Modelling the Microbial Quality and Safety of Foods

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Stellingen

1. De voorspellingen van kiemgetallen met het Gompertz-model op basis van variërende temperaturen als gedaan door Van Impe *et al.* (1995), geven een vertekend beeld van het verschil tussen dynamische en statische modellen.

   Van Impe, J.F. *et al.* 1995. Int. J. Food Microbiology **25**:227-249.

2. Optimaliteitscriteria voor de selectie van het beste mathematische model zouden naast de rest-variantie, het aantal parameters in het model en het aantal punten in de dataset, ook de intrinsieke niet-lineariteit van modellen mee moeten nemen.

   Hurvich, M., and Tsai, C.L. 1989. Biometrica **76**:297-307.

3. De term "predictive microbiology" zou veranderd moeten worden in "mathematical microbiology", omdat de ontwikkelde modellen mathematisch zijn en niet alleen bruikbaar voor voorspellingen (dit proefschrift).

4. Er is een fundamenteel verschil tussen validatie van modellen op basis van nieuw verzamelde data en validatie op basis van de dataset waarmee het model was ontwikkeld (dit proefschrift).

5. Parsimony is a non-parsimonious word for simplicity.

6. De huidige kennis van de mechanismen die groei en afsterving van micro-organismen bepalen maakt de tot dusver ontwikkelde modellen voor de voorspelling van groei en afsterving empirisch.

7. Voor het bepalen van groei-kinetische parameters met behulp van bacteriële groeicurves over een groot aantal log-schalen doet het aantal kolonies per plaat er niet zo veel toe, maar is het vooral belangrijk welke decimale verdunning er wordt geteld.

8. Gezien de toenemende reislust naar verre oorden is het in Nederland produceren en distribueren van kiemvrije levensmiddelen ongewenst voor de volksgezondheid.

9. Computerprogramma's geven vaak antwoord op vragen die je niet hebt gesteld.

10. Wanneer een computerprogramma uit eigener beweging kan promoveren zal er werkelijk sprake zijn van kunstmatige intelligentie.

11. De vraag hoe lang je met een fles perslucht onder water kunt blijven geeft aan dat de vrager de wet van Boyle (1662) niet heeft begrepen.

12. Het feit dat in Zeeland sportvissers wel en sportduikers geen vis mogen vangen, geeft duidelijk aan dat Gedeputeerde Staten van Zeeland vinden dat de ene vorm van recreatie de andere niet is.

Stellingen behorende bij het proefschrift "Modelling the microbial quality and safety of foods."

Taco Wijtzes
Gorinchem, 29 oktober 1996.

Aan Claudia, Pieter en Elly

Voorwoord

Veel van het werk dat in dit proefschrift beschreven is, is tot stand gekomen met medewerking van een groot aantal mensen. Ik wil graag alle studenten bedanken die geholpen hebben bij het verzamelen van data en het ontwikkelen van ideeën: Diederik Zwakenberg, Camiel Aalbers, Marty Boll, Erwin Heida, Anoesjka de Waard, Marc Bruggeman, Koen ten Hove, Linda Rutten, Viveta Kaliakatsou, Pradeep Malakar, Tamás Klenczner, Ingrid Derks en Gerhard Knol.

Ook de hulp van Jacora de Wit, Frans Rombouts, Marie Louise Kant Muermans, Ferry Stekelenburg, Anton van der Linden en Arjan Evers moet genoemd worden. Hun vakspecifieke kennis en adviezen tijdens de werkbesprekingen met TNO-Voeding en tijdens persoonlijke ontmoetingen zijn erg nuttig geweest. De door ons samen uitgedachte geesteskinderen zijn veelvuldig terug te vinden in dit proefschrift.

De directe begeleiding van mijn project was in handen van Klaas van 't Riet, Jos Huis in 't Veld en Marcel Zwietering. Zonder deze drie personen was er helemaal nooit een proefschrift gekomen. Gedurende alle fasen van het onderzoek hebben ze mij, soms met zachte, soms met harde hand proberen te sturen. Ik hoop dat het resultaat jullie meevalt.

Claudia, je moet op een gegeven moment helemaal gek zijn geworden van àl de dingen die tegelijk moesten. Het is aan jou te danken dat ik zèlf niet helemaal ben doorgedraaid. Je weet altijd weer dingen te bedenken waardoor mijn aandacht van het werk wordt afgeleid. Vooral je continue roep om Cocodrillo en vakantie is erg nuttig.

Als eerste wil ik natuurlijk de bewoners van kamer 720 bedanken, Ida Gunther, Rob van den Hout, Leonie Linders en Dirk Martens, en later Imke Leenen en Marian Vermue. Bedankt voor de discussies en de koffie. Het duurde wel erg lang voordat jullie mijn bureau durfden in te pikken. Daarnaast wil ik graag iedereen van de sectie Proceskunde bedanken. Jullie zijn een perfecte groep om in te werken.

Contents

Chapter 1

Models and modelling

ABSTRACT

Models are simplified descriptions of reality. The reality described by a model is only a part of entire reality, a so-called model space. This model space consists of all aware and unaware assumptions, all dependent and independent variables, relations between these, and all data gathered. Methods for choosing the appropriate model in the chosen model space are described, and worked out examples are given. Gathering data in the model space to equip models with parameter values usually results in an improved insight in the model space, therefore an initial model can be ameliorated. Setting up a model and gathering data is an iterative process. Different concepts for model validation are described. Furthermore, attention is given to the combination of models and different model spaces resulting in a model describing both model spaces. Finally, examples from this thesis are given by means of which the described ideas are illustrated.

INTRODUCTION

During our childhood we learned to understand the world by means of models. Model trains, model aeroplanes, and model cars are all models of reality. These models were useful since the real thing was too expensive, too big, too inaccessible or too dangerous. Simulations and models of situations appeared as games, such as playing cops and robbers. Adults create models for the same reasons. Scale model aeroplanes to assess flight behaviour in wind tunnels or scale models of river deltas to assess the water currents are examples of model building. All the mentioned models so far involve physical objects. The use of models can be extended if symbols instead of objects are used. By means of symbolic representations the location of high and low pressure fronts in weather can be calculated and the economic situation of a country assessed (1). Quality and safety of foods can also be predicted by means of models.

All models described in this thesis are symbolic representations of reality. The things that happen in reality are studied and models are created. Models should reflect what is happening and should be able to predict future and past states of the things they describe with adequate precision. It should be clear that models cannot give a full representation of reality. A particular model may describe some aspect very accurately whereas it completely fails to describe another. A toy car may look like a real car, but it will be impossible to sit in it, let alone drive it. Models are approximations of a very complex reality, and therefore are intrinsically erroneous (1, 4, 5).

The models developed in this thesis relate independent variables such as time, temperature, acidity, water activity to dependent variables such as bacterial numbers, growth rate and food composition. These models may vary from verbal (in words) to mathematical (with numbers). Complex problems usually have some solution that can only be attained through combination of verbal and mathematical models. Modelling quality of foods is a complex problem dealt with in this thesis.

In this chapter the rationale behind developing models and defining model systems is described. A system for the classification of models is given and worked out examples from different parts of this thesis are shown for clarification. The described system for classification provides for a basis to classify and combine the different models developed in this thesis. The rationale behind the development of the ideas in this chapter is that one starts without any idea about what to model, no ideas about any relations between any, yet unknown, dependent or independent variables. Before an actual model is developed, assumptions are needed to be able

to develop a model. The assumptions described are independent of any model or any model system. After describing the assumptions needed for developing models, attention is given to two ways of classifying models. After the assumptions and definitions are described, action can be undertaken to develop an understanding of relations between dependent and independent variables and an actual model can be developed. This is done by means of gathering data and validating models. Examples are provided for, but are only intended as illustrations rather than worked out models.

MODELLING ASSUMPTIONS

Model space. It is impossible to model everything, so a choice has to be made about the part of reality that is to be modelled. This part of reality is a sub-space of true reality. In modelling, it is assumed that this sub-space, model space from now on, has no connection to the rest of reality (Fig. 1) (1). Model space is defined as all factors that play a role in the determination of the phenomenon under study. This implies that both aware choices and unaware choices, made by the modeller, result in a model space. Some factors may play insignificant roles, but always are a part of the model space.

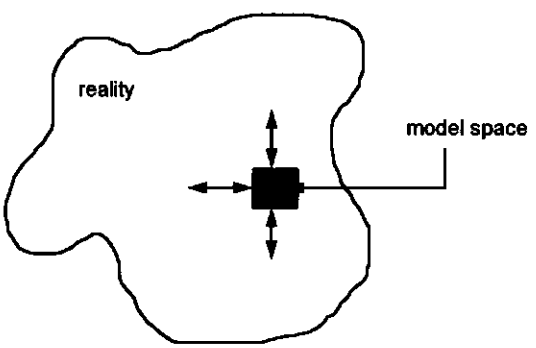

FIG. 1. Model space inside reality,
and the influence of the rest of reality.

Modelling bacterial growth as a function of acidity in a previously sterilised broth where only the amount of acid is thought to be of importance, can result in a model for bacterial growth as a function of initial pH. However, the composition of the broth may be affected by the production of acid by the microorganism, which may in its turn influence the growth characteristics of the microorganisms. The model, as mentioned, might not take this into

account, and is therefore not completely valid. If a model space is chosen well, the influence of other factors is small and may be neglected. However, since everything is connected, the choice of a model space implies automatically, that other factors, that can play a role in the determination of the model, may not be taken into account. Descriptions of chosen model spaces should always be present in materials and methods sections of scientific articles.

Phenomenon. Models are used to describe relationships between independent and dependent variables. The set of dependent and independent variables, and the relations between these, are called a phenomenon. To be able to model a phenomenon in a chosen model space, an understanding of the relations between the dependent and independent variables is needed. This understanding can be a result of experiments or logical thinking, both are described later. The understanding of the relations are the fundaments of a model. Understanding the relations between dependent and independent variables in a model space may help choosing the appropriate model. If a relation between independent and dependent variables can only be modelled with words, a model in words is most appropriate. If the model can be expressed in terms of mathematical equations, a mathematical model is most appropriate. A model should reflect the acquired understanding of the model space. This raises the issue of how well a phenomenon in a model space can be understood. Models are always interpretations of the phenomena in a model space, therefore models are always influenced by the ideas of the modeller.

Combining models. Models from related model spaces can be combined to obtain larger model spaces and wider applicability of the developed models. The combination of models from distinct model spaces can only be achieved if the combined model spaces touch or have some overlap. It is impossible to directly combine models from model spaces if they do not touch or overlap. In the case two model spaces A and B are combined ($A \cup B$), the resulting model space incorporates both model space A and model space B but is smaller than the sum of both model spaces with exactly the intersection of model space A and model space B, ($A \cap B$) (eq. 1).

$$A \cup B = A + B - (A \cap B) \tag{1}$$

Combining model spaces that do not touch or overlap, can be done by means of bridge model spaces. These bridges need to be set up carefully and have to have explicit meaning in both model spaces. If a bridge model space C is needed to combine two non-touching model

spaces A and B, the resulting model space can be represented symbolically by eq. 2. The intersection of A and B ($A \cap B$) does not exist.

$$A \cup B \cup C = A + B + C - (A \cap C) - (B \cap C) \quad (2)$$

MODEL CLASSIFICATION

Model verbality. Models can be divided in different classes ranging from purely mathematical to purely verbal. Mathematical models relate dependent variables to independent variables through explicit mathematical equations ($y_i = f(x_j)$). The influence of an independent variable on a dependent variable can be calculated by means of a mathematical equation. Verbal models relate the independent variables to the dependent variables through a description in words rather than mathematical equations. An example of a verbal model is a description of a tree. Other examples of verbal models can be found in many fields of research such as medicine or psychology, where classification takes place on generalised, verbal theorems. The mentioned model classes depict two extremes. Between these extremes, an infinite number of model classes can be distinguished. Several models in the range from verbal to mathematical will be described by means of examples from food microbiology.

A verbal model could be based on the observation from the past, that microorganisms grew on a particular food. The model would result in the prediction that the microorganism grows in the same type of product again. This type of model corresponds with growth or no growth for the dependent variable (Y-axis) whereas the independent variable (X-axis) is absent.

A less verbal model is based on the knowledge that microorganisms usually grow between refrigeration and hot temperatures. So the conclusion is that microorganisms can grow at room temperatures. Here, the Y-axis provides for two states again, growth or no growth and the X-axis is fuzzy (not explicitly numbered).

TABLE 1. Parameters and values for a match model

Microorganism	Food
T_{min} = 5 °C	
T_{opt} = 37 °C	T_{food} = 20 °C
T_{max} = 43 °C	

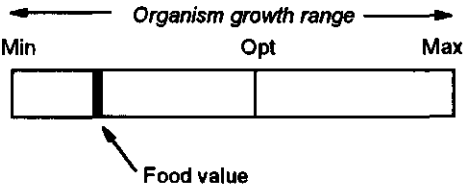

FIG. 2. Pattern matching to determine microbial growth at preset values for environmental parameters.

An even less verbal model is the following: A certain microorganism grows between a minimum temperature (T_{min}) and a maximum temperature (T_{max}) (Table 1). A food product has a specific temperature value, (T_{food}). If the value for T_{food} lies between T_{min} and T_{max}, the organism can grow on that food, if present. In this example, the X-axis is explicit, whereas the Y-axis only provides for two states, growth or no growth. This example of pattern matching is also shown in Fig. 2.

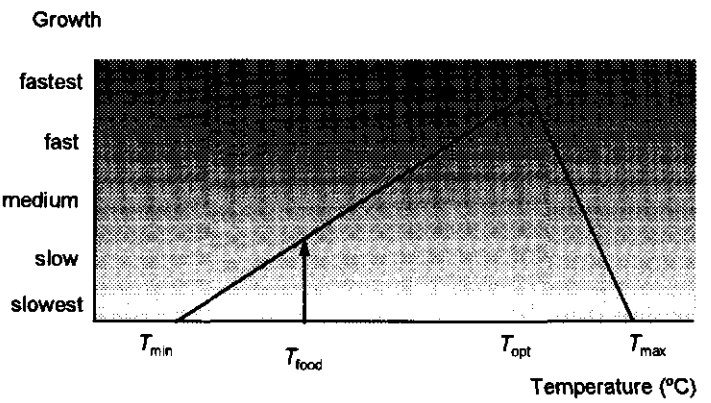

FIG. 3. Approximation of rate of increase of bacterial numbers using a fuzzy model with an explicit X-axis and a vague Y-axis.

A match model that would result in a more accurate estimate of the rate of increase of bacterial numbers is shown in Fig. 3. On the X-axis temperature is shown and on the Y-axis a qualitative representation of the rate of increase of biomass of a microorganism. Under optimal conditions (T=T_{opt}) the rate is fastest. If temperature becomes equal to the minimum or maximum (T_{min} and T_{max} respectively) the organism stops growing. This class of model gives more detailed values for growth rate of microorganisms, although these values are still qualitative. The model described here, can for instance be used for qualitative reasoning (i.e. a

microorganism grows faster than another). This model has an explicit X-axis and a fuzzy Y-axis. The previously described classes of models are also known as fuzzy models.

Finally, a mathematical equation relating temperature to bacterial growth rate (μ (h^{-1})) (Eq. 3) is described. T_{opt} is used to calculate one of the parameters (c) of the mathematical equation (3). The response curve is shown in Fig. 4. In this model both axes are explicit.

$$\sqrt{\mu} = b(T - T_{min}) \times \left\{1 - \exp\left[c(T - T_{max})\right]\right\} \quad (3)$$

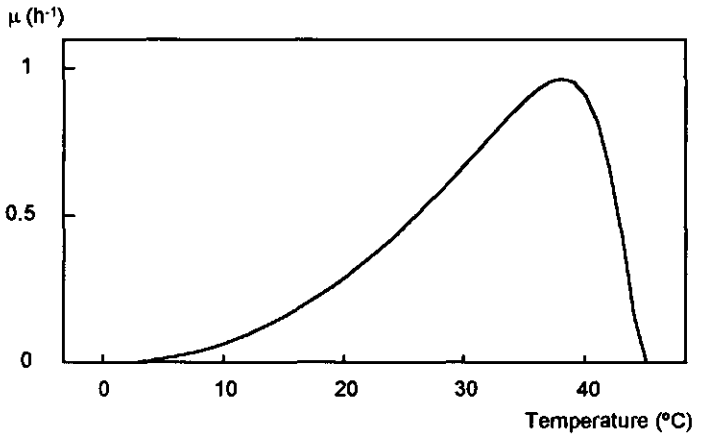

FIG. 4. Bacterial growth rate as a function of temperature.

Mathematical equations can be set up that mimic fuzzy, non-quantitative model spaces. This model class "upgrading" results in quantitative models and therefore quantitative predictions of a non-quantitative model space. The adequacy of a mathematical model in such a model space is doubtful. However, it may be useful to obtain quantitative predictions of such a model space, but the predictions should be used with great care. Mathematical equations that are useful for model class upgrading are polynomials and neural networks.

Underlying mechanisms. Some distinctions between models in general can be made, based on the underlying mechanisms of a model. Groups of models will be described as separate entities. In reality, these groups are characteristic spots in a continuum of model groups. The first group of models is based upon generally accepted, universal truths. This group of models is very small since, generally, reality cannot be fully understood. Models that may belong to this group are for instance the first and second law of thermodynamics, mass balances, impulse

balances, and definitions in mathematics. Models that also belong to this group are a model for an adequately functioning human hart, or the atomic structure of pure water (H_2O).

The second group of models is much larger. Models based on a good understanding of the model space can be placed here. Examples from this group are Newton's law for two attracting bodies, the Arrhenius equation for the effect of temperature on reaction rates of chemical reactions, or the development of microbial populations in time.

The last group of models consists of models that do not express any understanding of the phenomenon that is being studied. This group of models is also related to as black-box models.

Accuracy. The accuracy with which a model relates dependent variables to independent variables is irrespective of the group or class of model. A pure verbal model (class) based on general accepted truths (group) may describe the states of the model space in great detail, whereas a pure mathematical model (class) based on no understanding of the phenomenon in model space (group) may describe the states of a model space very poorly. Examples of verbal models describing their respective model spaces accurately are the first and second law of thermodynamics.

MODEL CHOICE and VALIDATION

Data gathering. After defining a model space, data can be collected. This sampling can take place in various ways. All possible states of the model space can be thought out, and some hypothesis as to the response of the dependent variables to the independent variables can be obtained. Einstein came up with the relativity theory, on the basis of imaginary experiments. Another extreme in sampling a model space is to set any possible combination of independent variables and sample the response of the dependent variables. The first method would require no experiments, the last requires an infinite number of experiments. Both methods can be combined and result in a sensible way of data gathering. Two ways of sampling model spaces for different classes of models are described below.

Statistical techniques are available to help sampling mathematical model spaces. Some statistical techniques require knowledge about the expected models and the model space, which means that previous experiences with the model space are necessary. Other statistical techniques help to set up models from scratch, but are much more laborious. Statistical

techniques can help to set up sampling schemes (experimental design) (1). After setting up a sampling scheme, the response of the dependent variables to the independent variables can be measured.

Interrogation techniques are developed to acquire knowledge from experts in a given model space. The boundaries of the model space have to be established first i.e. the determination of the field of expertise of the expert. An inventory of the independent and dependent variables is be made next. The model space is systematically filled by presenting combinations of independent variables to the expert, to which responses are noted.

Model choice. Through sampling the model space, an improved understanding of the relations between the independent and dependent variables develops. This understanding can be used to select the appropriate model class. The model should match the verbality of the studied phenomenon in model space. If, for instance, the dependency between independent and dependent variables is expressed in the form of distinct verbal facts i.e. descriptions in words, a verbal model is appropriate.

An improved understanding of the model space through sampling, usually results in an adjustment of the conceptual model. Therefore the model choice is influenced highly by gathering data. Gathering data and choosing the appropriate model are iterative processes that should finally result in the most adequate model for the studied phenomenon in model space.

Model validation. Before a model can be used for prediction, the quality of the predictions of the model should be assessed. Validation can be carried out on the basis of the same sampled data as the model was set up with. Here, this is called internal validation. External validation uses new data to assess the quality of the predictions of the model. These are two extremes in validation. External validation is considered the fairest way of validating the predictions of a model, but this can be argued against with. External validation requires extra data in a model space, therefore the sampled model space increases. The model may describe the contents of this changed model space accurately, but this is not a requirement of the model, since it was developed for a smaller model space. However, keeping in mind that a model must also predict new states of the model space, this method of validation is useful.

The examples described earlier in the data gathering section are used to present examples of validation. The validation of the expert knowledge based model can be done by the expert himself, which would be internal validation. External validation of the knowledge model could

be performed by another expert in the model space. In this case the earlier described problem of dissimilar model spaces arises again, because the validating expert may have different experiences with the model space. Quantitative criteria for a good or bad representation of the modelled model space are hard to give. The percentage of accurate predictions can be used although this does not reflect the complexity of the predictions and is therefore an awkward measure.

Statistical validation can be carried out if mathematical models were developed. Internal validation would use the data that was gathered for the model set-up. Several measures such as correlation coefficient of the data with the model predictions, or the variance of lack of fit compared with the variance of the measuring error by means of an F-ratio test can be used.

External validation of mathematical equations can be performed by means of collecting extra data. Dividing the measured data set in two, one for fitting the model and one for validating it, is also a commonly used method. The variance of the predictions of the model based on the original data set and the variance of the predictions of the model given the newly measured data set can be compared by means of an F-ratio test. Other methods of statistical validation can be looked up in statistical handbooks (1).

THESIS

General. Food quality and safety are determined by numerous factors that can be divided into several groups, physical, chemical, microbiological, food foreign and so forth. To influence food quality or safety, any of these factors can be altered, which results in a modified food composition or modified storage conditions (6). To assess the influence of a modification of food composition or storage conditions on food quality and food safety, key parameters have to be chosen. The key parameter should directly or indirectly influence food quality or safety. Furthermore, the key parameter should change significantly in the area where it is assumed to influence safety or quality (2, 3). An example of a reliable key parameter influencing food quality and food safety is the number of microorganisms. In foods, the number of microorganisms may change. If this number becomes too large, foods may spoil and food quality may decrease. If spoilage microorganisms are absent from a food, this may result in an improved food quality. Other examples of key parameters are colour, taste, texture, formation of chemical compounds, rate of creaming up of emulsions, presence of pesticides, and so on.

Since food quality and food safety are the sum of all key parameters, the relative importance of each key parameter should be weighted. A key parameter, such as microbial number, may play an important role in one food, such as meats, but may be trivial in another, such as candy. It is very hard to express quality or safety as fully defined entities, since they strongly depend on the studied food system. Therefore, quality and safety are often modelled by means of one separate key parameter (2, 3). The consequence of focusing on one key parameter only, may be that another, equally important key parameter can be neglected, and the predictions of food quality or food safety may be invalid.

Combining models. A structured method for predicting food quality and food safety is developed. The resulting model; a decision support system, FDSS (Food Decision Support System), combines models from several model spaces. Each of these models describes their respective model space sufficiently well. The rationale of the decision support system is described in chapter 5, but some models applied, are also exemplified in this chapter. Fig. 5 shows the flow chart of the production of a food product until its distribution. The final stage, shelf life calculation is not explained here.

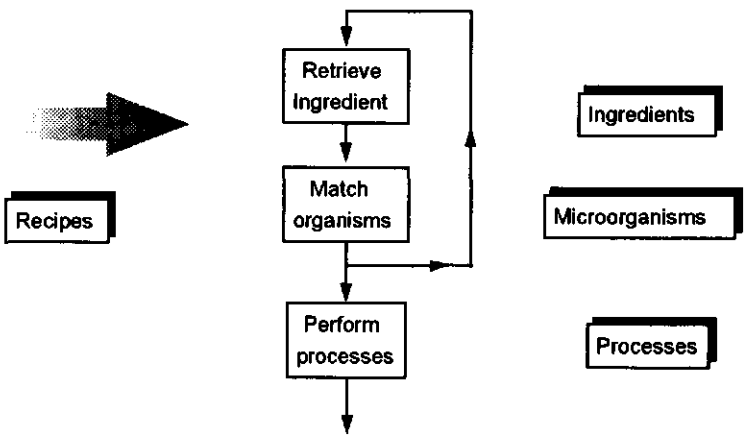

FIG. 5. Inference scheme FDSS.

The environmental parameters (T, pH, a_w, amount of O_2, etc.) of a large number of ingredients are stored in the ingredient database. Because of the large number of foods, it is impossible to collect the parameters of all existing ingredients. However, these parameters are required for the correct working of the decision support system. Lacking information of food

ingredients is therefore undesired and should be dealt with. A verbal model using hierarchical inference, i.e. an inverse tree structure (Fig. 6), is developed to overcome the problem of missing ingredient information. If, for instance, the parameters of skimmed milk are needed, but not stored in the tree structure, the products in the product group of skimmed milk are presented to the user and a judged approximation of the environmental parameters of skimmed milk can be made.

Some models used in FDSS are explicit mathematical models. The models for bacterial growth (e.g. eq. 3) in FDSS are chosen for the reason that most model parameters (e.g. T_{min}, T_{opt}, and T_{max}) can be looked up in literature or estimated by means of relatively easy experiments. The values of the model parameters were established experimentally for only two microorganisms. The required parameters for all other bacteria are looked up in literature or given by microbiological experts. The parameters are stored in the microorganisms database (Fig. 5). The applicability of the developed mathematical models is extended to all bacteria, which is a form of model extrapolation. The pattern match model described earlier in this chapter, is a bridge model used to combine microbial growth data with the environmental parameters of food ingredients.

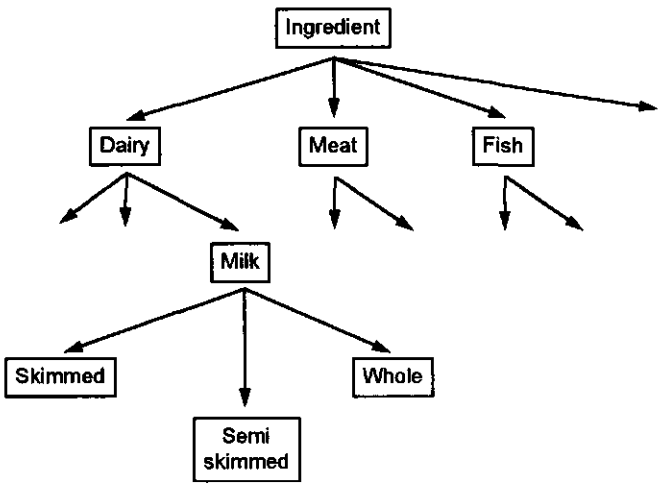

FIG. 6. Food ingredient tree structure.

Mathematical and verbal models are developed and used to describe the effects of different food production processes on bacterial growth. Temperature-time relations are coupled with models for the prediction of bacterial growth, developed in this thesis. The result of all

calculations is a prediction of shelf-life, stability and safety of foods. Predictions of the model are validated externally by means of comparison with literature values.

The mathematical bacterial growth models applied in FDSS are also described in this thesis. Bacterial growth as function of temperature, acidity, and water activity is modelled. Mathematical equations are developed after sampling the model space in two ways. Previous experience with the model space is used to set up hypothetical models that may describe the growth response as function of the independent variables. Then, a data set is measured in which a large number of combinations of independent variables (temperature, acidity and water activity) is set and the responses of the independent variables (growth rate, lag time and asymptotic number of microorganisms) are measured. Validation of the developed models is carried out both internally and externally.

Hypothetical mathematical models with independent variables temperature and acidity and dependent variables growth rate, lag time, and asymptotic number of *Lactobacillus curvatus*, are fitted to measured data and the best model is selected. This selection takes place by means of the mathematical properties of the hypothetical models. Statistical validation of the selected models is carried out by means of comparing the model predictions with the overall measuring error of the dependent variables.

A combination of two models is proposed to relate the effects of temperature, acidity, and water activity to bacterial growth rate of *Lactobacillus curvatus*. The model that is set up, is a result of the combination of two model spaces. One model space consists of the growth response of *L. curvatus* as a result of set temperature and set acidity and one model space consists of the same response to temperature and water activity. Since both model spaces overlap, the models can be combined into one. Both model spaces are merged and a new model is set up. The new model is fitted to the data and then used to predict states beyond the model space where new growth data are gathered. These data were not used to fit the parameters of the model, but only for model validation. It is shown that, with some care, model predictions can be made outside the model space, where growth data were gathered.

Bacterial growth of *Listeria monocytogenes* is modelled as a function of temperature, acidity and water activity. Model space is divided into separate parts where individual hypothetical models are set up. The hypothetical models are used to fit the measured growth data. In this case, validation of the models is performed using the comparison of the model predictions with the measuring error of the independent variables, so-called internal validation.

Furthermore, the predictions of the models are compared to values reported in literature, which is external validation.

Finally, a method for the identification of microorganisms that are present in foods is described. The model classes used for this are both verbal and mathematical. A first classification is made on the basis of a tree structure (verbal model) by means of which all bacteria are divided into separate groups. After reaching a separate group of organisms, characteristics of the organisms in this sub group are used to find the most likely match with the organism under study (mathematical model). If the likelihood is too low, a method of tracing identification errors can be used to find another sub-group of organisms (combined mathematical and verbal model). Validation of the model takes place by identifying a number of lactic acid bacteria. Since these measured data were not used to set up the model, this is a form of external validation.

CONCLUSIONS

A method for setting up models is described and examples are given. This method, however, is a model again. Since the model space belonging to this model is also pre-defined, choices were made as to what to incorporate and what to neglect. Because of the things we learned during and since our childhood, a modeller can never be unbiassed, which influences all the models we set up. This includes the described method for setting up models.

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Chapter 2

Modelling bacterial growth of *Lactobacillus curvatus* as a function of acidity and temperature

ABSTRACT

Models that describe the effect of acidity, temperature, and the combined effect of these variables on the growth parameters of *Lactobacillus curvatus* are developed and validated. Growth parameters (lag time, specific growth rate, and maximum population density) were calculated from growth data at different temperature acidity combinations. Experiments were set up to assess the quantitative effects of temperature and acidity on the growth parameters rather than for parameter estimation solely. The effect of acidity is monitored at several constant temperature values. Models are set up and fitted to the data. The same procedure is used at constant acidity values to model the effect of temperature. For lag time, specific growth rate, and maximum population density, the effect of temperature could be multiplied with the effect of acidity to obtain combinatory models that describe the effect of both controlling factors on the growth parameters. Lag time measurements showed large deviations, and therefore the lag time models developed can only be used to estimate the order of magnitude of lag time.

INTRODUCTION

The quality and shelf life of foods are often determined by the growth of microorganisms. Microbial growth is dependent on intrinsic factors such as water activity and acidity, and extrinsic factors such as temperature and oxygen availability. In recent years, the interest in developing mathematical models to describe the growth of microorganisms as a function of controlling factors has increased. Much work on combined effects of several controlling factors on bacterial growth was carried out; see, for example, papers by Buchanan (2), McMeekin *et al.* (3), and Sutherland *et al.* (9). The mathematical models can be used to predict the change in quality of a food in time and can therefore be applied to estimate the shelf life of foods. The models can help to decrease the required amount of costly and time consuming challenge tests, may help to design more efficient ways of challenge testing, and finally can be used for distribution chain optimisation. Another important feature of models is the acquisition of improved knowledge of the factors that determine food quality.

The combination of models for growth as a function of more than one controlling factor has been carried out by, for instance, McMeekin *et al.* (3), Adams *et al.* (1), and Wijtzes *et al.* (10). McMeekin *et al.* (3) suggested the multiplication of the effect of temperature on specific growth rate with the effect of water activity. Adams *et al.* (1) did the same for acidity and temperature. Wijtzes *et al.* (10) multiplied the effect of acidity, water activity, and temperature.

The authors mentioned above assume that the effect of one controlling factor does not influence the effect of other factors. Multiplication of the effects of temperature and acidity implies that neither the minimum nor the maximum pH at which growth takes place, is a function of temperature. Minimum and maximum temperature are also supposed to be independent of acidity. In this paper, the validity of the above assumptions will be evaluated and the models will be verified.

This paper provides for a structured way to model combinations of effects of various controlling factors. By using both microbiological expertise and practical modelling techniques, the mathematical relations between the controlling factors temperature and acidity (pH) and specific growth rate, lag phase, and maximum population density will be derived. For this purpose, an extensive data set is collected.

MODEL DEVELOPMENT

Theoretical growth curves. Zwietering *et al.* (12) derived a mathematical relation, based on the Gompertz model for the increase of a population over time, that relates population size over time to specific growth rate, lag time, and asymptotic level of organisms. The natural logarithm of the maximum number of microorganisms (N_{max}) is defined as the sum of the natural logarithm of the estimated initial number (N_0) and estimated asymptotic value (A) from the reparameterized Gompertz equation. Since direct estimates of $\ln(N_{max})$ were required for this data analysis, the reparameterized Gompertz equation was rewritten as:

$$\ln(N_t) = \ln(N_0) + \left\{\ln(N_{max}) - \ln(N_0)\right\}\exp\left\{-\exp\left[\frac{\mu_m e}{\left\{\ln(N_{max}) - \ln(N_0)\right\}}(\lambda - t) + 1\right]\right\} \qquad (1)$$

where t is time (in hours), N_t is the number of microorganisms at time t (in CFU per ml), N_0 is the asymptotic number of microorganisms at time zero (in CFU per ml), N_{max} is the maximum number of microorganisms (in CFU per ml), μ_m is the maximum specific growth rate (per hour), and λ the lag time (in hours).

Temperature effect on specific growth rate. Bélehrádek type models, as described below, were suggested for the first time in predictive microbiology by Ratkowsky *et al.* (7). The Bélehrádek type model known as the Ratkowsky equation describes the relation between specific growth rate and sub-optimal temperature for various microorganisms as:

$$\mu_m = b_2\left(T - T_{min,2}\right)^2 \qquad (2)$$

where b_2 (h^{-1} $°C^{-2}$), and $T_{min,2}$ (°C) are regression coefficients, $T_{min,2}$ representing the theoretical minimum temperature for growth; index 2 indicates the number of the equation. This model has the advantage of containing an easily interpretable parameter, T_{min}, the extrapolated temperature at which a microorganism cannot grow. All other models in this paper will contain as much of this type of parameter as possible, since these parameters may have biological meaning and can be interpreted easily.

Acidity effect on specific growth rate. Two models will be set up that may describe specific growth rate as a function of pH. It is well known that below the minimum pH and above the maximum pH, microorganisms stop growing and even die. At the optimum pH, the organisms

grow fastest. This behaviour can be described mathematically by using, for instance, a parabolic equation:

$$\mu_m = -b_3\left(\mathrm{pH} - \mathrm{pH}_{min,3}\right)\left(\mathrm{pH} - \mathrm{pH}_{max,3}\right) \quad (3)$$

where b_3 (h^{-1}) is a regression coefficient and $pH_{min,3}$ and $pH_{max,3}$ are the theoretical minimum and maximum pH for growth, respectively.

Another function that may be used to describe the mentioned curvature is based on the expanded Ratkowsky equation for temperature and specific growth rate (6). From the proposed equation, the square root is omitted, to result in equation 4:

$$\mu_m = b_4\left(\mathrm{pH} - \mathrm{pH}_{min,4}\right)\left\{1 - \exp\left[c_4\left(\mathrm{pH} - \mathrm{pH}_{max,4}\right)\right]\right\} \quad (4)$$

where c_4 is a new regression coefficient. The difference between equations 3 and 4 is the possibility of adjusting the location of the optimum pH. If the optimum pH is exactly in the middle between pH_{min} and pH_{max}, equation 3 can be used, if not, equation 4 applies. This can be verified by assessing the confidence interval and value of c_4. If the confidence interval overlaps zero, the curvature of equation 4, statistically, cannot be distinguished from symmetrical (see appendix). In that case, equation 3 is favoured, since it has one parameter less and describes the curvature as well as equation 4 does.

Temperature effect on lag time. From the observation that $\mu \times \lambda$ versus μ is uncorrelated, Zwietering *et al.* (11) proposed to use the inverse of equation 2 to model the length of the lag phase as a function of temperature:

$$\lambda = \frac{1}{b_5\left(T - T_{min,5}\right)^2} \quad (5)$$

Acidity effect on lag time. Here again, the reciprocal of the equation for specific growth rate as a function of pH is used. Since two different relations may apply, two lag phase models are proposed:

$$\lambda = \frac{-1}{b_6\left(\mathrm{pH} - \mathrm{pH}_{min,6}\right)\left(\mathrm{pH} - \mathrm{pH}_{max,6}\right)} \quad (6)$$

$$\lambda = \frac{1}{b_7(\mathrm{pH} - \mathrm{pH}_{\min,7})\{1 - \exp[c_7(\mathrm{pH} - \mathrm{pH}_{\max,7})]\}} \tag{7}$$

where all the parameters are as described above.

Temperature effect on maximum population density. A model to relate the maximum number of microorganisms (N_{max}) to temperature is proposed by Zwietering *et al.* (11) as:

$$\ln(N_{max}) = a_8 \frac{(T - T_{min,8})(T - T_{max,8})}{(T - T_{sub\ min,8})(T - T_{sup\ max,8})} \tag{8}$$

where a_8 is a regression coefficient, $T_{sub\ min,8}$ (°C) is a temperature just below the theoretical minimum temperature for growth, $T_{sup\ max,8}$ (°C) is a temperature just above the theoretical maximum temperature for growth and all other parameters are as defined above.

In this paper the effect of suboptimal temperature is modelled, and therefore the part of equation 8 that describes the temperature effect near the maximum can be omitted, resulting in equation 9, where the parameters are as defined above:

$$\ln(N_{max}) = a_9 \frac{(T - T_{min,9})}{(T - T_{sub\ min,9})} \tag{9}$$

If N_{max} values do not decrease with decreasing temperature, a constant value for N_{max} may describe the data correctly. Therefore another equation is proposed:

$$\ln(N_{max}) = c_{10} \tag{10}$$

Acidity effect on maximum population density. Similar N_{max} models for acidity can be suggested, pH is substituted for temperature to result in:

$$\ln(N_{max}) = a_{11} \frac{(\mathrm{pH} - \mathrm{pH}_{min,11})(\mathrm{pH} - \mathrm{pH}_{max,11})}{(\mathrm{pH} - \mathrm{pH}_{sub\ min,11})(\mathrm{pH} - \mathrm{pH}_{sup\ max,11})} \tag{11}$$

The maximum number of microorganisms can also be supposed to be constant over the entire acidity range:

$$\ln(N_{max}) = c_{12} \tag{12}$$

Combining acidity and temperature models for specific growth rate. If the effects of acidity and temperature can be multiplied, the combinatory effect on specific growth rate may have either of two forms. If the parabolic relationship between acidity and specific growth rate is preferred, equation 13 will be used.

$$\mu_m = -b_{13}\left(T - T_{min,13}\right)^2\left(\mathrm{pH} - \mathrm{pH}_{min,13}\right)\left(\mathrm{pH} - \mathrm{pH}_{max,13}\right) \tag{13}$$

If the Ratkowsky type curvature for pH is preferred, equation 14 may apply.

$$\mu_m = b_{14}\left(T - T_{min,14}\right)^2\left(\mathrm{pH} - \mathrm{pH}_{min,14}\right)\left\{1 - \exp\left[c_{14}\left(\mathrm{pH} - \mathrm{pH}_{max,14}\right)\right]\right\} \tag{14}$$

Combining acidity and temperature models for lag time. The model for the combinatory effect of temperature and pH on the length of the lag phase is dependent on the model developed for specific growth rate. Therefore, specific growth rate models will be ratified first, and lag phase models will result from these.

Combining acidity and temperature models for maximum population density. For each controlling factor, the most appropriate model will be chosen. Then, after checking the validity of the multiplication of the effect of temperature and acidity on the maximum number of organisms, the product of the two best models will be chosen.

MATERIALS and METHODS

Organism. A pure culture of *Lactobacillus curvatus,* a typical spoilage organism of different types of meat and meat products (4, 5), was used as a model organism.

Microbial experiments. At 8 different suboptimal temperatures between 6 and 31°C and various preset acidities between previously determined minimum and maximum pH values, 87 growth curves were determined. *L. curvatus* was grown in MRS broth (Difco Laboratories).

After sterilising the broth (121°C, 20 min), pH was set by using 2 N HCl (Merck) or 2 N NaOH (Merck) diluted in sterile demi water. The pH was measured with an electrode (Schott Geräte: N 5900 A) that was disinfected with 70% alcohol (Merck, aqua dest.).

Before the experiments, a frozen culture (-18°C) of *L. curvatus* was cultivated twice at 30°C, first for 24 h and then for 16 h. To prepare for the growth experiment, the last preculture was diluted to reach an initial level in the broth of 10^3 CFU per ml. The experiments were carried out in 50 ml tubes containing MRS-broth with preset pH and incubated at static, constant temperature.

Acidity was not controlled during the experiments. Acidity values remain constant in the range where growth rate and lag time are estimated. In the region where the growth curve bends towards the asymptote, pH drops until a value of 4.26 is reached in the asymptote. If the asymptote is reached, the glucose concentration drops to 0 mole per litre.

The need to incubate plates anaerobically (anaerobic jar with BBL-gaspack; Bekton Dickinson and Company) was investigated previous to the growth experiments. The colony counts did not vary by more than a factor of 3, between 3 and 5 days of incubation, and growth rate, lag time, and asymptote fits to the Gompertz equation indicated overlapping 95% confidence intervals for each parameter. Since, no significant differences were found between anaerobic and aerobic methods, for convenience, the aerobic incubation method was chosen.

At appropriate time intervals, a 0.1- or 1-ml sample was taken aseptically from the tube, and the time was noted. The number of microorganisms was determined in two ways. Part of the data set was acquired by the pour plate method, and part was acquired by using a spiral plate device (Spiral Systems Inc., model D). Growth rate, lag time and asymptote fits to the Gompertz equation indicated overlapping 95% confidence intervals for each parameter, and therefore no significant differences were found between the two counting methods. All plates were incubated aerobically for at least 3 days at 30°C. The plates were counted according to the appropriate method. The plates in the extreme growth conditions were incubated for 5 days and recounted. The colony counts did not vary more than by a factor of 3, between 3 and 5 days of incubation, and growth rate, lag time, and asymptote fits to the Gompertz equation indicated overlapping 95% confidence intervals for each parameter. Therefore, no significant differences in the number of colonies were found after 3 and 5 days of incubation, even under extreme growth conditions.

Clustering acidity data. Since the experiments were set up at constant temperatures and the pH was set at approximately the required value, a variation in pH values can be expected. The pH used was the calculated mean pH of a class. The deviation from the mean of the class was never more than 0.10 pH unit, so the deviation from the mean is about as large as the measuring error.

Statistical methods. All regressions were carried out with standard statistical software (SAS). A Marquardt algorithm was used for non-linear regression (8). Confidence intervals were calculated by using the estimated asymptotic covariance matrix and the Student t value.

The counts from the experiments at one set temperature and pH value were fitted to the rewritten Gompertz equation (eq. 1).

The dependent variables were related to the controlling factors without transforming the data first. Upon regression, the appropriate distribution of errors was used to fit the equations to the data. For specific growth rate and lag time data, a variance analysis showed that a gamma distribution of the dependent variable is most appropriate. A gamma distribution corresponds to taking the square root on both sides of the equation. For $\ln(N_0)$ and $\ln(N_{max})$ data, a Gaussian distributed error was found, and therefore no further transformation was required.

Lack of fit test. Model predictions were plotted against the observed values by using the appropriate transformation method. The residuals, the deviation from the diagonal, should be distributed homogeneously throughout the range of observed values and the values should not be too far away from the diagonal. F ratio tests provide a less subjective model validation (12). The deviations of the model predictions were tested against the mean of the measured values at clustered temperature-acidity combinations. The α value for the F ratio test was 0.05. The latter method was used to assess the statistical acceptability of a model.

Modelling procedure. Modelling of the data was carried out in several stages. First, temperature equations were fitted at constant pH values; then, pH equations were fitted to the data at constant temperatures. Then all estimated parameters from the models will be plotted against the other controlling factor to observe any trends. If trends are observed in the parameters that are supposed to be constant (pH_{min}, pH_{max}, T_{min}, etc.) the conclusion may be drawn that combinatory effects do exist and extension of the models is necessary.

RESULTS and DISCUSSION

Modelling the effect of temperature on specific growth rate. The growth rate data at constant acidity values are fitted with equation 2. The results from these fits are given in Fig. 1 and 2. In Fig. 1, $T_{min,2}$ is shown as a function of acidity. As can be seen, there is no significant trend in the data. The slope of the straight line fitted through the datum points of T_{min} was not significantly different from zero, and therefore, T_{min} can be supposed to be constant over the measured pH range. The mean $T_{min,2}$ resulting from the data in Fig. 1 equals -3.06°C.

The results from the estimations of b_2 are given in Fig. 2. The trend in the curve of Fig. 2 is a parabolic one, and therefore the effect of acidity on growth rate is reflected in the values for b_2.

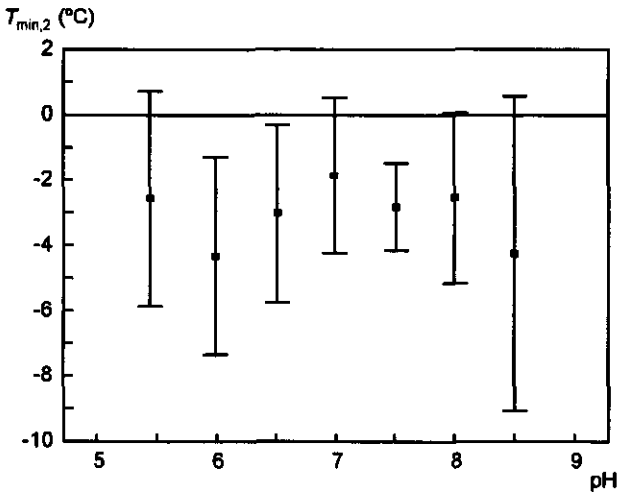

FIG. 1. $T_{min,2}$ as a function of acidity.

Modelling the effect of acidity on specific growth rate. The data set covers specific growth rate over the entire pH range for growth. Equation 3 is fitted to the data at various constant temperatures for which, at least, 6 datum points have been gathered, resulting in a minimum number of degrees of freedom of 3. In Fig. 3, the parameters $pH_{min,3}$ and $pH_{max,3}$ and their 95% confidence intervals are shown as a function of temperature. From these data it follows that neither $pH_{min,3}$ nor $pH_{max,3}$ shows a trend as a function of temperature.

In Fig. 4, the value of b_3 is displayed as a function of temperature; a quadratic curve can be drawn through the datum points. The conclusion may be drawn that only parameter b_3 is a function of temperature. Parameter b_3 as a function of temperature shows the same behaviour as specific growth rate as a function of temperature.

Equation 4 is fitted to the growth rate data at the same constant temperatures as equation 3. In Table 1, the resulting minimum and maximum pH, b_4, c_4, and their 95% confidence intervals at different temperatures are given. Again, no trend can be observed in $pH_{min,4}$ and $pH_{max,4}$. Therefore, the entire temperature effect must be present in the either of the two regression parameters b_4 and c_4. The values of c_4 are always close to zero, and the confidence intervals overlap zero, resulting in a close to symmetrical curve. The conclusion can therefore be drawn that, at least in this particular case, the parabolic equation for acidity (equation 3) can be used best to describe the measured specific growth rates. The large confidence interval values around b_4 and c_4 are an indication of overparameterisation of the model and are explained in the appendix.

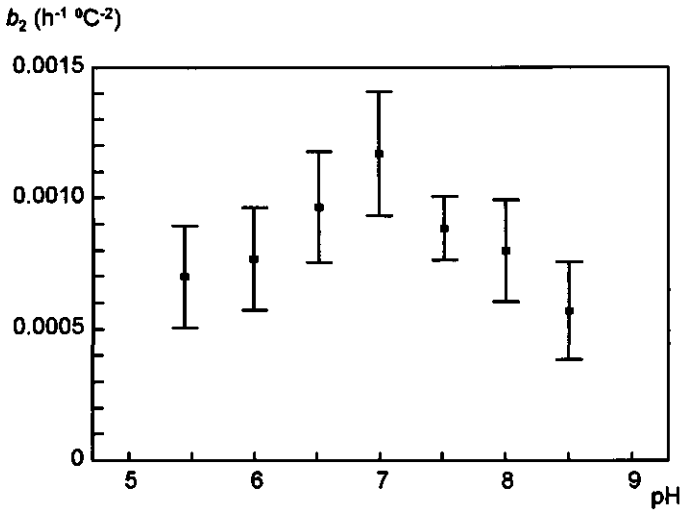

FIG. 2. b_2 as a function of acidity.

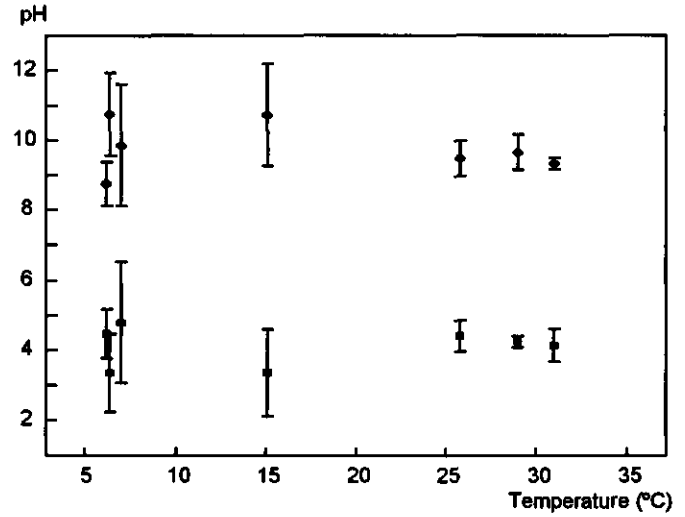

FIG. 3. $pH_{min,3}$ (■) and $pH_{max,3}$ (♦) as a function of temperature.

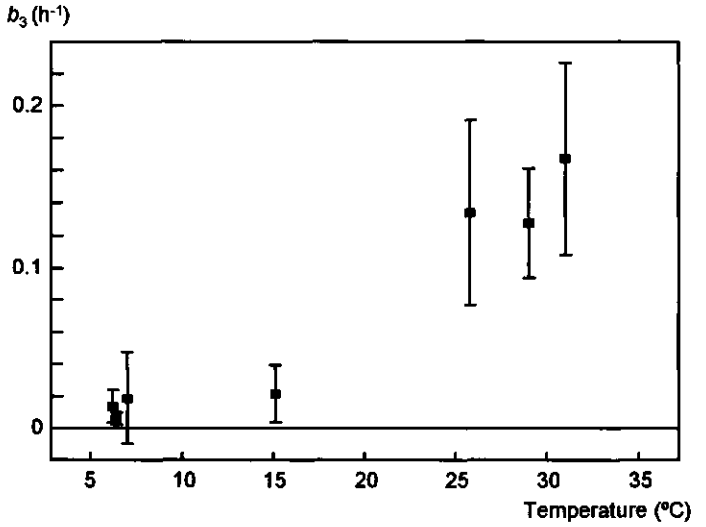

FIG. 4. b_3 as a function of temperature.

TABLE 1. Estimated parameters from equation 4 and their 95 % confidence intervals

T (°C)	$pH_{min,4}$		$pH_{max,4}$		b_4 (h^{-1})		c_4	
	fitted value	±95 %[a]	fitted value	±95 %[a]	fitted value	±95 %[a]	fitted value	±95 %[a]
6.2	3.07	5.99	8.34	6.80	-2.68×10^{-3}	4.81×10^{-2}	-0.36	18.05
6.4	4.28	1.33	14.08	32.22	1.95×10^{-2}	5.10×10^{-2}	1.33	4.60
7.0	4.79	7.71	9.86	8.95	3.56	4.15×10^{3}	5.32×10^{-3}	6.26
15.1	3.36	4.41	10.73	4.88	11.75	1.07×10^{5}	1.84×10^{-3}	1.68
25.8	4.39	1.54	9.45	1.77	3.93	1.45×10^{2}	3.58×10^{-2}	1.44
29.0	4.24	0.30	9.49	1.19	1.85	11×88	8.14×10^{-2}	0.61
31.0	4.14	2.04	9.52	1.35	11.70	1.01×10^{3}	1.46×10^{-2}	1.29

[a] The 95% confidence interval around the fitted value (e.g. a fitted value of 3.07 and a 95 % value of 5.99 means the 95 % confidence interval equals (-2.92, 9.06))

In conclusion, the fits of both equations 3 and 4 to the data support the conclusion that pH_{min} and pH_{max} are independent of temperature.

Combining the effect of temperature and acidity on specific growth rate. Fig. 3 shows that $pH_{min,3}$ and $pH_{max,3}$ are independent of temperature, and Fig. 1 indicates that $T_{min,2}$ is independent of acidity. Furthermore, Fig. 4 and Fig. 2 show an apparent parabolic relationship between b_2 and temperature as well as a parabolic relationship between b_3 and acidity. The suggested multiplication of the effects of temperature and acidity on specific growth rate is therefore allowed.

Since the parabolic equation (equation 3) for the relation between specific growth rate and pH is favoured, the combined model for the relation between specific growth rate and temperature and pH should be equation 13. The results from the fit are given in Table 2.

The square root of measured specific growth rate was plotted against the square root of the fits of the overall model for specific growth rate as a function of temperature and acidity (equation 13) and no trend was found. The calculated f value equals 1.05, whereas the reference 95% right-hand γ point F value equals 1.726, therefore the model is statistically accepted.

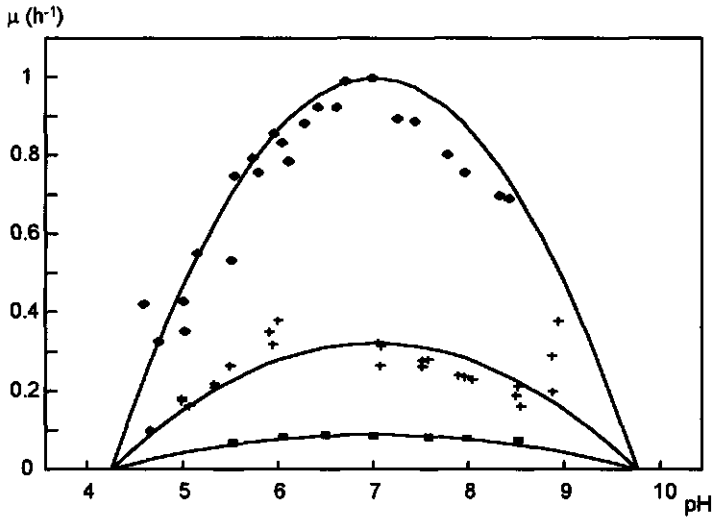

FIG. 5. Measured and fitted specific growth rates at 6°C (■), 15°C (+), and 29°C (♦).

The trend is described well at all temperatures. As an example, in Fig. 5, the measured data and the fit of the model are given at three temperatures (6, 15, and 29°C).

TABLE 2. Results of fit overall model for specific growth rate based on equation 13

Parameter	Estimated value	95% Confidence interval	
b_{13} (h^{-1} $°C^{-2}$)	1.263×10^{-4}	1.052×10^{-4}	1.474×10^{-4}
$T_{min,\ 13}$ (°C)	-3.27	-4.48	-2.06
$pH_{min,\ 13}$	4.26	4.13	4.38
$pH_{max,\ 13}$	9.77	9.53	10.01

Modelling the effect of temperature on lag time. The effects of temperature and pH on the length of the lag phase may be modelled as the reciprocal of the models for specific growth rate. However, at constant acidity values, a trend is visible in the $\mu \times \lambda$ versus μ graph. At constant pH values, lag time curves display hyperbolic behaviour as can be seen in Fig. 6, and therefore a hyperbolic type of model equation is required. Different hyperbolic equations were fitted to the data. It proved that, at all acidity values, equation 5 could best be fitted to the data.

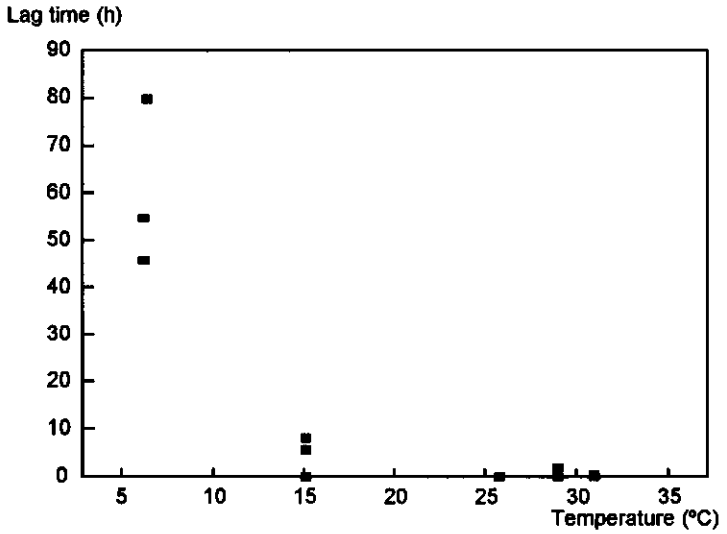

FIG. 6. Lag time as a function of temperature at pH = 5.45.

Parameter b_5 should be a function of acidity. As can be seen in Fig. 7, the relation between b_5 and acidity shows a parabolic curvature, and therefore the relation between acidity and lag time at constant temperatures can be modelled as such.

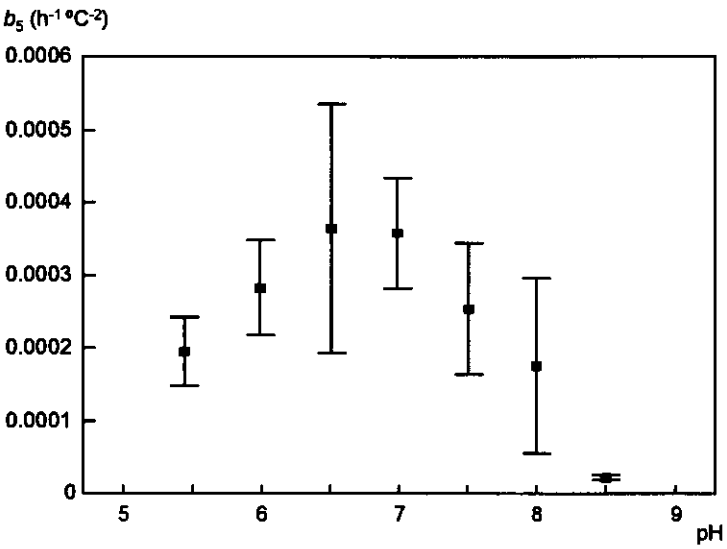

FIG. 7. b_5 as a function of acidity.

Modelling the effect of acidity on lag time. The lag time model as a function of temperature indicated that the pH effect took the form of an inverse parabola (equation 6). At constant temperature values, $\mu \times \lambda$ versus μ data were correlated again, invalidating the proposed inverse growth rate model. To retain the characteristic hyperbolic curvature, an exponent was introduced over equation 6.

To assess the value of the newly introduced exponent, an overall model had to be set up. The overall model describes the effects of both temperature and acidity on lag time, assuming that the effect of temperature and pH can be multiplied. This can be preliminarily concluded from the modelling of the temperature effect on lag time, although it still has to be proven. The exponent in this equation was then fitted along with the other regression coefficient.

The value for the exponent resulting from the overall model equals 2.14 and the confidence interval around this value ranges from 1.34 to 2.94. By analogy with equation 5, the value for the exponent over the acidity effect is set to 2.

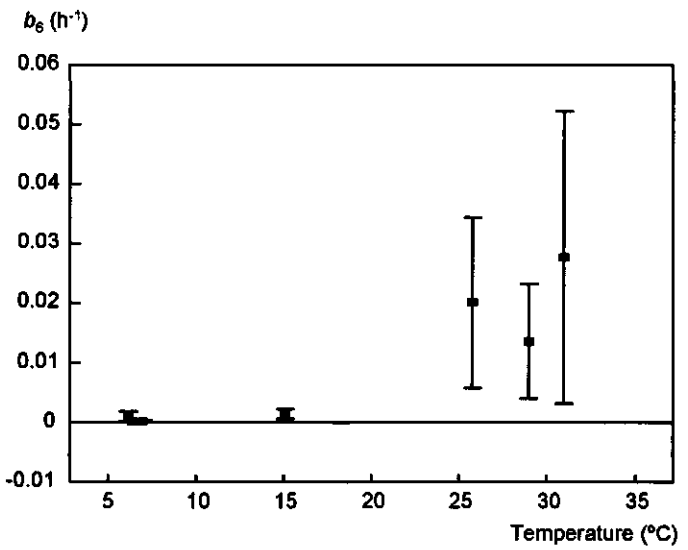

FIG. 8. b_6 as a function of temperature.

At constant temperatures, the newly developed model is fitted with constant parameters $pH_{min,6}$ and $pH_{max,6}$ taken from Table 2, and the exponent is set to 2. Upon regression of the new equation, the values of the regression coefficients b_6 were estimated and are shown in Fig.

8. In all cases the trend in the data was described well. Again, the regression coefficient b_6 takes the parabolic form of a temperature curve.

Combining the effect of temperature and acidity on lag time. For lag time, it can also be concluded that the effects of temperature and acidity can be multiplied. This results in an overall model, as given in equation 15:

$$\lambda = \frac{1}{b_{15}\left\{\left(\mathrm{pH} - \mathrm{pH}_{\min,13}\right)\left(\mathrm{pH} - \mathrm{pH}_{\max,13}\right)\left(T - T_{\min,13}\right)\right\}^2} \tag{15}$$

where $\mathrm{pH}_{\min,13}$, $\mathrm{pH}_{\max,13}$, and $T_{\min,13}$ are fixed, being the fitted values from the overall specific growth rate model (Table 2) resulting in a one-parameter model. The regression coefficient b_{15} is found to equal 4.18×10^{-6} (h^{-1} $°C^{-2}$) with a confidence interval value of $\pm1.19\times10^{-6}$ (h^{-1} $°C^{-2}$).

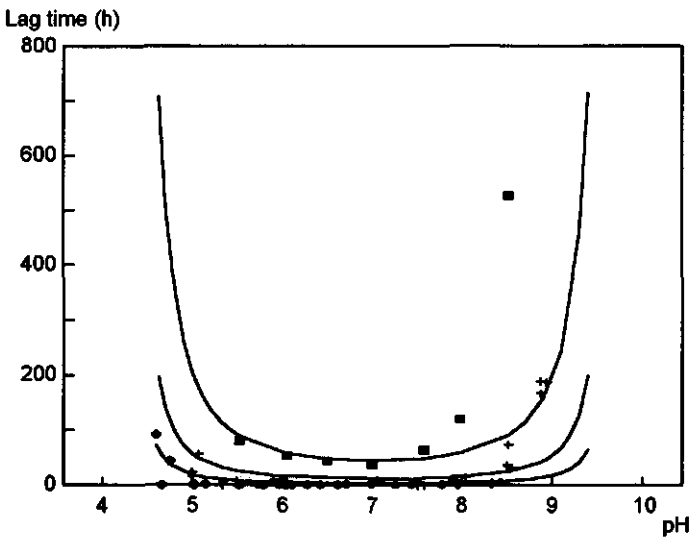

FIG. 9. Measured and fitted lag times at 6°C (■), 15°C (+), and 29°C (♦).

Validation of the overall lag time model. The reproducibility of the measured lag times is poor, and therefore, it is hard to model the effect of lag time. In the fitted versus observed plot, a trend is visible, and by using the F ratio test the model predictions are rejected (f = 6.909; F = 1.716); therefore, the conclusion can be drawn that the overall model does not describe the data adequately. The model, however, can be used to obtain an estimate of the order of

magnitude of lag time but not more than that. In Fig. 9, lag time data and model predictions at 6, 15, and 29°C are given. As can be seen, the overall trend is described well, but in individual cases, the model predictions can be quite far off; especially near the pH extremes. This can be an indication that pH_{min} and pH_{max} should not be fixed but fitted along with the other parameters of the model.

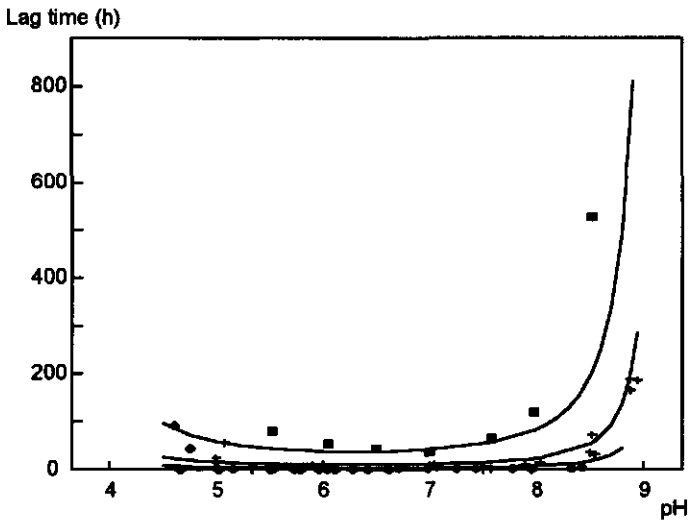

FIG. 10. Measured and fitted lag times at 6°C (■), 15°C (+), and 29°C (♦); Fitted pH_{min} and pH_{max}.

Fig. 10 shows the results when pH_{min} and pH_{max} have newly fitted values of 3.39 ± 1.15 and 9.23 ± 0.11 whereas b_{15} equals $4.00 \times 10^{-6} \pm 3.42 \times 10^{-6}$ (h^{-1} $°C^{-2}$). The significantly different values for minimum and maximum pH compared with the fitted values of the growth rate models may indicate that the mechanisms that control growth rate are different from those controlling lag time. The calculated f value was 3.799, whereas the reference F value was 1.720. Therefore, this model is also rejected statistically, but the predictions come closer than the model with fixed pH_{min} and pH_{max} taken from the growth rate model.

Modelling maximum population density. Four models were proposed to describe the effect of temperature and acidity on maximum population density. Here again, the effects of temperature and acidity were modelled separately first and then combined into one model. At constant temperatures, the maximum population density data did not deviate significantly from a constant value, and therefore equation 10 was used.

Near the extremes for acidity, i.e. the minimum and maximum pH, the maximum population density data become smaller, and therefore equation 11 was chosen to model this effect. The minimum and maximum pH were fixed at their fitted specific growth rate values, at the appropriate temperature. $pH_{sub\ min,11}$ and $pH_{sup\ max,11}$ were independent of temperature.

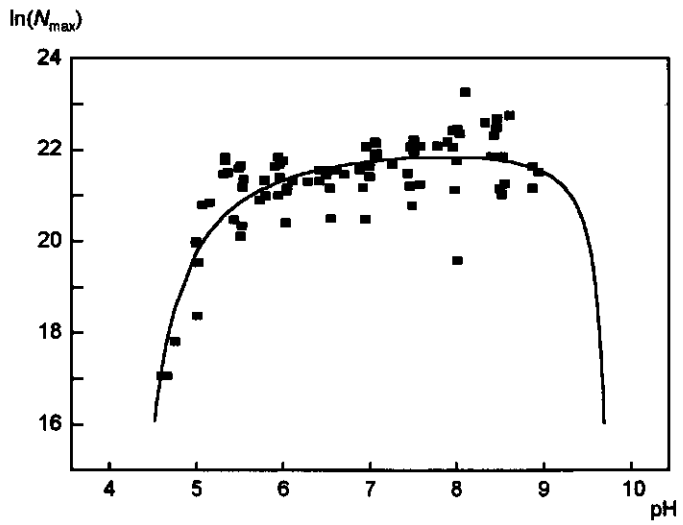

FIG. 11. Maximum population density as a function of acidity.

The overall model to describe the effect of both temperature and acidity on the height of the asymptote is therefore the product of equations 10 and 11, which makes it similar to equation 11. Upon fitting this equation, the values for $pH_{min,11}$ and $pH_{max,11}$ were fixed again, now at their fitted values resulting from the overall model for specific growth rate (Table 2). The results from this fit can be found in Table 3.

TABLE 3. Results of the fit of the overall model for the natural logarithm of the maximum population density based on equation 11

Parameter	Estimated value	95% Confidence interval	
a_{11}	22.87	22.30	23.44
$pH_{sub\ min,11}$	4.14	4.12	4.17
$pH_{sup\ max,11}$	9.80	9.76	9.83

Validation of the overall maximum population density model. In Fig. 11, the predictions of the overall model for maximum population density are given along with the datum points; the trend is described well. However, assessing the fit-versus-observed plot, the data did not appear uncorrelated. The F ratio test agrees with the latter, since f equals 3.47 against a reference F value of 1.72. Although the deviation from the required F value is small, the model is rejected statistically.

CONCLUSIONS

At suboptimal temperatures, the parabolic relationship between specific growth rate and temperature was found to apply. Over the entire pH range, the parabolic relationship between acidity and specific growth rate proved to be the most adequate model to describe the data. The combination of the parabolic model for acidity and temperature by multiplication into one resulted in a good model to describe the effect of temperature and acidity on specific growth rate.

The proposed models for lag time as a function of temperature described the trend well. The acidity effect was accounted for in the regression coefficient. Therefore, the effect of acidity could be multiplied with the effect of temperature. The model for lag time as a function of acidity needed alteration; a power was introduced and a new equation was set up. From the new equation, the effect of temperature could be multiplied with the effect of acidity. The overall model was rejected by the F ratio test.

The proposed models for maximum population density required no alteration on the basis of the measured data set. The overall model for maximum population density data predicts the trend well, although the model is statistically rejected.

A preliminary idea of the type of curve expected in growth data helps in modelling the effects of controlling factors on the growth parameters. It is useful to set up a hypothetical model before carrying out experiments, since from interpreting this model, it is possible to assess the range of controlling factors over which it is necessary to take enough measurements to verify the model.

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APPENDIX

The derivation of symmetry in the Ratkowsky equation (6),

$$\sqrt{\mu} = b(T - T_{\min})\{1 - \exp[c(T - T_{\max})]\} \tag{A1}$$

can be rewritten as

$$y = b(x - x_{\min})\{1 - \exp[c(x - x_{\max})]\} \tag{A2}$$

The first derivative of this equation can be written as

$$\frac{dy}{dx} = b\{1 - [c(x - x_{\min}) + 1]\exp[c(x - x_{\max})]\} \tag{A3}$$

At the optimum, the first derivative equals zero, and if b doesn't equal zero, then

$$[c(x_{\text{opt}} - x_{\min}) + 1]\exp[c(x_{\text{opt}} - x_{\max})] = 1 \tag{A4}$$

One of the properties of this type of symmetrical curve is that the optimum lies exactly in the middle between the minimum and the maximum, therefore

$$x_{\text{opt}} = \frac{x_{\min} + x_{\max}}{2} \tag{A5}$$

Substituting A5 into equation A4 results in

$$\left[\frac{c}{2}(x_{\max} - x_{\min}) + 1\right]\exp\left[\frac{c}{2}(x_{\min} - x_{\max})\right] = 1 \tag{A6}$$

Rewriting $\frac{c}{2}(x_{\max} - x_{\min})$ as Ω, the above equation becomes

$$[\Omega + 1]\exp[-\Omega] = 1 \tag{A7}$$

This equality only holds if Ω equals zero. Since x_{min} and x_{max} have different, fixed values, c has to become zero. However, if c equals zero, y and μ also equal zero (see equations A1 and A2). Finding the optimum using this method is defined as making x approach x_{opt}, and therefore c approaches zero instead of being zero.

Another property of symmetrical curves is that for every distance from the optimum x value, θ ($x_{opt}+\theta$, $x_{opt}-\theta$) the resulting y values of the function have to be equal. So

$$b\left(x_{opt} - x_{min} - \theta\right)\left\{1 - \exp\left[c\left(x_{opt} - x_{max} - \theta\right)\right]\right\} \tag{A8}$$

has to be equal to

$$b\left(x_{opt} - x_{min} + \theta\right)\left\{1 - \exp\left[c\left(x_{opt} - x_{max} + \theta\right)\right]\right\} \tag{A9}$$

Rewriting $\left(x_{opt} - x_{min} - \theta\right)$ as ν and $\left(x_{opt} - x_{min} + \theta\right)$ as κ the required equality can be written as

$$\nu\left\{1 - \exp\left[c\nu\right]\right\} = \kappa\left\{1 - \exp\left[c\kappa\right]\right\} \tag{A10}$$

which is similar to

$$\nu - \kappa = \nu\exp\left[c\nu\right] - \kappa\exp\left[c\kappa\right] \tag{A11}$$

From the foregoing, it was concluded that $c = 0$. In that case, the equality holds.

Fitting data. The closer c comes to zero, the closer y will be to zero. To compensate for this effect, b has to increase. Fitting this equation to nearly symmetrical acidity data will result in highly correlated b and c values and will give large asymptotic confidence intervals around b and c.

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Chapter 3

Validation of a combined temperature, water activity, acidity model for the description of bacterial growth of *Lactobacillus curvatus*

ABSTRACT

A model is set up to describe growth rate as a function of the controlling variables temperature, acidity and water activity. The model is based on two, earlier developed models, one for growth rate as a function of temperature and water activity and one for temperature and acidity. Based on the assumption that combinatory effects between acidity and water activity do not exist, the two models were multiplied to yield one model. The model was then fitted to data sets measured earlier, and the parameters of the model were determined. A new data set with values for controlling variables beyond the range of the model earlier developed, was then used to validate the newly developed model. The model was well able to predict outside the measured data range. Finally, the model was updated with all measured data. No significant changes in the parameters were observed.

INTRODUCTION

Quality and shelf life of foods is often determined by presence and growth of bacteria. Foodborne bacteria can be divided into spoilage and pathogenic species. Growth of bacteria is only allowed, to a certain extent, for spoilage organisms. Presence and growth of pathogenic organisms in foods, should be avoided as much as possible by inactivating the living cells or altering the product formulation in such a way that growth of these organisms is slow or even absent. In previous articles, growth of bacteria as functions of water activity, temperature or acidity (1, 4, 5, 7) was described. McMeekin *et al.* (4), for instance, describe the combined effect of water activity and temperature on bacterial growth rate. Wijtzes *et al.* (6) describe growth of *Lactobacillus curvatus* as a function of temperature and acidity. The effect of temperature, pH and water activity on bacterial growth rate of *Listeria monocytogenes* was modelled previously by Wijtzes *et al.* (7). The mentioned models contain fit parameters that have an interpretable meaning. The model proposed by McMeekin *et al.* (4) contains a theoretical minimal temperature and a minimal water activity. The model described by Wijtzes *et al.* (6), apart from theoretical minimal temperature, contains theoretical minimal and maximal pH. Estimates of the values for these parameters can be looked up in literature or assessed by relatively simple experiments; therefore, in this paper, this type of models will be used.

In this paper, models for bacterial growth rate as function of temperature and water activity, and growth rate as function of temperature and acidity are combined into one model describing growth rate as function of temperature, water activity and acidity. The parameters of the model are calculated on the basis of earlier measured data. The predictions of the model are then used to validate the model with a newly measured growth rate data set where the values of the controlling variables lie outside the earlier measured temperature, water activity, acidity data range.

Models. Growth rate as a function of suboptimal growth temperature was modelled by several researchers (1, 4, 5, 7) as (eq. 1)

$$\mu = b_1\left(T - T_{\min}\right)^2 \tag{1}$$

where μ (h^{-1}) is the specific growth rate of a microorganism at sub-optimal temperature T (°C), and b_1 (h^{-1} °C^{-2}) and $T_{\min}$ (°C) are regression coefficients. $T_{\min}$ represents the theoretical suboptimal temperature where growth rate just equals 0 (h^{-1}). Since the value of $T_{\min}$ was

found to be independent of acidity, Wijtzes *et al.* (6) propose to substitute the effect of acidity into parameter b_1 resulting in equation 2 where pH_{min} is the minimal pH, and pH_{max} the maximal pH at which growth stops. pH_{min} and pH_{max} were found to be independent of temperature. b_2 is a regression coefficient ($h^{-1}\,°C^{-2}$).

$$\mu = b_2(\mathrm{pH} - \mathrm{pH}_{min})(\mathrm{pH} - \mathrm{pH}_{max})(T - T_{min})^2 \qquad (2)$$

The same was done earlier by McMeekin *et al.* (4), a water activity model was substituted into b_1, resulting in equation 3 where $a_{w,min}$ equals the minimal water activity at which growth stops and b_3 is a regression coefficient ($h^{-1}°C^{-2}$).

$$\mu = b_3(a_w - a_{w,min})(T - T_{min})^2 \qquad (3)$$

By analogy with the above, an overall model is now set up that describes the effect of growth rate as a function of all three controlling variables, temperature, acidity and water activity (eq. 4), and b_4 ($h^{-1}°C^{-2}$) is a regression coefficient.

$$\mu = b_4(a_w - a_{w,min})(\mathrm{pH} - \mathrm{pH}_{min})(\mathrm{pH} - \mathrm{pH}_{max})(T - T_{min})^2 \qquad (4)$$

In this model, the value of pH_{min}, and pH_{max} are assumed to be independent of water activity, and the value of $a_{w,min}$ is assumed to be independent of acidity. In the range of controlling variables where the model is set up and validated, it is unlikely that the regression parameters are functions of other controlling variables, since none of the variables are at extreme values at the same time. Combination effects are more likely to occur near the extremes of the controlling variables.

MATERIALS and METHODS

Four data sets are used; three data sets are used to fit the parameters of the model and the fourth data set is used for validation. All data are based on plate counts, in order to measure the growth characteristics, where growth is not yet affected by macroscopic effects such as pH-changes, nutrient depletion and interactions between organisms. The first data set was measured to model the growth characteristics of *Lactobacillus curvatus* (LAB 962) as a function of temperature and acidity. The acquisition of this data set was described by Wijtzes

et al. (6). The measured water activity of this entire data set equals 0.992. A second data set was measured at a constant pH value of 6.2 over the entire water activity and suboptimal temperature range. Water activity was set using NaCl (Merck) and measured using a Novasina calibrated with different salt solutions. The same was done for the third data set; in this case however, the pH was set to 5.8 using 2 N sterile HCl (Merck). The aquisition of these data sets is described by Kant-Muermans *et al.* (2). The three data sets are combined into one, which consist of 164 growth curves. These combined data sets are called "old data set" from now on.

The fourth data set was measured outside the temperature, water activity, acidity range where the model parameters are fitted. MRS (Difco) was used as growth medium for *Lactobacillus curvatus* (LAB 962). Water activity was set using NaCl (Merck) and measured using a Novasina (ER84/3H/63T; sensors: enBSK-4) calibrated with different salt solutions. pH was set using 2 N sterile HCl (Merck) and 2 N sterile NaOH (Merck). The chosen combinations at which growth was followed were, for temperature, 7, 10, 15, and 28°C, for water activity, 0.95, 0.96, and 0.97, and for pH, 5, 6, 7, and 7.5. Various combinations were measured in replicate, resulting in a total number of datum points of 47. This data set is called "new data set" from now on.

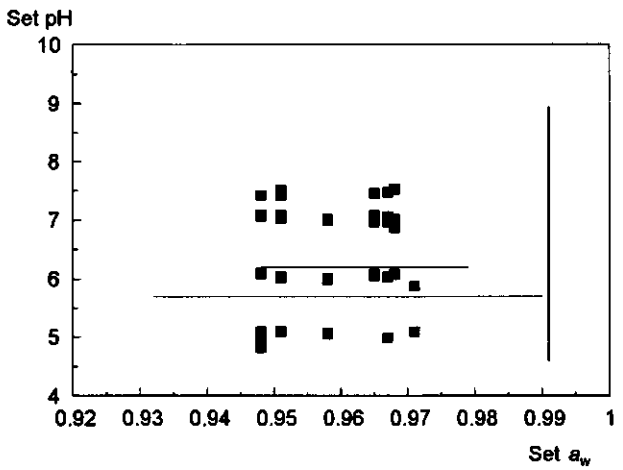

FIG. 1. Experimental setup; Earlier measured growth curves (—) and newly measured growth curves at variable temperatures (■).

Fig. 1 shows the experimental design at varying temperatures. The lines represent the experiments of the old data set, the datum points are the measured growth curves of the new data set.

In all cases numbers of microorganisms in time are fitted to the modified Gompertz equation (9) resulting in estimates for lag time, growth rate, asymptote and initial number of organisms. A non linear regression program with a Marquardt optimisation procedure was used.

Modelling procedure. Growth rates at different temperatures, water activities and acidities are fitted to equation 4 by means of a least square fitting routine that directly estimates the values of the parameters. To stabilise the variance of growth rate data, a square root transformation of growth rate was required. Since the model was set up for non-transformed growth rate, the right-hand side of equation 4 has to undergo the same transformation. The old data set is fitted to the model and the values for the parameters and 95 % confidence intervals are calculated. Model predictions (fitted) are plotted against the measured values using the square root transformation method. The residuals, deviations from the diagonal, should be distributed homogeneously throughout the range of observed values and the values should not be too far away from the diagonal. Furthermore, the 95% prediction interval of each of the fitted growth rates is calculated (3) and plotted in the figures. Finally, an F-ratio test is carried out to assess the statistical quality of the model predictions.

The comparison of the measured growth rates from the new data set with the predictions of the fitted model is performed next. The statistical acceptability is assessed in a predicted versus measured plot. The calculated 95% prediction interval of the equality line is used to assess the acceptability of the datum points of the new data set; if at least 95% of the measured datum points fall inside the plotted 95% prediction interval, the model is accepted statistically. Furthermore, an F-ratio test is used to calculate the statistical acceptability of the predictions of the model.

In the last stage of the modelling procedure, the growth rate model (eq. 4) is fitted to all measured data. A fitted versus measured plot gives a graphical representation of the statistical acceptability of the model predictions. An F-ratio test is used to calculate the statistical acceptability of the predictions of the model.

***F* ratio test.** To asses the statistical quality of a model, an F ratio test can be used. Two variances are compared, the variance of the fitted model and a typical $\sqrt{\mu}$ variance (5.11×10^{-3})

at 20 degrees of freedom, (8)). The model is found to apply if the variance of the model is not significantly larger than the typical growth rate variance. This can be assessed by means of an f ratio value, which is calculated as: $f = \frac{\text{var(model)}}{\text{var(reference)}} = \frac{\text{RSS}/\text{df}}{5.11 \times 10^{-3}}$, where RSS is the residual sum of squares of the fitted model and df are the degrees of freedom of the model. This f ratio value should be smaller than a reference 95% F_{20}^{df} value, to obtain statistically significant equal model prediction.

RESULTS and DISCUSSION

The old data set is fitted to the overall model resulting in an estimate for the parameters of the overall model (eq. 4) as given in Table 1. Fig. 2 shows the square root of the predicted growth rates versus the square root of the observed growth rate. As can be seen the distribution of the errors is homogeneous throughout the entire range of measured growth rates. The calculated 95% prediction interval of the fitted growth rates is also shown. Of the measured data set, 95% of the data has to fall within the prediction intervals. Of the old data set of 164 datum points, 156 points fall inside the 95% prediction interval which is exactly 95% of the datum points.

TABLE 1. Values of the estimated parameters from equation 4 and their 95 % confidence intervals; on basis of three data sets

Parameter	Estimated value	95 % lower confidence interval value	95 % upper confidence interval value
b_4 ($h^{-1}°C^{-2}$)	-2.34×10^{-3}	-2.74×10^{-3}	-1.94×10^{-3}
T_{min} (°C)	-3.63	-4.66	-2.61
pH_{min}	4.24	4.11	4.37
pH_{max}	9.53	9.33	9.73
$a_{w,min}$	0.928	0.925	0.931

The degrees of freedom of the fitted model are 159, the RSS of the fitted model equals 0.827, and the variance of the model is 5.20×10^{-3}. The calculated f value equals 1.02, whereas F_{20}^{159}=1.88, therefore, the variance of the model equals the variance of the measuring error so the model predictions are accepted statistically.

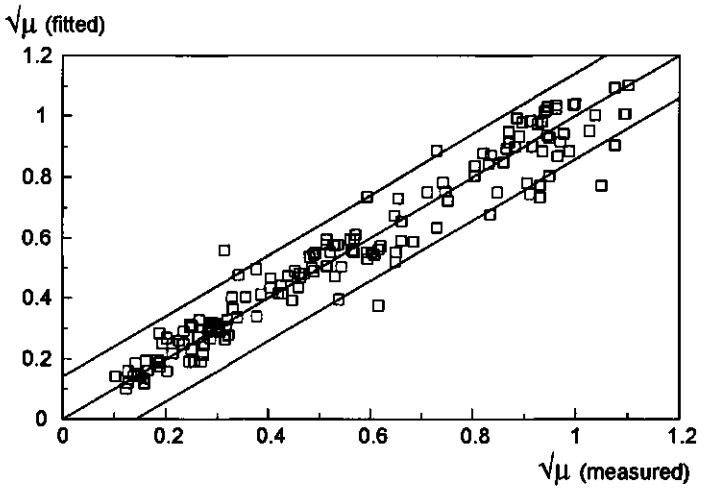

FIG. 2. Fitted versus measured transformed growth rate $\left(\sqrt{\mu}\right)$ and 95 % prediction interval of the model based on the old data set.

The model with the fitted parameters, based on the old data set, is now used to predict growth rates of the new data set. The measured growth rates from the old data set (□) and the new data set (■) are plotted against the predicted growth rates (Fig. 3). The closer the datum points are to the diagonal, the better the model predicts the newly measured growth rates. Only one datum point of the new data set falls outside the 95% prediction interval calculated with the model based on the old data set. This is only 2% of the newly measured data, whereas 5% is allowed for this prediction interval. The measurements of the new data set, therefore, can not be distinguished statistically from the predictions of the model based on the old data set.

The degrees of freedom for the prediction of the new data set are 47, since no parameters are estimated, the RSS of the model predictions equals 0.188, the variance of the model predictions, therefore, is 4.0×10^{-3}. The calculated f value equals 0.783, whereas $F_{20}^{42} = 2.03$. The model predictions are therefore accepted statistically.

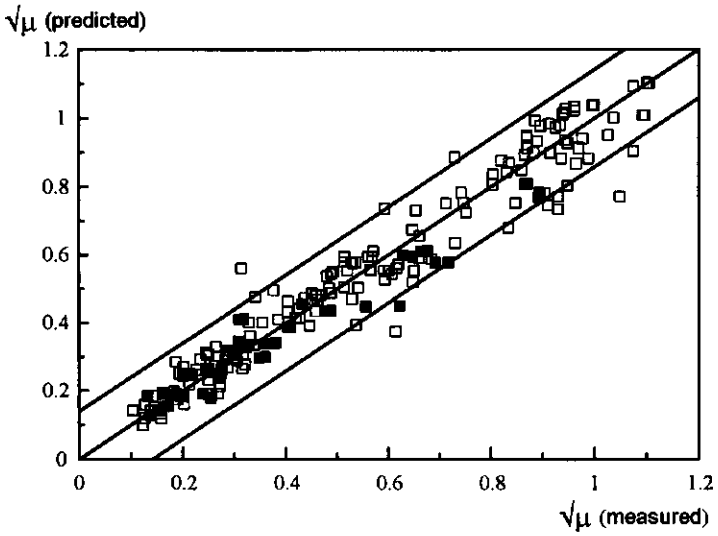

FIG. 3. Predicted (■) and fitted (□) versus measured transformed growth rate $\left(\sqrt{\mu}\right)$ and 95 % prediction interval of fitted model. Model extrapolation for new data set.

Growth rate from the new data set can be predicted with the model developed for the old data set. Extrapolation should not be considered good practice, since models are only valid in the range where actual data were gathered, although in this case it is statistically allowed to extrapolate.

TABLE 2. Values of the estimated parameters from equation 4 and their 95 % confidence intervals; all measured data sets

Parameter	Estimated value	95 % lower confidence interval value	95 % upper confidence interval value
b_4 ($h^{-1}°C^{-2}$)	-2.35×10^{-3}	-2.72×10^{-3}	-1.97×10^{-3}
T_{min} (°C)	-3.32	-4.20	-2.43
pH_{min}	4.23	4.10	4.35
pH_{max}	9.53	9.34	9.72
$a_{w,min}$	0.926	0.923	0.929

Finally, to refine the developed model, all data sets are used to re-estimate the value of the parameters. The parameter estimates are shown in Table 2. The degrees of freedom of the entire model are 206, the RSS of the model is 0.995, and the variance of the model equals 4.83×10^{-3}. The calculated f value equals 0.945 whereas the reference $F_{20}^{206}=1.87$, so the model is accepted statistically. Fig. 4 shows the square root of the fitted growth rate versus the square

root of the measured growth rate. All points are close to the diagonal, which was also indicated by the results of the F ratio test.

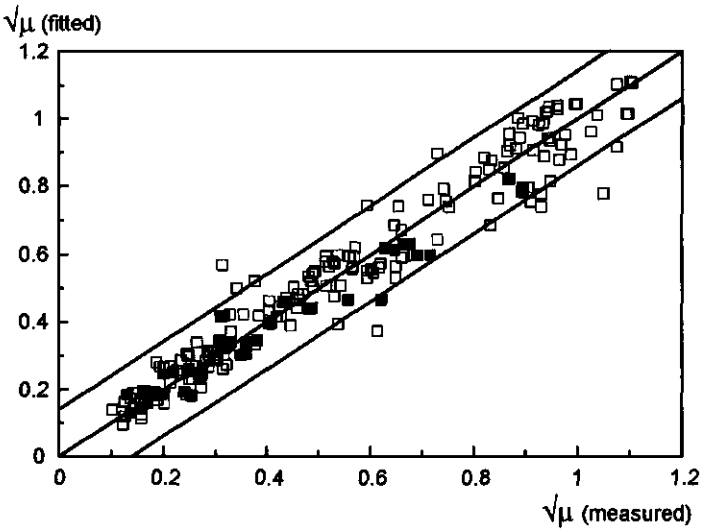

FIG. 4. Fitted versus measured transformed growth rate $\left(\sqrt{\mu}\right)$ for all measured data sets.

As can be concluded from Table 1 and 2, the estimated parameters nearly remain constant and the confidence intervals of all fitted parameters of the equation overlap. Because the values of the fitted parameters do not change significantly by the addition of the new data set, the measurement of this new data set was not neccesary for the estimation of the parameters. This leads to the conclusion that this type of model can be set up after performing a statistically satisfactory minimal number of experiments to estimate the parameters. Therefore, the determination of these parameters becomes easier and the interpretability of the model is enhanced.

The preliminary assumption, that effects of water activity, acidity and temperature on bacterial growth rate can be multiplied over a fairly wide range of the controlling variables remains likely.

ACKNOWLEDGEMENTS

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Chapter 4

Modelling bacterial growth of *Listeria monocytogenes* as a function of water activity, pH and temperature.

ABSTRACT

Temperature, pH and water activity are important factors controlling the microbiological safety of foods. To describe the growth rate of *Listeria monocytogenes* in relation to these factors, two equations have been developed. Both equations are based upon the Ratkowsky equation for temperature and growth rate. The first equation predicts growth rate at suboptimal pH values, suboptimal temperatures and suboptimal water activities, the second model predicts growth throughout the entire pH range. The first model may be used to predict growth rates between pH 4.6-6.7, temperature range 5-35°C and a water activity range of 0.95-0.997. The second model is valid throughout the pH range of 4.6-7.4 and the same temperature and water activity range as the first model.

INTRODUCTION

Microbial activity often influences the quality or safety of foods. To control microbial survival and outgrowth of microorganisms in foods, food preservation procedures are used. The most common preservation procedure is storing food at low temperatures. Other common ways to control microbial spoilage and growth of pathogens in foods are reduced pH in combination with low temperature and the adjustment of the water activity.

In recent years the interest in developing mathematical models that describe growth of microorganisms has increased. These models can predict changes in numbers of microorganisms in a product with time, dependent upon the physical and chemical condition of the product (8, 16).

Gompertz (7) developed a model that describes human mortality as a function of age. For microbiological use this model was reparameterized by Zwietering *et al.* (23) to include microbiologically significant parameters such as maximum growth rate (μ_m), lag time (λ) and asymptotic level of microorganisms (A) [e = exp(1)].

$$\ln\left(\frac{N}{N_0}\right) = A\exp\left\{-\exp\left[\frac{\mu_m e}{A}(\lambda - t)+1\right]\right\} \qquad (1)$$

where N is the number of microorganisms at time t and N_0 is the number of microorganisms at time t=0.

A model that describes the relationship between maximum specific growth rate (μ_m) of a microorganism and temperature at sub-optimal conditions for growth is the square root model of Ratkowsky *et al.* (15).

$$\sqrt{\mu_m} = b_1(T - T_{min}) \qquad (2)$$

where b_1 is a regression coefficient; T is the actual temperature (°C) and T_{min} is the extrapolated minimum growth temperature (°C) for the microorganism.

This equation has been modified by McMeekin *et al.* (11) to incorporate water activity (a_w).

$$\sqrt{\mu_m} = b_2\sqrt{(a_w - a_{w,min})}(T - T_{min}) \quad (3)$$

where b_2 is a new regression coefficient; a_w is the current water activity and $a_{w,min}$ is the extrapolated minimum water activity for growth of the microorganism.

Recently the square root model of Ratkowsky was modified by Adams *et al.* (1) to describe the growth rate of *Yersinia enterocolitica* in response to pH and temperature (equation 4):

$$\sqrt{\mu_m} = b_3\sqrt{(pH - pH_{min})}(T - T_{min}) \quad (4)$$

pH_{min} indicates the extrapolated minimum pH at which microorganisms will grow, pH indicates the actual acidity and b_3 is a regression coefficient. Equation 4 was validated for different acidulants; the value of the regression coefficient was different for each different acidulant, whereas the value for pH_{min} and the value for T_{min} were affected slightly by the type of acidulant.

Listeria monocytogenes is a pathogenic microorganism that is unique in its tolerance of factors that would normally prevent, or severely limit, growth. Walker *et al.* (20) found growth of *L. monocytogenes* in chicken broth at a temperature as low as -0.4°C. The lowest water activity for growth was reported by Petran and Zottola (12) to be 0.92. The minimum pH for growth is found to be 4.39 (6), the maximum pH for growth is reported to be 9.2 and the optimum pH for growth of *L. monocytogenes* is 7.0 (12).

The objective of this work is to describe growth of *L. monocytogenes* as a function of pH, water activity and temperature.

MATERIALS and METHODS

Organism. *Listeria monocytogenes* (NCTC 9863)

Media. NaCl was added to Tryptone Soya Broth (TSB, Oxoid CM 129; 30 gram per litre) during preparation to give final concentrations of 5-80 gram per litre. Media at each NaCl concentration were made in 1500 ml volumes, adjusted to the appropriate pH (4.6, 4.9, 5.3, 6.0, 6.3, 6.7, and 7.4) using 5 M HCl and dispensed in 300 ml volumes.

Tryptone Soya Agar (TSA) for plate counts was prepared using TSB (30 gram per litre) with 1% agar (Lab M, no.2). The pH was adjusted to 7.2. Maintenance medium (1 gram per litre bacteriological peptone (Lab M), 8.5 gram per litre NaCl, pH adjusted to 6.4) was used for all serial dilutions for plate counts and for dilution of the inoculum. The stock culture was maintained on Nutrient Agar (NA) slopes at 4°C. All media were autoclaved at 121°C for 15 min.

Inoculum. *L. monocytogenes* was grown in Heart Infusion Broth (HIB) (Difco) for 24 hours at 30°C (circa 10^8 cells per ml), diluted, and 1 ml used to inoculate each 300 ml volume of TSB, using a 1 ml repeating syringe (Eppendorf Multipette 4780, Baird and Tatlock Ltd, London) to give a final concentration of approximately 10^3 cells per ml.

Experimental procedure. TSB in 300 ml volumes was maintained overnight at the intended storage temperature prior to inoculation. Immediately after inoculation with *L. monocytogenes* and thoroughly mixing, TSB was aseptically dispensed in 10 ml volumes, using a peristaltic pump (Accuramatic-5, Jencons), into sterile 1 oz (28.4 ml) universal bottles with plastic screw caps. Viable numbers in each treatment combination were determined by direct plating of 20 μl on TSA immediately after dispensing and the time was noted. The remaining bottles were incubated at the appropriate temperature.

At appropriate time intervals during storage, decimal dilutions were made from separate bottles of TSB and 20 μl of each plated onto TSA. The plates were incubated for 24 hours at 30°C, colonies counted and viable numbers calculated according to the method of Farmiloe *et al.* (5) and expressed as ln(cfu) per ml.

Determination of pH and NaCl. Measurements of pH were made using a Whatman PHA 230 pH meter. NaCl concentrations of TSB were determined using the Official Method of the Society for Analytical Chemistry (9). The water activity was calculated from the NaCl concentration (13).

Experimental design. The limits of the experimental plan were chosen after a preliminary screening experiment to determine the growth limits of the strain used. Eighty two combinations of the following conditions were used:

storage temperature (°C): 5, 8, 10, 15, 20, 25, 30, 35.
pH: 4.6, 4.9, 5.3, 6.0, 6.3, 6.7, 7.4.
NaCl concentration (g/l) (a_w): 5 (0.997), 20 (0.989), 40 (0.977), 60 (0.964), 70 (0.957), 80 (0.950).

The combinations were chosen (with some replicates) between the experimental limits:

TABLE 1. Experimental plan; number of measured growth curves

a_w	pH	Temperature (°C)							
		5	8	10	15	20	25	30	35
0.997	4.6		1	1	1		1		1
0.977	4.6			1		1			1
0.977	4.9		1						
0.957	4.9				1		1		1
0.997	5.3	1		2					
0.989	5.3			3			2		
0.977	5.3			1		1			
0.964	5.3			3			2		
0.950	5.3			1					
0.997	6.0	3				1			1
0.989	6.0	2				2			
0.977	6.0	3		1		2	1	1	
0.964	6.0	2				2			
0.950	6.0			1		1			1
0.957	6.3	1							
0.997	6.7	1		1					
0.989	6.7			2			2		
0.977	6.7					1			
0.964	6.7			2			2		
0.950	6.7			1					
0.997	7.4	2		1	1		1		1
0.989	7.4	1							
0.977	7.4	1				1		1	
0.964	7.4	1							
0.950	7.4	1	1	1	1		1		1

Statistical modelling. Modelling was carried out in two stages. At each combination of NaCl (water activity), temperature and pH the bacterial counts were fitted to the Gompertz equation (equation 1) by a non-linear regression package using a Marquardt algorithm (23). The package estimates the maximum growth rate (μ_m), the final level of microorganisms (A = ln (N_∞/N_0)), the lag time (λ) and their 95 % confidence intervals. For the second stage a statistical

package for performing multiple non-linear regression was used to fit the growth rate to the controlling factors temperature, water activity and pH.

RESULTS

All growth curves gave good fits with the Gompertz model. In the second stage of the modelling, two different relations between the maximum growth rate, pH, temperature and water activity were used to fit the data. The first relationship is based upon the combination of equations 3 and 4. This model will only be valid in the suboptimal pH range.

$$\sqrt{\mu_m} = b_4\sqrt{(a_w - a_{w,min})}\sqrt{(pH - pH_{min})}(T - T_{min}) \quad (5)$$

where b_4 is a regression coefficient and the other parameters are as above.

Ratkowsky *et al.* (14) described a nonlinear regression model for fitting growth rate and temperature throughout the entire biokinetic temperature range. This relationship is adapted for pH instead of temperature in equation 6, where pH_{min} is the extrapolated minimum pH for growth, pH_{max} the extrapolated maximum pH for growth and b_5 and c are regression coefficients. This equation can be used to describe growth throughout the entire pH range.

$$\sqrt{\mu_m} = b_5(pH - pH_{min})\{1 - \exp[c(pH - pH_{max})]\} \quad (6)$$

The second relationship between growth rate, pH, temperature and water activity to apply in this case is derived from the combination of equation 3 and 6. Here b_6 and c_{pH} are regression coefficients.

$$\sqrt{\mu_m} = b_6\sqrt{(a_w - a_{w,min})}(pH - pH_{min})\{1 - \exp[c_{pH}(pH - pH_{max})]\}(T - T_{min}) \quad (7)$$

In Table 2 the results of the regressions are shown. The first two rows of the table show models that do not use the entire data set. The first uses only that part of the data set where the pH is less than or equal to 6.3, from now onwards to be called equation 5a. The second row shows a model that uses a larger pH range, here the pH is less than or equal to 6.7, from now onwards to be called equation 5b. Both pH ranges are sub-optimal (12). The last row in the table shows the values derived from literature.

TABLE 2. Regressed values for $a_{w,min}$, pH_{min}, pH_{max} and T_{min} for growth of *L. monocytogenes*

Equation	$a_{w,min}$	pH_{min}	pH_{max}	T_{min} (°C)
5a[a]	0.912	4.15	-[c]	-2.05
5b[b]	0.913	4.03	-[c]	-1.75
7[d]	0.916	3.84	9.82	-2.55
Refs.	0.92 (12)	4.39 (6)	9.2 (12)	-0.4 (20)

[a] : Not the entire data set; pH ≤ 6.3 (53 datum points).
[b] : Not the entire data set; pH ≤ 6.7 (65 datum points).
[c] : Not present in model.
[d] : Entire data set (82 datum points).

The predictions from both models were compared to the original data sets by comparing predicted growth rates at different constant conditions with the data set values. For this a 'general model' is introduced that uses the mean values of the measured data where the controlling factors are constant (equation 8). It describes the data sets as ".. at constant conditions with respect to one temperature, one pH value and one water activity value the growth rate is found to be ...". Therefore it cannot be used as a predictive model.

$$\overline{\mu}_m(i) = \sum_{j=1}^{n} \frac{\mu_m(i,j)}{n} \tag{8}$$

where $\mu_m(i,j)$ is the j^{th} growth rate at constant conditions i and $\overline{\mu}_m(i)$ is the mean growth rate at these constant conditions; n is number of replicates at constant conditions.

The statistical comparison was carried out using a F ratio test. Although this testing method is not valid for nonlinear equations it can give a good indication of the suitability of the model and how well it describes the data set. All models are tested against the general model that applies in the region of controlling factors. As stated by various workers this method must be seen as an approximation, not as a rigorous statistical analysis (19, 22).

The measuring error for the 'general model' is estimated by determining the deviation of the measured values from the mean value at a set of constant conditions. The residual sum of squares (RSS_1) is calculated from the deviation. The degrees of freedom (df_1) of this model are calculated as the total number of datum points minus the number of different constant conditions.

The error of prediction of a model is estimated by determining the deviation between the predictions from the model and the measured values. From these values the residual sum of squares (RSS_2) can be derived. The degrees of freedom (df_2) are calculated as the number of datum points minus the number of parameters in the model.

The lack of fit of the models can be estimated by taking the difference between RSS_2 and RSS_1. When this value is much smaller than the measuring error (RSS_1), the model is adequate. This comparison between the lack of fit and the measuring error can be quantified statistically by the f testing value:

$$f = \frac{(RSS_2 - RSS_1)/(df_2 - df_1)}{RSS_1/df_1} \tag{9}$$

The value for f is tested against a 95 % confidence $F_{df_1}^{df_2-df_1}$ value; if the value for f is smaller than the value for F the model is statistically acceptable.

In Table 3 the residual sum of squares of the general model (RSS_1), the degrees of freedom of the general model (df_1), the values for the residual sum of squares of the different models (RSS_2), the degrees of freedom of the models (df_2), the f and the F value are given. For all equations the value for f is smaller than the value for F, which indicates that all models are statistically acceptable.

TABLE 3. Statistical evaluation of the models

Equation	RSS_1	df_1	RSS_2	df_2	f	F
5a[a]	0.0495	16	0.2647	49	2.11	2.17
5b[b]	0.0923	20	0.3933	61	1.59	1.99
7[c]	0.0924	21	0.4204	76	1.36	1.91

[a] : Not the entire data set; pH ≤ 6.3 (53 datum points).
[b] : Not the entire data set; pH ≤ 6.7 (65 datum points).
[c] : Entire data set (82 datum points).

In Table 4 the predictions of the generation time of *L. monocytogenes* from the different models are compared with generation times from literature for various strains of *L. monocytogenes* in various growth media.

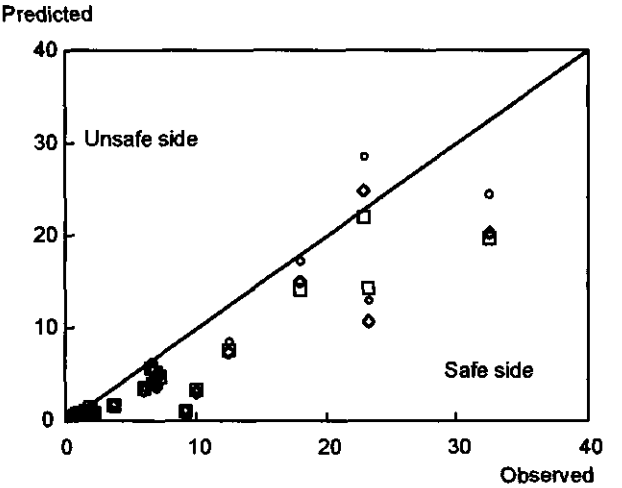

FIG. 1. Predicted generation time (h) versus observed generation time (h). Equation 5a (◊), Equation 5b (o), and Equation 7 (□).

Fig. 1, which is derived from Table 4, shows the predicted generation time versus the observed generation time. Whenever the predicted generation time and the observed generation time are equal, the result can be found on the diagonal in Fig. 1 which leads to an exact prediction. The 'unsafe', left-hand side of the diagonal, represents an area in which the predicted generation time is longer than the observed generation time (the organisms grow faster than predicted). When the predicted values are smaller than the observed values the shelf life of a food is estimated shorter than the real shelf life. This area can be found on the right-hand side of the diagonal indicated by 'safe side'.

TABLE 4. Comparison of predicted and observed values for generation time of *L. monocytogenes*, with observed values from literature

a_w	pH	T (°C)	Substrate	Strain	Generation time[a] (h)				Ref.
					Obs.	Eq. 5a	Eq. 5b	Eq. 7	
1.00	5.6	30	Unclarified cabbage juice	LCDC 81-861	9.16	1.06	1.13	1.04	(4)
1.00	6.2	30	Clarified cabbage juice	"	1.52	0.75	0.82	0.79	(4)
0.997	6.2	30	"	"	1.81	0.78	0.85	0.82	(4)
0.994	6.2	30	"	"	1.80	0.81	0.88	0.85	(4)
0.991	6.2	30	"	"	1.92	0.84	0.91	0.88	(4)
0.989	6.2	30	"	"	2.18	0.88	0.95	0.91	(4)
0.986	6.1	6	*Camembert* cheese	OH	18.0	15.0	17.3	14.1	(18)
0.997	7.2	5	Growth medium	109	23.25	10.7	13.0	14.3	(21)
0.997	7.2	10	Growth medium	109	6.93	3.69	4.27	5.18	(21)
0.997	7.2	25	Growth medium	109	1.58	0.73	0.82	1.07	(21)
0.995	6.2	30	Growth medium	109	0.91	0.52	0.58	0.77	(21)
0.995	6.4	10	Whole milk	Scott A	6.6	5.65	6.39	5.64	(10)
0.995	6.4	4	Skimmed milk	Scott A	32.5	20.3	24.5	19.7	(17)
0.995	6.4	8	Skimmed milk	Scott A	12.5	7.38	8.51	7.60	(17)
0.995	6.4	13	Skimmed milk	Scott A	6.0	3.30	3.71	3.49	(17)
0.995	6.4	21	Skimmed milk	V 7	1.9	1.41	1.56	1.53	(17)
0.995	6.4	35	Skimmed milk	V 7	0.7	0.55	0.60	0.60	(17)
0.98	5.9	15	Asparagus	Scott A	6.7	4.14	4.47	3.97	(2)
0.98	6.5	15	Broccoli	Scott A	10.0	3.08	3.39	3.32	(2)
0.98	5.6	15	Cauliflower	Scott A	7.2	4.99	5.32	4.70	(2)
0.99	5.6	5	Lean meat	Scott A	22.9	24.8	28.5	22.0	(3)
0.99	5.6	25	Lean meat	Scott A	3.7	1.70	1.81	1.65	(3)

[a]: Obs. = Observed generation time.
Eq. 5a = Predicted generation time by equation 5a.
Eq. 5b = Predicted generation time by equation 5b.
Eq. 7 = Predicted generation time by equation 7.

DISCUSSION

Mathematical models to predict growth of microorganisms are promising tools to safeguard foods from spoilage or pathogenic microorganisms, especially when the model consists of easily determinable parameters. We tried to develop models that use growth parameters of

microorganisms such as minimum pH, maximum pH, minimum temperature and minimum water activity and environmental variables pH, temperature and water activity.

The first two models based on equation 5 can only be used below the optimal pH whereas the pH range for the third model (equation 7) goes beyond the optimal pH. It should be noted that the models are valid in a temperature range from 5 to 35°C and a water activity range from 0.95 (80 gram per litre NaCl) to 0.997 (5 gram per litre NaCl) whereas the first model applies in a pH range from 4.6 to 6.3, the second model from 4.6 to 6.7 and the third model a pH range from 4.6 to 7.4.

The first two models (suboptimal pH and temperature) estimate the minimum water activity to support growth, very close to the reported minimum water activity for growth as can be seen in Table 2. According to Petran and Zotolla (12) the minimum value is 0.92 whereas the extrapolated value derived from equation 5a is 0.912 and the value derived from equation 5b equals 0.913.

The first model (equation 5a) estimates the minimal temperature 1.65°C lower than the reported value in literature by Walker *et al.* (20), and the estimated minimum pH is found to be 0.24 pH units lower than the reported minimum pH (6). Although this model cannot be used to describe growth throughout the entire pH and temperature range, it estimates the growth minima for pH, temperature and water activity close to the reported values.

The second model (equation 5b) estimates the minimum temperature a little better than the first model (1.4°C lower) (20). The minimum pH for growth is estimated 0.36 pH units lower than the value reported by George *et al.* (6).

The third model (entire pH range, suboptimal temperatures) also approximates the literature value for the minimum water activity very well, here the minimum water activity is 0.916. The estimated minimum growth temperature is 2.2°C lower than the literature value (20). The estimated minimum and maximum pH values are resp. 0.55 below (6) and 0.62 higher (12) than the reported values in literature.

The reason for the discrepancies between the reported values in the literature and the regressed values from the model may be found in the problem of detecting growth itself, close to the growth boundaries (e.g. minimum pH, minimum temperature etc.). Here, two factors play a very important role. First of all, lag time increases near the growth extremes; secondly,

the growth rate decreases near the extremes; at optimal values for pH and water activity the growth rate near the minimum for temperature approaches 0 divisions per h.

Considering Fig. 1 models 5a and 5b give one 'unsafe' prediction when used for the prediction of the generation time in lean meat at low temperature (Table 4). This temperature however is on the lowest temperature allowed for the usage of these models. Equation 7 gives a good estimate of the generation time in this case.

The predictions for the generation time of the models developed in this study are very good considering the facts that the models are developed for *L. monocytogenes* (NCTC 9863) whereas the observed generation times are for other species of *L. monocytogenes* (e.g. Scott A). Furthermore, most of the observed generation times are taken from foods whereas this study was carried out in broth. Most of the predictions are on the 'safe side' of the diagonal in Fig. 1. The predictions of equation 7 are always on the 'safe side' which is a good reason to choose for this model instead of the other two.

All models described are statistically accepted using the F ratio test. This means that an other criterion for the selection of the best model has to be formulated. The model that describes the full data set is the model that can be used in the largest range of controlling factors. The model that describes the full pH range (equation 7) therefore can be considered the best model.

In some cases the product is microbiologically far more stable than the models might predict. This problem might be related to the presence of other inhibiting factors not taken into account in these models.

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Chapter 5

Modelling bacterial growth and death rate as a function of acidity

ABSTRACT

In the first stage of modelling bacterial growth as a function of environmental parameters, bacterial numbers are related to time, and growth parameters, such as growth rate and lag time, are estimated. In the second stage, values for the growth parameters are related to the environmental parameters (temperature, pH etc.). A new equation for modelling bacterial growth in time under extreme conditions is described where attention is given to growth curves with initial death phases. In the second stage of the modelling procedure, models are developed that *contain interpretable parameters* (pH_{min}, pH_{max} and T_{min}). The *true interpretability* of the parameters pH_{min}, and pH_{max}, is given attention to in this chapter. The value of pH_{min} seems uniquely identified, however, some scatter is found around the value of pH_{max}. An explanation for the large scatter is sought in the composition of the MRS growth medium, and the physiology of the microorganisms.

INTRODUCTION

Mathematical modelling of growth of microorganisms takes place in two stages. In the first stage, a sigmoid curve is fitted to bacterial counts in time at constant environmental conditions, resulting in four parameters, initial numbers of microorganisms (N_0), lag time (λ), growth rate (μ), and asymptote (A) (1, 6, 8, 10, 11). Gathering growth data near the extremes of acidity and temperature is hard because growth is generally slow and sometimes initial death can be observed. Yet, evidence is needed to support the biological existence of a minimal and maximal pH value at which microorganisms stop growing. A different model should be used that takes into account the initial death phase.

In the second stage of modelling bacterial growth, mathematical models are developed that relate bacterial growth rate and lag time to the value of the environmental conditions (e.g. temperature, acidity, water activity) (2, 9, 12). Wijtzes *et al.* (10) related growth rate to environmental pH and environmental temperature (eq. 1).

$$\mu = b(\mathrm{pH} - \mathrm{pH}_{\min})(\mathrm{pH} - \mathrm{pH}_{\max})(T - T_{\min})^2 \qquad (1)$$

Wijtzes *et al.* (10) arrived at the conclusion that $\mathrm{pH}_{\min}$ and $\mathrm{pH}_{\max}$ were independent of temperature and $T_{\min}$ was independent of acidity. If temperature is kept constant, the temperature term can be omitted from the equation and a parabolic relation between growth rate and pH remains. The parameters ($T_{\min}$, $\mathrm{pH}_{\min}$ and $\mathrm{pH}_{\max}$) of the mathematical models were assumed to be fit parameters only and have no microbiological mechanistic meaning.

MATERIALS and METHODS

Organism. A pure culture of *Lactobacillus curvatus*, a typical spoilage organism of different types of meat and meat products (7, 8), was used as a model organism.

Microbial experiments. One constant temperature of 29°C was chosen to perform the growth experiments. Wijtzes *et al.* (10) previously determined the values for $\mathrm{pH}_{\min}$ and $\mathrm{pH}_{\max}$ for *L. curvatus* at 29°C to be respectively 4.24±0.30 and 9.49±1.19. The data of the experiments at 29°C (10) were used along with new data that were measured around the values of $\mathrm{pH}_{\min}$ and $\mathrm{pH}_{\max}$. *L. curvatus* was grown in MRS broth (Difco Laboratories). After sterilising the broth (121°C, 20 min), pH was set by using 2 N HCl (Merck) or 2 N NaOH (Merck) diluted in

sterile demiwater. The pH was measured with an electrode (Schott Geräte: N 5900 A) that was disinfected with 70 % alcohol (Merck, aqua dest.).

Before the experiments, a frozen culture (-18°C) of *L. curvatus* was cultivated twice at 30°C, first for 24 h and then for 16 h. To prepare for the growth experiment, the last preculture was diluted to reach an initial level in the broth between 10^5 and 10^7 CFU per ml, because an initial death phase was expected at extreme pH. The experiments were carried out in 50 ml tubes containing MRS-broth with pre-set pH and incubated at 29°C. Acidity was not controlled during the experiments.

BACTERIAL GROWTH CURVE under EXTREME CONDITIONS

During the lag phase and at extreme values of the environmental conditions, a decrease in bacterial numbers was observed. One example of this is presented in Fig. 1, where growth of *Lactobacillus curvatus* is followed at 29°C and pH=9.51. If initial death is observed, the earlier mentioned regular sigmoidal curve to relate numbers of microorganisms to time is not adequate. Therefore another equation is set up. It is assumed that a microbial population consists of two sub-populations (*A* and *B*). Population *A* dies immediately with a first order death rate constant of k (h^{-1}). First order inactivation kinetics in a batch system is used to relate microbial numbers with time.

$$N_{t,A} = N_{0,A} \times e^{-kt} \tag{2}$$

Population *B* is a part of the entire population that starts growing exponentially with growth rate μ (h^{-1}), with an initial number of population *B*, $N_{0,B}$. This population will also experience a lag time, λ_B (h). For the calculation of microbial numbers, first order growth in batch systems is used, where $t > \lambda_B$.

$$N_{t,B} = N_{0,B} \times e^{\mu(t-\lambda_B)} \tag{3}$$

The entire population consists of both population *A* and *B*. Therefore, $N_t = N_{t,A}+N_{t,B}$, which results in:

$$\ln(N_t) = \ln\left[N_{0,A} \times e^{-kt} + N_{0,B} \times e^{\mu(t-\lambda_B)}\right] \tag{4}$$

A cut off value is used for the asymptote. The value for this asymptote is determined by taking the average of the highest observed values. The proposed model is a mathematical representation of what is known as the intersection method. One line is drawn with slope $-k$, one line is drawn with slope μ, and one line is drawn through the asymptotic data. The time at intersection of the line with slope $-k$ and the line with slope μ is defined as the lag time of the overall population.

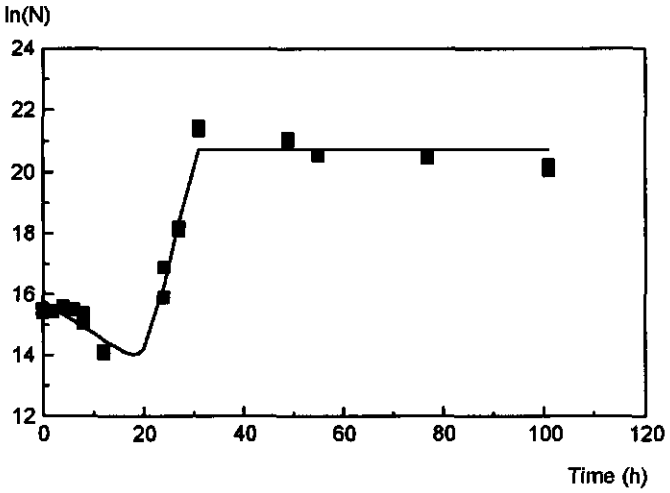

FIG. 1. Bacterial growth versus time with initial death phase.
Temperature 29°C; pH=9.51.

RESULTS and DISCUSSION

Population A. Equation 4 reduces into equation 2, if a population of microorganisms under extreme stress conditions does not contain microorganisms of population B, or if lag times of population B are very long (λ_B approaches infinity). In Fig. 2 an example is given of a population that only consists of dying organisms. The pH of this experiment was set to 10.

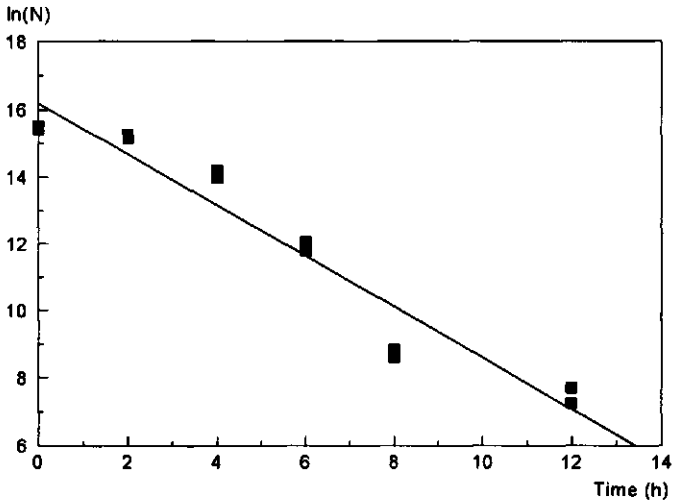

FIG. 2. Dying population at pH=10.

In Fig. 3, the fitted values of k of population A are given as a function of acidity. The highest pH near pH_{min} where initial death was observed equals 4.5, this is higher than pH_{min} but lies inside the 95% confidence interval. Near pH_{max}, death rate increases with increasing pH. The lowest pH at which initial death was observed is lower than pH_{max} but also lies inside the 95% confidence interval of pH_{max}. The values of pH_{min} and pH_{max} were determined for a growing population only.

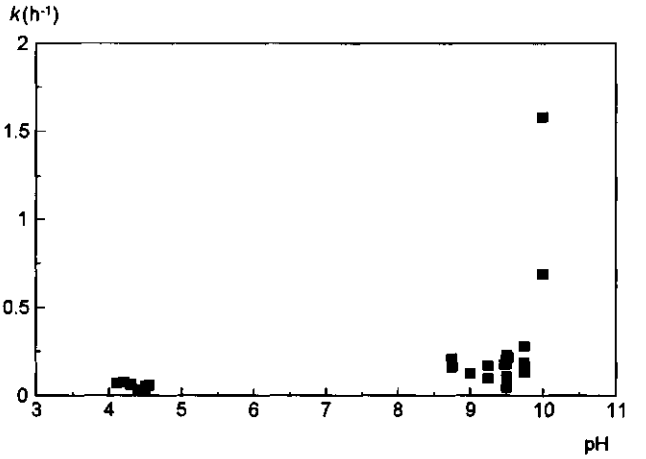

FIG. 3. Death rate k of population A as a function of pH.

Population *B*. In Fig 4 the growth rate of population *B* is shown as a function of acidity. As can be seen, below pH 4.5, no microbial growth is observed. This value is slightly higher than the theoretical minimum pH (4.24±0.30) for *Lactobacillus curvatus*, but lies inside the 95% confidence interval. This value might be slightly higher than pH_{min} because experiments close to pH_{min} result in initial death, as described earlier. If the initial death phase causes all bacteria to die, growth cannot be observed anymore, although, theoretically, growth might be possible.

Growth is observed even beyond pH_{max} (9.49±1.19), although the confidence interval around pH_{max} overlaps the range in which growth is observed. Near pH_{max} the scatter becomes very large, although a decline in growth rate is evident. Reasons for this scatter are sought below.

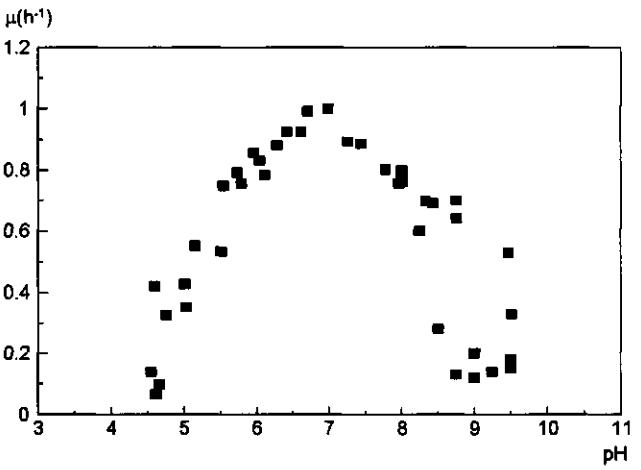

FIG. 4. Growth rate of population *B* as a function of acidity.

Combined model. The two population growth equation describes measured data well as can be seen in Fig. 1, which is typical for experimental data under extreme growth conditions. However, the equation contains two parameters that cannot be fitted independently, $N_{0,B}$ and λ_B. To be able to fit equation 4 to the data, the value of one of these parameters has to be fixed. It was assumed that $N_{0,B}$ equals one organism per tube, i.e. $N_{0,B} = 0.02$. In the case the 95 % confidence interval of the fitted value for k overlapped 0, the modified Gompertz equation (12) is used to fit the data.

The fitted values of both $-k$ and μ are shown in one graph in Fig. 5. As can be seen, a significant growth and death rate occur at one single pH near and above pH_{max} and below pH_{min}. The described parabola (eq. 1) is fitted to the data and new values for pH_{min} and pH_{max} are estimated. The fitted value for pH_{min} equals 4.38 ± 0.17, and pH_{max} equals 9.35 ± 0.10. The fitted value for pH_{min} nor pH_{max} change significantly (10).

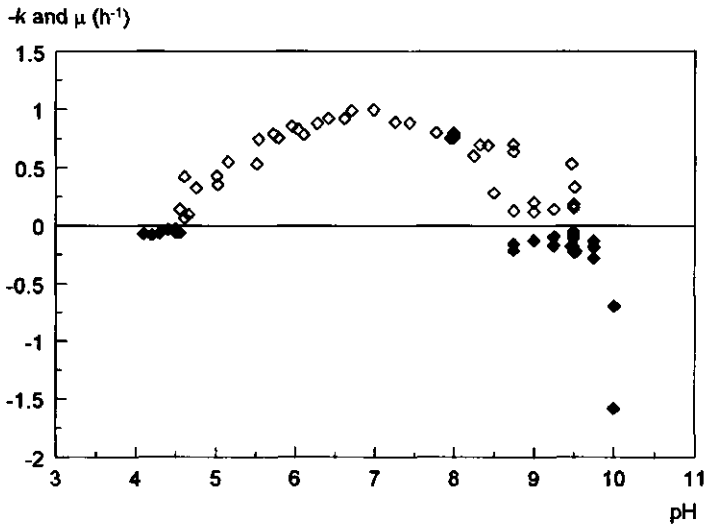

FIG. 5. Bacterial growth rate (✧), and death rate (◆) as a function of acidity.

Scatter. The scatter near pH_{max} might be a result of a poorly controlled pH throughout a single experiment. Therefore, pH was followed along with bacterial counts under extreme conditions. Fig. 6 shows that pH does not change significantly during the lag phase and the initial part of the exponential phase. Similar experiments at other pH values show the same results.

The pre-culturing method previous to the growth experiments may also be the cause of the observed scatter. The pre-culturing method is highly standardised (10), therefore the physiological state of the organisms prior to the growth experiments, is expected to be the same for each experiment. The microorganisms are then brought into the medium in which their growth is monitored. The shock microorganisms experience after inoculation in this growth medium should be constant every time the initial values of the environmental conditions are the same. However, an unexpected physiological inconsistency in the cultured cells may be another reason for the observed scatter.

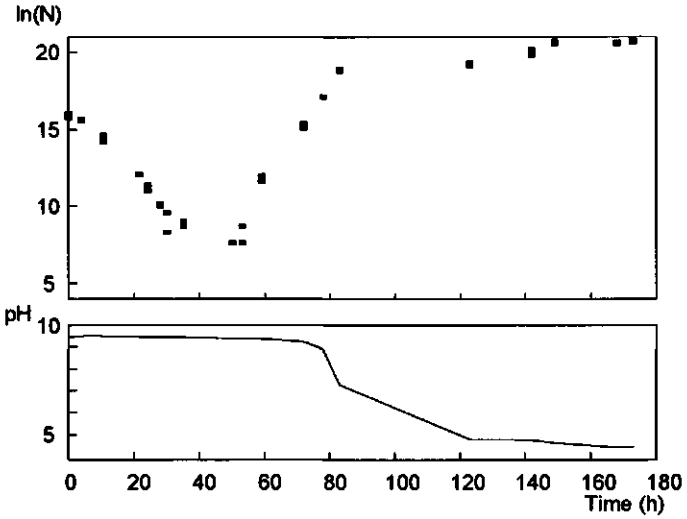

FIG. 6. Bacterial numbers (■) and acidity (–) as a function of time.
Initial pH=9.51.

Another explanation for the large scatter may be found in the composition of the MRS growth medium. The medium was developed for bacterial growth of lactic acid bacteria (5). Since these bacteria form acid, the medium is optimised for low acidity values. High acidity values may hydrolyse the proteins of the medium especially during sterilisation. However, the medium seems to have large enough buffer capacity, even at high acidity values (Fig. 6).

The existence and correct estimation of pH_{min} for bacterial growth, is of large importance in food technology, since foods and ingredients generally have pH values equal to neutral or below neutral (4). Adding acid to foods is a well known method for conservation, for instance to gherkins or onions. The existence of pH_{max} is of less importance to food technology, and more academic. There are no foods or ingredients that are stabilised at high pH values, because high pH values lead to saponification of fatty acids present.

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Chapter 6

A decision support system for the prediction of microbial food safety and food quality

ABSTRACT

Quality and safety of foods are often determined by the presence and growth of microorganisms. The development of a method to predict microbial food safety and quality is described. The construction of a food product from its ingredients is simulated, following a recipe. Food engineering heuristics are combined with models developed in predictive microbiology. Parameter values of ingredients of foods, such as water activity and acidity, and models for microbial growth and decay are used for the prediction of the microorganisms found in ingredients. The values of these parameters are collected in databases. If required information is lacking, reliable guesses of the parameters can be made. Food quality is calculated as function of fluctuating temperature in time. Several food distribution chains can be simulated to assess the influence of distribution chains on food quality. The described methods are implemented into a computerised decision support system that can be used in food production, product development and training. Furthermore, in future, it will be possible to apply expert knowledge in production and development of foods to improve the quality of prediction.

INTRODUCTION

Food quality is the result of numerous factors such as physical, chemical and microbiological characteristics. Rejection of a food on the basis of quality, can be a result of decay in the widest sense of the word. Presence or growth of microorganisms often cause problems with the quality of foods. Presence and growth of microorganisms are also important factors for the safety of foods. Other factors that play a role in the determination of food safety and food quality are, for instance, the presence of chemicals or food foreign substances such as glass fragments. Foods are usually recalled when they are considered unfit for consumption. Both decreased quality and product recall may have a large impact on credibility of food manufacturers and costs of food products (3, 6, 11).

At the moment standard procedures are in use that assess and control possible deviations from safety standards. These procedures, such as Good Manufacturing Practice (GMP), ISO, or the HACCP approach, are well known in food production. These procedures will be implemented more and more into food production, resulting in a better understanding of processes and connected possible problems or hazards (2, 3, 11). However detailed hazards are known and described, it proves very hard to actually control the possible hazards.

In predictive microbiology, the change in numbers of microorganisms in time can be calculated, given for example pH, temperature, or water activity of a food. The models that are developed in predictive microbiology can be used to calculate microbial numbers given a food composition, a food manufacturing line, or a food distribution chain. In combination with the above mentioned procedures, predictive microbiology gives improved, quantitative insight into the food properties and processes that are of importance to the safety and quality of foods (6, 16).

In this chapter the development of a computerised decision support system that simulates composition, production and distribution of foods is described. This computer system uses both mathematical models and expert knowledge of food technologists for the determination of the microbial numbers in foods. The developed decision support system can be used to find the microorganisms that grow in a specific food and the extent to which they do. The quality and safety of foods under production can be estimated. The microbiological quality of newly designed foods can be assessed without having to perform costly and time consuming challenge tests for each product formulation. Unfeasible new product formulations can be omitted from challenge and shelf life tests. The microbiological implication of small deviations

in set points of processing equipment can be calculated. Furthermore, the calculations can aid with the aquisition of improved insight in the processes that are of importance to the safety and quality of foods. Apart from some background in predictive microbiology, the layout and the workings of the decision support system are described and examples will be given.

MICROBIAL GROWTH

Bacteria, yeasts and moulds may occur and grow in foods. Foods can be seen as batch systems in which microorganisms can be present. Given a certain initial number of microorganisms and values for environmental conditions (temperature, acidity, humidity, etc.) favourable for growth, the number of microorganisms will increase as shown in Fig. 1. At the start of the curve, microorganisms have to adjust to the new environmental conditions (if applicable). This results in a period of time at which no growth occurs, the so-called lag phase (λ). In this adjustment period, the number of microorganisms may even decrease. After this adjustment phase, the microorganisms are accustomed to the prevailing environmental conditions and will start growing until a maximum rate is reached, the maximum specific growth rate (μ_m). After a certain time, growth will gradually slow down and finally stop upon which an asymptote (A) is reached. The reasons for reaching this plateau value can be several, varying from substrate depletion to the presence of inhibiting chemical compounds (6, 13).

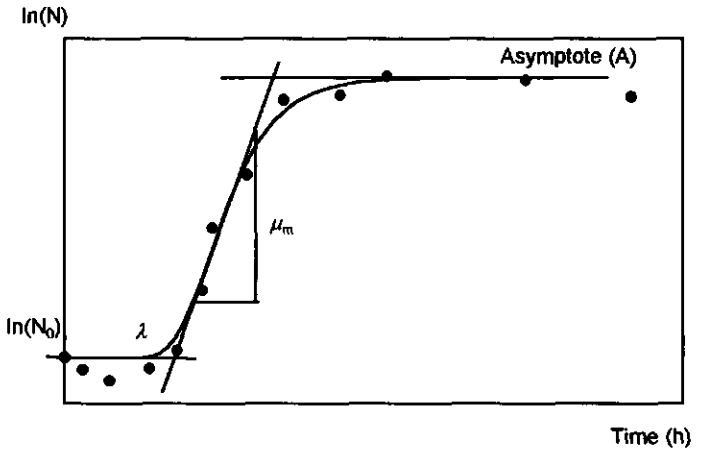

FIG. 1. Log of numbers of microorganisms in time.

For the prediction of food quality, usually, the first two stages play the most important role. Foods are usually spoiled if numbers of microorganisms become larger than 10^7 cells per gram product, which is generally lower that the asymptotic level of microorganisms. Furthermore, for pathogenic microorganisms safe numbers are generally far lower that the asymptotic number of microorganisms. Therefore, the asymptotic part of the curve is generally not very important (3).

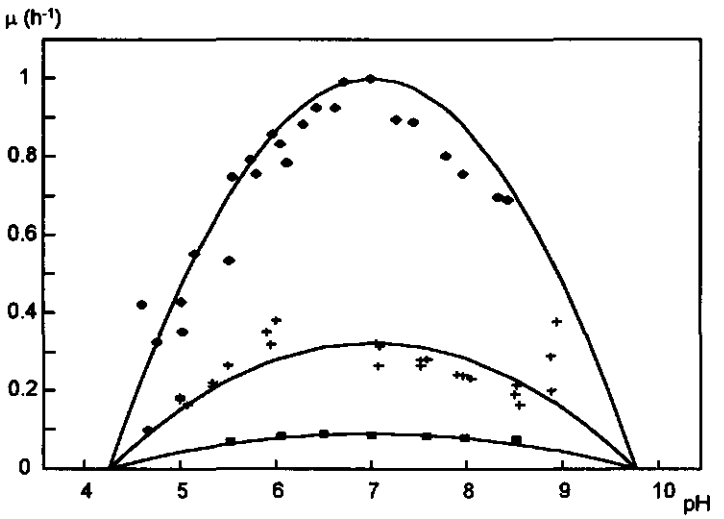

FIG. 2. Maximum specific growth rate of *Lactobacillus curvatus* as a function of acidity at 6 (■), 15 (+), and 29°C (◆) (13).

The growth rate and lag time of microorganisms depend on the environmental conditions (temperature, acidity, water activity). Growth rate is highest under optimal growth conditions and lag time is shortest. Mathematical models are developed that relate growth rate and lag time to the environmental conditions. As an example a model is described for acidity. The lower acidity the slower microorganisms grow (Fig. 2) and the longer it will take microorganisms to adjust to the environmental conditions. This effect takes place until the minimal growth acidity is reached, then growth rate becomes zero or lag time becomes infinite. At higher acidity, a point is reached where growth rate is maximal and lag time is shortest. Going beyond optimal acidity towards the maximum growth acidity, growth rate decreases, and the length of the lag phase increases again.

In earlier research, the effect of acidity on the growth parameters of microorganisms was quantified (13, 14) as well as the effects of several other environmental conditions (6, 8). Several mathematical equations were developed to describe the effect of these environmental conditions.

Under optimal acidity conditions (pH = pH_{opt}), growth rate will be maximal (μ_{opt}). For several microorganisms, the values for pH_{min}, pH_{max}, pH_{opt}, and μ_{opt} can be found in literature. A dimensionless growth rate γ can be used to describe the relative effect of each controlling factor (16):

$$\mu = \gamma \times \mu_{opt} \tag{1}$$

so that

$$\gamma(\mathrm{pH}) = \frac{\mu(\mathrm{pH})}{\mu(\mathrm{pH_{opt}})} = \frac{b(\mathrm{pH} - \mathrm{pH_{min}}) \times \left\{1 - \exp\left[c(\mathrm{pH} - \mathrm{pH_{max}})\right]\right\}}{\mu_{opt}} \tag{2}$$

If the value for pH_{opt} is known, c can be determined iteratively. After the value of c is determined, the b value can be derived, since at optimal conditions, (pH = pH_{opt}), γ has to equal unity, by definition.

The effect of multiple environmental conditions on growth rate has been investigated by several researchers (6, 8, 13, 14). It proved that separated effects of, for instance, temperature and water activity or temperature and acidity on growth rate could be multiplied. In dimensionless growth rate, γ(total) this can be represented as:

$$\gamma(\mathrm{total}) = \gamma(T) \cdot \gamma(\mathrm{pH}) \cdot \gamma(a_w) \cdot \gamma(\mathrm{O_2}) \tag{3}$$

In the case a value for μ_{opt} for a specific organism is unknown, the order of magnitude can be estimated for individual microorganisms. For bacteria the value of μ_{opt} is set to 2 h^{-1}, yeasts 0.75 h^{-1}, and moulds 0.25 h^{-1}.

The concept of dimensionless growth rates can also be used for the estimation of lag time (λ) since research proves that lag time is roughly inversely proportional to growth rate (1, 15). Eq. 4 relates actual lag time (λ) to lag time under optimal environmental conditions (λ_{opt}) and to the environmental conditions (γ(total)). If values for λ_{opt} are not known for individual

microorganisms, these values are set to 1 hour for bacteria under optimal conditions, 5 hours for yeasts and 10 hours for moulds.

$$\lambda = \frac{\lambda_{opt}}{\gamma(total)} \tag{4}$$

FOOD DESIGN SUPPORT SYSTEM

A computerised food decision support system is described in this paper which simulates the production of foods (Food Decision Support System, FDSS). The simulation takes place in different steps as shown in Fig. 3.

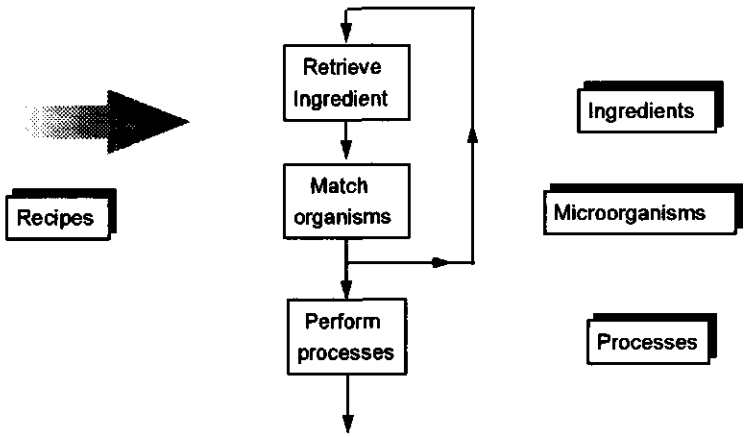

FIG. 3. Food design support system (FDSS), program flow.

Recipe. The composition of foods starts with the definition of the food recipe. The recipe database consists of records (single entities) with descriptions of recipes. A recipe is a description of ingredients and preparation methods for the simulation of the preparation of a food product, equivalent to recipes in a cook book. After a recipe is found, the production of the food is simulated.

TABLE 1. Record from the ingredient characteristics database

Semi-skimmed milk			
		minimum	maximum
Temperature (°C)	7	4	7
Acidity	6.2	6.1	6.5
Water activity	0.98	0.98	0.99
Aerobicity	Aerobic		

Ingredients. The characteristics of ingredients are present in the ingredient database. In the ingredient database, physical and chemical characteristics of food ingredients are stored. An ingredient is defined as a (part of a) food having a homogeneous physical and chemical composition on the scale of microorganisms. The acidity range (pH), water activity range, temperature range, and amount of oxygen of an ingredient were looked up from literature. An example of a record from the ingredient data base is given in Table 1.

Since there are large numbers of ingredients and the required values for the environmental conditions are not always known, the ingredients are grouped on the basis of their origin and physical composition. This grouping technique results in an inverse tree structure. At the top, a general ingredient can be found and going further into the branches of the tree structure, more and more specific ingredients can be identified. An excerpt of the tree structure is shown in Fig. 4.

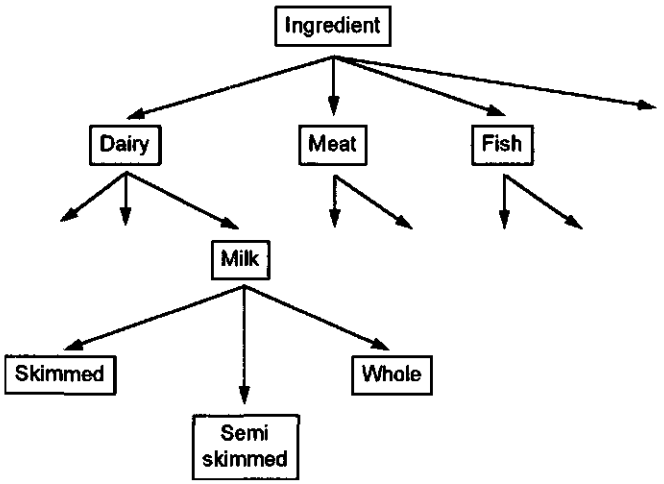

FIG. 4. Hierarchical ingredient database.

On the basis of the described tree structure, ingredient information can be inherited through hierarchical inference. If information is required for specific ingredients of which the values are not readily available in the database, FDSS presents the tree structure to the user to find related food ingredients of which the characteristics are known. This type of information inheritance was described earlier by Zwietering *et al.* (16).

TABLE 2. Record from the microorganism database

Lactobacillus curvatus			
Gram positive bacterium			
Fac. anaerobic			
No spores			
Not pathogenic			
Growth ranges:			
	minimum	optimum	maximum
Temperature (°C):	-3.00	35.00	42.00
pH:	4.29	6.88	9.28
a_w:	0.947		1.0
Growth kinetics parameters:			
μ_{opt}:	1.2 h^{-1}		
λ_{opt}:	1.0 h		
Thermal death kinetics parameters:			
D-value:	3.3 min at 61°C		
z-value:	6°C at 61°C		
Water activity kinetics parameter:			
a_w resistance:	moderate		
Cut off numbers:			
Spoilage level:	1×10^7 cfu per ml		

Microorganisms. After values of physical and chemical characteristics of ingredients are found, this information is coupled with kinetic parameters of microorganisms. In the microorganism database, properties of individual microorganisms are stored. For each microorganism, growth ranges with respect to environmental conditions such as temperature, water activity and acidity were looked up in literature and stored in FDSS. Moreover, of each organism in the database, the type (yeast, bacterium or mould), Gram-stain, spore forming capacity, decimal reduction times, *z*-values and pathogeneity are stored. An example record of

the microorganism database is given in Table 2. In total, the database consists of 57 different microorganisms that are of importance to food spoilage and food safety.

Individual models relating growth characteristics of microorganisms to environmental conditions, can be entered into this database. Microorganisms are only included in the database if the growth ranges with respect to temperature, acidity, water activity and oxygen are known.

Coupling ingredients and microorganisms is a two step operation. The program first determines which microorganisms can grow on the ingredient. This is done by a pattern match procedure (16). This first step is carried out for all known environmental characteristics of an ingredient except temperature. The second step uses experts' knowledge on incidence of microorganisms on known food ingredients. Both methods are described below.

Pattern matching. Growth of microorganisms can only take place between, for instance, minimum acidity and maximum acidity. If the value of a controlling environmental parameter (pH, a_w) of an ingredient lies between the boundary values for a microorganism, it is able to grow on the ingredient. An example is given in Fig. 5.

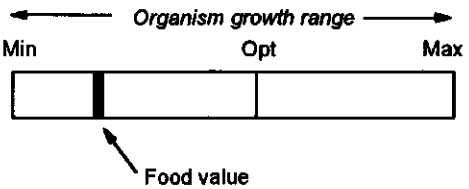

FIG. 5. Combining ingredient information with microbial growth ranges.

The selection on oxygen tolerance also takes place in this stage. For this selection no explicit mathematical models are available, so an expert model is applied in the form of a truth table. Facultatively anaerobic microorganisms can grow under any oxygen concentration. Anaerobic microorganisms can only grow under purely anaerobic conditions whereas aerobic microorganisms only grow under conditions where enough oxygen is present. If the food contains a small amount of oxygen (e.g. vacuum packed food), it contains too much oxygen for anaerobic microorganisms and too little air for aerobic microorganisms. Table 3 translates this description in $\gamma(O_2)$-values.

TABLE 3. Truth table for the calculation of $\gamma(O_2)$

$\gamma(O_2)$	Ingredient		
	No O_2	Small amount of O_2	O_2 present
Anaerobic organism	1	0	0
Fac. anaerobic organism	1	1	1
Aerobic organism	0	0	1

The selection of microorganisms in the pattern match phase involves the calculation of γ as function of acidity, water activity and amount of oxygen, only. If γ has a non-zero value after calculation, the organism is supposed to be able to grow in the product. The selection on temperature does not take place at this stage because temperature may fluctuate in later stages of food production.

Incidence of microorganisms. In the second step of finding the microorganisms that are present on an ingredient, expert knowledge on incidence of microorganisms in ingredients can be used. For several ingredients, knowledge exists in FDSS on the possible incidence of microorganisms on specific ingredients (such as spore forming microorganisms occurring frequently on herbs and spices). This knowledge can be used to fine-tune the list of selected growing microorganisms.

Because of the large numbers of ingredients, this knowledge cannot be available for all existing ingredients. Information on incidence of microorganisms is stored in the earlier described tree structure. Through hierarchical inference this information becomes available for most ingredients. If, for parts of the tree structure, no information on incidence of microorganisms is available at all, it is assumed that Beijerinck's rule applies. This rule implies that all microorganisms can be found everywhere and the environmental conditions determine incidence of microorganisms. This rule is implemented as the earlier described pattern match procedure. The most appropriate method can be chosen for any situation. If required, both methods can be tried for their outcome after processing.

Processes. The next step in the simulation of the production of a food is the processing of ingredients (Fig. 3). The preparation of foods always requires processing steps such as mixing, heating, stirring etc. Processes are methods to alter or combine ingredients. Results of processes usually are altered physical, chemical and microbiological properties of the processed products.

In this system, processes are built up of several so-called unit operations. Each unit operation has its own specific impact on the ingredients to which they are applied and to the microorganisms that undergo a unit operation. Unit operations can be combined to result into one overall process. Several unit operations are worked out in this paragraph.

Temperature related unit operations. Temperature has a large impact on growth characteristics of microorganisms. In this system it is assumed that between the minimum and maximum temperature for growth of a microorganism, the organism grows with the maximum specific growth rate belonging to the value of the environmental temperature. The response of microorganisms to temperature is shown in Fig 6.

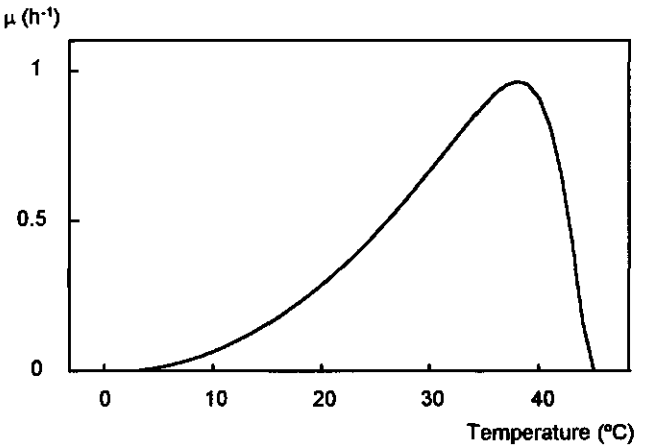

FIG. 6. Maximum specific growth rate as a function of temperature (8).

If temperature becomes larger than the maximum growth temperature, microorganisms start dying. First order inactivation kinetics is used to calculate the decrease in microbial numbers. Generally, D-values and z-values are found in microbiological literature (3). These values are calculated into the Arrhenuis parameters E_a and k_∞. Different temperature time relations can be applied to result in, for instance, sterilisation, freezing, pasteurisation, appertisation or cooling.

Water activity related unit operations. Drying foods is also a well known method of preventing microbial decay. In FDSS drying is defined as a modification of the amount of water in a product. Microorganisms are assumed to be not directly affected by altering the amount of water, but by changing the relative water pressure, water activity (a_w). A microorganism starts growing if the water activity of a product is higher than the minimum

water activity ($a_{w,min}$). Below $a_{w,min}$, microorganisms are inactivated. Inactivation of microorganisms as a result of drying consist of two distinct phenomena, dehydration inactivation (fast) and inactivation due to storage (slow), so called decay. Decay starts to play an important role after a microorganism was kept at low water activities for 24 hours or more (4). Since it is assumed that production processes usually last for shorter periods of time, this type of inactivation does not play an immediate role in this stage of the production of foods.

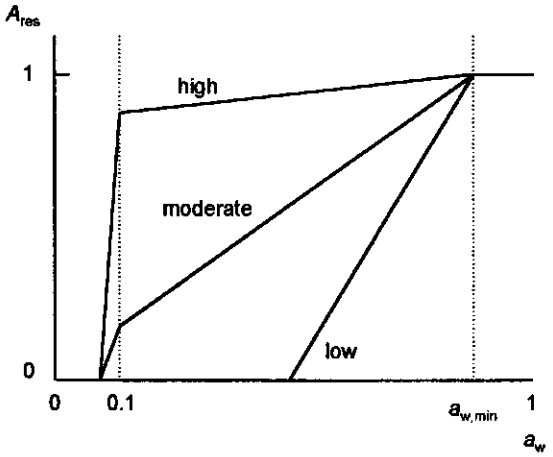

FIG. 7. Residual activities of three different types of microorganisms as functions of water activity (a_w).

Dehydration inactivation plays an immediate role in the determination of microbial numbers. The amount of surviving microorganisms is defined by means of a residual activity (A_{res}). The resulting number of microorganisms (N_t [cfu/ml]) after a drying step can be calculated as (4):

$$N_t = A_{res} \times N_0 \tag{5}$$

where N_0 [cfu/ml] is the number of microorganisms before drying.

Some microorganisms are known to have a high resistance to drying, such as *Saccharomyces cerevisiae*. This results in a high residual number after drying. Other microorganisms have very low residual activities (4). Residual activity of microorganisms as a function of water activity is shown in Fig. 7 for three types of microorganisms. Each type of organism has a different resistance to drying. If for specific microorganisms, no data is

available on resistance to drying, it is assumed that the resistance to water activity is moderate. All default settings and models can be changed if more or better information becomes available.

Acidity related unit operations. Acid can also be applied to lower acidity values of food products. After acidity is altered in a product, this affects the growth rate of microorganisms. Eq. 6 was developed to describe the effect of acidity and temperature on growth rate of *Lactobacillus curvatus*, a lactic acid bacterium (Fig 2) (13).

$$\mu_m = b(T - T_{min})^2 \times (pH - pH_{min})(pH - pH_{max}) \quad (6)$$

This response is generalised for all microorganisms. However, it is highly unlikely that all microorganisms have an optimum pH that lies exactly in the middle of pH_{min} and pH_{max}, therefore asymmetrical curves have also been implemented (14). The default type of curve used is asymmetrical, but equation 6 can be selected as well. If environmental acidity becomes smaller than pH_{min} or higher than pH_{max}, numbers of microorganisms decrease. The kinetics of the decrease of microorganisms in these regions is approximated by a simple line through (pH_{opt}, μ_{opt}) and (pH_{min},0) or (pH_{max}, 0). Improved models for death kinetics as a result of acidity can be implemented, whenever available.

Mixing unit operation. Mixing is defined as combining two or more ingredients. Mass averaged numbers of microorganisms of all involved ingredients are summed. Mass averaged temperature, pH and water activity are chosen as estimates of the resulting physical parameters. The amount of oxygen is approximated in the form of a truth table.

Better models for mixing food ingredients are needed, but since these are not available, these simple models are implemented. Approximations such as these, do not have a large impact on the calculation of the quality of foods since, in later stages of the simulation of foods, more precise information can be used for calculation.

Combining unit operations. A process consists of one or more, earlier described, unit operations. As a result of a unit operation, temperature, water activity and acidity may change in time. The mixing unit operation is assumed to be infinitely short. Time related unit operations can be performed simultaneously and individually. Mixing can take place in combination with a time related unit operation to make it time dependent. Process time is divided in time slots where unit operations can be combined. A pasteurisation process would

consist of three unit operations, heating up to the required temperature, keep at the required temperature for a certain time and then cool down.

For calculation, time slots are divided in small steps in which the conditions are assumed to be constant. The size of these time steps depends on the resulting increase or decrease of the changing environmental variable (temperature, pH and a_w). In a time step, temperature is allowed to change by a maximum value of 0.5°C, acidity is allowed to change 0.05 pH point, and water activity is allowed to change 0.005 unit. These default settings can be altered when needed. If unit operations are combined, the largest number of calculated time steps is used for the calculation of growth and decay.

In each small time step, the value of γ for each of the environmental parameters is calculated for a microorganism. Negative γ values override positive values, since microbial decay cannot take place at the same time as microbial growth. To assume worst case, lag time effects are not taken into account while processing foods. It is assumed that microorganisms start growing as soon as favourable environmental conditions for growth are reached. Numbers of microorganisms are calculated on the basis of immediate exponential growth or exponential decay.

DISTRIBUTION CHAINS

Changes in microbial numbers during distribution are calculated during various stages of product distribution. Scenarios are relationships between temperature and time after production of foods. The initial values of the environmental conditions of a food product are calculated by the food design program in the previous step. During distribution, acidity, water activity and amount of oxygen are assumed to remain constant. Different scenarios can be entered such as deep cooling, or temperature abuse. A typical temperature time relation is shown in Fig. 8.

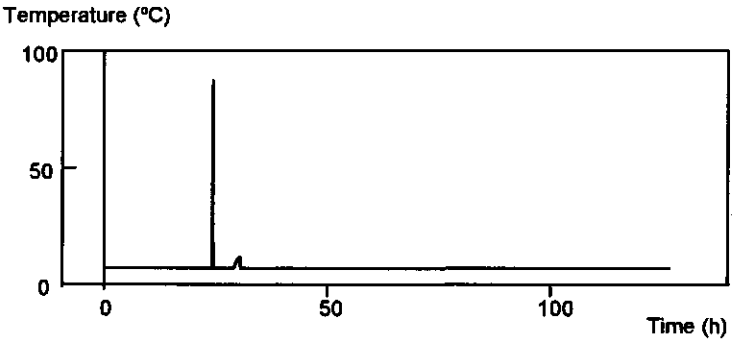

FIG. 8. Example of a temperature time relation for the processing and distribution of milk.

While calculating through a distribution scenario, microbial numbers may increase in one step, and remain constant in another. For each step in the scenario, the distribution chain program performs the final step of the pattern match procedure. Temperature is matched with the kinetic temperature parameters of each microorganism. If a certain organism cannot grow given the current temperature, the number remains constant until a favourable temperature for growth of this organism is reached.

Numbers of microorganisms in time, can be calculated if the values for lag time, growth rate and the asymptote are known at the prevailing values for the environmental conditions. For calculation a time delayed exponential growth model with asymptotic cut off value is used (eq. 7). Temperature is not constant, therefore, numbers of microorganisms under changing temperature conditions have to be calculated. The length of the lag phase (λ) and the microbial growth rate (μ) are calculated by means of the γ concept, at small time steps *i*. The size of these time steps is determined as described in combining unit operations. Lag time is only calculated if a microorganism has experienced stressful environmental conditions, where numbers of microorganisms decrease, such as drying, low acidity or high temperature conditions. The time step at which a temperature applies (t_i - t_{i-1}) is compared with the lag time at these conditions. *n* is the number of time steps needed to reach the end of the lag phase. As soon as enough time has passed for a microorganism to complete its entire lag time (Φ=1), exponential growth starts at a specific growth rate (μ) that belongs to the environmental conditions at time t_i. If Φ=1 is reached within a time step (t_i - t_{i-1}), the remaining time for exponential growth is calculated.

$$\begin{aligned} &\text{While} \quad \Phi \le 1 \quad \Rightarrow \Phi = \sum_{i=1}^{n} \frac{(t_i - t_{i-1})}{\lambda_i} \quad \text{and } N_i = N_0 \\ &\text{If} \quad \Phi > 1 \quad \Rightarrow \ln(N_i) = \ln(N_{i-1}) + \mu_i \times (t_i - t_{i-1}) \\ &\text{If} \quad N_i \ge A \Rightarrow N_i = A \end{aligned} \tag{7}$$

EXAMPLES

In this section two examples are described, one of the production and distribution of packaged pasteurised whole milk from raw milk, and storage of the product in refrigerated sale cabinets. Another example is the production of a dried, extruded food product, spaghetti.

Milk production. Dairy products such as milk, butter, cream, and cheese are all susceptible to microbial spoilage because of their chemical composition. Milk is an excellent growth medium for all common spoilage microorganisms, including yeasts and moulds. Raw, non-pasteurised milk contains varying numbers of microorganisms. The characteristics of raw milk are looked up in the ingredient database where the values for the parameters under aerobic atmospheric conditions are found to be pH = 6.6 and a_w = 0.993.

TABLE 4. Production and distribution schemes for the production of whole milk

	Scheme 1	Scheme 2	Scheme 3
Collection	5°C for 24 h	7°C for 24 h	5°C for 24 h
Pasteurisation	100°C for 0.01 s	89°C for 1 s	89°C for 1 s
Distribution			
- Factory	5°C for 4 h	7°C for 4 h	5°C for 4 h
- Truck	7°C for 2 h	12°C for 2 h	7°C for 2 h
- Shop/home	7°C for 96 h	7°C for 96 h	7°C for 96 h

After milking, raw milk is put at refrigeration temperatures (5-7°C) until it is collected and brought to the dairy company (Table 4). The simulation of three production and distribution schemes starts at this point. Scheme 1 describes a dairy company, keeping storage temperatures low and performing V-HTST (very high temperature, short time) pasteurisation (0.01 second at 100°C). Scheme 2 represents a dairy company that applies higher storage temperatures and lower pasteurisation temperatures. The HTST pasteurisation (1 second at 89°C) is a well known method for decreasing numbers of microorganisms in milk (3). Scheme 3 is equal to scheme 1 except for a HTST pasteurisation instead of V-HTST.

The pattern match procedure of microorganisms to the characteristics of raw milk results in a large list of bacteria, moulds and yeasts. Expert knowledge from literature is applied to roughly estimate initial numbers of microorganisms, where worst case is assumed in all scenarios. The total microbial count roughly equals 10^5 cfu per ml. No other expert knowledge is applied for removing non-applicable microorganisms. After performing the processing as described the main microorganisms and numbers are listed in Table 5.

TABLE 5. Result of three processing schemes for pasteurised milk

Processing	Stage of production	Organisms	Log_{10} (Numbers)/ml
Scheme 1	After collection	> 20 species	< 5
	After pasteurisation	*Bacillus* spp.	-3.2
Scheme 2	After collection	> 20 species	> 6
	After pasteurisation	*Bacillus* spp.	-0.2
Scheme 3	After collection	> 20 species	< 5
	After pasteurisation	*Bacillus* spp.	-2.9

The described distribution chain causes an increase in numbers of microorganisms (Table 6). In this table, the calculated shelf life of the product is also given at refrigeration temperatures (7°C). Shelf life is defined here as the time until a level of 10^3 cfu/ml *Bacillus* spp. is reached.

TABLE 6. Result of three distribution schemes for pasteurised milk

Distribution	Organisms	^{10}Log (Numbers/ml)	Calculated shelf life of product at 7°C
Scheme 1	*Bacillus* spp.	1.5	7 days
Scheme 2	*Bacillus* spp.	3.0	5 days
Scheme 3	*Bacillus* spp.	2.0	6 days

The decision support system calculates that the pasteurisation step in scheme 1 reduces the total microbial count from 10^5 per ml to a number below 1 per ml. In this system, the *Bacillus* spp. (family) is represented by three bacteria, *B. cereus*, *B. subtilis* (both slightly pathogenic microorganisms), and *B. licheniformis*. During pasteurisation, a slight increase in *B. subtilis* can be observed. Numbers of *B. cereus* and *B. licheniformis* decrease. After processing, the calculated count of *Bacillus* spp. equals 7×10^{-4} cfu per ml; so on average about 1 *Bacillus* can be found in 1.5 litre of pasteurised milk. *B. subtilis* has some species that can grow at low temperatures. In this case FDSS calculates worst case increase of *B. subtilis* which results in a final number of 30 per ml.

In scheme 2 the milk was collected at a higher temperature of 7°C. Microbial growth can be observed at this temperature, therefore microbial numbers increase slightly in the collection phase. The product is not spoiled yet, but requires an extensive decrease in microbial numbers. A slightly milder pasteurisation step is applied to the product which results in a larger number of *Bacillus* spp. present after pasteurisation. Since the distribution chain runs at temperatures of 7°C and 12°C, *Bacillus subtilis* grows throughout the entire distribution chain which results in 10^3 cfu per ml. The calculated remaining shelf life then changes to 5 days.

In scheme 3 a lower pasteurisation temperature results in a shelf life of 6 days, which is the regular reported shelf life of milk on milk cartons. These excersises show that both a low temperature during distribution and a high temperature for pasteurisation are very important factors influencing the shelf life of the product. In practice however, also spoilage organisms are found in milk, such as *Pseudomonas* spp (3). These organisms are removed from the milk during pasteurisation, so obviously, these organisms are reintroduced after pasteurisation (e.g. during filling of milk cartons). FDSS can also be used to simulate the introduction of spoilage organisms after pasteurisation. This, however, requires knowledge on final products and not on ingredients. This knowledge is not incorporated in the current system but can easily be added.

Spaghetti production. Spaghetti is an exceptionally dry product (a_w=0.7) and not very susceptible to microbial spoilage. However, the ingredients of spaghetti, eggs and flour, are known to cause microbial spoilage in products. Flour is also a very dry ingredient, but, as a result of the milling process, contains large numbers of spore forming bacteria, enterobacteriaceae, moulds, and microorganisms that are usually found on soil such as faecal streptococci (3). This knowledge cannot be derived from performing matching of microorganisms, but results from adding additional knowledge stored in the ingredient tree structure. Eggs are also known to bring along microorganisms. The other ingredients of which the product is composed are water, and salt.

The production process is a two step operation, extrusion and drying. The extrusion process is a combination of mixing, temperature, and water activity unit operations. The final water activity of the extruded spaghetti half product is approximately 0.75, the acidity of the product approximates 7 and the product can be stored at room temperature (25°C). The extrusion process lowers water activity slightly, and results in a mild pasteurisation step (2 min at 70°C). The product is then dried in an aseptic drying tunnel at an initial temperature of 50°C. In the drying tunnel, temperature drops to a value of 25°C. The entire process lasts 10 hours, in this time water activity drops to 0.7. This example is worked out here to see whether the

microorganisms present in the final product are pathogenic and may cause harm when present in a wet product such as prepared spaghetti. Since non of the microorganisms present in spaghetti are likely to grow because of the low water activity, a distribution chain is not simulated.

According to the data stored in FDSS, the number of microorganisms present on flour is the same as the amount of microorganisms present on grains, which equals about 10^6 per gram. The same holds for the amount of moulds present, which is recorded as 10^5 per gram and the amount of yeasts present is approximately 10^4 per gram. It is assumed that the numbers are equally distributed over all microorganisms selected. As an example: FDSS has records of 15 different mould species; the amount of moulds per gram equals 10^5 per gram, therefore each species inherits about 7×10^3 moulds per gram. In the next table the influence of the extrusion step on the amount of microorganisms present is shown, as well as the influence of the drying step.

TABLE 7. Results of the production of spaghetti

Processing	Organisms	Log_{10} (Numbers) per gram
Ingredients	> 20 species	6
Extrusion	7 species	1.2
Tunnel dryer	*Clostridium* spp.	0.4
	Bacillus spp.	0.3
	Salmonella spp.	-2.7
	Xeromyces spp.	-4.3

The extrusion process reduces the number of microorganisms present from 10^6 per gram to 15 per gram. The tunnel drying process reduces the number of microorganisms slightly, and alters the water activity of the product so that non of the remaining microorganisms can grow. A stable product with very low numbers of pathogenic microorganisms (*Clostridium* spp., *Bacillus* spp. and *Salmonella* spp.) (10) is produced. Rayman *et al.* (10) report the presence of *Salmonella* spp. in both egg containing and no-egg pasta in 4 out of 654 samples. Furthermore, the presence of *E. coli* is reported (10), however, in FDSS, this microorganism is eliminated during the extrusion process. Obviously, there are *E. coli* strains that are more heat resistant than the ones included in this system. In storage tests, *E. coli* is not found to survive in pasta after 10 days. Spicher (12) reports the presence of spores (1 to 1×10^3 cfu/gram), coliformes (absent to 24 cfu/gram), fecal streptococci (absent to 1.4×10^4 cfu/gram), *Staphylococcus aureus* (absent to 62 cfu/gram) and moulds (<10 to 1.1×10^4) in spaghetti. In

FDSS both the fecal streptococci and *Staphylococcus aureus* are eliminated during the extrusion process.

In practice, the extrusion process seems less able to eliminate reported microorganisms, therefore, the process parameters of the extrusion process need to be reassessed.

CONCLUSIONS

A computerised decision support system is set up that can be a helpful tool in developing and optimising food products. Several product formulations can be tested with respect to changes of food quality in time. Microbial consequences of temporary product formulations can also be calculated, so that on the spot decisions can be supported objectively. Consequences of different temperature settings in distribution chains can be calculated, which may result in more optimal chains with respect to microbial numbers and for instance costs of cooling.

Several types of models are used ranging from verbal (e.g. tree structure) to mathematical (e.g. microbial growth models). Resulting predictions will always be disturbed by inaccuracies resulting from the combination of these models and the inaccuracies of the models. However, the transparent way in which the calculations and predictions take place, and the possibility to alter information and models, results in a flexible system in which improved models or improved data can easily be implemented.

The predictions should be seen as an indication of possible occurrence and growth of microorganisms, rather than absolute numbers or predictions. The results of the prediction, can be used as indication, and also as reminder. Predictions of the computer system should be interpreted by food manufacturing experts to assess the relevancy to the food product. Furthermore, FDSS can be used for training production staff and quality engineers.

The HACCP concept requires insight in the relevant processes for decreasing and controlling certain risks in production and consumption of foods. The developed system can help quantifying effects of control measures on the microbial load of foods. Therefore this system can be a discussion partner for the team that implements the HACCP methodology into food production and distribution chains.

FURTHER RESEARCH

The results of the simulation of the production of a food should be interpreted by means of an expert system that assesses the numbers of microorganisms (quality) and possible deviations from safety regulations (safety). Knowledge of food microbiologists can be modelled and incorporated into an expert system shell that performs the checking. Interpretation of the results should take place in two stages. The first stage of the interpretation of the results takes place during the simulation of the production of a food. The second part assesses the shelf life of the developed product and the safety of the final product including the distribution chain phase. Each step in the entire production of the food should be assessed.

In each step the number of spoilage microorganisms should be lower than a general cut off value (10^7 microorganisms/gram) to provide for good quality intermediate products. The number of pathogenic microorganisms should be below the specific cut off value of each organism, for food safety reasons. If these values are exceeded, the program should warn the user and explain how modifications should be made to the simulated food.

Because of rapid developments in several fields of food technology, the information in the databases should be maintained and regularly updated, which is one of the ways FDSS can be updated and improved. At the moment part of the data and models in the databases, is validated, part still needs validation. However, the need for good models and data is evident. In predictive microbiology, more data sets are measured and more accurate models become available. FDSS uses the developed models and combines these with expert knowledge and engineering heuristics. The mathematically accurate predictions of the microbial growth models are influenced by the inaccuracies of the assumptions in the decision support system. Generalising models for microbial growth, that is using a model for one microorganism which was developed for another, also decreases the accuracy of the predictions slightly. Attention should be given to developing rules for more general application of models for groups of microorganisms. The accuracy of the assumptions in the decision support system also has to be assessed. If improved data and models become available, they can be incorporated into the databases of FDSS, although, at the moment, the system already performs well.

If modes of action should change because of new insights, FDSS is set up in such a way that parts can be taken out, reprogrammed and put back into the system. The system is programmed in different libraries. Each of these libraries performs its own task regardless of the rest of the system. To run one library, it is assumed that all required information is present,

if not, the entire system crashes. Because FDSS is set up this way, it is relatively easy to take one library out, alter it, and put it back into the system. The library that needs to be altered should be looked up and the conventions for that library should be followed, but calculations, inferences, and data handling can be altered at will.

The last way in which FDSS can be improved is by the addition of new libraries. Then the system could, for instance, be used for the prediction of chemical and physical spoilage of foods. Other types of models have to be developed, but the method of combination and prediction is largely equal to the those of the developed system. Chemical and physical spoilage play important roles in foods and food products in which no microorganisms can grow (e.g. dry foods), microorganisms grow slowly (e.g. dried meat), when microorganisms are not present (e.g. sterilised foods), or when large numbers of microorganisms or certain chemical compounds keep other microorganisms from growing (e.g. fermented foods). The characteristic times for each of these spoilage reactions should be calculated so that the most important decay reactions can be assessed. Models for prediction of chemical and physical spoilage can easily be added to the databases and libraries of FDSS, creating an even more versatile system for the assessment of quality of foods.

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Chapter 7

A computerised system for the identification of lactic acid bacteria.

ABSTRACT

A computerised system for the identification of bacteria was developed. The system is equipped with a key to the identification of lactic acid bacteria. The identification is carried out in two steps. The first step distinguishes classes of bacteria by following a decision tree with general identification tests. The second step in the identification is the distinction of species within a class on the basis of biochemical fermentation patterns. During group classification, probabilities for test failure are used. These probabilities can be used for assessing the quality of a given test answer. The probabilities are also used to select the most probable test answer in case of an inconclusive test result. The probabilities of test failure were determined by a group of experts and a group of potential users of the identification system. During species identification, similarity indices are calculated for all bacteria in a class. The described identification system has the ability to "learn" from different sessions in the species identification step, improving both identification speed and accuracy. Because of the versatile way in which the system is set up, it can very easily be expanded with identification keys to other organisms.

INTRODUCTION

Lactic acid bacteria play an important role in food technology, in various fermentations, in bacteriocin production, as probiotics, and in food spoilage. The group of lactic acid bacteria includes several genera of bacteria which differ considerably in morphological and physiological properties. Consequently, it is essential to have simple and effective methods for lactic acid bacteria identification (1). Identification can be separated in two stages, discrimination and confirmation. The discrimination procedure uses characteristics of organisms to distinguish between different species. Confirmation uses the same and other characteristics to ratify the organism that was distinguished from the others. In identification both strategies have to be balanced. The more confidence that is required for the identification, the more confirmatory questions have to be answered.

For the identification of lactic acid bacteria, different methods are in use, based on various techniques. A common method uses the microorganisms' ability to ferment 50 sugars, the API 50 CHL-test. The biochemical fermentation pattern can be analysed manually or with computer programs. The API LAB program is commercially available and it discriminates between species on the basis of a pattern matching principle. A library of previously identified bacteria is used as a reference for the identification of, yet unidentified, organisms. Another computerised identification system was described by Cox and Thomsen (1). This program also uses the results of the API 50 CHL-test kit for the identification of lactic acid bacteria. A different pattern matching procedure was used to discriminate between species. Both systems assess the entire group of lactic acid bacteria as a whole.

In both mentioned approaches the investigator must verify that the organism that is to be identified, belongs to the group of lactic acid bacteria. However, the described programs do not take the basic morphological and physiological variety within the heterogeneous group into account. Instead, a compensation is sought in the comparison of patterns comprising up to 50 traits. Consequently, numerous traits considered will not be of discriminatory value to the genus or species under study. In a group as heterogeneous as the lactic acid bacteria, this approach may lead to wrong species and even wrong genus results.

Our newly developed computerised identification system for lactic acid bacteria is based on a combination of general morphological and physiological characteristics, such as Gram-staining or other basic microbiological techniques, as well as a reasoned selection of carbohydrate fermentation tests from for instance the API-test strips. The identification is

carried out in two steps. First, the system distinguishes bacteria into classes, using straightforward microbiological tests such as Gram-staining and microscopy. This step is called group classification, consisting of discrimination and partly of confirmation. Secondly, carbohydrate fermentation patterns are used to discriminate between species; the species identification. The developed program establishes the name of an organism with as few questions as possible, taking into account that both discrimination and confirmation procedures are followed.

TABLE 1. Coded selected organisms for identification[1]

(A) *Lactobacillus helveticus* (NCIMB 8652)
(B) *Lactobacillus delbruekii* ss. *bulgaricus* (DSM 20080)
(C) *Lactobacillus halotolerans* (ATCC 35410)
(D) *Lactobacillus fermentum* (UOT 22-0-6)
(E) *Lactobacillus plantarum* (ATCC det. no number)
(F) *Lactobacillus curvatus* (LAB 962)
(G) *Leuconostoc lactis* (DSM 20192)
(H) *Leuconostoc mesenteroïdes* ss. *mesenteroïdes* (DSM 20343)
(I) *Pediococcus pentosaceus* (DSM 20336)
(J) *Pediococcus dextrinicus* (DSM 20335)
(K) *Streptococcus thermophilus* (NCFB 2075)
(L) *Enterococcus faecalis* (DSM 20478)
(M) *Lactococcus lactis* (LMG 2122)
(N) *Streptococcus mutans* (DSM 20523)

[1]: Abbreviations culture collections:
NCIMB: National Collection of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, Scotland, UK.
DSM: Deutsche Sammlüng von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.
ATCC: American Type Culture Collection, Rockville, USA.
UOT: University of Otago, Otago, New Zealand
LAB: Laboratorium voor Microbiologie, Rijksuniversiteit Gent, Gent, Belgium.
LMG: Laboratorium voor Microbiologie, Gent Culture Collection, Rijksuniversiteit Gent, Gent, Belgium.
NCFB: National Collection of Food Bacteria, Shinfield, Reading, UK.

MATERIALS and METHODS

To test the identification system, 14 stock cultures of lactic acid bacteria were selected, listed in Table 1. The selected organisms were coded (A .. N). All organisms were stored at -18°C in MRS broth (Oxiod) except for *Leuconostoc lactis* which was kept in milk. Prior to the identification procedure, all bacteria were grown aerobically on MRS agar (Oxoid) at 30°C for

3 days. The required tests are carried out according to the appropriate methods. The appropriate methods are described in standard microbiological handbooks (2).

Group classification. The key to the group classification is based on Bergey's Manual of Systematic Bacteriology (3). Lactic acid bacteria are distinguished from all other bacteria by performing Gram stains, absence of catalase and oxidase, morphology and growth under aerobic and anaerobic conditions. Lactic acid bacteria do not form spores except for *Sporolactobacillus*, so this is also a good preliminary criterion for identification. The decision tree resulting from the foregoing is given in Fig. 1.

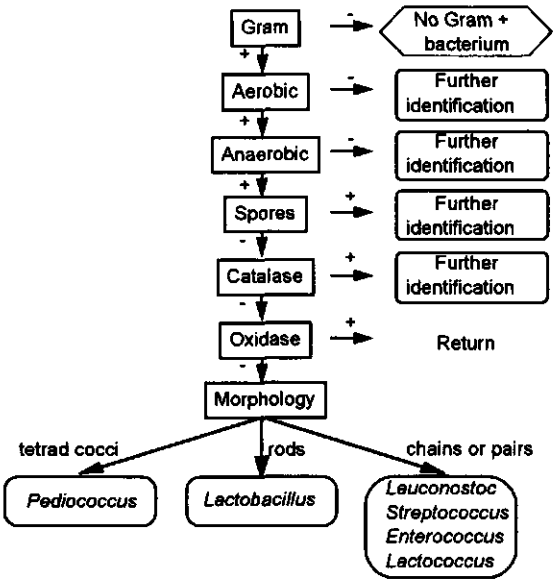

FIG. 1. Group classification decision tree.

The contents of the rectangles in the figure indicate tests to perform. Descriptions of these tests can be found in microbiological handbooks (2) and are documented in the program. The oval blocks indicate branches to further identification keys. The diamond shaped blocks represent classes or genera for which no further identification has been programmed, so called dead end identifications.

The bottom end of the key in Fig. 1 is extended with morphological characteristics that separate the entire group into three groups that are treated separately upon further

identification. The group, *Lactobacillus*, consist of rod shaped bacteria. The *Pediococcus* group, form cocci in a characteristic tetrad form. The group consisting of *Streptococcus*, *Lactococcus*, *Enterococcus*, and *Leuconostoc*, form cocci occurring as chains or pairs; which makes it difficult to distinguish these four genera from each other on a morphological basis. A detailed example for further discriminating the *Lactobacillus* group is given in Fig. 2. The *Lactobacillus* group is divided into three classes. All other groups are also differentiated using specific characteristics of each group. These classes are then characterised in the species identification step.

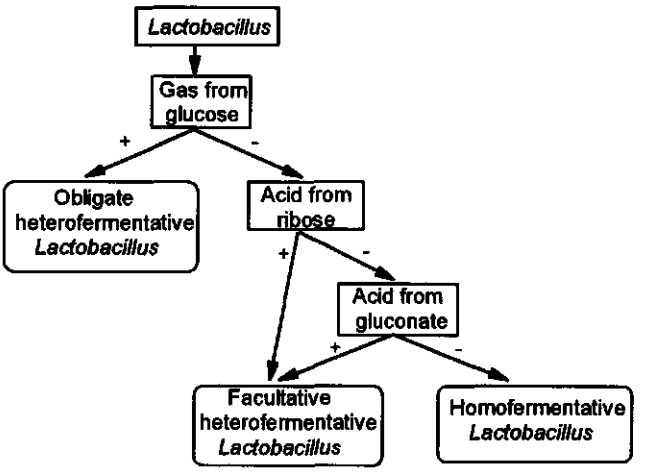

FIG. 2. Group classification key for *Lactobacillus* groups.

Species identification. In the foregoing stage, bacteria were distinguished into classes by various microbiological characteristics of each class. During the second stage of the identification, carbohydrate fermentation patterns are tested for each class. The patterns were extracted from Bergey's Manual of Systematic Bacteriology (3). Most class patterns can be matched with reactions available from the API 50 CHL test kit (bioMérieu, 69280 Marcy-l'Etoile, France). To discriminate between species in the *Streptococcus*, *Lactococcus*, *Enterococcus* class, the API 20 STREP test kit (bioMérieu) contains sufficient discriminative tests. Therefore, the use of either of these two is recommended.

The fermentation patterns are stored in tables of which the definite negative and positive reactions are being used for species discrimination. Negative reactions are defined as 90 % of the strains having a negative response to the test, whereas positive reactions indicate that 90 %

of the strains have a positive response. The remaining test characteristics (d = 11-89 % positive, and 0 = not tested) have been recorded in the tables but are not used for discrimination.

For each bacterium in the class a similarity index can be calculated. The value of the similarity index is based on the percentage positive or negative for each reaction. If the given answer equals the required result for a reaction, the similarity index for this reaction is set to 0.95. This value is the mean of 1.00 and 0.90; the value of a corresponding test result. If d-type or 0-type behaviour is recorded in the stored profile table, the index is set to 0.5. If the organism reaction is different from the given answer the similarity index of a test is set to 0.05. For all performed reactions, the similarity indices are summed and divided by the total number of performed tests.

Another index, the match index, can also be calculated. It is based on positive and negative reactions only; d-type and 0-type behaviour are not used. The match index is calculated as the number of corresponding reactions divided by the total number of used reactions. The maximum value of the match index is 1. In Table 2, an example of the calculation of both indices is given for *Lactobacillus curvatus* and user entered results for a few biochemical reactions.

TABLE 2. A hypothetical example for the calculation of the similarity and match index of the species identification of *Lactobacillus curvatus*.

Reaction	Required result for *L. curvatus*	User entered result	Similarity index	Match index
Amygdalin	Negative	Negative	0.95	1
Glucose	Positive	Negative	0.05	0
Gluconate	Positive	Positive	0.95	1
Lactose	Variable	Negative	0.50	Not used
Cellobiose	Positive	Positive	0.95	1
Mannitol	Negative	Negative	0.95	1
Mannose	Positive	Positive	0.95	1
		Calculated index	0.76	0.83

The most discriminative biochemical reaction is looked up in the biochemical profile table. This is defined as the reaction that separates the entire class of bacteria roughly in two parts, each containing about half of the total number. Upon entering the result of the test, the appropriate sub class is assessed to carry out a new search sequence. As soon as one specific

microorganism name is deduced, the results of a user defined number of other reactions is assessed to confirm the identification.

If the described method of species identification fails, i.e. the entered biochemical pattern does not completely match the pattern of one particular organism, similarity or match indices are used to choose the most likely organism from the group.

Conditional chances. In the group classification key, all test results are given a chance to be misinterpreted. Faculty members of the Food Microbiology department of the Wageningen Agricultural University were asked to predict the chance of misinterpretation of a given test. For instance: Given the fact that an organism is a non spore forming bacterium, estimate the chance that spores can be seen, using the proposed test method, i.e. phase contrast microscopy. Since experts usually remember cases where things went wrong, this technique is known to poorly reflect the actual chances of test failure. To obtain more reliable conditional chances, students from undergraduate practical classes were asked to carry out identifications of several pre identified species in different sessions. Per particular test, the participants were asked to record their findings. The results were compared with the required test results. This resulted in, for instance, a chance that a given answer is correct given an unquestionable (i.e. known) test outcome. For identification however, chances that test outcomes are correct given an entered (i.e. known) answer are needed. These last chances can be estimated from the collected chances by applying Bayes' theorem of conditional chances (5). The calculated chances are noted in the group classification key. In Table 3 the calculated chances are shown for each of the two collection methods. If the results were available from the undergraduate students, these are used, otherwise the conditional chances obtained from the experts are.

Using conditional chances, test outcomes can be suggested if the result of a performed test is not reliable or unavailable. The most reliable answer, that is, with the lowest conditional chance, is generated and group classification proceeds.

Certain combinations of test results can not lead to positive identifications for instance oxidase positive in the route of Fig. 1. Reaching such a point, the test that is most likely to have gone wrong is looked up. This is the test with the highest chance of failure. At this point, the most likely answer is generated and a new identification is carried out, retaining all other entered information. This procedure is called backtracking and is also called upon when the calculated similarity or match index from the species identification is lower than a user defined minimum value.

TABLE 3. Determined chances and conditional chances for the used microbial reactions; from expert opinion and undergraduate determination

Given that was seen	Experts chance to be		Undergraduate chance to be	
	correct	wrong	correct	wrong
Gram positive	0.85	0.15		
Gram negative	0.93	0.07		
Aerobic growth	0.90	0.10		
No aerobic growth	0.90	0.10		
Anaerobic growth	0.90	0.10		
No anaerobic growth	0.90	0.10		
Endospores present	0.72	0.28	0.61	0.39
Endospores not present	0.96	0.04	0.53	0.47
Oxidase positive	0.90	0.10	0.94	0.06
Oxidase negative	0.89	0.11	0.90	0.10
Catalase positive	0.92	0.08	1.00	0.00
Catalase negative	0.96	0.04	0.64	0.36
Gas formation from glucose	0.96	0.04		
No gas formation from glucose	0.96	0.04		
Acid formation from ribose	0.96	0.04		
No acid formation from ribose	0.96	0.04		
Growth at 15°C	0.98	0.02		
No growth at 15°C	0.96	0.04		
Growth at 45°C	0.98	0.02		
No growth at 45°C	0.94	0.06		
Morphology: Chains of cocci	0.73	0.27	0.91	0.09
Morphology: Tetrads	0.80	0.20	0.92	0.08
Morphology: Rods	0.92	0.08	0.92	0.08

Directed search. Directed search is based upon the idea that in a certain environment, specific microorganisms are found more frequently than others. If a certain organism is very often identified, the chance of it being for instance a house flora organism is quite large. Therefore, during species identification, the attention can be focused on finding this particular organism first by carrying out the most discriminative tests for this organism. The system may develop differently on different user sites, since using the system alters the species identification procedure. If the organism cannot be identified by means of the directed search method, the original species identification technique will be carried out.

RESULTS and DISCUSSION

The system was tested with 14 lactic acid bacteria. First, the group classification was carried out. Table 4 shows the tests proposed by the system and results for the coded organisms.

TABLE 4. Group classification, tests and results for coded bacteria

Test[1]	Encoded bacterium													
	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Gram	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Aerobic growth	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Anaerobic growth	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Spore formation	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase test	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase test	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Morphology[2]	1	1	1	1	1	1	2	2	3	3	2	2	2	2
Gas from glucose	-	-	+	+	-	-	+	+	-	-	-	-	-	-
Acid from ribose	-	-	nu	nu	+	+	nu	nu	nu	nu	nu	nu	nu	nu
Acid from gluconate	-	-	nu	nu	nu	nu	nu	nu	nu	nu	nu	nu	nu	nu
Growth at 15°C	nu	nu	nu	nu	nu	nu	+	+	nu	nu	nu	nu	nu	nu
Growth at 45°C	nu	nu	nu	nu	nu	nu	-	-	nu	nu	+	+	-	-

[1] Used symbols: + Positive or Yes; -: Negative or No; nu: Not used for identification.
[2] Morphology: 1: Rods; 2: Chains of cocci; 3: Tetrads.

The species identification following the group classification was carried on the basis of the API 50 CHL test kit for bacteria A to J, and the API 20 STREP for bacteria K to N. The number of confirmatory tests after a positive identification of a strain was set to 5. In Table 5 the results are shown of the API tests. For bacteria A to J of the API 50 CHL test (Table 5a) and for bacteria K to N the results of the API 20 STREP test (Table 5b).

In Table 6, the identified organism name for each of the coded organisms is given along with the calculated similarity and match index, between parenthesis. The presented indices have not been corrected for the accuracy of the group classification. The pilot identification of 14 bacteria resulted in the accurate identification of most strains. At bacterium code A (see Table 6), three bacteria with equal similarity indices were found. Based on the match index, however, the right organism was identified. In case of bacteria codes K, M, and N, bacteria with two to four similar match indices were deduced. In two instances (bacterium code K and M) the identification procedure based on the similarity index resulted in a wrong identification. In both cases the bacterium could not be positively identified solely by means of the match index, since

more than one bacterium name was deduced. The place where the appropriate organism was found is also shown in the table. These two cases are clear examples for the need of better discriminatory techniques for the species identification of bacteria in the classes of *Streptococcus*, *Enterococcus* and *Lactococcus*. In this respect the improved method for identification of Enterococci and fecal Streptococci as described by Knudtson and Hartman (4) could be introduced into the identification system. Other techniques such as DNA analysis (e.g. PCR techniques) can also be introduced into this computerised identification system.

CONCLUSIONS

The newly developed computerised system for the identification of lactic acid bacteria uses an established method for the identification of bacteria. The key to the identification of bacteria is set up in such a way that with the smallest number of tests, the highest possible certainty is accomplished. The identification key is split up into two parts, the part in which group classification is carried out and a part in which species are being identified.

The novelty of this system can be found in the possibility to reason with both the proposed tests and the test results. If information is not available, gaps can be filled in and answers generated. Furthermore, explanation is provided for in both the followed identification route and the way in which to carry out experiments. Well tried and easily practicable tests are suggested.

The described system has the ability to "learn" from different sessions using the principle of directed search. Especially when the system has run numerous sessions, the number of tests to carry out in the species identification can be reduced. This feature is particularly useful when specific house flora has to be identified very frequently.

The described identification program (MS-DOS) is available from the authors upon request.

ACKNOWLEDGEMENTS

The following strains were kindly provided by the Industrial Microbiology Group; University of Wageningen: *Lactobacillus helveticus*, *Lactobacillus delbrueckii* ss. *lactis*, *Lactobacillus halotolerans*, *Leuconostoc lactis*, and *Streptococcus thermophilis*.

TABLE 5a. Species identification tests and results for encoded bacteria A..J

Coded	API 50 CHL																																																	
Organism	0					5					10					15					20					25					30					35					40					45				
A	-	-	-	?	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	?	-	-	-	-	-	-
B	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	?	-	?	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
C	-	-	-	-	-	+	-	-	-	-	-	+	+	+	-	-	-	-	+	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
D	-	+	-	-	+	+	-	-	-	-	+	+	+	+	-	-	-	-	+	-	-	-	+	-	+	-	+	+	+	+	+	+	+	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	-
E	-	?	-	-	+	+	-	-	-	-	+	+	+	+	?	?	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	+	+	-	+
F	-	-	-	?	-	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
G	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+	+	+	+	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	-
H	-	-	-	+	+	+	+	-	-	-	+	+	+	+	-	+	-	-	+	-	-	+	+	-	-	+	+	-	+	+	+	+	+	-	-	+	-	-	+	-	+	-	+	+	-	-	+	+	+	-
I	-	+	-	+	+	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	+	?	-	+	-	-	-	+	?	-	+	-	-	-	-	-	-	-
J	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-

TABLE 5b. Species identification tests and results for encoded bacteria K..N

Coded	API 20 STREP																		
Organism	VP	HIP	ESC	PYRA	αGAL	βGUR	βGAL	PAL	LAP	ADH	RIB	ARA	MAN	SOR	LAC	TRE	INU	RAF	AMD	GLYG
K	+	-	-	-	-	-	+	-	+	-	+	-	-	-	+	-	-	-	-	-
L	+	-	+	+	-	-	-	-	+	+	+	-	+	+	+	+	-	-	+	-
M	+	-	+	-	-	-	-	-	+	+	+	-	-	-	+	+	-	-	-	-
N	+	-	+	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	-	-

TABLE 6. Coded organism name and identified organisms using similarity index and match index

Code and organism name	Identified organism	
	Similarity index	Match index
(A) *Lactobacillus helveticus*	*Lactobacillus helveticus* (0.86)	*Lactobacillus helveticus* (1)
	Lactobacillus delbruekii ssp. *bulgaricus* (0.86)	
	Lactobacillus amylophilus (0.86)	
(B) *Lactobacillus delbruekii* ssp. *bulgaricus*	*Lactobacillus delbruekii* ssp. *bulgaricus* (0.95)	*Lactobacillus delbruekii* ssp. *bulgaricus* (1)
(C) *Lactobacillus halotolerans*	*Lactobacillus halotolerans* (0.95)	*Lactobacillus halotolerans* (1)
(D) *Lactobacillus fermentum*	*Lactobacillus fermentum* (0.85)	*Lactobacillus fermentum* (1)
(E) *Lactobacillus plantarum*	*Lactobacillus plantarum* (0.91)	*Lactobacillus plantarum* (1)
(F) *Lactobacillus curvatus*	*Lactobacillus curvatus* (0.91)	*Lactobacillus curvatus* (1)
(G) *Leuconostoc lactis*	*Leuconostoc lactis* (0.95)	*Leuconostoc lactis* (1)
(H) *Leuconostoc mesenteroides* ssp. *mesenteroides*	*Leuc. mesenteroides* ssp. *mesenteroides* (0.74)	*Leuc. mesenteroides* ssp. *mesenteroides* (1)
(I) *Pediococcus pentosaceus*	*Pediococcus pentosaceus* (0.78)	*Pediococcus pentosaceus* (1)
(J) *Pediococcus dextrinicus*	*Pediococcus dextrinicus* (0.86)	*Pediococcus dextrinicus* (1)
(K) *Streptococcus thermophilus*	*Enterococcus salivarius* (0.73)	*Streptococcus thermophilus* (1)
	(*Streptococcus thermophilus* rated 9th (0.63))	*Streptococcus mitior* (1)
(L) *Enterococcus feacalis*	*Enterococcus feacalis* 2 (0.95)	*Enterococcus feacalis* (1)
(M) *Lactococcus lactis*	*Streptococcus milneri* 2 (0.84)	*Lactococcus lactis* (1)
	(*Lactococcus lactis* rated 16th (0.64))	*Streptococcus sanguis* (1)
		Lactococcus lactis ssp. *lactis* (1)
(N) *Streptococcus mutans*	*Streptococcus mutans* (0.905)	*Streptococcus mutans* (1)
		Streptococcus cricetus (1)
		Streptociccus sobrinus (1)
		Lactococcus piscium (1)

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Chapter 8

General discussion

INTRODUCTION

Growth of spoilage and pathogenic microorganisms in foods, is of importance to everyone involved in the production, distribution and consumption of foods and food products. Historically, microbial quality and safety of foods could only be determined by means of microbial shelf life tests. Shelf life and challenge testing of foods comprises inoculation of foods with microorganisms that are known to cause problems in the type of food developed. Some samples of the food are taken and the microorganisms are made to grow in the product under what are thought to be realistic product temperatures. The moment a product spoils, usually sensorically, is referred to as the shelf life of that product (3, 8).

Generally, the shortest shelf life value found in the survey is defined as the actual shelf life of the product. However, the variability in determined shelf life in one survey for one food is usually very high. Finally, the determined shelf life of a product is divided by an arbitrary number for safety reasons. Nowadays, product development has to be rapid, and shelf life testing is one of the time limiting steps that has to be undertaken for the production of safe foods. Shelf life testing is very time consuming and therefore expensive. An alternative method is described in this thesis, that relates shelf life with the composition of foods. This method uses models developed in predictive microbiology and recorded values of the environmental parameters of a food (temperature, acidity, and water activity) to calculate safety and shelf life of foods.

In predictive microbiology the relation between environmental parameters such as temperature or acidity and numbers of microorganisms is studied (5, chapter 2, 3, 4, 5). With the aid of models developed in predictive microbiology, new product formulations can be tested by computer calculation, before actually performing shelf life tests. On the basis of the predictions, unfeasible product formulations can be omitted from the shelf life tests. Furthermore, indications of shelf life can be given with less effort and with improved research data. However, in a final stage of product development, the predictions of the models need to be verified by means of rapid challenge tests or regular shelf life tests. The models developed in predictive microbiology can be used to speed up time consuming shelf life tests, but can not be used as a full alternative for shelf life testing (5, chapter 6).

In this last chapter of this thesis attention will be given to the requirements and possible extension of predictive microbiology. Attention will be given to modelling microbial growth of spoilage and pathogenic microorganisms.

PREDICTIVE MICROBIOLOGY

Requirements. The most important requirements in predictive microbiology are summed up below. An improved understanding of the factors that play a role in the determination of the safety, quality and shelf life of foods can be developed by addressing these requirements.

- Development of quantitative models,
- Development of qualitative models,
- Development of methods for combining quantitative and qualitative models,
- Development of methods for validation of models,
- Increasing accessibility of developed models and knowledge.

Most of these requirements have been worked out in this thesis, by assessing the aspects summed up in the following list. These aspects are given more attention in the rest of this chapter.

- Determination of the key parameters for modelling microbial food quality and safety,
- Counting techniques for low numbers of microorganisms,
- Models to relate growth parameters to the key environmental parameters,
- Methods to validate models, statistically or otherwise,
- Decision support system for the prediction of food safety and quality.

KEY PARAMETERS

A few of the key parameters for models for microbial safety and quality of foods are worked out below.

- The choice of the relevant microorganism,
- The environmental parameters (e.g. temperature, pH etc.),
- The medium in which growth is monitored.

Relevant microorganisms. The prediction of microbial quality and safety requires a choice of a relevant microorganism or a group of relevant microorganisms. The selection of relevant microorganisms is of utmost importance, since choosing the wrong organism might result in incorrect prediction of shelf life of foods and also in a wrong estimation of the quality and safety of the product. An identification system for bacteria is developed in this thesis (chapter 7) for the determination of the relevant spoilage organisms of a food. This identification system can currently be used to assess the name of, yet unidentified, lactic acid bacteria, but the

system is set up in such a way that it can be easily extended to other genera. Furthermore, the identification of lactic acid bacteria is important since apart from being spoilage microorganisms, they are important as starter cultures and claims exist that some species are active as probiotics.

For modelling microbial quality, generally, one of the predominant spoilage microorganisms is chosen. The choice of microorganism can also be guided by what is known of the properties of the microorganism related to the type of food product, and the conditions under which the food will be distributed. In chapter 6, a computerised system is developed that uses these properties for determining relevant organisms in a food. For modelling and insuring food safety, the response of each of the pathogenic organisms should be known. If the chance exists that the organism is present in a food, its growth or inactivation kinetics as a function of the environmental conditions should be known. By means of the models, combinations of environmental conditions can be chosen such that growth is severely inhibited, however, techniques should be developed to make sure that a possibly growing pathogenic microorganism is not present in a food.

For developing predictive microbiology models, two target organisms are chosen in this thesis, *Lactobacillus curvatus* (chapter 2, 3, and 5) and *Listeria monocytogenes* (chapter 4). *Lactobacillus curvatus* is chosen because of its relevance to the spoilage of meats and meat products. The strain of *Lactobacillus curvatus* used, was isolated from meats by Muermans *et al.* (6). *Listeria monocytogenes* is chosen as an example pathogenic organism because of its appearance throughout the entire food chain ranging from raw materials to end products. *Listeria monocytogenes* is able to grow at low pH-values, water activities, and at refrigeration temperatures. Therefore, the presence of *Listeria monocytogenes* in a product is likely and growth must be avoided by choosing adequate combinations of environmental conditions (3, chapter 3).

Environmental parameters. An environmental parameter that can be used for the development of predictive models should possess some basic characteristics. Firstly, it should affect microbial growth or death. Furthermore, it should be measurable both while setting up the model as well as in the (food) system to which the model is applied. Finally, to be able to influence microbial growth characteristics, the parameter should be controllable during production and/or distribution of the food.

Temperature is generally considered the most important environmental parameter determining growth of microorganisms, since it can be controlled during and after production and has little effect on the product itself (3, 5, 8). However, other environmental parameters can be equally important and should also be given attention to, such as acidity, water activity, amount of oxygen, redox potential, and concentration of growth inhibiting compounds (e.g. nisin or lactate). The effects of temperature and acidity on the bacterial growth parameters and the combined effect of these are studied in chapter 2. The combined effects of temperature, acidity, and water activity on bacterial growth rate are studied in chapter 3 and 4. The effect of acidity on bacterial growth and death rate is addressed in chapter 5.

The amount of oxygen and the redox potential also influence microbial growth. For instance, clostridia are strictly anaerobic. If this is not taken into account, predictions for growth of clostridia can be invalid. Therefore, these effects should be studied as well. Gas atmosphere or amount of oxygen present, can be modified and used for influencing shelf life of foods. In this thesis, however, the quantitative effect of oxygen was not studied since both target organisms (*Lactobacillus curvatus* and *Listeria monocytogenes*) are facultatively anaerobic. However, a qualitative model is described in this thesis that does relate bacterial growth rate to the amount of oxygen. The redox potential of a food system is also an important environmental parameter. However, redox potential can hardly be controlled in food systems. Therefore redox potential is not used as a controlling environmental parameter for predictive models. It should be mentioned that the validation of the *Listeria monocytogenes* model in chapter 4, showed that, in some cases, other factors have to be taken into account, such as the concentration of inhibiting chemical compounds.

Growth medium. The dependence of growth of microorganisms on environmental factors can be monitored in foods. However, since foods can be very complex, growth of microorganisms is usually followed in model systems. A model system can easily be controlled and all the factors that determine the growth characteristics of microorganisms can be manipulated. However, if the models have to be applied in real foods the predictions of the developed models also have to be checked in those foods. Some models are developed for foods, such as the model developed by Muermans *et al.* (6) for bacterial growth in meat products and the model developed by Dalgaard (1) for microbial growth in fish products.

Growth of *Lactobacillus curvatus* as well as growth of *Listeria monocytogenes* was monitored in model systems (see chapters 2-5). The growth characteristics of *Lactobacillus curvatus* were measured in MRS-broth, whereas the *Listeria monocytogenes* model was set up

in trypton soy broth. In both growth media it was found relatively easy to set water activity and acidity.

COUNTING MICROORGANISMS

Measuring microbial numbers of a developing population has been given attention to. The development of relevant models for spoilage microorganisms usually requires initial microbial numbers of 10^3 cfu per gram. Therefore, techniques with low detection limits should be used. The use, implication and drawbacks of several counting techniques are described in this paragraph. Furthermore, attention is paid to very low number counting, since this is one of the challenges for future predictive microbiology modelling.

In this paragraph, several aspects of counting microorganisms will be addressed:
- Plate counting and most probable numbers,
- Automated rapid methods,
- Limited growth and consequences for modelling,
- Extreme low numbers, probability modelling,
- Future developments.

Plate counting and MPN. A very laborious counting method, dilution in combination with plate counting, detects microbial numbers over the range of about 15 colony forming units (cfu) per gram to $> 10^{12}$ cfu per gram. On the basis of the most probable numbers (MPN) method even lower numbers (< 1 cfu per gram) per gram can be counted, although at higher values this technique is very laborious. In this thesis plate counting was used, for reasons explained in the rest of this paragraph.

Automated methods. Techniques are available that provide for quick, indirect measurement of microbial numbers, such as optical density, impedance or bioluminescence. These techniques are becoming very popular in predictive microbiology because of the possibility of automated generation of large quantities of data. The detection limits of these techniques differ from microorganism to microorganism, but the techniques are at best suited for microbial numbers higher than 10^5 cfu per gram (5). Direct counting techniques such as microscopy can be useful at low numbers of microorganisms, but are very laborious at high numbers. Flow cytometry or staining growing microorganisms and using automated microscopy with camera detection, yield expensive direct counting methods with low detection limits.

Limited growth. To develop preliminary ideas of the growth response of microorganisms to the environmental parameters, some of the described rapid automated detection techniques give quick and fairly accurate microbial data. However, these techniques should be used in combination with plate counting or microscopy, because especially near T_{min}, pH_{min}, pH_{max}, or $a_{w,min}$, growth is severely limited by the value of the environmental parameters and microorganisms do not reach the high numbers required by the indirect counting methods (chapter 2). The models developed in chapter 2 are used to illustrate the importance of low detection limits and well set up experiments. In this chapter, pH_{min} and pH_{max} were found independent of temperature, and T_{min} was found independent of pH. In chapter 3, the effects of temperature, acidity and water activity are multiplied resulting in an adequate description of microbial growth. This suggests that the regression parameters pH_{min} and pH_{max} are independent of water activity and $a_{w,min}$ is independent of acidity.

As an example, it is calculated what might happen if the extent of growth of *Lactobacillus curvatus* is studied with the aid of an automated indirect detection technique, such as optical density. An inoculum level of 10^4 cfu per gram is chosen which is just below the detection limit of 10^5 cfu per gram. Three fixed experimental time limits are chosen, reflecting the maximum duration of the experiments, being 10 days (240 hours), 25 days (600 hours), and 100 days (2400 hours). The number of microorganisms can be calculated by means of the reparameterized Gompertz equation (10). For this, growth rate, lag time, and asymptotic level of organisms are needed as a function of temperature and pH. The values for growth rate, lag time and asymptotic level of microorganisms can be calculated by means of the equations described in chapter 2, eq. 13, 15 and 11 respectively. The parameters for each of the models can also be found in chapter 2, see Table 2, page 29, and Table 3 respectively.

At a chosen temperature of 5°C, and an experimental time limit of 240 hours, a pH value of 4.66 can be found by means of trial and error (root finder), so that $N_{max} \approx 10^5$ cfu per gram. At this pH value, μ equals 0.0175 h^{-1}, λ equals 109 h, and $\ln(N_{max})$ equals 17.45. Therefore, the pH at which detection just does not occur at 5°C and an experimental time of 240 hours equals 4.66.

The calculated pH would be the observed pH_{min} given the chosen experimental set up. This exercise can be repeated for other temperatures by means of which a relation is found between temperature and pH_{min}. However, in reality, pH_{min} is found independent of temperature (chapter 2), so this is a theoretical exercise only. If another detection technique is used in

combination with a longer experimental time, or with a lower detection limit such as plate counting, this apparent relation between temperature and pH_{min} would not occur. The results of the calculations are given in Fig. 1.

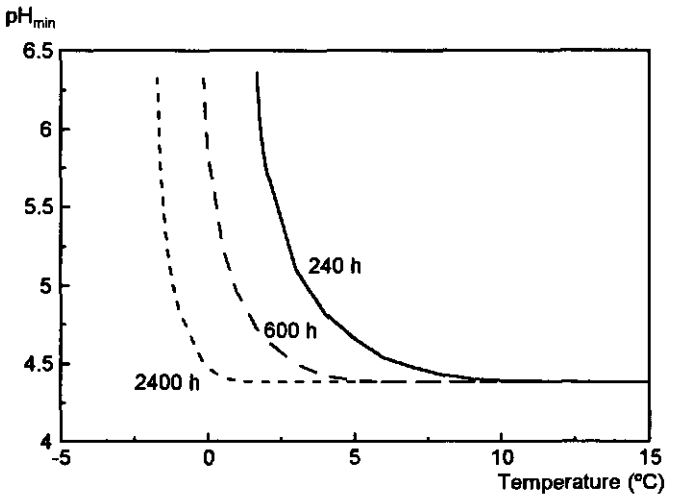

FIG. 1. Apparent pH_{min} as a function of temperature at several experimental time limits.

If the experimental time limit is set to 240 hours, the calculated pH_{min} approaches the upper boundary value of the 95% confidence interval of the fitted pH_{min} (4.38) (see chapter 2) at 14°C. If the experiments would last for 600 hours, the upper boundary value of the 95% confidence interval of pH_{min} is reached at 6°C. If the experiments last for 2400 hours, the calculated pH_{min} value reaches the 95% upper confidence boundary value at 2°C. Longer experiments lower the temperature at which the required calculated value of pH_{min} (4.38) is reached (e.g. 6 months, temperature = 0.5°C). These calculations emphasise the importance of well designed long term experiments and the use of detection techniques with low detection limits. The calculated lines in Fig. 1 are often referred to as the growth/no growth boundaries of a microorganism (7), but in fact are indications of the length of the experiments and the detection limits applied. In practice however, a product shelf life of 6 months can be satisfactory. Therefore, a product formulation can be chosen even within the growth ranges of the microorganism resulting in a semi-stable product.

TABLE 1. Infective dose of some food poisoning microorganisms (2)

Organism	Infective dose
Salmonella spp.	$10\text{-}10^6$
E. coli (O 157:H7)	10
E. coli (others)	$10^5\text{-}10^8$
Listeria monocytogenes	$10^7\text{-}10^8$
Campylobacter spp.	$10^3\text{-}10^5$
Shigella spp.	$10^2\text{-}10^5$

Probability modelling. For pathogenic microorganisms, even small numbers of microorganisms in foods may result in hazardous situations. The minimal infective dose of several food poisoning microorganisms is shown in Table 1 (2). The presence of 10 cfu *E. coli* (O 157:H7), in 100 gram of product can already cause illness (2). The currently available models in predictive microbiology are not set up to deal with these low numbers. Predictions of these models would therefore imply large extrapolations into an area where instead of actual microbial numbers, probabilities of presence of microorganisms play a role. Therefore, growth models should be used carefully for the prediction of the safety of foods. Limited growth modelling, and modelling growth-no growth boundaries, should be carried out at low bacterial numbers (e.g. 10 to 10^2 cfu per gram). The MPN-technique in combination with a very sensitive ELISA, lowering the detection limits even further, are the most sensible enumeration techniques.

However, target probabilities can become so small ($10^{-8}\text{-}10^{-15}$ cfu per ml) that large quantities of product are required. In practice, measuring becomes impossible. In this case, data can be derived from, for instance, epidemiological data. This is beyond the current scope of predictive microbiology, however, in future, this epidemiological or risk analysis type of data should also be modelled.

Current state and future developments. The most appropriate method for determination of microbial numbers for current and future predictive microbiology models are diluting and plate counting, flow cytometry, and automated camera detection. Since the use of flow cytometry also requires research for satisfactory fluorescent probes, which is outside the scope of this work, plate counting was chosen. Plate counting was automated as much as possible within the possibilities of the research. Serial dilutions of cultures were made manually. A spiral plate device was used to cover a number of serial dilutions. Counting the sectors of the plates was done manually. In future it seems logical to automate both the dilution and counting step. Reliable automated dilution equipment is now available in the market, and imagine analysis can

be used for determining the number of colonies on a plate. A combination of these three techniques would speed up data acquisition dramatically and should be investigated.

GROWTH MODELS

The kinetics of bacterial growth and decay need to be assessed to predict shelf life, quality, and safety of foods. The models developed need to be easily understandable and as generic as possible. In previous paragraphs some of the developed models were already mentioned and used. In this paragraph the development and quality of models will be addressed for which several aspects are worked out:

- General criteria for models,
- First stage models relating growth models to microbial numbers,
- Second stage models, for instance growth rate as a function of environmental conditions,
- Combining effects of environmental conditions of growth kinetics,
- Conclusions.

Criteria. There are a few criteria that may help to assess the quality of a mathematical model. First of all, the equation should describe the curvature of the measured data sufficiently well. This can be assessed by means of an *F*-ratio test as used in chapter 2, 3 and 4. Secondly, the parameters of the model need to be understandable and interpretable. Thirdly, there should be as few parameters as possible in an equation (parsimony). Lastly, the equation should be as generally applicable as possible. These criteria are used throughout this thesis to develop models and assess their quality.

First stage models. Several types of mathematical relations are used to model the development of populations in time. The Gompertz equation reparameterized by Zwietering *et al.* (10) has mostly been used for this purpose throughout this thesis except for in chapter 5. The modified Gompertz equation is used to estimate initial number, growth rate, lag time and asymptotic number of bacteria of a growing culture. After deriving values for these parameters, they can be related to the prevailing temperature, acidity, and water activity. The estimation of lag time, growth rate and asymptotic number is the first stage of the mathematical modelling.

Other models can be used to determine the value of growth rate, lag time and asymptotic number of microrganisms such as the logistic equation, the Verhulst equation, the Stannard equation, the Richards equation, the Schnutte equation, the differential equation of Baranyi,

and so on (5). All these equations can be used to fit sigmoid growth data. As mentioned earlier, the equation should contain meaningful parameters that can be fitted directly, preferably: lag time, growth rate, and under and upper asymptote. Also, the equation should not contain more parameters than needed. Typically, a sigmoid function needs four parameters, therefore, the function should not have more than four parameters. Lastly, the sigmoid function should be as generic as possible. If a sigmoid function possesses all of these characteristics, it can be used. The modified Gompertz equation satisfies all mentioned criteria in most cases, therefore, it is used to describe the increase of bacterial populations in time.

In chapter 5, initial decay of a bacterial culture is observed, therefore the modified Gompertz equation could not be used. Another equation is set up that could deal with this phenomenon. In this case, to describe the entire curvature, five parameters are needed, initial and final level, initial death rate, growth rate, and, possibly, lag time. Lag time might be an unidentifiable parameter since it can be derived from initial death rate and growth rate. The fits of the developed equation to the data in chapter 5, show that this is actually the case and a four parameter model proves satisfactory to describe the measured data. Therefore, one of the unidentifiable parameters is fixed, resulting in a four parameter model.

Second stage models. Mathematical equations relating the first stage parameters (growth rate, death rate, lag time, and asymptote) to the value of the environmental conditions are called second stage models. In this thesis, the emphasis is on the development of these types of models. The mathematical relation between temperature and the first stage parameters was studied by a large number of researchers and was assumed to be a given fact (5). Furthermore, the effect of water activity on bacterial growth was also investigated in great detail (4, 5), therefore a choice was made to investigate the effect of acidity. The three mentioned models, one for temperature, one for water activity and one, yet to be confirmed, model for acidity should be combined to result in one overall model for all these environmental parameters. This combination was already performed earlier for temperature and water activity where the effects of temperature and water activity were multiplied (4).

A pragmatic approach to modelling the combined effect of acidity and temperature on the growth characteristics of *Lactobacillus curvatus* is followed. At several individual temperatures, the entire growth response to acidity is measured and modelled. Intuitively it is expected that at low values for temperature, a microorganism would experience more problems in adjusting to low pH values than at higher temperatures. Therefore it was expected that the lowest pH at which microorganisms grow is influenced by temperature. However, the

parameters pH_{min} and pH_{max}, of the proposed acidity model, are found independent of temperature. The effect of temperature is considered a given fact, therefore, the parameters of the temperature model are estimated directly at several constant pH values. One of the parameters of the temperature model, T_{min}, is found independent of acidity, therefore, in chapter 2, it is concluded that the temperature and acidity models can be multiplied.

In chapter 3, a water activity model is multiplied with the temperature, pH model of chapter 2, to describe the effect of all three environmental parameters on bacterial growth rate. The resulting model is fitted to a combination of two independently measured data sets. One data set is measured for pH and temperature and another for water activity and temperature. After fitting the parameters of the resulting model, growth rates are predicted of another, newly measured data set. Since the resulting model predicts these new data well (see chapter 3) the multiplication of effects proves a good method for combining models for growth rate. The effects of temperature, pH and water activity are also multiplied for the development of a model for growth rate of *Listeria monocytogenes* (chapter 4).

A model for growth as a function of amount of oxygen is needed for the appropriate deduction of microorganisms able to grow in foods. Since no quantitative models are available, a qualitative model is developed. The model relates the amount of oxygen in a food, in the form of three distinct classes, to the ability of microorganisms to grow under different aerobic conditions. If a microorganism is able to grow (e.g. aerobic environmental conditions and aerobic microorganism), its growth rate is calculated as a function of temperature, water activity, and acidity. However, if the microorganism is not able to grow (e.g. anaerobic environmental conditions and aerobic microorganism), growth rate is set to zero. This is a simplified description of the effect of oxygen on microbial growth, and a more quantitative model is needed.

The length of the lag time influences the quality and safety of foods. During the lag time microbial numbers formally remain constant. Good second stage models for the length of the lag time are needed to make reliable estimates of the entire shelf life of foods. For pathogenic microorganisms, the importance of good models for lag time is evident. However, if the possibility exists that pathogenic organisms start growing, this is cause for concern. Lag time proves hard to model on the empirical basis as done in this thesis (chapter 2). A more physiologically oriented approach could maybe reveal the processes that are of importance to the amount of time microorganisms need to adjust to new environmental conditions. If the

physiological processes can be modelled mathematically, these models can be used to calculate shelf life of food products.

In chapter 6, it is assumed that the length of the lag phase is inversely proportional to growth rate as found by numerous researchers (9, pers. comm. T.A. Roberts, J. Baranyi, L. Rosso, T.A. McMeekin). In fact, it is noteworthy that in the case of *Lactobacillus curvatus*, this is not the case, as described in chapter 2. Unfortunately, this means that generic models for the length of the lag phase can not be developed with *Lactobacillus curvatus* as a model organism.

Combining effects. Extending the developed model with new environmental parameters should follow the same approach as used for the set up of the current model. The response of the organism to the new environmental parameter should be measured over the range of interest, at conditions that are close to optimal for the rest of the environmental parameters. Then, an individual model should be set up that describes the curvature of the measured data, and preferably consists of interpretable parameters. Next, two combination methods can be followed as demonstrated in this thesis. One approach is to vary the other environmental factors, in this case temperature, pH and a_w, and measure the full response curve of the new parameter each time (see chapter 2). With another approach, an overall model can be set up and used to predict new measured unmodelled data, where data should be gathered outside the region where the model was set up (see chapter 3).

The first method becomes increasingly more work if more environmental parameters are addressed. This can be explained by an example. Suppose the effect of temperature can be modelled by five growth curves (four parameter model) and the effect of acidity by four (three parameter model). At each temperature (of five), four growth curves for the effect of acidity, need to be gathered. So, in total at least 20 growth curves have to be collected. If this experimental approach is extended to water activity, and at least three growth curves for the appropriate description of the effect water activity are needed (two parameter model), in total, for the effect of temperature, pH and water activity, at least 60 growth curves have to be collected. Addition of a new environmental parameter of, say three growth curves per constant condition, requires at least 180 growth curves. This type of experimental design gives a high degree of certainty for the resulting model but results in an exponentially growing number of necessary experiments.

The second method gives less certainty, but is a lot less laborious. If the values for the environmental parameters for the auxiliary data are carefully chosen, the certainty of the accuracy of the model becomes larger. However, a combination of the first and second method is recommended. The full response of the "new" parameter can be sampled near the optima as well as halfway the minima and/or maxima of the other environmental parameters. Then, a model can be developed and fitted which in its turn can be used to predict data that were not used for setting up the model.

Conclusion. In this thesis the growth behaviour of *Lactobacillus curvatus* (chapter 2, 3, and 5) and *Listeria monocytogenes* (chapter 4) was studied as a function of acidity, water activity and temperature. For *Lactobacillus curvatus* the individual, separated effects of temperature, acidity and water activity could be multiplied. The same was done for *Listeria monocytogenes*. The resulting models for microbial growth rate showed good correlation with the measured data. The models for lag time can only be used to obtain rough estimates.

VALIDATION CONCEPTS

In chapter 1, two concepts of model validation are described, internal and external validation. It is also mentioned that both methods are used in this thesis. Internal validation can only be used to assess the descriptive quality of a model given the data on which it is based. The developed models of chapter 2, 3, and 4, are all validated internally. Some statistical approaches are used for this, such as *F*-ratio tests, predicted versus observed plots, constant variance test, and finally, but most importantly, looking at the results of the fits.

External validation is also used in chapter 3 and 4. In chapter 3, the predictions of the developed model for *Lactobacillus curvatus* are validated against data collected in MRS-broth, which was not used for setting up a model. The model that is set up describes the auxiliary data well as is shown by an *F* test, a predicted versus observed plot, and model prediction interval testing. In chapter 4, the predictions of the *Listeria monocytogenes* model are validated on the basis of generation times reported in literature. The model predicts generation times well for milk, cheese, growth medium, meats, and some vegetables. But the model predicts growth in cabbage juice poorly. This is probably caused by the presence of some chemical compounds that also influence bacterial growth of *L. monocytogenes*, apart from the factors used to develop the model. These chemical compounds are not present in any of the other foods, or do not play an important role.

FOOD DECISION SUPPORT SYSTEM

The use of mathematical models to obtain quick and easy estimates for shelf life of food products is very promising. However, the predictions of the models should be used with care. Before using the models developed, the user should know which microorganisms are of importance to the food that is being assessed. The models developed in predictive microbiology will predict the rate of growth of microorganisms, but will not tell whether microorganisms are present in foods. In chapter 6, a decision support system is developed that links the models developed in predictive microbiology with knowledge from food microbiology and food processing. With the aid of knowledge bases, heuristics, mathematical models, and fuzzy models, a judged calculation of shelf life and food safety is carried out, where those microorganisms that belong to the food product are considered. After running the system with the appropriate product formulation, the system will return the names of the key microorganisms and calculate their numbers during and after production and distribution.

The results obtained from the developed decision support system (FDSS) show that the system has promising potential to be used for product development and quality assessment of foods already under production. The concept of combining process engineering heuristics, microbiology, and knowledge of product development, results in a transparent method for the prediction of microbial food quality and safety. Microorganisms, ingredients, recipes and processes are stored in databases to which extra information can be added without altering the performance of the system.

The currently developed decision support system can be used to assess production steps that are critical for the growth or presence of microorganisms. Therefore, FDSS can assist with performing HACCP analyses of food production chains. The developed decision support system provides for objective predictions, however the predictions should be interpreted by the HACCP team for applicability to a specific situation. Currently, the developed system will be implemented into a commercial package that formalises the decisions taken by a HACCP team. The consequences of the decisions are calculated and, in future, strategic economical decisions can be based on the quantitative prediction of both shelf life, quality and safety of food products.

FINAL CONCLUSIONS

The identification of the microorganisms present in a food product can be carried out by means of an identification system for lactic acid bacteria that was developed in this thesis. This system helps determining the species name of the microorganisms that may have caused spoilage of the food product, using easily performable tests. At this time, the system works well for most groups of lactic acid bacteria and not so well for some other groups. Improved determination schemes are required for these microorganism groups. To obtain a system that works well for more species of bacteria, additional identification schemes to more genera can be implemented, which would enhance the applicability of the identification system. The method used for identification is generic and can easily be applied to other genera.

The influence of three important environmental conditions on microbial growth was studied where the final models were built up out of models for individual environmental conditions. By means of a transparent method, models were merged and the validity of the resulting models was tested both statistically and with a carpenter's eye. The described modelling techniques also showed that, in future, the parameters of the models can be determined by means of fairly simple experiments.

The feasibility of combining large quantities of food ingredient data, microbial growth models, process engineering heuristics and food technologist's expertise was tested by means of a decision support system. The developed system has become a promising tool for assessing quality of foods, calculating shelf life of foods, in training production staff, carrying out HACCP analysis of production chains and as a discussion partner that is not likely to overlook even complex interactions, as long as these are programmed into the system. One of the advantages of such a system is that it is accessible at all times, even if product experts are away. The system can easily be expanded with data and knowledge, therefore it can be tailored to work in specific food development and food production environments.

The most important advantage of the followed approach is that expertise from different sources, needed for the decision support system, is made explicit and made available to non-experts. The modelling procedure increases insight into the importance of factors influencing microbial food safety and food quality.

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Summary

Quality and safety of foods are often influenced by the presence and growth of microorganisms. Microorganisms in foods can be divided into two groups: pathogenic organisms, causing illness, and microorganisms that are not harmful to health, but that can spoil a product. Presence and growth of pathogenic organisms should be avoided as much as possible. Growth of spoilage organisms is allowed to a certain extent. Presence and growth of pathogenic microorganisms largely influences food safety, whereas growth of spoilage organisms, generally determines shelf life of a food product. Food quality is assumed to be influenced by both pathogenic organisms and spoilage organisms.

A method to predict microbial safety and quality of foods is presented. The construction of a food product from its ingredients is simulated, following a recipe. Food processing heuristics are combined with models developed in predictive microbiology. Parameter values of ingredients of foods, such as water activity and acidity, and models for microbial growth and decay are used for prediction. The values of these parameters are collected and present in databases. If required information is lacking, methods to make reliable guesses of the parameters are developed. Furthermore, expert knowledge in production and development of foods can be applied to improve the quality of prediction. Shelf life can be calculated as a function of fluctuating temperature in time. Several food distribution chains can be simulated to assess the influence of distribution chains on food quality. The described methods are implemented into a computerised decision support system.

Mathematical models for microbial growth, implemented in the decision support system, are given more attention to. Microbiological food quality is examined by modelling bacterial growth of a spoilage bacterium; *Lactobacillus curvatus*. Microbiological food safety is modelled by assessing growth behaviour of a pathogenic bacterium; *Listeria monocytogenes*.

Models that describe the effect of acidity, temperature, and the combined effect of these variables on the growth parameters of *Lactobacillus curvatus* are developed and validated. Growth parameters (lag time, specific growth rate, and maximum population density) are calculated from growth data at various temperature-acidity combinations. The effect of acidity is monitored at several constant temperature values. Models are set up and fitted to the data. The same procedure is used at constant acidity values to model the effect of temperature. For lag time, specific growth rate, and maximum population density, the effect of temperature can be multiplied with the effect of acidity. The models are equipped with parameters suggesting that organisms cease growing at minimal or maximal values for controlling variables (Temperature, pH, a_w). Evidence is presented for the existence of a lower and upper acidity boundary value for bacterial growth.

The effect of temperature, acidity, and water activity on bacterial growth rate of *Lactobacillus curvatus* is modelled in an extended model. The model is based on two, earlier developed models, one for growth rate as a function of temperature and water activity and the earlier mentioned model. It is assumed that combinatory effects between acidity and water activity do not exist. Therefore, the two models are multiplied to result into one model. The resulting model is fitted to data sets measured earlier, and the parameters of the model are determined. A new data set with values for controlling variables outside the data range where the model is developed, is used to validate the developed model. The model is found very well able to predict outside the measured data range.

Bacterial growth rate of *Listeria monocytogenes* is modelled as a function of temperature, acidity and water activity, for which two equations are developed. The first equation predicts growth rate at sub optimal acidity values, sub optimal temperatures and sub optimal water activities, the second model predicts growth throughout the entire acidity range. The models are validated statistically and by comparing model predictions with values reported in literature.

Finally, a computerised system for the identification of bacteria is developed. The system is equipped with a key to the identification of lactic acid bacteria. The identification is carried out in two steps. The first step distinguishes classes of bacteria by following a decision tree with general identification tests. The second step in the identification is the distinction of species within a class on the basis of biochemical fermentation patterns. During group classification, probabilities for test failure are used. These probabilities can be used for assessing the quality of a given test answer. The probabilities are also used to select the most probable test answer in case of an inconclusive test result. The probabilities of test failure are determined by a group of experts and a group of potential users of the identification system. During species identification, similarity indices are calculated for all bacteria in a class. The described identification system is able to "learn" from different sessions in the species identification step, improving both identification speed and accuracy. Because of the versatile way in which the system is set up, it can very easily be expanded with identification keys to other organisms.

Structured models and modelling methods are used to predict changes in quality and safety of foods. This thesis shows that even complex problems such as the prediction of the quality of foods, can be modelled through the combination of several models. Model systems are developed giving insight into the processes that are of importance in the determination of food quality and safety.

Samenvatting

Veiligheid en kwaliteit van levensmiddelen worden bepaald door een groot aantal factoren. Eén van deze factoren is het vóórkomen van microorganismen in levensmiddelen. Microorganismen kunnen worden onderverdeeld in twee groepen, te weten microorganismen die schadelijk zijn voor de gezondheid, zogenaamde pathogene microorganismen, en microorganismen die dat niet zijn. Schadelijke microorganismen moeten zoveel mogelijk afwezig zijn in levensmiddelen, of, indien ze toch aanwezig zijn, geremd worden in hun groei. Microorganismen die niet schadelijk zijn voor de gezondheid mogen aanwezig zijn in levensmiddelen en, tot op zekere hoogte, zelfs groeien. Wanneer deze onschadelijke microorganismen in te grote aantallen vóórkomen in een levensmiddel veranderen ze de eigenschappen van dit levensmiddel. In sommige gevallen is dat gewenst, zoals de produktie van bier, en andere gevallen is dit ongewenst, zoals bederf van vlees.

Om te kunnen voorspellen welke microorganismen gaan groeien en hoe hard ze groeien zijn wiskundige modellen ontwikkeld. In dit proefschrift zijn modellen beschreven om de groei van een niet-pathogeen microorganisme te beschrijven, te weten *Lactobacillus curvatus*. De snelheid waarmee *L. curvatus* groeit is onder andere afhankelijk van de temperatuur, zuurgraad en hoeveelheid water in een produkt. Naarmate de temperatuur lager wordt dan kamertemperatuur, gaat het organisme langzamer groeien totdat groei helemaal stopt. Hiervoor was in het verleden reeds een wiskundig model ontwikkeld. Dit wiskundige model is gecombineerd met een model wat in dit onderzoek is ontwikkeld, om de groei van *L. curvatus* bij verschillende zuurgraden te voorspellen. Naast een model voor *L. curvatus* is tevens een model ontwikkeld voor *Listeria monocytogenes*. Dit microorganisme is wel schadelijk voor de gezondheid.

De veiligheid en kwaliteit van levensmiddelen kan worden voorspeld met behulp van een computersysteem dat de ontwikkelde bacteriële groeimodellen gebruikt. Omdat er erg veel levensmiddelen zijn moest er een methode gevonden worden om levensmiddelen te groeperen. Daarnaast was het nodig om uit te kunnen rekenen wat er met de samenstelling van levensmiddelen gebeurt als ze bijvoorbeeld gemengd worden met een andere ingrediënt, of wanneer er water aan onttrokken wordt. Hiervoor waren wiskundige modellen beschikbaar of werden ontwikkeld. De ontwikkelde modellen kunnen gebruikt worden om uit te rekenen hoe lang het duurt totdat er een nog nèt acceptabel niveau van microorganismen bereikt wordt. Deze tijd wordt ook wel houdbaarheidstermijn genoemd.

Het ontwikkelde computersysteem kan worden gebruikt voor het berekenen van de houdbaarheid van reeds ontwikkelde en nog te ontwikkelen produkten. Daarnaast kan het gebruikt worden bij de opleiding van produktiemedewerkers, produkt-ontwikkelaars, studenten en zo voort. De voorspellingen van de systemen moeten worden gezien als indicatie,

niet als absolute uitkomsten. Eén van de voordelen van het ontwikkelde systeem is dat het altijd te raadplegen is, zelfs als produktexperts of leraren afwezig zijn. Doordat verschillende stappen van een produktieproces van levensmiddelen duidelijk op een rij gezet worden, geeft het systeem inzicht in welke stappen van belang zijn voor de produktie van veilige levensmiddelen.

In dit proefschrift heeft het combineren van verschillende soorten modellen centraal gestaan. Enerzijds zijn wiskundige modellen ontwikkeld en gebruikt, anderzijds zijn verbale, beschrijvende modellen toegepast om, wanneer wiskundige modellen ontoereikend waren, toch een voorspelling te kunnen doen. Zodra verschillende modellen worden gecombineerd bestaat de kans dat het resulterende model aangevuld moet worden met extra informatie. In dit proefschrift zijn methoden beschreven waarmee combinatie van modellen op een verantwoorde manier plaats kan vinden.

Curriculum vitae

Taco Wijtzes werd op 21 januari 1966 geboren te Groningen. In 1983 behaalde hij zijn HAVO-B diploma aan het Eindhovens Protestants Lyceum, gevolgd door het behalen van zijn VWO-B diploma in 1985. In datzelfde jaar begon hij aan een studie aan het Conservatorium van Utrecht, hoofdvak Fluit. Na een jaar aan het Conservatorium besloot hij te gaan studeren in Wageningen.

In januari 1992 studeerde hij af in de richting Levensmiddelentechnologie met als afstudeervakken Proceskunde en Levensmiddelen-microbiologie. Zijn stage-periode bracht hij door aan het Institute of Food Research in Reading, UK, bij Dr. T.A. Roberts. De stage resulteerde in een artikel dat is opgenomen in dit proefschrift. In datzelfde jaar ontving hij de Agridata informatica afstudeerscriptie prijs.

Vanaf februari 1992 tot en met april 1996 was hij als AIO verbonden aan de sectie Proceskunde van de Landbouwuniversiteit Wageningen en verrichtte hij het onderzoek wat leidde tot dit proefschrift.

Vanaf april 1996 tot heden is hij in dienst van Unilever, bij het Research Laboratorium te Vlaardingen, sectie Microbiology and Preservation.