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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 protonated forms of the acids, which are uncharged and may 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. Donnely 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 Romick (38). 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 medium, and *L. monocytogenes* F5069 was grown on tryptic soy agar (TSA) (Difco) supplemented with 1% glucose (Sigma). To select for antibiotic-resistant strains, chloramphenicol (M17-glucose agar) 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 (Ambi-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 KinetiCalc software, version 2.03 (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 McFeeters (32). An Aminex HPX-87H column was used along with 3 mM heptafluorobutyric 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 Innovations, Inc., Mountain View, Calif.) run on a 486/33 computer (Gateway2000, North Sioux City, S.D.). Protonated acid concentrations were calculated by using the Henderson-Hasselbach equation, based on the acid concentration and the pH of the medium. The pH values were determined by using a micro combination electrode (Accumet model 13-620-279; Fisher Scientific, Pittsburg, Pa.).

Statistics and programming. Predicted data and parameters for the nonlinear differential equation model (see Appendix A) were determined with simulation software written in C++ (Borland C++ for Windows, version 4.5; Borland International, Inc., Scotts Valley, Calif.) by using a 486/33 computer (Gateway2000). This simulation program runs under the Microsoft Windows 95 environment. It allows entry of model parameter values, carries out numerical integration, and then graphically displays the observed and predicted results. The algorithm used a fourth-order Runge-Kutta numerical integration method (see Appendix B). A constant step size of 0.05 on a time scale of 0 to 100 U was used; the rate parameters and time for the experiment were adjusted to this scale for calculations, but the values reported below were corrected to represent real time. The pH values were converted to free hydrogen ion concentrations for all calculations.

The initial parameter estimates were obtained by manual iterations of changing the parameters, calculating the predicted growth results, and viewing the predicted and experimental results with the simulation software. Further fitting of the five-equation model with the simulation program was based on minimizing the total sum of squared errors for the observed values minus the expected values (for all time points of observed and predicted data) for the variables in the model. To prevent the error term from being dominated by the high cell and hydrogen ion concentrations, the log of the cell concentration and pH values were used for this calculation. The error term was evaluated for a sequence of parameter values determined by using a random walk procedure, starting from the initial estimated parameter values. For each step in the random walk, the parameters were adjusted by a scaled increment, either increasing, decreasing, or not changing the current value, with equal probability. With the simulation program, user-selected parameter values and increments were used for the random walk. This allowed some parameters, such as those for specific growth rates or MICs, to be held constant, while other values were changed during the random walk. The least-squares function was then recalculated, and if the value decreased, the changes were accepted and the new values were used for the next step in the random walk. A goodness-of-fit value, similar to R^2 in linear regression, was also determined. For each variable in the model, this value was determined by using the equation 1 − (SSE/SST), where SSE (sum of squared errors) is the sum of squared errors as described above and SST (total sum of squares) is the sum of the squared deviations of the predicted values from the mean of the observed values. The mean of the five R^2 values for each set of variables in the model was determined for each set of initial starting conditions.

MIC determinations. MICs for the inhibition of growth by lactic acid were determined by measuring growth rates with different concentrations of acid in CJ broth medium. To determine the MICs for protonated acid, the pH and ionic strength of the medium were kept constant at 5.6 and 0.342 (equivalent to the ionic strength of 2% NaCl), respectively, while the concentration of protonated lactic acid was varied. The NaCl concentration was varied to maintain the constant ionic strength as the lactic acid anion concentration was increased. The contribution of malic acid ions (the major organic acid naturally present in CJ) to the ionic strength was included in the calculations to determine total ionic strength. The lactic acid used in these determinations was prepared from a concentrated stock solution (88% lactic acid; Sterling Chemicals, Inc., Texas City, Tex.). The 88% lactic acid solution was diluted 1:4 in deionized water. The diluted solution was then refluxed for approximately 16 h to hydrolyze lactic acid oligomers. A sample of the reflux solution was analyzed by HPLC by using an anion-exchange column (type HPX87-H; Bio-Rad Laboratories, Hercules, Calif.) at 75°C along with a refractive index detector (model 410; Waters Associates, Inc., Milford, Mass.). The eluent was 0.01 N sulfuric acid at a flow rate of 0.8 ml/min. By comparing the chromatograms obtained before and after refluxing, we determined that the solution was monomeric by the absence on the chromatogram of secondary peaks which were initially present.

To determine the minimum pH that allowed growth, the ionic strength was kept constant at 0.342, as described above, and 50 mM malic acid was added to increase the buffering capacity. Total acid concentrations were determined by HPLC as described above. The growth rates were determined by using the microtiter plate method described above and triplicate (or more) independent fermentations. For all MIC determinations, the regression equation and coefficient were determined from the entire data set, but only the mean values for the data are shown below. The intercept of the regression line (extrapolated to give a specific growth rate of zero) was used to determine the predicted MICs.

Competitive growth experiments. Cultures were prepared by growing cells overnight (for 16 h) at 30°C in CJ medium containing the appropriate antibiotic (chloramphenicol for LA221; erythromycin for B164) at a concentration of 5 μg/ml. The cells were harvested from these overnight cultures and resuspended in an equal volume of fresh CJ medium without antibiotics. The cells were diluted to the starting concentration by measuring the optical density at 600 nm (the preparations were diluted so that they were in the linear range of the spectrophotometer) and using a standard curve for optical density versus number of CFU per milliliter (data not shown). Twenty-milliliter portions of the cell suspensions containing mixed or pure cultures in CJ medium were injected aseptically through the septa of sterile Vacutainer tubes (16 by 165 mm; Becton-