Modeling the growth rates of *Escherichia coli* spp. and *Salmonella Typhimurium* LT2 in baby spinach leaves under slow cooling

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**ABSTRACT**

After field harvest, baby spinach leaves are transported to the packing shed where they are cooled by forced air systems. If contaminated, the temperature of spinach leaves will affect the number of pathogens in the leaves, and effective temperature control is critical to restrict their growth. Hence, the need to assess the impact of cooling practices on the growth of pathogens in leafy greens. The Baranyi model was used to describe the experimental data and build a dynamic model to predict microorganism growth rate in baby spinach leaves as function of temperature.

Baby spinach leaves, inoculated with 10^5 CFU/ml of *Salmonella Typhimurium* LT2 or 10^2 CFU/ml of an *Escherichia coli* cocktail, and were maintained at temperatures ranging from 10 to 37 °C for 30 h. At 10−30 °C, the *E. coli* strains grew significantly more (~2−4 log cycles) than the *Salmonella* strain (~0.11−2.4 log cycles) while at 37 °C, both bacterial populations increased by ~6 log cycles for 30 h. The growth kinetics of each microorganism followed the Baranyi model. The maximum bacterial population increased with temperature and the values were similar for both bacteria. The theoretical minimum temperature for growth was 5.88 °C and 4.76 °C for *Salmonella* and *E. coli*, respectively. The dynamic model was validated with an experimental linear cooling profile (slow cooling) and could be incorporated into a risk assessment tool to evaluate the growth of pathogens in baby spinach during processing and distribution.

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1. **Introduction**

During 1996 to 2008, eighty-two foodborne illness outbreaks were associated with the consumption of fresh produce (FDA, 2009). Consumption of leafy greens accounted for 34% of these outbreaks, i.e. 949 illnesses and 5 deaths. The foodborne illnesses in most of these outbreaks (86%) were caused by *Escherichia coli* O157:H7 though *Salmonella* have been implicated on outbreaks linked to leafy greens such as lettuce and spinach. For instance, although most cases of *Salmonella* poisoning are caused by undercooked eggs and chicken, several U.S. spinach producers had their bagged baby spinach recalled for potential *Salmonella* contamination in recent years (Consumer Reports.org, 2012).

Certain factors contribute to the microbiological contamination of fresh produce with pathogens. Contamination can arise because of the treatment of soil with manure and sewage sludge or from irrigation with contaminated water. Additionally, the application of technologies such as cutting, slicing, skinning, and shredding will remove the natural protective barriers of the intact plant and provide a suitable medium for the growth of contaminating microorganisms (EC, 2002).

An evaluation of the investigative report of the 2006 *E. coli* O157:H7 outbreak associated with Dole pre-packaged spinach (Grant et al., 2007) concluded that current practices were not sufficient to prevent the outbreak. It was suggested that variables such as initial inoculums (pathogen population levels), temperature fluctuation during processing and storage, and sanitation procedures (e.g., washing with chlorinated water) should be carefully evaluated for possible contamination and growth of pathogens in spinach leaves.

Quantitative risk assessment models can be a useful tool to determine the impact of different mitigation strategies (washing, irradiation) on the number of pathogens present in leafy vegetables. As part of the model development process, dynamic predictive models can be incorporated to evaluate pathogen growth in processing, transport, and distribution of leafy greens.

Predictive microbiology can be used to determine the growth of pathogens in foods during processing and storage (Roberts & Jarvis,
1983). A dynamic model is used to predict the population dynamics of pathogens in food systems at time-varying temperature profiles (non-isothermal conditions). To develop a dynamic model, a primary model is developed to predict microbial population levels at several isothermal conditions. Next, a secondary model will describe the effect of temperature, for instance, on parameters of the primary model such as growth rate. Finally, the dynamic model is developed by numerically integrating the primary and secondary models to predict microbial population at non-isothermal conditions (Van Impe, Poschet, Geeraerd, & Vereecken, 2005).

Although predictive modeling has been carried out for a variety of foods including eggs (Singh et al., 2011), meat products (Amézquita, 2005; Billoir, Denis, Commeau, Cornu, & Zuliani, 2011), tomatoes (Pan & Schaffner, 2010) and lettuce (Koseki & Isobe, 2005; Pérez Rodríguez et al., 2011), a growth model for Salmonella and E. coli in ready-to-eat baby spinach is not available. Such a model could be incorporated into risk assessment models to improve their performance in evaluating pathogen growth in the fresh produce at different steps of the processing line, during transportation, and distribution. Therefore, the objectives of this study were to evaluate the growth of Salmonella Typhimurium LT2 and an E. coli cocktail in baby spinach leaves under different temperatures and then use the Baranyi model to predict the growth of these microorganisms under slow temperature changes.

2. Materials and methods

2.1. Bacterial cultures

Rifampicin-resistant (80 µg/ml) cultures of S. Typhimurium LT2 and E. coli BAA-1427, E. coli BAA-1428, and E. coli BAA-1430, were acquired from Dr. Alejandro Castillo’s stock laboratory (Department of Animal Science, Texas A&M University) and maintained at −80 °C. Before being used, an inoculum was removed from the frozen culture with a loop, streaked onto Tryptic Soy Broth (TSB; Difco, Detroit, MI), and incubated for 24 h at 37 °C. Next, single colony isolates were obtained from Tryptic Soy Agar plates (TS; Difco) through two successive transfers on TSB. Colonies were stored on TSA slant at 25 °C as working cultures and used within 30 days (Moreira, Puerta-Gomez, Kim, & Castell-Perez, 2012). These bacteria have been already established as good surrogates for their equivalent pathogenic strains.

2.2. Inoculum preparation

The same procedure was followed for both the Salmonella and E. coli strains. A loop inoculum was transferred from the working culture at 25 °C to TSB test tubes and incubated at 37 °C for 18 h. After incubation, each test tube was centrifuged and washed three times (3000 × g for 15 min) with Difco buffered peptone water. Subsequently, each pellet was resuspended in 1 g/100 g PW. The OD₆₀₀ of the cell suspensions was adjusted to 0.5 of Absorbance (Milton Roy Spectronic 20D turbidity meter, Milton Roy Co, CA) for the bacterial growth at static temperature (isothermal) conditions: the model (Grijspeerdt & Vanrolleghem, 1999) was used to describe the lag-phase duration in hours. Data were used to run the temperature profile for the simulated cool down of the spinach leaves from 30 to 5 °C in 5 h. Among the different existing cooling methods used in Texas, such as ice, hydrocooling, forced-air, and room cooling, forced-air is commonly used in the fresh produce industry. However, this method is not very effective to rapidly cool the product because it takes from 1 to 10 h (Kader & Rolle, 2004) to achieve the target storage temperature. Therefore, a linear cooling profile was used to represent the slow cooling practice that could increase the risk of growing pathogenic bacteria thus compromising the safety of the leafy products. A programmable water bath with water circulation capability (RTE 740, Thermo Neslab, Portsmouth, N.H., U.S.A.) was used to run the temperature profiles (cooling rate, 0.087 °C/min). Four samples (in sterile 18 oz Whirl Pak® bags) were analyzed every 45 min for each microorganism enumeration. Precaution was taken to ensure the leaves were immersed in the water at all times.

2.5. Pathogen and growth modeling

Pathogen growth curves in baby spinach leaves as a function of temperature were fitted using the function of Baranyi and Roberts (1994). The modified Ratkowsky equation (1982) was used to describe the effect of temperature fluctuation on pathogen growth rate.

2.5.1. The predictive model

The reader is referred to Baranyi, Roberts, and McClure (1993) for the theoretical derivation of the model. The explicit form of the model (Grijspeerdt & Vanrolleghem, 1999) was used to describe the bacterial growth at static temperature (isothermal) conditions:

\[
y(t) = y_0 + \mu_{max} F(t) - \ln \left(1 + \frac{e^{\mu_{max} F(t)}}{e^{\mu_{max} y_0}}\right)
\]

with

\[
F(t) = t + \frac{1}{\nu} \ln (e^{-\nu t} + e^{-h_{yo}} - e^{-\nu t - h_{yo}})
\]

where, \(y(t)\) is the ln CFU/g of cells concentration at time \(t\); \(y_0\) is the initial cell concentration in ln CFU/g; \(\nu_{max}\) is the maximum cell concentration in ln CFU/g; \(h_{yo}\) is the maximum specific growth rate in terms of ln CFU/g in 1/h; \(\nu\) is rate of increase of the limiting substrate, assumed to be equal to \(\nu_{max} h_{yo} = \nu_{max} \times \lambda\); and \(\lambda\) is the lag-phase duration in hours. Data were fitted to the Baranyi model.
and the parameters \( y_o, y_{\text{max}}, \mu_{\text{max}}, \) and \( \lambda \) were estimated at each temperature using the MicroFit software program (Institute of Food Research (IFR), Norwich, UK). To evaluate the model, we used the \( R^2 \):

\[
R^2 = 1 - \frac{\text{SSE}}{\text{SST}}
\]  

where, SSE is the sum of squares of residuals and SST is the total sum of squares. The root mean squared error (RMSE) was also used to evaluate the model’s performance:

\[
\text{RMSE} = \sqrt{\frac{\text{SSE}}{N-p}}
\]  

where, \( N \) is the number of observations, and \( p \) is the number of model parameters (i.e. 4). The above two statistical quantities were calculated for each temperature after fitting the growth data into Eq. (1).

2.5.2. Temperature effect on the maximum growth rate

The effect of temperature on the maximum growth rate was modeled as (Ratkowsky, Olley, McMeekin, & Ball, 1982):

\[
\sqrt{\mu_{\text{max}}} = a(T - T_{\text{min}})
\]

where, \( T_{\text{min}} \) is the theoretical minimum temperature beyond which growth is not possible and \( a \) is a regression coefficient calculated using MATLAB 7.2 (MathWorks, Natick, MA). Equation (5) provides the microbial growth rate up to the optimum growth temperature for any temperature change (dynamic model). Error estimates on the parameters were obtained from the curve-fitting software MicroFit\textsuperscript{a} Version 1.0 (Institute of Food Research, UK, 2011, p. 21) by consideration of the Jacobian matrix.

3. Results and discussion

In most cases, the growth curves for \( S.\) Typhimurium LT2 or \( E.\) coli cocktail inoculated on baby spinach leaves showed little lag time. This finding is similar to the findings of Pan and Schaffner (2010), who reported that \( S.\) Enteritidis serotypes growing in fresh-cut tomatoes have no lag time when cultured at temperatures between 10 and 35 \(^\circ\)C. The parameter \( h_s \) describes the potential growth of inocula in a medium (Baranyi & Roberts, 1994). The calculated \( h_s \) values for \( S.\) Typhimurium LT2 or \( E.\) coli in this study were 0.5 and 1.2, respectively, close to the values of 1.00 and 1.31 found for \( S.\) Enteritidis and \( E.\) coli O157:H7, respectively, in iceberg lettuce (Koseki & Isobe, 2005). Similar results were found for \( S.\) Typhimurium in fresh eggs (0.72–1.93) for a temperature range of 10–43 \(^\circ\)C (Gumudavelli, Subbiah, Thippareddi, Velugoti, & Froning, 2007). The average values for the estimated \( h_s \) for \( S.\) Typhimurium LT2 and for \( E.\) coli were 3.6 log CFU/g and 2.3 log CFU/g, respectively. Although we were careful in maintaining a constant initial inoculum level between the two replicates, a small difference in these values was observed for all temperatures, as explained by Gumudavelli et al. (2007).

Table 1 presents the estimated maximum growth rate and the maximum cell concentration values using the Baranyi model (Eqs. (1) and (2)) for growth of \( S.\) Typhimurium LT2 or \( E.\) coli in baby spinach leaves over the temperatures range of 10–37 \(^\circ\)C. In general, the growth rate of \( S.\) Typhimurium LT2 was slower than that of the \( E.\) coli cocktail. At 10–30 \(^\circ\)C, the \( E.\) coli strains grew significantly more (~2–4 log cycles) than the \( S.\) Enteritidis strain (~0.11–2.4 log cycles) while at 37 \(^\circ\)C both populations of bacteria increased by ~6 log cycles for 30 h. The RMSE values ranged from 0.01 to 0.12 log CFU/g for \( S.\) Typhimurium LT2, and from 0.11 to 0.22 log CFU/g for \( E.\) coli. The \( R^2 \) values were greater than 0.85 for all temperatures and microorganisms, with exception for \( E.\) coli (0.78) and \( S.\) Typhimurium LT2 (0.60) at 20 \(^\circ\)C. Hence, the Baranyi model (Eqs. (1) and (2)) predicted the growth rate of \( S.\) Typhimurium LT2 and the \( E.\) coli cocktail in baby spinach leaves.

For \( S.\) Typhimurium LT2, the maximum cell concentrations, \( y_{\text{max}} \), ranged from 3.71 to 8.85 log CFU/g as temperature increased from 10 to 37 \(^\circ\)C, respectively (Table 1). For the \( E.\) coli cocktail, those values varied from 4.38 to 8.28 log CFU/g. Koseki and Isobe (2005) and Singh et al. (2011) found similar \( y_{\text{max}} \) values for temperatures between 10 and 25 \(^\circ\)C. On the other hand, Gumudavelli et al. (2007) reported that the maximum cell concentration for \( S.\) Enteritidis growth in egg yolk was constant (about 8.65 log CFU/g) for temperatures ranging from 10 to 43 \(^\circ\)C with the initial inoculums of 2.5 log CFU/g. The discrepancy with our results may be due to the different types of microorganisms and food substrates used. Proliferation of microorganisms is primarily influenced by temperature (Baranyi & Roberts, 1994) when enough nutrients are available (Aruscavage, Lee, Miller, & Lejeune, 2006). In a competitive situation, when only a limited amount of nutrients is available, the maximum cell concentration seems to be influenced by other factors. An organism that grows quickly, presents a competitive advantage, establishing dominance when nutrient levels are high or it is able to grow when there are few nutrients remaining. Additionally, competitors who have the ability to produce antimicrobial compounds have a competitive advantage (Aruscavage et al., 2006; Schuenzel & Harrison, 2002).

Fig. 1 shows the effect of temperature on the maximum growth rate, \( \mu_{\text{max}} \), for \( S.\) Typhimurium LT2 and \( E.\) coli in baby spinach leaves calculated using Eq. (5) with the coefficients shown in Table 2. The theoretical minimum \( T_{\text{min}} \) temperatures beyond which growth is not possible, for \( S.\) Typhimurium LT2 and \( E.\) coli inoculated in baby spinach were 5.88 \(^\circ\)C and 4.76 \(^\circ\)C, respectively. These values are within the range of those found by others. For instance, experimental minimum growth temperatures for different \( S.\) Enteritidis serotypes inoculated on the surface of agar were reported to vary from 5.5 to 6.8 \(^\circ\)C; with a value of 6.2 \(^\circ\)C for \( S.\) Typhimurium LT2 (Matches & Liston, 1968). Theoretical \( T_{\text{min}} \) values varied from 4.1 \(^\circ\)C for a \( S.\) Enteritidis cocktail (Typhimurium, Newport, Javiana, and Braenderup) inoculated in fresh red tomato cuts (Pan & Schaffner, 2010), 4.96 \(^\circ\)C for \( S.\) Enteritidis spp in iceberg lettuce (Koseki &

Table 1

<table>
<thead>
<tr>
<th>Temperature [(^\circ)C]</th>
<th>( y_{\text{max}} ) [log CFU/g]</th>
<th>( \mu_{\text{max}} ) [log CFU/g/h]</th>
<th>RMSE</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3.71 ± 0.11*</td>
<td>0.01</td>
<td>0.01</td>
<td>0.99</td>
</tr>
<tr>
<td>20</td>
<td>4.31 ± 0.05</td>
<td>0.04</td>
<td>0.04</td>
<td>0.60</td>
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<tr>
<td>30</td>
<td>6.10 ± 0.68</td>
<td>0.49</td>
<td>0.06</td>
<td>0.88</td>
</tr>
<tr>
<td>37</td>
<td>8.85 ± 0.12</td>
<td>0.35</td>
<td>0.12</td>
<td>0.97</td>
</tr>
</tbody>
</table>

\* Error estimates were obtained using MicroFit\textsuperscript{a} (IFR, 2011).
Isobe, 2005) to 6.13 °C for S. Enteritidis in shell eggs (Gumudavelli et al., 2007). Theoretical $T_{\text{min}}$ for $E. coli$ growth in iceberg lettuce was 4.54 °C (Koseki & Isobe, 2005).

The maximum growth rate, $\mu_{\text{max}}$, ranged from 0.01 to 0.6 log CFU/g/h for the $E. coli$ cocktail and from 0.01 to 0.4 log CFU/g/h for the $S. Typhimurium$ LT2 at 10–37 °C (Table 1). These values are similar to those found by Koseki and Isobe (2005) for $E. coli$ O157:H7 and Salmonella spp. on the surface of iceberg lettuce (0.02–0.44 log CFU/g/h) at 10–25 °C.

A second-order polynomial was used to predict the values of $y_{\text{max}}$ as a function of temperature:

$$y_{\text{max}} = A_1 T^2 + A_2 T + A_3$$  \[6\]

where the values of the coefficients $A_1$ to $A_3$ for each microorganism are presented in Table 2.

A dynamic model was built by incorporating Eqs. (5) and (6) into the Baranyi model (Eqs. (1) and (2)) to predict the growth of both microorganisms at different temperatures. The value of $y_0$ was fixed to 3.6 log CFU/g for $S. Typhimurium$ LT2 and 2.3 log CFU/g for $E. coli$ to compare with experimental values. Unlike egg yolk (Gumudavelli et al., 2007), where the $y_{\text{max}}$ value was constant, the value of $y_{\text{max}}$ for baby spinach increased as the temperature increased (Fig. 1).

Figs. 2 and 3 show the results from predicting the growth rate data with the dynamic models (Eqs. (5) and (6)). We should note that the traditional microbial plating method has an imprecision of 0.5 log CFU/g. For the 30 °C data set for Salmonella, the dynamic model tends to over-predict the experimental data because the maximum growth rate, $\mu_{\text{max}}$, was over-estimated (Fig. 1). Inclusion of growth data at 25 and 35 °C into the model would improve its accuracy. The $R^2$ values showed the same trend with all the values greater than 0.86, except at 30 °C (0.78) for $S. Typhimurium$ LT2. In the case of $E. coli$, the dynamic model fitted the data well, with RMSE values between 0.12 and 0.28 and $R^2$ values larger than 0.92 (data not shown).

### 3.1. Model validation

Although a slow cooling procedure was used in this study, low growth was observed in both types of microorganisms. Fig. 4 shows that the dynamic model predicted well the growth of both microorganisms during the linear cooling scenario, showing that $E. coli$ and $S. Typhimurium$ LT2 populations increased only by 0.46 log CFU/g and 0.42 log CFU/g, respectively. However, $E. coli$ spp. showed higher growth compared to $S. Typhimurium$ LT2. A similar result was observed on the growth of $E. coli$ O157:H7 compared to Salmonella Enteritidis on the surface of iceberg lettuce (Koseki & Isobe, 2005). A slower cooling process would be even more unacceptable to control the growth of these microorganisms in baby spinach leaves.

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### Table 2

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>$\mu_{\text{max}}$</th>
<th>$\sigma$</th>
<th>$T_{\text{min}}$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S. Typhimurium$</td>
<td>0.002396</td>
<td>0.000296</td>
<td>5.88</td>
<td>0.93</td>
</tr>
<tr>
<td>$E. coli$</td>
<td>0.00605</td>
<td>0.000605</td>
<td>4.76</td>
<td>0.97</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>$y_{\text{max}}$</th>
<th>$A_1$</th>
<th>$A_2$</th>
<th>$A_3$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S. Typhimurium$</td>
<td>0.0090</td>
<td>-0.2369</td>
<td>5.2411</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>$E. coli$</td>
<td>0.0075</td>
<td>-0.2059</td>
<td>5.6769</td>
<td>0.99</td>
<td></td>
</tr>
</tbody>
</table>

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![Fig. 1. Maximum growth rate ($\mu_{\text{max}}$) and maximum concentration ($y_{\text{max}}$) for Salmonella Typhimurium LT2 and $E. coli$ on baby spinach as a function of temperature.](image)
Fig. 2. Prediction of growth rate of *E. coli* at different temperatures using the dynamic model.

Fig. 3. Predicted growth rate of *Salmonella Typhimurium* LT2 at different temperatures using the dynamic model.
leaves were laid on the shade without cooling (harvesting is crucial to ensure microbial safety. If the baby spinach stationary phase. Hence, faster cooling of baby spinach leaves after decreased the growth rate of both microorganisms, close to the retical minimum temperature was estimated as 5.88 °C and 4.76 °C for the Baranyi model. The effect of temperature on the square root of C14 experiment isothermal growth data for both microorganisms followed the Baranyi model. The effect of temperature on the square root of the growth rate was described as a linear function and the theoretical minimum temperature was estimated at 5.88 °C and 4.76 °C for S. Typhimurium LT2 and E. coli, respectively. The maximum population increased with temperature and the value was similar for both bacteria.

The dynamic model adequately describes the growth behavior of S. Typhimurium LT2 and E. coli in baby spinach leaves as a function of time and temperature. The model was validated with data for a linear cooling profile, showing that baby spinach leaves should be rapidly cooled down to 5 °C to reduce the growth rate of both microorganisms to the stationary phase.

The data reported in this study could be used to evaluate the impact of cooling practices at the processing facility, during handling or distribution of fresh produce, on the growth of S. Typhimurium LT2 and E. coli. Furthermore, the predictive model could be incorporated into a risk assessment model to enhance the ability to evaluate the growth of pathogens in baby spinach processing and distribution.

4. Conclusions

These results clearly show that cooling the spinach leaves to 5 °C decreased the growth rate of both microorganisms, close to the stationary phase. Hence, faster cooling of baby spinach leaves after harvesting is crucial to ensure microbial safety. If the baby spinach leaves were laid on the shade without cooling (T ~ 20 °C), after 12 h, the microorganism would still be in the exponential phase (Fig. 5). In this scenario, intervention strategies such as washing with chlorinated water or irradiation treatments, should be implemented (Moreira, Klutke, Castell-Perez, Puerta-Gomez, & Kim, 2011).

Fig. 4. Validation of the predictive model to describe the experimental data of S. Typhimurium LT2 and E. coli population growth in function of a linear cooling profile (water cooling).

Fig. 5. Simulation of E. coli and Salmonella Typhimurium LT2 population growth in baby spinach leaves under a linear cooling profile (water cooling) with final temperature equal to 20 °C.

4. Conclusions

The results of this study illustrate that the growth rate of S. Typhimurium LT2 was slower (T > 10 °C) than that of E. coli in baby spinach leaves for the temperature range of 10–37 °C. The experimental isothermal growth data for both microorganisms followed the Baranyi model. The effect of temperature on the square root of the growth rate was described as a linear function and the theoretical minimum temperature was estimated as 5.88 °C and 4.76 °C for S. Typhimurium LT2 and E. coli, respectively. The maximum population increased with temperature and the value was similar for both bacteria.

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