Intermediary report - January 2003

**MICROBIAL FOOD SAFETY ASSESSMENT: DEVELOPMENT AND INTEGRATION OF GENERIC PREDICTIVE MODELLING TOOLS**
**CP-31**

KUL - RUG

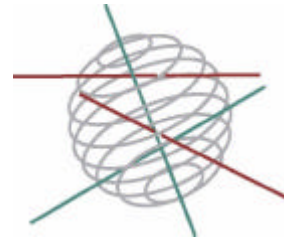# SPSD II

**PART 1**
SUSTAINABLE PRODUCTION AND CONSUMPTION PATTERNS

- GENERAL ISSUES
- AGRO-FOOD
- ENERGY
- TRANSPORT

**This research project is realised within the framework of the Scientific support plan for a sustainable development policy (SPSD II)**

**Part I "Sustainable production and consumption patterns"**

The appendixes to this report are available at :
http://www.belspo.be (FEDRA)

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# Microbial Food Safety Assessment: Development and Integration of Generic Predictive Modeling Tools

# First Year Report

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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 21$^{st}$ 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 (Temmerman, 2001).

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 behaviour of micro-organisms in foods, taking into account their complex microbial ecology, as generic tools for microbial food safety assessment.*

The <u>scientific research objectives</u> of this Research Project are threefold.

1.  Development and integration of (both *macroscopic* and *microscopic*) building blocks into a *widely applicable novel generation predictive modelling methodology*.
    Transferability (i.e., the generic nature of the approach) will be assessed at different levels.
a.  To transfer model structures from one micro-organism 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 modelling framework generates an application driven optimal modelling procedure (*modelling 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 modelling 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 modelling 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 24.5 billion € (FEVIA, 2001), 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

## 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 modelling methodologies

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

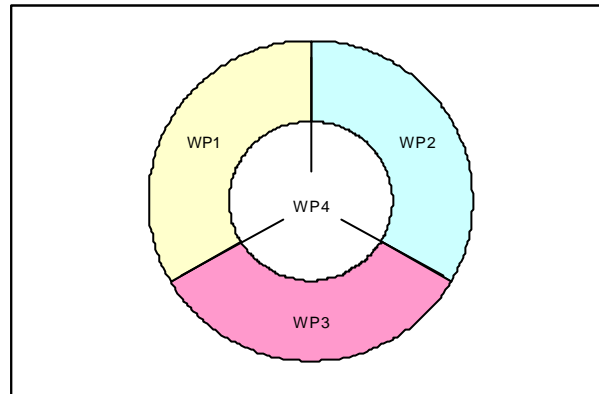The relationship between these Work Packages is presented schematically in Figure 1.



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

The novel predictive modelling 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 $\leftrightarrow$ WP1 and WP3 $\leftrightarrow$ 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 modelling attempts are the driving force for designing and performing more informative experiments that will yield models with a higher predictive value.

    ✓  WP1 $\leftrightarrow$ 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 centre 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 modelling 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 modelling approach, the alternative *individual-based modelling* (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 modelled 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.

## 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 modelling viewpoint, it is therefore recommended to start with a simple, well-defined case study, based on a single mechanism (Type A), which is complexified during the course of the project (Type B).

*Type A*: 1 antagonist → 1 metabolite (lactic acid) → 1 pathogen (Task 2.1)

*Type B*: 1 antagonist → 2 metabolites (lactic acid + bacteriocin) → 1 pathogen (Task 2.2)

*Task 2.1: Antagonistic phenomena through a single metabolic product*

**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.

*Task 2.2: Antagonistic phenomena through multiple metabolic products*

The experimental and modelling expertise built up in Task 2.1 will be fully employed in this task.

**Step 1: Experimental Protocol and Data Generation.** Since bacteriocins are not active towards gram- bacteria, L. innocua is selected as model pathogenic organism. To help in selecting an appropriate bacteriocinogenic lactic acid bacterial strain, the Advisory Committee will be consulted. Note that the exact type of interaction (i.e., (i) inhibition, (ii) inhibition following inactivation, or even (iii) only inactivation) has to be verified experimentally and probably depends on the selected antagonist and bacteriocin. The monitoring procedure of the bacteriocin concentration will depend on its molecular structure and may involve agar diffusion techniques or chromatographical techniques.

**Step 2: Model Construction.** The models designed in Task 2.1 are modularly extended to include antagonistic effects induced by bacteriocin production.

**Step 3 for Task 2.1 & Task 2.2: Model validation** The applicability of the developed models on new experimental data will be thoroughly evaluated.

## Work Package 3: Developing and integrating predictive modelling 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 modelling 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.

### *Task 3.2: Macroscopic predictive modelling*

Macroscopic predictive modelling 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 modelling*

**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 behaviour. This is the domain of Individual based modelling (IBM). The first step in this approach is to devise a set of rules, consistent with observation, which govern the behaviour 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) behaviour 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 behaviour (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 centre 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 modelling 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 modelling 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 modelling 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 modelling 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.

4   A searchable overview of literature of predictive microbiology, microbiological risk assessment, HACCP, …

4   An overview of the results and conclusions of this project.

4   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 modelling data and client-specific guidance throughout all stages of predictive model generation and exploitation).

4   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 mode lling methodologies**

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

**Work Package 4: Towards a national centre 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 the intermediary results, preliminary conclusions and recommendations *with regard to the Tasks indicated for Year 1* will be presented in the next section (Section 3).

10

| | Year 1 | | Year 2 | | Year 3 | | Year 4 | |
|---|---|---|---|---|---|---|---|---|
| Months | 0 to 6 | 7 to 12 | 13 to 18 | 19 to 24 | 25 to 30 | 31 to 36 | 37 to 42 | 43 to 48 |
| **Work Package 1: Exploring the boundaries of microbial evolution** | | | | | | | | |
| *Task 1.1: Quantifying lag phenomena due to temperature variations* | | | | | | | | |
| Data generation | █ | █ | █ | █ | █ | █ | | |
| Model development | | █ | █ | █ | █ | █ | █ | |
| Model validation | | | | | | | █ | |
| *Task 1.2: : Quantitative study of the effect of the history of contaminating cells on their lag phase* | | | | | | | | |
| Data generation | █ | █ | █ | █ | █ | █ | | |
| Model development | | █ | █ | █ | █ | █ | █ | |
| Model validation | | | | | | | █ | |
| **Work Package 2: Quantifying inhibition and inactivation phenomena due to microbial interaction** | | | | | | | | |
| *Task 2.1: Antagonistic phenomena through a single metabolic product* | | | | | | | | |
| 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 | | | | █ | | | | |
| *Task 2.2: Antagonistic phenomena through multiple metabolic products* | | | | | | | | |
| Sub-Task 2.2.1: Inhibition phenomena in model system Type B | | | | | | | | |
| Data generation | | | | | █ | █ | | |
| Model development | | | | | | █ | █ | |
| Model validation | | | | | | | █ | |
| Sub-Task 2.2.2: Inactivation phenomena in model system Type B | | | | | | | | |
| Data generation | | | | | █ | █ | | |
| Model development | | | | | | █ | █ | |
| Model validation | | | | | | | █ | |

# 3. DETAILED DESCRIPTION OF THE INTERMEDIARY RESULTS, PRELIMINARY CONCLUSIONS AND RECOMMENDATIONS

## 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.**

Initially, a reproducible experimental protocol has been formulated as follows. Experiments are performed in a computer-controlled bioreactor (*New Brunswick Scientific Inc., USA*) where *E. coli* K12 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 heating rate that could be realised in the bioreactor is equal to $0.62 \pm 0.17°C/min$. The inoculum is prepared by subculturing 5 μL 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. In all these experiments, the pH is kept constant at 7.55 by adding base (1N KOH) or acid (1N $H_2SO_4$) to the medium. 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 (*Spiral Systems Inc., Cincinatti, Eddyjet, USA*). 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*).

This experimental setup is used to characterise 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. It is known that the lag phase is not only affected by the environmental conditions, but also by the physiological state of the cells at the moment of the environmental change. Because the physiological state of the cell (depending on the growth stage) can be defined by cell properties, like cell mass, protein content or RNA, we have started with measuring the protein content to discover more on the trigger of microbial lag.

Two series of bioreactor experiments with positive temperature shifts have been performed. Besides temperature, no environmental changes have taken place. In a first series, the initial temperature was kept constant at 15°C and temperature jumps with amplitudes of 5, 7.5, 8.8, 10, 15 and 25°C were applied to cells with the same pre-history (exponential growth phase) (Swinnen *et al.*, 2002). A summary of the resulting intermediate lag phases in function of the amplitude is presented in Figure 1 (see Appendix 1). It can be deducted that temperature jumps with amplitude of minimum 8.8°C cause a lag phase with a constant duration of (approximately) one hour. In the second series, temperature shifts with a constant magnitude ΔT of 10°C starting at an initial temperature of 10, 12.5, 15, 20, 25 and 30°C were implemented at the same cell density. Figure 2 (Appendix 1) gives an overview of the resulting intermediate lag phases in function of the initial temperature of the temperature change. Temperature shifts starting at 12.5, 15 and 20°C result significantly in an intermediate lag phase, while in case of shifts starting at 25 and 30°C immediate adjustment of the growth rate was observed. For the shift starting at 10°C, further testing is needed to determine if the occurrence of a lag phase is significant. By combining the results of both series of experiments, it can be concluded that the lower boundary of the *normal physiological range* lies between 22.5°C and 23.8°C (Swinnen *et al.*, 2003, *submitted*). This *normal physiological range* can be defined as the linear part of the Arrhenius plot (ln ($\mu_{max}$) 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.

The evolution of the measured glucose and acetate concentrations for the experiment with a temperature shift from 15 to 25°C are presented in Figure 3 (Appendix 1). 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 were obtained until now.

Additional experiments are being performed to further characterize the lag phenomenon and the normal physiological range. Furthermore, the influence of negative temperature shifts on the lag phase will be investigated.

**Step 2: Model Construction.**

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 a*l., 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* is being conducted, mainly focussing on the influence of temperature and culture history on the lag phase during growth of bacteria.

In predictive microbiology, a two-step modelling 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 micro-organisms, 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.

With regard to the starting phase of *microscopic modelling approaches*, reference is made to results reported under Task 3.3, and its associated Appendix 8, where some of the model indicated above are further analysed.

*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 was developed to isolate single cells in the cup of a microtiter plate based on standardised 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 $10^3$ cfu/ml. This cell count was controlled by plate counting on 200µl inoculum on TSA, with incubation at 30°C for 24h. Further dilution was performed by adding 200 µl of inoculum to 200µl broth in each cup of the first row of a microtiter plate. These cups were used to make further ½-dilution series ending up with single cells isolated in the cup of a microtiter plate.

This procedure was repeated for 9 plates, resulting in 72 ½-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 comparing these results to McKellar & Knight (2000), who observed the last well of each row showing growth, 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). When comparing these results to the method of Robinson et al. (2001) this method gives a higher chance of having single cells (80% vs.

70%). *For more details, reference is made to Francois et al. (2003), which is currently submitted. A copy of this article can be found in Appendix 2.*

The Solid Phase Cytometry experiments (as indicated in Section 2.2) will not be performed as test conditions reveal to be too different from the OD measurements (liquid vs. solid).

In a second step the individual lag phase of *Listeria monocytogenes* was investigated at different temperatures using OD measurements. Microtiterplates were filled as previously described, and incubated at the fixed temperature. 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). At least 100 replications were made for each set of conditions.

At the moment, the combined effects of pH, $a_w$ and temperature are investigated using optimal pre-culture conditions (24h in BHI @ 30°C). The data collection combining different temperatures and pH is almost finished and data are currently processed. Data collection combining previous factors with $a_w$ will be performed during spring-summer 2003. In a further step, these results will be compared to more stressful pre-culture conditions.

**Step 2: Model Construction.**

In this research, a simulation model is constructed that represents the serial dilution process described in Step 1. The aim of the modelling process is (*i*) to gain insight in the governing mechanisms of the dilution process, (*ii*) to confirm experimental findings, and (*iii*) to enable the prediction of an average outcome of future microtiter plate experiments. *For a detailed description of the model building process, reference is made to Appendix 3, of which an abbreviated version is currently under submission (Standaert et al., 2003).*

The starting point for a simulation is the measurement of the cell concentration of the original inoculum suspension based on plate counts (ideally $10^3$ CFU/mL). The model is based on a unit operation in which all cells inoculated in a well of the first column of the plate are transferred randomly and individually to the well in the second column with a 50% chance. This cycle represents one dilution step and is repeated until all subsequent wells are filled and the dilution series is complete. Statistical analysis shows that this basic simulation scheme is a fully random process and all columns show a Poisson-type cell number distribution. The experimental data, on the other hand, follow a different pattern: the left-hand columns of a plate seem to be better described by a lognormal distribution, while the right-hand columns do correspond to a Poisson distribution. The lognormal nature shows that the process has *contagious* characteristics and indicates the presence of an aggregational aspect (see, e.g., Jarvis, 1989) that gradually disappears as the dilution series progresses towards the right side of the plate. The simulation procedure is adjusted to account for these observations. Inoculum numbers are now randomly selected from a lognormal distribution based on the mean inoculum concentration measurement and a randomly selected variance. This version of the model is used to quantify the performance of the experimental process. Simulation results indicate that the wells considered by the experimental protocol (wells showing growth in the last five columns) have a probability of 77-78% of actually containing exactly one cell.

Visual comparison of simulation and experimental data shows that the introduction of the lognormal distribution alone cannot account for the extent of the aggregational character of the dilution process. For this reason, a *cell clumping* mechanism is added to the simulation model: transfer now happens at the level of cell clumps, not of individual cells. Simulation results show that the clumping algorithm can explain the *contagious* nature of the dilution series. Individual simulated plate experiments show more irregular patterns, which is also observed in the experimental data. Future research includes (*i*) an investigation of the predictive performance of the model based on the available experimental data, and (*ii*) an evaluation of the clumping algorithm, which, for the time being, remains fully hypothetical.

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

*Task 2.1: Antagonistic phenomena through a single metabolic product*

## 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 *1* antagonist, a lactic acid bacterium, interferes through *1* antimicrobial metabolite, lactic acid, with *1* target, a foodborne pathogen. Two examples of this *1:1:1* 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 et al., 2003).*

*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, and are summarised in Appendix 4.

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 $10^3$ 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 behaviour 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).

| | Case study #1 | Case study #2 |
|---|---|---|
| Inoculum ratio *antagonist / target* [cfu/mL] | $10^3$ / 0 <br> 0 / $10^3$ <br> $10^3$ / $10^3$ <br> $10^4$ / $10^3$ <br> $10^5$ / $10^3$ <br> $10^6$ / $10^3$ <br> $10^7$ / $10^3$ | |
| Temperature [°C] | 35,12 | 37, 22,12,7,4 |

Table 1: Experimental plan.

All experiments are performed under anaerobic atmosphere at an initial pH of 6.2. At regular time instances, the cell concentration of antagonist and pathogen, the total lactic acid concentration, the pH and the glucose concentration are monitored. The latter variable is measured to verify the absence of glucose exhaustion. All experiments are performed in duplicate.
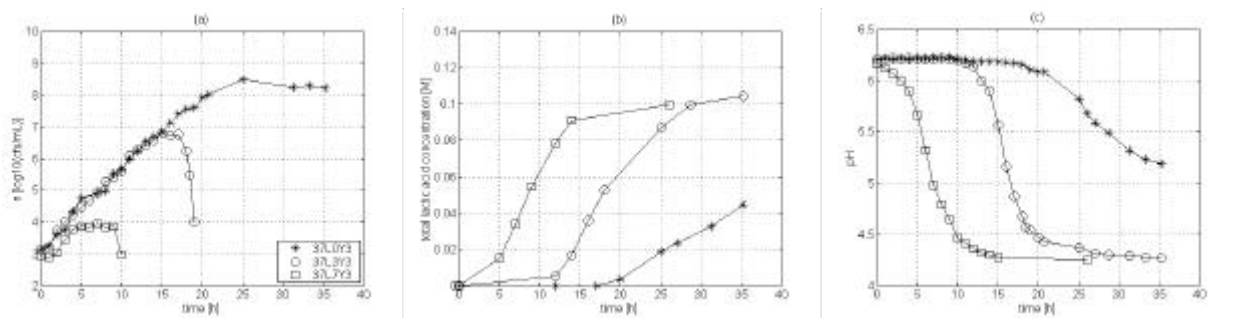
### Experimental results



Figure 2: Cell concentration, total lactic acid concentration and pH as a function of time for *Y. enterocolitica* in mono- and coculture with *Lact. sakei* at 37°C.

Figure 2 represents the cell concentration, the total lactic acid concentration and the pH as a function of time for some experiments at 37°C, consisting of (i) *Y. enterocolitica* in monoculture (37L0Y3), (ii) *Y. enterocolitica* in coculture with *Lact. sakei*, the latter inoculated at $10^3$ cfu/mL (37L3Y3), and (iii) *Y. enterocolitica* in coculture with *Lact. sakei*, the latter inoculated at $10^7$ cfu/ml (37L7Y3). These experiments can be regarded as a representative sample of the entire collection and allow deducing some general qualitative trends. A more comprehensive overview of the data of the case studies is provided in Appendix 5. For the monoculture experiment, a clear exponential growth phase and a stationary phase can be observed, whereas a lag phase is barely present. Significant acid production (and corresponding pH reduction) is only apparent from the late exponential phase on. In the coculture experiments, 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. 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 the *Yersinia* growth curves (mono- and coculture), the onset of the stationary phase coincides with the large increment in the lactic acid production curve. In contrast to the monocultures, the stationary cell concentration is not maintained in the cocultures. 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. Note however that 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 3, 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)].
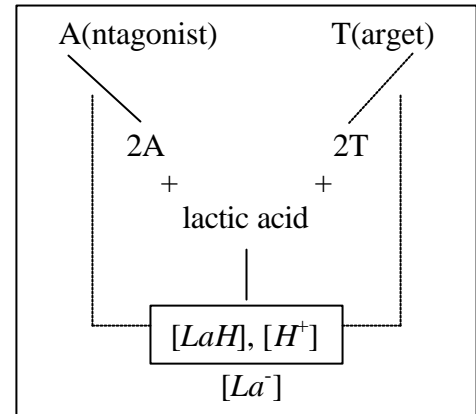


Figure 3: Reaction scheme of the experimental system.

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*):

$$\frac{dN_i}{dt} = \boldsymbol{m}_{max,i} \cdot \boldsymbol{m}_i([LaH],[H^+]) \cdot N_i$$

$$\frac{dLaH_{tot}}{dt} = \sum \boldsymbol{p}_{max,i} \cdot \boldsymbol{p}_i([LaH],[H^+]) \cdot N_i$$

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

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

with $t$ [h] the time, $N_i$ [cfu/mL] the cell concentration, $\boldsymbol{m}_{max,i}$ [1/h] the maximum specific growth rate, $\boldsymbol{p}_{max,i}$ [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.

Further model development will concentrate on each of the subprocesses separately. In a first phase, the chemical subprocess of lactic acid dissociation is taken into account. To start, two mechanistic models out of literature 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. The equations provide an accurate

description of the acidifying profiles measured in the media of the two case studies. Full details can be found in Appendix 6.

Next to this global modelling framework for the two case-studies at hand, an extended literature review was made concerning different strategies for modelling *chemical inhibition and inactivation* of micro-organisms (Devlieghere et al., 2003, submitted).

## Work Package 3: Developing and integrating predictive modelling methodologies

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

As underlined in Bernaerts and Van Impe (2003a, *submitted*), bioprocess modelling presents a challenging subject that requires a meticulous modelling strategy. During the modelling 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.

In the first place, research activities of Task 3.1 have been conducted in close collaboration with WP 1.1, 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).

In the second place, 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). Thus far, 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). However, more model parameters are probably involved in the description of

the studied lag phenomena. As a proper model structure is currently under development (see Tasks 1.1 and 3.3), an existing *four*-parameter model has been selected to work out the mathematical framework. In Bernaerts *et al.* 2003b and 2003c (*submitted)* and Gysemans *et al.* (2003, *submitted*), a model output sensitivity analysis has been performed and optimal experiment designs have been computed for two cardinal values models (Rosso *et al.*, 1995). As a starting point, an optimal selection of the independent variables has been aimed for. Optimal experimental plans are distinct from the arbitrary placement of independent variables which is commonly applied in factorial designs. Further research will investigate the applicability of *dynamic* inputs for optimal parameter estimation.

*Task 3.2: Macroscopic predictive modelling*

The close interface between WP3 and WPs 1 and 2, is illustrated above, where (intermediary) results on macroscopic modelling techniques, directly related with aforementioned tasks, is reported (see Task 1.1., Step 2 and Task 2.1, Step 2). For both approaches, *balance models* and *kinetic modelling approaches are indicated.*

Task 3.2 has also deliverables on his own, namely concerning *methodological modelling developments*.

**Step 1: Balance models.** Description of microbial cell (population) behaviour influenced by dynamically changing environmental conditions intrinsically asks for dynamic mathematical balance models. In Bernaerts *et al.* (2003d, *submitted*), 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) modelling approaches (see Task 3.3.) are explained using the case studies addressed in this project (see WPs 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).

**Step 2: Kinetic models.** A second development is related with *advanced black box modelling approaches* for use as *kinetic models.* In Geeraerd et al. (2002) and (2003), 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 modelling 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.

**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 variability.* For these purposes, a confidence related with a model prediction is indispensable, and a transition form 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 (Poschet et al., 2003). A normal distribution over the experimental data was considered. This probabilistic approach, incorporating experimental variation, is applied to experimental growth data of *Escherichia coli* K12 and *Listeria innocua* ATCC 33090. For more details, reference is made to Appendix 7.

Variability, inherent in the production and use of microtiter-experiments in order to characterize the lag fase of individual cells, is also taking into account as described in the research reported under Task 1.2, Step 2 and its associated Appendix (Appendix 3).

### Task 3.3: Microscopic predictive modelling

In Task 1.1, more information is given 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 merely 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 environment 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. The purpose of this work package is to reveal the underlying mechanisms of the lag phenomena as much as possible 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 must be studied on an individual based level. Although this 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). As a consequence, cell mass at division (and also average cell mass) is an exponential function of growth rate and $(C + D)$ period: $m_d = 2m_l \exp[ \mu(C + D) ]$. 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 behaviour 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 $\mu$ and $(C + D)$, the lag time $\lambda$ can be predicted.

This theory is then the basis for a critical evaluation of the existing modelling 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 behaviour, 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 and Van Impe (2003) (see also Appendix 8).

Future work will focus on the application of the above-mentioned theories on the experimental results reported under Task 1.1 and WP 2.

## 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 homepage of the project can be consulted at

- ✓ http://www.agr.kuleuven.ac.be/lmt/biotec/index.htm
- ✓ Research
- ✓ Projects and Research Cooperations
- ✓ DWTC CP-31

The home page summarises the project objectives, current applications of predictive microbiology in the food industry, the two partners of the project and a direct link to the homepage of nine members of the users' committee.

A first session of the hands-on course on predictive modelling courses offered at all members of the users' committee has taken place at November 8, 2002.

In the near future (March 15, 2003), the second session of this hands-on course will be held.

The homepage will be enlarged in order to include more links on research and developments related with the basics and application of predictive modelling for the food industry.

It is also worth mentioning that several (joint) BioTeC and LFMFP research (indicated above at the related tasks), and, as such, directly related with this project, is currently under submission for the *Fourth International Conference on Predictive Modelling in Foods*, which will be held in Quimper, June 15-19, 2003, and which is a co-organisation of the Université de Bretagne Occidentale (Prof. Pierre Mafart) and KULeuven/BioTeC (Prof. Jan Van Impe).

## 4. FUTURE PROSPECTS AND FUTURE PLANNING

For the future planning of this Research Project, reference is made to the timetable reported in Section 2.4, indicating that all tasks will continue in the second year of the project.

As the previous section contains *intermediate results, conclusions and recommendations*, it was decided to report specific future activities for each task at the end of each subsection.

# 5. ANNEXES

## 5.1 References

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Bernaerts K. and Van Impe J.F. 2002. Optimal dynamic experiment design for estimation of microbial growth kinetics at sub-optimal temperatures: modes of implementation. In: B. O'Connor and D. Thiel (Eds.), Proceedings of the Second International Conference on Simulation in Food and Bio-industry, 212-216 [The Second International Conference on Simulation in Food and Bio-industry (FOODSIM' 2002), Blarney (Ireland), June 17-18, 2002]

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## 5.2 Publications

This list covers all publications that have been *published* during the first year of the project's lifetime (i.e. December 15, 2001 – December 14, 2002). A number of research results have currently been accepted for publication or submitted, as indicated in previous sections.

### International Conferences with review - full paper

F. Devlieghere, A. Geeraerd, J. Van Impe and J. Debevere 2002. Combined preservation factors and modelling. Frontiers in microbial fermentation and preservation. Society for Applied Microbiology (SFAM) & The Dutch Society for Microbiology, 29-31
[January 2002 Meeting of SFAM, Wageningen (The Netherlands), January, 9-11, 2002]

K. Bernaerts and J.F. Van Impe 2002. Optimal dynamic experiment design for estimation of microbial growth kinetics at sub-optimal temperatures: modes of implementation. In: B. O'Connor and D. Thiel (Eds.), Proceedings of the Second International Conference on Simulation in Food and Bio-industry, 212-216
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A.H. Geeraerd, V.P. Valdramidis, F. Devlieghere, H. Bernaert, J. Debevere and J.F. Van Impe 2002. Development of a novel modelling methodology by incorporating a priori microbiological knowledge in a black box modelling approach. In: L. Axelsson, E.S. Tronrud and K.J. Merok (Eds.) Proceedings and abstracts of the 18th International ICFMH Symposium, 135-138
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K.M. Vereecken, M. Antwi, M. Janssen, A. Holvoet, F. Devlieghere, J. Debevere and J.F. Van Impe 2002. Biocontrol of microbial pathogens with lactic acid bacteria: evaluation through predictive modelling. In: L. Axelsson, E.S. Tronrud and K.J. Merok (Eds.), Proceedings & Abstracts of the 18th Symposium of the International Committee on Food Microbiology and Hygiene (ICFMH), 163-166
[Food Micro 2002, *Eighteenth International Symposium of the International Committee on Food Microbiology and Hygiene* (ICFMH), Lillehammer (Norway), August 18-23, 2002]

### International Conferences with review - abstracts

Annemie H. Geeraerd, David Marquenie, Carine Soontjens, Chris W. Michiels, Bart M. Nicolaï and Jan F. Van Impe 2002. Predicting the combined effect of a mild heat and pulsed light treatment on the inactivation of *Botrytis cinerea* and *Monilinia fructigena*. Postharvest Unlimited, Book of Abstracts, Poster Presentation PT-8, 2p.
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F. Devlieghere, K. Francois, K.M. Vereecken, A.H. Geeraerd, J.F. Van Impe and J. Debevere, 2002. Modeling of chemical inhibition and inactivation. 1st International Conference on Microbial Risk Assessment: Foodborne Hazards, University of Maryland, Book of abstracts, Abstract 26. (*invited lecture*)
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J.F.M. Van Impe 2002. Modeling microbial evolution under dynamic conditions. 1st International Conference on Microbiological Risk Assessment: Foodborne Hazards, University of Maryland, Book of abstracts, Abstract 28. (*invited lecture*)
[1st International Conference on Microbiological Risk Assessment: Foodborne Hazards, Adelphi (USA), July 24-26, 2002]

F. Poschet, K. Bernaerts, A.H. Geeraerd, N. Scheerlinck, B.M. Nicolaï and J.F. Van Impe 2002. Investigation of the sensitivity of microbial growth parameter distributions to data quality and quantity by means of Monte Carlo analysis. 1st International Conference on Microbiological Risk Assessment: Foodborne Hazards, University of Maryland, Book of abstracts, Abstract 55.
[1st International Conference on Microbiological Risk Assessment: Foodborne Hazards, Adelphi (USA), July 24-26, 2002]

L. Vermeiren, F. Devlieghere, A. Assi, and J. Debevere, 2002. Inhibitory spectrum of bacteriocin producing lactic acid bacteria as a primary screening of their potential use as protective cultures for meat preservation. In: L. Axelsson, E.S. Tronrud and K.J. Merok (eds.), Proceedings & Abstracts of the 18th Symposium of the International Committee on Food Microbiology and Hygiene (ICFMH), 304
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F. Poschet; K.M. Vereecken, A.H. Geeraerd and J.F. Van Impe 2002. Towards a new generation of simple models for microbial growth. In: L. Axelsson, E.S. Tronrud and K.J. Merok (eds.), Proceedings & Abstracts of the 18th Symposium of the International Committee on Food Microbiology and Hygiene (ICFMH), 340
[Food Micro 2002, Eighteenth International Symposium of the International Committee on Food Microbiology and Hygiene (ICFMH), Lillehammer (Norway), August 18-23, 2002]

K. Bernaerts and J.F. Van Impe 2002. An advanced data processing protocol for optimal parameter estimation of thermal microbial growth kinetics (PART I). In: L. Axelsson, E.S. Tronrud and K.J. Merok (Eds.), Proceedings and abstracts of the 18th international ICFMH Symposium, 341
[Food Micro 2002, *Eighteenth International Symposium of the International Committee on Food Microbiology and Hygiene* (ICFMH), Lillehammer (Norway), August 18-23, 2002]

I.A.M. Swinnen, K. Bernaerts, K. Gysemans, E.J. Dens and J. Van Impe 2002. Characterisation of microbial lag induced by a sudden rise in temperature. In: L. Axelsson, E.S. Tronrud and K.J. Merok (eds.), Proceedings & Abstracts of the 18th Symposium of the International Committee on Food Microbiology and Hygiene (ICFMH), 342
[Food Micro 2002, Eighteenth International Symposium of the International Committee on Food Microbiology and Hygiene (ICFMH), Lillehammer (Norway), August 18-23, 2002]

V.P. Valdramidis, K. Bernaerts, A.H. Geeraerd and J.F. Van Impe 2002. Application of a novel procedure to quantify thermal inactivation kinetics (PART II), 343
[Food Micro 2002, *Eighteenth International Symposium of the International Committee on Food Microbiology and Hygiene* (ICFMH), Lillehammer (Norway), August 18-23, 2002]

**Other conference proceedings - abstract**

I.A.M. Swinnen, K. Bernaerts, K. Gysemans, E.J. Dens and J. Van Impe 2002. Characterisation of microbial lag induced by a sudden rise in temperature. Poster presentation. 1 p.
[IPA/PAI/IUAP-P5 *Interuniversity Pole of Attraction on Modelling, Identification, Simulation and Control of Complex Systems*, *Study Day*, Leuven, May 15, 2002]

A. Geeraerd, D. Marquenie, C. Soontjens, A. Schenk, C. Michiels, B. Nicolaï and J. Van Impe 2002. A model prototype describing the effect of a subsequent UV-C and heat treatment on the survival of conidiospores of *Botrytis cinerea*. Poster presentation. 1 p.
[IPA/PAI/IUAP-P5 *Interuniversity Pole of Attraction on Modelling, Identification, Simulation and Control of Complex Systems*, *Study Day*, Leuven, May 15, 2002]

A.R. Standaert, A.H. Geeraerd, K. Bernaerts; K. Francois, F. Devlieghere, J. Debevere and J.F. Van Impe 2002. Analysis and evaluation of a serial dilution experimental protocol by means of a simulation model. , Poster presentation. 1 p.
[IPA/PAI/IAP-P5 *Interuniversity Attraction Pole on Dynamical Systems and Control: Identification and Modelling*, Study Day, Louvain-la-Neuve (Belgium), November 26, 2002]

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[IPA/PAI/IAP-P5 *Interuniversity Attraction Pole on Dynamical Systems and Control: Identification and Modelling*, Study Day, Louvain-la-Neuve (Belgium), November 26, 2002]

## 5.3 Detailed results

Appendixes with detailed results are provided in separate documents, with the following subdivision
- Work Package 1, Task 1.1, Step 1: Appendix 1
- Work Package 1, Task 1.2, Step 1: Appendix 2
- Work Package 1, Task 1.2, Step 2: Appendix 3
- Work Package 2, Task 2.1, Step 1, Experimental plan: Appendix 4
- Work Package 2, Task 2.1, Step 1, Experimental results: Appendix 5
- Work Package 2, Task 2.1, Step 2: Appendix 6
- Work Package 3, Task 3.2, Step 3: Appendix 7
- Work Package 3, Task 3.3: Appendix 8