

SPSD II

MICROBIAL FOOD SAFETY ASSESSMENT: DEVELOPMENT AND INTEGRATION OF GENERIC PREDICTIVE MODELING TOOLS

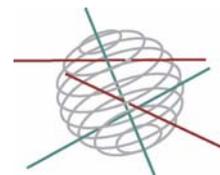
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PART 1

SUSTAINABLE PRODUCTION AND CONSUMPTION PATTERNS

-  GENERAL ISSUES
-  AGRO-FOOD
-  ENERGY
-  TRANSPORT



Part 1:
Sustainable production and consumption patterns

FINAL REPORT



**MICROBIAL FOOD SAFETY ASSESSMENT:
DEVELOPMENT AND INTEGRATION OF GENERIC
PREDICTIVE MODELING TOOLS**

CP/31

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1 INTRODUCTION

1.1. Context and summary

Modern consumers demand safe, natural, untreated, high quality, and wholesome food products, within the framework of sustainable development. The search for an economically feasible combination of all these issues is a major challenge for the food industry in the 21st century, and can only be attained by safeguarding the food chain *from farm to fork*.

A key element of the on-going implementation of the FAO/WHO Risk Analysis framework and principles is Microbiological Risk Assessment. Risks in the food chain are specified as related to pathogenic micro-organisms on the basis of sound science, combining qualitative and quantitative data in the areas of epidemiology and pathogenicity of micro-organisms with food production and handling (Klapwijk *et al.*, 2000). It is indicated that Europe is lagging behind North America both in terms of quantity of ongoing work and the depth of activity when referring to published Microbiological Risk Assessment studies. The urgency to catch up is illustrated in the European Commission's strategic objectives, priorities and work programme in relation to food safety in particular, and food law in general as outlined in the *White Paper on Food Safety* (COM (1999) 719 final, 12 January 2000). This document elaborates the Commission's commitment to develop *a comprehensive integrated approach* for regulating the food supply chain. In particular it proposes the establishment of a *European Food Authority* and an overarching set of definitions, principles and measures to ensure a high level of protection and the effective functioning of the internal market in food. Food laws should be based on the principles of *Risk Analysis*, and *Risk Assessment* should be based on the available scientific evidence and undertaken in an independent, objective and transparent manner (COM (2000) 716 final, 8 November 2000).

The (Belgian) Federal Agency for Safety of the Food Chain is officially established on February 4, 2000, as a direct consequence of the dioxin crises in June 1999. The Agency has two principal tasks: analysis of the risks related to food products and control of the complete food chain *from the stable to the table*. Hereto, the Agency should establish measures related to the analysis and control of risks that could endanger the health of consumers.

This Research Project is to be framed within the context of the above mentioned Risk Assessment. More precisely, this projects' overall objective is the development and integration of generic predictive modeling tools in the area *exposure assessment*, based on *predictive microbiology*, to enhance microbial food safety.

The (relatively young) discipline of *predictive microbiology* deals with the design and analysis of quantitative relations (mathematical models) aiming at the prediction of the evolution (growth, inactivation, survival, ...) of pathogenic or spoilage microorganisms (the so-called target-organisms) during subsequent stages of production, distribution and storage of food products. The project focuses on the development and integration of a collection of generic predictive modeling tools for predictive microbiology, hereby aiming at standardizing and consolidating the promising use of mathematical modeling techniques in the framework of risk analysis of foods. As a vehicle to demonstrate their intrinsic generic nature and applicability, two case studies (that are challenging from both the scientific and technological/economical point of view) will be used for development and validation purposes: (i) exploring the boundaries of microbial evolution, and (ii) quantifying interactions between micro-organisms.

1.2. Objectives

The overall aim of this Research Project is to design and exploit new generation predictive models able to predict the behavior of microorganisms in foods, taking into account their complex microbial ecology, as generic tools for microbial food safety assessment.

The scientific research objectives of this Research Project are threefold.

1. Development and integration of (both *macroscopic* and *microscopic*) building blocks into a *widely applicable novel generation predictive modeling methodology*.
Transferability (i.e., the generic nature of the approach) will be assessed at different levels.
 - a. To transfer model structures from one microorganism to another.
 - b. To extend model structures to describe more complex phenomena. For example: (i) to extend single species balance models to multiple species balance models describing interaction, and (ii) to extend kinetic models to incorporate the effect of multiple environmental factors.
This modular extension property also implies that models can be reduced in complexity in a natural way if certain conditions are satisfied (e.g., no competing species, environmental factor not limiting).
2. To increase the *fundamental insight in mechanisms underlying microbial lag phenomena*, with particular emphasis on (i) (sudden) changes in environmental conditions during microbial evolution, and (ii) the previous history of the cells.
3. To increase the *fundamental insight in antagonistic interaction phenomena occurring in mixed microbial cultures*, with particular emphasis on interactions caused by (i) a single metabolic product, or (ii) multiple metabolic products.

Technological research objectives can be formulated as follows.

Realization of scientific objective 1 results in a *standardized collection of rules, concepts, and techniques* helpful in building an appropriate model for the application under study. In other words, the modeling framework generates an application driven optimal modeling procedure (*modeling recipe*), ranging from experimental design and data collection, over data processing and model identification, up to protocols for model validation and validation in real food products.

As a *first technological research objective*, the microbial phenomena that form the subject of scientific objectives 2 and 3 will serve as vehicles to *demonstrate and validate the applicability of the novel modeling methodology*. Transferability assessment is important in this respect.

As a *second technological research objective*, a user-friendly portal site will be created on the Internet providing state-of-the-art electronic knowledge transfer to the food industry in Belgium. Within this project's lifetime, a one-way communication from the research consortium towards the Belgian food industry, academic institutions and regulatory bodies will be established.

1.3. Expected outcomes

Details on the expected technical outcomes will be presented in Section 2.

In the following paragraphs, two issues are dealt with: (i) the way this project is in the public interest, and (ii) industrial application possibilities.

Food-borne pathogenic bacteria are currently responsible for significant illnesses. The yearly number of cases of food poisoning in the Netherlands has been estimated on 2,000,000/year. As a result, there

is world-wide a substantially increasing interest in predictive microbiology that is expected to offer in the very near future an essential contribution to the improvement of the microbial safety and quality of foods. The availability of a user friendly, standardised predictive modeling methodology offers a front seat view on the behavior of micro-organisms in foods in response to changes in intrinsic, extrinsic or processing factors, or to changes in the formulation of food ingredients. Therefore, it enables to deal carefully with energy and time consumption, food ingredients and production of waste, contributing to the application of the *precautionary/preventing principle* in the frame of sustainable development. In addition, it opens interesting opportunities for *education and training*. Finally, the acceptance by the social instances of this research related to *food quality* and *public health aspects* is guaranteed.

With its annual turnover of 30 billion € (FEVIA, 2002), the food industry lies in third place for total Belgian industrial output. The food industry employs about 87,000 people (2001) which make it the second largest industrial employer in Belgium. More and more the presence of a sound risk assurance system in a food company, in which predictive microbiology can be an important tool, will be essential to deliver to important customers like distribution chains. As such, the development of knowledge regarding predictive microbiology contributes to *competitiveness*. Especially larger companies, that have in-house knowledge of food microbiology, will be able to assimilate the technique of predictive microbiology. As consolidation is one of the major trends within the European food industry, specialists are more and more available within the group a food company belongs to. The Advisory Committee associated to this project consists of a large number of companies and organisations in the food area. Its members are well divided over the different sectors: representatives of the meat industry, dairy industry, fish industry, ingredients and additives industry, savoury industry, prepared meals industry, handling and processing of fruit and vegetables, household and body care products, pharmaceutical industry, and (last but not least) the Federal Agency for Safety of the Food Chain and the Flemish Centre for Postharvest Technology. Both large companies and SMEs are represented in the Committee.

2 DETAILED DESCRIPTION OF THE SCIENTIFIC METHODOLOGY COVERING THE WHOLE PROJECT DURATION (DECEMBER 2001 – JUNE 2006)

This description is a fusion of the original project proposal (covering the period December 2001-December 2005) and the scientific description provided to motivate the project prolongation (covering the period January-June 2006).

2.1. Research strategy

The project objectives listed in Section 1.2 will be reached by carrying out four major Work Packages (WPs):

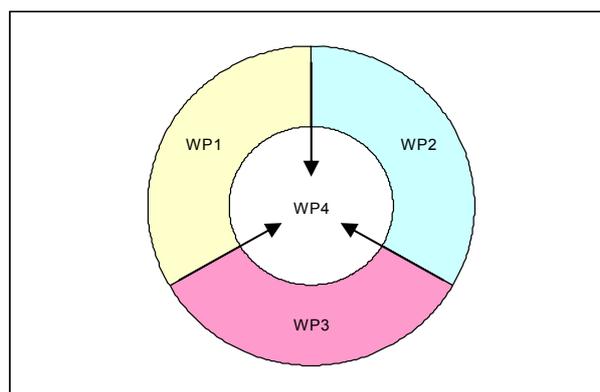
Work Package 1: Exploring the boundaries of microbial evolution

Work Package 2: Quantifying inhibition and inactivation phenomena due to microbial interaction

Work Package 3: Developing and integrating predictive modeling methodologies

Work Package 4: Towards a Belgian center for predictive microbiology/risk assessment

The relationship between these Work Packages is presented in the next scheme.



Scheme 1: Overview of the interface between the different Work Packages (WP's).

The novel predictive modeling methodology is developed in WP3, while microbial lag phenomena and microbial interactions are the subject of WPs 1 and 2 respectively. While WP3 has its own deliverables, it also serves as the mathematical foundation (see figure above) for the quantitative work to be performed in WP1 and WP2. The Work Packages 1 to 3 continuously interact at their interface.

✓ WP3 ↔ WP1 and WP3 ↔ WP2

The different steps in the model building cycle (namely, data generation, model development, and model validation) are to be performed in an iterative scheme. Optimally designed experiments (calculated based on methods developed in WP3) are needed to generate informative data sets of microbial lag phenomena in WP1 and of antagonistic microbial interaction phenomena in WP2. These data, in combination with available *a priori* mechanistic knowledge, will allow for proper model structure selection (based on building blocks developed in WP3) and model parameter estimation (including uncertainty assessment using techniques developed in WP3). Models in predictive microbiology are usually of the *grey box* (hybrid) type, combining mechanistic (*white box*) and regression (*black box*) elements. Validation of the model is a crucial step within this cycle: less

successful modeling attempts are the driving force for designing and performing more informative experiments that will yield models with a higher predictive value.

✓ WP1 ↔ WP2

While WP1 concentrates on the initial (lag) phase which delays initiation of microbial evolution, WP2 investigates the termination of microbial growth by inhibition or even inactivation effects. Proper integration of the elementary model building blocks describing these individual phenomena will result in new generation predictive models valid over the entire time domain from lag over exponential growth up to inhibition (and possibly inactivation).

Finally, all knowledge generated in WP 1 to 3 is integrated in WP4 to contribute to the establishment of a national center for predictive microbiology/quantitative microbial risk assessment.

2.2. Detailed description of the work program

Work Package 1: Exploring the boundaries of microbial evolution

In this study two approaches regarding modeling the lag phase are explored. In Task 1.1 lag phases induced by a sudden temperature change are studied, while in Task 1.2 the influence of the history of a contaminating cell population on the distribution of individual lag phases is examined.

Task 1.1: Quantifying lag phenomena due to temperature variations

Step 1: Experimental Protocol and Data Generation.

This task consists in formulating a reproducible experimental protocol and analytical methods for optimal determination of the existence and length of the lag phase caused by a change in temperature as well as for the identification of relevant influencing variables. In order to avoid interference of any change of the medium composition on the temperature-dependent lag, temperature shifts are applied during growth (lag, exponential or stationary phase). The experimental protocol implies the standardization of the pre-culturing conditions, the composition of the growth medium and the process conditions (other than temperature). A series of well-thought experiments will be designed from which the occurrence and the amplitude of the lag phases (in relation with the previous history as defined above) can be quantified.

The experiments with stepwise temperature changes will be performed in computer-controlled bioreactors equipped with sensors and programmable control systems for temperature, pH and dissolved oxygen. The following variables are determined off-line: (i) population density, (ii) substrate concentration, and (iii) metabolite concentration possibly affecting the lag phase. As a model organism, *Escherichia coli* K12 grown in a nutritionally rich medium is used.

Step 2: Model Construction.

In contrast with most predictive models to date, the *macroscopic* (i.e., population level) model will be *dynamic*, i.e., it will consist of a set of differential equations enabling continuous description of the lag dynamics. The qualitative knowledge derived from the rigorous experimental study is directly included within the structure characterization step. Next to the macroscopic modeling approach, the alternative *individual-based modeling* (cell level) techniques investigated in WP 3 will be applied.

Task 1.2: Quantitative study of the effect of the history of contaminating cells on their lag

Step 1: Experimental Protocol and Data Generation.

In a first step a protocol will be developed to determine the lag phase of an individual cell and in this way the distribution of the individual lag phase of cells within a contaminating population. *Listeria monocytogenes* is chosen as model organism. The lag phase of individual cells will be determined by following the individual growth in the wells of microtiterplates, containing one cell/well, by turbidity measurements in an ELISA reader. The protocol will be validated by comparing the obtained results with Solid Phase Cytometry (SPC) measurements.

In a second step, the influence of the history of contaminating *L. monocytogenes* cells on their individual lag phase (and on the distribution within a cell population) will be investigated for cells which are contaminating ideal but chilled substrates (7°C). This distribution will be determined for different histories of the contaminating cells (together with its influence on the apparent lag phase). The influence of individual factors (T, pH and water activity) as well as their interactive influence on (the distribution of) the individual lag phases within a cell population of *L. monocytogenes* will be quantified.

In a third step, the influence of the history of contaminating *L. monocytogenes* cells on their individual lag phase will be investigated for cells that are contaminating non-ideal chilled food substrates.

Step 2: Model Construction.

The obtained data will be used to develop models that are able to predict the influence of history factors on the apparent lag phase and on the distribution of the individual lag phase of a cell population at 7°C for *L. monocytogenes*. Parameters describing the observed distribution will be modeled as a function of history determining parameters (temperature, water activity and pH). The obtained data of individual lag phases within a cell population will be used to validate microscopic models developed in WP3.

Step 3 for Task 1.1 and Task 1.2: Model validation and transferability.

The model structure and the corresponding parameter estimates obtained from the previously mentioned experimental designs in Tasks 1.1 and 1.2 will be validated on new growth data. The validation tests for Task 1.1 will be generated under time-varying temperature conditions not tested during model development. For Task 1.2, the applicability of the proposed model structure will be tested for other microbial strains and other substrates.

Task 1.3. Growth behaviour of *L. monocytogenes* at the growth/no growth boundary

In Task 1.2, the individual lag phase of *L. monocytogenes* is investigated in conditions where growth was obvious. At the growth/no growth boundary, lag phases evolve towards infinite. Little is known about the kinetics of *L. monocytogenes* at this boundary although many food products are having properties in the range of this boundary. In this task, which is considered during the project prolongation (January – June 2006), lag phases are determined in the growth/no growth area first at high inoculation levels and then at low inoculation level. The dependency of the growth/no growth boundary of environmental conditions is also determined for low and high inoculum levels.

Work Package 2: Quantifying inhibition and inactivation phenomena due to microbial interaction

The primary aim is the development of dynamic predictive models for antagonistic interaction phenomena in mixed microbial cultures, consisting of 1 antagonist and 1 pathogen. Several mechanisms can simultaneously cause antagonistic phenomena. From a modeling viewpoint, it is therefore recommended to start with a simple, well-defined case study, based on a single mechanism: 1 antagonist → 1 metabolite (lactic acid) → 1 pathogen

Task 2.1: Antagonistic phenomena through a single metabolic product

Subtasks 2.1.1 and 2.1.2 Inhibition and inactivation phenomena

Step 1: Experimental Protocol and Data Generation.

Two different antagonist-pathogen systems will be investigated, namely *Lactococcus lactis/Listeria innocua* (co-culture 1) and *Lactobacillus sakei/Yersinia enterocolitica* (co-culture 2). Both selected antagonists are homofermentative and bacteriocin negative. During preliminary research of BioTeC and LFMFP, two interaction phenomena could be observed: (i) inhibition of the pathogen by the antagonist (bacteriostatic effect occurring in co-culture 1), and (ii) inactivation (after inhibition) of the pathogen by the antagonist (bactericidal effect occurring in co-culture 2). A subsequent characterization of co-culture 1 and 2 will enable to gradually incorporate both interaction effects in an appropriate model structure (see further). Experiments will be performed in a rich medium. Factors to be investigated are (i) temperature, (ii) the ratio of initial cell concentration antagonist/pathogen, (iii) initial medium pH, and (iv) medium buffer capacity. During experiments, the following variables will be monitored: viable/total cells (through plate/microscopic counting), medium components (through chromatography) and pH.

Step 2: Model Construction.

Two different strategies will be explored. In a first strategy, available single species models are used to fit experimental data of the pathogen in both pure and mixed cultures. Differences in estimated parameter values are quantified through statistical techniques. In a second strategy, a novel model is developed, which -in contrast to the previous approach- incorporates antagonism from the model structure characterization step on. Essential building blocks are (i) a set of dynamic balance equations for pathogen and antagonist and for all variables influencing or influenced by the microbial proliferation, and (ii) related kinetic models, describing the specific rates of the balance equations as function of state variables and other influencing factors.

Subtask 2.1.3 Uncoupling the individual lactic acid and pH effects

Step 1: Experimental Protocol and Data Generation.

The inactivation of *L. innocua* will be investigated at different combinations of initial pH (pH_0) and initial concentration undissociated lactic acid $[LaH]_0$. Two series of experiments will be performed. (i) To simulate the effect in co-cultures, the evolution of *L. innocua* is followed at an artificially created initial total lactic acid (i.e., both dissociated and undissociated forms) concentration. As such, *L. innocua* inactivation is investigated at (pH_0 , $[LaH]_0$)-combinations determined by the buffering capacity of the experimental medium. (ii) Inactivation of *L. innocua* will also be investigated at (pH_0 ,

$[LaH]_0$)-combinations not situated on the trajectory determined by the buffering capacity of the experimental medium, but forming an approximately rectangular shape in the (pH , $[LaH]$)-plane. In contrast to the inactivation in (i) and, for example, in co-culture 2 where pH and undissociated lactic acid are interrelated, the individual effects of both factors on the *L. innocua* inactivation can be separated.

During the project prolongation phase, and taking into account the actual project results (see, for example, the Third Year Report), much attention is paid on the further delineation of experiments enabling to test the variability in response observed during the previously performed experiments. Repetitions (originating from one pre-culture) are performed simultaneously, with multiple sampling at each time point, and microscopic cell evaluation. Other analyses can be considered if necessary to explain the variability.

Next to this fundamental issue, the kinetic experiments will be completed.

Step 2: Model Construction.

Available and, where needed, newly developed primary inactivation models will be used to describe the experimental data of *L. innocua* in both series of experiments. After selection of the most suitable primary model, a secondary model describing the variation of the primary model parameters with pH_0 and $[LaH]_0$ will be developed.

Step 3: Model Validation

The constructed model is validated during the project prolongation phase on a set of co-culture experiments, where a dynamic profile of pH and LaH is naturally occurring. As such, the model can be validated on its performance and, by confronting the model predictions with the newly developed experimental data, possibly induced dynamic effects can be identified and evaluated.

Work Package 3: Developing and integrating predictive modeling methodologies

While having deliverables on its own, WP 3 also provides methodological support for both WPs 1 and 2. Three tasks can be distinguished. First, Optimal Experimental Design techniques for microbial kinetic studies are explored in Task 3.1. This will allow obtaining informative, high quality experimental data in WPs 1 and 2. Further, generic macroscopic and microscopic model building blocks are developed and integrated in Tasks 3.2 and 3.3, respectively. In WPs 1 and 2, their applicability (and possible limitations) to model the specific phenomena under study are evaluated.

Task 3.1: Optimal Experimental Design (OED) of kinetic studies

Experiments are to be designed in order to perform the following steps in the modeling cycle.

Step 1: Model structure discrimination.

This involves the selection of a model structure out of a pre-defined set of candidate model structures (either newly developed or taken from literature).

Step 2: Parameter estimation of kinetic models.

Accurate parameter estimates can be obtained by application of dynamic profiles of experimental conditions during experiments. Optimal dynamic profiles can be designed by application of the

Optimal Experimental Design methodology, in which basically a scalar function of the so-called Fisher information matrix is optimized.

Step 3: Model validation.

Attention will be paid to OED for both mathematical and product validation.

For the project prolongation phase, the following aims are put forward (i) Validation of a series of optimally designed temperature profiles for optimal estimation of the CTMI parameters for the selected model organism (*E. coli* K12 MG1655). (ii) Transfer of the OED/PE methodology to the CPM (cardinal pH model) will be evaluated. The applicability of the *dynamic* pH profiles for parameter estimation will hereto be evaluated based on a literature review and a well-selected set of dynamic bioreactor experiments.

Task 3.2: Macroscopic predictive modeling

Macroscopic predictive modeling consists of the specification of a set of differential equations to describe the evolution of a system on a macroscopic, i.e., population level.

Step 1: Balance models.

Dynamic models will be preferentially applied. For both Work packages 1 and 2, it can be expected that the vector of state variables needs to be extended in order to include extra information on the microbial metabolism. This approach may contribute to accurately describe the microbial evolution aspects studied in WPs 1 and 2, for which the classical predictive models, based on the living cell concentration only, are not sufficient.

Step 2: Kinetic models.

Kinetic models to quantify the influence of environmental factors will be maximally based on available and newly collected (i.e., in WPs 1 and 2) mechanistic knowledge, completed with advanced black box models like artificial neural networks.

Step 3: Implementation of variability.

The uncertainty on model parameters and predictions will be assessed by computation of asymptotic standard errors and joint confidence regions, and by Monte Carlo analysis.

Task 3.3: Microscopic predictive modeling

Step 1: Individual based models.

The fundamental unit of bacterial life, encapsulating action, information storage and processing, as well as variability, is the cell. It therefore seems appropriate to construct models in terms of individual cells and their behavior. This is the domain of Individual based modeling (IBM). The first step in this approach is to devise a set of rules, consistent with observation, which govern the behavior of the microscopic entities and their responses to changing external conditions. This set of rules comprises the system model. The output of a well-designed simulation of the model should be comparable with the real (macroscopic) behavior that the model is attempting to explain.

Step 2: Implementation of variability and object oriented programming.

It is interesting to develop IBMs in an object-oriented programming language. The principle of the latter is to represent each simulated bacterial cell electronically as an object instance of an object-oriented program. These objects are called agents since they are independent entities with their own state (set of parameter values) and behavior (rules). As each simulated cell has its own set of parameters, which is an independent copy of the list of default parameter values, variation is straightforward. New values can be obtained by, e.g., random draws from a (Gaussian) distribution with a chosen coefficient of variation.

Work Package 4: Towards a national center for predictive microbiology/risk assessment

Task 4.1: One-way communication with the national food industry - Development of an Internet Portal Site

In this task an informative Internet Portal Site (i.e., in a first phase, a compilation of interesting links) will be constructed, forming a generic interface between regulatory bodies, industry and academic institutions in Belgium, focusing on the applicability of predictive modeling methodologies in the food industry. It should be noted that the actual trend of consolidation in the European food industry opens interesting perspectives towards the level of in-house knowledge of food microbiology and careful use of predictive microbiology. As such, the exploitation of predictive modeling methodologies in the framework of a quantitative microbiological risk assessment becomes more and more feasible for the Belgian industry, increasing the applicability of such a Portal Site.

The Advisory Committee of this Research Project consists of representatives of the major food industries (meat industry, dairy industry, fish industry, ingredients and additives industry, savoury industry, prepared meals industry, handling and processing of fruit and vegetables) as well as the recently established Federal Agency for the Safety of the Food Chain and the Flemish Centre for Postharvest Technology. An active input will be requested as to the structure of the Portal Site, as well as to the kind of information and the level of detail. This will be thoroughly discussed at the occasion of introductory (hands-on) predictive modeling courses offered to the members of the Advisory Committee during the early lifetime of the project.

As a starting point, the generic results of this project will be made available for the Advisory Committee through restricted access on this Portal Site. General information, however, e.g., an up-to-date exhaustive summary of freeware and commercial software packages for predictive microbiology, will be available to all interested companies and research institutions.

Task 4.2: Two-way communication with the national food industry - Towards the development of Active Server Pages

Exploitation of the predictive modeling framework is part and parcel of this research project. Therefore, in this task, the needs of the Belgian food industry in the form of specifications for the development of an Internet based interactive predictive microbiology tool (based on Active Server Pages) will be categorized and summarized. Active Server Pages have the important property that they are dynamically created, i.e., on the basis of a specific request. The active input of the Advisory Committee is requested, delineating (i) the most pertinent questions when using predictive microbiology methodologies arising in the different branches of the food industry, and (ii) the actual functionality and structure of the Active Server Pages. The following topics arise.

- A searchable overview of literature of predictive microbiology, microbiological risk assessment, HACCP, ...
- An overview of the results and conclusions of this project.
- An e-mail service enabling to post questions to one of the two research partners of this project after narrowing his search based on the actual knowledge database (including electronic communication of predictive modeling data and client-specific guidance throughout all stages of predictive model generation and exploitation).
- An electronic newsletter on industrial applications.

Note that the actual implementation of these Active Server Pages is out of the scope of this Research Project.

2.3. Distribution of tasks among the two partners of the Research Project

Work Package 1: Exploring the boundaries of microbial evolution

Both research groups aim at a profound experimental study and accurate quantification of lag phenomena in microbial dynamics.

Data Collection.

Within the scope of the available infrastructure in both teams, bioreactor experiments are only performed at BioTeC, while microtiter experiments take place at LFMFP. To establish a suitable experimental design for both experimental techniques, the large experience of BioTeC in this discipline will be exploited.

Model Construction.

With respect to model construction, BioTeC will develop macroscopic and microscopic models to describe the population lag and the individual cell lags, as influenced by temperature gradients (Task 1.1) and cell history (Tasks 1.1 and 1.2). LFMFP focuses on quantifying the effect of the history of contaminating cells on the distribution of the individual cell lags (Task 1.2).

Model Validation.

Both teams together will design, perform and evaluate a series of validation experiments. If necessary, model adaptations are accomplished.

Work Package 2: Quantifying inhibition and inactivation phenomena due to microbial interaction

Both research groups have two main objectives: (i) a fundamental contribution to the microbiological/experimental knowledge of microbial interactions, and (ii) the incorporation of this knowledge in the discipline predictive microbiology by means of appropriate mathematical models.

Data collection.

Given the numerous factors involved in the experimental study, both research groups perform experiments, after a thorough concertation with respect to the experimental protocol, taking into account the previous mentioned requirements. Within the scope of the available infrastructure in both

teams, bioreactor experiments are only performed at BioTeC, while the experiments with the pathogen *Y. enterocolitica* take place at LFMFP only. In general, LFMFP co-ordinates the experimental part, based on the huge experimental knowledge available within that research group.

Model construction.

With respect to the model building step, LFMFP focuses on statistical data processing (Sub-Task 2.1.1), while BioTeC develops innovative models (all subtasks). The efficacy of both strategies is critically evaluated. BioTeC co-ordinates this part, based on its vast experience as mentioned above and the relation of this Research Proposal with the EU-project PREMIUM.

Validation.

Validation experiments are designed, performed and evaluated by both teams together. If necessary, the developed models are further refined.

Work Package 3: Developing and integrating predictive modeling methodologies

Methodological modeling developments are conducted and guided by BioTeC, in close interaction with the research performed at LFMFP.

Work Package 4: Towards a national center for predictive microbiology/risk assessment

The homepage of the project is maintained at BioTeC, while the content is decided upon by both partners of the project. An active input of the Advisory committee is requested as well.

2.4. Timetable of Work

On the next page, details about the time schedule of this Research Project are given.

A detailed description of final results of this project will be presented in the next section (Section 3).

Months	Year 1		Year 2		Year 3		Year 4		Year 5	
	0 to 6	7 to 12	13 to 18	19 to 24	25 to 30	31 to 36	37 to 42	43 to 48	49 to 54	
Work Package 1: Exploring the boundaries of microbial evolution										
<i>Task 1.1: Quantifying lag phenomena due to temperature variations</i>										
Data generation	█									
Model development		█								
Model validation							█			
<i>Task 1.2: Quantitative study of the effect of the history of contaminating cells on their lag phase</i>										
Data generation	█									
Model development		█								
Model validation							█		█	
<i>Task 1.3: Growth behaviour of L. monocytogenes at the growth/no growth boundary</i>										
							█	█	█	
Work Package 2: Quantifying inhibition and inactivation phenomena due to microbial interaction										
<i>Task 2.1: Antagonistic phenomena through a single metabolic product</i>										
Sub-Task 2.1.1: Inhibition phenomena in model system Type A										
Data generation	█									
Model development		█								
Model validation			█							
Sub-Task 2.1.2: Inactivation phenomena in model system Type A										
Data generation		█								
Model development			█							
Model validation				█						
Sub-Task 2.1.3: Uncoupling the individual lactic acid and pH effects										
Data generation				█						
Model development				█						
Model validation								█		
Work Package 3: Developing and integrating predictive modeling methodologies										
<i>Task 3.1: Optimal Experimental Design</i>										
	█				█				█	
<i>Task 3.2: Macroscopic modeling</i>										
	█				█					
<i>Task 3.3: Microscopic modeling</i>										
	█									
Work Package 4: Towards a national center for predictive microbiology/risk assessment										
<i>Task 4.1: One-way communication</i>										
	█									
<i>Task 4.2: Two-way communication</i>										
					█					

3 DETAILED DESCRIPTION OF THE FINAL PROJECT RESULTS AND CONCLUSIONS

Work Package 1: Exploring the boundaries of microbial evolution

Task 1.1: Quantifying lag phenomena due to temperature variations

Step 1: Experimental Protocol and Data Generation.

Experimental protocol.

Experiments are performed in a computer-controlled bioreactor (*New Brunswick Scientific Inc., USA*) where *E. coli* K12 MG1655 is grown under aerobic conditions in 4.5 L Brain Heart Infusion broth (Oxoid). A recirculation chiller (*Neslab instruments Inc., US*) is connected to the bioreactor to obtain low temperatures. The inoculum is prepared by subculturing 5 μ L of a frozen (-80°C) bacterial culture twice in 20 mL BHI at 18°C on a rotary shaker (175 rpm), subsequently for 24h and 18h. Temperature and pH are on-line monitored and controlled during the experiments. In all these experiments, the pH is kept constant at 7.55. The heating rate that could be realised in the bioreactor is equal to $0.875 \pm 0.13^{\circ}\text{C}/\text{min}$. At regular time instants during the experiments, samples are taken aseptically. To determine the intermediate lag accurately, a higher sampling frequency was applied around t_{shift} , i.e., the moment of the temperature change. The cell density [CFU/mL] is determined by plate counting (on BHI) by means of a spiral plater (*Eddy Jet IUL Instruments s.a., Spain*). To determine the duration of the intermediate lag phase λ_s , the experimental data are fitted both with the growth model of Baranyi and Roberts (1994) and the model of Rosso (1995).

For some experiments, additional measurements were performed. Glucose concentration (substrate) is measured enzymatically (*Granutest*). Concentration of acetate (metabolite) is measured by means of a Gas-Chromatograph-Flame Ionization Detector. Protein concentration of the cells is measured by the method of Lowry. To obtain these proteins, the cells have to be washed, centrifuged and lysed by an extraction reagent (*BugBuster, Novagen, Inc., Germany*). Subsequently, the protein concentrations are measured spectrophotometrically (*RC DC Protein Assay, Bio-Rad Laboratories, Inc., USA*).

Experimental design and data generation.

A factorial design is outlined within this study. A graphical representation of the complete factorial design is shown in Figure 1. This experimental design is used to characterize the effect of (i) the amplitude of the temperature shift, (ii) the pre-shift temperature level, and (iii) the post-shift temperature level on the occurrence and length of an intermediate lag phase. Within the temperature range of interest, namely the suboptimal temperature range of *E. coli*, i.e., $10\text{-}40^{\circ}\text{C}$, a matrix of experiments with different pre-shift temperatures and temperature shift amplitudes (two factors) has been identified. For the initial temperatures, this range has been subdivided using intervals of 2.5°C yielding 13 levels. The temperature shift amplitudes range between -30 and 30°C with 2.5°C intervals. In this way, 25 amplitude levels are included. As constant temperature experiments (no lag induction) and experiments in which the temperature rises above 40°C or decreases below 10°C are not relevant for this research and will thus not be taken into account, the complete factorial design involves 156 experiments. The vertical lines in the matrix (Figure 1) indicate experiments with constant initial temperatures T_1 , while the horizontal lines represent experiments with constant temperature shift amplitudes ΔT . The centre horizontal line indicates

the experiments at constant temperature. Temperature up- and downshifts are positioned below and above this horizontal line, respectively.

T_1 ΔT	10	12.5	15	17.5	20	22.5	25	27.5	30	32.5	35	37.5	40
-30													
-27.5													
-25													
-22.5													
-20													
-17.5													
-15													
-12.5													
-10													
-7.5													
-5													
-2.5													
0													
2.5													
5													
7.5													
10													
12.5													
15													
17.5													
20													
22.5													
25													
27.5													
30													

Figure 1: Graphical representation of the experimental plan. This design embeds a complete factorial design. Initial temperature (T_1) and amplitude (ΔT) of the temperature step are indicated on the first row and column, respectively. The grey horizontal line illustrates the experiments at constant temperature. Other series of experiments (see text) are marked by the filled blocks.

Not all experiments within this factorial design have been performed within this study. This would namely present a very time-consuming and labor-intensive job. In order to reduce the number of experiments, this factorial design has been approached in a *systematic* way. In zones where the dynamics (i.e., the presence or absence of a lag phase) change abruptly, the experimental plan has been refined. For example, in view of characterizing the normal physiological range accurately, a denser grid was selected in the temperature zone 20-25°C, which is suggested to play a critical role in this phenomenon (Ng *et al.*, 1962).

The state of the cells has been kept constant at the moment of the temperature shift by applying the shifts to the same cell density. More specifically, temperature up- and downshifts were applied at a cell density of approximately 12 ln(CFU x mL⁻¹) and 15 ln(CFU x mL⁻¹), respectively. Hence, cells were in the same physiological state at the moment of the shift in all experiments with temperature up- or downshifts. Hereto, the moment of the temperature shift t_s has been adjusted according to the initial temperature.

The sets of experiments performed within this factorial design can be classified in the following groups of experiments.

- ✓ *Temperature upshift with T_1 constant at 15 °C.* A large set of experiments with the same initial temperature, i.e., 15°C, was performed (Figure 1, black boxes). Herein, temperature shifts with amplitudes of 0, 5, 7.5, 10, 15 and 20°C were applied after 18.67 hours (corresponding with a cell density of approximately 12 ln (CFU/mL). Later on, additional experiments with temperature shifts with intermediate amplitudes of 6, 6.8, 8, 8.8 and 9.5°C were performed in order to better define the lag/no lag boundary.
- ✓ *Temperature upshift with ΔT constant.* One extensive set and two smaller sets of experiments with temperature upshifts with a constant magnitude ΔT of 10°C (Figure 1, left down to right up diagonally lined boxes), 5°C (Figure 1, horizontally lined boxes), and 15°C (Figure 1, crossed boxes), respectively, were completed. The initial temperatures were varied from 10°C to 25, 30 and 35°C, respectively, with intervals of 2.5 or 5°C. The time to grow at T_1 was adjusted in each experiment in order to obtain approximately the same cell density at the moment of the temperature shift.
- ✓ *Temperature downshift with T_1 constant.* Two limited series of experiments with temperature downshifts starting at constant initial temperatures T_1 of 25°C and 35°C were also implemented (Figure 1, left up to right down diagonally lined boxes and vertically lined boxes, respectively). Temperature downshifts were applied after 2.25 and 4.5 hours (corresponding with a cell density of approximately 15 ln (CFU/mL), respectively).

Qualitative data description and simulation results.

Temperature upshift with T_1 constant at 15 °C. The initial temperature T_1 was kept constant at 15°C and temperature jumps with various amplitudes after 18.67 hours (i.e., at a cell density of approximately 12 ln(CFU/mL) were applied. A summary of the resulting intermediate lag phases as function of the amplitude is presented in Figure 2.

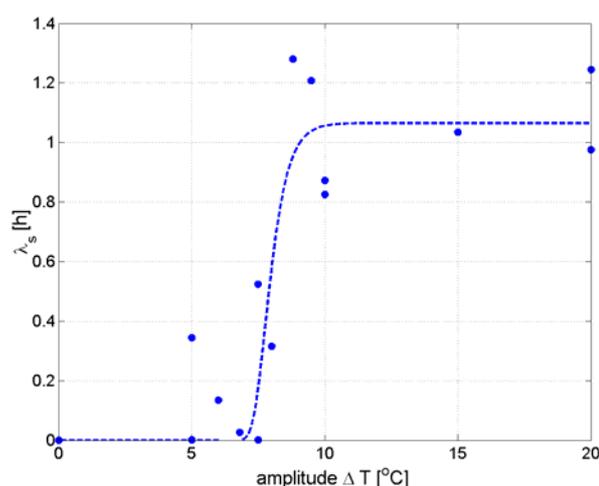


Figure 2: Intermediate lag phase λ_s [h] as function of the temperature shift amplitude ΔT [°C] for temperature shifts starting at the same initial temperature of 15 °C. (●) values of λ_s obtained by fitting with the model of Baranyi and Roberts (1994). A fit with Equation (1.1.1) is represented by the dashed line.

Roughly spoken, a temperature jump of maximum 5°C causes no intermediate lag phase, whereas temperature jumps with amplitudes larger than 8.8°C cause a lag phase with a constant duration of (approximately) one hour. It can be concluded that, in between, the duration of the lag phase seems to gradually increase with increasing amplitude.

For more details, reference is made to the research reported in Swinnen et al. (2003a,b ;2005).

Temperature upshift with ΔT constant. Temperature shifts with a constant magnitude ΔT of 10°C were implemented. The initial temperatures were varied from 10°C to 30°C with intervals of 2.5°C. The time to grow at T_1 was adjusted in each experiment in order to obtain the same cell density at the moment of the temperature shift (same as in the first set of experiments). Figure 3 gives an overview of the resulting intermediate lag phases as function of the initial temperature of the temperature change. Temperature shifts starting at 10, 12.5, 15, 17.5 and 20°C result in a significant intermediate lag phase. Observe that this value is the same as in the first set of experiments. Starting from 25, 27.5 and 30°C only a small lag phase was observed which moreover can be attributed to the time needed for the temperature rise (see Swinnen et al., 2005). The temperature shift from 22.5°C to 32.5°C caused a lag phase of medium length. The set of experiments was completed with some additional experiments to refine the relation between λ_s and T_1 .

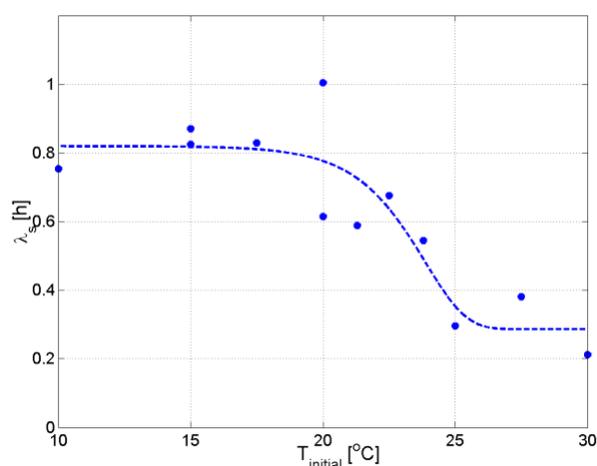


Figure 3: Intermediate lag phase λ_s [h] as function of the initial temperature of the temperature shift [$^{\circ}\text{C}$] for temperature shifts with a constant amplitude of 10°C. (●) values of λ_s obtained by fitting with the model of Baranyi and Roberts (1994). A fit with Equation (1.1.2) is represented by the dashed line.

By combining the results of both series of experiments, it can be concluded that the lower boundary of the normal physiological range lies between 22.79°C and 23.86°C (Swinnen et al., 2005). This normal physiological range can be defined as the linear part of the Arrhenius plot ($\ln(\mu_{\text{max}})$ versus $1/T$). It has been observed by Ng et al. (1962) that temperature shifts (positive or negative) within this region cause no lag phase, while shifts starting at a temperature below this range to a temperature within the range result in an adaptation period. We also observed an effect of the amplitude of the temperature shift on the lag phase duration.

Additional experiments with temperature shift amplitudes constant at 5°C and 15°C have been performed to further characterize the lag phenomenon and the normal physiological range. Experimental data

complied with the above-outlined observations. The large data set allowed construction of an overall model for lag (see below).

Temperature downshift with T_1 constant. A series of experiments imposing a negative temperature shift to an exponentially growing *E. coli* K12 MG1655 culture has been performed. On the basis of an extensive simulation study using the growth model of Baranyi and Roberts (1994) and the model of Hills and Wright (1994), it can be concluded that no significant adaptation period can be identified. The bioreactor and cooling system used in this research could not establish sudden temperature drops, whereas very high rates for temperature increases could be realized. Therefore, it can be derived that temperature decreases with a moderate rate in temperature change do not induce (visible) adaptation phenomena.

Metabolite and protein measurements. The evolution of the measured glucose and acetate concentrations proved that the lag phase was not caused by an exhaustion of the substrate (glucose) or an inhibitory concentration of acetate. Protein concentrations were measured but no reliable results could be obtained (e.g., due to the too small amount of cell material at the moment of the temperature shift).

Step 2: Model Construction.

Review on predictive modeling of microbial lag phenomena on a macroscopic level.

Factors influencing the lag time duration are the (changes in) environmental conditions (e.g., Whiting and Bagi, 2002), the identity and the phenotype of the bacterium (Buchanan and Cygnarowicz, 1990), the growth stage or physiological history of the cells (McMeekin *et al.*, 1993) and the inoculum size at the moment of the environmental change (e.g., Augustin *et al.*, 2000b). A survey of *predictive modeling of microbial lag phenomena on a macroscopic scale* was being conducted, mainly focusing on the influence of temperature and culture history on the lag phase during growth of bacteria.

In predictive microbiology, a two-step modeling approach is currently being used.

Primary models describe the evolution of microbial numbers with time and can be subdivided into deterministic and stochastic models. Primary *deterministic* models describe the evolution of microorganisms, using one single (deterministic) set of model parameters. The heterogeneous population model of McKellar (1997) is a static model, while the model of Baranyi and Roberts (1994) is already dynamic, i.e., the model is represented by differential equations (*balance models*). Both models cannot describe intermediate lag phenomena. The dynamic model of Hills and Wright (1994) is able to describe intermediate lag phases and makes a distinction between biomass and cell number. Baranyi and Roberts (1994) and Hills and Wright (1994) have included an extra (fitting) parameter to describe the physiological state of the cells. In primary *stochastic* models, the model parameters are distributed or random variables. The model of Buchanan *et al.* (1997) is a static model, but makes a difference between growth of biomass and cell number. McKellar (2001) has expanded his static model (McKellar, 1997) to a dynamic continuous-discrete-continuous model. Finally, Baranyi (1998) defines the relation between the individual cell's lag times and the population lag time. These three stochastic models cannot describe intermediate lag phases. Overall shortcomings are that assumed mechanistic concepts of the models are not experimentally validated. The influencing factors are mostly not included or lumped into one (fitting) parameter.

Secondary models (*kinetic models*), e.g., Augustin *et al.* (2000), describe the influence of the environmental conditions on the primary model parameters.

This survey of *predictive modeling of microbial lag phenomena on a macroscopic scale*, mainly focusing on the influence of temperature and culture history on the lag phase during growth of bacteria, is published in Swinnen *et al.* (2004a).

With regard to *microscopic modeling approaches*, reference is made to results reported under Task 3.3.

Modeling microbial lag due to a sudden rise in temperature

Models have been developed for the lag time data resulting from temperature upshift experiments. The following modeling approaches have been adopted.

(1) Modeling the independent effect of T_1 and ΔT on the lag phase duration (λ_s).

The effect of temperature shift amplitude and the effect of initial temperature have been assessed individually. Series of experimental data, i.e., either with constant initial temperature or constant temperature shift amplitude, were considered. The experimental data were presented above (see Figures 2 and 3) and are described by appropriate model equations (presented by the dashed lines) (Swinnen *et al.*, 2004b, 2005). The selected model structures describe the plateaus of “lag” and “no lag” observed within the data using an appropriate rate of transition. Note that the data reflect the normal physiological temperature range concept as reported by, e.g., Ng *et al.* (1962).

For the evolution of λ_s as function of the amplitude ΔT for the experiments starting at a constant initial temperature of 15°C, the following equation was proposed (as based on Van Impe *et al.*, 1992):

$$\lambda_s(\Delta T) = \lambda_{\max} [\exp(-\exp(\alpha(\Delta T_{\text{trans}} - \Delta T)))] \quad (1.1.1)$$

with λ_{\max} [h] the maximum value of λ_s , ΔT_{trans} the mid-transition value [°C], and α the transition velocity [°C⁻¹]. The relation between λ_s and the initial temperature T_1 for the experiments with a constant temperature shift amplitude of 10°C can be described by a modification of the former equation :

$$\lambda_s(T_1) = [\lambda_{\max} - \lambda_{\min}] \cdot [\exp(-\exp(-\alpha(T_{\text{trans}} - T_1)))] + \lambda_{\min} \quad (1.1.2)$$

with λ_{\max} and λ_{\min} , the maximum and minimum values of λ_s [h], T_{trans} the mid-transition temperature [°C], and α the transition velocity [°C⁻¹].

(2) Secondary model structure for λ_s as function of T_1 and ΔT .

The combined effect of T_1 and ΔT was integrated within a secondary model. The full set of λ_s data was subdivided into an identification data set and a (small) validation data set. First, the identification data were used to derive a set of candidate model structures. Next, the proposed model structures were put to trial using the validation set.

The present modeling results can be summarized as follows. Both a linear and nonlinear model structure was identified to the data. Identification of a response surface model (linear in its model parameters) results in a second-order polynomial relation embedding both quadratic and cross-product terms (interaction effect). At some points, the response surface model fails to describe the data, as the following unrealistic trends appear: (i) negative λ_s -values are predicted for small amplitudes and low initial temperatures, (ii) at high initial temperature levels λ_s decreases with increasing temperature shift amplitude. Inspired on the above-derived model equations ((1.1.1) and (1.1.2)), a nonlinear model structure could also be derived. Opposed to the response surface model, the nonlinear model embeds the normal physiological temperature range concept and better describes the trends observed in the experimental data. However, as both models have a comparable descriptive and predictive quality when computing performance criteria for the identification and validation data, model selection is not yet possible. Moreover, a large parameter estimation uncertainty may point at over-parameterization.

(3) Modeling the work to be done as function of ΔT .

A widely accepted *working hypothesis* in predictive microbiology, formulated by Robinson *et al.* (1998), states that lag is determined by two (hypothetical) quantities, namely, (i) the amount of work that a cell has to perform to adapt to its new environment, and (ii) the rate at which it can perform this work. Mathematically, this hypothesis can be formulated as

$$\langle \text{lag} \rangle \times \langle \text{rate} \rangle = \langle \text{work} \rangle$$

Typically, *<lag>* is identified with the lag parameter λ (in this research λ_s) and the *<rate>* is identified with the maximum specific growth rate μ_{\max} dictated by the present environmental conditions. The *work to be done* by the cells upon a change from one environment to another is given by the product $\lambda \times \mu_{\max}$. For several existing deterministic and stochastic population models, Swinnen *et al.* (2004a) has derived the relation between $\lambda \times \mu_{\max}$ and the physiological state parameter of the models considered.

In this research, the *work to be done* to adapt to a sudden temperature upshift when the culture is growing exponentially, expressed by $\lambda_s \times \mu_{\max}$ (where μ_{\max} corresponds to the maximum specific growth rate at the post-shift temperature level T_2), is modeled. Out of a series of model structures, a linear relation with translation was selected. This model structure reflects the normal physiological temperature range concept, and shows that the *work to be done* is proportional to the temperature shift amplitude. The obtained results were critically compared with other publications on this matter.

The latter work has been published in Swinnen et al. (2006).

Task 1.2: Quantitative study of the effect of the history of contaminating cells on their lag

Protocol development for isolation of individual cells

In a first step a protocol was developed to isolate single cells in the cup of a microtiter plate based on standardized dilution principles. The bacteria were subcultured twice to eliminate variance in the pre-cultural conditions. Afterwards the cell count was standardized to 10^8 CFU/ml using OD measurements at 600 nm. Starting from the standardized inoculum a classical dilution series was made ending up with 1000 cfu/ml. This cell count was controlled by plate counting of 200 μ l inoculum on TSA, incubation at 30°C for 24h. Further dilution was performed by adding 200 μ l of inoculum to 200 μ l broth in each cup of the first row of a microtiter plate. These cups were used to make further $\frac{1}{2}$ -dilution series ending up with single cells isolated in the cup of a microtiter plate (Figure 4). This procedure was repeated for 9 plates, resulting in 72 $\frac{1}{2}$ -dilution series. The content of each cup was plated on TSA and incubated for 24h @ 30°C to control the dilution pattern and to locate the single cells.

From the results, it was clear that individual dilution series do not follow the expected pattern from the theoretical mean values. Sometimes it can even be seen that empty cups are followed by cups containing one or even two cells. In contrast, the mean values do follow the normal expected dilution pattern.

Single cells are mainly located in the last 5 columns, so these columns are taken into account. In the last 5 columns, 75 cups containing cells were counted giving a yield of 75/72. From these 75 cups, 60 contained one single cell, while 15 cups did contain two or more cells, resulting in a chance of 80% having a single cell. When compared with protocols obtained in literature, a higher chance of having single cells (80% VS 37%) was combined with a slightly higher yield (using the new method it is possible to use several cups from one row).

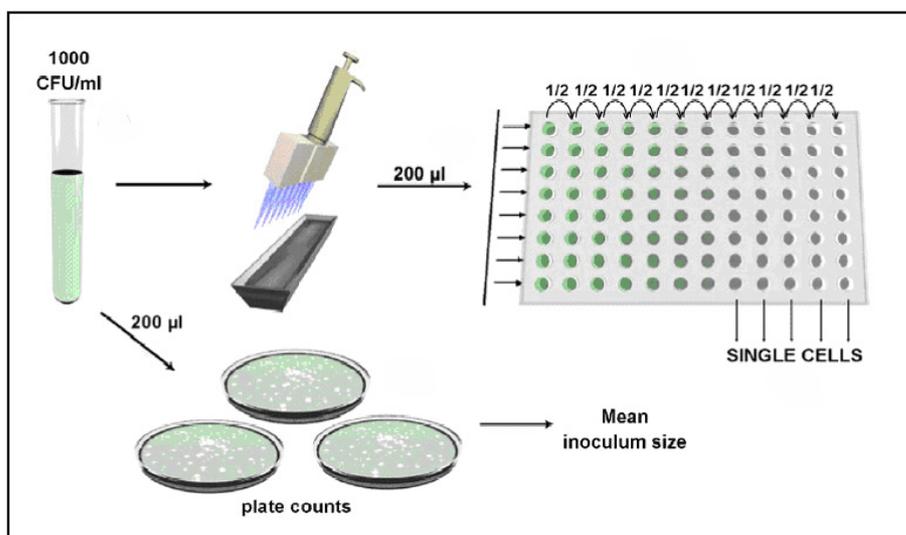


Figure 4: Overview of the dilution protocol

For more details reference is made to Francois et al. (2003) and Standaert et al. (2005).

Protocol development for measuring the lag phase of individual cells

For measuring the lag phase of individual cells (isolated in the cups of microtiter plates as previously described), a protocol was developed using optical density measurements in microtiter plates. Microtiter plates were filled as previously described, and incubated at the fixed conditions. The cell density was measured at regular intervals using OD measurements at 600 nm (Versamax microplate reader, Molecular devices, Sunnyvale, CA, USA). The upper part of the growth curve was generated by calculating the cell counts out of the OD values using a calibration curve. By extrapolating the linear part of the curve, the individual lag phase is cut off at the inoculation level (1 CFU/200µl = 5 CFU/ml) (Figure 5). At least 100 replications were made for each set of conditions.

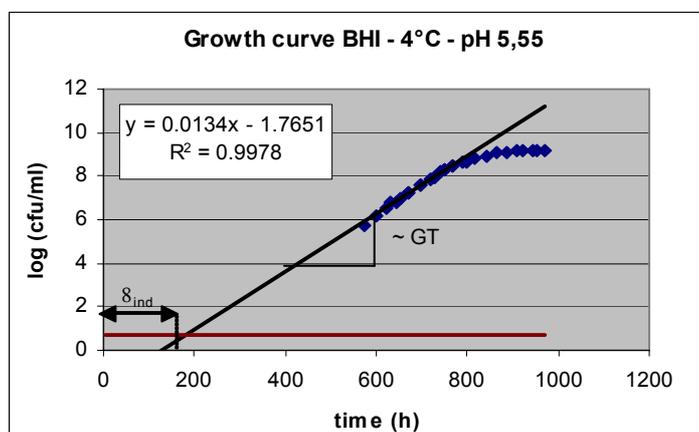


Figure 5: Linear extrapolation method to calculate individual cell lag phases (λ_{ind}) and generation times (GT)

The results from the optical density were recalculated as colony forming units as a function of time, using a calibration curve. Therefore, a dataset was generated containing 96 points starting from a cell density of about 5×10^9 CFU/ml and diluted in a $\frac{1}{2}$ way to a cell density of about 5×10^6 CFU/ml. Differences in OD between the blanks and the samples occurred starting from a cell density of 1×10^7 CFU/ml. A logarithmic transformation was done for both the OD values and the cell counts to equalize the differences between the data points. These transformed data were used to fit a linear regression curve. A good correlation was observed ($R^2 = 0,972$).

Using these data, the upper part of the *L. monocytogenes* growth curve could be constructed, consisting of a linear part (the exponential growth zone) moving over to the stationary phase.

The method assumes that once the cell lag phase has passed, a cell immediately grows at its maximum growth speed (μ_{Max}) until the stationary phase is reached. By extrapolating this linear zone, the individual lag phase is cut off from the inoculation level line and the generation time can be calculated from the slope. The data points for linear regression were selected by maximizing the adjusted R^2 . The linear extrapolation method was preferred over the sigmoidal curve fittings as high correlations were obtained for an easy to use method.

However, during the project it was noticed that environmental factors do have an influence on the relation between the optical density and the cell count.

Therefore, the effect of environmental stress factors on the relationship between the optical density measured at 600 nm, and the plate count results was investigated. Different Temperature levels (between 2°C and 30°C), pH levels (7.4 – 4.8), and a_w levels (0.995 – 0.946) were investigated as separate stress factors, and as combined conditions. Nineteen different combinations were tested. *L. monocytogenes* cells were grown in BHI, adjusted to the appropriate growth conditions. When the turbidity in the tube was maximal, a ½ dilution series was made in a microtiter plate, resulting in twelve consecutive dilution steps with eight replicates of each dilution. The optical density of the cups was measured at 600 nm using a Versamax™ microplate reader (Molecular devices, Sunnyvale, CA, USA) and consecutively the cell count was determined by classical plate counting on TSA. A logarithmic transformation was performed for both the OD values and the cell count data to standardize the variability and equalize the differences between the data points. These transformed data were used to fit a linear regression curve.

Different stress factors were shifting the calibration curve parallel to the optimal curve. Especially pH was having a main effect (Figure 6), while the pure effect of temperature and a_w was less pronounced, although these environmental factors played a more important role when different environmental factors were combined. The parallelism between the different calibration curves was statistically proved by an F-test. As the curves were assumed to be all parallel, a forced regression procedure was performed on all nineteen datasets: all regression lines were “forced” to have the same slope, while the intercept of the regression line was variable as a function of the environmental conditions.

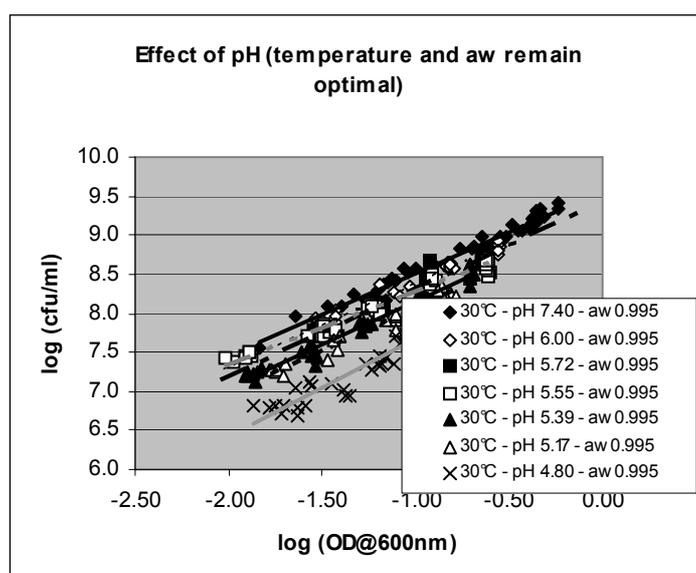


Figure 6: Effect of low pH-values on the calibration curve between OD and viable counts for *Listeria monocytogenes*

The forced calibration curve results were used to model the calibration curve shift as a function of the environmental parameters temperature, pH and a_w .

Microscopic viability tests showed a viability decrease with increasing stress levels, causing a shift of the calibration curve.

In a last step, a model was developed describing the effect of environmental factors on the calibration curve, making use of a constrained polynomial approach developed in Task 3.2.

For more details reference is made to Francois et al. (2005a).

Data collection evaluating the effect of environmental parameters on the individual lag phase of *L. monocytogenes*

In a third step, the individual cell lag time of *Listeria monocytogenes* was investigated as a function of temperature, pH and a_w . To isolate the single cells in the cup of a microtiter plate, the protocol that was previously developed was used.

In a first step the growth curves of *Listeria monocytogenes* were determined, starting from individual cells, measuring the optical density at 600 nm in a Versamax™ microplate reader (Molecular devices, Sunnyvale, CA, USA) as a function of time. A factorial experiment design was made incorporating temperature, pH and water activity.

For all performed experiments, generation times (GT) and individual lag phases (λ_{ind}) were calculated using the linear extrapolation method. High adjusted correlation factors were obtained for the linear regression (0,99 – 0,98). The results were examined at three levels: firstly the mean values of the individual lag phase and generation time were calculated for each set of environmental conditions and these results were compared to the predictions from the Pathogen Modeling Program (US Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, Pennsylvania, USA, <http://www.arserrc.gov/mfs/pathogen.htm>); secondly, histograms were made describing the data per set of environmental conditions; and thirdly, a distribution was fitted to the data using @RISK 4.5.2 Professional Edition (Palisade Corporation, Newfield, NY, USA).

All three factors had a significant influence on the distribution of the individual lag phases of a contaminating population of *L. monocytogenes*. The influence of temperature is illustrated in Figure 7 while the influence of pH is illustrated in Figure 8.

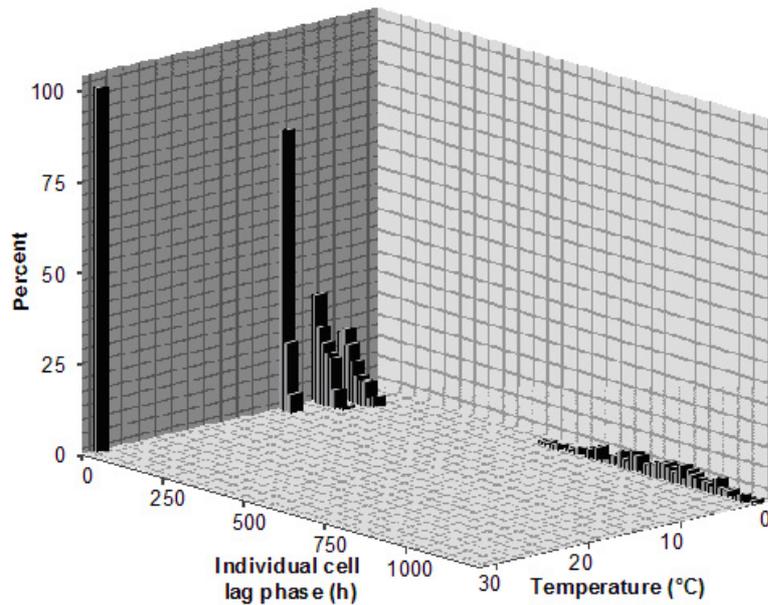


Figure 7: The effect of temperature stress on the distribution of the individual cell lag phase of *L. monocytogenes*

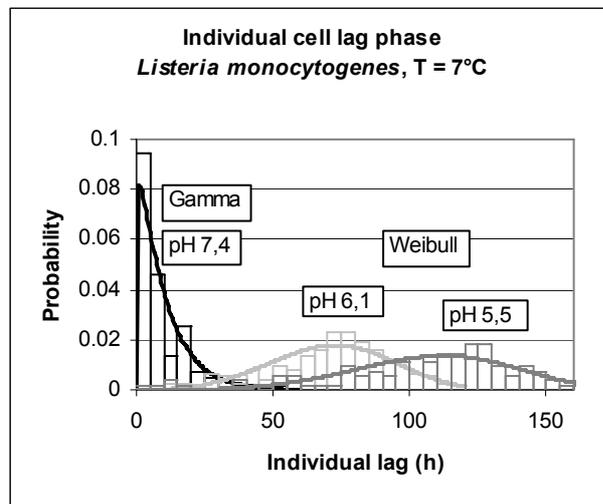


Figure 8: Distributions fitted on the histograms of the individual cell lag phases of *L. monocytogenes* cultivated at 7°C in BHI. pH was adjusted to 7.4 (no acid added), 6.1 and 5.5 using HCl.

Two types of distributions were necessary to cover the whole range of observed datasets: when dealing with low and intermediate stress levels, the gamma distribution fitted best to the data, while for higher stress levels a Weibull distribution is proposed (Figure 8). When dealing with rather low stress levels – which was often the case if only one type of stress was applied - the gamma distribution should be applied; when only temperature stress was applied, using the non-acidified growth medium, the gamma distribution was applicable for temperatures down to 7°C. When, on the other hand, the effect of pH was

tested for individual lag phases at 30°C, the gamma function was valid for pH values down to 5.0, although at such high stress levels the distribution fit was rather poor. When more severe stress conditions were applied (2°C or combined pH-temperature stress) the Weibull distribution delivered more acceptable fits for all combinations. This distribution was able to handle the right density shift in the distribution, and was proposed in @RISK as one of the best distributions for all combinations.

For more details reference is made to Francois et al. (2005b) and Francois et al. (2006a).

Modeling individual cell lag time distributions for *L. monocytogenes*

In this part of the project, the distributions that were collected in the previous part were modeled as a function of the environmental parameters temperature, pH and a_w . An integrated modeling approach was proposed and applied to an existing dataset of individual cell lag time measurements of *Listeria monocytogenes*. In a first step, a logistic modeling approach was applied to predict the fraction of zero-lag cells (which start growing immediately) as a function of temperature, pH and water activity. For the non-zero-lag cells, the mean and variance of the lag time distribution were modeled with a hyperbolic-type model structure. This mean and variance allow identifying the parameters of a 2-parameter Weibull distribution, representing the non-zero-lag cell lag time distribution. The integration of the developed models allows predicting a global distribution of individual cell lag times for any combination of environmental conditions in the interpolation domain of the original temperature, pH and water activity. These distributions are further applied to refine the risk assessment concerning *L. monocytogenes* by incorporating intercellular variability.

For more details reference is made to Standaert et al. (2006a).

Effect of pre-cultural conditions on the individual cell lag phase of *L. monocytogenes*

In this part of the project, the impact of the precultural temperature and pH on the individual cell lag phase of *L. monocytogenes*, incubated at 7°C, is assessed.

In a first step, the pure temperature effect (37, 15, 10, 7, 4 and 2°C) was investigated on a subsequent growth at 7°C and pH 7.4. In a second step, low precultural temperatures (10, 7 and 4°C) were combined with a controlled pH at 7.4 and 5.7 with a subsequent growth at 7°C and different pH values (7.4, 6.0 and 5.5). Growth was monitored by OD measurements at 600 nm using a microplate reader.

For all temperature-pH combinations, the individual cell lag phase and the subsequent growth rate were determined using a three phase linear growth model. Around 100 replications were made for each set of conditions. The results were shown as histograms, and distributions were fitted to those data. In most cases the exponential distribution gave the best fitting results.

It was observed that at low precultural temperatures, a high proportion of *L. monocytogenes* cells were able to grow with almost no lag phase. The lower the precultural temperature, the shorter the mean lag phase and the higher the proportion of cells showing no lag phase. Regarding to the pH effect, the pH transition from the precultural to a growth media was proportional to the mean values of the lag phases. There was no remarkable effect observed on the generation time.

For more details, reference is made to Francois et al. (2007).

Practical implications of the individual cell approach on the level of challenge tests

In this part of the project the focus of this research shifted from experiments in broths to tests in real food data: the variability in growth between individual *L. monocytogenes* cells was investigated on pâté and

ham. These results were compared to simulations based on previous data obtained in the project. Single cells were isolated by a dilution protocol which was a slight modification from the protocol previously described and inoculated on 15g samples of pâté and ham, pasteurized in the packaging. 250 samples were inoculated of each product, of which 50 samples were analyzed on each analysis day. The results are illustrated for pâté in Figure 9. Results were compared to Monte Carlo simulations performed in @RISK 4.5, based on distributions that describe the variability of the individual cell lag phases and generation times of *L. monocytogenes*. Based on the same simulation techniques, the variability effect was investigated for different inoculum levels (10, 100, 1000 and 10000 cells). It was demonstrated that the expected variability of the outgrowth of *L. monocytogenes* in a challenge test is very high for low inoculum levels.

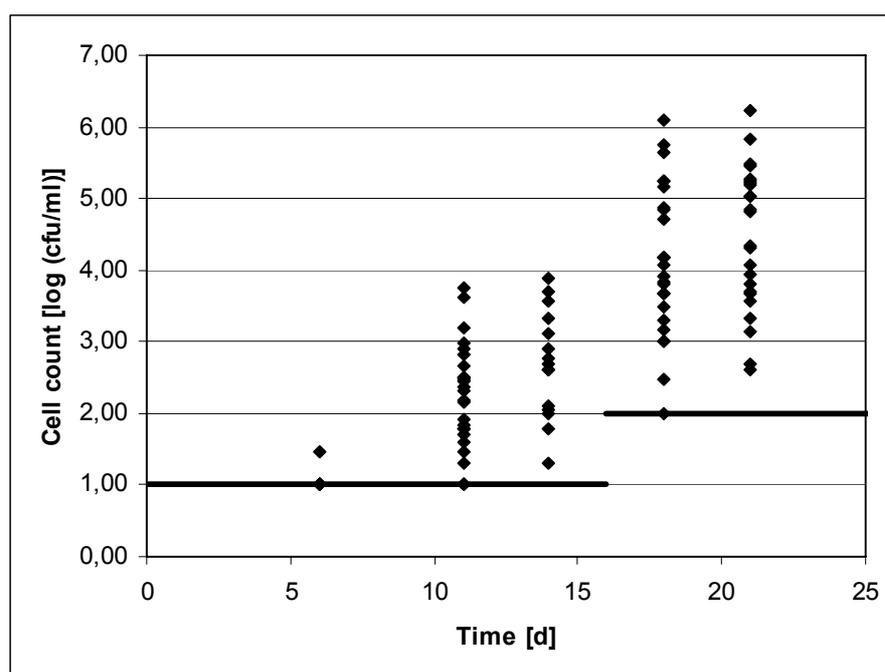


Figure 9: Cell density of *L. monocytogenes*, grown in liver pâté at 7°C, as a function of time. Single cells were used to inoculate samples of 15g of liver pâté. 50 samples were analyzed per day, except for day 14 (35 samples) and day 21 (48 samples)

The variability in growth characteristics observed between different single *L. monocytogenes* cells on foods appeared to be very large. The simulations based on the previously collected OD data in broths, could be confirmed by foods inoculated with single *L. monocytogenes* cells. The large variability between different individual *L. monocytogenes* cells has serious consequences for the experimental design of a challenge test. 1000 cells have to be inoculated to a food sample in order to reduce the variability to acceptable levels and quantify the behavior of the pathogen consistently.

For more details, reference is made to Francois et al. (2006b).

Practical implications of the individual cell approach on the risk assessment level

In this part, the effect of the individual cell lag phase variability was encapsulated in a risk assessment study for *L. monocytogenes* in pâté. A basic framework was designed to estimate the contamination level of the pâté at the time of consumption, taking into account the incidence levels and the initial contamination levels at retail. Growth was calculated on pâté units of 150g, comparing an individual

based approach to a classical population based approach. The two different protocols were compared using @RISK 4.5 simulations.

If only the individual cell lag variability was taken into account, while the other variables like the inoculum level or the time of consumption were fixed at their most likely value, important differences were observed between the individual based approach and the classical approach, especially at low inoculum levels, resulting in high variability when using the individual based approach. Although, when all variable factors, like inoculum level or time of consumption, were taken into account, no significant differences were observed between the different approaches, concluding that the individual cell lag phase variability was overruled by the global variability of the exposure assessment framework. Even if the simulated conditions became harsher, by lowering the inoculum level and lowering a_w , no differences were created between the individual based approach and the classical approach.

This means that the individual cell lag phase variability of *L. monocytogenes* has important consequences when studying specific growth cases, especially when the applied inoculum levels are low, but when performing more general exposure assessment studies, the variability between the individual cell lag phases is too limited to have a major impact on the total exposure assessment.

For more details, reference is made to Francois et al. (2006c).

Task 1.3. Growth behaviour of *L. monocytogenes* at the growth/no growth boundary

It should be remarked that a large part of this work was already accomplished during a previous two-year project financed by the Belgian Federal Public Service of Public Health, Food Chain Safety and Environment, while some scientific issues were dealt with during the prolongation phase of this project (January – June 2006). For reasons of clarity, all project results are summarized below.

In Task 1.3, more stringent conditions (in comparison with the conditions of Task 1.2), where the lag phase evolves towards infinity, were examined. The position of the growth/no growth boundary, i.e., the interface between conditions that allow microbial growth (finite lag phase) and conditions that inhibit growth (lag phase \approx infinity) was determined as a function of environmental conditions. If growth was possible, the detection time, i.e., the time elapsed before growth was detected, was estimated as measure for the lag phase. In a first series of experiments high cell numbers were considered; in a second series low cell numbers were examined.

To evaluate the growth behavior at the growth/no growth boundary, different media were made based on Nutrient Broth (NB). The media differed in their pH (between 5.0 and 6.0), a_w (between 0.960 and 0.990) and/or acetic acid concentration (between 0 and 0.8 % (w/w)). The NB media were buffered additionally by adding 2.5 g/L Na_2HPO_4 in order to be able to fine-tune the pH. To assure that the pH, a_w and acetic acid concentration did not change anymore after the preparation of the media, the media were sterilized by filter sterilization (\O 0.2 μm , Nalge Nunc International) instead of autoclaving.

The media were divided over different microtiter plates and inoculum was added to each medium. Since the inoculum was cultured (for 24 h at 30 °C) in standard NB instead of the pH-, a_w - and acetic acid adapted media, it was necessary to wash the cells prior to inoculation into the microtiter plates. This washing procedure consisted of a centrifugation (4600 g) of the 24 h culture. Afterwards the supernatant was discarded; the cells were resuspended in a saline solution and centrifuged again. Finally the saline solution was discarded and the cells were resuspended in the appropriate adapted medium.

Standardized inoculum cell densities were used in the experiments. Standardization was accomplished by optical density (OD) measurements, using a calibration curve for Nutrient Broth (NB) (OD-measurements at 380 nm in function of cell count) (using the protocols developed in **Task 1.2**). The initial cell density in the experiments at high inoculation levels was approximately 10^6 CFU/mL. The initial cell densities in the experiments at lower inoculation levels ranged from circa $2 \cdot 10^5$ CFU/well to 1 CFU/well (one well contains 200 μ L). This range of levels was obtained by making successive $\frac{1}{2}$ dilutions starting from $\pm 2 \cdot 10^5$ CFU/well. The applied dilution protocol was based on the protocol of Francois *et al.* (2003), since this protocol has the advantage of obtaining single cells with a high certainty combined with a sufficient yield. There is however a small difference between the protocol of Francois *et al.* (2003) and the applied protocol. In the latter, the same pipette points were used throughout the complete dilution series. Due to this small difference, adjustments to the simulation model of Standaert *et al.* (2005) were necessary to accurately describe the applied protocol.

Growth at the different pH, a_w and acetic acid levels was examined at 7 °C during 90 days. All media were tested in twenty replicates (high inoculation levels) or in forty replicates (low inoculation levels) to get accurate information about the variability in growth behavior of *L. monocytogenes* at the boundary between growth and no growth.

Growth was determined by measuring at regular time intervals the OD of the inoculated media with a VERSAmax microtiter plate reader at 380 nm. Growth was recorded if the OD increase was higher than three times the standard deviation of the OD of the blank. Determination of growth by OD measurements was feasible for high inoculation levels, even in stress conditions, because the inoculation levels were close to the detection limit. At lower cell densities, however, it was possible that the detection limit was not obtained even though growth had occurred.

If growth had occurred, the time to detection was also determined in the experiments at high inoculation levels. The time to detection was defined as the time at which the OD was higher than the noise on the signal ($OD_{\text{blank}} + \text{three times the standard deviation of the } OD_{\text{blank}}$) and was determined for each of the replicates of each medium in which more than 50 % of the replicates showed growth. The time to detection can be seen as a measure for the lag phase since the inoculum level is close to the detection limit.

The growth/no growth data at higher cell densities were used to develop a growth/no growth model that describes the interface between growth and no growth conditions as a function of pH, acetic acid concentration and a_w . Two types of models were considered, namely the ordinary logistic regression model (e.g., applied by Koutsoumanis *et al.*, 2004) and the square root-type logistic regression model (Ratkowsky and Ross, 1995). For the present case study, the former proved to be the better of the two.

The growth/no growth data (and model) at high cell density levels showed that the transition from growth to no growth was gradual (see Figure 10), meaning that there was no abrupt transition from the growth zone (i.e., growth in all replicates) to the no growth zone (i.e., no growth in all replicates). Instead, there was a transition zone between the growth and no growth zone in which the media showed growth in only some of the replicates. This gradual transition could be observed due the fact that the intervals between the levels were quite narrow and by the high number of replicates (20).

A major decrease in growth probability was noticed at the transition from pH 6.0 to 5.8 for all acetic acid concentrations (Figure 10). At constant acetic acid concentrations a major shift in the growth/no growth

boundary was seen at the transition from 0 % acetic acid to 0.2 % acetic acid (Figure 11). In the studied region for a_w (0.960-0.990), this factor had a minor influence on the position of the growth/no growth boundary (data not shown).

From the growth/no growth data (and model) at lower cell densities similar conclusions were drawn with respect to the abruptness of the growth/no growth interface and the importance of pH, acetic acid and a_w on the position of the interface. In addition it was concluded that the influence of the cell density is strong. A decrease of cell density goes along with a shift of the growth/no growth interface to milder conditions.

The time to detection results indicate that the time before growth was detected, globally increased when the combinations of environmental factors were more stringent. The biggest increase always seemed to take place at conditions very near to the no growth region.

It was also seen from the results that in general the confidence interval on the mean of the individual time to detection became larger under more stringent conditions (close to the no growth area). This is probably due to a larger variation in the lag phases. In some wells a subpopulation of resistant cells can be selected upon which subsequent growth of populations will depend.

Most of the above results are discussed in more depth in Gysemans et al. (2004a, 2006a,b,c), Vermeulen et al. (2006a,b) and Geeraerd et al. (2006). Publications of the more recent results are in preparation.

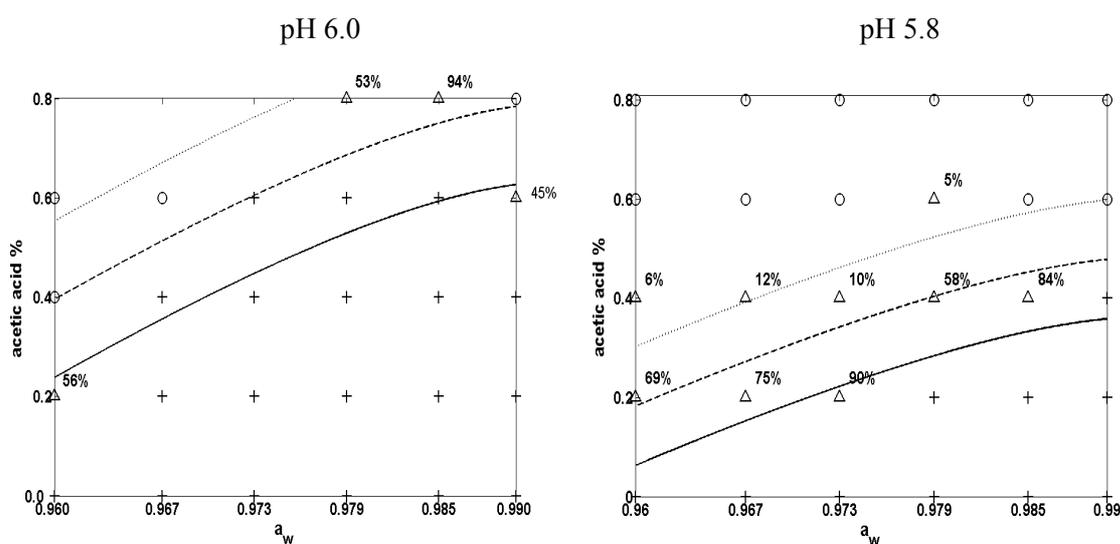


Figure 10: Probability of growth (p) at two different pH levels (A: pH 6, B: pH 5.8). (+) $p = 1$ (growth was observed in all of the 20 replicates), (o) $p = 0$ (growth was observed in none of the 20 replicates) and (Δ) $p \in]0,1[$ (growth was observed in some of the 20 replicates). In the latter case the measured percentage of growth is indicated. Lines represent the ordinary logistic regression model predictions; $p = 0.9$ (-), $p = 0.5$ (- -), $p = 0.1$ (· · ·).

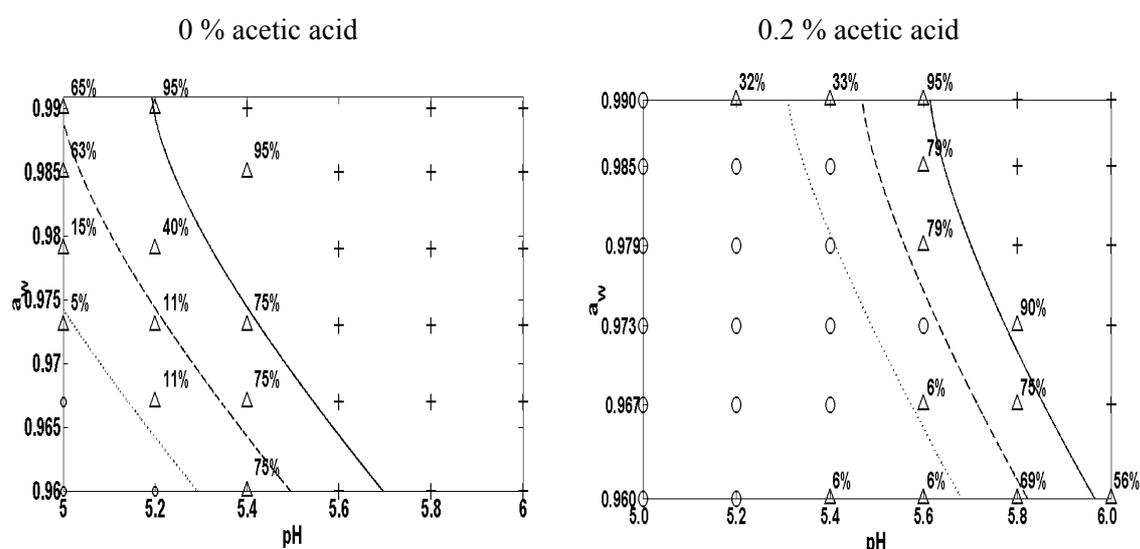


Figure 11: Probability of growth (p) at two different acetic acid concentrations (A: 0 % (w/w) acetic acid, B: 0.2 % (w/w) acetic acid). (+) $p = 1$ (growth was observed in all of the 20 replicates), (o) $p = 0$ (growth was observed in none of the 20 replicates) and (Δ) $p \in]0,1[$ (growth was observed in some of the 20 replicates). In the latter case the measured percentage of growth is indicated. Lines represent the ordinary logistic regression model predictions; $p = 0.9$ (-), $p = 0.5$ (- -), $p = 0.1$ (· · ·)

Work Package 2: Quantifying inhibition and inactivation phenomena due to microbial interaction

Task 2.1: Antagonistic phenomena through a single metabolic product

Subtask 2.1.1 Inhibition phenomena

This task was performed during the first and second year of the project.

Step 1: Experimental Protocol and Data Generation.

Experimental system

To allow for an unambiguous qualitative and quantitative analysis, we choose to start from a well-defined experimental system, designed as simple as possible (see Section 2.2). This system involves a two species population, in which I antagonist, a lactic acid bacterium, interferes through I antimicrobial metabolite, lactic acid, with I target, a foodborne pathogen. Two examples of this $I:I$ system are considered:

- *Lactococcus lactis* and *Listeria innocua* (Case study #1), and
- *Lactobacillus sakei* and *Yersinia enterocolitica* (Case study #2).

The selection of the antagonistic and pathogenic species, the metabolite and the further experimental implementation is guided by the following considerations.

Antagonist. The casting of a lactic acid bacterium as antagonist is self-evident, on the basis of its safety and antimicrobial potential. To preserve the single mechanism aspect, the lactic acid bacterium must be

homofermentative, producing lactic acid as a sole metabolite. In addition, it may not produce bacteriocins or other metabolites that may be toxic towards the pathogen.

Pathogen. For the same reason as the antagonist, the pathogen must operate a homolactic metabolism. The non pathogenic *L. innocua* is chosen as a model for the foodborne pathogen *L. monocytogenes*.

Metabolite. Since the antagonist is a homofermentative lactic acid bacterium, the single antimicrobial metabolite is automatically lactic acid. Lactic acid is produced by all lactic acid bacteria. Next, it is the only compound that appears as a single metabolite: production of other metabolites is always accompanied by lactic acid formation.

Medium. For reasons of convenience and reproducibility, the use of a commercially available undefined rich growth medium is evident. Such media can often be considered as representatives of foods, in which (essential) nutrients are usually abundant. In view of the desired homofermentative metabolism, the use of glucose as a carbon source is desirable. Further, to preclude competition for available nutrients, a possible second interaction mechanism, these nutrients must be present in excess at all times during mono- and coculture incubation. *In this project, considerable effort has been devoted to the establishment of a medium meeting these requirements (Vereecken, 2002).*

Environmental conditions. In order to maintain the homofermentative metabolism of *L. innocua*, experiments are performed in an anaerobic atmosphere. Other environmental factors are not critical with respect to the 1:1:1 system and are indicated further in the text.

Experimental plan

Experiments with the *L. lactis/L. innocua* case study are performed at BioTeC. Experiments with the *Lact. sakei/Y. enterocolitica* case study are conducted at the LFMFP. Prior to this experimental study, an experimental plan and protocol have been agreed upon between the two laboratories. The materials and methods applied for both case studies are thus merely the same. Briefly, growth curves of the *L. lactis/L. innocua* case study were collected in 1L erlenmeyer flasks, filled with 550 mL of medium, which were closed with screw caps containing a septum. The medium used was a modified Brain Heart Infusion (BHI) medium, containing 37 g/L BHI, 18 g/L glucose, 4 g/L yeast extract, 1mL/L Tween 80, 0.2 g/L MgSO₄·7H₂O and 0.04 g/L MnSO₄·H₂O. Before inoculation, the medium was flushed with N₂ to obtain anaerobic conditions and pH correction to a value of 6.2 was performed with HCl 4N. Flasks were incubated in a cooling incubator. At regular time intervals, samples were taken with a sterile syringe and needle through the septum. In these samples, growth of both antagonist and pathogen was quantified through determination of cfu/mL by selective plate counting. After filtration of the sample to remove the cells, the total lactic acid concentration, the pH and glucose concentration are monitored. pH was determined with a pH sensor. Total lactic acid was measured through gas chromatographic analysis of the methyl ester. Glucose concentration was determined enzymatically (Granutest, Merck) to verify the absence of glucose exhaustion.

All experiments are performed in duplicate. The experimental plan is illustrated in Table 1. As can be seen from this table, an assessment of the influence of (i) the inoculum concentrations of antagonist and pathogen, and (ii) the temperature on the interaction effects is aimed at. The selected levels of the influencing factors can be motivated as follows.

Inoculum concentration. For the monoculture experiments, a fixed inoculum level of 10³ cfu/mL is selected. It is widely accepted that for single species growth, the maximum specific growth rate and the maximum cell concentration, which are important growth parameters in this research (see further), are not (or only negligibly) influenced by the inoculum size (if not too low or too close to the maximum cell concentration) [see, e.g., Buchanan and Phillips (1990)]. An investigation of different initial cell concentrations is thus not necessary. However, it is uncertain whether this precept can be extrapolated to

the *coculture* proliferation. For example, many research reports mention the influence of the antagonist's inoculum on the interaction effect experienced by the pathogen [see, e.g., Skyttä *et al.* (1991)]. Therefore, we have opted to test a number of antagonist/pathogen inoculum ratios. The selected ratio levels differ from each other with respect to the cell concentration of the antagonist.

Temperature. It is widely accepted that temperature is a main factor determining the microbial behavior in food. Each set of mono- and coculture experiments, with initial cell concentrations as specified in the table, is performed at different temperatures. The tested temperature levels include values typical for fermentation processes on the one hand (37, 35 and 22°C), and for cool storage -with a possible temperature abuse- on the other hand (12, 7 and 4°C).

Table 1: Experimental plan for Subtask 2.1.1

	Case study #1	Case study #2
Inoculum ratio <i>antagonist / target</i> [cfu/mL]	10 ³ / 0 0 / 10 ³ 10 ³ / 10 ³ 10 ⁴ / 10 ³ 10 ⁵ / 10 ³ 10 ⁶ / 10 ³ 10 ⁷ / 10 ³	
3.1.1.1 Temperature [°C]	35,12	37, 22,12,7,4

Experimental results

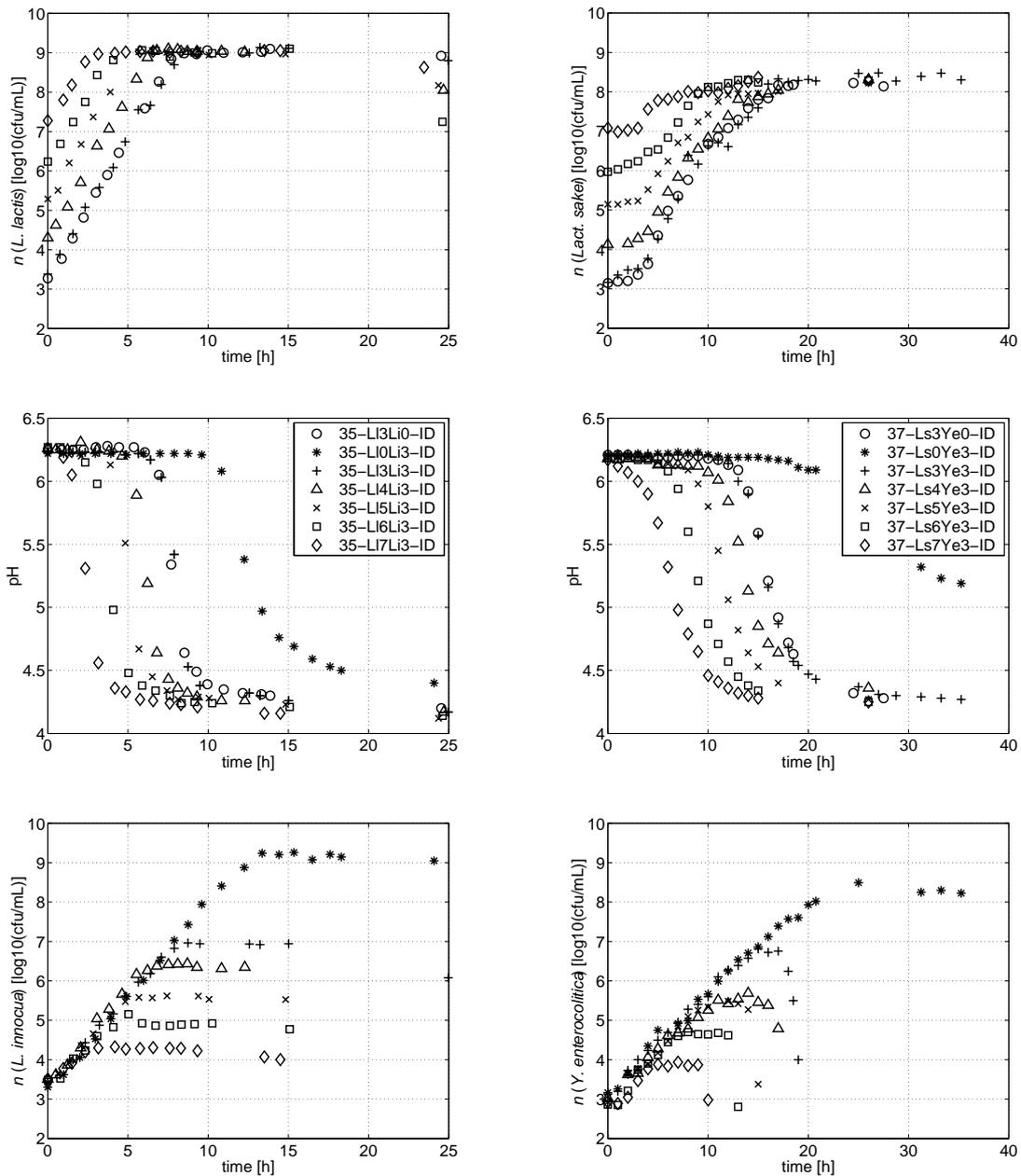


Figure 12: Cell concentration and pH versus time for *L. lactis*/*L. innocua* at 35°C (left) and *Lact. sakei*/*Y. enterocolitica* at 37°C (right). Each experiment is referred to with a code, denoting the temperature and inoculum ratio, e.g., 37-L4Y3-ID indicates the experiment at 37°C with an inoculum of 10⁴ cfu/mL of *Lact. sakei* and 10³ cfu/mL of *Y. enterocolitica*.

Figure 12 represents the cell concentration and the pH as a function of time for Case study #1 at 35°C (left panel) and for Case study #2 at 37°C (right panel). For the monoculture experiment, a clear exponential growth phase and a stationary phase can be observed, whereas a lag phase is barely present (except for *Lact. sakei*). Significant acid production (not shown) (and corresponding pH reduction) is only apparent from the late exponential phase on. In the coculture experiments, lactic acid is formed in a larger amount as compared to the monoculture experiments because of the additional production by the lactic acid bacterium. For both case studies, two distinct antagonistic effects emerge, namely, an early initiation of the stationary phase and a decline phase, where the cell concentration is reduced to beneath the detection level.

For Case study #1, the growth of *L. lactis* (Figure 12, left panel) always proceeds at the same rate, evolving to the same stationary level, irrespective of the co-incubation with *L. innocua*. On the other hand, in the different experiments, the inhibition of *L. innocua* occurs at a gradually earlier time instant for increasing antagonist inocula, but always synchronous with the abrupt increase in lactic acid (not shown in the figure) and decrease in pH. In addition, a complete inactivation (i.e., to below the detection limit of $10^{2.8}$ cfu/mL) is obtained at the end of some experiments. For the *Lact. sakei*/*Y. enterocolitica* cocultures in Case study #2 (Figure 12, right panel), the same features as for the *L. lactis*/*L. innocua* curves can be recognised: the growth characteristics (i.e., lag phase duration, maximum specific growth rate and maximum cell concentration) of *Lact. sakei* remain unchanged, while the *Y. enterocolitica* growth curves are distorted by inhibition and inactivation effects. As for the previous case study, the stationary phase of *Y. enterocolitica* starts earlier when the initial cell concentration of *Lact. sakei* increases. However, in contrast to *L. innocua* in Case study #1, *Y. enterocolitica* cannot maintain the stationary cell level for a long period. In all experiments, a pronounced inactivation takes place quite rapidly after inhibition. Furthermore, from the curves, it is clear that the inactivation rate is significantly larger than the preceding growth rate.

It appears that two threshold concentrations of lactic acid exist, of which the first one is bacteriostatic, and the second one -only attained in the coculture- is bactericidal. It should be noticed here that the data of glucose concentration reveal that there is never substrate limitation (data not shown). By consequence, all intra- and interspecific interaction effects, in casu induction of the stationary phase and/or the decline phase, can only be ascribed to the increasing lactic acid concentration, which is in agreement with the particular intoxication mechanism, postulated above.

Step 2: Model Construction.

A *first approach* consists of exploiting predictive modeling knowledge for pure cultures in order to quantify interaction phenomena in mixed cultures [see, e.g., Buchanan and Bagi (1999)]. A classical single species model, namely, the model of Baranyi and Roberts (1994) is used to fit the experimental data of the pathogenic organism both in pure and mixed culture. Discrepancies in the estimated values for the growth parameters (lag phase, maximum specific growth rate and maximum cell concentration) are quantified by means of statistical techniques and can be regarded as a measure of the degree of interaction.

Application of this method to the experimental data reveals, as expected, a pronounced reduction of the parameter denoting the maximum cell concentration in coculture as compared to its value in monoculture (results not shown).

Positive aspects of this approach are its simplicity and descriptive quality. However, interaction effects are only *reflected* in the numerical values of the parameters and not (mechanistically) *explained*. Therefore, a *second approach* is proposed, in which interaction effects are embodied in the model's structure.

As a first step, a reaction scheme for the 1:1:1 type interaction is outlined in Figure 13, including available mechanistic knowledge. In this scheme, the full line arrows indicate the different subprocesses, i.e., the growth and lactic acid production by the antagonist and the pathogen, and the equilibrium dissociation reaction of lactic acid in the applied medium. The dashed line arrows express the negative influence of the undissociated form of lactic acid $[LaH]$ [M] and the protons $[H^+]$ [M] on the growth and production processes. From literature, it is known that the toxic activity of lactic acid is mediated through these components in particular [e.g., Russell (1992)].

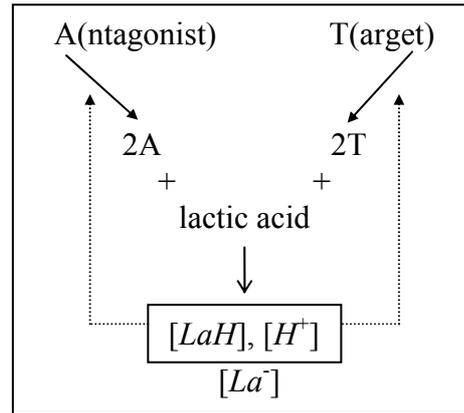


Figure 13 : Reaction scheme of the experimental system.

As a global *modeling framework* in which the reaction scheme can be enclosed, we propose the following set of differential (*balance models*) and algebraic equations (with $i=A,T$):

$$\frac{dN_i}{dt} = \mu_i \cdot N_i = \mu_{\max,i} \cdot \mu_{Q,i}(Q) \cdot \mu_{LaH,H^+,i}([LaH], [H^+]) \cdot N_i \quad (2.1)$$

$$\frac{dLaH_{tot}}{dt} = \sum \pi_i \cdot N_i = \sum \pi_{\max,i} \cdot \pi_{LaH,H^+,i}([LaH], [H^+]) \cdot N_i \quad (2.2)$$

$$[H^+] = f(LaH_{tot}, \text{buffer}) \quad (2.3)$$

$$[LaH] = g(LaH_{tot}, [H^+]) \quad (2.4)$$

with t [h] the time, N_i [cfu/mL] the cell concentration, $\mu_{\max,i}$ [1/h] the maximum specific growth rate, $\pi_{\max,i}$ [mmol/(cfu · h)] the maximum specific production rate and LaH_{tot} [M] the total lactic acid concentration (i.e., $[LaH] + [La^-]$). In this set, the *differential equations* quantify the growth of and the lactic acid production by the organisms, comprising the *biochemical* subprocesses of the experimental system. Since the specific growth and production rates are dependent on $[LaH]$ and $[H^+]$, interaction effects will be described as a consequence of an increasing concentration of $[LaH]$ and $[H^+]$ in the environment. In contrast to the differential equations, the *algebraic equations* account for the purely *chemical subprocess* of the experimental system, i.e., the dissociation of lactic acid in the aqueous medium.

In a first modeling phase (in the first year of the project), the chemical subprocess of lactic acid dissociation is taken into account [equations (2.3) and (2.4)]. To start, two mechanistic models out of literature (Nicolai *et al.*, 1993 and Passos *et al.*, 1994) are analysed and compared. Although these models -which are based on classical chemical equilibria, mass and charge balances- are not directly applicable to the experimental data, they have played an inspiring role in the establishment of an alternative method. This novel method, which builds upon the results reported in Vereecken and Van Impe (2001) and (2002), consists of two reversible algebraic equations, relating $[LaH]$ to LaH_{tot} , and pH to $[LaH]$ respectively:

$$[LaH] = \alpha_1 \cdot LaH_{tot} - \frac{\alpha_1 \cdot \beta_1}{2 \cdot (\beta_1 - \gamma_1)} \cdot \left[(LaH_{tot} + \beta_1) - \sqrt{(LaH_{tot} + \beta_1)^2 - 4 \cdot (\beta_1 - \gamma_1) \cdot LaH_{tot}} \right] \quad (2.5)$$

$$pH = \frac{1}{2 \cdot \alpha_2 \cdot \gamma_2} \cdot \left[(\beta_2 - 2 \cdot \gamma_2) \cdot [LaH] - \sqrt{\beta_2^2 \cdot [LaH]^2 + 4 \cdot \alpha_2 \cdot \beta_2^2 \cdot \gamma_2 \cdot [LaH]} \right] + pH_0 \quad (2.6)$$

Starting from the increasing LaH_{tot} -values, the equations provide an accurate description of the acidifying profiles measured in the media of the two case studies.

In a second phase (during the second year of this project), the main achievements are the development of model components for the biochemical subprocesses of (i) growth and (ii) lactic acid production of antagonist and target.

The specific *growth rate* in equation (2.1) incorporates an inhibition function dependent of $[LaH]$ and $[H^+]$ (or equivalently, their negative logarithms, $pLaH$ and pH), describing the stationary phase in the mono- and coculture growth curves. A suitable inhibition function is selected out of a set of candidate equations found in literature. Further, the function is adapted in such a way that it includes consecutively a decreasing phase and a zero phase when the lactic acid concentration increases:

$$\mu_{pLaH,pH,i}(pLaH,pH) \quad (2.7)$$

$$= \begin{cases} \left(1 - \frac{10^{-pLaH}}{10^{-pLaH_{min,i}}}\right)^\alpha \cdot \left(1 - \frac{10^{-pH}}{10^{-pH_{min,i}}}\right)^\beta & \text{if } pLaH \geq pLaH_{min,i} \text{ and } pH \geq pH_{min,i} \\ 0 & \text{if } pLaH < pLaH_{min,i} \text{ or } pH < pH_{min,i} \end{cases}$$

with $pLaH_{min,i}$ and $pH_{min,i}$: the values of $pLaH$ and pH , respectively, at which growth ceases; parameters α and β are free (but constrained to $\alpha > 1$, $\beta > 1$) or fixed (at a value of $1 + 10^{-6}$).

Next, the submodel for the growth model is applied to the experimental data of the two case studies. The resulting parameter estimates and confidence intervals (not shown) indicate that not all parameters can be estimated in a reliable way. It is postulated that this is caused by the correlation between the independent variables of the specific growth rate function $pLaH$ (or $[LaH]$) and pH (or $[H^+]$), which is inherently present in (natural) fermentation processes. The problem can be relaxed by using a reduced version of the novel model, containing four free parameters, namely the initial cell concentration N_0 , the lag phase duration λ , the maximum specific growth rate $\mu_{max,i}$ and a growth limiting concentration of undissociated lactic acid $pLaH_{min,i}$ (which corresponds to the negative logarithm of $[LaH]_{max,i}$, i.e., $-\log([LaH]_{max,i})$). Parameters α and β are fixed at a value of $1 + 10^{-6}$ during the estimation procedure. For parameter $pH_{min,i}$, two methods are suggested. In the first, $pH_{min,i}$ is put equal to the minimum pH for growth at the ambient temperature in a rich medium acidified with a strong acid, as available in literature. For the second method, the parameter $pH_{min,i}$ is related to $pLaH_{min,i}$ by means of equation (2.6). In this case however, the model is only appropriate if acidification results from lactic acid production only (and not, for example, from addition of a strong acid). For more details on the model development for this subprocess, reference is made to Vereecken *et al.* (2003).

The specific *production rate* in equation (2.4) comprises growth and non-growth associated (maintenance) production of lactic acid, and the negative influence of $[LaH]$ and pH on these metabolic processes. It is demonstrated that the experimental system under study does not obey the classical linear law, which is based on a constant maintenance related production rate. Therefore, a novel expression for the maintenance is proposed, in which the experimentally observed decreasing production rate at higher lactic acid concentrations is accounted for.

$$\pi_{pLaH,pH,i}(pLaH,pH) = Y_i \cdot \mu_i(pLaH,pH) + m_{\max,i} \cdot m_i(pLaH,pH)$$

$$m_i(pLaH,pH) = \begin{cases} \left(1 - \frac{10^{-pLaH}}{10^{-pLaH_{\min,M,i}}}\right)^\gamma \cdot \left(1 - \frac{10^{-pH}}{10^{-pH_{\min,M,i}}}\right)^\delta & \text{if } pLaH \geq pLaH_{\min,M,i} \text{ and } pH \geq pH_{\min,M,i} \\ 0 & \text{if } pLaH < pLaH_{\min,M,i} \text{ or } pH < pH_{\min,M,i} \end{cases}$$

The descriptive power of this model is illustrated by means of the experimental data of the case studies. Key parameters in the submodel for lactic acid production are the yield of lactic acid over cfu Y_i [mmol/cfu], the maximum maintenance $m_{\max,i}$ [mmol/(cfu·h)], and a metabolism limiting concentration of undissociated lactic acid $pLaH_{\min,M,i}$ [corresponding to $-\log([LaH]_{\max,M,i})$]. Similar to α and β in the model for growth and lactic acid induced inhibition, parameters γ and δ are kept constant at a value of $1 + 10^{-6}$ during the parameter optimization procedure. And again, the parameter $pH_{\min,M,i}$, corresponding to $-\log([H^+]_{\max,M,i})$, is related to $pLaH_{\min,M,i}$ by means of equation (2.6). For further details on the model development for this subprocess, reference is made to Vereecken (2002).

In summary, combination of the submodels for growth, lactic acid production and dissociation enables to quantify the lactic acid induced inhibition effect on growth *and* metabolism. By means of the unified model, a precise description of the experimental data of the cell concentration, the lactic acid concentration and the pH is obtained for both case studies (see, for example, Figure 14). Next, it is demonstrated that the complete model, in combination with the estimated parameter values yields an accurate prediction of the experimental data of the validation set.

For more details, reference is made to Vereecken (2002), Vereecken and Van Impe (2002), Vereecken et al. (2002) and Vereecken et al. (2003).

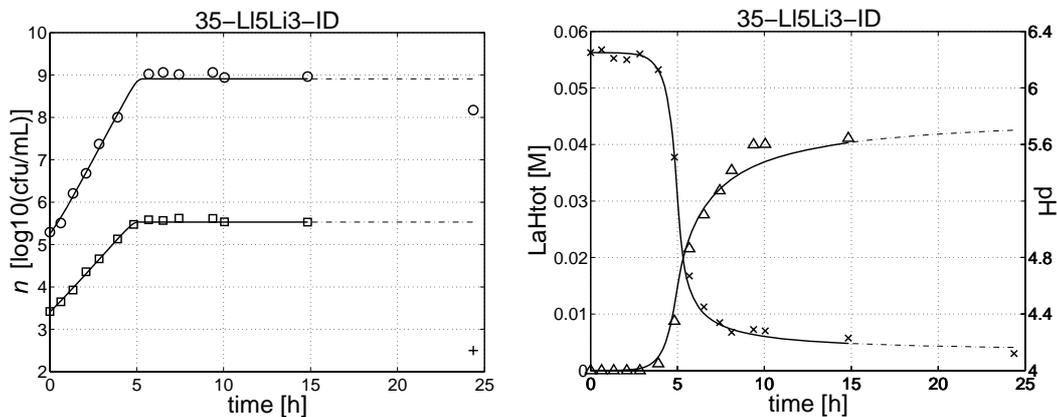


Figure 14: Application of the unified model to Case study #1, experiment with inoculum ratio *L. lactis*/*L. innocua* = $10^5/10^3$ cfu/mL

Next to this global modeling framework for the two case studies at hand, an extended literature review was made concerning different strategies for modeling *chemical inhibition and inactivation* of micro-organisms.

This research is currently published in Devlieghere et al. (2004)..

Subtask 2.1.2 Inactivation phenomena

The modeling steps for this subtask were performed during the third year of the project.

Step 1: Experimental Protocol and Data Generation.

Experimental data for Case study #2, as generated in Subtask 2.1.1 were used in this subtask. In the model development steps, the data of the coculture experiments performed at 12°C were explored. Similarly to the experimental data of the evolution of *Y. enterocolitica* for the cocultures performed at 37°C presented in Figure 2.1, the evolution of *Y. enterocolitica* for the cocultures performed at 12°C also show growth, early induction of the stationary phase (i.e., inhibition) and finally inactivation of the target organism. Data of the experiments 12-Ls0Ye3-ID and 12-Ls7Ye3-ID were not suitable as inactivation of *Y. enterocolitica* in monoculture did not occur within the observed time range. On the contrary, it proceeded too fast in the coculture experiment 12-Ls7Ye3-ID to obtain some data points in the inactivation phase.

Step 2: Model Construction.

The differential equation for growth and inhibition of the pathogen as developed in Subtask 2.1.1 [equations (2.1) and (2.7)] was extended to describe the subsequent, experimentally observed, inactivation phase of *Y. enterocolitica* in Case study #2 as function of the influencing factors pH and undissociated lactic acid. An important structural model requirement is the reduction to growth and inhibition of the pathogen when no inactivation takes place.

Modular extension of the existing model [equations (2.1) and (2.7)] to inactivation can be done in two ways: (i) the population N can be divided into a viable and death fraction, or (ii) the reaction kinetics can be extended with additional terms (i.e., additive) and/or factors (i.e., multiplicative) in such a manner that it describes the three subsequent growth phases (i.e., growth, inhibition and inactivation). As measurements of the total cell concentration (i.e., viable and death cells) were lacking in the current study, method (ii) was preferred. The reaction kinetics in the newly developed model comprises two parts: one for growth and inhibition of the target organism, and one for the subsequent inactivation process.

The selection of a suitable model structure for inactivation is driven by data of the variation of the *specific evolution rate* for *Y. enterocolitica* μ_{Ye} with pH and the concentration undissociated lactic acid [LaH]. The data for μ_{Ye} were constructed by linear regression of every three subsequent data points of the cell concentration. In contrast to the specific growth rate in equation (2.1) of Subtask 2.1.1, μ_{Ye} comprises both growth *and* inactivation. The evolution of μ_{Ye} with pH and undissociated lactic acid is presented in Figure 15. As a low pH corresponds to a high [LaH], the evolution of μ_{Ye} with pH and [LaH] show opposite behavior. This graphical profile is useful for the identification of critical points in the evolution of μ_{Ye} . Initially, the pH of the medium equals pH_0 ($[LaH]_0 = 0$). As pH decreases to pH_{inhib} (or [LaH] increases to $[LaH]_{inhib}$), the specific evolution rate μ_{Ye} decreases from a positive value towards zero. Then μ_{Ye} remains zero over a certain range. From pH_{inact} (or $[LaH]_{inact}$) on, μ_{Ye} decreases to negative values. These parameters are preferably incorporated into the function describing μ_{Ye} as they are easily interpretable.

For simplicity, a suitable model structure is initially developed for only one of the two toxic components. Because of the diffuse evolution of μ_{Ye} with [LaH] at high concentrations (Figure 15, right), the factor pH is preferred. The factor [LaH] does not appear in the equations, but is implicitly taken into account as undissociated lactic acid is directly related to pH by the chemical equilibrium and mass and charge balances for a specific medium having a fixed buffer capacity [equations (2.3) and (2.4), or in full form,

equations (2.5) and (2.6) developed in the previous subtask]. Afterwards, the second toxic component [LaH] is taken into account.

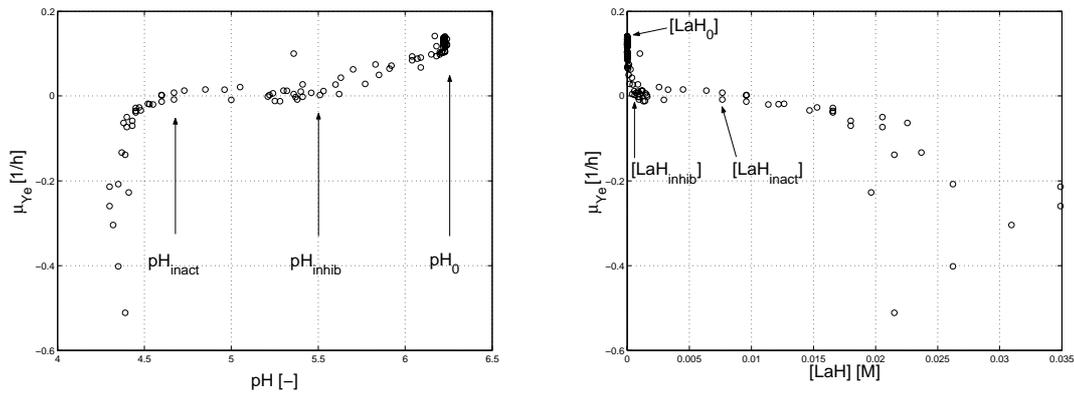


Figure 15: Specific evolution rate μ_{Ye} of *Y. enterocolitica* as function of pH (left) and the concentration undissociated lactic acid [LaH] (right) with pH_0 and $[LaH]_0$: the starting pH and concentration undissociated lactic acid, pH_{inhib} and $[LaH]_{inhib}$: their values at which growth ceases, pH_{inact} and $[LaH]_{inact}$: their values at which inactivation starts.

Based on the thermal model structure of Van Impe *et al.* (1992) the reaction kinetics μ_{Ye} finally consists of two parts: $\mu_{growth}(pH, pLaH)$ for description of the growth and inhibition (i.e., positive values of μ_{Ye}), and $\mu_{inact}(pH, pLaH)$ for description of the inactivation (i.e., μ_{Ye} at negative values). Both parts were formulated as being negatively influenced by the undissociated lactic acid concentration [LaH] (or its negative logarithm, $pLaH = -\log([LaH])$) and pH .

$$\mu_{Ye}(pH, pLaH) = \mu_{growth}(pH, pLaH) \cdot F_{trans}(pH) + \mu_{inact}(pH, pLaH) \cdot [1 - F_{trans}(pH)]$$

An expression for $\mu_{growth}(pH, pLaH)$ describing the exponential growth phase and early induction of the stationary phase is equal to $\mu_{max, i} \cdot \mu_{pLaH, pH, i}(pLaH, pH)$. An expression for the latter is taken from equation (2.7).

The transition function $F_{trans}(pH)$ with values between 0 and 1 ensures the smooth transition from growth to inactivation. The function, given in the equation below, has been applied similarly in, for example, Van Impe *et al.* (1992), and has also similarities with the well-known modified Gompertz equation for microbial growth in Zwietering *et al.* (1990).

$$F_{trans}(pH) = \exp\{-\exp[\alpha \cdot (pH_{trans} - pH)]\} \quad \text{with } \alpha > 0$$

with pH_{trans} [-]: the pH-value at which transition takes place, α [-]: the parameter describing the curvature of the transition. Depending on the value of α being finite or infinite, $F_{trans}(pH)$ responds to a continuously differentiable equation or a step function respectively. As a consequence, according to the shape of $F_{trans}(pH)$, the functions $\mu_{growth}(pH, pLaH)$ and $\mu_{inact}(pH, pLaH)$ have to fulfil different conditions to ensure a smooth transition. In contrast to μ_{growth} and μ_{inact} who both are dependent on pH and $pLaH$, F_{trans} remains dependent on pH only since it guarantees the transition between $\mu_{growth}(pH, pLaH)$ and $\mu_{inact}(pH, pLaH)$.

Parameter optimization studies based on experimental data of the cell concentration of *Y. enterocolitica* were performed, in a first phase for *pH* only, but in a second phase the second influencing factor undissociated lactic acid was included. Analogous to Subtask 2.1.1 where $pLaH_{\min,i}$ was related to $pH_{\min,i}$ by equation (2.6), the lactic acid associated parameters in the model structures for the inactivation phase were related to the pH-parameters to circumvent the strong correlation between them. This finally led to the selection of one possible structure for a good and reliable prediction of the inactivation phase (L_1 [-], L_2 [-] and c_B [-]: parameters).

$$\mu_{inact}(pH, pLaH) = -\exp\left(-\frac{pH}{L_1} - \frac{pLaH}{L_2} + c_B\right)$$

Parameters L_1 [-] and L_2 [-] can be interpreted as a pH- and pLaH-value, respectively. Consequently, as already mentioned, the parameter L_2 was related to L_1 by means of equation (2.6).

Until this point, model equations were applied to each of the cocultures separately. The resulting parameter values (not shown) for each coculture were comparable, but not exactly equal. However, when considering undissociated lactic acid and pH as the influencing factors in the current system, there should exist *one unique parameter set valid for all the cocultures*. Additionally, the present results do not enable the prediction of the inhibition and inactivation of the target organism in cocultures with intermediary inoculum concentrations for the antagonist (i.e., intermediary concentrations in the range of $10^3 - 10^6$ cfu/mL). Because of these reasons, the final set of equations was applied simultaneously to the experimental data of all cocultures showing an inactivation phase. A global model valid for all the cocultures was obtained. Comparison of the model simulation based on the optimal parameter values and the experimental data is illustrated in Figure 16.

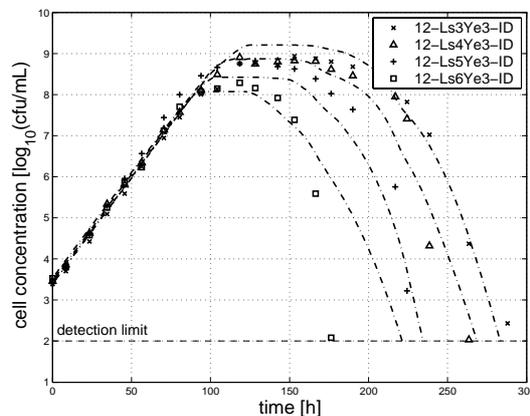


Figure 16 : Simulation of the evolution (growth, inhibition and inactivation) of *Y. enterocolitica* in coculture with *Lact. sakei* by means of the global model with one unique parameter set valid for all the cocultures.

The global model and its optimal parameter values gave satisfying results when used to predict the experimental data of the validation set. For more details of this extended model, reference is made to Janssen *et al.* (2003, 2004b and 2006b).

Observe that it could be anticipated that other lactic acid mediated coculture experiments could also be described using the developed model structure. The resulting overall model can also be seen as a basis for other models describing microbial interactions, as it can be assumed to be transferable to, for example, other organic acids, bacteriocins, etc. Additionally, when no microbial interaction occurs (e.g., no antagonist), the equation for growth of the antagonist and the term for lactic acid production by the antagonist can be omitted. As such, the model reduces in a natural way to growth and lactic acid production and (intraspecies) inhibition in monoculture of the target organism.

Subtask 2.1.3 Uncoupling the individual lactic acid and pH effects

The one-to-one interrelationship between pH and undissociated lactic acid and the correlation between their parameters mentioned in Subtasks 2.1.1 and 2.1.2 are not to be seen as an artefact of the experiments performed, but are inevitably related to each lactic acid production process. To circumvent these difficulties, more knowledge of the *individual (separate)* effects of pH and undissociated lactic acid is desirable. Indeed, by studying the individual effects of pH and undissociated lactic acid the model structure could be refined. This information can be obtained by studying the effects of pH and undissociated lactic acid not only at conditions determined by the one-to-one relationship but also at conditions outside this relationship (see e.g., Buchanan *et al.*, 1993).

This subtask was started in the third year of the project.

Step 1: Experimental Protocol and Data Generation.

Experimental plan

The inactivation of *L. innocua* of Case study #1 was investigated at controlled (static) conditions of pH and undissociated lactic acid ([LaH]). Similarly to the coculture experiments performed for Case study #1, inactivation experiments were performed in 1 L erlenmeyer flasks filled with 550 mL of a rich, modified Brain Heart Infusion medium. This medium was flushed with N₂ to obtain anaerobic conditions. However, in the current subtask, no lactic acid bacterium was used. Before inoculation, a combination of initial pH (i.e., pH_0) and initial concentration of undissociated lactic acid (i.e., $[LaH_0]$) was set by addition of the appropriate volumes of strong acid (HCl) or base (KOH), and lactic acid. No extra buffers were added. At the moment, 30 combinations were tested, which are graphically presented in Figure 17:

- 11 (pH_0 , $[LaH_0]$)-combinations situated *on* the traditional (pH , $[LaH]$)-trajectory: to simulate the effect of co-cultures the evolution of *L. innocua* at artificially created initial total lactic acid concentrations (i.e., both dissociated and undissociated forms) ranging from 0.04 to 0.12 M was followed. Before the addition of lactic acid, the pH was set to 6.20 for these experiments. As such, the (pH_0 , $[LaH_0]$)-conditions obtained are determined by the same relationship as for the cocultures in Case study #1.
- 19 (pH_0 , $[LaH_0]$)-combinations situated *outside* this trajectory: to be able to separate the effects of pH and $[LaH]$ in contrast to the mixed microbial system in Subtasks 2.1.1 and 2.1.2, combinations forming a rectangular shape in the (pH , $[LaH]$)-plane were tested. Values of pH_0 are ranging from 3.43 to 4.5, while $[LaH]_0$ ranges from 0 to 0.05 M. Differences in inactivation curves with equal pH_0 can be ascribed to the variation in $[LaH]_0$, and vice versa.

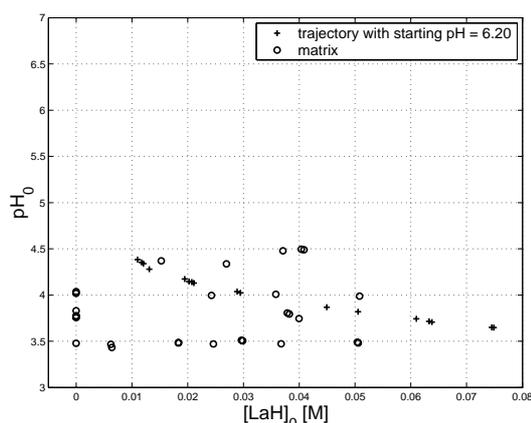


Figure 17 : Overview of the $(pH_0, [LaH]_0)$ -combinations tested in Subtask 2.1.3

L. innocua was inoculated at a concentration of 10^8 cfu/mL and all experiments were performed at 12°C .

Experimental results

In the evolution of the cell concentration as function of time, two phases could be distinguished: (i) a period with a constant cell concentration, i.e., a *shoulder period*, followed by (ii) a period in which the cell concentration decreased to values below the detection limit, i.e., a *descent phase*. Depending on the pH_0 and $[LaH]_0$ -conditions, the latter phase consisted of one or two loglinear part(s) with respective slope(s), or took a concave or convex shape. During the experiments, the glucose concentration remained constant, indicating that nutrient depletion cannot be the cause of the inactivation. As anaerobic conditions prevent *L. innocua* from producing acetic acid next to lactic acid (if any production would occur at all, given the inactivation of the microbe) (Kelly and Patchett, 1996), the prevailing lactic acid and pH conditions were the only explaining factors for the observed inactivation process.

For the experimental conditions situated on the trajectory corresponding to the conditions in coculture, it can be concluded that when increasing the initial total lactic acid concentration $LaH_{tot,0}$ (i.e., increasing $[LaH]_0$ and decreasing pH_0), the length of the shoulder period was reduced, while the inactivation rate increased. This is illustrated in Figure 18.

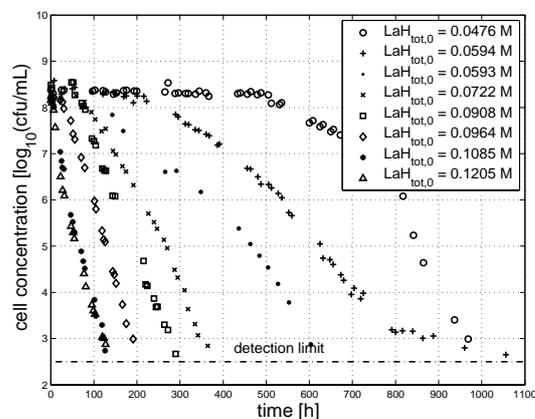


Figure 18 : *L. innocua* inactivation at $(pH_0, [LaH]_0)$ -combinations on the trajectory

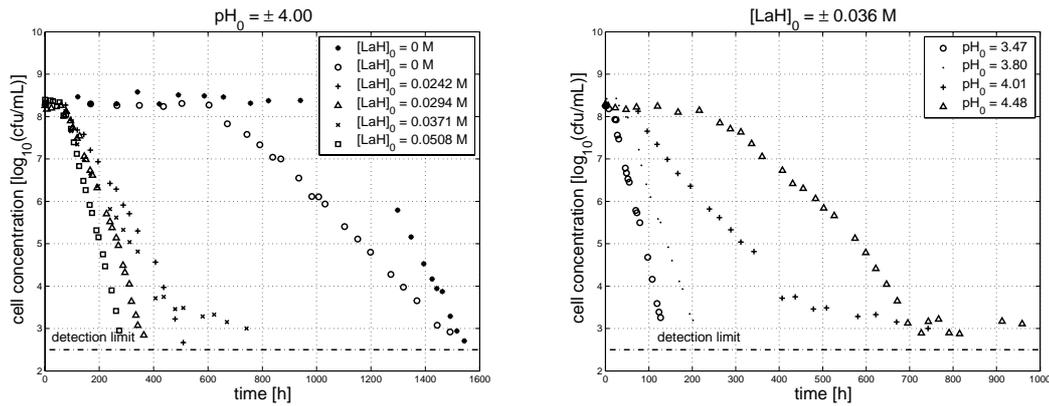


Figure 19 : Inactivation of *L. innocua* at $(pH_0, [LaH]_0)$ -combinations situated in the rectangular shape for a constant $pH_0 = 4.00$ (left) and $[LaH]_0 = 0.036$ M (right)

For the $(pH_0, [LaH]_0)$ -conditions forming the approximately rectangular shape in the $(pH, [LaH])$ -plane, it appeared that $[LaH]_0$ and pH_0 have an influence on both the length of the shoulder period and the inactivation rate when considering results at a constant pH_0 and $[LaH]_0$ respectively. The latter conclusions are illustrated by means of Figure 19. Part of these results is published as Janssen *et al.* (2004a, 2005).

After comparing the collected inactivation curves, a certain variation became visible: (i) inactivation curves for identical conditions (for experiments performed in duplicate) did not show an identical evolution (results not shown), and (ii) in the vicinity of the growth/no growth interface the inactivation process seemed to be a rather contradictory process (e.g., for $pH_0 = 4.00$ and $[LaH]_0 = 0$ M in Figure 19). Attempts to locate the cause of this variation did not led to any conclusive results. However, it seems acceptable that an increased variance in the bacterial response due to less favorable conditions might serve as the main reason for the observations made.

Step 2: Model Construction.

In the model development steps, no further distinction is made between the two series of experimental data as the final model has to be valid for *all* $(pH_0, [LaH_0])$ -combinations, on the trajectory with conditions corresponding to the cocultures of Subtasks 2.1.1 and 2.1.2 as well as for those outside this trajectory, forming the approximately rectangular shape. In addition, because of the observed variability in the inactivation process for some $(pH_0, [LaH_0])$ -conditions, all inactivation curves (singular or plural) were taken into account.

First, four types of primary inactivation models were calibrated on the experimental data by means of the Microsoft[®] Excel Tool GInaFiT (Geeraerd *et al.*, 2005). For most of the experimental data, either the loglinear model with shoulder or a Weibull-type model gave the best result, with the frequency of 'most suitable model' for both being about 50 %. As there was no preference for one or the other model based on the goodness-of-fit criterium, other factors were to be taken into account. The Weibull-model was preferred as primary inactivation model as it was able to describe the various inactivation shapes.

Next, a secondary model was developed to describe the evolution of the parameters of the primary Weibull-type model as function of pH_0 and $[LaH_0]$. Suitable model structures and parameter values are identified. Based on combination of the calibrated primary and secondary models, one can predict which conditions of pH_0 and $[LaH_0]$ are necessary to obtain a predetermined inactivation within a predetermined time range.

The results obtained in this subtask were recently presented in a poster on *International Satellite Congress of the Fifth International Symposium on Hormone and Veterinary Drug Residue Analysis: Platform for Scientific Concertation: Food Safety*, organised in the frame of the cluster project *Platform for Scientific Concertation: Food Safety*, financed by the Belgian Federal Science Policy Office (Janssen *et al.*, 2006a). In addition, a manuscript about the findings reported in Subtask 2.1.3 is currently being finalised.

As a validation step within this subtask, it has to be checked whether the combined (primary and secondary) model structures developed for the controlled static conditions are applicable to the inactivation of *L. innocua* under dynamic conditions as, for example, in Case study #1. Hereto, an artificial lactic acid profile will be created to mimic the lactic acid production by the antagonist.

Work Package 3: Developing and integrating predictive modeling methodologies

Task 3.1: Optimal Experimental Design (OED) of kinetic studies

As underlined in Bernaerts and Van Impe (2004), bioprocess modeling presents a challenging subject that requires a meticulous modeling strategy. During the modeling process, *experimental data* form a key ingredient during structure characterisation (SC) and parameter estimation (PE). Accurate *system identification* can only be guaranteed if the experimental data contain sufficient *information* on the process dynamics. In this respect, sufficient effort should be spent on *optimal experiment design* (OED) in order to maximise the information that can be extracted from data; especially because experimental data generation for bioprocesses usually presents a time-consuming, labour-intensive and costly job.

Experiment design for Task 1.1 (first year)

Research activities of Task 3.1 have been conducted in close collaboration with **Task 1.1**. Previous dynamic experiments in the context of OED/PE (see, e.g., Bernaerts *et al.*, 2000, Bernaerts *et al.*, 2002, Bernaerts and Van Impe 2002) have shown that sudden temperature rises (without changing other environmental conditions) yield a small but significant lag phase (or delayed growth response). As extensive data sets on this intermediate lag phenomenon are lacking, a first step is to generate experimental data (i.e., **Task 1.1**) which enable us (i) to identify the *causal relationship* between the microbial dynamics and applied temperature conditions, and (ii) to *characterise* a (set of) candidate *model structure(s)*. Hereto, the set of relevant influencing factors has been identified (see **Task 1.1**), in casu the effects of (i) the step amplitude, (ii) pre- and post-shift temperature, and (iii) state of the cells.

As reported by, e.g., Box and Draper (1971), Davies (1993), it is wise to choose values of these influencing variable(s) *equally distributed* within the region of interest *when the model structure is unknown*. Suffice to say that extrapolation, i.e., making model predictions outside the studied region, is out of the question. The scale of the design should thus include the region within which predictions are to be made. If *a priori* information on the kinetics is available, it is recommendable to space more treatment levels at regions where rapid changes of the dependent variable(s) are expected (e.g., Davies, 1993, Walker and Jones 1993).

Based on this knowledge, a *full factorial design* has been outlined for **Task 1.1**. Full factorial designs refer to experimental plans which encompass all possible combinations of the levels of the factor(s) (i.e., independent variables) under study (Anderson and McLean, 1974). Given k the number of factors and l levels of each factor, a complete factorial design contains l^k experiments of different treatment

combinations. Hence, the number of treatments increases rapidly as the number of factors and/or levels increases. But, Anderson and McLean (1974) note that sufficient levels are necessary to investigate non-linear trends correctly.

Here, the suggested factorial design encompasses a number of experiments that enable the characterisation of (i) the effect of the temperature step amplitude, and (ii) the pre- and post-shift temperature. Within the temperature range of interest, i.e., 10-40°C, a matrix of experiments (with different pre- and post-shift temperatures) has been identified. The temperature range has been subdivided using intervals of 2.5°C (i.e., 13 levels). As constant temperature experiments are not included (no lag induction), the complete factorial design involves 156 experiments. However, in view of characterising the *normal physiological range* accurately a more dense grid is selected in the temperature zone, i.e., 20-25°C, which is suggested to play a critical role in this phenomenon (e.g., Ng *et al.*, 1962). Details on the established experiments have been presented under **Task 1.1**. Based on the collected data, the experimental plan shall be revised for further research (e.g., selection of most informative experiments for SC).

Optimal experiment design for four-parameter problems

Research activities have focussed on optimal experiment design for parameter estimation. Once a suitable model structure has been selected, the methodology of optimal experiment design for parameter estimation (based on the Fisher information matrix) can be addressed to improve the parameter estimation accuracy by maximising the information that can be extracted from the experimental data (see, e.g., Walter and Pronzato, 1997). Whereas the framework for OED/PE in the field of predictive microbiology has been well-established for a *two*-parameter estimation problem (see, e.g., Bernaerts *et al.*, 2000, Bernaerts *et al.*, 2002), a *four*-parameter estimation problem has been investigated in the framework of this project. Predictive models mostly encompass more than two model parameters when more complex phenomena are being modeled (see, for example, the model reported in Task 2.1).

Two existing *four*-parameter models have been selected to work out the mathematical framework. In Bernaerts *et al.* (2003a, 2003b, 2005) and Gysemans *et al.* (2003, 2004b), a model output sensitivity analysis has been performed and optimal experiment designs have been computed for two cardinal values model, namely, the Cardinal Temperature Model with Inflection point (CTMI) and the Cardinal pH model (CPM) (Rosso *et al.*, 1995).

1) Optimal *static* experiment design (first, second and third year)

An optimal selection of the independent variables (temperature and pH, respectively) has been aimed for. Optimal experimental plans are distinct from the arbitrary placement of independent variables which is commonly applied in factorial designs. The following results are published in Bernaerts *et al.* (2005).

Several optimal design criteria based on the Fisher information matrix were applied during optimization. All but one of the criteria optimize an aspect of the parameter estimation quality, e.g., the E-criterion minimizes the largest parameter estimation error. One criterion, the so-called G-criterion, minimizes the prediction error variance. This criterion was considered as for non-linear models (as under study) the precision of the model prediction is not necessarily directly correlated to parameter estimation accuracy. Depending on the criterion, different optimal experimental plans were computed.

The following main results were obtained. Firstly, all design criteria aiming for parameter estimation quality yield experimental designs including four different temperatures (pHs) near the maxima of the sensitivity functions (i.e., partial derivatives of the model output with respect to each model parameter) with replicates at some of these temperature levels. G-optimal designs, on the other hand, have no specific structure: informative temperatures (pHs) are scattered. Secondly, the inclusion of more temperature (pH)

inputs increases the information content of the data. However, at a certain number of experiments saturation occurs for some criteria.

Within the above-mentioned results, temperature (pH) values were optimally selected within the region of growth. In some cases this resulted in optimal designs including optimal treatments (temperature or pH) positioned at the boundaries of this region (i.e., the minimum or maximum temperature/pH for growth). However, these boundary conditions do not yield growth. Such experiments are impractical and design errors (e.g., erroneous nominal values) have drastic effects (e.g., inactivation may be observed when the nominal value for the maximum growth temperature was selected too high). In this respect, OED/PE has also been executed with lower and upper constraints. Results are published in Bernaerts *et al.* (2003b, 2005) and Gysemans *et al.* (2004b). Constrained designs lead to decreased information content (optimality) of final designs. This is more pronounced for the CPM-optimal designs than for the CTMI-designs because two informative points are excluded in the CPM-designs, namely the minimum and maximum pH for growth, while only one informative point, i.e., the maximum growth temperature is excluded in the CTMI-designs. In the latter case, the set of informative temperatures is shifted to the left (i.e., to lower values).

2) Optimal *dynamic* experiment design (*third, fourth year and project prolongation*)

The CTMI model

Building upon the above-stated results, the work on optimal *dynamic* experiment design for estimation of the CTMI model parameters has been initiated. As the four-parameter estimation problem becomes computationally complex and time-consuming, the problem is reformulated as an iterative experiment design encompassing a series of optimally computed temperature inputs for estimation of parameter couples. Thoughtful selection of the parameter couples and the optimal design scheme allows an amelioration of the model parameters twice (starting from the nominal model parameters) within a series of four experiments.

The following results have been achieved.

(i) Continuing the work with *E. coli* K12 MG1655 (see **Task 1.1** and Bernaerts *et al.*, 2000, 2002), nominal values for the cardinal temperature model parameters have been determined. Hereto, experiments in the super-optimal temperature range for growth have been performed. Nominal model parameters are required to compute optimal designs. As a side-result irregular growth dynamics of *E. coli* K12 (in BHI) have been detected at elevated temperatures, especially, close to the maximum temperature for growth. At 44°C and more pronounced at 45°C, the exponential growth phase is disrupted by some kind of *apparent* lag phase. Log-linear growth becomes multiphasic. At 46°C, a short period of fast growth is followed by first-order inactivation. Several possible causes of these phenomena have been put forward and experimentally tested. Temperature history (referring to the (pre)adaptation to elevated temperature) as well as medium composition (referring to the presence of chaperones avoiding protein denaturation) exerts a significant effect on the dynamics near the maximum temperature for growth. Given a pre-adaptation at 45°C, the maximum temperature limit even shifts to nearly 47°C. Results have been submitted for publication in Van Derlinden *et al.* (2006a,b). Application of the pre-adaptation protocol produced acceptable specific growth rate estimates enabling nominal parameter estimation for the CTMI. However, the observed effects (i.e., irregular growth and effects of temperature history) have a major impact for OED. During optimization, temperature profiles should be upper-bounded by 43 ~ 43.5°C.

(ii) During optimal design, model validity should be guaranteed at all times. Necessary temperature constraints need to be specified. Abrupt temperature changes disrupt exponential growth (see WP 1.1.) and may not occur in optimal temperature designs. Additional experiments have been performed to explore the rate of adaptation of the microbial population when temperature changes are moderate.

Dynamic experiments implementing temperature profiles with moderate rates of temperature changes and spanning the complete growth temperature range have been performed. As in **Task 1.1**, experiments have been performed in bioreactors under controlled conditions. Experimental data have been analyzed via Monte Carlo simulation. Temperature ramps with a slope of 5°C per hour seem to guarantee an immediately changing specific growth rate as prescribed by the model structure.

(iii) In the onset to optimal dynamic experiments, the CTMI model parameters of *E. coli* K12 MG1655 have been estimated from a series of *four* dynamic experiments, the temperature profiles of which varied linearly within (parts of) the growth temperature range. Kinetic parameters are deduced in different ways, (i) directly from the cell density data (one-step-identification) and (ii) on the basis of the specific growth rates, obtained as the local derivatives of the cell density data, as function of temperature (two-step-identification). Experiments were performed in a computer-controlled bioreactor with *E. coli* K12 MG1655 as in **Task 1.1**.

Temperature profiles are heuristically derived. The complete growth temperature region was subdivided into two zones covering sup-optimal and optimal-super-optimal temperatures, respectively. Temperature profiles were selected such that the culture grew (most of the time) exponentially while temperature was changing. Temperature was not allowed to closely approach the temperature boundaries of growth to ensure significant growth rates. Furthermore, only very moderate temperature gradients were applied as rapid changes violate the model structure (see, e.g., Swinnen *et al.*, 2005; Bernaerts *et al.*, 2002 and earlier in this report).

Two-step-identification refers to (i) the computation of the specific growth rate, obtained as the local derivatives of the cell density data, as function of temperature, followed by (ii) the identification of the CTMI on the specific growth rate estimates as function of temperature. The specific growth rate as function of time for each experiment can be derived in multiple ways, e.g., (i) central differentiation of the experimental data, (ii) approximation of the data points using a cubic spline which is subsequently numerically differentiated, (iii) approximation of the data points using a smoothing cubic spline which is subsequently numerically differentiated. Negative values for the specific growth rate are put equal to zero. In one-step-identification, all experimental data are joined in a global parameter estimation step during which the CTMI parameters are considered as global model parameters.

Although temperature profiles were not optimized, good parameter values with low uncertainties could be derived from four dynamic data sets via a one-step-identification. The model parameters from the CTMI can be derived with high accuracy and in a time-efficient way from four dynamic experiments. Amongst the various scenarios for parameter estimation, the one-step-identification clearly excels. During the one-step-identification, the information content of the dynamic experiments is maximally exploited thanks to the use of the dynamic growth model.

These results have been presented in Bernaerts *et al.* (2006).

(iv) Finally, optimal temperature inputs have been computed according to the OED/PE framework using the Fisher information matrix and associated design criteria. Biological restrictions imposed by the model structure have been taken into account through a *constrained* design. Designs are based on the growth model of Baranyi and Roberts encompassing the CTMI model. The above-outlined 2-by-2 parameter estimation strategy has been followed. Thus far, linear time-varying temperature inputs are optimized with respect of the D- and modified E-criterion. One may highlight this optimization problem is multimodal. Global optima could be found through ample random initialization but would be greatly facilitated using stochastic algorithms. The latter as well as the experimental validation of optimal linear profiles is currently being investigated.

To be complete, publications explaining the concepts of optimal experiment design for bioprocesses (Bernaerts and Van Impe, 2004; Bernaerts and Van Impe, 2005) have been realized in the context of this

research project. Theory and methodologies are well-illustrated by means of case studies addressed in this project.

The CPM model (part of the fourth year, and project prolongation)

Transferability of the OED/PE methodology to the CPM (cardinal pH model) is being evaluated. Optimal *static* experiment design could already be successfully applied to the CPM model (see Bernaerts *et al.*, 2005). The applicability of *dynamic* pH profiles for parameter estimation is evaluated based on a literature review and a well-selected set of dynamic bioreactor experiments.

The growth dynamics of *E. coli* K12 MG1655 have been determined at various pH levels within the pH range of growth. Two types of experiments were performed: (i) uncontrolled pH experiments (starting from an initial pH, the pH is allowed to dynamically change), (ii) controlled pH experiments (pH is kept constant by controlled addition of base or acid). Cell density, glucose and acetic acid were measured. When *E. coli* K12 MG1655 grows in BHI, the micro-organism first consumes glucose producing biomass and acetic acid, and subsequently internalizes acetic acid to form biomass. This metabolism is known as diauxic growth (e.g., Wolfe, 2005) and causes the pH to dynamically vary. In literature reported pH experiments are always uncontrolled experiments. Hence, models like the CPM model are built on the initial pH (of uncontrolled experiments). The performed bioreactor experiments were performed (i) to validate the CPM model based on initial pH values for the applied strains, (ii) to compare to growth under controlled and uncontrolled pH conditions, and (iii) to model the effect of (moderate) dynamic temperature changes on the growth rate.

The following conclusions can be drawn on the basis of a large series of experiments between pH 4.5 and pH 9.5. (i) Maximum specific growth rate estimates (μ_{\max}) derived from uncontrolled pH experiments could not be modelled by the CPM model. A pH range within which pH had little effect on μ_{\max} is observed. An additional model parameter characterizing this plateau was introduced in the CPM. A similar observation could only be retrieved in Presser *et al.* (1997), where data were limited to the suboptimal pH range. (ii) Comparing results from uncontrolled and controlled experiments, μ_{\max} under uncontrolled condition is consistently higher than μ_{\max} from controlled experiments. Most extreme is the effect at the pH boundaries of growth. For example, keeping the pH constant 9 does not allow growth initiation (after a period of survival even inactivation is observed) whereas starting at an initial pH 9 and leaving it free to change, supports growth at a relatively high μ_{\max} . The strain is able to initiate growth and acidify the medium, thereby lowering the pH to more optimal condition. Within uncontrolled experiments, the pH drop (caused by acetic acid production) is more or less 2 pH units. At the lower pH limit for growth, the lowering of the pH in combination with the produced acetic acid exhibits an inhibitory effect (expressed by a smaller maximum population density in the stationary phase).

Within a simulation study, it is currently investigated if the dynamic growth curves can be predicted by a mathematical model embedding effects of pH (in the form of $[H^+]$) and acetic acid (in its un-dissociated form). The model structure could be equivalent with the model developed in Subtasks 2.1.1 and 2.1.2.

Given the current information, application of dynamic experiments for CPM model parameter estimation seems a complicated task as the cell metabolism must be taken into account.

Task 3.2: Macroscopic predictive modeling

The close interface between Work Package 3 and Work Packages 1 and 2 is illustrated above, where results on macroscopic modeling techniques, directly related with aforementioned tasks, is reported :

- Task 1.1., Step 2, “**Modeling microbial lag due to a sudden rise in temperature**”

- Task 1.2, “**Modeling individual cell lag time distributions for *Listeria monocytogenes***”
- Task 1.3 “**Growth behaviour of *L. monocytogenes* at the growth/no growth boundary**”
- Task 2.1, Subtask 2.1.1, Step 2 “**Model construction**” (related with microbial inhibition phenomena through a single metabolic product)
- Task 2.1, Subtask 2.1.2, Step 2 “**Model construction**” (related with microbial inactivation phenomena through a single metabolic product)
- Task 2.1, Subtask 2.1.3, Step 2 “**Model construction**” (related with the uncoupling of the individual lactic acid and pH effects).

The model developments are various and range from *balance models* and *kinetic modeling approaches* over *logistic type model approaches* to *distribution model types*.

Task 3.2 has also deliverables on its own, namely, concerning *methodological modeling developments*.

Step 1: Balance models.

As already indicated in the first year scientific report, description of microbial cell (population) behavior influenced by dynamically changing environmental conditions intrinsically asks for dynamic mathematical balance models. In Bernaerts *et al.* (2004), Bernaerts and Van Impe (2004) and Bernaerts and Van Impe (2005), a *general dynamic model building concept* describing microbial evolution under dynamic conditions is presented. Starting from an elementary model building block, the model structure can be gradually complexified to incorporate increasing numbers of influencing factors. The fundamental concepts of dynamic macroscopic (population level) and microscopic (individual based) modeling approaches (see Task 3.3.) are explained using the case studies addressed in this project (as covered by Work Packages 1 and 2). With respect to current and future research trends, the need for (i) more advanced measurement techniques, (ii) measurements under dynamic conditions, and (iii) more complex model structures, is pointed out. In the context of quantitative risk assessment, the mathematical model complexity needs to be kept under control. An important challenge for the future is therefore the search for a satisfactory trade-off between predictive power and manageability of mathematical models: *When is simple good enough?* (after Buchanan *et al.*, 1997).

Next to this, a novel generation of balance type models is developed during the second year of this project. The principal underlying reason for this development is the observation that in order to increase the predictive power of mathematical models, more mechanistic (i.e., (micro)biological) knowledge should be incorporated from the on-set in the mathematical model structure. For microbial growth, research is in a first phase focused on modeling the stationary phase of the growth curve. This stationary phase is the result of (i) substrate *S* depletion and/or (ii) toxic product *P* inhibition. Both phenomena cannot be described with the classical logistic model types. In order to overcome this drawback, a novel class of more mechanistically inspired so-called S&P models is being developed and analysed (see also Task 3.3). More details can be found in Van Impe *et al.* (2005) and Poschet *et al.* (2005a).

Step 2: Kinetic models.

A novel development is related with *advanced black box modeling approaches* for use as *kinetic models*. Research about this issue has already been reported in the previous scientific report, and is published in 2004 (Geeraerd *et al.*, 2004). Herein, a novel procedure is developed, consisting of three steps: (i) careful formulation of the available microbiological information, both from literature and from the experimental case study at hand, (ii) translation of these requirements in mathematical terms under the form of partial derivatives throughout the complete interpolation region of the experimental design, and (iii) determination of parameter values with suitable optimisation techniques for a flexible black box modeling approach, e.g., a polynomial model or an artificial neural network model. As a vehicle for this procedure,

the description of the maximum specific growth rate of *Lactobacillus sakei* in modified BHI-broth as influenced by suboptimal temperature, water activity, sodium lactate and dissolved carbon dioxide concentration is under study. The procedure results in a constrained polynomial model with excellent descriptive and interpolating features in comparison with an extended Ratkowsky-type model and classical polynomial model, by combining specific properties of both model types. The developed procedure is illustrated on the description of the lag phase as well. It is stressed how the confrontation with experimental data is very important to appreciate the descriptive and interpolating capacities of new or existing models, which is nowadays not always carefully performed. Alternatively, the first two steps of the novel procedure can be used as a tool to demonstrate clearly (possible) interpolative shortcomings of an existing model with straightforward spreadsheet calculations.

The procedure has found immediate application in the project in Task 1.1 during the “**Protocol development for measuring the lag phase of individual cells**”, namely to model the calibration curve shift, relating OD measurements and viable counts measurements, as a function of environmental conditions. This is reported as Francois et al. (2005a).

Step 3: Implementation of variability.

A third development is related with the application of predictive microbiology in the context of hazard analysis and critical control points and risk analysis studies, in other words, with *the implementation of variation*. For these purposes, a confidence related with a model prediction is indispensable, and a transition from classical deterministic models towards stochastic models is essential. Such models predict a probability mass function for the microbial load at a certain time instant. Monte Carlo, which is a general tool to compute statistical characteristics, is used to generate, starting from the experimental observations and a deterministic growth model, probability density functions for (i) the model parameters and (ii) the predictions as a function of time. Next to this, also the optimal experiment setup can be determined by means of this approach. This probabilistic approach, incorporating experimental variation, is applied to experimental growth data and thermal enzyme inactivation data.

For more details, reference is made to Poschet et al. (2004) and (2005b).

Variability, inherent in the production and use of microtiter-experiments in order to characterize the lag phase of individual cells, is also taking into account as described in the research reported under **Task 1.2**. Reference is also especially made to (i) “**Practical implications of the individual cell approach on the level of challenge tests**”, where Monte Carlo simulations are implemented to characterize the challenge test results on pâté, and, (ii) “**Practical implications of the individual cell approach on the risk assessment level**”. The latter research concludes that the individual cell lag phase variability of *L. monocytogenes* has important consequences when studying specific growth cases, especially when the applied inoculum levels are low, but when performing more general exposure assessment studies, the variability between the individual cell lag phases is too limited to have a major impact on the total exposure assessment.

For more details, reference is made to Francois et al. (2006b) and Francois et al. (2006c).

Task 3.3: Microscopic predictive modeling

Traditionally, predictive microbiology has adopted a *macroscopic* approach to modeling microbial dynamics. Population-level variables like the total cell concentration N or the total biomass X are

modelled in function of environmental conditions. Also, experiments are usually performed under standardised and strictly controlled laboratory experiments, using relatively high inoculum levels.

For some aspects of microbial growth, such as the microbial growth rate μ_{max} , this approach gives satisfactory results, but for others it is clearly lacking in its ability to provide accurate predictions. The bacterial lag phase has proven to be quite difficult to predict, especially when stochastic effects come in to play because of the presence of low cell numbers (Bridson and Gould, 2000). Also, more realistic environments (i.e., real food products) clearly cause more complex bacterial dynamics to occur than can be modelled with these classical model structures.

To tackle these difficulties, two main approaches can be pointed out. First, classical models are being extended with stochastic elements (Baranyi, 2002; Koutlik *et al.*, 2005), which provides them with a basis to model inter-cell variability within the modelled population. Second, new modeling approaches such as individual based modeling (IbM) are being explored and applied to predictive modeling case studies (Dens *et al.*, 2005a,b; Ginovart *et al.*, 2002; Prats *et al.*, 2006).

The concept of IbM can be summarized by the following defining criteria (Breckling *et al.*, 2006).

- a population is represented by a number of entities or agents which are separately accessible and which have at least one quantitative or qualitative property that differs from each other;
- the model describes the change of these characteristics over time.

The IbM methodology intrinsically differs with the traditional, macroscopic modeling approach, often based on differential equations. Instead of modeling a global characteristic of the population as a whole, IbM is based on a (more or less) simple *microscopic* model of an individual cell constituting that population, describing its substrate uptake, cell division, metabolite production, interaction with its local micro-environment, and so on. By simulating a large number of these individual cell models in parallel, their dynamics and local interactions will give rise to a higher-level population-scale macroscopic behaviour with its own characteristics, which is often referred to as *emergence*. This approach avoids the need to formally define relationships at the macroscopic level.

The *macroscopic* equation based approach is well suited to describe homogeneous populations in homogeneous environments, where the number of individuals is high enough to characterise the population as a whole in terms of continuous variables. The characteristics of individual agents (cells) are lumped together in the form of averages, providing an efficient and economical way to describe the dynamics. The IbM approach is complementary in the sense that it provides the following intrinsic advantages.

- IbM model structures allow a more natural mapping of the biological reality onto the model structure.
- Agent-to-agent heterogeneity and interactions between individuals can be incorporated in a natural way.
- Spatial heterogeneity can be easily added due to the modular structure of IbM implementations.
- Mechanistic knowledge, available in literature, is usually specified at the level of the individual agent and can be directly implemented in the model.

Of course, IbM also has arguments against it.

- IbM models rapidly attain a high level of complexity with lots of parameters and have no formally defined mathematical structure. This complicates the documentation of, and communication about the models.
- Simulating large numbers of agents requires a considerable amount of computing power.
- IbM lacks a solid theoretical framework for model analysis, as is available for macroscopic, equation-based modeling techniques.
- IbM models can require detailed biological knowledge, which may not be available. The high number of parameters may lead to an increased inherent susceptibility to internal errors and a high sensitivity to small parameter changes. Some counterarguments to this can be found in Breckling *et al.* (2006).

IbM modeling of the individual cell lag phase

A first basis for individual based modeling (IbM) was laid in the 1940s by Leslie, who proposed a modeling approach for population age distributions based on subgrouping the population based on age. This differentiation approach climaxes to the level of individual agents in IbM modeling. The first true IbM implementations followed in the 1970s (see, e.g., Kaiser (1979)) but it is only in the 1990s that IbM modeling has seen a greater adoption. The main application area is ecology with important publications like DeAngelis and Gross (1992) but since about ten years, the application of IbM modeling is spreading to various other scientific disciplines. Examples can be found in social sciences (e.g., Helbing *et al.*, 2000), economics (e.g., LeBaron, 2000; Winker and Gilli, 2002), microbial ecology (Kreft *et al.*, 1998, 2001; Picioreanu *et al.*, 2004), (predictive) microbiology (Dens *et al.*, 2005a,b; Almaas *et al.*, 2004), and so on.

In **Task 1.1**, and more specifically, in Swinnen *et al.* (2004a), research is described on lag phenomena in general, and on how these phenomena are currently being modelled in the domain of predictive microbiology. It can be concluded that the models available until now remain largely empirical and thus have limited predictive value in environmental conditions other than those for which the model was developed and for which the parameters were estimated. McKellar and Knight (2000) designate this problem to the poor understanding of the physiological events taking place during adaptation of cells to new environments. They also emphasise that empirical equations have a limited ability to enhance the knowledge concerning the physiological stages of bacterial adaptation to new environments and subsequent growth. Especially when the purpose of the models is predictive rather than descriptive, like is the case in *predictive* microbiology, it is important to incorporate as much physiological knowledge as possible into the model in order to make it more generally valid. One of the topics of this work package is the study of the underlying mechanisms of the lag phenomena such that we would be able to give the parameters some mechanistic meaning, including its temperature dependency.

To learn more about the basic mechanisms behind lag phenomena, the theory on cell division is studied on an individual based level. Although some literature (e.g., Cooper and Helmstetter (1968) and Donachie (1968)) dates from 30 to 40 years ago, and provides a large amount of information on how cells would likely respond to changing environmental conditions, it has up till now hardly been introduced in the field of predictive microbiology. It appears that initiation of DNA replication is regulated by the increase in cell mass and that a constant time is needed for DNA replication and cell division (the so-called *C* and *D* period respectively). This is illustrated in Figure 20.

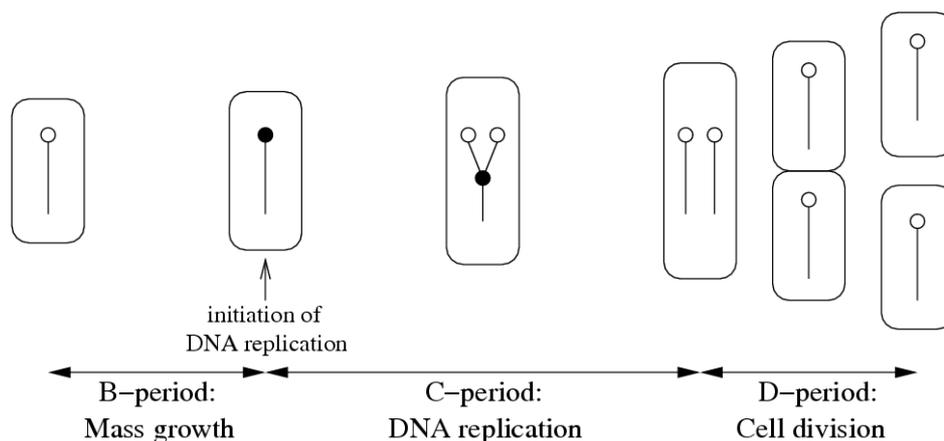


Figure 20 : Schematic representation of the cell cycle (after Keasling et al., 1995). The volume of the cell grows continuously at a certain growth rate μ . At a particular time, DNA replication is initiated. An uninitiated chromosome origin is represented by an open circle. A chromosome origin at which DNA replication is initiated is represented by a filled circle. The time required for a replication fork to proceed from one end of the genome (origin) to the other (terminus) is denoted by the C-period. The time needed for cell division (after replication is terminated) is denoted by the D-period.

As a consequence, cell mass at division (and also average cell mass) is an exponential function of growth rate and $(C + D)$ period: $m_d = 2m \cdot \exp[\mu(C + D)]$. The repercussion of this theory on the evolution of a cell population after a medium shift predicts *rate maintenance* of cell number. Based on published experimental evidence and the theory of Donachie (1968) and Cooper and Helm-stetter (1968) on DNA replication and cell division, we proposed a similar theory for the behavior of cell populations at a temperature shift and at a (more general) combination of medium and temperature shift. From the obtained theory, and the temperature dependence of μ and $(C + D)$, the lag time λ can be predicted. Simulation results for a temperature shift from 15°C to 35°C with the resulting intermediate lag phase are shown in Figure 21.

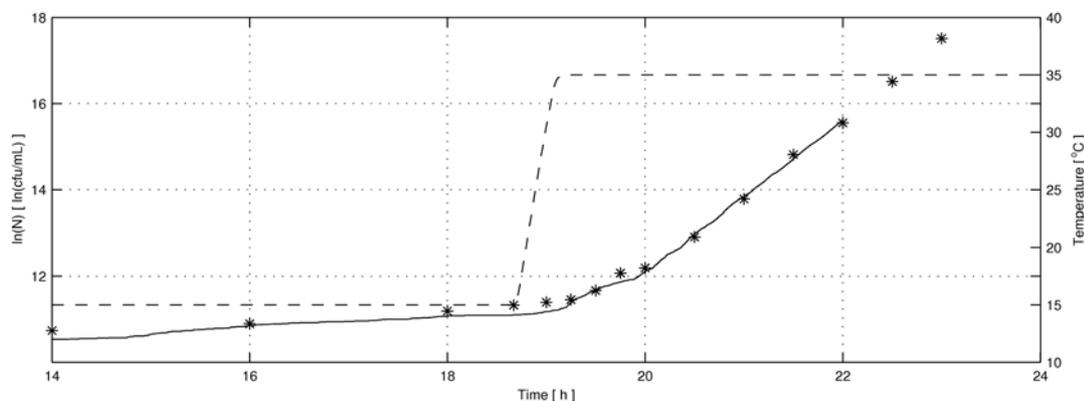


Figure 21 : Fit of the BacSim model simulation results on an experimental dataset of *Escherichia coli* K12 (*), subject to a sudden temperature increase from 15 to 35°C (dashed line) (as performed in **Task 1.1**). The model is capable of describing the intermediate lag phase dynamics.

This theory is then the basis for a critical evaluation of the existing modeling concepts on lag in predictive microbiology. An important conclusion is that the so-called physiological state of the cells which is very important in the prediction of lag behavior, can now be identified with measurable cell parameters like cell mass, DNA, RNA and protein content per cell. The physiological state of the cells evolves exponentially as a function of growth rate and interval between initiation of DNA replication and cell division ($C + D$). Furthermore, from this definition, it emerges that the work defined by the product $\lambda \cdot \mu$ corresponds exactly to the change in physiological state between the two environments.

For more details, reference is made to Dens et al. (2005a,b).

The individual-based modeling methodology has been studied extensively in this research. The modeling framework used for these modeling efforts, is BacSim (see, a.o., Kreft *et al.*, 1998). Considerable effort has been put into documenting and debugging the source code of this framework, in order to gain a better understanding of the object-oriented structure of the program and assure an efficient continuation of this research. The model that was used for this research was based on an older version of BacSim. The model was modified to use a newer BacSim version, implying the adaptation of the code to a new, more logical object-oriented structure. This updating process guarantees that future model extensions will be straightforward to integrate, and eases our communication and collaboration with the original BacSim developers.

IbM implementation of S&P type models

In a second part of this work package, BacSim has been used to develop a generic platform for the implementation of S&P type microbial growth models (Van Impe *et al.*, 2005 and Poschet *et al.*, 2005a), where the onset of the stationary phase is modelled in a more mechanistic way, incorporating the effect of metabolite and substrate concentrations. An overview can be found in Standaert *et al.* (2004). The developed model serves as a platform to work towards more specific case studies.

One of those specific case studies is the implementation of an antagonistic two-species system, which is described in Task 2.1. In essence, the IbM model is a translation of the macroscopic model structures presented in Task 2.1 to the level of the individual cell. This way, the model can be extended to incorporate inter-cell variability and, on the long term, effects of spatial structure on the behaviour of the microbial populations.

Exploration of IbM parameter estimation techniques

IbM parameter identification is also one of the topics in Task 3.3. IBMs can only be simulated when individual model parameters and their stochastic properties are known. Opposed to macroscopic variables, cell-based characteristics are much more difficult to determine experimentally. Estimation of individual parameters from macroscopic data stands for an attractive supplement to the time-consuming, labor-intensive, and error-prone individual measurements. Moreover, parameter estimation of IBMs is still a relatively unexplored area but becomes more feasible given the incessant progress in computational capacity.

Individual growth rate and the associated variance have been estimated from macroscopic cell density measurements of the 2-species antagonist-target system, described in **Task 2.1**. Parameter estimates are obtained via least squares estimation and based on a grid-search approach. Practical issues on simulation limitations, minimizing Monte Carlo variance, choice of the objective function and others, have been resolved. Results show that the average individual growth rate and its associated variance can be estimated uniquely though with difficulty from exponential growth data. The effect of number of data points and the sample spacing has been investigated. Opposed to population models, parameter estimation

uncertainty is less influenced by the number of data points (only large datasets can lower the variance significantly) and grouping of samples (or replaced sampling at the same time instants) lowers the variance.

A journal publication covering this research has been submitted (Standaert et al., 2006b).

Work Package 4: Towards a national centre for predictive microbiology/risk assessment

Task 4.1: One-way communication with the national food industry - Development of an Internet Portal Site

The project website (see Figure 22) can be consulted at <http://cit.kuleuven.be/biotec/research/projects/podwb/index.php>. The website contains an introduction, a project summary, an overview of the scientific partners, an overview of the members of the advisory committee. The Internet Portal site is visualized through “Microbiology Links” covering general subjects and more specific ones like HACCP, risk assessment, micro-organism specific information, predictive microbiology software and (European) government guidelines regarding food safety (see an example in Figure 23).



Figure 22: Homepage of the project website.

As part of the project’s dissemination, **four hands-on sessions** were organized for the members of the users’ committee of the project, comprising people from the Belgian Federal Science Policy, for policy supporting purposes, as well as people from the food industry. *In these sessions, the basics of predictive microbiology as a condensed knowledge base for pro-active food safety preservation was elaborated upon (Sessions I and II), while in Session III and IV more application-oriented examples were treated in detail.*

Every session was followed by a short survey for having guidance for the organisation of the next session, and it can be stated that the users' committee was appreciating very much these workshops.

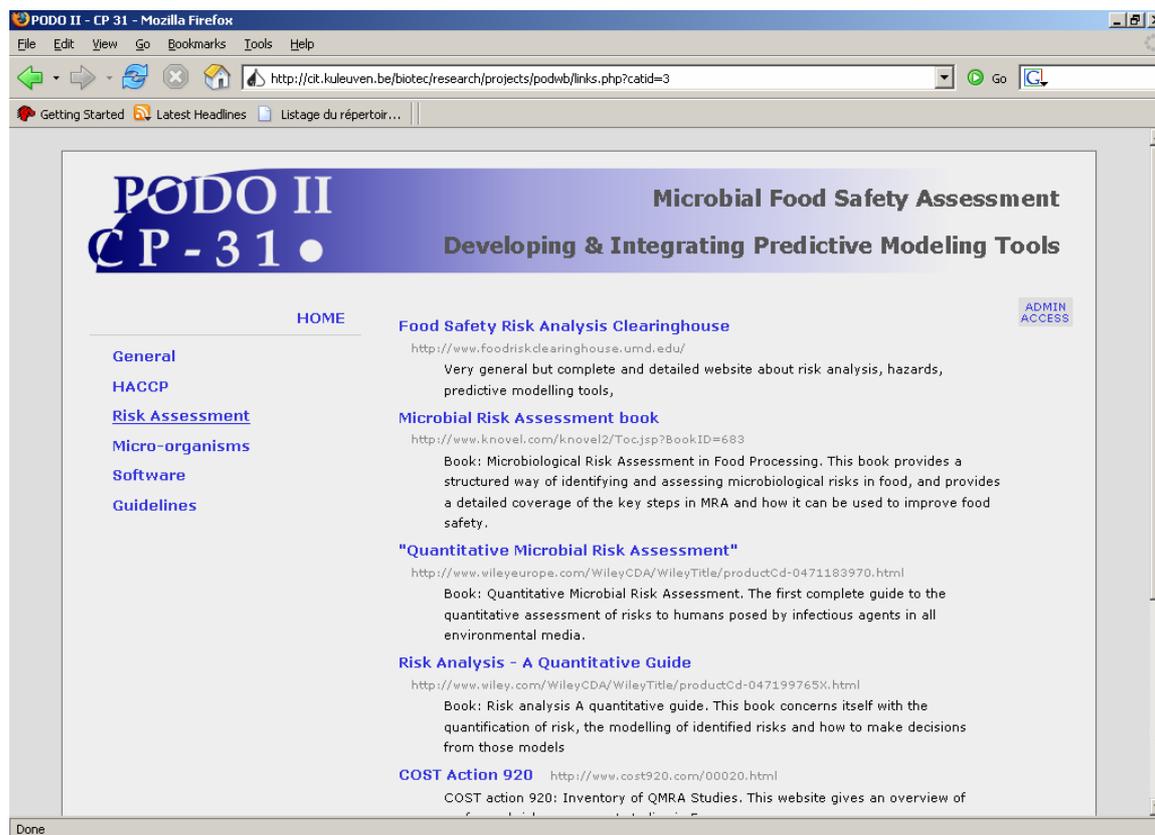


Figure 23: A screenshot showing part of the Risk Assessment links provided and shortly commented upon.

The user committee had eight meetings concerning the scientific results of the project:

- May 3, 2002
- November 8, 2002
- March 14, 2003
- September 26, 2003
- March 19, 2004
- September 24, 2004
- March 18, 2005
- June 9, 2006

An important dissemination activity of the project has been the *Fourth International Conference on Predictive Modeling in Foods* took place in Quimper (France) in a co-organization of the Université de Bretagne Occidentale (France) and K.U.Leuven/BioTeC (Van Impe J.F.M., Geeraerd A.H., Leguérinel I. and Mafart P. 2003. Predictive Modeling in Foods - Conference Proceedings. K.U.Leuven/BioTeC, Belgium (ISBN 90-5682-400-7), 347 p.). This conference was the fourth world wide conference in the discipline and KULEuven/BioTeC is also invited to co-organize the next world wide conference, taking place in Greece in 2007.

Task 4.2: Two-way communication with the national food industry - Towards the development of Active Server Pages

Suggestions made by members of the user committee, at the occasion of the scientific meetings or the hands-on sessions, or made by e-mail, and covering both questions and suggestions related with the website, where all taken into account during the continuous update of the project website.

A direct technological realization of the project is GInaFiT – a freeware add-in for Microsoft® Excel (see Figure 24) related with the identification of suitable mathematical models for the inactivation of micro-organisms under varying environmental conditions (see also the publication of Geeraerd et al., 2005). The user-friendly tool bridges the gap between people developing predictive modeling approaches and end-users in the food industry not familiar with advanced non-linear regression analysis tools. The current version of the tool contains nine inactivation models. The tool can freely be downloaded from the K.U.Leuven/BioTeC-homepage <http://cit.kuleuven.be/biotec/> at the topic “Downloads”.

At this moment, about 200 people from around the globe have downloaded the tool, and positive reactions were received. People downloading the tool are generally affiliated with universities, other research institutes or food industry, but also with the chemical and pharmaceutical industry. This illustrates the versatility of GInaFiT. Support for end-users having questions related with the direct or indirect application possibilities of GInaFiT is delivered on a case-by-case basis.

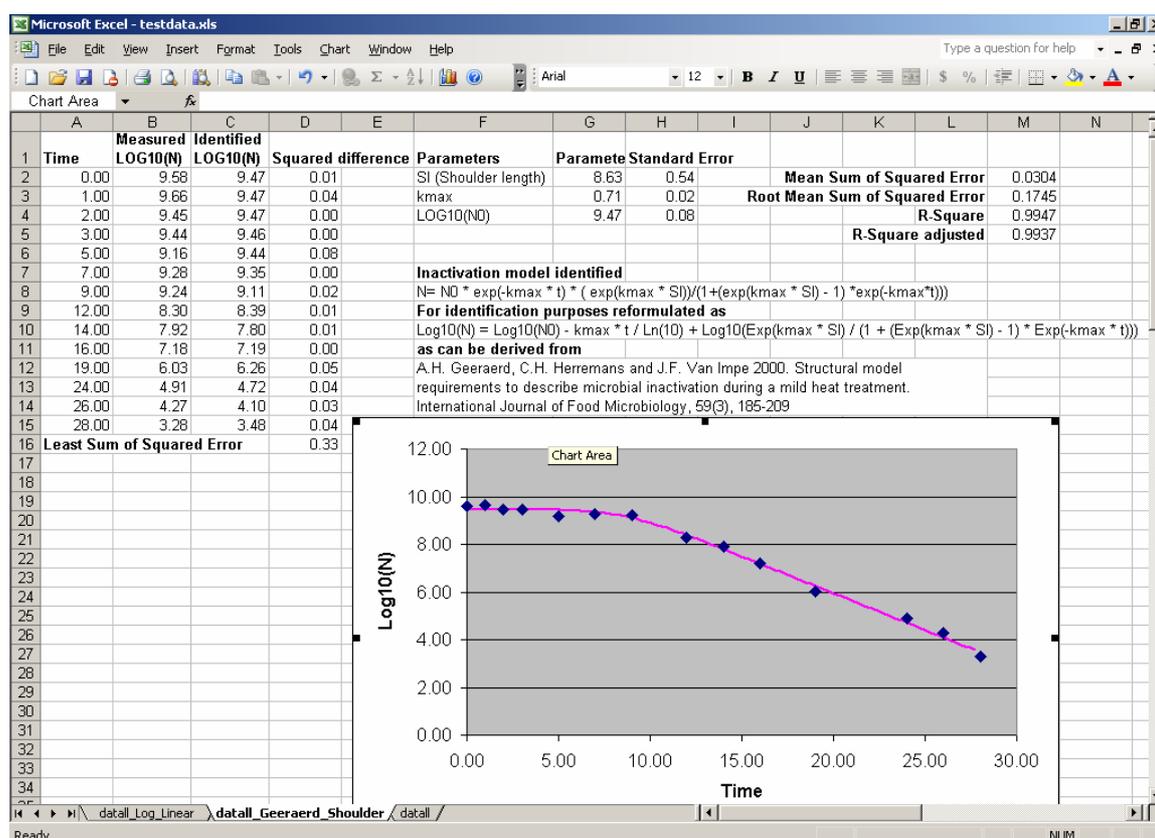


Figure 24: A GInaFiT screenshot: an example of a microbial inactivation model identification (parameter estimates in column F, standard errors in column G, figure, statistical measures in columns L and M) based on the experimental data included in columns A and B.

4 ANNEXES

4.1. References

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4.2. Publications

Books –Editor

2003

J.F.M. Van Impe, A.H. Geeraerd, I. Leguérinel and P. Mafart 2003. Predictive Modeling in Foods - Conference Proceedings. KULeuven/BioTeC, Belgium (ISBN 90-5682-400-7), 347 p.

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