Survival of *Salmonella* and *Enterococcus faecium* in high fructose corn syrup and honey at room temperature (22 °C)

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**ARTICLE INFO**

Keywords: Honey
High fructose corn syrup (HFCS)
Survival
*Salmonella*
*Enterococcus faecium*
Osmotic pressure

**ABSTRACT**

Salmonellosis has been frequently associated with the consumption of high-sugar, low-moisture foods. Honey and high fructose corn syrup (HFCS) are widely used liquid sugars that are added as humectants in low-moisture foods. The objective of this study was to determine the ability of *Salmonella* and its presumable surrogate, *Enterococcus faecium* NRRL B-2354, to survive in honey and HFCS during storage at room temperature (~22 °C). Using freeze-dried and lawn grown bacteria, the survival of *Salmonella* and *E. faecium* in honey and HFCS was determined. Regardless of the inoculation methods, more than 5 log reductions were observed in both bacteria in honey and HFCS after 21-days of storage at 22 °C. The pathogens and surrogate in honey and HFCS fell below the detectable limit after 28-days of storage. Thus, the tested bacteria are not able to survive in honey and HFCS over one-month storage at room temperature. The similar level of bacterial reduction in honey and HFCS in storage suggests that the main cause was not the commonly perceived antimicrobial agents in honey. In addition to high acidity of these liquid sugars, the extremely high osmotic pressure is likely the main reason for bacterial death in honey and HFCS during storage. The data provided useful information in developing effective microbial-safe strategies to be incorporated in the preparation or storage of low-moisture food and ingredients.

1. Introduction

Foodborne disease is one of the biggest public health concerns globally. In the United States, *Salmonella* causes around 11% of foodborne diseases annually (Scallan et al., 2011). *Salmonella* outbreaks were frequently associated with high-moisture poultry and meats, such as eggs, poultry and beef (CDC, 2018, 2020; Tauxe, 1991), as well as vegetables and fruits, such as onions and peaches (CDC, 2020a & b). Additionally, there are increasing outbreaks caused by *Salmonella* associated with low-moisture foods, such as spices, nuts, cereal, coconut, peanut butter, milk powder, and dried fruits (CDC, 2008, 2009, 2016). In a low water activity (aw) environment, *Salmonella* cannot grow or multiply, but it can survive for a long time and cause safety issues for human beings. For example, *Salmonella* were detected in dried fruits, including dried cranberries, raisins, and strawberries after 42 days; and in date paste after 126 days of storage under ambient conditions (Beuchat & Mann, 2014; Podolak, Enache, Stone, Black, & Elliott, 2010).

Low-moisture food products containing high concentrations of sugar have also been associated with salmonellosis outbreaks. These foods include chocolate bars (60% sugar content) (Eun et al., 2019; Werber et al., 2005), halva and high sugar, sesame seed-based product (Brockmann, 2001; De Jong et al., 2001), and honey smacks cereal (30–50% of sugar content) (USDA-FDA, 2019). For some *Salmonella* serotypes such as *Salmonella* serovars Eastbourne, Napoli and Typhimurium isolated from chocolate, a very low infection dose (<10^1–10^2 CFU/g) of bacteria counts in low-moisture products was enough to cause salmonellosis infections (EU, 2014).

Liquid sugars such as high fructose corn syrup (HFCS) and honey are added as an ingredient for sugars in most of the low-moisture foods. HFCS is a fructose-glucose liquid sweetener which is used as an alternative to sucrose, due to its low cost and desired physical and functional attributes to food and beverage applications, including sweetness, flavor...
enhancement, color and flavor development, and osmotic stability (White, 2014). HFCS is as generally recognized as safe (GRAS), primarily due to the addition of enzymes during preparation that were affirmed as GRAS (FDA, 2017). The sugar composition (i.e. glucose to fructose ratio) is nearly the same as that of honey, invert sugar, or sucrose which were previously declared as GRAS (Stavanga et al., 2006; USFDA, 1996). HFCS is used extensively in baked goods, canned fruits, jams and jellies, chocolate syrups and many other processed foods (Hanover & White, 1993).

Honey, a naturally sweet substance, is consumed as a healthy food ingredient and applied toward the treatment of a broad spectrum of diseases (Ajibola, Chamunorwa, & Erwanger, 2012). Honey is known to be a complex product with its main ingredients being fructose and glucose and its minor components including vitamins, minerals, amino acids, organic acids, enzymes, and polyphenols (Clechovská & Vorlova, 2001). Honey is known for its antimicrobial activities against various acids, organic acids, enzymes, and polyphenols (Olaitan, Adeleke, & Ola, 2007). Currently, information on the survival of Salmonella in HFCS and honey alone are limited. Therefore, this study aimed to (1) determine the survival of Salmonella and Enterococcus faecium NRRL B-2354 in HFCS and honey stored at ambient temperature (~22 °C), (2) verify the E. faecium is an appropriate surrogate strain of Salmonella in these liquid sugars, and (3) study the influence of osmotic pressure on survivability of Salmonella and E. faecium.

2. Materials and methods

2.1. Sample preparation

Organic raw honey (Great Value Brand) was purchased from a local Walmart store (Pullman, WA). High fructose corn syrup (HFCS-55) containing 55% fructose & 45% glucose was obtained from Golden barrel (Honey brook, PA). The aw of honey and syrup was measured at 23 °C with an Aquameter (Aqualab Series 3, Decagon Devices, Inc., Pullman, WA). The moisture content of samples was measured on wet basis according to an AOAC method, using a gravimetric method with an ADP-31 vacuum oven (Yamato 116 Scientific, Inc., Santa Clara, CA) set at 70 °C with a vacuum pressure of 0.08 MPa for 24 h. The pH, moisture content, density, and sugar content (on wet basis) were determined according to the International Honey Commission (Stefan, 1984). The viscosity of the samples was measured using a Discovery Hybrid Rheometer HR-3 (159 Luken's Drive, New Castle, DE). All samples were measured in triplicate.

2.2. Determination of osmotic pressure of HFCS and honey

Osmotic pressure is the hydrostatic pressure exerted across a semi-permeable membrane due to osmosis. The osmotic pressure in a solution of low solute concentration can be determined using Eq. (1) (Foster & Spector, 1995; Spector & Kenyon, 2012):

\[
\pi = i \text{MRT} \tag{1}
\]

where,

\[\pi = \text{Osmotic pressure (atm)}\]
\[i = \text{Van't Hoff’s factor (this is the number of ions that will form when a solute is dissolved in water)}\]
\[M = \text{Osmolarity or osmotic concentration (mol/L)}\]
\[R = \text{Gas constant (0.08206 L atm. mol}^{\text{-1}} \cdot \text{K}^{-1})\]
\[T = \text{Temperature in Kelvin (K)}\]

Osmolarity is the number of osmoles of solute per liter of solution. It is expressed as mOsmol/L. (Erstad, 2003). Osmolarity was determined either experimentally or calculated by using Eq. (2) as described below.

\[M = \frac{n}{V} \tag{2}\]

where,

\[M = \text{Osmolarity (mol/L)}\]
\[n = \text{number of moles of solute (mole)}\]
\[V = \text{volume of the solution in liters}\]

Table 2 lists: (a) Density (g/L), (b) Concentration of solute (g), (c) Estimated molecular weight (g/mole), (d) Calculated volume of solution (v), (e) Calculated number of moles of solute (n), and (f) Calculated molarity for 100% honey and HFCS.

The experimental osmolarity was determined using the osmometer analyzer (Osmette S model 4002 Precision Systems, INC, Natick, MA) (Ali, Alqarni, Owayss, Hassan, & Smith, 2017; Erstad, 2003). Due to the narrow measurement range (0–2.000 mOsmol/L) of this instrument and high osmolarities of honey and HFCS, samples were diluted with distilled water to 5, 10, 15, 20, 25, and 30%. The diluted samples were then used to measure the osmolarity. A correlation between sample concentrations and the measured osmolarities was developed, and the osmolarity for 100% honey and HFCS were extrapolated (Fig. 1). Finally, the osmotic pressure was determined using Van’t Hoff’s equation (Eq. (1)).

2.3. Bacterial strains

Three Salmonella strains, (S. Enteritidis PT30, S. Tennessee K4643, & S. Agona 447967), were used in this study to prepare a three-strain cocktail. S. Enteritidis PT30 was obtained from Dr. Linda Harris (University of California, Davis). S. Tennessee K4643 and S. Agona 447967 were kindly gifted by Dr. Nathan Anderson (USDA, Greater Chicago, Illinois). E. faecium NRRL B-2354 strain was obtained from the USDA Agricultural Research Service (USDA-ARS) from Peoria, Illinois. All the strains were stored in a stock solution of trypticase soy broth supplemented with 0.6% (w/v) yeast extract (TSBYE) (Hardy Diagnostics, Santa Maria, CA) and 20% glycerol at ~80 °C until use.

2.4. Culture and inoculum preparation

2.4.1. Dry inoculation method

For dry inoculation method, a loop of culture stock of each Salmonella strain and E. faecium was transferred to 9 mL of TSBYE and incubated at 37 °C for 24 h. Three mL of each culture were transferred to 30 mL of TSBYE and incubated at 37 °C for 24 h. Then, 4 mL of these previous cultures were transferred to 400 mL of TSBYE in a conical flask and placed in an incubated shaker (Thermo Scientific™ MaxQ4000 Benchtop Orbital Shakers, Marietta, OH, USA) with a constant shaking speed at 230 rpm at 37 °C for 24 h. The cultured bacteria was transferred to centrifuge tubes and then centrifuged at 6000 × g for 15 min at 4 °C (Centrifuge 5810 R®, Eppendorf North America, Hauppauge, NY). The pellets were washed three times and re-suspended in 2.0 mL of sterilized distilled water before use. For freeze-drying, a 250 μL sample of the prepared suspension was transferred into a sterile 1.5 mL Snaplock Microtube, pre-frozen immediately in liquid nitrogen and transferred to a freeze-drying system (Labconco Corporation, Kansas City, MO, USA) where it was frozen dried at ~90 °C for 48 h (Xu et al., 2015).

Liquid honey at room temperature was highly viscous. It was difficult to thoroughly mix bacterial culture with honey samples. Thus, before inoculation, 20 g of liquid honey was transferred to 50 mL beaker, heated at 45 °C for 5 min in a convection oven (Yamato Scientific
Fig. 1. Experimental molarity at different concentrations of high fructose corn syrup (HFCS) (A) and honey (B) at 22 °C.

*The green and red dots are the molarity of glucose and fructose adopted from (Lide, 2004). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

America Inc., CA, USA), mixed with 100 mg of freeze-dried bacteria, and then cooled immediately to room temperature (22 °C). For HFCS, a 100 mg of freeze-dried bacteria was added to a 20 g of HFCS without heating. The inoculated samples were vortexed (Fisher Scientific, Standard Vortex Mixer, USA) for at least 1 min to allow for sufficient mixing. In our preliminary study, we found pre-heating honey at 45 °C for 10 min and further addition of freeze-dried bacteria did not reduce the population when enumerated immediately when inoculated honey reach to 22 °C. Also, studies indicated Salmonella when subjected to sublethal heat treatments at 45–48 °C for 30 min did not reduce its population (Running, Crawford, Tierney, & Peeler, 1990; Mackey & Derrick, 1986). The initial bacterial populations were quantified immediately following inoculation into honey and HFCS.

2.4.2. Lawn inoculation method

For the lawn inoculation method, cultures were regrown twice by successively sub-culturing in TSAYE at 37 °C for 24 h. Three hundred microliters of each strain were plated onto sterile tryptic soy agar with 0.6% yeast extract (TSAYE) (Hardy Diagnostics, Santa Maria, CA) in a 100 × 15 mm plate and incubated at 37 °C for 24 h. The bacterial lawn was collected from TSAYE using a plastic hockey-stick spreader and flooding with 5 mL of 1 × phosphate-buffered saline buffer (PBS), and then centrifuged at 8000×g at 4 °C for 15 min (Centrifuge 5810 R®, Eppendorf North America, Hauppauge, NY). The resulting pellets were re-suspended in sterile PBS to achieve ~10^9–10^10 CFU/mL, then combined in an equal volume to obtain the Salmonella cocktail. In addition to the three-cocktail strains, this inoculation method was also used to compare the survival of S. Enteritidis PT-30 and E. faecium in honey and HFCS.

One-hundred grams of honey or HFCS were placed in a 250 mL DURAN® brand glass bottle with a magnetic stirrer and 1 mL each of either the 3-strain Salmonella cocktail, S. Enteritidis PT30, or E. faecium was added to the samples and stirred for 3 min to achieve ~10^9 CFU/mL. These inoculated samples were stirred until sufficiently mixed, and the initial bacterial populations were immediately quantified.

2.5. Survival of bacteria during storage

To determine the survival of bacteria in honey or HFCS sample, 1 mL of inoculated samples were added to 9.0 mL of sterile PBS. Samples were repeatedly diluted this way to obtain a series of 10-fold serial diluted. The appropriate dilutions were spread plated in duplicate on TSAYE plates followed by incubation at 37 °C for 48 h for enumeration. The survival testing of bacteria was performed weekly for up to four weeks.

2.6. Statistical analysis

The survivability of E. faecium and Salmonella in honey and HFCS for both inoculation methods were analyzed with one-way ANOVA with a confidence interval of 95% (α = 0.05) using Minitab software (version 19.2, Minitab, LLC, PA). Three independent experiments were performed. Each experiment had two Duran bottles-where two subsamples were serially diluted and plated in duplicates. Results were represented as Mean ± standard deviation.

3. Results

3.1. Physicochemical properties

The comparison of physicochemical properties of honey and HFCS are listed in Table 1. There were no significant differences (P > 0.05) between honey and HFCS in terms of moisture content, water activity (a_w), density, and sugar (“Brix). However, the pH and viscosity of HFCS was lower than the honey samples (Table 1).

### Table 1: Physicochemical properties of honey and HFCS at room temperature (~22 °C).

<table>
<thead>
<tr>
<th>Physicochemical property</th>
<th>Honey</th>
<th>HFCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture Content (%)</td>
<td>15.3 ± 0.4^a</td>
<td>14.9 ± 0.1^b</td>
</tr>
<tr>
<td>pH</td>
<td>3.8 ± 0.1^a</td>
<td>3.4 ± 0.1^b</td>
</tr>
<tr>
<td>Dynamic Viscosity (N.s.m^-2)</td>
<td>8.67 ± 0.39^a</td>
<td>2.00 ± 0.1^b</td>
</tr>
<tr>
<td>Water Activity (a_w)</td>
<td>0.55 ± 0.08^a</td>
<td>0.55 ± 0.03^a</td>
</tr>
<tr>
<td>Density (g/mL)</td>
<td>1.40 ± 0.02^a</td>
<td>1.38 ± 0.01^a</td>
</tr>
<tr>
<td>Sugar (%)</td>
<td>83.1 ± 0.5^a</td>
<td>82.4 ± 0.7^a</td>
</tr>
</tbody>
</table>

^a,b Mean within a row in different letters mean significantly different (P < 0.05).  
^a Water activity was measured at room temperature. Mean ± standard deviation. HFCS: High fructose corn syrup. Moisture and sugar contents are on wet basis.
Thus, it is assumed that the molecular weight of honey as ~182 g/mol based on their respective proportions, and in comparison, the molecular weight of HFCS as provided by the company was 185 g/mol (Marshall, Goff, & Hartel, 2012). The calculated molarity for honey and HFCS are 6.5 M and 6.3 M, respectively. The osmotic pressure for 100% honey and HFCS were 157.4 atm and 152.5 atm, respectively (Table 2).

The measured molarity and osmotic pressure of the diluted honey and HFCS are shown in Table 3. The molarity for 100% honey and HFCS, calculated from the linear equation Fig. 1, was 6.6 M and 6.7 M, and the osmotic pressure was 160.2 atm (16.3 MPa) and 161.5 atm (16.4 MPa), respectively. These values of molarity and osmotic pressure are comparable to the calculated and experimental results (Fig. 2).

The molarity of glucose solutions with a concentration up to 60% and fructose solution of a concentration up to 48% were previously reported (Lide, 2004); and these data are comparable with our experimental data for the diluted honey and HFCS with concentrations up to 30% (Fig. 1). It should be noted that the Van’t Hoff osmotic pressure equation (Eq. (1)) is only accurate for diluted solutions. When applying it to highly concentrated solutions, the results would be inaccurate. According to the theoretical equation derived by (Chaplin, 2011; Huang & Xie, 2012), the actual osmotic pressures should be 3 times higher than the calculated using the Van’t Hoff’s equation. Thus, the true osmotic pressure in the pure honey and HFCS samples should be more than 3 times of the calculated value of 160 atm.

### 3.2. Survival of bacteria using the lagoon inoculation method

The survival of Salmonella cocktail, S. Enteritidis PT30 and E. faecium prepared by lagoon-based inoculum in honey and HFCS during stored at 22 °C for 4 weeks is shown in Table 5. The initial population of S. Enteritidis PT30 and E. faecium in honey was ~9.6 log10 CFU/mL. There was 2.3 log10 CFU/mL reduction during the first week of storage, and ~5.0 log10 CFU/mL reduction of E. faecium or S. Enteritidis PT30 after three weeks of storage at 22 °C in honey (Table 5). The survivability of E. faecium was greater in honey after 28 days of storage compared to S. Enteritidis PT30 which were not detectable. Similarly, in HFCS, from initial ~9.9 log10 CFU/mL-the population of E. faecium and S. Enteritidis PT30 was reduced by >5.0 log10 CFU/mL after a 3-week storage at 22 °C and neither bacteria were not detectable after 28-days of storage at 22 °C (Table 5).

### 4. Discussion

Sugar plays an important role in maintaining the functional property of foods, by providing an essential carbohydrate source, increasing the food’s sweetness, and enhancing its flavors. It is also helpful for flavor balance, color formation, bulkiness and texture maintenance, fermentation and preservation (CFIA, 2018). Due to their hygroscopic nature, sugars can easily dissolve in water by forming hydrogen bonds with water molecules, which helps in preserving and extending the shelf-life of food products (Syamaladevi et al., 2016). High-sugar products such as fruit preserves, syrups, confectons, and dried fruits are not generally thought to pose a microbiological hazard. It is hypothesized that high concentrations of sugars exert an osmotic shock, which is not suitable for the growth of most microorganisms or causes cell death (Pena-Meléndez, Perry, & Youssef, 2014).

The results from this study suggest that Salmonella and E. faecium were not able to survive in honey after 28 days of storage at 22 °C, regardless of the inoculation methods. In support of our finding, Tyssen and Durand (1973) reported a 9-log reduction of S. Enteritidis PT30 in honey stored at 18–20 °C for 34 days. Many studies speculate that honey has an antibacterial effect, mainly caused by total phenolic compounds such as methyl syringate (Al-Waili, Salom, Al-Ghamdi, & Ansari, 2012; Almassaudi et al., 2017). It was also suggested that the presence of amino acids, phenol antioxidant, antibiotic-rich proteins, as well as kynurenic acid contribute to the antibacterial effect of honey products (Boretta, Gelmini, Lodì, Piazzalunga, & Facino, 2010; G Vaillioun, 2014). However, other researchers postulated that the bacterial inhibition of honey was due to the non-peroxide and osmotic effect (Al Somal, Coley, Molan, & Hancock, 1994). In our study, the reduction of S. Enteritidis PT30 and other Salmonella serovars in HFCS was similar to those in honey.
bacterial populations in honey and HFCS were reduced by more than a 5-log reduction after 21–28 days storage at 22 °C. HFCS and honey have a similar concentration of sugar (around 83%) with a ratio 1.2:1 of fructose and glucose, respectively, but HFCS does not have notable antimicrobials. This suggests that the reduction of Salmonella in honey might not be due to antimicrobial agents in honey, but have been mainly caused by the high osmotic pressure. Studies have shown that short time (~10 min) high pressure processing (HPP) ranging 300–500 MPa resulted in complete reduction of S. Enteritidis population on chicken fillets, beef (Argyri, Papadopoulou, Nisiotou, Tassou, & Chorianopoulos, 2018; Rodrigues et al., 2016; Tananuwong, Chitsakun, & Tattiyakul, 2012) and ~3.3 log reduction in almonds (Goodridge, Willford, & Kalchayanand, 2006). Also HPP validation of E. faecium in strawberry juice producing 5-log reduction (Yildiz, Pokhrel, Unluturk, & Barbosa-Cánovas, 2019). In our study, the estimated osmotic pressures in honey and HFCS are about one order of magnitude smaller than that used in HPP processing. It is likely that in the presence of high osmotic pressure (i.e., 50 MPa) in honey and HFCS, the vegetative bacterial cells might gradually lose their viability during 28 days of storage. It can also be postulated that in addition to high osmotic pressure, low pH of HFCS and honey (pH 3.4 and 3.8 respectively) contributed for gradual reduction of high density of bacteria during 4-week of storage. The bactericidal effect of honey and sugar was significantly reduced when increasing the pH 3.4 to 7.0 (Kwakman et al., 2010). The hygroscopic nature of honey and HFCS can draw the moisture out of the environment
of the bacteria and cause cell death. High osmotic pressure causes ribosomal changes and protein denaturation in the bacteria (Abe, 2007).

Salmonella can survive in a dry product for an extended duration of time. For example, Salmonella was found to survive for 52–61 days in intermediate moisture foods such as brioche (a_w 0.88) at 20 °C (Kapetanakou et al., 2019). In a previous study conducted by Beuchat and co-workers on the survival of Salmonella in granulated sucrose, regardless of inoculation level (2.2 or 5.2 log_{10} CFU/g), wet or dry inoculation, a_w 0.54 or 0.24, and storage temperature 5 or 24 °C, Salmonella was able to survive over 52 weeks of storage (Larry R. Beuchat, Mann, Kelly, & Ortega, 2017). In our study, Salmonella was completely inhibited during 4 weeks of storage in honey and HFCS with a_w 0.55. This suggests that a_w is not the main factor that had caused inactivation of Salmonella in liquid sugars. In addition, the observed similar level of reduction of Salmonella between honey and HFCS indicate that the perceived antimicrobials in honey were not the main cause for microbial reduction; whereas, osmotic pressure and high acidity among these sugars exerted in a similar fashion. On the other hand, inoculation methodology has a great impact in the reproducibility and survival of bacteria in low a_w food studies. Different inoculation methods have been used to inoculate various dry foods-carriers such as sand or talc, use of a dry or wet bacterial inoculum in order to represent the route of contamination. Our study showed neither inoculation method (i.e. wet or dry) nor Salmonella strain had an impact on the survival of Salmonella or E. faecium in honey or HFCS.

In this study, with few exceptions, the survival of E. faecium in both honey and HFCS was similar to Salmonella either with lawn grown or dry inoculation (Tables 4 and 5). This suggests that E. faecium can be considered as an appropriate surrogate for determining the survivability of Salmonella in liquid sugars. E. faecium was shown as a suitable surrogate in determining survival and thermal resistance of different low a_w food such as date paste (Ozturk et al., 2019), toasted oat cereals (Deen and Diez-Gonzalez, 2019), coconut (Dhowlaghar, Zhu, & Ballom, 2019), wheat flour (Xu et al., 2019) and cocoa powder (Tasi et al., 2019).

5. Conclusion
Salmonella and E. faecium die off in honey and HFCS stored at 22 °C for 28 days, regardless of the inoculation method. Besides the antimicrobial compounds existed in honey, the high osmotic pressure in high sugar products determined in this study is likely another main killing factor responsible for the observed bacterial reduction. These results indicate that honey and HFCS are fairly safe as compared to other low-moisture foods or sugars. More systematic studies are still needed to investigate the survival of other pathogenic foodborne bacteria in high concentration liquid sugar products.

Author Contributions
Jaza Alshammari and Nitin Dhowlaghar prepared the first draft of the manuscript, designed the experiments, collected data, and analyzed data. and assisted with preparation of the manuscript. Yucen Xie contributed in preparation of the experiments and in editing. Junming Tang and Meijun Zhu supervised the project, contributed in interpreting results and editing the manuscript. Also, Jie Xu, Shyam Sablani, and contributed in editing the manuscript. Authors declare no competing interests in this study.

Declaration of competing interest
The authors declare there is no conflict of interest in this research.

Acknowledgments
This research was funded with the USDA-SAS 2020-68012-31822, USDA National Institute of Food and Agriculture, Hatch project 1016366. The authors acknowledge the financial support from the government of Saudi Arabia for Jaza Alshammari to pursue his Ph.D. degree at Washington State University. Also, we would like to thank Dr. Linda Harris, University of California, Davis, for providing us the stock culture of S. Enteritidis PT 30 and E. faecium, and Dr. Nathan Anderson (FDA, Illinois) for providing us the stock culture of S. Tennessee K4643, & S. Agona 447967.

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