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A Research Note

Thermal Resistance and Growth of *Bacillus licheniformis* and *Bacillus subtilis* in Tomato Juice

J. H. RODRIGUEZ, M. A. COUSIN*, and P. E. NELSON

Department of Food Science, Purdue University, West Lafayette, Indiana 47907

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ABSTRACT

Bacillus species are common contaminants of soil and can be found with tomatoes and other vegetables and fruits. This research was done to assess the thermal stability of spores of acid tolerant *Bacillus licheniformis* and *Bacillus subtilis* in tomato juice. The D values at 90, 95, and 100°C were 29.5, 15.8, and 5.7 min for *B. subtilis* and 29.9, 12.2, and 5.9 min for *B. licheniformis*, respectively. The z value for *B. subtilis* was 14°C and for *B. licheniformis* was 14.2°C. Aerobically, *B. subtilis* spores could germinate and outgrow in tomato juice (pH 4.4) within 4 d at 35°C at inoculum levels as low as 1 spore per ml; however, *B. licheniformis* spores could only germinate and outgrow if levels were 10^4 spores per ml or higher. Both *Bacillus* species could raise the pH of tomato juice to above 4.8 when grown aerobically. Neither *B. licheniformis* nor *B. subtilis* could germinate and outgrow anaerobically in tomato juice (pH 4.4).

Bacillus spores are common contaminants of raw fruits and vegetables. The presence of *Bacillus* spores and vegetative cells in thermally processed foods will depend upon the thermal treatment given to the foods. Fields et al. (*8*) isolated *Bacillus* species from 30% of the home-canned tomatoes evaluated. Mundt et al. (15) isolated *Bacillus licheniformis* from home-canned tomatoes and tomato juice. When these reports appeared, the United States Department of Agriculture and Food and Drug Administration expressed concern over the adequacy of the thermal processing recommendations for home and commercially canned tomato products, respectively. Montville and Sapers (*14*) evaluated the thermal resistance of spores of *B. licheniformis* [reclassified as *B. subtilis* by Rodriguez et al. (*22*)], grown in trytic soy broth and added to tomato puree (pH 4.4) and found that they had $D_{100°C}$ of 2.0 min.

The thermal resistance of bacterial spores is affected by many factors, such as species, strain, cationic environment of the sporulation medium, and pH (*19-21*). Since spores found in nature are often more heat resistant than spores produced in the laboratory, the medium used to produce the spores is important. Calcium and magnesium ions affected both the cationic concentration of spores and the thermal resistance of spores (*1,2,5-7,13,24,26*). Conditions during the heating of spores also will affect their thermal resistance. Roberts and Hitchins (*20*) reported that the pH of the heating medium influenced the thermal resistance of bacterial spores. Generally, thermal resistance decreases as the pH becomes more acid.

In addition to the concern over thermal resistance of these *Bacillus* species, there was concern that they could grow in acid foods and raise the pH above 4.8 where *Clostridium botulinum* could initiate growth. Species of *Bacillus* (*8,12*) and several mold species (*10,17*) raised the pH of tomatoes and tomato juice to greater than pH 4.8 [normal tomato products have a pH ranging from 4 to 4.7 (*16*)]. Therefore, the survival and growth of *Bacillus* species in tomato products can result in pH elevation to the low acid range.

For growth of *Bacillus* species to occur in thermally processed tomato products, four criteria must be met: activation, germination, outgrowth of spores, and vegetative growth. The thermal process could serve as the activation step if it were not lethal to the spores. Tomato products contain sufficient nutrients and can be stored under conditions that allow germination, outgrowth, and vegetative growth. The purpose of this research was to study the thermal resistance in tomato juice of spores of *B. licheniformis* and *Bacillus subtilis*, which were isolated from acid foods, and to determine the initial inoculum level that was necessary for aerobic and anaerobic growth in tomato juice at pH 4.4.

MATERIALS AND METHODS

Microorganisms

Spores of *B. subtilis* (075-T-09, T. J. Montville, U.S. Department of Agriculture, Philadelphia, PA), *B. subtilis* (042-T-04, M. L. Fields, University of Missouri, Columbia) and *B. licheniformis* (64-83-46, National Food Processors Association, Berkeley, CA) were obtained from frozen stock solutions according to Rodriguez et al. (*22*). Originally, *B. subtilis* spores were isolated from home-

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canned tomatoes and *B. licheniformis* spores were isolated from acidified onions.

The strains were streaked onto nutrient agar (Difco Laboratories, Detroit, MI) after receipt and incubated at 32°C for 24 h. Isolated colonies were selected, inoculated into nutrient broth (Difco), incubated at 32°C for 24 h, and then streaked onto nutrient agar slants for maintenance. All cultures were stored at 4°C and transferred to fresh slants every 3 months.

Spore production

Spores were produced in tryptic soy broth (TSB, Difco) and mineral medium of Donnellan et al. (*7*) according to Rodriguez et al. (*22*). Final spore suspensions were frozen and stored at -20°C until used.

Inoculum of spores needed for aerobic and anaerobic growth in tomato juice

Frozen spores were thawed, diluted to levels of 10^0 to 10^6 spores per ml at intervals differing by a factor of 10, and heated for 10 min at 80°C. Seven inoculum levels from 10^0 to 10^6 spores per ml were used for *B. licheniformis* and six inoculum levels from 10^0 to 10^5 spores per ml were used for *B. subtilis* 075-T-09. The spores were inoculated into sterile commercially produced tomato juice (pH 4.4) and were incubated at 35°C for 60 d. For anaerobic growth, the tubes of tomato juice were steamed for 15 min, cooled, inoculated, placed into anaerobic jars, and incubated at 35°C. Ten and nine replicate tubes of tomato juice were used for each inoculum level for aerobic and anaerobic growth, respectively. Uninoculated tubes of tomato juice served as controls.

The aerobic tubes were visually examined every day for 60 d for pellicle formation. Anaerobic tubes were examined after 60 d. After 60 d, the pH of the medium in each tube was taken. Plate counts on nutrient agar of the medium from some tubes showing no growth after 60 d were made to determine if the cells had increased without any obvious signs of growth or if the cell number had decreased.

Thermal resistance

Frozen spore suspensions were quickly thawed, mixed, and diluted to 10^7 to 10^8 spores per ml in 0.1% peptone (Difco) water. Nine milliliters of commercial tomato juice (pH 4.4) preequilibrated to 90, 95, and 100°C, in a water bath, was inoculated with 1 ml of spores of *B. licheniformis* and *B. subtilis* 075-T-09. Every 2 to 7 min (depending on the temperature) over a 33-min period, 1 ml was removed from each tube and added to 9 ml of Butterfields phosphate buffered sterile water held in an ice bath (*9*). Serial dilutions were made in duplicate and spread onto nutrient agar (Difco) plates which were incubated at 35°C for 24 to 36 h. Two trials were done for each species and each temperature. Survivor data were plotted using the least squares linear regression (excluded time 0), the line of best fit was established, the slope was determined, and the D and z values were calculated.

RESULTS AND DISCUSSION

Thermal resistance of Bacillus *spores*

Bacillus subtilis 075-T-09 and 042-T-04 and *B. licheniformis* spores were used to determine if they could withstand thermal processing of tomato juice. These strains were chosen because they were able to grow aerobically in tomato juice and raise the pH to above 4.8. As seen in Fig. 1, when the spores of *B. subtilis* 042-T-04 were produced in TSB, they were much less heat resistant than the same spores produced in the mineral medium of Donnellan et al. (*7*). This suggests that spores produced in foods that contain cations, such as calcium and magnesium, will be more

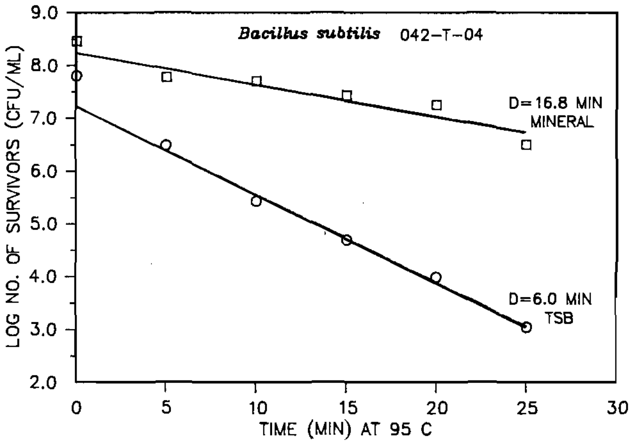

Figure 1. *Survivor curves for* Bacillus subtilis *042-T-04 spores produced in mineral medium (□) and tryptic soy broth (○) and subsequently heated in tomato juice.*

heat resistant than those produced in laboratory media. Cations that were present in media caused an increase in heat resistance by *Bacillus* spores (*6,13,25*). The D values were calculated from the survivor curves:

$$D = \frac{-1}{\text{slope}}$$

The D_{95} for spores of *B. subtilis* 042-T-04 produced in TSB was 6 min compared to a D_{95} of 16.8 min for spores produced in the mineral medium. Many spores found in foods come from soil and a medium with minerals would tend to approximate conditions of the soil more than a medium such as TSB. In evaluating a spore for its heat resistance, a medium that is closest to natural conditions would be desired.

The survivor curves for spores of *B. subtilis* 075-T-09, produced in the mineral medium and heated at 90-100°C, are shown in Fig. 2. Although this *B. subtilis* strain was the same one used by Montville and Sapers (*14*) for their thermal resistance studies using spores produced in tryptic soy broth (D values of 7.5, 5.1, and 1.9 min at 90, 95, and 100°C, respectively), D values in tomato juice for spores produced in the mineral medium were 29.5, 15.8, and 5.7 min at 90, 95, and 100°C, respectively. This discrepancy could be due to the use of the medium with cations which affords thermal resistance.

The survivor curves for spores of *B. licheniformis* produced in mineral medium and heated at 90-100°C are shown in Fig. 2. The $D_{90\text{-}100}$ are close to those for spores of *B. subtilis* 075-T-09. The z values $\left(\frac{-1}{\text{slope}}\right.$ from thermal death time curves, data not given$\left.\right)$ for spores of *B. subtilis* 075-T-09 and *B. licheniformis* were 14 and 14.2 °C, respectively. Montville and Sapers (*14*) calculated the z value for *B. subtilis* 075-T-09 as 14.9°C. Based on the D and z values calculated in this study, spores of both *B. subtilis* 075-T-09 and *B. licheniformis* could survive commercial thermal processing (10 to 60 min at 100°C) of tomato juice depending upon the initial pH of the tomato juice, the initial spore load, and the can size, and the time-temperature of processing.

Inoculum of spores needed for aerobic and anaerobic growth in tomato juice

Because spores of both *B. subtilis* and *B. licheniformis* survived the thermal processing, it was necessary to deter-

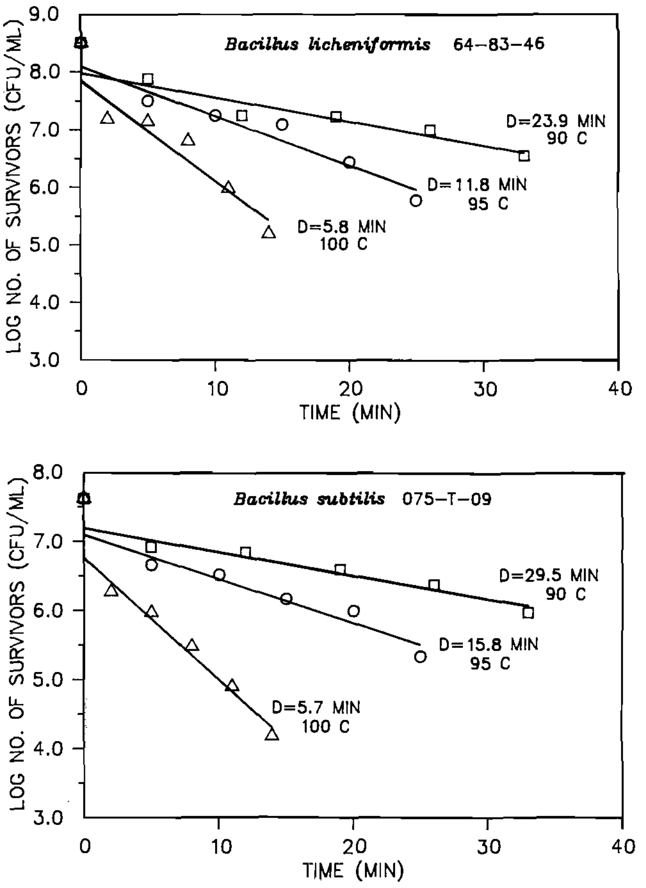

Figure 2. *Survivor curves for* Bacillus subtilis *075-T-09 and* Bacillus licheniformis *64-83-46 spores produced in mineral medium and heated at 90°C (□), 95°C (○), and 100°C (Δ) in tomato juice.*

mine what inoculum level of spores was needed for activation, germination, outgrowth, and vegetative growth in tomato juice. *B. subtilis* 075-T-09 spores showed outgrowth aerobically by pellicle formation in tomato juice (pH 4.4) at levels of 10^2 through 10^5 spores per ml after 2 d at 35°C. After 4 d at 35°C, pellicles were seen on tomato juice tubes inoculated with 10^0 and 10^1 spores per ml. After 60 d, the final pH of the tomato juice had been raised to 8.12 to 8.78 for all replicates in tomato juice at all inoculum levels.

B. licheniformis spores took longer than *B. subtilis* 075-T-09 spores to initiate growth aerobically in tomato juice at 35°C. After 8 d, only three of 10 tubes showed pellicles when 10^6 spores per ml were used as the inoculum. By 35 d, all replicates had pellicles for the 10^6 spores per ml inoculum. Only 50% of the tomato juice tubes with 10^5 spores per ml and 20% of those at 10^4 spores per ml had pellicles after 60 d at 35°C. Cells were not recoverable from tubes where there was no pellicle. The pH was also raised to 6.9 to 9.1 in the tomato juices when there was a pellicle formed. The longer the *B. licheniformis* grew, the higher was the final pH.

Because only a fraction of the *B. licheniformis* spore population is able to germinate and outgrow at pH of 4.4, the inoculum must be larger for the probability of germination and outgrowth in tomato juice. A small number of cells was able to initially grow in tomato juice and change the pH so that other cells could grow. The results for these spores of *B. licheniformis* agree with those reported by other researchers for spores of *B. cereus* and *C. botulinum* where the initial load of spores was important to determine whether growth occurred at a given pH (*11,18,23*). At lower pH values, a higher inoculum level was needed to assure initiation of growth in either food or laboratory medium.

Anaerobically, there was no growth in any tubes for *B. licheniformis* or *B. subtilis* 075-T-09 spores. *B. subtilis* strains are strict aerobes, but *B. licheniformis* strains are facultative anaerobes. Rodriguez et al. (*22*) also showed that *B. licheniformis* vegetative cells could not grow anaerobically in tomato juice. Bell and DeLacy (*3*) concluded that *B. licheniformis* needed oxygen for outgrowth of germinated spores in chub luncheon meats. Further research with tryptone - yeast extract - glucose medium showed no anaerobic outgrowth of germinated spores (*4*). The research presented here shows that *B. licheniformis* and *B. subtilis* will not grow in properly canned tomato juice; therefore, pH elevation postprocessing is not of concern in properly canned tomato juice.

CONCLUSIONS

If spores of *B. licheniformis* and *B. subtilis* can survive processing temperatures used for thermally processed tomato juice, they would not be able to grow anaerobically in tomato juice at a pH of 4.4 or below. In fact, tomato juice seemed to be bactericidal to *B. licheniformis* spores, and they would only outgrow if there were very high spore levels. The inoculum level was not critical for aerobic outgrowth of *B. subtilis* spores; however, *B. licheniformis* needed 10^4 spores per ml or higher to outgrow aerobically in tomato juice at pH 4.4. Spores of *B. subtilis* and *B. licheniformis* can be activated by the thermal processing of tomato juice, but they can only outgrow if aerobic conditions are present in the can.

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