reflected in the increasing percentage reductions in $D_{130°C}$ values observed for the 2,000- and 4,000-IU/ml treatments. At a pH of 6.4, the mean reduction in $D_{130°C}$ value was 19.3%, whereas at pH 5.7 and 5.0, the reductions averaged 30 and 38.2%, respectively. Data were analyzed for interaction effects between pH and nisin, and only individual effects were found.

Effect of nisin and pH on thermal resistance of *B. licheniformis* spores. Table 3 summarizes the mean $D_{100°C}$ values and percentages of reduction in $D_{100°C}$ values calculated between the control and two nisin-containing treatments for *B. licheniformis* spores heated in a CMMS (10 to 12% fat cocoa powder) at pH values of 6.4 and 5.0. $D_{100°C}$ values were 3.3, 2.8, and 2.8 min for the 0-, 2,000-, and 4,000-IU/ml treatments, respectively, at pH 6.4. The reduction in $D_{100°C}$ was 15.2% for both the 2,000- and 4,000-IU/ml treatments.

The effects of pH and nisin on *B. licheniformis* spores are also shown in Table 3. $D_{100°C}$ values for spores in the higher-acid CMMS (10 to 12% fat cocoa powder) yielded significantly (P ≤ 0.15) lower $D_{100°C}$ values than the more basic CMMS of 1.0, 0.8, and 0.8 min for the 0-, 2,000-, and 4,000-IU/ml nisin concentrations, respectively. These reductions in $D_{100°C}$ values reflected a 20% decrease in heat resistance of the nisin-treated spores. *B. licheniformis* data were also analyzed for interaction effects between pH and nisin, and only individual effects were found.

Effect of nisin on spore germination. The effect of nisin on the germination of *B. stearothermophilus* spores in the presence of a germinant (sucrose at a concentration of 6%), nisin (2,000 IU/ml), or a combination of both was evaluated in SPB (pH 6.4). Germination in spores (phase dark; initial germination percentage of 62.3%) after 5 and 10 min of heating was greatest in the treatment containing both sucrose and nisin (84.3 ± 2.08% and 94.3 ± 3.60%, respectively) (data not shown). Germination percentages for nisin alone (87.0 ± 4.58%) and germinant alone (87.3 ± 2.89%) after 10 min were similar but higher than those of spores heated in unsupplemented SPB (data not shown).

## DISCUSSION

Spores of *B. stearothermophilus* ATCC 12980 and *B. licheniformis* ATCC 14580 heated in a CMMS supplemented with nisin were more sensitive to heat (lower $D$ values) than spores in the same medium without nisin. The mechanism of action of nisin against spores is a controver-

### TABLE 2. $D$ values and $z_D$ values for thermal inactivation trials (130, 135, and 137°C) of *Bacillus stearothermophilus* spores in a CMMS* supplemented with three concentrations of nisin*

<table>
<thead>
<tr>
<th>Nisin concentration (IU/ml)</th>
<th>$D$ values (s)</th>
<th>$z_D$ value (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>130°C</td>
<td>135°C</td>
</tr>
<tr>
<td>0</td>
<td>21.7 <em>A</em></td>
<td>5.5 <em>C</em></td>
</tr>
<tr>
<td>2,000</td>
<td>17.2 <em>B</em></td>
<td>3.9 <em>E</em></td>
</tr>
<tr>
<td>4,000</td>
<td>17.8 <em>B</em></td>
<td>3.3 <em>E</em></td>
</tr>
</tbody>
</table>

* CMMS, chocolate milk model system (10–12% fat cocoa powder).

* Spores were held in the CMMS with nisin for 5 h before heating.

* Mean $D$ values followed by different letters are significantly different (P ≤ 0.10, n = 3).

### FIGURE 4. Thermal inactivation of *B. stearothermophilus* spores at 130°C in a standard CMMS (10 to 12% fat cocoa powder) at pH 5.0 to 6.4 exposed to one of three levels of nisin. Mean $D$ values with different letters (a through f) are significantly different (P ≤ 0.05). Spores were held in the CMMS with nisin for 5 hours before heating.

1. Reductions are calculated as the percentage difference in $D$ values between the control (0 IU of nisin per ml) and nisin-containing treatments ($D_{nisin} - D_{0 nisin} \times 100$). Mean of three trials (n = 3).

2. 0 IU of nisin per ml; □, 2,000 IU of nisin per ml; ■, 4,000 IU of nisin per ml.

### TABLE 3. $D$ values and percent reduction in $D$ values for *Bacillus licheniformis* spores preexposed for 5 h to the control and nisin-containing treatments in a CMMS* adjusted to pH 6.4 and 5.0 before heating at 100°C

<table>
<thead>
<tr>
<th>pH</th>
<th>Nisin concentration (IU/ml)</th>
<th>$D$ values (min)</th>
<th>Reduction of $D$ values (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nisin-containing treatments</td>
</tr>
<tr>
<td>6.4</td>
<td>0 (control)</td>
<td>3.3 <em>A</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2,000</td>
<td>2.8 <em>B</em></td>
<td>15.2</td>
</tr>
<tr>
<td></td>
<td>4,000</td>
<td>2.8 <em>B</em></td>
<td>15.2</td>
</tr>
<tr>
<td>5.0</td>
<td>0 (control)</td>
<td>1.0 <em>C</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2,000</td>
<td>0.8 <em>D</em></td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>4,000</td>
<td>0.8 <em>D</em></td>
<td>20</td>
</tr>
</tbody>
</table>

* CMMS, chocolate milk model system (10–12% fat cocoa powder).

* Reductions are calculated as the percentage difference in $D$ values between the control and nisin-containing treatments: ($D_{nisin} - D_{0 nisin} \times 100$).

* Mean $D$ values followed by different letters are significantly different (P ≤ 0.15, n = 3).
sial topic, and research in this area has been inconclusive. However, current research supports the idea that the activity of nisin against spores is predominantly bacteriostatic and not bacteriocidal (7, 27). Nisin activity against spores has been shown to occur at the stage of preemergent swelling, leading to prevention of spore outgrowth (1, 14). Limited molecular studies have suggested that this action is caused by the binding of some unusual dehydroalanine groups contained in nisin with sulfhydryl groups on protein residues of freshly germinated spores (28). Our data are in agreement with those of previous studies using nisin in the thermal processing of chocolate milk. These studies demonstrated that less severe heat treatments were needed to obtain shelf-stable products by using nisin as an adjunct to thermal processing (12, 17). By reducing the heating time, these milks are likely to have improved quality and nutritional value (15).

D values did not vary significantly between the treatments containing 2,000 and 4,000 IU of nisin per ml regardless of exposure time, suggesting that treatment costs could be reduced without compromising efficacy by using 2,000 IU/ml. The nisin concentrations used in our studies are comparable with those of other studies that have used nisin (3,200 IU/ml) as an aid to thermal processing of chocolate-flavored milk (17) or that observed the effect of nisin (4,000 and 3,200 IU/ml) on the keeping quality of pasteurized milk and half-and-half (18, 24).

Increasing the exposure time of spores in the standard CMMS (10 to 12% fat cocoa powder) from 5 to 15 h increased spore sensitivity to heat in the control and nisin treatment groups (Fig. 2). This finding may indicate that increasing spore exposure time in the standard CMMS before thermal processing may promote spore germination and thus lead to an increase in heat sensitivity of the germinated spores. Furthermore, germinated spores would also be more sensitive to nisin activity. As described in Materials and Methods, B. stearothermophilus spores were initially heat-shocked before each experiment to produce more homogeneous spores. The possibility exists that this initial heat-shock treatment may have induced some spore germination, although the ratio of phase-dark to phase-bright spores did not change appreciably (data not shown). Furthermore, spore germination may also have been initiated upon reaction with nisin. Any or all of these factors may have contributed to the reduction in sensitivity of spores to heat. Although addition of nisin to a chocolate milk drink 15 to 24 h before pasteurization would be expected to reduce thermal resistance of contaminating spores, current production scenarios may preclude such extended exposure times before thermal treatment. Shorter exposure times after product formulation, such as 5 h or less, would be more commercially acceptable.

Two CMMS were evaluated in this study to provide baseline data of model dairy beverage formulations of different fat concentrations (from cocoa) at a constant pH. SPB was included to provide a nutrient-free (germinant-free), nonfat system reference and to analyze the effects of sucrose as an ingredient. When comparing the control (0 IU of nisin per ml) CMMS (10 to 12% fat cocoa powder) to a lower fat CMMS (0 to 1% fat cocoa powder), a significant (P ≤ 0.05) difference in D130°C values was clearly detected (Fig. 3). Although cocoa powder made up only 1% (by weight) of the CMMS, the higher fat system did protect the spores from heat, as shown by the higher D130°C values. Some previous studies attributed this protection by fat to the poor heat conductivity of lipids (22). Correlations have also been made between the water content of lipids and spore heat resistance. Lipids saturated with higher concentrations of water provided less protection of spores to heat. Other studies (26) disregarding the possibility of poor heat conductivity and water content of lipids suggested that increasing free fatty acids in lipids may provide better protection of spores from heat. Similar findings were also observed in the treatment with 4,000 IU of nisin per ml, in which D130°C values of spores heated in the 0 to 1% fat cocoa powder CMMS were significantly (P ≤ 0.05) lower than those derived from heating trials involving the 10 to 12% fat cocoa powder CMMS. Previous investigators have hypothesized that fat may bind nisin, rendering it unavailable to react with spores or vegetative cells (6). Nisin is a hydrophilic molecule that would be attracted to lipid components in foods; thus, molecules of nisin may not be uniformly distributed in foods, which may reduce its antibacterial activity (30). Studies performed by Jung et al. (21) on Listeria monocytogenes compared the effect of nisin in fluid milk containing various amounts of fat. It was found that nisin activity decreased 33% when added to skim milk and 50% when added to milk containing 1.29% fat. The investigators concluded that nisin molecules may actually be bound to milk fat globules.

No significant (P ≥ 0.05) differences in D130°C values were detected in the SPB systems regardless of the amount of nisin added (Fig. 3). This suggests that nisin has a minimal or no effect on enhancing inactivation of spores in a nutrient-free or very simple (sucrose) nutritional system. It appears that nisin requires the presence of specific germinants or nutrients for spore activity and that this activity does not extend to dormant spores. Another explanation might be that spores are more sensitive to heat (lower D values) in a nutrient-free system in comparison to any effect caused by nisin and/or sucrose.

The zD values reported for B. stearothermophilus spores in this study are typical of those cited in the literature, which are generally between 8 and 10°C (36). Larger zD values in control treatments (0 IU of nisin per ml) indicate a greater temperature dependence for spores inactivated in a CMMS (10 to 12% fat cocoa powder) without nisin versus those in nisin-containing treatments (Table 2). This study also demonstrated that the higher the process temperature (130 vs. 137°C), the greater the impact of nisin on reducing spore resistance. These data are promising for the dairy industry, because with the addition of nisin, processors may be able to heat chocolate drinks at lower temperatures and for shorter times, resulting in more acceptable quality attributes, such as functionality, viscosity, flavor, and similar or better shelf life.

Previous studies have documented an increase in nisin activity against spores as the pH of the medium is reduced.
(33). This observation is associated with the greater stability and activity of nisin in more acidic environments (6, 30). In the CMMS (10 to 12% fat cocoa powder), spores were more sensitive to heat as pH decreased (Fig. 4). This observation is clearly supported by numerous investigators (2, 9, 10, 19, 23) and is the basis of current regulatory requirements for canning of acid and low-acid foods. Regardless of pH, the $D_{130^\circ C}$ values derived for B. stea\thermophilus spores heated in the nisin-containing CMMS (10 to 12% fat cocoa powder) treatments were significantly ($P \leq 0.05$) lower than those from the corresponding controls. Furthermore, significant ($P \leq 0.05$) differences in $D_{130^\circ C}$ values within CMMS treatments were detected with all three pH variables. For B. stea\thermophilus, a reduction in CMMS pH of 1.4 units resulted in significant ($P \leq 0.05$) reductions in $D_{130^\circ C}$ values of approximately 15 and 24% in the CMMSs containing 2,000 and 4,000 IU of nisin per ml nisin, respectively. Similar reductions in $D_{100^\circ C}$ values related to the pH of the medium were also observed for B. licheniformis (Table 3).

The role of nisin in germination is not clearly defined. Mazzotta and Montville (25) recently evaluated the progerminant activity of nisin on Clostridium botulinum 169B spores. Their investigations showed a 1.6-fold increase in the germination rate of spores exposed to 1,000 IU of nisin per ml. Germination was measured by the decrease in absorbance at 600 nm, phase-contrast microscopy, dipicolinic acid efflux, and thermal resistance studies. Our preliminary studies show an increase in germination from 62.3% at time 0 to 94.3% at 10 min using nisin in the presence of sucrose (data not shown). Moreover, our results seem to indicate an additive effect on germination in the buffers containing nisin alone and sucrose alone and a possible synergistic effect between nisin and sucrose when added in combination. Further studies using spore crops with a lower initial percentage of germinated spores to increase the sensitivity of this method are warranted. Starting with a spore crop with fewer germinated spores would allow for more nisin-mediated germination to occur.

In conclusion, this study clearly verified that addition of Nisaplin to dairy-based beverages, such as a chocolate milk drink, or other foods intended to be heated reduces the thermal resistance of selected bacterial spores. Increased sensitivity of spores to heat may provide food processors with an opportunity to reduce thermal processes and expenses while maintaining product quality, functionality, and shelf stability.

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