Thermal Inactivation of Bacterial Pathogens and Fungal Spores Under Post-Process Contamination Scenarios in Maple Syrup Processing

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THERMAL INACTIVATION OF BACTERIAL PATHOGENS AND FUNGAL SPORES UNDER POST-PROCESS CONTAMINATION SCENARIOS IN MAPLE SYRUP PROCESSING

By

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B.S. Virginia Tech, 2015

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Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science (in Food Science and Human Nutrition)

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Maple syrup is produced by the extensive thermal evaporation of maple sap traditionally collected from the *Acer saccharum* tree and other maple varietals. The resulting low water activity product (Aw $\leq 0.85$) is classified by the FDA as a low risk food commodity, due to the moisture limitations which inhibit the growth of pathogenic microorganisms. However, recent outbreaks associated with other designated low risk products, including peanut butter and wheat flour, now challenge the current understanding of factors required to induce human illness. Among the most notorious bacterial pathogens, as few as ten bacterial cells, have been cited to cause infection. In addition to bacteria-based risks, fungal contaminants have also been noted to jeopardize safety due to the potential for mycotoxin production, penetrating beyond the immediate product surface.

The extensive heating process required to produce syrup from sap is sufficient to eradicate the majority of present microflora. However, post-process contamination scenarios, which are augmented by producer behaviors, can introduce microorganisms into the finished product. Among these risk factors include direct product contamination due to insufficient heating temperatures, or contamination of bottles, as a result of improper container storage. Therefore, the objectives of this work are to (i) determine the thermal inactivation (D-value) of
predominant bacterial pathogens (STEC, *Listeria monocytogenes*, *Salmonella*) in maple syrup heated to 180°F, a common bottling temperature, (ii) assess the effectiveness of commonly used bottling temperatures (180°F and 190°F) in the inactivation of bacteria (previously described) and fungal spores (*Aspergillus* and *Penicillium*) desiccated on the interior of several types of retail containers, (iii) determine the efficacy of applying a boiling water pre-fill treatment in eradicating microbial hazards, and (iv) assess the survival or growth capabilities of both bacteria and fungi in maple sap and syrup held under normal storage conditions.

The data from this work showed that when the syrup is heated to 180°F for at least 23 seconds, this heating method is sufficient to achieve a 5-log reduction (pasteurization) in the three bacterial pathogens we identified. However, when contaminants were desiccated on the interior of bottles, even a fill temperature of 190°F, is ineffective in eradicating all contamination risks. Although a boiling water pre-fill treatment reduced the likelihood of microbial survival, it did not eradicate all populations we studied across every container type. However, due to reduced heat retention capacity and bottle shape, utilizing a fancy glass bottle results in the greatest likelihood of microbe survival. Therefore, the largest plastic bottle at the highest fill temperature possible, is recommended to reduce product contamination risks. If contamination does occur, bacterial pathogens are capable of survival for up to 30 days in ambient maple syrup and up to 60 days in refrigerated maple sap. Producers may consider retaining finished product prior to sale in order to reduce the potential for bacterial food safety risks. Fungal (*Aspergillus* and *Penicillium*) contaminated syrup demonstrated continuous growth in both products. Therefore, it is recommended that fungal-contaminated syrup must be discarded due to the potential risks of mycotoxin production that could pose harm to consumer health.
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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Maple syrup is a popular food commodity inherent to Midwest and Northern regions in the United States and Canada. Despite its strong geographical roots, maple syrup has gained popularity amongst European and Asian consumers (Levinson-King & Murphy, 2017). In 2016, the maple industry was valued at $147 million dollars in the United States, with Vermont and New York being the largest producers by volume. This projection was a 17% increase from 2015, suggesting that maple syrup continues to grow in global popularity (USDA National Agricultural Statistical Service, 2017).

To produce maple syrup, sap of the sugar maple (Acer saccharum), or other maple tree varietals, is collected and then evaporated until the required sensory and quality standards are reached (Perkins & van den Berg, 2009). Although a conceptually simple process, there are complex considerations to ensuring the final product is high quality and safe from pathogenic bacteria and molds. This literature review serves to introduce the production and characteristics of maple syrup, to address the prominent microbiological hazards that can possibly contaminate maple syrup throughout the various production steps, and establish a better understanding of the safety concerns for this traditionally “low risk” food product.

1.1.1 History

Maple syrup production is believed to have originated with the early Native American tribes of the Northeast. Although the timing of the first maple syrup production is unknown, there are countless legends for how it may have been initiated. The likely scenario is that Native Americans observed animals, such as the red squirrel, sucking sap from the twigs of
the maple trees (Heinrich, 1992). The indigenous people’s curiosity prompted them to collect sap for themselves, and when they boiled it, saw that it produced a brown aromatic liquid that we know today as maple syrup (Heinrich, 1992).

European settlers began to adopt these practices, and refined the process through the introduction of more advanced equipment, such as iron kettles for boiling (Henshaw, 1890). Although maple syrup existed, the primary product made from maple sap during these times was granulated maple sugar (Pearsall, 2015). Granulated maple sugar was popular amongst northerners throughout the civil war era because it was an alternative to exorbitantly priced white sugar, and provided a sweetener alternative that did not rely on slave labor. Today, syrup dominates the market although a wide variety of other maple-based products exist.

1.1.2 Health Benefits

Prior research on phenolic compounds present in maple syrup has been mostly focused on sensory impacts. However, beyond the vast contributions to flavor, aroma, and color, these compounds have been correlated with additional health benefits in the human diet. (Edwards, Rossi, Corpe, Butterworth, & Ellis, 2016). Specifically, the antioxidant and antiradical properties have been linked to reduced instances of cardiovascular disease, degenerative diseases, including multiple sclerosis, and cancer (Edwards et al., 2016). Although these health-mediated effects have not been correlated with syrup consumption directly, antioxidant and antiradical presence suggests an additional advantage over other sweeteners, such as sucrose. Minerals found in maple syrup include calcium, iron, magnesium, zinc, and potassium (Lagacé, Leclerc, Charron, & Sadiki, 2015; Yoshikawa, Tani, Baba, & Hashimoto, 2013). When compared to table sugar calorically, maple syrup is slightly higher at 52 calories per tablespoon, compared to sugar’s 45 calories per tablespoon. Its lower glycemic index
score (54 compared to 65 in cane sugar), phenolic compound composition, as well as mineral content, provides additional unique benefits (Levy, 2015). Despite its several advantages, this natural sweetener should only be consumed in moderation (Kannall, 2017).

1.1.3 Sap Composition

Sap is considered sterile while inside the tree xylem, but once sap exits the tree, it is susceptible to microbial contamination (Filteau et al., 2011). Sap composition is inconsistent, as a result of changing environmental and microbial factors. Sap typically contains 95-99% water, with sugar mixtures comprising the rest of the remaining sap composition. Sucrose is the primary carbohydrate, although glucose and fructose are also present in minor amounts. As a result of environmental exposure and storage conditions, the concentration of these minor sugars will increase. This increase is due to microorganisms hydrolyzing sucrose into these constituents during fermentation, reaching to as much as 0.25% by the end of the maple season (Perkins & van den Berg, 2009). Soil nutrients have also been associated with increased sugar levels. Wild and Yanai (2015) for example, determined soil nitrogen to be a major contributor to increasing photosynthesis rates, ultimately resulting in sweeter sap. The remaining organic constituents include phenolic compounds, amino acids, and proteins (Ball, 2007). Primary phenols include vanillin, syringaldehyde, coniferol, and sinapic acids among others (Filipic, Underwood, Willits, 1965; Underwood, Willis, Lento, 1961; Clément, Lagacé, & Panneton, 2010). Organic acids in sap include malic, acetic, citric, fumaric, and lactic acids (Lagacé, 2015). Potassium and calcium are the largest mineral contributors, although several others such as magnesium, iron, and zinc are present in much smaller amounts (Lagacé 2015; Perkins & van den Berg, 2009). The pH levels of sap are approximately neutral, but have been sampled in ranges spanning from 3.9 to 7.9 (Ball, 2007).
1.1.4 Syrup Composition

Finished syrup has a typical Brix level of 66-68° and water activity levels between 0.83-0.86, attributing to its low risk product status (Ball, 2007; Frasz & Miller, 2015). Finished syrup has a pH level between 6.3-6.8, although like sap, these values depend on microbial and environmental factors (Frasz & Miller, 2015; Perkins & van den Berg, 2009; Sair & Snell, 1939). Maple syrup contains all the compounds previously mentioned in sap. However, the ratio of these constituents will determine the degree of formation of additional compounds, which develop during the evaporation process. These include flavor compounds such as alkylpyrazines, phenols, and carbonyls, which all contribute to the sensory attributes of the finished product (Akochi-K, Alli, & Kermasha, 1997). Phenolics are most abundant, with twenty-five already identified in maple syrup (Panneton, Clément, & Lagacé, 2013). They are derived from the breakdown of lignin within sap, whereas pyrazines are likely produced during Maillard reactions during evaporation (Ball 2007; Clément et al., 2010).

Metals such as copper, tin, or zinc may also be found in syrup, as a result of equipment contact during processing (Perkins & van den Berg, 2009).

1.2 Processing

1.2.1 Tapping

Tree tapping is the first step in collecting maple sap for the later production of maple syrup. Taps (also commonly referred to as spiles) are inserted into the tree as a means to expel sap for subsequent collection and processing. In order to tap a maple tree, a producer will drill a hole approximately four to five feet above ground level (Blumenstock & Hopkins, 2007; Walters & Shigo, 1978). The depth of the tap hole typically ranges from 1.5” to 2.5” (Wilmot, 2014). However, there has been evidence to demonstrate that a reduced taphole depth (less
than 2.5”) may deliver comparable sap yields while also limiting internal tree damage. For example, a study conducted at the University of Vermont Proctor Maple Center during the 1998 sugaring season, indicated that drilling to the 1.5” depth using either a 5/16” or 7/16” spout, obtained 98% of the sap yield (determined by volume) of the 2.5” alternative. Whereas, holes that were drilled to a ¾” depth only produced 86% of the sap volume obtained from the 1.5” taphole. Wilmot (2014), also determined that when a taphole was drilled to the 2.5” depth, the drill accessed damaged brown wood (formed from a previous tap site), which resulted in increased internal tree damage. Therefore, it is beneficial to maintain a reduced drill depth as it results in less internal tree damage (Wilmot et al., 2007).

Size is a second consideration when analyzing the spout selected for tree tapping. As suggested from the previously mentioned study, commercially, 5/16” and 7/16” taps are used as industry standards. Beyond tap hole depth, an additional study conducted at the University of Vermont Proctor Research Center analyzed the effects of tap hole size on internal tree damage and overall sap yield. The researchers assessed the area of stained wood as an indicator of tree damage and concluded that the 5/16” hole produced on average 20% less damage, and similar sap yields, compared to its larger alternative. (Wilmot et al., 2007).

The number and type of taps (plastic vs. stainless steel material) selected are dependent on the size of the tree and the processing methods employed by the syrup producer. There are varying recommendations regarding the appropriate number of taps per tree size, however, the conservative recommendation proposed by University of Maine Cooperative Extension suggests that a tree less than 10” diameter should not be tapped, 1 tap is suitable for a 12” to 20” tree, and an additional tap can be added to trees greater than 20” in diameter (Blumenstock & Hopkins, 2007). Stainless steel spouts are typical to traditional sap collection
methods, whereas plastic is more common in modern vacuum tubing operations (Wilmot et al., 2007).

1.2.2 Sap Collection

Sap from the sugar maple tree is preferred for syrup production, although red and silver maples (*Acer rubrum* and *Acer saccharinum*) are also widely used (Farrell, 2013; Freedman, 2014). Sap release and quality is primarily dependent on climate variables, which are characteristic to the northern hemisphere’s late winter and early spring temperature fluctuations. More specifically, the series of freeze thaw cycles that typically occur throughout the “sugaring season” (late February to early April in New England, although this can fluctuate based on location), promote sap release from the tree for further processing (Roberge, 2018). The sub-freezing nighttime temperatures cause the sap to freeze, which creates a negative pressure inside of the tree relative to the outside environment. The above freezing temperatures the following day cause the frozen sap to return to a liquid creating a positive pressure differential inside of the tree. This positive pressure forces the sap out of the nearest possible exit, which is the tap site that has been inserted by the syrup producer. This negative to positive pressure transition is a result of carbon dioxide expansion and contraction inside of the tree as a function of temperature variation (Walters & Shigo, 1978). The following night, temperatures return to freezing, and the sap supply is restored as more water is up taken from the tree roots (Matthews & Iverson, 2017).

In a typical sugaring season, an average five to fifteen gallons of sap are collected per tap from healthy maple trees. However, this yield estimate fluctuates in response to tapping methodology, seasonal variation, as well tree size (Blumenstock & Hopkins, 2007; Perkins & van den Berg, 2009). Traditional methods include the use of plastic or metal buckets often
accompanied with covers to prevent light-induced oxidation, debris, water, snow and animals from contaminating the sap. During the 1950s, producers began to implement tubing systems that relied on gravity to feed the sap downhill from the tap hole directly to the sugaring house (also referred to as a sugar shack) or sap collection tank (Wessel, 2015; Wood, 2011). More recently, producers have integrated vacuum pumps to increase sap flow. With a vacuum tubing network, a producer can anticipate to extract nearly 25 gallons of sap per tree tap (Perkins & van den Berg, 2009).

1.2.3 Evaporation

After collection, the sap is brought to the sugarhouse where it undergoes an extensive evaporation process. This thermal process develops the majority of sensory attributes in the finished syrup, including color, flavor, and texture (Findlay & Snell, 1935; Willits, Porter & Buch, 1952). Raw sap composition varies, but is typically between 2-3% sugar (Blumenstock & Hopkins, 2007). However, sugar sap content has been reported to exceed 4%. Sap is evaporated until it reaches a minimum 66% soluble solids (66º Brix) concentration, which is the federal standard of identity for maple syrup in both the United States and Canadian markets (USDA, 1979; OMAFRA, 2016a) and can range up to 68.9 º Brix. Syrup below 66 and above 68.9 º Brix is not considered “syrup”.

A typical evaporation system shown in Figure 1.1 is comprised of a “front” and “back” pan, each of which further compartmentalizes the sap as the density continues to increase throughout the heating process. The shallow pan depth and bottom grooved surface facilitates maximum heat transfer.
Other common features of syrup evaporation systems include oil or wood fuel sources. Wood-based fuel is popular in small-scale operations, which comprise the majority of syrup producers. However, oil-based heat is common among larger commercial operations because it is less labor intensive and more cost effective (Sanford, 2003). Commercial evaporators are typically insulated and contain heat exchangers. Heat exchangers collect the hot steam from the evaporator pans, and allocate it to pre-heat the unprocessed sap before it enters the evaporator, further reducing production time and energy consumption. A hood is also found above the evaporator pans to create a safe exit for steam that is not collected in the heat.
exchanger. The hood will also form a physical barrier from foreign particles entering the product (Garret et al., 1977; Perkins & van den Berg 2009).

It is important to mention that not all evaporation systems include these features. Small-scale operations in particular will often omit a hood, but utilize an alternative venting method such as a stack out of the sugarhouse. It should be noted that absence of a methodology to prevent steam from exiting the sugar shack, may increase microbial contamination of the product. Steam accumulation allows for condensation to form in the processing area, including above the evaporator pan. This is a concern for finished product safety because both bacterial and fungal contaminants thrive in moist environments. Condensation dripping into the product, into open syrup bottles, or onto a processing surface presents a viable route of entry for bacterial and fungal introduction.

1.2.3.1 Scale

Scale is a mineral deposit that clings to the surfaces of the evaporator pan during heating (Isselhardt, van den Berg, & Perkins, 2012). It is formed as a result of minerals exceeding their saturation point, which causes the compounds to be expelled from solution (Perkins & van den Berg, 2009). The composition of scale varies depending on initial sap chemistry and equipment, but primarily consists of calcium and magnesium, with phosphorus, manganese, aluminum, iron, and copper also present in lower amounts. If not removed entirely, scale can reduce heat transfer rates during evaporation. Aside from being extremely difficult to remove, it is a problem for syrup production because it can be the source of off-flavors in the finished product and interrupts the even thermal distribution in the evaporator (Isselhardt et al., 2012).
1.2.3.2 Reverse Osmosis and Sap Concentration

Commercial operations will often implement reverse osmosis (RO) filtration to pre-concentrate the sap prior to evaporation. RO technology forces the sap under pressure through a finite filter to remove approximately 75% of the water from raw sap (Bouchard & Lebrun, 1999). Due to high equipment costs, the degree of concentration is often dependent upon the operation size. The midrange RO equipment typically allows sap concentration up to 8-15° Brix, and more advanced (costly) equipment can concentrate sap as high as 25° Brix. (Perkins & van den Berg, 2009). The University of Vermont Proctor Research Center analyzed the effects of varying levels of pre-concentration on overall syrup composition and sensory perception. The studies determined that there are only minor variations in finished syrup composition (compared to syrup produced with non-concentrated sap), such as minor color changes. None of these differences, however, were large enough to contribute to a change in syrup grade or were perceived during sensory analysis testing by trained panelists. Utilizing this equipment provides significant fuel cost reductions to heat the evaporator, as well as reducing overall production time (van den Berg, Perkins, Isselhardt, Godshall, & Lloyd, 2015).

1.2.4 Filtration

After evaporation, syrup is filtered before bottling to ensure that the final product meets clarity and grade expectations. Sugar sand, also commonly referred to as niter or loose scale, is the mineral deposit that remains suspended in the syrup liquid (Isselhardt et al., 2012). The composition of this material is nearly identical to scale. Snell reported that sugar sand is primarily composed of calcium malate, followed by silica, with lesser amounts of manganese, magnesium, phosphorus, and iron (Snell & Lochhead, 1914). Davis et al. (1963)
presented similar findings when assessing sugar sand composition. This study also determined that malic acid, as well as calcium content, within the raw sap are the primary indicators for determining the amount of sugar sand that will be present in the syrup (Davis, et al., 1963). A variety of filtration systems to remove sugar sand are used in industry, but the most popular commercial methodology uses diatomaceous earth filtration through a filter press (Perkins & van den Berg, 2009).

### 1.2.5 Grades of Syrup

A grading scale was developed to provide sensory and quality standards of finished syrup, which is beneficial to both the producer and consumer. From the producer’s perspective, it promotes quality standards and creates effective marketing strategies to sell the product. Likewise, the consumer can have increased assurance that the product they are purchasing is of a certain expected flavor characteristic or quality (Drake & James, 1992). Prior to 2015, the USDA grading system was entirely voluntary, although individual states such as Vermont, implemented their own set of standards. The revised system aims to remove the ambiguity of the previous scales, so that sensory descriptors are more approachable to consumers, and better defined for producers. The updated grading system is also standardized to be the same for both United States and Canadian markets, which previously utilized different systems of characterization and labeling. Table 1.1 references the common flavor descriptors of syrups classified into each of the revised retail grades. Generally, Grade A product must have uniform coloring, and contain only anticipated flavors and aromas. Processing grade syrup (not shown in Table 1.1) however, is not intended for retail markets and can thus contain any percentage of light transmittance, as well as possess off-flavors including: “burnt”, “fermented” or “buddy”. Despite the flavor and color differences, all
specified grades must be clean and void of turbidity, with a minimum 66° Brix, not to exceed 68.9° Brix (Agriculture Marketing Service USDA, 2015; USDA, 1979).

**Table 1.1: U.S. Maple Syrup Grade Characteristics**

<table>
<thead>
<tr>
<th>Grade</th>
<th>Flavor Descriptor</th>
<th>% Light Transmittance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Golden</td>
<td>Delicate</td>
<td>&gt; Or equal to 75%</td>
</tr>
<tr>
<td>Amber</td>
<td>Rich</td>
<td>50-74.9%</td>
</tr>
<tr>
<td>Dark</td>
<td>Robust</td>
<td>25-49.9%</td>
</tr>
<tr>
<td>Very Dark</td>
<td>Strong</td>
<td>&lt; 25%</td>
</tr>
</tbody>
</table>

a. Adapted from Final Notice United States Standards for Grades of Maple Sirup (Syrup). by Agriculture Marketing Services, USDA. 2015.

Microorganisms have a significant impact on syrup grade determination, as microflora can alter sap composition, due to the hydrolysis of sucrose into reducing sugars, such as glucose and fructose (Filteau, Lagacé, LaPointe, & Roy, 2011). Accumulation of these reducing sugars influences the finished product flavor and color, because invert sugars have increased reactivity with free amino acids at higher temperatures, which can result in a darker colored product upon boiling. (Filteau et.al, 2011; Lagacé, Girouard, Dumont, Fortin, & Roy, 2002; Naghski & Willits, 1957). Sap released at the start of the sugaring season generally produces lighter, more delicately flavored syrup, because it contains fewer microorganisms due to reduced environmental temperatures and limited bacterial accumulation (Van den Berg, Perkins & Isselhardt, 2015). For this reason, it is recommended that sap is stored at refrigerated temperatures and used as soon as possible after collection.

It should be mentioned however, that controlled microorganism fermentation can also result in some positive product attributes. *Pseudomonas geniculata* for example, a constant sap
inhabitant, has been noted to significantly enhance maple flavor without sacrificing color due to malic acid utilization for carbon sources. (Lagacé et al., 2004).

However, some research has been unsuccessful in consistently correlating syrup chemical composition with product grade. Thirty-five Ontario syrup samples were analyzed in 2014, and demonstrated no significant differences in reducing sugar, pH levels, or density among all of the graded samples (Singh et al., 2014). Ninety-nine syrup samples of varying grades and production locations during the 2015 season also did not demonstrate consistent results for conductivity, pH levels or carbohydrate composition (Van den Berg et al., 2015). These findings indicate that finished syrup attributes are specific to a particular sample, and are largely influenced by sap terroir and processing attributes including microbial presence, lag period before sap boiling, sap storage conditions, as well as process sanitation methods (Lagacé et al., 2002).

1.3 Packaging

1.3.1 Fill Temperature

It is common for syrup producers to cool syrup after evaporation in bulk containers (50-55-gallon drums). This practice necessitates reheating the syrup before dispensing into retail packaging for consumers. A minimum bottle fill temperature of 180°F (82.2°C) is the current recommendation to sufficiently reduce risks from pathogenic bacteria and mold for all container types (Dumont, Saucier, Allard, & Aurouze, 2007; Whalen & Morselli, 1984). After filling and capping, it is common practice for smaller scale producers to invert or tilt the bottles in order to sanitize the headspace and underside of the cap (Fifield, 2013). Other producers will immediately hot fill from the finishing pan after filtration to fill directly into retail containers. This recommendation was developed because room temperature packaging
of syrup had proven to be inadequate. One study aseptically packaged syrup in glass bottles, and found that after 16 months of ambient storage, 42% of product filled at 77°F (25°C) had distorted containers, likely as a result of microbial gas production inside of the bottles. This same study did not observe mold or yeast growth in samples stored at room temperature if they had been previously heated to 180°F (82.2°C) (Dumont et al., 2007).

However, there is evidence to suggest temperatures beyond this current recommendation are necessary to ensure product safety and quality. Whalen and Morselli analyzed the association between mold growth and bottle fill temperature and found that 7% of syrup filled at 180°F (82.2°C), and stored at 75°F (23.9°C) and 86°F (30°C) respectively, were contaminated with a visible mold layer on the product surface, regardless of bottling material. Although refrigerated storage (39°F, 3.8°C) immediately after bottling prevented any visible mold growth, this is often not the environment where consumers, producers, or distributors will keep unopened product (Whalen & Morselli, 1984). The investigators also determined that filling the syrup at 200°F (93.3°C) was sufficient to kill all molds, as none were detected in any of the product samples under all the previously mentioned storage conditions (Whalen & Morselli, 1984). These results for mold eradication were consistent with a prior study, which also used a 200°F (93.3°C) fill temperature, and storage temperatures of 43°F (6.1°C) and 77°F (25°C) with and without fluorescent light exposure (Morselli & Sendak, 1980).

Although higher syrup temperatures have been proven to eliminate mold and bacteria, there may be an upper limit to what producers are willing to use in their process. Fill temperatures of 200°F (93.3°C) or higher are often cautioned against because it can darken the
color of the finished syrup, as well as create additional sugar sand, which will ultimately reduce product clarity and manipulate grade (Hopkins, n.d.; Fifield, 2013).

1.3.2 Container Material and Storage

Tin cans were the most common retail container during early maple syrup production, but this container type has primarily been replaced due to oxidation-induced rust formation (Morselli & Sedak, 1980; Winch & Morrow, 1975; Wood, 2011). Today, retail products can be found in plastic, glass, or “fancy” glass bottles of varying sizes. Glass has high consumer appeal, because unlike tin or plastic, it allows product visibility. Plastic, on the other hand, is relatively inexpensive and can be the surface of a unique product label (Winch & Morrow, 1975).

Few studies have determined that bottle material and storage conditions have an impact on finished syrup quality. Sendak, Morselli, and Bee (1978), compared the quality degradation of syrup stored in: a glass bottle, a plastic jug (two samples each made from different types of plastic material), a polypropylene bottle, a high-density polyethylene squeeze bottle, and a tin can. They found that all container types were sufficient to maintain initial product quality for up to six months at 75°F (23.9°C). However, storage beyond six months produced changes in syrup color, pH levels, flavor, and density with the plastic material being most adversely affected. A similar study conducted by Underwood et al. (1974) reported that syrup stored in plastic across refrigerated, ambient, and elevated temperatures had the most significant color darkening over time compared to glass and tin under the same environmental conditions. The color change was so drastic in some samples that it lowered the product grade.
Although changes to finished syrup composition can indicate product quality loss, further research is needed to understand the effects of retail container sizes and materials on finished product safety over storage time.

1.4 Cleaning and Sanitization

Proper equipment sanitization ensures finished product safety from pathogenic bacteria and mold. At minimum, processing equipment should be cleaned immediately following the last sap run or syrup production of the season. However, it is also recommended that the equipment is then sanitized and rinsed before the first sap collection of the following season (NYS Maple Producers’ Association & NYS Department of Agriculture, n.d.).

Equipment cleaning methods are dependent on the type of equipment and processing stage. For example, the removal of scale, a mineral rich compound which is often present in the evaporator, will likely require a more aggressive cleaning regimen compared to removal of syrup residues in the finishing pan, which primarily contains sugars (OMAFRA, 2016b). When only maple syrup residues are present, hot water is often adequate to clean the product contact areas (Graham & Milo, 2018). The cleaning methods employed in practice, however, are highly variable. A 2011 survey conducted by Perkins and van den Berg analyzed common vacuum tube cleaning methods among United States syrup producers, and determined that the majority implement an air and water flush technique. In this procedure, the collection lines are thoroughly rinsed with water and air under pressure, and then allowed to drain and dry (Perkins & van den Berg, 2012). An air only methodology is the second most common practice. This approach removes any remaining sap found in the tubing via air suction without rinsing at the end of the sugar season. Surprisingly, 6% of the producers surveyed indicated
that they do not employ any cleaning procedure for the collection tubing (Perkins & van den Berg, 2012).

Chemical based sanitizers are uncommon in syrup operations for a few reasons. First, regulations require that all pure syrup products are free from additives, including sanitizer residues, or they must be labeled (USDA, 1979). Additionally, sap and syrup have been shown to easily absorb residues, and it is therefore necessary that chemicals used in production do not leave any remnants on the equipment or in the collection tubing (Hopkins, n.d.; Morselli, Whalen & Bagget, 1985). Such compounds can produce off-flavors and colors within the finished product. For example, Morselli et al. (1985) conducted a sensory analysis on syrups produced using equipment sanitized with varying concentrations of chlorine-based solutions. From all the chlorine samples, panelists described the syrups as either “salty” (at the highest level) or containing “undefined off flavors” when compared to the control, which used only a water flush method. A color difference of two grades was also detected in syrups with high chlorine concentrations.

Approximately twenty percent of syrup producers reported using chemical sanitization methods to flush their tubing systems (Perkins & van den Berg, 2012). These food grade solutions are regulated by the Environmental Protection Agency (EPA), and are found in 21 CFR Part 178 Indirect Food Additives: Adjuvants, Production Aids, and Sanitizers (Perkins, Roberge, Childs, Graham, & Farrell, 2016; Pennsylvania Department of Agriculture, 2016). When chemicals are used in processing, chlorine, hydrogen peroxide, and alcohol-based sanitizers are most common (Perkins & van den Berg, 2012). Although permitted in the Canadian market, isopropyl alcohol (IPA) is currently not allowed for use in the United States due to its non-compliance with pesticide labeling laws (Perkins et al., 2016). With respect to
IPA usage, concerns regarding plastic constituent degradation from collection tubing has been suggested as a potential contamination source in maple syrup and sap. A recent study evaluated plastic residue presence (particularly Octabenzone which had previously been isolated from preseason sap samples, and phthalates a plasticizer) from sap collection tubing systems after sanitization with IPA solution. Although no plastic residues were detected in the finished product, the researchers recommend that all syrup operations, regardless of IPA usage, rinse the collection tubing with the first sap collection of the year to ensure plastic compounds from tubing do not enter the product (Lagacé, Charron, & Sadiki, 2017).

Additional requirements for sanitizer selection include effectiveness, approved application, safety, and non-corrosiveness. An ideal sanitizer has a target pH level between 6.5-7.5. A pH level below 5 is extremely corrosive and will degrade equipment over time. An additional consideration for highly acidic chlorine-based sanitizers is that they can generate dangerous compounds, such as chlorine gases, which are unsafe for employees. Conversely, a sanitizer with pH levels greater than 8 will not be successful in combating harmful microorganisms (NYS Maple Producers’ Association & Department of Agriculture, n.d.). In all cleaning and sanitization procedures, the water used for mixing solutions is required to be clean and sanitary (Ohio Administrative Code - 901:3-46-09 Water supply, 2017).

1.5 Sap Microbiota

As previously mentioned, maple sap provides ample nutrients to sustain microbial populations. Although carbon is a major nutrient source, the nitrogen content in sap has also been proven to be a critical component to potential microbial growth, and is primarily influenced by soil constituents (Stevens, 1908; Nikolajeva & Zommere, 2018). Britten and Morin (1995) found maximum exopolysaccharide production by *Enterobacter agglomerans* in maple sap when
the nitrogen content was unrestricted. When this nutrient was not limited, the maple sap exhibited an extremely diverse microbial community comprising both bacterial and fungal species. Many microorganisms in sap are derived from the tree wood itself, which enters the tap hole after tapping (Consenza, 1970). However, microbial growth can be amplified due to improper process sanitation, such as in collection tubing, warm environmental temperatures, or sap storage conditions.

Of the bacteria found in maple sap, Gram-negative bacteria predominate including *Pseudomonas, Enterobacter, Flavobacterium*, and *Achromobacter*. Less frequently reported Gram-negative bacteria include *Rahnella, Ralstonia, Janthinobacterium*, and *Sphingomonas*. Additionally, Gram–positive bacterial genera including *Bacillus, Leuconostoc, Micrococcus* and *Staphylococcus* have also been routinely recovered in maple sap (Consenza, 1970; Filteau, 2010, 2011, 2012; Edson et al., 1912). The *Pseudomonas* genus is the largest contributor to sap microflora, with the remaining groups having been isolated in varying amounts. However, these populations will fluctuate as a result of nutrient depletion, acid accumulation due to microbial fermentation, and environmental temperatures. Nikolajeva & Zommere (2018) isolated several microbial groups from birch sap, which was stored at 68°F (20°C) and 39.2°F (4°C) for 58 days, respectively. However, only *Burkholderia cepacia* was identified as a predominant member of the microbial community throughout the entire storage period. These findings were attributed to the reduction in pH levels from 5.41 to 3.41, due to fermentation throughout storage. However, it is necessary to mention that despite the changing dynamics of sap microflora, psychrotrophic bacteria are always represented in the greatest quantity due to the increased growth potential under normal sap storage conditions (typically 25-45°F if stored outside).
In addition to bacteria, yeast and molds are also present in maple sap. Predominant yeasts include *Candida*, *Cryptococcus*, and *Rhodotorula* (Cosenza, 1970; Filteau et al., 2011; Labbe et al., 2001; Sheneman & Costilow, 1959; Sheneman et al., 1959) although several others have also been identified. Some filamentous molds are regularly recovered from sap, which include *Aspergillus*, *Penicillium*, *Phoma*, *Hormodendreum*, *Fusarium*, *Alternaria*, *Cephalosporium*, *Rhizoctonia*, and *Coniothyrium* (Sheneman & Costilow, 1959). However, these fungal groups are often outcompeted by bacteria due to the slower growth rate of these microorganisms. In fact, when the microbial community becomes so significant, visible defects in the sap become apparent. Often, a producer will become aware of bacterial-based defects before mold growth can be observed. Common sap defects are ropiness, which results in polymerization of sugars due to bacterial activity; buddiness, which produces a characteristically green liquid with an undesirable off-flavor; and discolored red or gray sap due to pigmented organism presence, such as *Micrococcus roseus* (Edson, 1910, 1912; Pelletier, 2018; Wasserman, 1963).

**1.6 Syrup Microbiota**

The majority of maple sap microflora will not survive the extensive thermal processing temperatures required to produce syrup. Additionally, the concentration of sugars (and corresponding high osmotic pressure) and the resulting low water activity would also not sustain the bacterial microbiota. However, despite the absence of vegetative bacteria, the effects of these organisms and sap quality can significantly influence the finished syrup quality. For example, exopolysaccharide production in ropy sap will also result in ropy syrup, which reduces syrup quality because the sugar chains will become concentrated during the boiling process. Similarly, defective flavors produced in buddy sap are typically transferred to the finished product (Lagacé et al., 2018; Pettier, 2018).
Fungi on the other hand, are notoriously more osmotolerant and can live in environments with reduced water availability, such as maple syrup. Like bacteria, mold spores are generally incapable of surviving the extensive evaporation process. However, post-process contamination can introduce these organisms, such as use of improper bottling temperatures or lack of effective sanitation programs. Therefore, it is unsurprising that fungi are regularly found in spoiled maple syrup (Annis et al., 2016; Calder et al., 2011). In fact, syrup is frequently contaminated with surface fungal mats, and a wide range of genera have been isolated from the finished product, including those previously mentioned in the sap microbiota section of this review (Annis et al., 2016; Calder et al., 2011; Sheneman & Costilow, 1959). Aside from anticipated microflora in maple sap and syrup, it is necessary to review the primary hazards associated with maple syrup safety.

1.7 Food Safety Modernization Act (FSMA)

Foodborne illness affects an estimated forty-eight million Americans annually (CDC, 2018a; FDA, 2018). The Centers for Disease Control and Prevention (CDC) estimates that three thousand of these reported foodborne illness cases are fatal (CDC, 2018a). These statistics reinforce the food industry’s inherent obligation to implement programs that prevent the occurrence of these outbreaks.

The Food Safety Modernization Act (FSMA), signed in 2011, is considered a major reform to prior food safety laws. Broadly, any food producer required to register with the FDA as a manufacturing facility (defined in section 415 of the Federal Food, Drug, and Cosmetic Act), is required to comply with this legislation. In understanding this definition of a manufacturing facility, the regulation applies to any establishment which processes, packages, or stores food intended for human consumption. There are a few exemptions (based on
company size and low risk activities) and exceptions to this definition, which includes some farms and retail food establishments.

The shifted objective of this legislation is to implement more food safety prevention tactics, as opposed to reactive planning after an outbreak has already occurred. This approach is accomplished by developing an effective food safety plan, which requires conducting a hazard analysis. The hazard analysis must address the most probable biological, chemical, and/or physical risks associated with a food product and process, and identify appropriate preventative controls such as processing, sanitation, or allergen contamination program parameters, as well as methods to monitor these identified controls (FDA, 2018). In doing so, FSMA increases producer accountability, and provides the FDA with increased authority to ensure food producer compliance with safety regulations. Although the maple industry is in some cases not required to follow all aspects of this law, FSMA has nonetheless created increased pressure to ensure compliance with food safety prevention planning and documentation (Saucier-Choate & Bryant, 2018). The law is subdivided into five different focus areas, summarized in Table 1.2 (FDA, 2018):
Table 1.2: FSMA Focus Areas and Objectives

<table>
<thead>
<tr>
<th>Focus Area</th>
<th>Main Objective</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevention-based control of the entire food</td>
<td>Producers are required to implement written safety programs to prevent accidental and intentional product contamination.</td>
</tr>
<tr>
<td>supply</td>
<td></td>
</tr>
<tr>
<td>Inspection and Compliance</td>
<td>The frequency of facility inspections is increased. Producers must now write food safety plans and monitoring procedures. FDA is granted access to compliance records.</td>
</tr>
<tr>
<td>Response – Mandatory Food Recalls</td>
<td>Recalls are no longer voluntary. FDA can require food recalls and suspend facility registration. Improved record keeping through product and ingredient traceability.</td>
</tr>
<tr>
<td>Imported Food Safety</td>
<td>Imported foods must now meet U.S standards. FDA can execute inspections on imports.</td>
</tr>
<tr>
<td>Enhanced partnerships</td>
<td>FDA is to collaborate with local, state, and federal agencies to ensure compliance at all levels</td>
</tr>
</tbody>
</table>

a. Adapted from FDA Food and Drug Modernization Act (FSMA) by Food and Drug Administration. 2018.

1.8 Low Risk Foods and Outbreaks

A low risk food product either has a pH value below 4.6 or a water activity below 0.85, both of which would presumably not support the survival of pathogenic microorganisms, if an adequate thermal process is applied (Virginia Cooperative Extension, 2012). It is therefore assumed that these products are less likely to be implicated in a foodborne outbreak. Maple syrup is designated by the FDA as a low risk food due to its reduced water activity level between 0.83-0.85 (University of Maine Extension, n.d). In reference to FSMA, maple syrup producers are required to follow certain record-keeping protocols and Current Good Manufacturing Practices (cGMPS), but are exempt from the hazard analysis and risk-based preventive control requirements, assuming no other value-added products, such as maple-based sauces, are produced at the facility (Saucier-Choate and Bryant, 2018).
Although no formal foodborne outbreak investigations have occurred in regards to maple syrup products, it is necessary to consider the product composition and potential hazards associated with the process. Regardless of the assumptions around low risk foods, more and more outbreaks have occurred connected to products given this designation. For example, the Soy Nut Butter Company recently recalled all butter spreads (estimated Aw = 0.45) and granola products (estimated Aw = 0.20) contaminated with *Escherichia coli* O157:H7 due to unsanitary facility conditions (Barbosa-Cánovas, 2007; FDA, 2017b; Ma et al., 2009). Similarly, Diamond Pet Foods’ dry dog food product was contaminated with *Salmonella* Infantis, which caused forty-nine human illnesses after contact with a pet that had previously consumed the infected product (CDC, 2012). It is also critical to note that although the risk of illness due to pathogenic contamination typically rises as the pathogen population increases, several bacteria are capable of causing illness in doses as few as 10 cells. Thus, a better understanding of the survival of these pathogens, even in the absence of growth, is needed to better ensure consumer safety. Moreover, it has been hypothesized that stresses posed by adverse environmental conditions may induce metabolic patterns that increase the virulence of some species upon ingestion (House et al., 2009; Humphrey et al., 1996; O'Driscoll, Gahan & Hill, 1996).

Like bacteria, fungi are a potential threat to contaminating low risk food commodities, as a result of the ubiquitous nature of these organisms in the environment. Molds typically require even less water availability for growth than bacteria, and are also capable of mycotoxin production, making them a viable hazard in maple syrup production. Many of these mycotoxins, or secondary metabolites, are carcinogenic and have been associated with a wide range of ailments including kidney failure, liver cancer, and bladder cancer (Montville et
al., 2012d; Montville et al., 2012e). Moreover, the reduced capability of bacteria to grow at low water activities means that xerotolerant fungi face significantly reduced competition in these environments. Magnoli et al. (2006) analyzed the presence of ochratoxin A in peanut seed crops, a low water activity food, in Córdoba, Argentina. The researchers determined that 32% of the crop samples tested positive for the toxin as a consequence of contamination with various Aspergillus species. Similarly, Geremew et al. (2016) isolated mycotoxin producing fungi (primarily ochratoxin-producing Aspergillus species) in 87% of coffee arabica samples (maximum 16% moisture content) collected from six Ethiopian coffee producing regions. Therefore, a deeper understanding of the survival capabilities of these microorganisms in maple syrup is critical in preventing a potential future outbreak.

1.9 Bacterial Food Safety Risks

1.9.1 Salmonella

Salmonella is a pathogenic bacterium that causes an estimated 15% of all foodborne illness cases in the United States (Carson-DeWitt & Cataldo, 2015). The onset of symptoms occurs within 48 hours of ingestion, but has been reported as early as 6 hours, with the average illness duration between 5-7 days (Carson-DeWitt & Cataldo, 2015; Kunwar, Singh, Mangla, & Hiremath, 2013). Common salmonellosis symptoms include: nausea, diarrhea, vomiting, abdominal cramping, and headache (Kunwar et al., 2013). For most people, the infection will clear on its own, however, the pathogen’s ubiquity in the environment and the possibility of additional health complications are the reasons this bacteria is the leading cause of foodborne illness deaths annually in the United States (Andino & Hanning, 2015). Salmonellosis occurs due to ingestion of the organism itself, and in some cases, requires as few as ten bacterial cells to induce illness (Beuchat et al., 2013).
*Salmonella* is a rod shaped, Gram-negative, facultative anaerobe (Andino & Hanning, 2015). It belongs to the *Enterobacteriaceae* family and is subdivided into two main species: *Salmonella enterica* and *Salmonella bongori* (Montville, Matthews, & Kniel, 2012c). There are greater than 2,500 distinguishing serovars, the majority of which belong to the *enterica* species (Jones et al., 2008; Montville et al., 2012c). Each serovar has unique properties, such as most *Salmonella* have flagella, but some strains such as the Gallinarum serovar, are non-motile (Montville et al., 2012c). *S. enterica* is responsible for the majority of foodborne illnesses in the United States, with serovars Typhimurium and Enteritidis most frequently implicated in outbreaks (Hendriksen et al., 2011; Jones et al., 2008).

Although *Salmonella* is often linked to the consumption of under-processed poultry and dairy, it has been the cause of several foodborne outbreaks of low risk food products. For example, a 2009 *S. Typhimurium* outbreak of King Nut peanut butter, a product with a typical water activity of 0.45 and a pH level of 5.12, caused 714 illnesses and 9 fatalities, as a result of unsanitary facility conditions (CDC, 2009; Ma et al., 2009). Kellogg’s Honey Smack cereal, another low water activity food, caused 135 illnesses and 34 hospitalizations after contamination with *S. mbandaka* (CDC, 2018b). Hometown Food Company also recalled an estimated 490,000 pounds of Pillsbury Unbleached All-Purpose Flour, (Aw = 0.70) due to possible *Salmonella* contamination (Carter n.d; Hoffman, 2019).

1.9.1.1 *Salmonella* Heat Resistance

The reason for this pathogen’s survival in low risk foods is because of its varied stress responses when faced with adverse environmental conditions. The ideal growth conditions for all *Salmonella* serovars is a pH level between 6.5-7.5, water activity greater than 0.93, and temperature of 95°F (35°C) (Montville et al., 2012c). However, this versatile bacterium has
been isolated in a pH range between 3.8-9.5, temperatures spanning 35.6-129.2°F (54°C), and water activity levels significantly below 0.85 (Beuchat et al., 2013).

*Salmonella* has been shown to exhibit increased heat resistance in low water activity foods. For example, Ma et al. (2009) observed a biphasic inactivation curve of *Salmonella* populations in inoculated commercial peanut butter samples ($A_w = 0.45$) which were heated to 159.8°F (71°C), 170.6°F (77°C), and 181.4°F (83°C), respectively. A sharp population decline followed by a more gradual reduction rate after the initial heating period, suggests that the microorganism is capable of adapting to the changing environmental conditions.

Even more concerning, a presumed lethal heat treatment may not be adequate for the eradication of this bacteria under normal storage conditions. He et al. (2011) analyzed the survival of *S. enterica* in inoculated peanut butter ($A_w = 0.40$) and found less than a two-log reduction in the bacterial population after 30 days of ambient storage followed by a subsequent 1 hour 72°C water bath heat treatment. Similarly, Kataoka et al. (2014) found that *Salmonella* enterica serotypes Tennessee and Typhimurium survived in inoculated (5-6 log CFU/g) and heat treated (75°C for 25-50 minutes) peanut paste samples for over one year of ambient (defined as 20°C) storage. The increased heat resistance may be due to a possible synergistic effect of low product water activity and fat content, although increase in fat between paste formulations (47% increased to 56%) alone, was not a significant factor in survival duration.

1.9.2 *Shiga toxin-producing E. coli (STEC)*

STEC is a pathogenic bacterium that causes an estimated 265,000 foodborne illnesses in the United States annually (CDC, 2014). The onset of gastroenteritis symptoms occurs within four days after ingesting the microorganism, and is marked by severe abdominal
cramping, diarrhea, and bloody stools. The duration of illness is one week and will resolve in most instances without treatment (CDC, 2017; Montville et al., 2012a). However, the ease of transfer, potential complications and severity of some STEC infections are what makes this microorganism a significant public health concern. More specifically, an estimated five percent of reported illnesses resulted in hemolytic uremic syndrome (HUS), a potentially life-threatening kidney disease (CDC, 2017; Feng, Weagant & Jinneman, 2011). As with Salmonella, some strains require as few as ten bacterial cells to induce illness (Feng et al., 2011)

E. coli is a Gram-negative, rod shaped facultative anaerobe (FDA, 2012). The complex classification system for this bacterium is based on surface antigen composition, the method of infection, in addition to virulence factors. For example, STEC O157:H7 indicates that the bacteria is enterohemorrhagic and contains both somatic (O) and flagellar (H) surface antigens (Montville, 2012a). When assessing the virulence factors of STEC, all are capable of producing cytotoxins Stx1 and/or Stx2, which restrict normal protein functions within the host cell. These bacteria also contain the eae gene, which allows for adherence to the intestinal wall surface, which in combination with the cytotoxin, results in illness (Aidar-Ugrinovich et al., 2007).

The majority of STEC research is focused on O157:H7 survival, however, this strain only constitutes 36% of reported E. coli implicated foodborne illness cases (CDC, 2014). Additionally, most of the published literature associated with this pathogen is focused on leafy vegetables and meat contamination, as it is part of the normal intestinal microflora of beef cattle (Aidar-Ugrinovich et al., 2007). But beyond meat and produce, STEC has been the cause of outbreaks associated with several low risk foods. In 2016, General Mills executed a
voluntary recall of bulk flour, a product with a water activity below 0.70, contaminated with 
*E. coli* O121 and *E. coli* O26, which had infected sixty-three people across twenty-four states 
(Carter n.d; FDA, 2017a).

### 1.9.2.1 STEC Heat Resistance

The pathogen’s resistance to high processing temperatures is contingent on the 
composition of the food matrix, as well as prior environmental adaptations, such as exposure 
to reduced water activity or high levels of acid. Acid-adapted STEC strains have been shown 
to exhibit increased heat resistance compared to their non-adapted counterparts. For example, 
Topalcengiz and Danyluk (2017) concluded STEC growth in tryptic soy broth (TSB) media 
with 1% glucose (to induce acid adaptation response) significantly increased heat resistance 
(d-values) in inoculated orange juice samples heated to 133°F (56.1°C) - 140°F (60°C).

Hiramatsu et al. (2005) also analyzed the heat resistance of thirty-five STEC strains on dried 
paper discs at 95°F (35°C) for twenty-four hours. The researchers determined that the 
majority of STEC strains survived, with a maximum 4-log reduction, even after refrigerated 
storage for twenty-two months. This rate of survival was dramatically enhanced when paper 
disks contained sucrose. The rate of survival was seventy-nine times higher when 36% 
sucrose discs were introduced. The bacteria on sucrose discs survived after an additional heat 
treatment of 158°F (70°C) for 5 hours. The survival is likely because sucrose has been shown 
to stabilize cell membranes (through temperature reduction), which in turn provides a 
protective barrier to the inner cell contents, such as proteins, when exposed to heat (Leslie, 
Israeli, Lighthart, Crowe, & Crowe, 1995). Daryaei et al. (2018) also investigated the heat 
tolerance of a STEC cocktail of O157 and non-O157 strains in several low moisture foods 
with water activities between 0.26-0.63. This study indicated that in order to achieve a five-
log reduction in the lowest water activity range (chicken meat powder with $A_w = 0.25$), holding the product at 176°F (80°C) for 15.5 minutes, was required. However, a confectionary formula with a slightly higher water activity ($A_w = 0.42$) had a similar heat resistance as the chicken meat powder, likely due to the increased sucrose content protecting the bacteria. These studies each suggest that this microorganism will show a similar tolerance in a high sucrose - low water activity matrix, such as maple syrup.

### 1.9.3 *Listeria monocytogenes*

*Listeria monocytogenes* infection results in approximately 1,660 illnesses annually in the United States. The majority of cases require hospitalization, and the extremely high mortality rate (16%), makes this type of infection a grave public health concern (Montville et al., 2012b). The incubation period before symptom onset ranges between one to four weeks, which also creates difficulties in identifying the contaminated food source (CDC, 2016; Montville et al., 2012b). Symptoms are highly variable, but are often expressed as “flu-like”, as in listerial gastroenteritis cases. Immune-compromised populations, such as pregnant women, young children and the elderly, are more susceptible to listeriosis, which is the more severe form of the infection. Additional health complications can arise in these severe cases including: septicemia, meningitis, and spontaneous abortions in pregnant women (CDC, 2016).

*Listeria* is a Gram-positive, rod shaped, facultative anaerobe with flagella to allow mobility. There are several identified species, however, only *Listeria monocytogenes* is considered a human pathogen capable of causing listeriosis. The majority of listeriosis cases are caused by *L. monocytogenes* serotypes 1/2a, 1/2b, and 4b (Pan, Breidt, & Kathariou, 2009). The bacteria’s pervasive nature in the environment, and the unknown infectious
dosage, are additional challenges when assessing the risk of food contamination. For these reasons, the United States has implemented a zero-tolerance policy, which requires immediate recall for *Listeria* presence in all foods (USDA-FSIS, 2014).

RTE (ready-to-eat) foods are frequently implicated in reported outbreaks associated with *Listeria monocytogenes*, as there is often no final kill step during consumer preparation. The psychrotrophic nature of this bacteria increases the risk of growth during refrigerated storage (CDC, 2018c). Although there have been no reported *Listeria* outbreaks associated with low risk food products, such as maple syrup, it is necessary to address its potential survival due to the severity of illness, ubiquity in processing environments, heat resistance, and adaptability of the pathogen.

### 1.9.3.1 *Listeria monocytogenes* Heat Resistance

The ideal growth conditions for this microorganism are temperatures between 86°F (30°C) - 98°F (36.7°C), water activity values greater than 0.97, and pH ranges between 4.5 - 9.6 (Montville, 2012b). However, *Listeria* has been shown to survive in environments far exceeding these values, with growth at temperatures as low as 32°F (0°C) and survival in minimum water activity and pH values of 0.83 and 4.3, respectively (Francois et al., 2006; Montville, 2012b; Schwartzman, Belessi, Butler, Skandamis, & Jordan, 2011).

*Listeria* is highly adaptive and can withstand otherwise adverse environmental conditions (Koseki, Nakamura, & Shiina, 2015). Shen et al. (2014) investigated the stability of heat adaptive responses of known low, medium, and highly-heat resistant *Listeria monocytogenes* serotypes after an initial 118.4°F (48°C) for 0.5 or 1 hour, and subsequent 140°F (60°C) for 10 minutes, heat treatment. Although results were strain specific, the researchers found that the highly-heat resistant serotypes exhibited an average five-log
regrowth in CFU/ml of tryptic soy broth supplemented with 0.6% yeast, compared to identical strains, which were only subjected to the initial heat treatment. These values were only stable at room temperature for two hours, but were maintained for up to twenty-four hours under refrigerated storage.

Interestingly, most of the temperature-focused research associated with *Listeria* is based on its survival during refrigerated and frozen storage. There are limited published thermal inactivation studies analyzing the behavior of this microorganism in low-risk food media. However, *Listeria* has been isolated from these foods, and therefore, presents a viable hazard requiring further investigation (Kimber, Kaur, Wang, Danyluk, & Harris, 2012; Koseki et al., 2015). Kimber et al. (2012) reported a very gradual population reduction in *Listeria* inoculated on pistachios and almonds. In ambient conditions, nuts which had a maximum water activity of 0.50 throughout storage, only demonstrated a 0.86 CFU/g reduction per month of storage time. However, no population reductions were reported in product kept in frozen and refrigeration temperatures for one year. A second study observed the survival of *L. monocytogenes* in powdered baby formula (A_w = 0.28) after one year at refrigerated and ambient storage (Koseki et al., 2015). Therefore, additional information is needed to better understand the heat tolerance of this pathogen in high sucrose compositions, such as maple syrup.

**1.9.4 Viable but non-culturable (VBNC)**

Each of the previously mentioned pathogens are non-spore forming bacteria, but are capable of entry into a viable, but non-culturable state (VBNC), which is a survival tactic that can potentially increase heat resistance (Liao et al., 2018). VBNC allows the bacteria to enter a dormant-like state, marked by a reduction in metabolic reactions, alterations in gene
expressions, and changes to the cell structure (Coutard et al., 2007; Roszak, Grimes & Colwell, 1984). The bacterium is often able to return to its vegetative form assuming favorable conditions are met, and there is sufficient nutrient restoration within the potential revival period (Liao et al., 2018; Roszak et al., 1984). Environmental changes that can induce this adaptive stress response include: extreme temperatures, acidity, lack of nutrients, or sanitizer and preservative contact (Roszak et al., 1984). It can therefore be suggested that any of these organisms will remain on a surface or in a product, such as maple syrup, under unfavorable environmental conditions, even if cells are unable to execute all normal functions for extended periods of time.

1.9.5 Biofilm Formation

All microorganisms mentioned in this review are also capable of biofilm formation. A biofilm is formed when the bacteria excrete an extracellular matrix, which can then attach to a range of materials including metal, glass, and plastic (Stepanović, Ćirković, Ranin, & Švabić-Vlahović, 2004). Biofilms are particularly detrimental to food manufacturing environments because they have been shown to have increased resistance to cleaners and sanitizers, and provide a potential source for recontamination if not effectively removed from the processing surface (Joseph, Otta, Karunasagar, & Karunasagar, 2001).

With respect to maple syrup manufacturing, biofilms are difficult to remove from sap collection tubing networks, sap collection tanks, and syrup bottles. Sap collection tubing is often the most susceptible to biofilm formation, because bends in the lines and elevated temperatures from sun exposure provide an ideal medium for bacterial attachment and growth (King & Morselli, 1985). Lagacé, Jacques, Mafu, & Roy (2006a), for example, produced a Pseudomas marginalis biofilm using inoculated maple sap. The researchers found that the
biofilm produced at 30°C, as opposed to 15°C, contained a greater number of bacteria, as well as a more adherent film. These results indicate that increased environmental temperatures enhance bacterial attachment and growth. This is supported by a second study which analyzed collection tubing used during the 2002-2003 Québec sugaring season. The researchers found that biofilms had greater adhesion and contained more bacteria later in the season compared to samples which were collected earlier in the production period. These findings were attributed to an increase in environmental temperature and the accumulation of biofilm populations throughout the sugaring season (Lagacé, Jacques, Mafu, & Roy, 2006b).

Unfortunately, syrup producers are often not aware of biofilm formation until the microbial community is so large that it causes a visible block in sap flow. Although *Pseudomonas* species comprise the majority of the biofilm plaques extracted from sap collection networks, *Salmonella*, STEC, and *Listeria* are threats to syrup production as a result of each organism’s adaptability to adverse environmental conditions and ability to form biofilm (King & Morselli, 1985).

The multiple routes of possible pathogen entry into a syrup operation creates a considerable product safety concern. Unlike many food commodities, a significant portion of syrup production (such as tree tapping and sap collection) occurs in an open-air environment, which promotes transfer of bacteria between human, animal, and environmental sources (Andino & Hanning, 2015; Feng, Weagant, & Jinneman, 2011; Montville, 2012). *Listeria monocytogenes* is a soil microbe, whereas *Salmonella* and STEC are commonly transferred via direct human or animal contact, providing an obvious access points for syrup contamination. This may occur at multiple processing stages such as when inserting a spile, collecting sap, or if an animal chews on the collection tubing (which creates an opening for
transmission). Employees can potentially transfer viruses and/or pathogens into the sugaring house if an infected person directly contacts the product or a processing surface. This transmission may occur as a result of poor personal hygiene (hands and/or clothes), lack of running potable water/handwashing access, lack of proper equipment sanitation, or absence of good manufacturing practices. Instances of outbreaks due to water leakage (e.g. from damaged roofing or ceilings) contaminated with animal feces have been recorded by U.S public health agencies. This route of entry is a particular concern in the maple industry due to the consistent presence of moisture and condensation that may allow such a problem to go undetected. Therefore, further research is needed to address the survival of these organisms if present in the finished product.

1.10 Fungal Food Safety Risks

Beyond bacterial food safety risks, fungi including Aspergillus and Penicillium spp., are also considerable health threats to the maple syrup supply. Although neither of these molds have been linked to a food safety incident involving maple syrup, the frequent association of these genera with syrup quality loss suggests further investigation is necessary to ensure consumer safety.

Between 2010-2014, researchers at the University of Maine analyzed fifty-two samples of syrup contaminated with a visible surface mass and determined that 94% of these defects originated from fungal growth. The majority of these samples were within the required °Brix and water activity ranges, contradicting the previous assumption that fungal growth only occurs in syrup deviating from targeted specifications. Through DNA extraction techniques, the researchers determined the majority of these samples were contaminated with Aspergillus followed by Penicillium and Wallemia (Calder, Hopkins, Marshall & Annis, 2011; Annis,
Hopkins, Calder & Garcia, 2016). In order to identify potential risk factors for this type of quality loss, a similar 2014 study examined sixty-eight samples of Ontario maple syrup for fungal growth. Although one third of these defects were associated with high product water activity levels and the syrup was filled at room temperature; additional samples were filled using typical bottling temperatures, and were also found to be contaminated. The investigators predicted this was the result of poor process sanitization practices. Of the contaminated samples in this study, twenty-three mold species were identified, predominately Aspergillus and Penicillium (Frasz & Miller, 2015).

1.10.1 Aspergillus

Aspergillus is primarily a saprotrophic, mildly xerophilic mold which is ubiquitous in the environment and commonly found in the air and soil. The name Aspergillus is derived from its structural features, with swollen vesicle and surrounding conidia reminiscent of an aspergillum; a tool used to mist holy water during religious ceremonies (Scizzocchio, 2009). The vast majority of species within this genus do not pose any human health risks in otherwise healthy individuals because only spores are commonly ingested (Montville et al., 2012d).

The majority of human health-related reports stems from immunocompromised people who have inhaled Aspergillus spores and developed secondary illnesses, such as allergic bronchopulmonary aspergillosis (ABPA) or chronic pulmonary aspergillosis (CPA) (CDC, n.da). These invasive fungal infections can quickly become fatal, by spreading to other organs such as the liver, kidney, heart, and brain (Montville et al., 2012d; Scizzocchio, 2009). In 2014, the CDC reported 15,000 Aspergillus related hospitalizations in the United States. Most of these cases were the result of construction at a hospital site or a medical procedure which used an Aspergillus contaminated device. The symptoms for these mycoses related illnesses are highly
variable and depend on the type of infection. Symptoms of ABPA include shortness of breath and coughing, whereas the symptoms for an *Aspergillus*-induced sinus infection include excess mucus in the nasal cavity and headache (CDC, n.da).

Foodborne illnesses associated with *Aspergillus* are linked to mycotoxins, or secondary metabolites, which are harmful to human health. Typically, agricultural commodities such as nuts, corn, rice, and cornmeal are implicated in these cases (Missouri Department of Agriculture, n.d; Montville et al., 2012d). More specifically, risk factors in the harvesting of these products include insect or animal damage (providing an access point for the mold), changes in climate, as well as improper storage and handling. The *Aspergillus* species, which produces these toxins under ideal conditions, are considered to be opportunistic pathogens which feed on food materials where they grow. Aflatoxin, often associated with *A. flavus*, *A. parasiticus*, and *A. nomius*, is the most notable mycotoxin produced by this genus because it is a known liver carcinogen (Montville et al., 2012d; Scazzocchio, 2009).

Literature indicates that the optimal conditions for aflatoxin production are a water activity of 0.99 and a temperature of 91°F (32.8°C) (Murphy, Hendrich, Landgren & Bryant, 2006). However, the metabolite has been isolated in conditions far exceeding these values. Del Palacio, Bettucci, and Pan (2016) isolated the compound in 220 wheat samples (Aw = 0.693-0.709) stored in silos for up to 120 days (the duration of the study). Although potential oxygen exposure in the silo and warm environmental temperatures may have contributed to these findings, it is likely that improperly packaged or stored food products have increased susceptibility to toxin production. Therefore, toxin production is possible in other low water activity commodities, including maple syrup.
There is a much higher occurrence of aflatoxin-related foodborne illnesses in underdeveloped countries where individuals frequently harvest their own food supplies or there are less established food regulations. The most notable of these was in Kenya (2004) in which maize was heavily contaminated with the metabolite and resulted in over 300 illnesses, with nearly 40% of these reports resulting in fatalities (CDC, 2004; Montville et al., 2012d; Scaggocchio, 2009). Similarly, a 1974 aflatoxin outbreak in India, which caused hundreds of cases of hepatitis, resulted in 289 illnesses and 108 deaths. This disastrous outbreak can also be attributed to maize contaminated with the mycotoxin (Krishnamachari, Bhat, Nagarajan & Tilak, 1975). Although aflatoxin is arguably the most dangerous of these compounds, other species within the genus are also capable of ochratoxin A, citrinin, and patulin production (Montville et al., 2012d; Scaggocchio, 2009).

1.10.1.1 *Aspergillus* Heat Resistance

Mold spores are notoriously less sensitive to reduced water activity environments compared to bacterial pathogens. Although there are water requirements for mold growth, unlike bacteria, osmotic stress alone may not kill the spore but rather prevent spore germination. In turn, when conditions are more favorable, mold is capable of growth. As previously noted, *Aspergillus* is regularly found in agricultural commodities with naturally low water contents such as peanuts, wheat grains, and maple syrup. Effective heat treatments are thus necessary to ensure that the mold spores are killed, and presumably unable to emit mycotoxins.

In a preliminary study conducted by Annis et al. (2016), the researchers were unable to eradicate all *Aspergillus* spores in maple syrup until a five-minute heat treatment at 179.6°F (82°C) was applied. When compared to other low water activity foods, a 2018 study analyzed the effects of temperature on the decimal reduction time of *A. flavus* spores in peanut kernels with
varying water activities. The study determined that heating kernels with a water activity of 0.846, the approximate specification of maple syrup, for 3.69 minutes at 149°F (65°C) was necessary to kill 90% of the spore population. The study also determined that an even lower water activity of 0.783 increased the reduction time to 7.25 minutes at this same temperature (Zang et al., 2018). Therefore, a deeper understanding of the heat resistance capabilities of this mold in maple syrup on a larger scale and under typical processing conditions is critical.

Mycotoxins, such as aflatoxin, are much more heat stable, persisting in the product even after the spores are eradicated. The heat stability of the toxin itself is dependent on product composition, such as moisture and acidity. Mann, Codifer, and Dollear (1967) were only able to remove 75% of aflatoxin B1 and B2 present in cottonseed meal with a 30% moisture content (the approximate moisture of maple syrup) after heating to 212°F (100°C) for 60 minutes (Stuckel and Low, 1996). These conditions would be well beyond an appropriate heating profile to maintaining maple syrup quality.

1.10.2 *Penicillium*

*Penicillium*, like *Aspergillus*, is a saprophytic and relatively xerophilic mold, prevalent in the environment and a frequent food contaminant (Montville et al., 2012d; Yin et al., 2012). Agriculture-based products such as wheat, barley, and fruits are more susceptible to this contaminant (Cabañes et al., 2010; Montville et al., 2012e). The name of the fungus is a tribute to its’ structural features with long philades, extending from the conidiophore, similar to a paintbrush or the Latin penicillus (Yin et al., 2012). The genus is comprised of over three hundred species, with greater than eighty species identified as harmful to human health (Montville et al., 2012e; Yin et al., 2012).
There are few known illnesses caused by ingesting the mold spores directly. An acute *Penicillium* exposure in an otherwise healthy individual can result in several allergy-related symptoms including running nose, coughing, sore throat, or sinus infection (CDC, n.db). Immunocompromised populations, however, have a greater susceptibility to illnesses with increased severity in symptoms. The most notable of these is Talaromycosis (previously termed Penicilliosis), caused by *T. marneffei* (formally *P. marneffei*), a dimorphic opportunistic pathogen (yeast-forming upon entering the body). Talaromycosis typically infects patients with HIV. Inherent to Southeast Asian populations, the illness can cause a rash, difficulty breathing, and swelling in the liver, spleen, and lymph nodes. Antifungal treatments dramatically reduce the mortality rate of this illness with less than 25% of deaths when treated (Cao et al., 2011; CDC, n.dc).

As with most fungal-based instances of foodborne illness, products contaminated with *Penicillium* can result in mycotoxicoses. With respect to all toxigenic species within the genus, more than forty harmful mycotoxins have been identified. The most significant of these include ochratoxin A (produced by *P. verrucosum* and *P. nordicum*), patulin (commonly associated with *P. expansum*), citrinin (produced by *P. citrinum* and others) and mycophenolic acid (a metabolite of several species, including *P. brevicompactum*) (Cabañes, Bragulat, & Castellá, 2010; Montville et al., 2012e; Murphy et al., 2006; Patel et al., 2016).

The symptoms for these intoxications are highly variable and depend on the compound causing the illness. Ochratoxin A is a carcinogen, which has been associated with Balkan endemic nephropathy, a condition prevalent in rural communities of Eastern Europe. The disease typically results in kidney failure and potential bladder cancers after prolonged exposure to the metabolite via food sources (Montville et al., 2012e). The ideal conditions for ochratoxin A
production are $A_w$ of 0.90-0.98 and a temperature of 77°F (25°C) (Murphy et al., 2006; Northolt, Van Egmond & Paulsch, 1979). However, literature has shown that the mold is capable of producing the compound under conditions which deviate from these specifications. Dhungana et al. (2019) extracted the metabolite from grain samples, which were inoculated with *P. verrucosum*, that was modified to a $A_w$ of 0.85, the approximate value of maple syrup. Although the amount of toxin produced was significantly less than that emitted in other treatments within the study ($A_w$ of 0.90), it suggests that additional understanding of the organism’s growth potential in syrup is necessary.

Similarly, mycophenolic acid exposure can result in immune system suppression (via inhibition of T and B cell reproduction) and has also been associated with miscarriages in pregnant women (Annis et al., 2016; Great Plains Laboratory Inc., n.d). In fact, Annis et al. (2016) monitored mycophenolic acid production of three *P. brevijactum* isolates which were obtained from maple syrup samples. When grown on Sabouraud yeast agar (a sugar rich media), all of the isolates were capable of producing the toxin, with one of the strains emitting more than 1.4 mg/mL of the compound after 15 days of room temperature (68°F, 20°C) incubation. The study also determined that two of the isolates were capable of producing the toxin when the mold was grown directly in maple syrup. One of the strains produced 8.8 ug/mL after 55 days of room temperature incubation (Annis et al., 2016). Thus, additional data regarding the fungus’ survival under typical maple syrup processing conditions is necessary to establish pointed processing recommendations to producers.

1.10.2.1 *Penicillium* Heat Resistance

As with the other organisms discussed in this review, *Penicillium* is a frequent contaminant of various food products and an adequate heat treatment is needed for spore
eradication. In the previously mentioned work conducted by Annis et al. (2016), researchers also tested the thermal inactivation of spores from several *Penicillium* species (including *P. chrysogenum*, as well as *P. brevicompactum*) in maple syrup. Inoculated syrup was heated to 149°F (65°C) in 2-minute increments until the majority of the spores were not recovered. Among the most heat resistant strains, a 10-minute exposure at this temperature was required to kill 90% of the present population. The survival rate at higher temperatures was also analyzed as syrup was heated for three minutes at 140 (60°C), 149 (65°C), 158 (70°C),167 (75°C), and 179.6°F (82°C), respectively. It was not until a three minute 179.6°F (82.2°C) treatment was used that all spores were eradicated (Annis et al., 2016). Like aflatoxin, mycotoxins produced by *Penicillium* are more heat stable than the spores themselves and are thus a threat to maple syrup safety if the fungus is present. A 2016 study, assessed the thermal inactivation of ochratoxin A by inoculating citrate-phosphate-borate buffer, modified to pH 7 with the metabolite (10 ng/mL). The study determined that even after a heat treatment of 392°F (200°C) for 60 minutes, 10% of the toxin still remained in the buffer solution (Dahal, 2016). Similarly, Pérez De Obanos, González-Peñas, and López De Cerain (2005), recovered an average 33.5% of the toxin present across coffee bean samples that were roasted for 5 minutes at 500°F (260°C). Although not a direct comparison to how maple syrup is processed, the excessive heat treatments described in both of the mentioned studies would far exceed what would be acceptable to maintain syrup integrity. Therefore, thermal inactivation parameters of the fungal spores under typical processing conditions is essential. In order to ensure consumer safety, opened syrup should always be stored under refrigerated storage. In the event of visible mold growth, a common misconception is that simply scraping off a fungal mass deems the product once again safe after reboiling the syrup. However,
due to mycotoxin production, which can penetrate the product beyond the visible surface mold, fungal-contaminated syrup should never be consumed.

1.11. Conclusions and Experimental Objectives

Current research on the microbial contamination of maple syrup is largely centered on product quality loss (such as grade and organoleptic changes) and process optimization. To our knowledge, there have been no prior studies that analyzed the tolerance capabilities of both bacterial and fungal pathogens in maple syrup. Although there are currently no reported foodborne illness cases associated with syrup consumption; it is necessary to better understand the thermal inactivation requirements of major pathogens to ensure consumer safety. Despite the designation as a low risk food, improper processing practices such as low bottle fill temperatures, cross contamination with unsanitary packaging materials or surfaces, are potential sources of pathogen introduction and survival. By increasing awareness of the microbial risks and establishing specific processing guidelines, the maple industry can provide greater assurance to regulators and the public that the syrup supply will remain safe.

Therefore, the objectives of this research are to (i) determine the survival of each organism under typical storage conditions, (ii) determine the decimal reduction time of STEC, *Salmonella*, and *Listeria monocytogenes* species at 180°F (82.2°C), a common bottle fill temperature, (iii) assess the survival capabilities of both bacterial and fungal (*Penicillium* and *Aspergillus*) pathogens in simulated bottle surface contamination using various retail containers, and (iv) determine the effectiveness of a boiling water pre-fill treatment for mitigating the risks of bottle surface contamination.
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CHAPTER 2

THERMAL INACTIVATION OF STEC, SALMONELLA, AND LISTERIA MONOCYTOGENES IN MAPLE SYRUP

2.1 Abstract

It has long been the assumption that adequate pathogen growth is required to induce foodborne illness. Low water activity environments, such as maple syrup ($A_w = 0.85$) were previously thought to inhibit such growth. However, more frequent instances of foodborne illness associated with low risk food products including wheat flour, peanut butter, and dry pet foods, have now challenged this belief (CDC, 2012; FDA, 2017; Hoffman, 2019). Low infectious doses, or the absence of growth, have been attributed to causing these illnesses, hence a deeper assessment for pathogen inactivation within these products is necessary to ensuring consumer safety. Thus, the aim of this study was to determine the decimal reduction times (D-value) of shiga toxin-producing Escherichia coli (STEC), Listeria monocytogenes and Salmonella, in maple syrup. Particular attention was given to a heat treatment at 180°F (82.2°C), a common bottling and reheating temperature. The thin-walled capillary tube method was used in this analysis. A $z$-value curve was constructed using the experimentally determined D-values for each pathogen at 140°F (60°C), 150°F (65.6°C), and 160°F (71.1°C). Our results indicate that of these pathogens, L. monocytogenes is the most heat resistant in maple syrup, as expected. The D-values for STEC, L. monocytogenes and Salmonella in 180°F (82.2°C) maple syrup are 0.47, 4.63 and 1.56 seconds, respectively. Pasteurization, defined as a minimum five-log reduction, in all three bacterial species, can be achieved by holding syrup at this temperature for 23 seconds. These findings suggest that current maple syrup heating practices are adequate to mitigate the risk of bacterial pathogen contamination.
2.2 Introduction

Frequent outbreaks associated with low water activity foods has raised questions regarding the presumed safety of these products. In 2017, Soy Nut Butter products ($A_w = 0.45$) were implicated in a multistate outbreak of *E. coli* O157 due to insanitary processing conditions (Carter, n.d; FDA, 2017). More recently, Hometown Food Company voluntarily recalled over 12,000 cases of all-purpose flour ($A_w = 0.70$) as a result of potential *Salmonella* contamination (Carter n.d; Hoffman, 2019). In 2019, Brand Castle LLC similarly recalled many brands of wheat flour products ($A_w = 0.70$) contaminated with *E. coli* O26, which resulted in twenty-one reported illnesses (CDC, 2019).

Maple syrup is produced in the Northeast and Midwest region of the United States and Canada by concentrating the sap of the sugar maple tree (*Acer saccharum*), and other maple varietals, via extensive thermal evaporation. The high °Brix and correspondingly low water activity levels of maple syrup are assumed to inhibit pathogenic bacterial survival. For this reason, the FDA classifies syrup as a low risk food commodity (CFSAN, 2015). Although maple syrup has yet to be associated with a documented foodborne illness, the way in which it is processed can amplify risk for pathogen introduction.

As with any agriculturally-based food, bacterial introduction in some respects is inevitable. Sap once removed from the tree is susceptible to microbial colonization and growth, as a result of environmental exposure and high-water content (95-99%) (Perkins & van den Berg, 2009). For this reason, spoilage microbiota, such as *Pseudomonas fluorescens* and *Rahnella*, are commonly associated with changes to sap chemistry and subsequent finished syrup grade (Filteau, Lagacé, LaPointe, & Roy, 2012). Various manufacturing practices, however, can also contribute to bacterial accumulation. Sap storage conditions and duration, equipment cleaning
procedures, producer behaviors, processing environment conditions (such as condensation over the processing area), and post-processing practices can further augment these risks. Therefore, an adequate heat treatment is necessary to ensure both spoilage, and pathogenic bacterial populations are effectively killed. A minimum bottling temperature of 180°F (82.2°C), which is applicable to both reheating of bulk syrup for retail packaging, as well as post-filtration filling, is the current industry standard for microbial risk prevention (Dumont, 2007; Whalen & Morselli, 1984).

Pathogenic bacteria, including shiga toxin-producing *Escherichia coli* (STEC), *Salmonella* and *Listeria monocytogenes*, pose the greatest risk to maple syrup safety. All three bacterial genera are ubiquitous in the environment, have low to unknown infectious doses, and exhibit increased heat resistance in low water activity foods. STEC, which can be spread through human and animal sources, is associated with gastroenteritis symptoms and hemolytic uremic syndrome in more severe cases. This pathogen was reduced by 5-logs in dried chicken meat powder \((A_w = 0.25)\) only after a 176°F (80°C) for a 15.5-minute treatment was applied (CDC, 2017; Daryaei et al., 2018). Similarly, *Salmonella* is also transferred by animals and people and can induce cramping, vomiting and diarrhea. *Salmonella* has been isolated in peanut butter samples, which were produced from inoculated peanuts that received a blanch and roast process, utilizing temperatures of 212°F (100°C) and 320°F (160°C) (Kunwar et al., 2013; Nascimento et al., 2018). *L. monocytogenes*, the most heat resistant of these pathogens, is a soil microbe and can cause flu-like symptoms, spontaneous abortions in pregnant women and death in an estimated 16% of reported cases (CDC, 2016; Montville et al., 2012b). *L. monocytogenes*, which has demonstrated survival in a variety of low water activity foods, such as powdered baby formula \((A_w = 0.28)\) and inoculated nuts, is also psychrotrophic, and can grow under refrigerated
conditions where sap and syrup are often stored (CDC, 2018c; Kimber et al., 2012; Koseki et al., 2015).

Quantitative data for the eradication of these pathogens in maple syrup has yet to be produced. Therefore, the objective of this research was to assess the validity of the current bottle filling recommendations by determining the decimal reduction time (D-value) of STEC, *Listeria monocytogenes*, and *Salmonella* (two-strain composites) at 180°F (82.2°C) in maple syrup.

2.3 Materials and Methods

2.3.1 Syrup Analysis

Grade A Amber maple syrup (Highland Sugar works, Websterville, VT) was used in this analysis. The pH (Edge Model, HI2020 Probe Hanna Instruments, Woonsocket, RI), water activity (Aqua Lab Series 3 TE, Decagon Devices Inc., Pullman, WA), and °Brix levels (Pocket Refractometer PAL-3, ATAGO, Tokyo, Japan) were recorded in triplicate readings prior to syrup inoculation for each trial. Syrup specifications are detailed below (Table 2.1).

Table 2.1: Grade A Amber Maple Syrup Specifications

<table>
<thead>
<tr>
<th>Specification</th>
<th>Mean ± Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.60±0.24</td>
</tr>
<tr>
<td>Water Activity</td>
<td>0.858±0.00</td>
</tr>
<tr>
<td>°Brix</td>
<td>66.14±0.16</td>
</tr>
</tbody>
</table>

a. pH n = 36, Water Activity n = 34, °Brix n = 30

2.3.2 Bacterial Inoculum Preparation

Bacterial colonies were streaked for isolation on selective media and incubated at optimal growth temperatures (detailed below). A single colony of *E. coli* O111:H8 ATCC BAA-184 (American Type Culture Collection, Manassas, VA), *E. coli* O26:H11 ATCC BAA-1653 (American Type Culture Collection, Manassas, VA), *Salmonella* Enteriditis ATCC BAA-1045
(American Type Culture Collection, Manassas, VA), *Salmonella* Braenderup (a hazelnut isolate), as well as *L. monocytogenes* ATCC 19111 (American Type Culture Collection, Manassas, VA) and *L. monocytogenes* ATCC 19115 (American Type Culture Collection, Manassas, VA) were used in this analysis.

Each strain was individually propagated in Luria Miller broth (Alpha Biosciences, Baltimore, MD). All inoculated broths were incubated for 24 hours at optimal growth temperatures for each bacterium (Table 2.2). After the 24-hour incubation period, Luria broth cultures were centrifuged (Centrifuge 5810 R, Eppendorf, Hauppauge, NY) for 10 minutes at 15.550 x g and resuspended in 5 mL of 0.1% peptone (Difco, Sparks, MD). Bacterial cultures of like species were combined and diluted as appropriate in 0.1% sterile peptone.

### 2.3.3 Capillary Tube Preparation and Heating

The prepared stock culture was diluted to approximately 5.5 log CFU/ml in Grade A Amber maple syrup (Highland Sugar works, Websterville, VT). A 50-μl aliquot of the inoculated syrup was then dispensed into sterilized (121°C, 15 minutes) thin-walled capillary tubes (Fisher Scientific, Waltham, MA) and heat-sealed using a Bunsen burner flame (Figure 2.1). To ensure even heat distribution, particular caution was taken when filling the tubes so that no air bubbles were present. For consistent sample removal, capillary tubes for each time point assessment were placed in custom mesh screen baskets (Saint-Gobain, Grand Island, NY) (Figure 2.2). The baskets were then submerged into a preheated 140°F (60°C), 150°F (65.6°C), or 160°F (71.1°C) circulating water bath (Precision CIR19, Thermo Fisher Scientific, Newington, NH). The location of baskets for each time point was randomized across replicate experiments. To promote adequate water circulation, test tube racks were arranged in the water bath so that capillary tubes did not directly contact the equipment. Timing was monitored using a digital timer with no
come-up time assumed. At the conclusion of each time point, capillary tubes were immediately submerged in ice. After sufficient cooling, capillary tubes were submerged in 95% ethanol for 10 minutes to inactivate surface contaminants, then transferred to a biological safety cabinet to air dry. Once there was no detectable ethanol on the outer surface (approximately 2 minutes), each capillary tube was placed into individual sterile 15 ml conical tubes. To release the syrup, capillary tubes were broken using the sterilized handle of a cell spreader. The conical tube contents were then diluted in 0.1% peptone and well vortexed to ensure the entire syrup volume was separated from the glass until further analysis. Three capillary tubes were analyzed at each time point and all experiments were performed in triplicate.

Figure 2.1: Capillary Tubes Filled with Inoculated Maple Syrup and Flame-Sealed Prior to Water Bath Submersion
Figure 2.2: Submerged Capillary Tubes in Water Bath Using Custom Mesh Screen Cups
Test tube racks were placed in the water bath to ensure even temperature distribution across samples.

Figure 2.3: Capillary Tubes Submerged in Ice (A) and Transferred to 95% Ethanol Solution for Surface Decontamination (B) Prior to Hood Aeration
2.3.4 Microbial Enumeration

For bacterial recovery, 100 µl of the diluted syrup sample was then spread plated onto the appropriate selective media (Table 2.2). All plates were then overlaid with 5 ml of tempered (55°C Isotemp 105 water bath, Fischer Scientific, Dubuque, IA) soft Brain Heart Infusion (BHI) overlay. The overlay was prepared using Brain Heart Infusion Broth (Acumedia, Lansing, MI) and 0.6% Bacteriological Agar (Alpha Biosciences, Baltimore, MD). Once the overlay was evenly dispersed and solidified, plates were inverted and incubated at the previously described optimal incubation temperatures for an additional 48 hours (Table 2.2). Characteristic bacterial colonies from each plate were counted for enumeration of the surviving population.

Table 2.2: Selective Media and Incubation Temperature Used

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Selective Agar</th>
<th>Incubation Temperature °C (48 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STEC</td>
<td>MacConkey Sorbitol Agar a</td>
<td>37</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>PALCAM b</td>
<td>32</td>
</tr>
<tr>
<td>Salmonella</td>
<td>XLD c</td>
<td>37</td>
</tr>
</tbody>
</table>

a. MacConkey Sorbitol Agar (Alpha Biosciences, Baltimore, MD)
b. PALCAM (Polymyxin Acriflavine Lithium Chloride ceftazidime Aesculin Mannitol, BD Difco, Sparks, MD)
c. XLD (Xylose Lysine Deoxycholate agar, BD Difco, Sparks, MD)

2.3.5 Decimal Reduction Time (D-value) Determination

Individual log CFU/mL versus time (minutes) inactivation curves were created for each of the three experimental replicates per temperature. This was performed by determining the mean, then log transforming the three capillary tube samples from each time and temperature combination. The initial shoulder (lag phase) and stationary phase portions were removed from each curve, in order to capture only the logarithmic death-phase of the bacteria. The three death-
phase lines (one for each replicate) were consolidated and outlier data points were removed to establish one representative linear regression for pathogen survival at the corresponding temperature. Decimal reduction time (D-value) was determined by calculating the negative inverse slope of the calculated line equation [A] (Microsoft Excel Version 16.29.1, Microsoft, Redmond, WA)

$$\log_{10}(X) = \log_{10}(X_i) - \frac{t}{D} \quad [A]$$

where \(X = \frac{cfu \ bacteria}{ml \ syrup}\) at \(t\) (minutes), \(X_i\) is the initial \(\frac{cfu \ bacteria}{ml \ syrup}\) observation in the regression model, and \(D\) is the decimal reduction time (minutes).

2.3.6 z-value Curve

The D-value, at each of the three temperatures, was then used to generate a z-value equation. The z-value was determined by plotting the \(\log_{10}\) of the D-values against the corresponding temperature. The \(D_{180^\circ F}\) was calculated by determining the antilog of the z-value regression at the target temperature [B].

$$\log_{10}(D) = \log_{10}(D_i) - \frac{F}{z} \quad [B]$$

Where \(D = \) decimal reduction time (minutes) at temperature \(F\) (°F), \(D_i\) is the decimal reduction time at 180°F, and \(z\) represents the z-value (°F).

2.4 Results and Discussion

Decimal reduction time (D-value) is an imperative tool to ensuring a thermal process is sufficient in effectively inactivating the microbial hazards of greatest concern in any given food system. This provides confidence to both producers and consumers alike, that a particular product is safe from pathogen-related health risks. Determination of a unique D-value is required for each individual microbial population (species), at a given temperature, and within a specific food medium. It is a labor intensive, timely, and often a costly endeavor. Therefore, it is seldom calculated for low risk commodities, such as maple syrup. The low water activity of maple syrup,
as well as the extensive thermal process, have been assumed to adequately mitigate the threat of bacterial pathogens as potential sources of foodborne illness. However, such information is necessary considering our changing knowledge of pathogen behavior in previously assumed low risk products. No prior reports have determined the D-value for STEC, *L. monocytogenes*, or *Salmonella* in maple syrup. Therefore, the objective of this work was to validate the current bottle fill temperature (180°F, 82.2°C) by determining the D-value of each of the three pathogens in maple syrup and provide data to be used by industry members for the validation of their processing practices.

The thermal inactivation curves for STEC, *L. monocytogenes*, and *Salmonella* at 140°F (60°C), 150°F (65.6°C), and 160°F (71.1°C) in maple syrup are shown in Figure 2.4. The detection limit in our procedure is 2 log CFU/mL syrup, and therefore, all figures demonstrate reductions to this threshold. Our results showed that *L. monocytogenes* was the most heat resistant at 140°F (60°C), 150°F (65.6°C), and 160°F (71.1°C) °F, surviving more than 2 times longer than the remaining pathogens. This result was anticipated, due to the thick peptidoglycan layer surrounding the cytoplasmic membrane, a structural feature of all Gram-positive bacteria, such as *L. monocytogenes*. This thick outer layer facilitates greater survival under thermal stress compared to Gram-negatives, such as STEC and *Salmonella* (Knabel, 1989). Our findings are also consistent with other literature that drew comparisons in survival capabilities between the three species (Bunning, Crawford, Tierney & Peeler, 1990; Huang, 2004; Kimber et al., 2012; Koseki et al., 2015; Muriana, 1997; Li, Sheldon & Ball, 2005).
2.4A

![Graph showing the relationship between pathogen log CFU/mL in syrup and minutes at 140°F.](image)

- **A**: $y = -0.2519x + 4.6233 \quad R^2 = 0.9515$
- **B**: $y = -0.142x + 3.4412 \quad R^2 = 0.955$
- **C**: $y = -0.0643x + 4.9267 \quad R^2 = 0.9969$

2.4B

![Graph showing the relationship between pathogen log CFU/mL in syrup and minutes at 150°F.](image)

- **A**: $y = -1.3787x + 4.7225 \quad R^2 = 0.9487$
- **B**: $y = -0.5856x + 4.0359 \quad R^2 = 0.9395$
- **C**: $y = -0.1877x + 5.4139 \quad R^2 = 0.9573$
2.4 C

Figure 2.4: D-value Determination of STEC, *Listeria monocytogenes*, and *Salmonella* at 140°F (A), 150°F (B), and 160°F (C) in Maple Syrup. Data depicts mean log CFU/mL syrup after submersion in circulating water bath for predetermined time points. Line equations for STEC (a), *Listeria monocytogenes* (b), and *Salmonella* (c) are shown next to the corresponding deactivation curve.

The ability for *L. monocytogenes* to withstand extended thermal processing is also believed to be the result of various heat tolerance mechanisms including cell membrane stabilization and synthesis of thermal shock proteins. The induction of heat shock proteins is thought to not only sustain cellular structures from becoming compromised during heating, but also prevent cell protein denaturation (Doyle et al., 2001; Ellis & van der Vies, 1991; Georgopoulos & Welch, 1993; Sergelidis & Abrahim, 2009). Osmotic stress caused by a high sucrose composition, such as that found in maple syrup, can both trigger and amplify these responses. Sumner, Sandros, Harmon, Scott, and Bernard (1991) observed an increase in the D-value of *Listeria monocytogenes* Scott A strains exposed to sucrose solutions adjusted to various water activities. Specifically, the researchers found the $D_{65.6\, ^\circ C}$ increased from 0.36 to 3.8
minutes, in solutions formulated to $A_w$ 0.98 and 0.90, respectively. Jørgensen, Stephens and Knøchel (1995) similarly observed a maximum 8-fold increase in heat resistance among *L. monocytogenes* strains that were grown and subsequently heated in media with increased NaCL concentrations. It is thus unsurprising that this trend was observed in heated maple syrup under osmotic stress. It is important to note that our study was designed to simulate incidental contamination during processing, so cells were not adapted to the low water activity or high sucrose concentration of the syrup. It is unlikely that *L. monocytogenes* became acclimated to the high sucrose environment during the short lag time between capillary tube filling and heat exposure (approximately 1 hour). An extended habituation in this environment would be expected to enhance the ability to assess these survival strategies. This concept of increased thermal tolerance in *L. monocytogenes* strains due to environmental adaptations has been well reported in several food commodities, such as cured meat, dairy, and egg products (Table 2.3).

In addition to osmotic stress, *L. monocytogenes* exposed to sublethal heat treatments have also demonstrated increased heat resistance in subsequent thermal processes (Bunning et al., 1990; Farber & Brown, 1990; Linton et al., 1992; Skandamis et al., 2008). This is an important consideration in maple syrup production because reheating of bulk syrup into retail containers is common practice for many operations. Therefore, complete inactivation in the initial heating is necessary, as *L. monocytogenes* is a psychrotrophic bacterium, capable of regrowth under typical maple syrup storage conditions. Additionally, the unknown infectious dose for the pathogen suggests that any survival of the most resistant cells may induce illness. In order to account for this potential regrowth in heat shocked cells, BHI overlay was used in our procedure to recover injured cells and avoid overestimating the effectiveness of the applied treatments.
Table 2.3: Previous Studies Demonstrating Increased Thermal Tolerance of *Listeria monocytogenes* Due to Environmental Adaptation in Foods

<table>
<thead>
<tr>
<th>Authors</th>
<th>Product(s)</th>
<th>Environmental Adaptation</th>
<th>D-value or Primary Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schoeni, Brunner &amp; Doyle, 1991</td>
<td>Ground beef and Fermented beaker sausage</td>
<td>Curing salts, Fat</td>
<td>Sausage (55% fat, and salt) $D_{54.4^\circ C} = 20.1$ minutes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ground Beef (70% fat)                                                                 $D_{54.4^\circ C} = 22.4$ minutes</td>
</tr>
<tr>
<td>Casadei, Esteves de Matos, Harrison &amp; Gaze, 1998</td>
<td>Liquid broth compared to half cream, Double cream, Butter</td>
<td>Fat content</td>
<td>Maximum 7.15x longer in dairy than broth grown strains</td>
</tr>
<tr>
<td>Fain et al., 1991</td>
<td>Ground beef</td>
<td>Fat content</td>
<td>$D_{135^\circ F} = 2.6$ minutes in lean (2%) and 5.8 min in fat (30.5%)</td>
</tr>
<tr>
<td>Palumbo, Beers, Bhaduri, &amp; Palumbo, 1995</td>
<td>Egg yolk, Liquid egg products</td>
<td>Water activity (adjusted with sugar or salt)</td>
<td>$D_{64.4^\circ C} = 0.44$ minutes in plain yolk, 8.26 min in 10% salt or 5% sugar adapted yolk</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$D_{64.4^\circ C} = 27.3$ minutes in 20% salt adapted yolk treatment</td>
</tr>
<tr>
<td>Tsai et al., 2019</td>
<td>Unsweetened cocoa powder</td>
<td>Water activity</td>
<td>$D_{70^\circ C} = 21.9$ minutes at Aw 0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$D_{70^\circ C} = 7.3$ minutes at Aw 0.45</td>
</tr>
<tr>
<td>Taylor, Tsai, Rasco, Tang, &amp; Zhu, 2018</td>
<td>Wheat flour</td>
<td>Water activity</td>
<td>$D_{70^\circ C} = 37.1$ minutes at Aw 0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$D_{70^\circ C} = 17.44$ minutes at Aw 0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$D_{70^\circ C} = 16.85$ minutes at Aw 0.60</td>
</tr>
</tbody>
</table>
Like *L. monocytogenes*, both *Salmonella* and STEC, also activate various stress mechanisms, such as reduced or redirected metabolic activity in response to adverse environmental conditions (He, 2014, Jozefczuk et al., 2010; Spector, 1998). However, both pathogens are mesophilic and generally demonstrate greater sensitivity to environmental factors, such as acidity and heat. Thus, *Salmonella* and STEC are generally regarded as being more susceptible to such stressors compared to other bacterial species, such as *L. monocytogenes* (He, 2014). It is therefore unsurprising that *Salmonella* and STEC had similar D-values. The slightly increased tolerance generally exhibited by *Salmonella* relative to STEC at most temperatures is also in agreement with other published literature which compared the thermal inactivation of the two species in other low water activity environments (He, 2014; Koseki et al., 2015). However, these results are not universal, as other studies found some pathogenic *E. coli* strains to have increased thermal tolerance under varied environmental stressors, suggesting that these findings are both strain and situation specific (Mazzotta, 2001; Topalcengiz & Danyluk, 2017; Vasan, Ingham & Ingham, 2017). Cocktails of two strains for each pathogen were used in this analysis, but this study cannot truly represent the heterogeneity of either species. Such differences can be attributed to other external factors including bacteria growth conditions, bacterial strain, product composition, and product storage temperatures.
As expected, the calculated D-value for *L. monocytogenes* at 180°F (82.2°C) was more than 2.5 times the reduction times of both STEC and *Salmonella* (Figure 2.4). We determined the predicted decimal reduction times for STEC, *L. monocytogenes* and *Salmonella* at 180°F (82.2°C) in maple syrup to be 0.47 seconds, 4.63 seconds, and 1.56 seconds, respectively (Table 2.4). These findings are encouraging as it indicates that heating syrup to 180°F (82.2°C) for at least 23 seconds is sufficient to provide a minimum 5-log reduction of all three bacterial pathogens we tested under this specific post-process contamination scenario. Our data therefore suggests that the current thermal processing parameters used by the maple industry are sufficient in limiting these bacterial safety risks during potential occurrences of post-process contamination.
Table 2.4: Summary of Decimal Reduction Times (D-values) of STEC, *L. monocytogenes*, and *Salmonella* at Corresponding Temperature Assessments. 180°F is represented in green due to the calculation using z-value equations in Figure 2.5.

<table>
<thead>
<tr>
<th></th>
<th>MINUTES</th>
<th>SECONDS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$D_{140^\circ F}$</td>
<td>$D_{150^\circ F}$</td>
</tr>
<tr>
<td>STEC</td>
<td>7.04</td>
<td>0.73</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>15.55</td>
<td>5.33</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>3.96</td>
<td>1.71</td>
</tr>
</tbody>
</table>

It is necessary to mention that the D-value determination for *Aspergillus* and *Penicillium* composites, the predominant fungal species associated with maple syrup quality loss, was also attempted in this study. However, inconsistent recovery within both the unheated control and heat-treated capillary tubes created unexpected challenges in developing reliable inactivation curves. This made quantitative determination of each species’ heat resistance in maple syrup unreliable. We hypothesize that this was attributed to the hydrophobic nature of the mold spores, which caused aggregation in the syrup and subsequent issues in even application of heat and data consistency. Understanding the thermal tolerance of *Aspergillus* and *Penicillium*, however, is necessary in developing a more comprehensive recommendation to mitigate all microbiological hazards, as both species are often isolated from syrup (Calder, Hopkins, Marshall & Annis, 2011; Frasz & Miller, 2015). As with the bacterial pathogens we analyzed, although neither fungal species has been implicated in a food safety incident involving maple syrup, the ability to produce mycotoxins, or harmful secondary metabolites, can cause a wide range of health problems in consumers. These potential health conditions at high enough concentrations includes liver cancer, bladder cancer, and organ failure (Montville, Matthews & Kniel, 2012c). We would recommend analyzing additional inactivation procedures to obtain this data.
2.5 Conclusions

The goal of this study was to assess the inactivation kinetics for prominent bacterial and fungal pathogens in maple syrup, and to assess the effectiveness of common bottling temperatures against these pathogens. Our results indicate that heating maple syrup at the industry standard of 180°F for a minimum of 23 seconds is adequate to provide a five-log reduction for STEC, *Salmonella*, and *L. monocytogenes* populations. Therefore, current industry heating practices appear to be sufficient in mitigating these pathogenic bacterial risks. The absence of thermal inactivation data for fungal pathogens presents a considerable gap in validating this processing standard. Producers concerned with fungal contaminants should seek alternative methodologies for obtaining this data.

2.6 Acknowledgements

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serotypes, Listeria monocytogenes, and Staphylococcus aureus in high solids liquid egg 

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CHAPTER 3
INACTIVATION OF STEC, SALMONELLA, LISTERIA MONOCYTOGENES, 
ASPERGILLUS AND PENICILLIUM SURVIVAL BY FILLING OF RETAIL BOTTLES 
WITH 180°F AND 190°F HEATED MAPLE SYRUP

3.1 Abstract

Recent outbreaks have generated increased concern regarding the presumed safety of low
water activity products. Maple syrup is one such commodity, with no previous reports associated
with foodborne illness. To ensure product safety and quality, filling of retail bottles with syrup
heated to a minimum 180°F (82.2°C) is the current industry standard. However, this
recommendation is applied without consideration of highly variable package volume and
geometry, and has yet to be validated for efficacy in killing prominent bacterial and fungal
species. Thus, the goal of this study was to investigate the efficacy of filling bottles with syrup
heated to 180°F (82.2°C) or 190°F (87.8°C) in reducing pathogenic contaminants, and the effect
of bottle type and size on this inactivation. Specifically, survival frequency in two strain
composites of STEC, Listeria monocytogenes, Salmonella, as well as Aspergillus spp. and
Penicillium spp., were assessed. Bacterial pathogens were selected due to the severity of
illnesses, low to unknown infectious doses, and potential for introduction as a cross contaminant
from processing environments or personnel. Fungal genera selected are commonly associated
with maple syrup and specific strains used in this study were isolated from contaminated maple
syrup samples. Our research consisted of filling fancy glass (50, 100, 250 mL), standard glass
(237, 355 mL), and plastic (237, 473, 946 mL) retail containers inoculated to ~3 log CFU/mL
syrup per bacterial, or ~ 4 log spores /mL per fungal, contaminant with either 180°F (82.2°C) or
190°F (87.8°C) heated syrup. The three bacterial species were inoculated in one pathogen
cocktail, but analyzed separately from fungi-inoculated bottle trials. Bacterial recovery was accomplished by enriching aliquots of the syrup samples in selective enrichment broths incubated for 24 hours at optimal growth temperatures. Syrup from bottles inoculated with fungi was transferred to sterile storage containers and then allowed to incubate undisturbed for up to 30 days at ambient temperature (~70°F, 21°C). Selective agars and optimal incubation temperatures were used in the subsequent recovery of both bacteria and fungi. The probability of survival for each organism was then analyzed using best fit logistic regression models in R studio. We found that for both bacterial and fungal isolates, the probability of microorganism survival significantly (p < 0.05) increased when a fancy glass bottle was used, regardless of volume. An increase in bottle fill temperature or bottle volume significantly (p < 0.05) decreased the probability of pathogen survival in most, but not all populations. Due to the failure of heated syrup to fully inactivate most inoculated pathogens, exploration of extended syrup hold times, using higher fill temperatures, or prefill sanitization treatments are recommended to effectively mitigate these food safety risks.

3.2 Introduction

Low water activity foods (defined as $A_w < 0.850$), including maple syrup, are considered lower risk commodities because the limited water accessibility within the food matrix is assumed to hinder microbial survival or growth to infectious dosage levels (Virginia Cooperative Extension, 2012). For these reasons, there are often fewer regulations in maple syrup manufacturing compared to the production of many other foods (USNARA, 2019). For example, when only maple syrup is made at a sugaring facility, producers are required to follow appropriate record-keeping and good manufacturing practices, but are exempt from preventative HACCP plan documentation (Saucier-Choate & Bryant, 2018).
Despite these assumptions and consequential eases in regulations, low water activity (A\text{w}) foods have been implicated in many cases of foodborne illness. For example, Salmonella Typhimurium was the cause of over 700 illnesses in the United States in 2009 after consumption of King Nut peanut butter products (A\text{w} = 0.45) (CDC, 2009). More recently, General Mills voluntarily recalled wheat flour (approximate A\text{w} of 0.70) contaminated with Escherichia coli O126 and E. coli O26, which was linked to sixty-three illnesses (Carter, n.d; FDA, 2017). Listeria monocytogenes’ pervasive environmental presence, frequent occurrence in manufacturing settings, ability to grow at water activity levels lower than both E.coli and Salmonella, also present valid concerns to maple syrup safety (Montville, Matthews, & Kniel 2012b). L. monocytogenes has also been associated with food safety incidents involving low A\text{w} products, such as the 2014 multistate outbreak involving wax coated apples used in caramel apple production (CDC, 2015).

Fungi, including Aspergillus and Penicillium, are also potential health threats. Mycotoxins, or secondary metabolites, are produced by some fungal species include aflatoxin, ochratoxin A, and mycophenolic acids (Montville, Matthews, & Kniel, 2012a). These compounds are extremely heat stable and have been connected to various health conditions including kidney failure, liver and bladder cancers (Montville et al., 2012a; Montville, Matthews, & Kniel, 2012c; Scaccocchio, 2009). Although neither Aspergillus nor Penicillium has been linked to foodborne illnesses caused by maple syrup consumption directly, both fungal species are frequently associated with maple syrup quality loss. Researchers at the University of Maine, for example, analyzed fifty-two spoiled maple syrup samples between 2010-2014 and found the majority to be contaminated with a visible fungal mat (Calder, Hopkins, Marshall & Annis, 2011; Annis, Hopkins, Calder & Garcia, 2016).
To mitigate the risk of these microorganisms in syrup, the current bottle filling recommendation is to fill bottles to 90% capacity with syrup heated to a minimum 180°F (Dumont, 2007; Whalen & Morselli, 1984). Our previous study (Chapter 2) proved that this recommendation is sufficient for significantly reducing or eliminating bacterial pathogen risks when the syrup itself is directly contaminated. The presumptive decimal reduction times for STEC, *L. monocytogenes*, and *Salmonella* in maple syrup at 180°F were found to be 0.47 seconds, 4.63 seconds, and 1.56 seconds, respectively (Chapter 2). Therefore, heating syrup to 180°F for at least 23 seconds is required to achieve a 5-log reduction in all three of the bacterial pathogens. However, despite these encouraging findings, there are several gaps in the current bottle filling recommendations. There are several routes of pathogen introduction within a syrup operation. Beyond direct syrup contamination, interior bottle surfaces are also susceptible to pathogen contact due to several risk factors, including employee personal hygiene or container storage conditions. Like most instances of cross contamination, producers are likely to be unaware when a bottle has become compromised. Additionally, the recommendation is applied to all retail bottle types, regardless of size or packaging material, which is a concern due to differences in insulation capabilities. (Engineering Toolbox, 2011; OMAFRA, 2015; Summer, 2018).

Therefore, the objective of this study was to validate the effectiveness of the current bottle filling recommendations by assessing the survival of both bacterial pathogens (STEC, *L. monocytogenes*, and *Salmonella*) and fungal contaminants (*Aspergillus* and *Penicillium*), when desiccated on interior retail container surfaces before bottling.
3.3 Materials and Methods

3.3.1 Bacterial Culture Preparation

Bacterial colonies were streaked for isolation on selective media and incubated at optimal growth temperatures (detailed below). A single colony of *E. coli* O111:H8 ATCC BAA-184 (American Type Culture Collection, Manassas, VA), *E. coli* O26:H11 ATCC BAA-1653 (American Type Culture Collection, Manassas, VA), *Salmonella* Enteriditis ATCC BAA-1045 (American Type Culture Collection, Manassas, VA), *Salmonella* Braenderup (a hazelnut isolate), as well as *L. monocytogenes* ATCC 19111 (American Type Culture Collection, Manassas, VA) and *L. monocytogenes* ATCC 19115 (American Type Culture Collection, Manassas, VA) were used in this analysis.

Each strain was individually propagated in a sterile 15 mL conical tube (Fisher Scientific, Waltham, MA) containing 10 ml of sterile Luria Miller (LB) broth (Alpha Biosciences, Baltimore). All inoculated broths were incubated for 24 hours at optimal growth temperatures for each bacterium (37°C for *E. coli* and *Salmonella* cultures; 32°C for *L. monocytogenes* strains). After the 24-hour incubation period, Luria broth cultures were centrifuged (Centrifuge 5810 R, Eppendorf, Hauppauge, NY) for 10 minutes at 10,000 rpm /15.550 x g. Supernatant from the centrifuged cultures was decanted from each conical tube, and the resulting pellet was resuspended in 5 mL 0.1% peptone (Difco, Sparks, MD). Bacterial cultures of like species were combined and diluted in 0.1% sterile peptone solution. The three diluted bacterial cultures were then combined to create an inoculum cocktail. Each bottle was inoculated with ~3 log CFU/mL per bacterial pathogen using the prepared mixture. The inoculum cocktail concentration was verified by determining the log CFU/mL of triplicate overnight cultures, grown in LB broth, and plated on selective agar for each bacterium.
3.3.2 Mold Culture Preparation

Mold isolates previously isolated from contaminated maple syrup samples, *Penicillium brevicompactum* (SNH6A), *Penicillium* sp. (SNH9B), *Aspergillus* sp. (SNY1A), and *Aspergillus pseudoglacus* (SME2A), were used in the fungal analysis. Each isolate was individually plated on APDA (Acidified Potato Dextrose Agar). The media was prepared using potato dextrose agar (Alpha Biosciences, Baltimore, MD) supplemented with 10% tartaric acid (Sigma-Aldrich, St. Louis, MO). Mold cultures were incubated at ambient temperature (between 20-25°C) for approximately 8-9 days until there was visible sporulation for each isolate.

To produce a spore suspension, the surface of each sporulating culture was lightly scraped using a sterilized spreader and added to sterile 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO) solution. The suspensions for like species were combined and the concentration of each was determined using a Neubauer improved hemocytometer (Hausser Scientific, Horsham, PA). *Penicillium* and *Aspergillus* spore suspensions were then separately diluted in 0.1% peptone, then combined to create an inoculum cocktail. Each bottle was inoculated with ~ 4 log spores/mL for each mold species.

3.3.3 Bottle Inoculation

The retail containers (purchased from Bascom Maple Farms, Acworth, NH) used in this study are displayed in Figure 3.1. Once the inoculum was dispensed, bottles were rotated to ensure even distribution of inoculum liquid. The inoculated bottles were allowed to dry via circulating air in a sterilized biosafety cabinet overnight (~18 hours) prior to syrup filling (Figure 3.2). Four of each inoculated bottle type were filled with unheated syrup (control treatment) and six of each bottle type were filled with heated syrup. Two unheated control treatment bottles and
three experimental bottles were used in each mold replicate. All experiments were performed in triplicate for each temperature validation.

Figure 3.1: Various Retail Bottles Used in Bottle Filling Temperature Analysis
A. Fancy Glass 50 mL, B. Fancy Glass 100 mL, C. Fancy Glass 250 mL, D. Regular Glass 237 mL, E. Regular Glass 355 mL, F. Plastic 237 mL, G. Plastic 473 mL, H. Plastic 946 mL.
Both bacteria and mold inoculated bottles were dried overnight (~18 hours) in a sterile biosafety cabinet prior to bottle filling. No liquid inoculum was visible in bottles prior to syrup filling.

### 3.3.4 Syrup Analysis

Grade A Amber maple syrup (Maine Maple Products, Madison, ME) was used in this study. The pH (Edge Model, HI2020 probe, Hanna Instruments, Woonsocket, RI), water activity (Aqua Lab Series 3 TE, Decagon Devices Inc., Pullman, WA), and °Brix levels (Pocket Refractometer PAL-3, ATAGO, Tokyo, Japan) of the syrup were recorded in triplicate readings prior to syrup inoculation for each trial. Syrup specifications are detailed below (Table 3.1).

**Table 3.1: Grade A Amber Maple Syrup Specifications**

<table>
<thead>
<tr>
<th>Specification</th>
<th>Mean ± Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.52 ± 0.22</td>
</tr>
<tr>
<td>Water Activity</td>
<td>0.858 ± 0.00</td>
</tr>
<tr>
<td>°Brix</td>
<td>66.36 ± 0.21</td>
</tr>
</tbody>
</table>

a. pH and water activity n= 18, °Brix n= 14
3.3.5 Syrup Bottle Filling

Maple syrup was heated to 180°F or 190°F in an 8-gallon, insulated kettle (Edelmetall Brü Kettle 8 gallon, Midwest Supplies, Roseville, MN) using a single burner hot plate (Walmart, Bentonville, AR). The kettle was fitted with a custom water heater jacket (Frost King, Mahwah, NJ) to maintain heat. A 5/8” clear vinyl tubing (Everbilt, Home Depot, Atlanta, GA) was also fitted to the front ball valve for ease in syrup dispensing. The syrup temperature was monitored to ± 2°F using a type K-style thermocouple (OMEGA, Norwalk, CT) positioned in the center of the kettle throughout the heating period. Inoculated bottles were selected at random and filled with the heated syrup to a minimum 90% bottle capacity, not to exceed the target bottle volume. The bottle fill volumes were confirmed by weight using a gram scale (Accuris Instruments, Edison, NJ) and capped. To avoid heat exchange between samples, filled bottles were placed 6 inches apart throughout the cooling period. Bottles were allowed to cool overnight before subsequent analysis. The cooling curve for each bottle type was also recorded during all experimental replicates.
Figure 3.3: Custom Syrup Dispensing Apparatus for Bacteria and Mold Inoculated Maple Syrup Bottles
Edelmetall Brü Kettle was placed on a single burner hot plate until target fill temperature was reached. A thermocouple was inserted into the center of kettle to monitor syrup temperature throughout heating.
Figure 3.4: Cooling Set Up for Syrup-Filled Bottles
After sealing, the syrup filled bottles were placed 6 inches apart overnight to ensure adequate cooling before inoculum enumeration.

3.3.6 Syrup Bottle Bacterial Recovery Analysis

After cooling, filled bottles were inverted and rotated by hand for one minute to homogenize the syrup samples. Approximately one half of the syrup in each bottle was then divided into thirds and weighed into three separate homogenizer blender bags (Nasco Whirl Pak, Fort Atkinson, WI). The above steps were repeated for the same bottle until all syrup was dispensed into the homogenizer bags.

For each homogenizer bag, 2X the selective enrichment broth was added 1:1 to the syrup weight. Sample bags were homogenized by hand for 1 minute prior to incubation. All selective enrichment broths were incubated for 24 hours at optimal growth temperatures. After the incubation period, stomacher bags were again agitated by hand, and 100 µL was spread-plated onto selective agar media. The selective enrichment broths, selective agars, and incubation
temperatures corresponding to each pathogen are listed in Table 3.2. The selective agar plates were incubated for an additional 48 hours and observed for presence or absence of characteristic colony morphology.

**Table 3.2: Selective Agar and Incubation Temperatures used in Bacterial Recovery Analysis**

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Selective Enrichment Broth</th>
<th>Enrichment Broth Incubation Temperature °C</th>
<th>Selective Agar</th>
<th>Agar Incubation Temperature °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>STEC</td>
<td>EEB&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37</td>
<td>MacConkey Sorbitol&lt;sup&gt;d&lt;/sup&gt;</td>
<td>37</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>LEB&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32</td>
<td>PALCAM&lt;sup&gt;e&lt;/sup&gt;</td>
<td>32</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>RV&lt;sup&gt;c&lt;/sup&gt;</td>
<td>42.5</td>
<td>XLD&lt;sup&gt;f&lt;/sup&gt;</td>
<td>37</td>
</tr>
</tbody>
</table>

a. Enterobacteria Enrichment Mossel Broth, Himedia Laboratories, Mumbai, India  
b. *Listeria* Enrichment Broth, Alpha Biosciences, Baltimore, MD  
c. Rappaport Vassiliadis Broth, Millipore Sigma, Burlington, MA; Sigma-Aldrich, St. Louis, MO  
d. Alpha Biosciences, Baltimore, MD  
e. Polymyxin Acriflavine Lithium Chloride ceftazidime Aesculin Mannitol, BD Difco, Sparks, MD) prepared with 2% PALCAM supplement (BD Difco, Sparks, MD) and incubated at 32°C.  
f. Xylose Lysine Deoxycholate agar, BD Difco, Sparks, MD; Alpha Biosciences, Baltimore, MD; Sigma-Aldrich, St. Louis, MO)

### 3.3.7 Syrup Bottle Mold Recovery Analysis

After cooling, mold-inoculated bottles were briefly agitated and the entire syrup volume was transferred to a sterilized (UV 15 minutes) plastic Rubbermaid Take Along food storage container (Rubbermaid Inc., Huntersville, NC). Table 3.3 lists the storage container corresponding to the syrup bottle size used to ensure a minimum 1-inch headspace. The syrup in plastic quart bottles was divided evenly between two containers to meet this headspace requirement. The containers were allowed to sit undisturbed for 30 days at ~70°F(21°C) until further analysis.
Table 3.3: Bottle Type with Corresponding Storage Container (Mold Regrowth Incubation)

<table>
<thead>
<tr>
<th>BOTTLE TYPE:</th>
<th>RUBBERMAID CONTAINER SIZE:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plastic 237 mL</td>
<td>2.9 Cup Square (669 mL)</td>
</tr>
<tr>
<td>Plastic 473 mL</td>
<td>3.2 Cup Small Bowl (760 mL)</td>
</tr>
<tr>
<td>Plastic 946 mL</td>
<td>5.2 Deep Square (1.2 L) (two)</td>
</tr>
<tr>
<td>Glass 237 mL</td>
<td>2 Cup Twist and Seal (473 mL)</td>
</tr>
<tr>
<td>Glass 355 mL</td>
<td>2.9 Cup Square</td>
</tr>
<tr>
<td>Fancy Glass 50 mL</td>
<td>0.5 Cup Mini (118 mL)</td>
</tr>
<tr>
<td>Fancy Glass 100 mL</td>
<td>1.2 Cup Twist and Seal (284 mL)</td>
</tr>
<tr>
<td>Fancy Glass 250 mL</td>
<td>2.1 Cup Mini Deep Dish (500 mL)</td>
</tr>
</tbody>
</table>

After the storage period, containers were visually examined for mold growth. Colony observations were noted for presumptive recovery (based on color and colony shape) then transferred to APDA and incubated for 5 days at 68°F (20°C). Post incubation, wet mount slides were prepared using lactophenol cotton blue dye (Hardy Diagnostics, Santa Maria, CA) to ensure spore-producing structures of surviving colonies were characteristic of *Aspergillus* and/or *Penicillium* spp.

3.3.8 Cooling Profile Monitoring

During both bacteria and mold bottle inoculation trials, the cooling profile for each bottle type/size was recorded. This was accomplished by filling one uninoculated bottle with heated syrup per replication. Upon filling to the target syrup volume, the bottles were immediately sealed using a needle-punctured cap to fit a single K-style thermocouple probe into the approximate bottle center (determined by height). Bottles were placed 6 inches apart, and temperatures were recorded on an SD card in five-minute increments until all bottles reached room temperature, defined as 77°F (25°C). Cooling curves were prepared by averaging data observations from both bacteria and mold replicates at both of the analyzed fill temperatures.
(180°F and 190°F). This method was used because differences in cooling rates between 180°F and 190°F were negligible.

Figure 3.5: Bottle Cooling Set Up
Caps for retail containers were punctured to insert a single thermocouple probe in the center of the bottle. Once the probe was inserted, the caps were tightly covered with Play-Doh (Hasbro, Pawtucket, RI) to avoid premature heat loss. All bottles were allowed to reach a minimum 25°C prior to thermocouple removal.

3.3.9 Statistical Analysis
Data were analyzed to determine the significant differences (p < 0.05) in the survival probability of each pathogen in response to bottle shape, bottle volume, and syrup fill temperature using best fit logistic regression models (glm function) in R studio. To determine the best fit logistic regression model, the ANOVA function in R studio was used.

3.4 Results and Discussion
The aim of this study was to assess the effectiveness of commonly used maple syrup bottle fill temperatures in reducing bacterial and fungal presence across several types and sizes of inoculated retail containers. The interior surfaces of various bottles were inoculated and dried overnight to simulate a plausible bottle surface contamination scenario during package storage,
as producers are less likely to be aware of this type of contamination. Additionally, bacterial pathogens especially, have demonstrated increased heat resistance upon desiccation (Almatroudi, 2018; Daryaei, 2018; Ma et al., 2009; Shen et al., 2014). However, it is necessary to mention that across all bottle types, the initial bacterial and to the lesser extent fungal spore populations were reduced prior to syrup filling, as a result of mortality due to inoculum desiccation during the overnight incubation period. Although the degree of inoculum reduction was inconsistent across bottles, the fancy glass containers generally demonstrated greater recovery of bacteria especially, in the unheated syrup control treatment.

Little research regarding the food safety risks associated with maple syrup processing has been completed to date. The limited water availability within the food matrix, as well as the elevated bottling temperatures used during processing have been assumed to adequately mitigate the risk of microbial hazards. Additionally, the absence of any documented foodborne outbreaks associated with this low risk commodity has also resulted in few research initiatives around maple syrup safety. However, recent outbreaks associated with other low water activity foods, such as peanut butter and wheat flour, now challenge the current understanding of pathogen survival, infectious dosage, and microbe adaptability in these products.

3.4.1 Bacterial Survival in Retail Bottles Filled with 180°F and 190°F Heated Syrup

Figures 3.6 – 3.8 illustrate the percentage of survival for each individual bacterial population after filling various bottles with the unheated control treatment, 180°F or 190°F maple syrup. Figures 3.9-3.11 demonstrates the predictive survival probabilities of each bacterial population when a change in bottle volume is continuous and a particular bottle shape and syrup fill temperature (unheated or 180°F) are used (obtained from logistic regression). The predictive plots for the 190°F fill temperature were nearly identical to 180°F and thus not presented.
Figure 3.6: Percentage of Bottles with Positive STEC Recovery.

Figure represents survival after filling bottles with unheated (control treatment n = 24), 180°F (n = 18) or 190°F (n = 18) maple syrup. Among bottle types, FG indicates use of a fancy glass bottle, G indicates use of a regular glass bottle, and P denotes use of a plastic bottle. Milliliters (mL) corresponding to each bottle volume are also listed within bottle type. Error bars indicate standard deviation between replicates (n=3).
3.7

**Figure 3.7: Percentage of Bottles with Positive *L. monocytogenes* Recovery.**

Figure represents survival after filling bottles with unheated (control treatment n = 24), 180°F (n = 18) or 190°F (n = 18) maple syrup. Among bottle types, FG indicates use of a fancy glass bottle, G indicates use of a regular glass bottle, and P denotes use of a plastic bottle. Milliliters (mL) corresponding to each bottle volume are also listed within bottle type. Error bars indicate standard deviation between replicates (n=3).

3.8
Figure 3.8: Percentage of Bottles with Positive Salmonella Recovery.

Figure represents Survival after filling bottles with unheated (control treatment n = 24), 180°F (n = 18) or 190°F (n = 18) maple syrup. Among bottle types, FG indicates use of a fancy glass bottle, G indicates use of a regular glass bottle, and P denotes use of a plastic bottle. Milliliters (mL) corresponding to each bottle volume are also listed within bottle type. Error bars indicate standard deviation between replicates (n=3).

Bottle shape impacted the survival probability of all the bacterial populations we analyzed, regardless of the syrup fill temperature or bottle volume. More specifically, utilizing a fancy glass bottle significantly (p < 0.05) increased the survival probability of most populations, whereas plastic bottles significantly reduced (p < 0.05) the bacterial pathogen probability of survival, regardless of bottle volume. These findings may have been attributed to differences between the bottle surfaces, which resulted in varied desiccation prior to the heat treatment.

Beginning with the plastic bottles, a possible explanation for this finding is that there was potentially reduced desiccation of the bacteria as a result of the polyvinylidene chloride “XL” coating lining the bottle interior, which is used to prevent product oxidation. This coating may have inadvertently created some beading in the dispersion of the inoculum liquid, which when compared to regular glass, improved survival in the unheated control treatments, but significantly (p < 0.05) reduced the probability of survival following the heat treatment as a result of limited stress responses. Although the degree of drying was not explicitly determined in our analysis, the smooth surfaces of the regular glass bottle on the other hand, may have promoted inoculum desiccation and was therefore, generally recovered less in the unheated control treatments due to excessive mortality. As a result, there was non-significant reduction in survival probability for any of the bacteria we analyzed using a regular glass bottle, due to the limited recovery in the unheated control treatments for most of the populations. Conversely, use of fancy glass bottles significantly (p < 0.05) increased the probability of survival. We suspect that the pointed corners of the fancy glass bottle interior, which provides the appearance of a maple leaf, may have
facilitated an ideal degree of desiccation to increase pathogen resistance for the heat treatment, while also preventing excessive die-off in the inoculum procedure. The leaf points likely allowed for minor pooling of the inoculum liquid, which when combined with the generally smaller bottle opening compared to the remaining container types, may have reduced the air flow in the packaging, thus potentially limiting the degree of drying. As a result of these observations, the fancy glass bottles are inherently greater risk than the remaining bottle alternatives.

3.9

Figure 3.9: Effects of Container Volume and Shape on Predictive STEC Survival Probability After Filling with Unheated (A) or 180°F (B) Heated Maple Syrup. F indicates use of a fancy glass bottle, G indicates use of a regular glass bottle, and P denotes use of a plastic bottle, mL = Milliliters.
3.10

Figure 3.10: Effects of Container Volume and Shape on Predictive \textit{L. monocytogenes} Survival Probability After Filling with Unheated (A) or 180°F (B) Heated Maple Syrup. F indicates use of a fancy glass bottle, G indicates use of a regular glass bottle, and P denotes use of a plastic bottle, mL = Milliliters.

3.11

Figure 3.11: Effects of Container Volume and Shape on Predictive \textit{Salmonella} Survival Probability After Filling with Unheated (A) or 180°F (B) Heated Maple Syrup. F indicates use of a fancy glass bottle, G indicates use of a regular glass bottle, and P denotes use of a plastic bottle, mL = Milliliters.
Beyond bottle shape, syrup volume and fill temperature also both had obvious impacts on the probability of bacterial pathogen survival. Although not universally consistent for all temperatures and bottle types, most bacterial populations demonstrated a significant (p < 0.05) reduction in survival probability, as a result of an increase in syrup temperature and container volume. This conclusion is reflected in the cooling rate of the various bottles we analyzed (Figure 3.12). Despite filling with syrup heated to either 180°F or 190°F, the starting temperatures were relatively consistent across trials due to premature heat loss during capping. Generally, within bottle type, the larger bottle size had a slower rate of cooling compared to smaller bottles of the same material, as expected. For example, the largest fancy glass bottle (250mL) reached room temperature (defined 77°F) 3.5 hours after filling, whereas the 50mL counterpart reached the room temperature threshold by 1.9 hours. Similarly, the largest plastic bottle did not reach 77°F until 6 hours after bottle filling, whereas the 237mL plastic bottle reached this temperature at 4.5 hours. This difference is due to the larger quantity of syrup which presumably retains heat longer than the smaller volume alternatives.

Additionally, comparisons across the various bottle types demonstrate obvious differences in the cooling rates. As a result of this, the duration of time spent above presumably lethal temperatures was variable between containers. Previous literature has demonstrated a considerable decline in vegetative bacterial populations exposed to a minimum 125°F, and thus for the purposes of our analysis, we have defined temperatures above this threshold as a lethal temperature range (Bryan, 2004; Goodfellow & Brown, 1978; Makukutu & Guthrie, 1986). Plastic containers generally cooled more slowly, with the largest plastic bottles (473 and 946 mL) spending a significantly (P < 0.05) longer duration in the defined lethal temperature range compared to all other bottle types. Unsurprisingly, this further supports our findings that plastic
bottles generally demonstrated the lowest bacterial survival rates relative to all the other containers we tested. Specifically, as can be seen in Figure 3.12, the largest plastic bottle remained above 125°F for the first 85 minutes of cooling, whereas the largest regular glass bottle maintained a temperature above 125°F for only 45 minutes. An interesting observation regarding the plastic bottle cooling however, is that although most bottle types were at room temperature after 4 hours of cooling, the larger plastic bottles maintained a temperature between 85-90°F, and gradually reduced throughout the remainder of the monitoring period. These temperatures are optimal growth conditions for the mesophilic bacteria used in this analysis. Therefore, it can be suggested that dwell times at these ideal growth conditions may have enhanced the recovery for any surviving bacterial cells. For this reason, Figures 3.10 and 3.11 demonstrate a slight upward trend for survival probability in response to increased volumes for the plastic bottles. This is a considerable contrast from the two smallest fancy glass bottles, which spent significantly (p < 0.05) less time under presumed lethal conditions, compared to the remaining bottle types. As can be seen in Figure 3.12, both the 50 mL and 100 mL fancy glass bottle reached below 125°F within 25 minutes post syrup filling. This observation is also in support of our findings for the limited bacterial inactivation within these containers. A summary of the duration of time each retail bottle remained above the lethal temperature threshold is shown in Table 3.4.
Table 3.4: Average Duration of Time Each Retail Bottle Remained Above Lethal (≥125°F) Temperature Threshold. Temperatures were recorded in 5-minute increments throughout the cooling period. FG indicates use of a fancy glass bottle, G indicates use of a regular glass bottle, and P denotes use of a plastic bottle. mL = Milliliters corresponding to each bottle volume. Numbers depict temperature in°F± Standard Deviation between replicates.

<table>
<thead>
<tr>
<th>MINUTES</th>
<th>BOTTLE TYPE</th>
<th>FG50mL</th>
<th>FG100mL</th>
<th>FG250mL</th>
<th>G237mL</th>
<th>G355mL</th>
<th>P237mL</th>
<th>P473mL</th>
<th>P946mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>FG50mL</td>
<td>151±9.80</td>
<td>158±13.3</td>
<td>152±11.8</td>
<td>160±12.2</td>
<td>166±11.9</td>
<td>162±11.1</td>
<td>169±12.6</td>
<td>172±11.4</td>
</tr>
<tr>
<td>15</td>
<td>FG50mL</td>
<td>130±7.77</td>
<td>134±9.18</td>
<td>137±7.88</td>
<td>145±13.0</td>
<td>151±9.46</td>
<td>150±8.31</td>
<td>156±11.2</td>
<td>160±8.23</td>
</tr>
<tr>
<td>25</td>
<td>FG50mL</td>
<td>121±8.38</td>
<td>125±7.76</td>
<td>130±7.61</td>
<td>138±9.63</td>
<td>144±9.31</td>
<td>144±6.66</td>
<td>150±9.41</td>
<td>155±7.88</td>
</tr>
<tr>
<td>35</td>
<td>FG50mL</td>
<td>108±9.35</td>
<td>117±7.66</td>
<td>124±6.90</td>
<td>131±6.94</td>
<td>136±7.73</td>
<td>138±5.40</td>
<td>144±8.63</td>
<td>149±7.37</td>
</tr>
<tr>
<td>45</td>
<td>FG50mL</td>
<td>102±8.74</td>
<td>111±7.44</td>
<td>118±6.79</td>
<td>125±10.7</td>
<td>130±7.15</td>
<td>132±4.93</td>
<td>139±8.04</td>
<td>144±6.89</td>
</tr>
<tr>
<td>55</td>
<td>FG50mL</td>
<td>96.6±8.29</td>
<td>105±7.28</td>
<td>113±6.64</td>
<td>119±6.50</td>
<td>124±7.00</td>
<td>127±4.85</td>
<td>134±7.75</td>
<td>139±6.46</td>
</tr>
<tr>
<td>65</td>
<td>FG50mL</td>
<td>90.7±7.98</td>
<td>100±7.06</td>
<td>109±6.43</td>
<td>114±6.30</td>
<td>120±6.82</td>
<td>122±5.10</td>
<td>129±7.72</td>
<td>134±6.89</td>
</tr>
<tr>
<td>75</td>
<td>FG50mL</td>
<td>86.6±7.77</td>
<td>96.0±7.00</td>
<td>105±6.49</td>
<td>112±6.01</td>
<td>115±6.95</td>
<td>118±5.48</td>
<td>126±7.52</td>
<td>130±6.96</td>
</tr>
<tr>
<td>85</td>
<td>FG50mL</td>
<td>83.5±7.57</td>
<td>92.2±7.01</td>
<td>102±6.78</td>
<td>109±6.16</td>
<td>111±7.02</td>
<td>114±5.78</td>
<td>122±7.54</td>
<td>126±6.96</td>
</tr>
<tr>
<td>95</td>
<td>FG50mL</td>
<td>80.9±7.47</td>
<td>89.0±6.95</td>
<td>98.7±6.96</td>
<td>104±6.39</td>
<td>108±7.07</td>
<td>110±6.08</td>
<td>118±7.40</td>
<td>122±6.55</td>
</tr>
</tbody>
</table>

a. n = 12
b. n = 11
c. n = 10

It should be noted that these variation of cooling rates between the containers, is more precisely a function of the differences among the surface area to volume ratio of the bottles, and not exclusively the result of syrup quantity. As expected, bottles with a larger surface area to volume ratio would lose heat more rapidly. Although this ratio has yet to be determined for every bottle we analyzed, it has been confirmed for a portion of the maple syrup containers used in the present study. As suspected, the fancy glass bottles (all volumes) have a larger surface area to volume ratio compared to the two largest plastic bottles used in our analysis (S. Annis, personal communication, April 23, 2020). A summary of the available surface areas is listed below in Table 3.5.
Table 3.5: Surface Area to Volume Ratio of Various Maple Syrup Bottles

<table>
<thead>
<tr>
<th>BOTTLE TYPE:</th>
<th>Surface Area: Volume (cm²/mL)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mL Maple Leaf</td>
<td>4.52</td>
</tr>
<tr>
<td>100 mL Maple Leaf</td>
<td>3.35</td>
</tr>
<tr>
<td>250 mL Maple Leaf</td>
<td>1.91</td>
</tr>
<tr>
<td>473 mL Plastic</td>
<td>0.94</td>
</tr>
<tr>
<td>946 mL Plastic</td>
<td>0.63</td>
</tr>
</tbody>
</table>

¹ Data obtained from S. Annis (personal communication, April 23, 2020).

The effects of these differences between the cooling rates of the various bottles, may also be influenced by the bottle material. It is well known that high density polyethylene, the material used in plastic maple syrup bottles, has a lower thermal conductivity (0.48 Watt/(meter*Kelvin)) compared to glass (1.82 Watt/(meter*Kelvin)) (Engineering Tool Box, 2011; OMAFRA, 2015; Summer, 2018). Additionally, the XL coating in the plastic bottles may have also provided additional heat insulation, ultimately extending heat exposure and increasing pathogen lethality. This conclusion is apparent when comparing the 237 mL regular glass and plastic bottles. Despite identical container volumes, across populations there were generally less survival when a 237 mL plastic bottle was used compared to the 237 mL regular glass bottle counterpart (Figures 3.6-3.8). This observation is also likely a factor of bottle shape, with the assumed smaller area of the regular glass bottle potentially losing heat more quickly than the wider plastic bottle alternative. Although as previously stated, the surface area to volume ratio of the glass bottles has yet to be validated.

Despite this general trend in survival probability reduction in response to increased bottle volume and increased syrup temperature, our results indicate that neither of these factors significantly (p <0.05) lowered the survival probability for STEC. As a result, there was generally greater STEC survival across all bottle types following the heat treatment compared to both *L. monocytogenes* and *Salmonella*. A potential explanation for this finding may be a
superior cross-protective stress response of STEC, that is induced once the bacteria is exposed to a sublethal stressor, such as desiccation on a surface or osmotic stress in a syrup environment. This cross protection would improve the thermal tolerance of the pathogen once exposed to a subsequent stressor, such as heat. Therefore, neither the increase in syrup fill temperature, which is amplified by the syrup volume, significantly reduced the probability of STEC survival. However, the use of a plastic bottle significantly (p <0.05) reduced the likelihood of STEC compared to either of the glass bottle types. As previously suggested in the unheated control bottles, there was possibly less desiccation of the bacteria in the inoculum procedure, which likely resulted in less expression of virulence factors to promote cross resistance in the subsequent heat treatment.

Figure 3.12: Mean Bottle Temperature Across 180°F and 190°F Bottle Filling Replicates. Temperatures were recorded in 5-minute increments for up to 6 hours or until syrup reached room temperature (defined as 77°F or less). FG indicates use of a fancy glass bottle, G indicates use of a regular glass bottle, and P denotes use of a plastic bottle. mL = Milliliters corresponding to each bottle volume.
The enhanced cross protective response of gram-negative bacteria has been well reported in previous literature. For example, STEC strains grown in 1% glucose induced an acid adaptation response, which drastically increased the thermal tolerance when the pathogen was inoculated into orange juice samples and heated (56.1-60 °C) (Topalcengiz and Danyluk, 2017). Similarly, an additional study determined that 15 STEC strains which were previously dried on paper discs (35°C) for 24 hours, survived a subsequent heat treatment of 158 °F for five hours, when the paper discs contained 36% sucrose, an osmotic stressor. The recovery of the STEC populations were considerably higher than the STEC isolates which were dried on discs without the sucrose modification (Hiramatsu et al., 2005).

This finding is a stark contrast from our results in the thermal inactivation comparison of the bacterial pathogens when the maple syrup was directly inoculated (Chapter 2). Under the contamination scenario presented in Chapter 2, Listeria monocytogenes was proven to be the most heat resistant of the three pathogens. This result is likely attributed to the structural advantages of gram-positive bacteria, which generally improves thermal tolerance. Particularly, the thick peptidoglycan layer of the cell wall creates a heat shock barrier compared to the thin peptidoglycan alternative of gram-negative organisms including STEC and Salmonella spp. used in the present study (Salton & Kim, 1996). However, the bacteria were only exposed to the maple syrup for a limited period (less than 1 hour) prior to the heat treatment in our procedure. This observation suggests that it was likely insufficient time for the gram-negative organisms, to induce these cross protective mechanisms which would have drastically improved heat resistance.
3.4.2 Fungal Spore Survival in Retail Bottles Filled with 180°F and 190°F Heated Syrup

Aspergillus and Penicillium spores were recovered much more readily across all bottle types in both the unheated control treatment and heat-treated syrup samples, compared to the bacterial pathogens we analyzed (Figures 3.13 and 3.14). This is further illustrated in Figures 3.15 and 3.16, which demonstrates the predictive survival probability of each fungal genus when volume is continuous, a particular bottle shape is used, and bottles are filled with either unheated or 180°F syrup. The higher likelihood of fungal spore survival compared to bacteria, is likely attributed to the greater desiccation tolerance of fungal spores in the inoculum drying procedure, as well greater xerotolerance once exposed to a maple syrup environment.

However, as mentioned the variability in inoculum drying was not determined in our procedure. In order to assess the perceived differences in desiccation among the syrup bottles, perhaps separate testing to measure the variations in air flow among containers would have verified this theory. For example, collecting data regarding air velocity and volume using a thin probe digital anemometer for each bottle under the conditions specified in the inoculum drying procedure, may have been an effective strategy. An additional methodology for assessing the degree of drying within each container may have been accomplished by using a “non-invasive” or pinless moisture meter. Although typically used for identifying moisture accumulation in non-visible surfaces such as subflooring, obtaining several measurements for each bottle type post inoculation (such as one inoculated bottle per trial replicate), would have effectively documented the unique characteristics of each bottle. The greater moisture content within the bottle would be indicative of less inoculum desiccation.

Like bacteria, bottle shape was a significant factor in the survival probability of fungal spores, regardless of syrup fill temperature and bottle volume. More specifically, the use of a
fancy glass bottle significantly (p < 0.05) increased the survival probability of both Aspergillus and Penicillium isolates compared to the remaining bottle types. This observation may be a factor of facilitated spore aggregation in the grooves of the fancy glass bottle, which improved survival capabilities upon desiccation of the inoculum in reference to the inoculum drying and subsequent exposure to a heat treatment. Although an inadvertent observation of their analysis, Doyle and Marth (1975) for example, found a slight lag in the linear thermal inactivation curves of Aspergillus flavus and Aspergillus parasiticus conidia inoculated in solutions of various pH and ion concentrations. The researchers suspected this was a result of potential spore clumping prior to heating in some of their samples. This may offer a potential explanation of why both fungal genera were isolated slightly, but not significantly, more frequently pre-and post-heat treatment using a fancy glass bottle in our study.
Figure 3.13: Percentage of Bottles with Positive *Aspergillus* Recovery After Filling with Unheated (control n = 12), 180°F (n = 9) or 190°F (n = 9) Heated Maple Syrup.
Among bottle types, F indicates use of a fancy glass bottle, G indicates use of a regular glass bottle, and P denotes use of a plastic bottle. Milliliters (mL) corresponding to each bottle volume are also listed. Error bars indicate standard deviation between replicates (n=3).
Figure 3.14: Percentage of Bottles with Positive *Penicillium* Recovery After Filling with Unheated (control n = 12), 180°F (n = 9) or 190°F (n = 9) Heated Maple Syrup.

Among bottle types, F indicates use of a fancy glass bottle, G indicates use of a regular glass bottle, and P denotes use of a plastic bottle. Milliliters (mL) corresponding to each bottle volume are also listed. Error bars indicate standard deviation between replicates (n=3).
3.15

Figure 3.15: Effect of Container Volume and Shape on Predictive Survival Probability of *Aspergillus* spp. After Filling with Unheated (A) or 180°F (B) Heated Maple Syrup. F indicates use of a fancy glass bottle, G indicates use of a regular glass bottle, and P denotes use of a plastic bottle, mL = Milliliters.

3.16

Figure 3.16: Effect of Container Volume and Shape on Predictive Survival Probability of *Penicillium* spp. After Filling with Unheated (A) or 180°F (B) Heated Maple Syrup. F indicates use of a fancy glass bottle, G indicates use of a regular glass bottle, and P denotes use of a plastic bottle, mL = Milliliters.
In addition to bottle shape, it is unsurprising that an increase in syrup fill temperature significantly (p < 0.05) reduced the survival probability for both *Aspergillus* and *Penicillium* spores. Like bacteria, there was a general trend in reduced survival probability as syrup volume increased. However, the reduction in survival probability in response to volume increase was only significant (p < 0.05) for the *Penicillium* population. This result may be attributed to the presumed greater thermal resistance of the *Aspergillus* isolates we tested compared to *Penicillium* strains, in which the variations in heat retention as a result of syrup volume were insignificant. Therefore, a more sensitive isolate, such as the *Penicillium* species used in this analysis, have a greater susceptibility to extended exposure of elevated temperatures causing more expansive die off. Although results are largely situation specific, this conclusion is supported by previous studies which assessed the thermal inactivation of the two fungal genera under various heat treatment scenarios and found *Aspergillus* spp. to demonstrate greater heat tolerance compared to *Penicillium* spp. (Beuchat, 1981; Kamil, Lupuliasa, Draganescu, & Vlaia, 2011; Shearer, Mazzotta, Chuyate & Gombas, 2002). However, despite these trends, it should also be noted that the survival capabilities of fungal spores are contingent upon a combination of factors including temperature exposure, environment water activity, and the strain used in thermal inactivation analysis. Buerman, Worobo, and Padilla – Zakour (2019) identified an inverse relationship between medium A\textsubscript{w} and *Aspergillus* spp. thermal resistance (defined by D-value) in confectionary formulations of fructose and evaporated milk adjusted to various water activities. Specifically, the D-values of *A. fischeri* spores at 90°C and A\textsubscript{w} 0.75 and 0.80 were predicted to be 11.54 and 9.29 minutes, respectively. However, there were limitations to this trend, as the researchers also determined that reducing A\textsubscript{w} to 0.70 resulted in a D-value of only 5.01 minutes at 90°C. Similarly, this same work also analyzed the thermal inactivation of *A.*
pseudoglaucus, and found that this species was far less heat resistant than A. fischeri, with a predicted D-value of 0.52 minutes at 82°C and a A_w of 0.80, the approximate specifications of maple syrup processing. Therefore, the survival data obtained from our study is likely strain and situation specific, and thus the effectiveness of an applied heat treatment in a syrup operation will be largely dependent on these particular factors.

3.4.3 Risk Assessment of Bacterial and Fungal Pathogens in Maple Syrup Operations

As a result of these conclusions regarding the effectiveness of an applied heat treatment due to bottle shape and volume, a risk comparison among the different microbial populations is necessary to provide context for specific recommendations to syrup producers. The inoculation protocol used in this analysis simulates realistic bottle surface contamination scenarios. With respect to bacterial pathogens, gaps in processing best practices can increase the risk for product contact surface contamination. Condensation accumulation above an evaporator pan for example, can drip into the interior of a bottle and dry. Fungal spore contamination, however, is a much more likely contamination scenario because mold spores are largely airborne and can easily transfer to a bottle opening via air circulation. This observation is reflected in the greater fungal spore recovery across all bottle types in comparison to bacterial pathogens in our data. However, despite this greater recovery and increased likelihood of contamination, fungal spores pose a relatively lower health risk to consumers for a few reasons. Firstly, consumers are often aware of fungal contamination due to obvious surface mold growth, and as a result are more likely to discard compromised product. Bacterial growth on the other hand, cannot be visualized in the product, making it an inherently greater risk to consumer health if it occurs. Additionally, the infectious dosages of these microbial groups are starkly different. As previously mentioned, fungal mycotoxins, as opposed to direct spore consumption, will induce illness in humans. Our
work does not address whether, or to what extent, mycotoxins are formed in the syrup. Additionally, the infectious dosages of these metabolites are widely variable and reported by using a variety of metrics. The World Health Organization for example, estimates that acute aflatoxin B1 exposure of 20-120 µg/ kg of consumer body weight over a minimum 1-week period can be fatal (WHO, 2018). Whereas 720 mg of mycophenolic acid exposure twice daily has been reported to inhibit immune system responses in adults (Annis et al., 2016). Infectious doses of bacterial pathogens, however, are much lower and relatively consistent, with as few as ten Salmonella cells inducing illness as one example (Beuchat et al., 2013).

Therefore, based on these results, we suspect that STEC is the greatest risk to syrup safety, followed by L. monocytogenes, and finally Salmonella. The order of this ranking is based on predominance of survival following heat treatments, as well as susceptibility to reduction by syrup volume and fill temperature. As mentioned, neither an increase in temperature nor volume were significant in reducing the probability of STEC survival, making it the greatest threat to consumers. L. monocytogenes generally demonstrated a greater probability of survival than Salmonella post-heat treatment, and is reasonably the second biggest concern to consumer health. After bacterial pathogens, Penicillium followed by Aspergillus pose the greatest risk to safety. Penicillium was recovered in 100% of unheated control treatment syrup samples, unlike Aspergillus, therefore it more likely to compromise the product under this probable contamination scenario. It warrants mentioning, however, that from a producer perspective, the greatest potential for economic loss comes from fungal contaminants. Because of their ubiquitous presence in the environment, and the potential for spores to be transmitted by air, fungi are more likely to come into contact with syrup and/or packaging materials, and their capability to germinate in finished packaging is well documented. As clearly demonstrated by
this work, current syrup reheating practices cannot be presumed to eliminate this risk, regardless of the package used.

However, despite this generalized ranking, as suggested, the probability of survival among these pathogens is augmented by bottle shape, bottle volume, and fill temperature selection. Fancy glass bottles, regardless of size, present the greatest concerns to consumer safety due to perceived enhanced attachment capabilities among organisms. Following fancy glass, regular glass, then plastic bottles, pose the biggest hazards to consumer health due to the differences in thermal holding capacities. With consideration of fungal contamination, visual examination of glass bottles can quickly alert a consumer or producer that a product has become compromised. However, despite this advantage the heat retention of the plastic bottles reduces the risk of spore germination making it a lesser concern to product safety. Therefore, we recommend the usage of the largest plastic bottle filled with the highest syrup temperature possible to mitigate risk of contamination. When a glass bottle is required, the use of a regular glass container (largest size) as opposed to fancy glass will also reduce the likelihood of pathogen survival.

3.5 Conclusions

The goal of this study was to assess the effectiveness of common bottle filling parameters in reducing the most prevalent bacterial and fungal safety risks to maple syrup safety. Among all populations, the use of a fancy glass bottle significantly (p < 0.05) increased the survival probability of all microbial pathogens, suggesting this container has inherently greater risk to consumer safety compared to the remaining containers we studied. Additionally, although the use of heat treatments generally reduced the probability of microbial population survival, neither increase in temperature or volume eliminated the pathogens we analyzed. In fact, in our data for
the majority of genera, there was still recovery after filling with 190°F maple syrup in some containers. Therefore, due to potential product quality losses that could occur due to heating syrup above 190°F, other bottle pre-fill treatments such as sanitization should be further explored in reducing these foodborne safety hazards.

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APPENDIX A

EFFECTIVENESS OF USING A BOILING WATER TREATMENT IN REDUCING STEC, SALMONELLA, LISTERIA MONOCYTOGENES, ASPERGILLUS AND PENICILLIUM ON INTERIOR SURFACES OF MAPLE SYRUP BOTTLES

A.1 Abstract

The current practice in the maple industry is to fill retail bottles with syrup heated to a minimum 180°F. However, our previous work (Chapter 3) demonstrated that use of a 180°F or 190°F bottle fill temperature were inadequate in the complete eradication of both bacterial pathogens (STEC, *Listeria monocytogenes*, *Salmonella*) and fungal spores (*Aspergillus*, *Penicillium*) desiccated on interior bottle surfaces. Therefore, the objective of this study was to determine the effectiveness of a boiling water prefill treatment in mitigating microbial safety risks since sanitizer use may impact maple syrup sensory qualities and product labeling. A boiling water prefill treatment would be relatively simple for producers to implement due to low cost and ease of application. Our data indicates that application of a boiling water pretreatment significantly (p < 0.05) reduced the survival probability of most of the microbial populations we analyzed. However, the effectiveness of the treatment is largely dependent upon the bottle shape and bottle volume in many cases. Specifically, use of a fancy glass bottle significantly (p < 0.05) increased the survival probability compared to the remaining bottle types. An increase in bottle volume generally reduced the pathogen survival probability, however, this reduction was significant (p < 0.05) only for the *Salmonella* and *Aspergillus* populations. The procedure was generally more effective in reducing bacterial food safety risks compared to fungal spore contaminants, although complete eradication was not observed for several of the pathogen/bottle combinations. Therefore, we do not recommend this treatment parameter for the maple industry as a sole approach to microbial safety risk prevention. Future research to assess the sequential
application of a boiling water prefill treatment followed by syrup hot fill process may better
determine the effectiveness of this methodology.

A.2 Material and Methods

A.2.1 Microbial Culture Preparation

Reference section 3.3.1 and 3.3.2 for procedure description.

A.2.2 Bottle Inoculation

Reference section 3.3.3 for procedure description.

A.2.3 Boiling Water Bottle Filling

Boiling water (212°F) was prepared using several single burner hot plates until a visible
rolling boil was observed. Inoculated bottles were selected at random and filled to capacity with
the boiling water. Capacity was confirmed by weight using a gram scale (Accuris Instruments,
Edison, NJ). The water was allowed to sit undisturbed in the bottles for 10 seconds before
decanting and subsequent analysis.

A.2.4 Bacterial Survival Analysis

Immediately after the boiling water was removed, each bottle was filled to half capacity
(confirmed by weight) with sterile 0.05% SDS (Sodium Dodecyl Sulfate; Fisher scientific,
Fairlawn, NJ) solution. Bacterial enrichment was then performed using the procedure previously
described in section 3.3.6 of this thesis, however, the SDS liquid was used in lieu of maple syrup.

A.2.5 Mold Survival Analysis

Like the bacterial analysis method, after the boiling water was decanted, each bottle was
filled to half capacity with sterile 0.05% SDS solution. Mold recovery was preformed using SDS
solution in lieu of syrup following the procedure outlined in section 3.3.7. However, after the 30-
day incubation period, fungal growth remained suspended in the SDS (Figure A.1a). To ensure
all fragmented colonies were collected, samples exhibiting growth were passed through a funnel filter with a vacuum pump attachment (Maxima Dry Oil Free Vacuum Pump, Fisher Scientific, Waltham, MA). The funnel was then fitted with 5.5 centimeter circular filter paper (Whatman International Ltd., Maidstone, England). Once the entire sample was passed through the filter, the filter paper was removed and positioned face down on APDA (Acidified Potato Dextrose Agar, Alpha Biosciences, Baltimore, MD) agar plates (Figure A.1c). The media was prepared with 10% tartaric acid (Sigma-Aldrich, St. Louis, MO) according to manufacturer instructions. All APDA plates were then incubated for 5 days at 68°F. Following incubation period, species were identified by examining spore-producing structures. This was performed by preparing wet mount slides using lactophenol cotton blue dye (Hardy Diagnostics, Santa Maria, CA).
**Figure A.1: Mold Recovery Procedure Following Boiling Water Treatment.** (A) Depicts non-sporulating fungal growth in SDS liquid after the 30-day ambient incubation period. (B) Vacuum pump fitted with a filter funnel to isolate entire fungal population suspended in the sample liquid. (C) Filter paper positioned face down on prepared APDA agar and incubated for 5 days at 68°F.

A.2.6 Statistical Analysis

Reference section 3.3.8 for description.

A.3 Results and Discussion

The purpose of this work was to determine the effectiveness of applying a boiling water prefill treatment to reduce bacterial and fungal pathogens desiccated on the interior of commonly used retail maple syrup bottles. The proposed procedure is highly cost effective, requiring no additional equipment except access to boiling water. Therefore, it would be easy to implement in
the majority operations as a preventative measure against post-process contaminants. As previously mentioned in Chapter 3, the intent of this procedure was to simulate a realistic post-process contamination scenario applicable to compromised packaging. To accomplish this, inoculated bottles were dried overnight (~18 hours) in a sterile biosafety cabinet prior to applying the boiling water treatment. Literature has shown that by desiccating the inoculum, pathogens may demonstrate an increased resistance to normal process interventions, such as heat treatments (Daryaei et al., 2018; Ma et al., 2009). Additionally, when there are no visible signs of contamination, a maple producer is likely to be unaware when a bottle has become compromised. However, like the temperature filling analysis (Chapter 3), it is necessary to note that the overnight desiccation procedure killed a portion of the inoculum (bacterial and fungal) prior to applying the boiling water treatment. As expected by the previous bottling work, the degree of inoculum die-off was rather inconsistent among bottle types, however, fancy glass bottles generally demonstrated the greatest survival rates in the unheated (no boiling water) control treatments across most pathogen groups.

A.3.1 The Effects of Bacterial Survival Among Retail Bottles After Boiling Water Treatment

Figures A.2 – A.4 demonstrates the percentage of survival for each bacterial population pre- and post-boiling water treatment application. To supplement this data, Figures A.5-A.7 illustrates the predictive survival probability of each bacterial genus, as a result of continuous changes to bottle volume before and after the boiling water treatment (obtained from logistic regression). As expected, application of the boiling water treatment significantly (p < 0.05) reduced the probability of survival for all bacterial pathogens we analyzed. However, survival probability was contingent on bottle shape for most of the populations.
Particularly, the use of a fancy glass bottle significantly (p < 0.05) increased the likelihood of survival for both *Salmonella* and *Listeria monocytogenes* in both the unheated control and heat-treated samples, compared to other bottle types. However, as expected, the survival probability for all pathogens was greatest for fancy glass bottles in the unheated control treatments. As suggested in Chapter 3 of this work, although the degree of dessication was not explicitly determined in our analysis, minor pooling in the crevices of the bottle interior, combined with reduced airflow as a result of the smaller bottle opening, may have resulted in an optimal desiccation of the inoculum. These factors may have increased the survival probability of the organisms, contributing to a larger initial population. Specifically, the degree of desiccation may have been sufficient to improve thermal resistance among microorganisms in bottles exposed to the boiling water treatment, but not excessively to result in increased inoculum die-off prior to the treatment (unheated controls). This explanation is also applicable to the larger initial population in the plastic bottles (compared to regular glass) among the unheated control treatments, which can be seen in Figures A.2 and A.4. Although surfaces in the plastic bottle are smooth, pooling of the inoculum liquid is possible. Specifically, the interior of the plastic bottles is lined with a polyvinylidene chloride coating, which we observed reduced the spread of the inoculum by inadvertently creating a beading effect. Compared to the flat and coating free surfaces of the regular glass bottle, this in turn, may have reduced the degree of desiccation, improving the recovery in the non-heated, control treatment samples.

In addition to shape, the effectiveness of the applied treatment was also affected by bottle volume. As can be seen in Figures A.5-A.7, there was an overall trend in predictive survival probability reduction for all pathogens, in response to an increase in bottle volume (and therefore increase in boiling water presence), as expected. Therefore, an increase in volume, presumably
retained a higher temperature for a longer period of time during the heat treatment, which resulted in reduction of the surviving population. More specifically, the effects of heat retention in response to boiling volume are directly associated with the surface area to volume ratio of the individual bottles. This statement is supported by previous data collected by S. Annis (personal communication, April 23, 2020), which determined that fancy glass maple bottles (all sizes) have a larger surface area to volume ratio compared to the 473- and 946-mL plastic bottles. Although the surface area to volume ratio has not been determined for either of the regular glass bottles used in our study, this observation reinforces that generally smaller volume bottles loose heat more rapidly. Beyond the effects of volume and surface area (and thus duration of thermal exposure) as suggested in Chapter 3, heat capacity of the bottling material also likely contributes to our findings. As mentioned, plastic has a lower thermal conductivity compared to glass, and therefore, it is unsurprising that the plastic bottles exhibited some of the lowest survival rates across all the bacterial genera we analyzed in this study (OMAFRA, 2015; Summer, 2018).

However, it should be noted that although there was a general trend in reduction for all bacteria, only the survival probability for *Salmonella* was significantly (*p* < 0.05) reduced in response to an increase in bottle volume. The reason for this could be due to a greater survival of *Salmonella* across the unheated control treatment bottles, and thus the degree of reduction is relative to what was initially recovered. This result is particularly interesting, because it speaks to the enhanced attachment capabilities of the bacteria (Cui, Walcott & Chen, 2017; Jain & Chen, 2007). In the syrup bottle-filled treatments (Chapter 3), which used only viscous maple syrup to remove adherent bacteria, *Salmonella* was the least recovered pathogen across all bottle types. However, application of a surfactant liquid in the present procedure dramatically improved the removal of the pathogen from the bottle surfaces.
Interestingly, no differences in STEC survival probability (p < 0.05) was detected due to either bottle shape or syrup volume. As suggested in Chapter 3, it is possible that the pathogen is exhibiting an enhanced cross resistance compared to the remaining bacterial genera. The initial desiccation of the inoculum potentially created a sublethal stressor, which in turn may have improved the heat resistance of the bacteria once exposed to the boiling water treatment. Examples of this cross-resistance mechanism has been well reported in previous literature. Topalçengiz and Danyluk (2017) for example, observed increased heat resistance in STEC inoculated orange juice samples in which the bacteria had previously been acid adapted. Hiramatsu et al. (2005), similarly observed an increased thermal tolerance in STEC strains which were dried under osmotic stress (36% sucrose paper discs) compared to samples dried on none sucrose containing paper discs. This finding is a considerable difference from the thermal inactivation work conducted in Chapter 2, in which Listeria monocytogenes, the only gram-positive bacteria used in this analysis, demonstrated the greatest heat tolerance.

Therefore, with consideration of the bottle shape, none of the unique bottle attributes significantly impacted the probability of STEC survival. Particularly, neither the assumed optimal desiccation of the fancy glass bottles, perceived increase in inoculum desiccation of the regular glass bottles, or reduced thermal conductivity of the plastic material had a considerable effect on survival, likely due to the microorganism’s competitive genetic advantages. As a result, the bottle volume amplified the effects of the heat treatment, but did not significantly reduce the survival probability of the pathogen. It should be noted that an increase in bottle volume also did not significantly reduce L. monocytogenes survival. We suspect that this observation is due to a reduced cell dispersion in the SDS liquid compared to the maple syrup matrix which reduced the recovery of the pathogen. Additionally, although the cooling rates for each bottle using the
boiling water treatment was also not determine, this finding may also be due to the consistent heat exposure among every bottle types. By applying the heat treatment for only 10 seconds, the rate of bottle cooling was likely more consistent between containers compared the previous work using maple syrup, and therefore differences were marginal.

A.2

Figure A.2: Percentage of Bottles with Positive STEC Recovery Before and After the Boiling Water Treatment. Control unheated treatment (n = 12) and boiling water treatment (n = 18). Among bottle types, FG indicates use of a fancy glass bottle, G indicates use of a regular glass bottle, and P denotes use of a plastic bottle. Milliliters (mL) corresponding to each bottle volume are also listed within bottle type. Error bars indicate standard deviation between replicates (n=3).
Figure A.3: Percentage of Bottles with Positive *L. monocytogenes* Recovery Before and After the Boiling Water Treatment. Control unheated treatment (n = 12) and boiling water treatment (n = 18). Among bottle types, FG indicates use of a fancy glass bottle, G indicates use of a regular glass bottle, and P denotes use of a plastic bottle. Milliliters (mL) corresponding to each bottle volume are also listed within bottle type. Error bars indicate standard deviation between replicates (n=3).
A.4

Figure A.4: Percentage of Bottles with Positive Salmonella Recovery Before and After the Boiling Water Treatment. Control unheated treatment (n = 12) and boiling water treatment (n = 18). Among bottle types, FG indicates use of a fancy glass bottle, G indicates use of a regular glass bottle, and P denotes use of a plastic bottle. Milliliters (mL) corresponding to each bottle volume are also listed within bottle type. Error bars indicate standard deviation between replicates (n=3).
Figure A.5: Effect of Container Volume and Shape on Predictive STEC Survival Probability Before (Unheated) (A) and After Boiling Water Treatment (B). F indicates use of a fancy glass bottle, G indicates use of a regular glass bottle, and P denotes use of a plastic bottle, mL = Milliliters.

Figure A.6: Effect of Container Volume and Shape on Predictive L. monocytogenes Survival Probability Before (Unheated) (A) and After Boiling Water Treatment (B). F indicates use of a fancy glass bottle, G indicates use of a regular glass bottle, and P denotes use of a plastic bottle, mL = Milliliters.
A.3.2 The Effects of Fungal Spore Survival Among Retail Bottles After Boiling Water Treatment

Consistent with the previous bottling work, *Aspergillus* and *Penicillium* spores were generally recovered more readily across bottle types in the unheated control treatments samples compared to the unheated controls of most of the bacterial pathogen bottle combinations we analyzed (Figures A.7 and A.8). As suggested in Chapter 3, the increase in fungal spore survival among the control treatments compared to bacteria, is potentially attributed to the greater desiccation tolerance of fungal spores in the inoculum drying procedure due to the protective structures of the spores. An example of these protective features includes hydrophobins, which are small proteins secreted by the fungus suspected to improve desiccation tolerance among other stressors (Beauvais & Latgé, 2018; Girardin et al., 1999). Although as mentioned, these differences in the dessication of the inoculum was not explicitly determined in our study.
Figure A.8: Percentage of Bottles with Positive *Aspergillus* Recovery Before and After the Boiling Water Treatment. Control unheated treatment (n = 6) and boiling water treatment (n = 9). Among bottle types, FG indicates use of a fancy glass bottle, G indicates use of a regular glass bottle, and P denotes use of a plastic bottle. Milliliters (mL) corresponding to each bottle volume are also listed within bottle type. Error bars indicate standard deviation between replicates (n=3).

Figure A.9: Percentage of Bottles with Positive Penicillium Recovery Before and After the Boiling Water Treatment. Control unheated treatment (n = 6) and boiling water treatment (n = 9).
Unlike the previous bottling chapter, however, none of the same individual factors (temperature, bottle shape, or bottle volume) significantly impacted the survival probability of both fungal genera regarding the boiling water treatment. These somewhat variable trends are illustrated in Figures A.10 and A.11, which demonstrates the predictive survival probability of each fungal genus before and after the boiling water treatment, when volume is continuous, and a particular bottle shape is used. Beginning with temperature, although application of the boiling water treatment demonstrated a trend in survival probability reduction for both Aspergillus and Penicillium, the degree of reduction based on temperature alone was only significant ($p < 0.05$) for the *Penicillium* population (Figure A2.5). This finding was somewhat surprising, because *Aspergillus* was seldom recovered following the boiling water treatment. Although, there was greater survival of *Penicillium* across all control treatment bottles (> 80%), and thus the degree of reduction is relative to what was initially recovered.
A.10

Figure A.10: Effect of Container Volume and Shape on Predictive *Aspergillus* Survival Probability Before (Unheated) (A) and After Boiling Water Treatment (B). F indicates use of a fancy glass bottle, G indicates use of a regular glass bottle, and P denotes use of a plastic bottle, mL = Milliliters.

A.11

Figure A.11: Effect of Container Volume and Shape on Predictive *Penicillium* Survival Probability Before (Unheated) (A) and After Boiling Water Treatment (B). F indicates use of a fancy glass bottle, G indicates use of a regular glass bottle, and P denotes use of a plastic bottle, mL = Milliliters.
As expected, an increase in volume reduced the survival probability of the mold spores (such as in fancy glass bottles, Figure A.8). However, survival probability as a result of volume increase was only significantly \((p < 0.05)\) reduced for the *Aspergillus* population. More specifically, our results indicate that the interaction of volume and temperature together resulted in a significant \((p < 0.05)\) reduction in *Aspergillus* survival likelihood. The effects of the heat treatment are presumably amplified by the bottle volume, with larger volumes retaining higher temperatures for longer, killing the spores. Although this correlation of the two factors is logical, it was somewhat surprising that the same conclusion was not observed for *Penicillium*, considering previous literature has shown *Penicillium* isolates to be less heat resistant than *Aspergillus* species (Beuchat, 1981; Kamil, Lupuliasa, Draganescu, & Vlaia, 2011; Shearer, Mazzotta, Chuyate & Gombas, 2002). *Penicillium* was recovered in all bottle types following the heat treatment, however, Figure A.9 demonstrates that there was only a marginal difference in spore survival following the boiling water treatment in response to an increase in volume. As mentioned in the bacterial analysis, the duration of thermal exposure was limited to 10 seconds, which may have resulted in these limited differences between the bottle volumes.

Finally, bottle shape also influenced the survival probability of the mold spores. Use of a fancy glass bottle increased the survival of both fungal genera, however, this increase was only significant \((p < 0.05)\) for the *Penicillium* population. Within the unheated control treatment, there was 100% *Penicillium* recovery across all sizes of fancy glass bottles. As suggested, the geometrical differences of this bottle may have created optimal dessication, facilitating survival in the unheated controls, as well as survival following the heat treatment (Harding, Marques, Howard & Olson, 2009). Whereas the smooth surfaces of the regular glass bottle and large opening in the 946mL plastic bottle, improved airflow to promote desiccation in the unheated
control bottles, which may have slightly reduced the recovery prior to the heat treatment (Figure A.9). With respect to the survival following the boiling water application, as mentioned in the bacterial analysis of this work, the larger surface area to volume ratio of the fancy glass bottles may have also contributed to the increased recovery of this container type (S. Annis, personal communication, April 23, 2020). Interestingly, *Penicillium* was recovered within all plastic bottle sizes following the heat treatment, which may have been the result of spore aggregation for reasons previously described (Chapter 3). With reference to spore survival among regular glass bottles, because fungal spores are less sensitive to desiccation, it is unsurprising that there was also some recovery in this bottle type for both genera following the boiling water exposure.

Despite these differences among the bottle attributes, it is necessary to mention that fungal spores were generally recovered more readily in the syrup fill treatments (Chapter 3) compared to the boiling water treatment. The increased nutrient availability of the syrup compared to the SDS solution likely attributed to this finding. Aside from the abundant sucrose source, nitrogen which has previously been proven to be a limiting factor in fungal growth, is also present (approximately 0.03%) in maple syrup (Brzonkalik et al., 2011; Perkins & Van den Berg, 2009; Prendes et al., 2017). Therefore, this finding suggests that our results from the boiling water treatment may underestimate the contamination potential of fungal spores using typical maple processing conditions.

**A.4 Conclusions**

The results from both the bacterial and fungal spore analyses demonstrate that although a boiling water prefill treatment reduced pathogen populations, this methodology was not entirely effective in removing all the contaminants within several of the bottle/pathogen combinations in our analysis. Like the syrup fill treatments in Chapter 3, success of the boiling water procedure
was largely dependent on container volume and shape. As expected from our previous work, there is inherently greater risk of pathogen survival in fancy glass bottles compared to the remaining container types due to differences in desiccation, reduced volume, and high thermal conductivity of the material. Additionally, the use of a larger bottle volume generally results in a lower probability of pathogen survival. Therefore, application of a prefill treatment does not entirely negate the risks associated with the smaller fancy glass bottles. We therefore continue to recommend that producers use the largest plastic bottle filled with the highest syrup temperature possible to mitigate microbial-based food safety risks. Perhaps sequential analysis of a boiling water heat treatment in combination with hot syrup filling may be effective in complete eradication of microbial hazards associated with maple syrup bottling. Producers concerned with this type of product contamination should further investigate the parameters of a boiling water treatment (for a longer heat exposure duration) in addition to other process interventions, including sanitization to reduce the risks of pathogen contamination.

A.5 References

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APPENDIX B

SURVIVAL OF STEC, LISTERIA MONOCYTOGENES, SALMONELLA, ASPERGILLUS AND PENICILLIUM DURING NORMAL STORAGE CONDITIONS OF MAPLE SYRUP AND MAPLE SAP

B.1 Abstract

In the absence of good manufacturing practices, post-process contamination in maple syrup operations can threaten product safety or quality. Therefore, the goal of this study was to assess the survival of bacterial pathogens and growth of spoilage fungi in refrigerated (~4°C) maple sap and ambient (~22°C) maple syrup, the typical storage conditions for both products. Maple sap and syrup samples were inoculated to approximately 2 log CFU/mL with STEC, Listeria monocytogenes, Salmonella, and separate Aspergillus and Penicillium two-strain composites. Our research consisted of conducting monthly assessments for bacterial detection, performed through selective enrichment and plating procedures, or biweekly visual examination of fungal growth. We detected no bacterial species in refrigerated maple sap after 60 days of storage or beyond 30 days in ambient maple syrup. Fungal growth in refrigerated maple sap was evident after 19 days of storage and exhibited continuous hyphal growth for the duration of the 72-day monitoring period. In maple syrup, most fungal replicates exhibited sporulation with the exception of one Grade A Dark Aspergillus spp. replicate exhibiting only hyphal growth. For these reasons, it may be possible that maple syrup can be retained by the producer for 30 days before sale in order to better ensure consumer safety. However, mold-contaminated product should always be discarded due to potential for mycotoxin production.
B.2 Materials and Methods

B.2.1 Bacterial Culture Preparation

Bacterial colonies were streaked for isolation on selective media and incubated at optimal growth temperatures for 24 hours (Table B.1). A single colony of *E. coli* O111:H8 ATCC BAA-184 (American Type Culture Collection, Manassas, VA), *E. coli* O26:H11 ATCC BAA-1653 (American Type Culture Collection, Manassas, VA), *Salmonella* Enteriditis ATCC BAA-1045 (American Type Culture Collection, Manassas, VA), *Salmonella* Braenderup (a hazelnut isolate), as well as *L. monocytogenes* ATCC 19111 (American Type Culture Collection, Manassas, VA) and *L. monocytogenes* ATCC 19115 (American Type Culture Collection, Manassas, VA) were individually propagated in Luria Miller broth (LB; Alpha Biosciences, Baltimore, MD).

Inoculated broths were then incubated for 24 hours at optimal growth temperatures for each pathogen (Table B.1). A 200 µl aliquot of each overnight culture was plated onto LB agar, which was prepared by combining LB broth with 1.5% bacteriological agar (Alpha Biosciences, Baltimore, MD). Inoculated LB agar plates were incubated for an additional 24 hours at the previously described temperatures. The resulting bacterial lawns were then scraped with 5 mL of 0.1% peptone (Difco, Sparks, MD) using a sterilized spreader, and placed into separate 15 mL conical tubes (Fisher Scientific, Waltham, MA). The scraped lawn cultures were then centrifuged (Centrifuge 5810 R, Eppendorf, Hauppauge, NY) for 10 minutes at 15,550 x g and the supernatant was decanted. The resulting bacterial pellet was resuspended in 5 mL of autoclaved maple sap (collected from commercial maple syrup operations in Somerset and Penobscot Counties in Maine during the spring of 2018 and 2019). Like bacterial species were combined into one conical tube and again vortexed. The bacterial cultures were appropriately diluted in sap then the three diluted bacterial species were combined to deliver a targeted 2 log CFU/mL sap or
syrup per pathogen in each sample (Table B.2). To verify the volume required to deliver the target cell concentration in the sap or syrup, the concentration of the starting suspension was confirmed using an optical density reader (Biotek International, Winooski, Vermont).

Specifications for sap and syrup samples can be found in Table B.3

**Table B.1: Selective Enrichment Broth and Agar with Optimal Incubation Temperatures used in Bacterial Recovery Analysis**

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Selective Enrichment Broth</th>
<th>Enrichment Broth Incubation Temperature °C</th>
<th>Selective Agar</th>
<th>Agar Incubation Temperature °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>STEC</td>
<td>EEB&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37</td>
<td>EMB&lt;sup&gt;d&lt;/sup&gt;</td>
<td>37</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>LEB&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32</td>
<td>MOX&lt;sup&gt;e&lt;/sup&gt;</td>
<td>32</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>RV&lt;sup&gt;c&lt;/sup&gt;</td>
<td>42.5</td>
<td>XLD&lt;sup&gt;f&lt;/sup&gt;</td>
<td>37</td>
</tr>
</tbody>
</table>

- a. Enterobacteria Enrichment Mossel Broth, (Himedia Laboratories, Mumbai, India)
- b. *Listeria* Enrichment Broth, (Alpha Biosciences, Baltimore, MD)
- c. Rappaport Vassiliadis Broth, (Millipore Sigma, Burlington, MA; Sigma-Aldrich, St. Louis, MO)
- d. EMB (Eoisin Methylene Blue, BD Difco, Sparks, MD)
- e. MOX (Modified Oxford Agar, Alpha Biosciences, Baltimore, MD)
- f. XLD (Xylose Lysine Deoxycholate agar, BD Difco, Sparks, MD)

### B.2.2 Mold Culture Preparation

Mold isolates *Penicillium brevicompactum* complex (SNH6A), *Penicillium* sp. 4 (SNH9B), *Aspergillus* sp. 2 (SNY1A), and *Aspergillus pseudoglaucus* (SME2A) were plated onto APDA (Acidified Potato Dextrose Agar). The media was prepared using potato dextrose agar (Alpha Biosciences, Baltimore, MD) supplemented with 10% tartaric acid (Sigma-Aldrich, St. Louis, MO). Mold cultures were incubated at room temperature (between 20-25°C) for approximately 8-9 days until there was visible sporulation for all isolates. To produce a spore suspension, the surface of the incubated cultures were lightly scraped using a sterilized spreader.
and 0.05% Tween 20 solution (Sigma-Aldrich, St. Louis, MO). The suspensions for like species were combined, and the concentration was determined using a Neubauer improved hemocytometer (Hauser Scientific, Horsham, PA). Suspensions were then diluted into sterile maple sap to deliver 2 log spores/mL of sap or syrup. The starting inoculation levels for each sample are also listed in Table B.2.

**B.2.3 Bacterial Sample Analysis**

Individual conical tubes containing either 5 mL of sterile maple sap or syrup were used during each storage assessment in order to avoid exposing samples to potential environmental contaminants. Three grades of maple syrup and one sap sample were evaluated for this analysis. It is necessary to note that Grade A Golden syrup was used in only one *Listeria monocytogenes* replicate. This is because upon confirmation of the initial inoculum level for the *L. monocytogenes* Grade A Very Dark replicate, the population was higher (by 1 log CFU/mL) than the targeted concentration. Therefore, to have consistency between replicates, the Grade A Golden sample was produced.

Syrup samples were stored at room temperature (~22°C) and sap was held in refrigerated storage (~4°C) throughout the analysis period. On Day 0, 1 mL of the inoculated sap or syrup sample was spread-plated on selective media (300, 300, 400 µl onto three separate agar plates in duplicate) and counted to confirm the starting concentration of the culture. Subsequent testing was performed every 30 days for detection only. During shelf life monitoring, 1.5 mL of sap or syrup from a single conical tube was enriched 1:10 in one of the three selective enrichment broths and incubated at optimal growth temperatures for 24 hours prior to plating. Incubated enrichment bags were then streaked for isolation in duplicate onto selective media (Table B.1).
Selective agars were incubated for an additional 48 hours before examination. When no pathogen was detected, two subsequent checks were sequentially performed for confirmation of results.

**Table B.2: Initial log CFU/mL Sap or Syrup During Storage Study Analysis**

<table>
<thead>
<tr>
<th>Sample</th>
<th>STEC log CFU/mL</th>
<th>L. Monocytogenes log CFU/mL</th>
<th>Salmonella log CFU/mL</th>
<th>Aspergillus spp. log CFU/mL</th>
<th>Penicillium spp. log CFU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sap a</td>
<td>2.14</td>
<td>2.21</td>
<td>2.41</td>
<td>2.47</td>
<td>2.69</td>
</tr>
<tr>
<td>Grade A Amber b</td>
<td>2.25</td>
<td>2.00</td>
<td>2.56</td>
<td>2.52</td>
<td>2.34</td>
</tr>
<tr>
<td>Grade A Dark c</td>
<td>2.21</td>
<td>2.10</td>
<td>2.54</td>
<td>2.50</td>
<td>2.50</td>
</tr>
<tr>
<td>Grade A Very Dark d</td>
<td>1.96</td>
<td>N/A</td>
<td>2.31</td>
<td>2.62</td>
<td>2.24</td>
</tr>
<tr>
<td>Grade A Golden e</td>
<td>N/A</td>
<td>2.37</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

a. Obtained from commercial maple syrup operations in the Somerset and Penobscot Counties (Maine) during 2018 and 2019 sugaring season
b. Highland Sugar works, Websterville, VT
c. Ben’s Pure Maple Products LLC. Temple, NH
d. Bemis Family Farm, Corina, ME
e. Merrifield Maple Farm, Gorham, ME

**Table B.3: Mean °Brix and Water Activity Levels of Syrup and Sap Samples Used In Storage Analysis**

<table>
<thead>
<tr>
<th>Specification Mean± Standard Deviation</th>
<th>Sap b</th>
<th>Grade A Amber c</th>
<th>Grade A Dark d</th>
<th>Grade A Very Dark e</th>
<th>Grade A Golden f</th>
</tr>
</thead>
<tbody>
<tr>
<td>°Brix a</td>
<td>2.70 ± 0.14</td>
<td>67.40 ± 0.00</td>
<td>66.95 ± 0.07</td>
<td>66.70 ± 0.00</td>
<td>68.05 ± 0.07</td>
</tr>
<tr>
<td>Water Activity a</td>
<td>1.00 ± 0.00</td>
<td>0.855 ± 0.00</td>
<td>0.843 ± 0.00</td>
<td>0.849 ± 0.00</td>
<td>0.856 ± 0.00</td>
</tr>
</tbody>
</table>
Table B.3 Continued

a. \( n = 2 \)

b. Obtained from commercial maple syrup operations in the Somerset and Penobscot Counties (Maine) during 2018 and 2019 sugaring season

c. Highland Sugar works, Websterville, VT

d. Ben’s Pure Maple Products LLC. Temple, NH

e. Bemis Family Farm, Corina, ME

f. Merrifield Maple Farm, Gorham, ME

B.2.4 Mold Sample Analysis

For both mold species, three inoculated syrup tubes and one inoculated sap tube was prepared for monitoring. *Aspergillus* and *Penicillium* spp. samples were prepared separately to facilitate easier identification. Shelf life samples were monitored every two weeks and any visual change in growth was recorded. Unlike obvious surface contamination of syrup samples, mold growth was observed at the bottom of the sap tubes. For this reason, fungal samples were streaked onto APDA and incubated for an additional 5 days at room temperature (~22 °C) for species confirmation at the end of the study. Genera were confirmed by visual examination of colony color and spore producing structures. The conclusion of analysis was determined by no notable changes in fungal growth after three consecutive sample checks.

B.3 Results and Discussion

There are several potential post-process contamination sources within a maple syrup operation. Prior to syrup production, maple sap is highly susceptible to microbial inhabitation, and can quickly become contaminated when not stored appropriately. After the syrup is prepared, inadequate filling temperatures when a bottle surface is compromised, improper sanitation procedures, or absence of good manufacturing practices can contribute to this risk. Therefore, in light of these potential deviations from best practices, a time estimate for pathogen survival in either product under typical storage conditions is necessary. In this study, the survival
of both bacterial pathogens and fungal spores was assessed in syrup held at ambient temperatures (~22 °C) and sap stored under refrigeration (~4 °C).

As mentioned in prior sections of this thesis, bacterial contamination can result in a range of adverse health conditions including nausea, diarrhea, vomiting, cramping, bloody stool, or hemolytic uremic syndrome and death in the most severe cases (CDC, 2017; Kunwar et al., 2013; Montville et al., 2012a). Our results show that both *Listeria monocytogenes* and *Salmonella* composites are capable of survival in refrigerated sap for a maximum of 60 days. However, STEC was not recovered in inoculated maple sap after 30 days of refrigerated storage. This result may be attributed to heightened sensitivity of the bacteria to psychrotrophic conditions compared to the remaining strains we tested. When inoculated in three different grades of maple syrup, all three bacterial pathogens were undetected after 30 days of ambient storage (Table B.4). It is unsurprising that the bacterial pathogens we analyzed survived longer in maple sap compared to maple syrup. Maple sap is a moisture rich environment, similar to irrigation water, and irrigation water has been found to be contaminated with the three genera (Falardeau, Johnson, Pagotto, & Wang, 2017; Steele, M. & Odumeru, 2004). Although not identical to pure water, the sucrose composition in maple sap is marginal, and has only been reported in concentrations as high as 5%. This results in limited water binding, creating increased moisture accessibility and a useable carbon source to sustain the present microbial population (Perkins & van den Berg, 2009). However, nutrient limitation or absence of growth from other external factors, such as temperature, causes a decline in the microbial community over time. On the other hand, the reduced survival duration of bacteria in maple syrup suggests that the high sugar composition consequently binds the available water for the bacterial inoculum, creating osmotic stress and limiting nutrient resources. This likely inhibits the growth of the population.
resulting in eventual die off of the surviving population. Therefore, retention of the finished product by a producer before sale may provide additional security that the products are in fact safe to consume.

**Table B.4: Maximum Days Estimation of Bacterial Pathogen Survival in Refrigerated Maple Sap and Ambiently Stored Maple Syrup**

<table>
<thead>
<tr>
<th></th>
<th>STEC</th>
<th><em>Listeria monocytogenes</em></th>
<th><em>Salmonella</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade A Amber</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Grade A Dark</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Grade A Very Dark</td>
<td>30</td>
<td>N/A</td>
<td>30</td>
</tr>
<tr>
<td>Grade A Golden</td>
<td>N/A</td>
<td>30</td>
<td>N/A</td>
</tr>
<tr>
<td>Sap</td>
<td>30</td>
<td>60</td>
<td>60</td>
</tr>
</tbody>
</table>

Fungal contamination by mycotoxin-producing molds can also jeopardize consumer safety. As discussed, a wide range of mycotoxins or secondary metabolites, including ochratoxin A, aflatoxin, and mycophenolic acid, can be formed by either *Aspergillus* or *Penicillium* species, when conditions such as pH, oxygen, water activity, and nutrients such as nitrogen content are favorable (Brzonkalik, Herrling, Syldatk, & Neumann, 2011; Prendes et al., 2017). Mycotoxicosis may result in several adverse health conditions, such as liver or bladder cancers and even organ failure (Montville et al., 2012b; Montville et al., 2012c). Thankfully, unlike bacterial contaminants, fungal growth creates a visual product defect alerting the producer or consumer that the sap or syrup has become compromised. Simply removing surface mold and reheating the product, however, is not acceptable practice for mitigating this risk, due to possible toxin penetration beyond the immediate product surface. Removal of these secondary
metabolites requires extensive high processing temperatures to entirely eradicate. Such practices would be unlikely implemented by a producer or consumer, as it would significantly reduce product quality.

In our study, a visible fungal mat for both *Aspergillus* and *Penicillium* inoculated sap samples was evident after 19 days of refrigerated storage. Although the fungal mats continued to exhibit hyphal growth throughout the 72-day monitoring period, neither sample sporulated until the colony was transferred to APDA. Visible sporulation, as well as examination of spore producing structures after 5 days of ambient (~22°C) incubation on APDA plates, confirmed that hyphal masses were in fact the result of intended inoculum growth rather than inadvertent sample contamination. Other studies have demonstrated that fungal sporulation and mycotoxin production are closely correlated, therefore, hyphal growth alone does not necessarily indicate fungal toxin production (McDonald et al., 2004; Reverberi et al., 2010). However, when environmental conditions become more favorable risk of illness may be increased (Brzonkalik, et al., 2011; Prendes et al., 2017). Therefore, any fungal presence in sap may introduce health complications for consumers. Interestingly, in our work, mold growth was evident at the bottom of the sap samples rather than the product surface. Perhaps ample head space in the conical tubes facilitated increased oxygen exposure to allow for fungal growth without the need for spore germination at the product surface. For this reason, fungal growth was monitored by surface area coverage at the base of the container and measuring the increase in height of the fungal mass in reference to the milliliter (mL) markers on the side of the conical tube. The growth rate of the hyphal masses in sap are shown in Table B.5. with pictures in Figure B.1.
Table B.5: Fungal Growth Rate in Refrigerated Maple Sap Defined by Surface Area Coverage and Height in Milliliters (mL)

<table>
<thead>
<tr>
<th>Day</th>
<th>Aspergillus spp.</th>
<th>Penicillium spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>30</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>45</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>59</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>72</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

+ 25% coverage with height growth to ~ 3 mm or less
++ 25-49% coverage with height growth to ~ 4-4.9mm
+++50-75% coverage with height growth to ~ 5-5.9mm
++++75%-100% coverage with height to > ~ 6 mm

A visible fungal mat was also evident for both species in maple syrup after 19 days of ambient storage. Inoculated syrup samples were retained for a maximum of 128 days in order to observe the growth rate of each species throughout the monitoring period. The observations for this analysis are shown in Table B.5 with additional pictures in Figures B.2 and B.3. Unlike the inoculated sap, sporulation was obvious in the majority of the syrup samples. The exception to this was Aspergillus inoculated Grade A Dark syrup, which demonstrated only hyphal growth. Although much less sensitive to low water activity environments, both fungal species demonstrated spore germination in the syrup samples in order to survive environmental stress (Beuchat, 1983).

As previously suggested, sporulation can be indicative of mycotoxin production, however, it is necessary to mention that these two factors are not mutually inclusive. In fact, the conditions required for toxin production are varied compared to sporulation alone. For example, some Aspergillus and Penicillium species require a minimum water activity of 0.77 for growth,
however, are only capable of ochratoxin production when the water activity is a minimum 0.83 (Beuchat, 1983). Nonetheless, the conditions within maple syrup generally meet this minimum threshold for mycotoxin production, therefore, a lag period before product sale as suggested by the bacterial inoculated samples, is not recommended for fungal contaminated product.

Table B.6: Fungal Growth Rate in Three Grades of Ambient Maple Syrup Samples Over 128 Days

<table>
<thead>
<tr>
<th>Day</th>
<th>Aspergillus spp.</th>
<th>Penicillium spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Grade A Amber</td>
<td>Grade A Dark</td>
</tr>
<tr>
<td>19</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>45</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>59</td>
<td>++++</td>
<td>*</td>
</tr>
<tr>
<td>72</td>
<td>++++</td>
<td>++*</td>
</tr>
<tr>
<td>86</td>
<td>++++</td>
<td>++*</td>
</tr>
<tr>
<td>100</td>
<td>++++</td>
<td>++*</td>
</tr>
<tr>
<td>114</td>
<td>++++</td>
<td>++*</td>
</tr>
<tr>
<td>128</td>
<td>++++</td>
<td>++*</td>
</tr>
</tbody>
</table>

- no growth
* indicates hyphal growth only
+ minimal evidence of growth
++ Sporulation with 25-49% surface coverage
+++ Sporulation with 50-74% surface coverage
++++ Sporulation with 75-89% surface coverage
+++++ Complete fungal mat or > 90% surface coverage
B.4 Conclusions

The aim of this work was to assess the survival of pathogenic bacteria and growth of mycotoxin producing molds in contaminated maple sap and syrup stored in refrigerated and ambient temperatures, respectively. Our results show that bacteria are capable of surviving in maple sap for a maximum 60 days, but are only detected before 30 days of storage in maple syrup. Both *Aspergillus* and *Penicillium* composites demonstrated continuous growth in both maple sap and syrup throughout the monitoring period. However, only sporulation was visible in mold inoculated maple syrup samples. These findings suggest that bacteria have limited survival in maple syrup and sap, whereas both food environments provide ample nutrient sources to sustain mold populations.
<table>
<thead>
<tr>
<th>Day</th>
<th>Aspergillus spp.</th>
<th>Penicillium spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>59</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**CONFIRMATION**

![Image](image5.png)  ![Image](image6.png)

*Figure B.1: Photographs of *Aspergillus* and *Penicillium* spp. Growth In Refrigerated Maple Sap During Storage Study Analysis*
<table>
<thead>
<tr>
<th>Day</th>
<th>Grade A Amber</th>
<th>Grade A Dark</th>
<th>Grade A Very Dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
</tr>
<tr>
<td>59</td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>86</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
</tr>
<tr>
<td>114</td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure B.2: Photographs of *Aspergillus* spp. Growth In Ambient Maple Syrup During Storage Study Analysis
<table>
<thead>
<tr>
<th>Day</th>
<th>Grade A Amber</th>
<th>Grade A Dark</th>
<th>Grade A Very Dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
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<tr>
<td>59</td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>86</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
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<tr>
<td>114</td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
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</tbody>
</table>

Figure B.3: Photographs of *Penicillium* spp. Growth In Ambient Maple Syrup During Storage Study Analysis
B.5 References

   http://jfoodprotection.org/doi/pdf/10.4315/0362-028X-46.2.135

   doi:10.1016/j.ijfoodmicro.2011.03.016

   https://www.cdc.gov/ecoli/ecoli-symptoms.html

   https://doi.org/10.1371/journal.pone.0185437

   https://doi.org/10.1016/j.mjafi.2013.01.005


Maria Fiore was born in Boston, MA on August 7, 1992. She was raised in the greater Boston area and graduated from Winchester High School in 2011. She attended Virginia Polytechnic Institute and State University, where she graduated with a bachelor’s degree in Food Science in 2015. Upon graduation, she returned to New England where she worked as a research technologist in private industry until August 2018. Maria plans to remain in New England to pursue a food safety-oriented career. Maria is a candidate for the Master of Science Degree in Food Science and Human Nutrition from the University of Maine in May 2020.