Modeling of the Competitive Growth of *Listeria monocytogenes* and *Lactococcus lactis* in Vegetable Broth

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Current mathematical models used by food microbiologists do not address the issue of competitive growth in mixed cultures of bacteria. We developed a mathematical model which consists of a system of nonlinear differential equations describing the growth of competing bacterial cell cultures. In this model, bacterial cell growth is limited by the accumulation of protonated lactic acid and decreasing pH. In our experimental system, pure and mixed cultures of *Lactococcus lactis* and *Listeria monocytogenes* were grown in a vegetable broth medium. Predictions of the model indicate that pH is the primary factor that limits the growth of *L. monocytogenes* in competition with a strain of *L. lactis* which does not produce the bacteriocin nisin. The model also predicts the values of parameters that affect the growth and death of the competing populations. Further development of this model will incorporate the effects of additional inhibitors, such as bacteriocins, and may aid in the selection of lactic acid bacterium cultures for use in competitive inhibition of pathogens in minimally processed foods.

The presence of pathogenic microorganisms on minimally processed refrigerated (MPR) vegetable products and the ability of these microorganisms to grow during storage have been documented (6, 25, 30, 33, 41, 43). Current trends are to extend the shelf life of MPR vegetable products by reducing the microbial load through washing or sanitizing procedures, modified-atmosphere packaging, and other methods (1, 5, 6, 17, 37). Development of these technologies has raised some concerns about how the microbial ecology of the products may be affected, and questions concerning the potential for growth of pathogens (17, 21, 23, 25, 43) have arisen. Jay (26) has argued that the success of sanitation procedures used to eliminate pathogenic bacteria from foods may have encouraged the emergence of *Listeria monocytogenes*, *Escherichia coli* O157: H7, and other organisms as food-borne pathogens by reducing the competitive microorganism populations.

The use of competitive microflora to enhance the safety of MPR products has been proposed by a number of authors (reviewed in references 20, 24, and 44). It has been suggested that lactic acid bacteria (LAB) could be used for this, in part because of their “generally regarded as safe” (GRAS) status and because they are commonly used in food fermentations. LAB species in refrigerated food products can produce a variety of metabolites, such as lactic and acetic acids (which lower the pH), hydrogen peroxide, bacteriocins, etc., which are inhibitory to competing bacteria in foods, including psychrotrophic pathogens (15, 28, 36, 49). The safety of traditional fermented products has not been questioned, and the objective of using biocontrol cultures is not to ferment foods but to control microbial ecology if spoilage does occur. An example of the use of LAB biocontrol cultures is the Wisconsin process for ensuring the safety of bacon (45, 46). Recent studies of this type have included the use of protective cultures in a variety of refrigerated meat (4, 14, 40, 53) and vegetable (10, 38, 50, 51) products. While these studies have shown that the use of LAB as competitive cultures may be effective in preventing the growth of pathogens in foods, a detailed investigation into the mechanisms by which this competitive inhibition occurs has not been carried out.

We chose a modeling approach to examine the dynamic nature of the interference type of competition or amensalism, in which one bacterial culture inhibits the growth of another (and itself as well) by producing inhibitory metabolites. To our knowledge, no models of this type have been described previously. This type of bacterial competition is associated with biocontrol applications in foods, as well as food fermentations or spoilage, where there is usually an excess of nutrients. While models for other types of competition between species have been described, including parasitism, predation, competition for nutrients, etc. (reviewed in references 16 and 18), the mathematics and ecology literature on amensalism is very limited. Frederickson (18) concluded that “amensalism, interference-type competition, and indirect parasitism should be studied both mathematically and experimentally, since the sum total of quantitative knowledge concerning these interactions is near zero.” A long-term goal of this research is to develop a theoretical foundation for the use of biocontrol cultures in foods by determining the factors important in the predominance of biocontrol bacteria over pathogenic microorganisms.

A number of models have been developed to predict the growth of bacteria in foods (for reviews see references 3, 35, 42, and 54). Several common types of growth models, including the logistic, Gompertz, and Richards curves, have been shown to be special cases of a more general model (35, 47, 48). These models may be classified as empirical models; they describe sigmoidal functions that approximate bacterial growth curves of cell concentration versus time. A modified Gompertz curve (9, 19, 54), which may be used to predict the logarithm of cell concentration over time, has been found to most closely approximate bacterial growth (54). It has been argued, however, that the usefulness of empirical models is limited and that a more fundamental understanding of the changes that take place during batch growth of bacteria will require the use of mechanistic models (2, 34, 52). Mechanistic models may be developed from theoretical or experimentally determined data.

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describing the cause or mechanism behind the dynamic changes observed in an experimental system. Our model may be classified as partially mechanistic, based on our use of organic acid and pH as variables that affect the growth and death of the competing cultures. As our understanding of how these factors affect bacterial growth increases, we may approach our goal of a fully mechanistic model.

Our primary model system consists of an LAB, Lactococcus lactis subsp. lactis NCK401, in competition with a pathogen, L. monocytogenes F5069B, in a vegetable broth extract. In this system, lactic acid is the main inhibitory compound that affects the growth of the competing bacteria. Both of these organisms carry out homolactic fermentation. The inhibitory properties of organic acids, such as lactic acid, have been attributed to the protonation of the acids, which are not included and therefore cross biological membranes. The resulting inhibition of growth may be due to the acidification of the cytoplasm and/or accumulation of acid anions inside the cell (39). In general, LAB are much more resistant to low pH values than other bacteria are. McDonald et al. (31) found that the low-limiting internal pH of selected LAB correlated with the ability of these organisms to survive in vegetable fermentations. Important criteria for choosing LAB for use as biocontrol cultures should, therefore, include such factors as protonated acid sensitivity, pH sensitivity, and acid production rate. By incorporating these factors as parameters into our model, we were able to determine estimated values for these parameters and to gain insight into their relative importance in the competitive growth process.

MATERIALS AND METHODS

Bacterial strains and media. Strain LA221 (NCK403 transformed with pGK12 [see below]), a non-nisin-producing derivative (22), was obtained from the USDA Food Fermentation Lab culture collection (Raleigh, N.C.). L. monocytogenes B164 (F5069, serotype 4b, transformed with pGKE [see below]) was obtained from C. Donnelly of the University of Vermont. Plasmids pGKC and pGKE were derivatives (6a) of pGK12 (27) and carried the genes encoding either chloramphenicol resistance (pGKC) or erythromycin resistance (pGKE). LA221 was transformed with pGKC by electroporation by using a modification of the method of Luchansky et al. (29), as described by Breidt and Fleming (7). L. monocytogenes B164 was similarly transformed with pGKE by Ronick (30). Both plasmids were determined to have stably transformed the bacteria (6b, 38). L. lactis LA221 was grown on M17 (Difco Laboratories, Detroit, Mich.) broth containing 1.5% agar (Difco) and 1% glucose (Sigma Chemical Co., St. Louis, Mo.) for plate media. L. monocytogenes F5069 was grown on tryptophan-free Todd-Hewitt agar (TSA) (Difco) supplemented with 1% glucose (Sigma). To select for antibiotic-resistant strains, chloramphenicol (M17-agar-glace) or erythromycin (TSA-glucose-agar) was added at a concentration of 5 μg/ml. Cucumber juice (CJ) medium containing 60% cucumber juice in water supplemented with 2% NaCl was prepared as described by Daeschel et al. (12).

Measurement of bacterial growth kinetics. Bacterial growth rates were determined by using a microtiter plate reader, as described by Breidt et al. (8). Cells were grown in 200-μl fermentation volumes in a temperature-controlled microtiter plate reader (model EL312; Bio-Tek Instruments, Inc., Winooski, Vt.) placed inside a heating-cooling incubator (Amhii-Hi-Low Chamber; Lab-Line Instruments Inc., Melrose Park, Ill.). Incubation of the microtiter plate reader in the environment chamber allowed the microtiter plates to be incubated at constant temperatures above or below room temperature, as indicated below. The 200-μl culture broth preparations were overlaid with mineral oil to prevent evaporation during extended incubation. The microtiter plate reader was controlled with KineticBase software, version 2.01 (Bio-Tek), which allowed optical density readings to be taken every 1.5 h for up to 99 h. The resulting ASCII text data file was processed by using Regress software (8). In the competitive growth experiments, bacterial cell counts were determined by using a spiral plater (Autoplate 3000; Spiral Biotech, Inc., Bethesda, Md.) and a colony counter (Protos Plus; Bioscience International, Rockville, Md.).

Biological assays. High-performance liquid chromatography (HPLC) analyses of organic acids and sugars were carried out by using the single-injection method of Martin et al. (24). An HPLC with an HPLC-3025 pump, a Shimadzu SPD-10AV UV detector, a Shimadzu C18 (5 μm) heptfluorobutyric acid (Aldrich Chemical Co., Inc., Milwaukee, Wis.) as the mobile phase. Organic acids were detected with a conductivity detector (model CDM-2; Dionex Corp., Sunnyvale, Calif.), and sugars were detected in-line following NaOH addition with a pulsed amperometric detector (model PAD-2; Dionex). Data were collected by using Chrom Perfect software (Justice Innova-
Dickinson and Co., Franklin Lakes, N.J.) that contained no additive. The tubes were incubated at 10°C in a heating-cooling water bath (MGW Lauda model RC2; Brinkmann Instrument Co., Westbury, N.Y.). Samples were obtained from the tubes (after mixing to ensure that the cells were evenly suspended) at different times by aseptically removing 1-ml portions with a syringe. Each 1-ml sample was used to determine the number of CFU per milliliter by diluting it as needed and plating it onto antibiotic-containing media with the spiral plater. The remaining sample was frozen at $-20^\circ$C and saved for use in pH and HPLC analyses. Plates were incubated at 30°C for 48 h, and the number of CFU per milliliter in each sample was determined with the automated plate counter (as described above).

**RESULTS**

**Dynamic growth model.** To characterize the potential of *L. lactis* as a biocontrol culture, competitive growth studies were carried out. The ability of *L. lactis* LA221 to inhibit the growth of *L. monocytogenes* F5069B in a mixed culture was investigated at 10°C. This temperature was chosen as an abuse temperature, like the temperatures that may occur when there is improper refrigeration of minimally processed foods. To understand the factors that allow one culture to predominate over another, we developed a model that incorporated the variables that directly affected the growth of each organism in the mixed culture (see Appendix A). The rate equations in the model were similar in form to the logistic equation for bacterial growth (16). Because *L. lactis* LA221 (an organism that does not produce nisin) and *L. monocytogenes* both carry out homolactic fermentation of glucose, the primary regulators of growth were assumed to be (protonated) lactic acid and the low pH of the medium during growth of these bacteria. Malic...
acid concentration was included as a variable in the model because our CJ medium contained malate (concentration, approximately 8 mM), which is naturally found in cucumbers. *L. lactis* ferments malate via a malolactic enzyme (11), which raises the pH of the medium and affects the growth of the cells.

The cell growth functions in the model allowed for separate parameters controlling the inhibition of growth (for example *k*₃ and *k*₅) and metabolism (*k*₄ and *k*₆). This is because the bacteria can continue to metabolize and produce lactic acid during the stationary phase when (we assume) growth has ceased, as measured by the number of CFU per milliliter. At some point, however, the metabolism of the microorganisms can no longer be maintained as the protonated acid concentration increases and the number of CFU declines. The lag phase was modeled in the computer simulation (data not shown) as a Heaviside function, which forced the specific growth rate to zero for the duration of this phase. For this model, protonated lactic acid and pH were assumed to be the only effectors of growth. Both *L. monocytogenes* and *L. lactis* produce lactic acid by homolactic fermentation. The *L. lactis* strain did not produce nisin. Further development of the model will include the effects of the bacteriocin nisin and possibly additional inhibitors of growth, such as hydrogen peroxide, which may be produced by LAB.

**Mixed-culture growth experiments.** Figure 1 shows the observed and predicted results for growth of *L. lactis* and *L. monocytogenes* in pure culture, respectively. In the mixed-culture experiments we used different ratios of initial cell concentrations for the mixed cultures; the ratios of *L. lactis* in competition with *L. monocytogenes* were 10⁶:10⁶ (Fig. 1B), 10⁷:10⁷ (Fig. 1C), and 10⁸:10⁷ (Fig. 1D). The mean pseudo-R² values for growth both separately and in mixed culture were 0.940 (Fig. 1A), 0.922 (Fig. 1B), 0.896 (Fig. 1C), 0.832 (Fig. 1D), and 0.929 (Fig. 1E). The malic acid data was not used for the R² calculation for the data shown in Fig. 1E because the predicted values did not change. Figure 1D shows that the *L. lactis* culture was inhibited by *L. monocytogenes* to a greater extent than predicted. This could have been due to some inhibitory effect of the *L. monocytogenes* culture not included in the model.

**Determination of parameter values.** Table 1 shows the parameter estimates obtained with the model. To determine if the parameter values used in the model accurately reflected the parameter values for the bacterial cells, independent measurements were made for selected model parameters. Figure 2 shows the lowest pH value, pH 4.68, that allowed growth of *L. monocytogenes* in buffered CJ medium. The ionic strength of CJ medium was kept constant at 0.342, as described above. Figure 3 shows that the MIC of protonated lactic acid was 6.43 mM for *L. monocytogenes* in CJ medium when the ionic strength was 0.342 and the pH was kept constant at 5.6. Figure 4 shows similar data for the protonated acid MIC for *L. lactis*, which was found to be 5.3 mM. In addition, the specific growth rates for *L. lactis* (0.0932 h⁻¹) and *L. monocytogenes* (0.1011 h⁻¹) were measured independently in pure culture, and the resulting data, along with a summary of observed and estimated values from the model, are shown in Table 2. The parameter values for the inhibition of growth of *L. monocytogenes* by protonated acid were not accurately predicted by the model because in all cases, pH was found to be the limiting factor for growth for both mixed-culture growth and growth of *L. monocytogenes* in pure culture (as shown in Fig. 1B through E). Because regulators of pH and protonated acid were modeled as independent regulators of growth, only the most limiting of these factors can be predicted. While the pure-culture system can be modeled by using protonated acid as the sole growth-limiting factor (by changing the parameter values), the parameters used in this case do not allow the model to accurately predict the outcome of the competitive growth experi-

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol(s)</th>
<th>Units</th>
<th>Estimated values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific growth rate</td>
<td>α, β</td>
<td>Hour⁻¹</td>
<td>0.1049</td>
</tr>
<tr>
<td>Protonated acid production rate</td>
<td>γ, δ</td>
<td>Millimoles CFU⁻¹ hour⁻¹</td>
<td>1.7 × 10⁻¹⁰ 2.95 × 10⁻¹⁰</td>
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<tr>
<td>MIC (growth) of protonated acid</td>
<td>k₆, k₇</td>
<td>Millimolar</td>
<td>5.2</td>
</tr>
<tr>
<td>MIC (metabolism) of protonated acid</td>
<td>k₈, k₉</td>
<td>Millimolar</td>
<td>8.907</td>
</tr>
<tr>
<td>Maximum protonated acid concn</td>
<td>k₃, k₄</td>
<td>Millimolar</td>
<td>11.5</td>
</tr>
<tr>
<td>MIC (growth) of pH</td>
<td>k₁₀, k₉</td>
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<tr>
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<td>pH units</td>
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<tr>
<td>Minimum pH</td>
<td></td>
<td></td>
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<tr>
<td>Malate utilization rate</td>
<td>k</td>
<td>Millimoles CFU⁻¹ hour⁻¹</td>
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<tr>
<td>Buffering due to malate utilization</td>
<td>θ</td>
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<td>−5.33</td>
</tr>
<tr>
<td>Proton concentration change rate</td>
<td>ρ</td>
<td>Moles CFU⁻¹ hour⁻¹</td>
<td>−5.472</td>
</tr>
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</table>

- a For mixed cultures, this parameter takes the lowest pH value of the two listed parameter values.
- b These values were estimates only; growth was apparently controlled by pH, as described in the text.
The mean values from five independent determinations of growth rate for each concentration of protonated acid are shown (C). The regression line for the entire data set (solid line) and the 95% confidence limits (dashed lines) for the regression line (dashed lines) are also shown.

FIG. 3. Limiting protonated acid concentration for the growth of L. monocytogenes. The mean values from five independent determinations of growth rate for each concentration of protonated acid are shown (C). The regression line for the entire data set (solid line) and the 95% confidence limits (dashed lines) for the regression line (dashed lines) are also shown.

DISCUSSION

Traditional bacterial growth models in food microbiology had the advantage of simplicity, and explicit solutions of the equations were possible. However, to understand the dynamic changes in the competitive growth of bacteria, more complex models may be needed. We used a series of nonlinear differential equations, which cannot be solved unless numerical methods are used. The Runge-Kutta algorithm which we used for numerical integration is widely used and relatively simple to program (13a). When a numerical approach is used, fewer limiting assumptions need to be made, and a mechanistic model can be used. The primary difficulty lies in picking the parameter values that allow the numerical solution to fit the observed data. As the complexity of the model grows and a number of data sets which use different initial starting conditions are generated, this problem becomes more difficult. To identify parameters, we developed computer software to carry out the numerical integration and graphically display the observed and predicted results, which allowed repeated trials of different parameter sets. A random search of the parameter space was then employed to find the best fit of the parameter values to the data. This random walk method was chosen for reasons of computational simplicity and because a complete search of all possible parameter combinations for even a very limited set of values was not possible for the 21 parameters of the model. Use of conventional minimization programs was confounded by the difficulty of programming a minimization algorithm to call a complex C++ function consisting of numerical integration of the model, followed by calculation of the sum of squared errors for the observed and predicted data. Further refinement of the parameter estimation algorithm will be the subject of future research. The model was validated by independent measurements of selected parameter values and by comparison of observed and predicted results. Because the parameter values for the model represent physical properties of the L. lactis and L. monocytogenes cells, they can aid in understanding how the growth of the competing cultures was controlled.

The parameter values obtained for the L. lactis and L. monocytogenes cultures were, in general, similar to each other, except that the acid production rate for L. lactis was faster than that for L. monocytogenes and the L. monocytogenes culture was more sensitive to low pH than the L. lactis culture was (Table 1). It was observed that the growth and death of the L. monocytogenes culture could be accurately predicted by the model only if pH was assumed to be the limiting variable. In every case (Fig. 1), growth of the L. monocytogenes culture ceased before the protonated acid concentration reached the independently determine MIC. This suggests that pH was the primary factor limiting the growth of L. monocytogenes for all of the initial starting conditions used in the model. An effective biocontrol culture for L. monocytogenes may, therefore, be one that produces a small amount of acid quickly to lower the pH, and large amounts of organic acid may not be needed.

It is interesting to note that as shown in Fig. 1D, the L. lactis culture did not grow as much as expected based on the pre- 100 times smaller than the initial cell number of the target pathogen), this may indicate that the parameter values for the L. lactis culture are not optimized. An alternative explanation is that the L. monocytogenes culture produced some inhibitory metabolite not included in the model. Further research will include incorporating the effects of additional inhibitory metabolites of LAB, such as bacteriocins and hydrogen peroxide.

APPENDIX A

The model consists of a system of five differential equations with variables for the two cell types (Nv and Nc), the protonated acid concentration (C), the concentration of hydrogen ions (P), and the malate concentration (M). Malate was included because L. lactis LA221 fer-
ments malate by means of the malolactic enzyme, which raises the pH. The parameters are defined in Table 1.

\[
dN_i/dt = g_i(C, P, \alpha)N_i
\]  
(A1)

\[
dN_j/dt = g_j(C, P, \beta)N_j
\]  
(A2)

\[
dC/dt = \gamma[N_i(1-C/kp_j)] + [8N_i(1-C/kp_j)]
\]  
(A3)

\[
dP/dt = \rho(1-P/kp_i) - \kappa(dM/dt)
\]  
(A4)

\[
dM/dt = -\delta N M
\]  
(A5)

with

\[
g_i(C, P, \alpha) = \alpha \times \min \left\{ \left[ 1 - C/H(C_i) \right], \left[ 1 - P/H(P_i) \right] \right\}
\]

\[
g_j(C, P, \beta) = \beta \times \min \left\{ \left[ 1 - C/H(C_i) \right], \left[ 1 - P/H(P_i) \right] \right\}
\]

The growth functions \(g_1\) and \(g_2\) modeled the inhibitory effects of protonated lactic acid or pH independently. This assumption was based on the work of Passot et al. (34), who modeled the growth of LAB in cucumber fermentations and found that the effects of pH, protonated lactic and acetic acids, and NaCl concentration could be modeled independently. The growth rate was modified by the minimum (min) value for a growth-limiting function. The functions \(H_i(C)\) and \(H_j(P)\) are discontinuous forcing functions (Heaviside functions) of the protonated acid and free hydrogen ion concentrations, respectively:

\[
H_i(C) = kp_i^*H(kp_i - C) + C^*H(C - kp_i)^*H(kp_i - C) + P^*H(P - kp_i)
\]

\[
H_j(P) = kp_j^*H(kp_j - P) + P^*H(P - kp_j)^*H(kp_j - C) + C^*H(C - kp_i)
\]

\[
H_i(C) = kp_i^*H(kp_i - C) + C^*H(C - kp_i)^*H(kp_i - C) + P^*H(P - kp_i)
\]

For \(H_1\) and \(H_2\), when \(C = kp_i\), \(C = kp_j\), \(P = kp_j\), or \(P = kp_i\), the value of the parameter was returned (similarly for \(H_1\) and \(H_2\)). For any other value of \(C\), the function is calculated as shown.

**APPENDIX B**

For numerical integration, a Runge-Kutta single-step fourth-order method was used. The simulation program was based on the general algorithm (reviewed in reference 13):

\[
f_0 = f(x_0, y_0)
\]

\[
f_1 = f(x_0 + (1/2)y_0, y_0 + (1/2)f_0)
\]

\[
f_2 = f(x_0 + (1/2)y_0, y_0 + (1/2)f_1)
\]

\[
f_3 = f(x_0 + y_0, y_0 + f_1)
\]

\[
y(x_0 + h) = y_0 + (1/6)(f_0 + 2f_2 + 2f_3 + f_1)
\]

The simulation program is available electronically. For information see http://www4.ncsu.edu/unity/users/fac/brceed/web/simwin.htm or contact the corresponding author.

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