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

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Abstract
Salmonelliosis 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 NEB, 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 log10 robustness 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 activity of these liquid sugars, the extremely high osmotic pressure is likely the main cause for bacterial death in honey and HFCS during storage. The data provided useful information in developing effective microbiological safety strategies to be introduced in the preparation or storage of low-moisture food and ingredients.

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

1. Introduction
Foodborne disease is one of the biggest public health concerns globally. In the United States, salmonellosis causes around 11% of foodborne diseases annually (Grencis et al., 2011). Salmonella outbreaks were frequently associated with high moisture poultry and meats, such as eggs, poultry and beef (CDC, 2018b, 2020; Tauxe, 1991), as well as vegetables and fruits, such as onions and peaches (CDC, 2009a & 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, 2009b, 2016a). In a low water activity (aw) environment, Salmonella cannot grow or multiply, but it can survive for a long time and pose 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 (Bruchet & Mann, 2014; Podolak, Enache, Stone, Black, & Elliot, 2010).

Low moisture food products containing high concentrations of sugar have also been associated with salmonellosis outbreaks. These foods include chocolate bars (40% sugar content) (Eom et al., 2019; Werber et al., 2010), halva and high sugar, sesame seed based product (Brockmann, 2001; De Jong et al., 2001), and honey snacks cereal (36-50% of sugar content) (USDA FSA, 2019). For some Salmonella serotypes such as Salmonella enterica Kamburuz, Napoli and Typhimurium isolated from chocolate, a very low infection dose (<10^3 CFU/g) of bacteria could in low moisture products was enough to cause salmonellosis infections (EEOI, 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 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 (Dover & et al., 2006; USDA, 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 (Alhaddad, Chuma, & Elweisng, 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 (Chereches & Verones, 2001). Honey is known for its antimicrobial activities against various types of bacteria (Ros, Kobrnitz, Sevilo, & Gas, 2015; K柬埔寨 & Oliver, 1993). It is also well known that young and bacterial spores survive in honey and are able to withstand the acidity and concentrated sugar (Sevin & Oliver, 1998). These contaminants may come from primary sources such as pollen, beekeeping’s digestive tracts, dust, dirt, air, flowers, or secondary sources of contamination, such as humans, equipment, and containers (Ghobian, Afshahi, & Ola, 2007). Currently, information on the survival of Salsamella in HFCs and honey alone are limited. Therefore, this study aimed to (1) determine the survival of Salsamella and Enteroxoccus faecalis NERL B-2954 in HFCs and honey stored at ambient temperature (−22 °C), (2) verify the E. faecalis is an appropriate surrogate strain of Salsamella in those liquid sugars, and (3) study the influence of osmotic pressure on survivability of Salsamella and E. faecalis.

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 (HCS-55) containing 50% fructose & 40% glucose was obtained from Golden barrel (Honey brook, PA). The moisture of honey and syrup was measured at 23 °C with a Refractometer (Aquatech Series 3, Devcon Devices Inc., Pullman, WA). The moisture content of samples was measured on a Series 70 vacuum oven (Thermo Scientific, Inc., Santa Clara, CA) set at 70 °C with a vacuum pressure of 0.08 MPa for 24 h. The moisture content, density, and sugar content (on wet basis) were determined according to the International Honey Commission (Borlin, 1964). The viscosity of the samples was measured using a Discovery Rheometer HR-3 (119 Lukens Drive, New Castle, DE). All samples were measured in triplicates.

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 & Spencer, 1995; Spencer & Kenyon, 2012).

\[ \pi = \frac{M}{V} \]  

\[ \pi = \text{Osmotic pressure (atm)} \]

\[ \pi = \text{Van’t Hoff's factor (this is the number of ions that will form when a solute is dissolved in water)} \]

\[ M = \text{Osmolarity or osmotic concentration (mol/L)} \]

\[ V = \text{Gas constant (0.08206 L atm to mol}^{-1} \text{ K}^{-1}) \]

\[ T = \text{Temperature in Kelvin (K)} \]

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

\[ M = \sqrt{\frac{V}{n}} \]

\[ M = \text{Osmolarity (mol/L)} \]

\[ n = \text{number of moles of solute (mole)} \]

\[ V = \text{volume of the solution in liters} \]

2.3. Bacterial strain

Three Salsamella strains, S. Enteroxids PT30, S. Tennessee K46f43, and S. Agona 447967, were used in this study to prepare a three-strain cocktail. S. Enteroxids PT30 was obtained from Dr. Linda Harris (University of California, Davis), S. Tennessee K46f43 and S. Agona 447967 were kindly gifted by Dr. Nathan Anderson (UNDA, Greater Chicago, Illinois). E. faecalis NERL B-2954 strain was obtained from the USDA Agricultural Research Service (USDA-ARS) from Peoria, Illinois. All the strains were stored in a stock solution of tryptic soy broth supplement with 0.6% (w/v) yeast extract (TSBYE) (Becton Dickinson, 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 Salsamella strain and E. faecalis 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 (Thomas Scientific MMOXQ000 Benchmark Orbital Shakers, Marietta, OH, USA) with a constant shaking speed at 200 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., 2018). 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 (Tomato Scientific
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 lower 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, smudges indicated Salmonella when subjected to sublethal best treatments at 45–48°C for 30 min did not reduce its population (Bunning, Crossland, Tierney, & Preller, 1999; Mackey & Derrick, 1996). 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 grown twice by successively sub-culturing in TSBYE at 37°C for 24 h. Thirteen milliliters of each strain were plated onto sterile tryptic soy agar with 0.05% yeast extract (TSYE) (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 TSYE 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 ~10^6–10^7 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 PT30 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 ~10^7 CFU/ml. These inoculated samples were sterilized 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 (aw), density, and sugar (Brix). However, the pH and viscosity of HFCS were lower than the honey samples (Table 1).

3.1.1. Molarity and osmotic pressure

The calculated molarity and osmotic pressure of the 100% honey and HFCS are presented in Table 2. Honey is a complex food system that contains varying amounts of compositions, which varies with the sources that beets collected. Ninety-five percent of these molecules are dispersed with water and carbohydrates, and the rest are associated with proteins and other chemicals (Bagdastrov, 2009). The carbohydrates in honey are mostly found in the form of fructose (~38%) and glucose (~32%). Because fructose and glucose are the main components in honey, and both having the same molecular weight (180.16 g/mol).

Table 1

<table>
<thead>
<tr>
<th>Physicochemical property</th>
<th>Honey</th>
<th>HFCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture Content (%)</td>
<td>14.9 ± 0.1^a</td>
<td>14.9 ± 0.1^a</td>
</tr>
<tr>
<td>pH</td>
<td>3.8 ± 0.1^a^</td>
<td>5.4 ± 0.1^a</td>
</tr>
<tr>
<td>Dynamic Viscosity (mPa⋅s)</td>
<td>9.67 ± 0.39^b</td>
<td>3.00 ± 0.13</td>
</tr>
<tr>
<td>Water Activity (aw)</td>
<td>0.92 ± 0.05^b</td>
<td>0.92 ± 0.05^b</td>
</tr>
<tr>
<td>Density (g/ml)</td>
<td>1.40 ± 0.035</td>
<td>1.39 ± 0.035</td>
</tr>
<tr>
<td>Brix (%)</td>
<td>83.1 ± 0.3^b</td>
<td>82.4 ± 0.7^b</td>
</tr>
</tbody>
</table>

^a, b: Mean within a row in different letters mean significantly different (P < 0.05).

^c: Water activity was measured at room temperature, mean ± standard deviation. HFCS: High fructose corn syrup. Moisture and sugar contents are on wet basis.
Table 2
Calculation of osmotic pressure for honey and HFCs at ~22 °C.

<table>
<thead>
<tr>
<th></th>
<th>Honey (osmotic pressure)</th>
<th>HFCs (osmotic pressure)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (g/ml)</td>
<td>1.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Concentration (g/ml)</td>
<td>10.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Osmotic pressure</td>
<td>59.5</td>
<td>41.0</td>
</tr>
</tbody>
</table>

Therefore, it is assumed that the muscle weight of honey as ~162 g/ml based on their respective proportions, and in comparison, the muscle weight of HFCs as provided by the company was 185 g/ml (Brooke, 2012). The calculated osmolarity for honey and HFCs are 84.5 M and 8.9 M, respectively. The osmotic pressure for 100% honey and HFCs were 157.4 atm and 152.5 atm, respectively (Table 2).

The measured osmolarity and osmotic pressure of the diluted honey and HFCs are shown in Table 3. The osmolarity for 100% honey and HFCs, calculated from the linear equation, was 6.5 M and 6.7 M, and the osmotic pressure was 160.2 atm (16.5 MPa) and 161.5 atm (16.4 MPa), respectively. These values of osmolarity and osmotic pressure are comparable to the calculated and experimental results (Fig. 2).

Table 3
Experimental osmotic pressure of diluted honey and HFCs at ~22 °C.

<table>
<thead>
<tr>
<th>Concentration (g/ml)</th>
<th>Osmotic pressure (atm)</th>
<th>Osmotic pressure (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>20.0 ± 0.1</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>10</td>
<td>125.8 ± 0.2</td>
<td>12.5 ± 0.2</td>
</tr>
<tr>
<td>15</td>
<td>181.3 ± 0.2</td>
<td>18.1 ± 0.2</td>
</tr>
<tr>
<td>20</td>
<td>205.5 ± 0.6</td>
<td>20.6 ± 0.6</td>
</tr>
<tr>
<td>25</td>
<td>245.1 ± 3.3</td>
<td>24.5 ± 3.3</td>
</tr>
<tr>
<td>30</td>
<td>294.5 ± 5.0</td>
<td>29.5 ± 5.0</td>
</tr>
</tbody>
</table>

Mean ± standard deviation (n = 3).

The osmotic pressure calculated from the extrapolated equations in (Fig. 2).
Fig. 2. The calculated osmotic pressure from calculated and experimental osmotic data for honey and high fructose corn syrup (HFCS).

Table 4
Survival counts of Salmonella and E. faecium in honey and HFCS using frozen inoculation method.

<table>
<thead>
<tr>
<th>Days</th>
<th>Honey (log_{10} CFU/ml)</th>
<th>HFCS (log_{10} CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. faecium</td>
<td>S. enteritidis PT-30</td>
</tr>
<tr>
<td>0</td>
<td>10.6 ± 0.1</td>
<td>7.6 ± 0.1</td>
</tr>
<tr>
<td>7</td>
<td>8.4 ± 0.1</td>
<td>7.6 ± 0.1</td>
</tr>
<tr>
<td>14</td>
<td>7.5 ± 0.1</td>
<td>6.0 ± 0.1</td>
</tr>
<tr>
<td>21</td>
<td>5.6 ± 0.1</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>28</td>
<td>5.5 ± 0.1</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND: Not-detectable. Mean ± standard deviation, averaged from three independent studies, 3 replicates per treatment at each sampling day within each independent study. HFCS: high fructose corn syrup.

Table 5
Survival counts of S. enteritidis PT-30 and E. faecium in honey and high fructose corn syrup using freeze-dried inoculation method.

<table>
<thead>
<tr>
<th>Days</th>
<th>Honey (log_{10} CFU/ml)</th>
<th>HFCS (log_{10} CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. faecium</td>
<td>S. enteritidis PT-30</td>
</tr>
<tr>
<td>0</td>
<td>9.6 ± 0.1</td>
<td>9.6 ± 0.1</td>
</tr>
<tr>
<td>7</td>
<td>7.6 ± 0.2</td>
<td>6.7 ± 0.2</td>
</tr>
<tr>
<td>14</td>
<td>6.0 ± 0.3</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>21</td>
<td>4.5 ± 0.0</td>
<td>3.6 ± 0.9</td>
</tr>
<tr>
<td>28</td>
<td>2.6 ± 0.7</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND: Not-detectable. Mean ± standard deviation, averaged from three independent studies, 3 replicates per treatment at each sampling day within each independent study. HFCS: high fructose corn syrup.

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 (Argeri, Papadopoulos, Nistou, Tasous, & Chatzirizopoulou, 2018; Rodrigues et al., 2016; Tanasuwongs, Chulsakun, & Tatiyakul, 2012) and ~3.3 log reduction in almonds (Goodridge, Willford, & Kelchmann, 2006). Also, HPP validation of E. faecium in strawberry juice producing 5-log reduction (Yilmaz, Polkriel, Unlukur, & Barbosa-Coutos, 2019). In our study, the estimated osmotic pressure 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 3.6 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 from 3.4 to 7.0 (Kozminski et al., 2019). 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 brieche (n=0.088) at 20 °C (Gopalan, et al., 2019). In a previous study conducted by Bechaert and co-workers on the survival of Salmonella in granulated sugar, regardless of inoculation level (2.2 or 5.2 log_10 CFU/g), wet or dry inoculation, n=0.54 or 0.24, and storage temperature 5 or 24 °C, Salmonella was able to survive over 52 weeks of storage (Larry R Bechaert, Manu, Kelly, & Oregil, 2017). In our study, Salmonella was completely inhibited during 4 weeks of storage in honey and HFCs indicates that the perceived anti-microbial 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 n=0 food studies. Different inoculation methods have been used to inoculate various dry foods carriers such as sand or n=0, 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 linear growth 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 n=0 food such as date paste (Ozturk et al., 2018), instant oatmeal (Deniz de Díaz-Gómez, 2019), coconut (Bhoslewar, Zhu, & Balbon, 2019), wheat flour (Ozturk et al., 2019) and cocoa powder (Tazi 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 existing in honey, the high osmotic pressure in high sugar products determined in this study is likely to be a critical 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
Jazz Alhammam and Nithi Dhoslewar prepared the first draft of the manuscript, designed the experiments, collected data, and analyzed data, and wrote with the exception of the manuscript. Yusme Xi contributed in preparation of the experiments and in editing. Junying Tang and Meijun Zhu supervised the project, contributed in interpreting results and editing the manuscript. Xue, Niyi Sanlasi, 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.

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