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Final report on progress with the already existing package modifications (if necessary)

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Summary

A number of existing software packages are available today to predict the growth/survival of spoilage and pathogenic microorganisms in/on foods. Two different approaches are being developed in the available softwares, i.e. polynomial and cardinal modular simulation tools. *Combase Predictor* polynomial simulation tool enables good fitness of fit but requires numerous polynomial parameters which have to be determined for each given conditions. While *Sym'Previus* cardinal modular simulation tool is specifically applied to food and enables the development of several moduli, modelling the influence of different factors with biological significance parameters, *i.e.* temperature, pH, water activity, organic acid.

After describing the different approaches used in the already existing predictive microbiology softwares, growth simulations are compared to growth kinetics obtained by AUA for *L. monocytogenes* artificially contaminating vanilla cream stored in different conditions (two dynamic (fluctuating) temperature profiles). Results showed that there could be certain degree of over/under-estimation of the responses of the pathogen from both softwares in the temperature scenarios assayed. In the case of *Combase Predictor* a correction factor was defined based on the bias factor (B_f) calculated index indicating the average deviation between predictions and observations in order to reduce the over-estimation of the software. The correction factor was effective in producing more realistic predictions of the pathogen growth. In parallel, *L. monocytogenes* growth kinetics and *Sym'Previus* growth simulations during food shelf-life have been compared for artificially inoculated meat-based products during storage under several dynamic temperature scenarios, i.e. with or without cold chain break. An attempt has been made to add values, when the determination of growth rate was possible, to *L. monocytogenes* kinetics provided by WP2A using *Sym'Previus* software. The differences observed between *L. monocytogenes* experimental bacterial counts and simulations could be associated to the effect of the presence of cheese consortia microbial interactions. However, simulating microbial interactions is a complex phenomenon that requires modelling of each successive step based on measurements of associated parameters (e.g., lactic acid gradients, bioactive compounds diffusion). *Sym'Previus* decision-making tool takes into account food batch and intra-species

variability via industrial control data and allows simulation of *L. monocytogenes* growth for static and dynamic conditions of pH, a_w and temperature as reported in the probabilistic approach presented in the case of *L. monocytogenes* contamination of smoked salmon. Based on the developments performed within WP3, the impact of gelatine and casein texturing agents has been integrated in the modular approach developed for *Sym'Previus* existing tool. A revised version of the software is now available; it is dedicated to research in order to further investigate the impact of food texture and microbial interactions on pathogen growth simulation.

1. Background and rationale

The concept of predictive microbiology stipulates that a detailed knowledge of microbial responses to environmental conditions enables objective evaluation of the effect of processing, distribution and storage operations on the microbiological quality and safety of foods. It involves the accumulation of knowledge on microbial behavior in foods and its implementation into mathematical models. If responses are observed under a sufficient number of combinations of the environmental factors, then it is possible to predict how the organism would respond to any conditions in the environmental region where these observations were made.

Predictive models are divided into primary, secondary and tertiary based models. *Primary models* describe the change of bacteria numbers with time under particular environmental and cultural conditions. These models are used for the calculation of kinetic parameters of growth such as maximum growth rate, generation time, lag phase duration and maximum population density. *Secondary models* are used for the quantification of the effects of various environmental factors such as temperature, pH, water activity, redox potential, for each and every parameter of growth. Two main approaches are commonly used and developed, i.e. polynomial models and modular cardinal models. Polynomial approach provides excellent goodness of fit due to the high number of parameters used for given conditions. Nevertheless, the polynomial approach presents a low robustness, which makes difficult a general approach. In contrast, modular cardinal models use parameters with biological significance such as minimal optimal and maximal values of pH, a_w, temperature, growth inhibitor concentrations (e.g., organic acids) which are the main factors having major effects on bacterial behaviour. All other minor factors are incorporated in the food matrix effect. For example, using a primary model the growth rate can be calculated for the corresponding temperature. Then, the growth rates corresponding to different temperatures are adapted in a secondary model so that the effect of temperature is expressed quantitatively with a mathematic equation allowing the user to determine the growth rate at any temperature. *Tertiary models* take modelling to its final form. These models combine primary and secondary models in a friendly software packages. The final users of such systems (e.g., the industry) are not required to have prior knowledge of microbiological techniques and mathematical expertise. Tertiary models will change predictive food microbiology into an accessible and useful tool for the industry and any other related field.

A modern quality and safety assurance system should rely on prevention rather than end product testing and control. To establish such a system, it is necessary to acquire a thorough knowledge of the relation between storage conditions and spoilage/safety, expressed in quantitative terms, i.e. effective and accurate predictive models. A number of mathematical

software packages already exist for pathogenic and spoilage bacterial growth/survival/destruction prediction such as *Combase*, *Pathogen Modelling Program* and *Sym'Previus*. *Combase Predictor* and *Pathogen modelling* program are based on the polynomial approach simulation. In contrast, *Sym'Previus* database and simulation tool is specifically applied to food and based on modular cardinal models. Generally, polynomial simulation tool enables good fitness of fit but requires numerous polynomial parameters which has to be determined for each given condition. In contrast, cardinal modular simulation tool enables the development of several moduli modelling the influence of different factors with biological significance parameters. It must be stressed that these predictive software programs are based on numerous experimental datasets expressing various combinations of environmental factors. Access to these data has become important for (i) validating the robustness of models, (ii) bringing transparency to microbial risk assessment, and (iii) advancing modelling techniques. Recent initiatives, such as the *ComBase* database (www.combase.cc), developed by the UK Institute of Food Research, Norwich, and US Department of Agriculture-Agricultural Research Service, are compiling tens of thousands of predictive microbiology data sets to describe the growth, survival, and inactivation of microorganisms, and to accelerate model development and validation. The probabilistic approach of *Sym'Previus* (www.sympREVIUS.org) developed by ADRIA Développement, Quimper, France accounts for contamination probabilities at the end of shelf-life based on industrial data. Strong attention has been brought to user-friendly software interfaces in order to assist food industrials and scientists who might not have a strong knowledge in predictive microbiology. Such decision-making tools represent a modern quality and safety assurance system complying with the EC regulation No 2073/2005 on microbial criteria for foodstuff.

The main objective of this task is to evaluate developed and existing predictive models in order to investigate the applicability of the later models to traditional European foods and propose potential improvements, if necessary.

2. Steps in the development of predictive models

There are numerous strategies in model development for the prediction of microbial growth; however, the development of mathematical models is basically oriented to the following four steps, namely (i) experimental planning, (ii) data collection and analysis, (iii) mathematical description, and (iv) validation.

Experimental planning

A number of factors should be taken into consideration in the planning of experiments and data collection, such as:

- *The selection of the independent variable(s) of the models*

Following selection of the independent variable(s) of the model, it is useful to check whether there are interactions between the examined environmental factors. It is also necessary to determine the experimental range of the variables, which depends on the specific product(s) under investigation. The choice of the range should be selected with great care since a mathematical model will not give good predictions outside the initially defined range.

- *Response variable*

The response variable of concern is usually the change in bacterial population density over time, expressed as growth rate, generation time, lag time, etc. This variable is further related to environmental factors (e.g. pH, a_w, temperature, etc.) in secondary modelling.

- *The preparation of inoculum*

For the preparation of the inoculum different factors should be considered, including: (i) the conditions of inoculum pre-culture (e.g. temperature, pH, a_w, etc.) that could influence the later growth stages, particularly lag time, (ii) the size of the inoculum, (iii) mono or co-culture, and (iv) preparation of the inoculum for application to foods or laboratory media.

- *Growth medium*

Great attention should also be given to the growth medium. This can be either a laboratory medium or some type of food. The use of laboratory media allows the application of a wider range of monitoring methods for the determination of microbial growth (optical density, impedance etc.) and the generation of a plethora of data within short-time intervals. However, in the last few years there has been some criticism on the use of laboratory media for the development of mathematical models. This is due to the fact that laboratory media do not take into consideration important factors that influence microbial growth, such as the food matrix and the interactions with the indigenous microflora.

- *The quantity of data*

There is a minimum amount of data required to generate a reliable mathematical model. Usually ten to fifteen data points per independent variable are enough to fit a kinetic model. It is also essential to have an even distribution of data points in all the phases of the growth curve.

Data collection and analysis

The collection of suitable data is very important for the development of mathematical models. Kinetic models require the determination of the growth rate of the microorganism under investigation. Since there is no direct way for the calculation of the growth rate, the determination of microbial population over time is essential. Various methods can be used; these can be direct, when the cells are counted (e.g. viable counting, microscopy) or indirect, when the microbial density is determined via quantification of certain attributes of the cells, e.g. DNA, dry weight, optical density, or impedance.

Mathematic description of models

After data collection, a sigmoid equation is fitted and the kinetic parameters of growth are estimated (generation time, lag time, etc.). These values are then used for the development of models that describe the effect of various environmental factors (e.g. temperature, pH, water activity, etc.) on microbial growth.

In order to determine the equation that best describes the data set collected, regression techniques are implemented taking into consideration the standard criteria for regression analysis, which are:

- the precision of adaptation
- the ability to forecast combinations of factors that have not been examined
- the incorporation of all relative factors
- the utilization of the least possible parameters
- the determination of prediction error
- the biological meaning and realistic values of parameter estimates
- the mathematical modification (transformation) of parameters if this improves their statistical attributes.

Model validation

Model evaluation is a very important stage in the overall process. Developed models should be evaluated under real conditions to show whether microorganisms behave in a similar way to that predicted using the mathematical models for the same conditions. Users of certain models should be aware of the performance limits as well as the applicability range of the model. In practice, the precision of a model does not depend on the quality of data fitting but on its ability to simulate the microbial growth. The stage of model evaluation has often been characterized as the weakest point in the process of model development as there are no standard methods for the

evaluation. However, certain indicators are widely employed to determine the predictive potential of models, namely the bias (B_f) and the accuracy (A_f) factors. The bias factor provides indication for the mean variation between the predicted and observed values, whereas the accuracy factor provides an indication of the size of variation between predicted and observed values.

3. Combase Predictor 2.0

ComBase Predictor is a ready-to-use predictive microbiology tool that comprises a set of new models predicting the response of the organisms as a function of the environmental factors, including temperature, pH, and water activity. Some models also include an additional, fourth factor, such as the concentration of carbon dioxide or acetic acid. Survival and death models are also included in *ComBase Predictor. ComBase Predictor* has a number of notable features:

- it can be used to make predictions in either a static temperature or under fluctuating (changing) temperature conditions
- it can provide up to four simultaneous predictions
- it includes growth and survival curves as well as thermal and non-thermal death curves

Table 1. Summary of raw datasets used to generate *ComBase Predictor*

Growth	(3 factors*)		(4th factor**)	
	No. of curves in the initial set	**No. of newly included curves**	**No. of curves in initial set**	**No. of newly included**
Aeromonas hydrophila	125	**33**	**-**	**-**
Bacillus cereus with CO_2 (%)	86	**109**	58	-
Bacillus licheniformis	53	-	-	-
Bacillus subtilis	68	-	-	-
Clostridium botulinum (non-prot.)	52	-	-	-
Clostridium botulinum (prot.)	77	-	-	-
Clostridium perfringens	51	-	-	-
Escherichia coli with CO_2 (%)	80	**89**	78	-
Listeria monocyto genes / innocua with CO_2 (%)	251	**279**	75	**9**
Listeria monocytogenes / innocua with nitrite (ppm)	251	**279**	63	**118**
Listeria monocytogenes / innocua with lactic (ppm)	251	**279**	129	-
Listeria monocyto genes / innocua with acetic (ppm)	251	**279**	52	-
Staphylococcus aureus	93	-	-	-
Salmonellae with CO_2 (%)	146	**113**	23	-
Salmonellae with nitrite (ppm)	146	**113**	28	-
Shigella flexneri with nitrite (ppm)	**-**	**193**	**-**	**125**
Yersinia enterocolitica with CO_2 (%)	282	**35**	31	-
Yersinia enterocolitica with lactic (ppm)	282	**31**	77	-
Brochothrix thermosphacta	44	-	-	-
Pseudomonas spp	**-**	**144**	**-**	**-**

* 3 factors: temperature, pH and water activity

** 4 factors: additional factor to the above (either carbon dioxide, lactic acid, acetic acid or nitrite)

How to use Combase

Registration

Before using *ComBase Predictor,* registration is necessary to use the ***ComBase Modelling Toolbox,*** which is a collection of Internet-based, freely available predictive microbiology software tools (Figure 1).

Figure 1. Registration form of *Combase*

Once the user's account has been created, the login process must be followed by typing the user's email address and password as shown in Figure 2. Before clicking on the 'Login' button, you need to accept the terms and conditions by clicking on the checkbox.

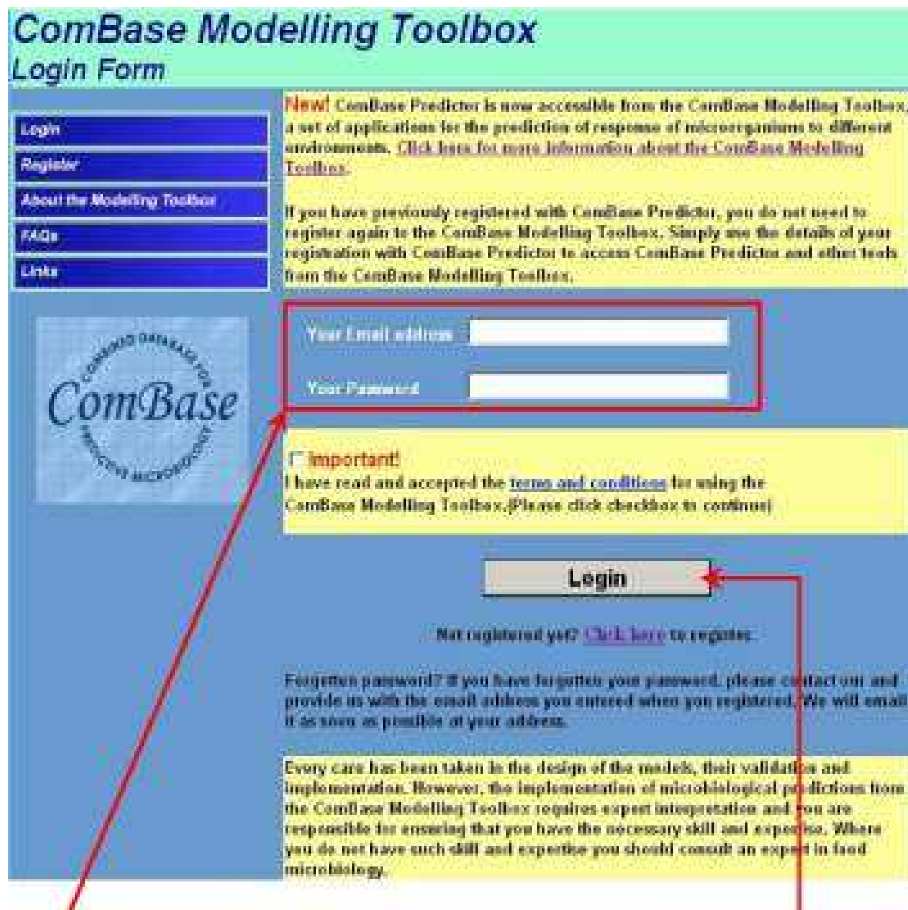
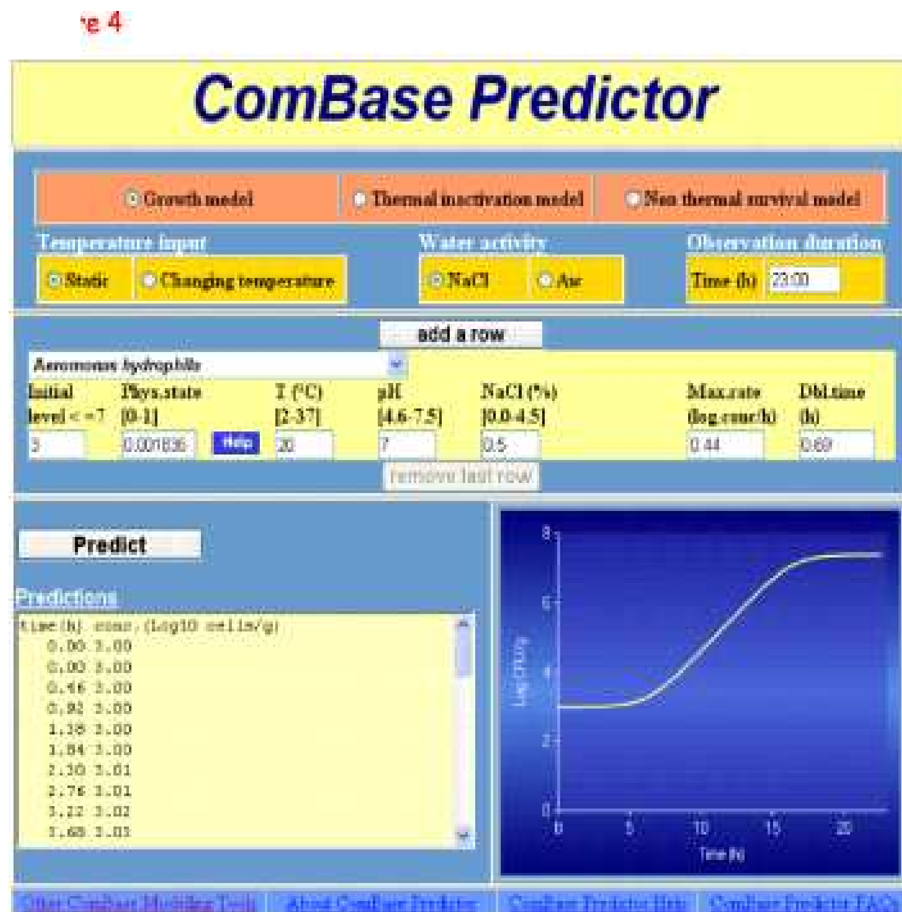


Figure 2. Login page of *Combase*

Login opens the *Combase Predictive Tools* page (Figure 3).

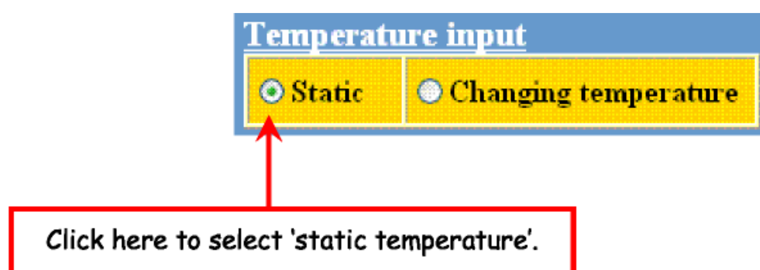
The *Combase Predictor* page is assessed by selecting the ‘Combase Predictor’ link. The general layout of the page is the following:



Using *Combase Predictor*

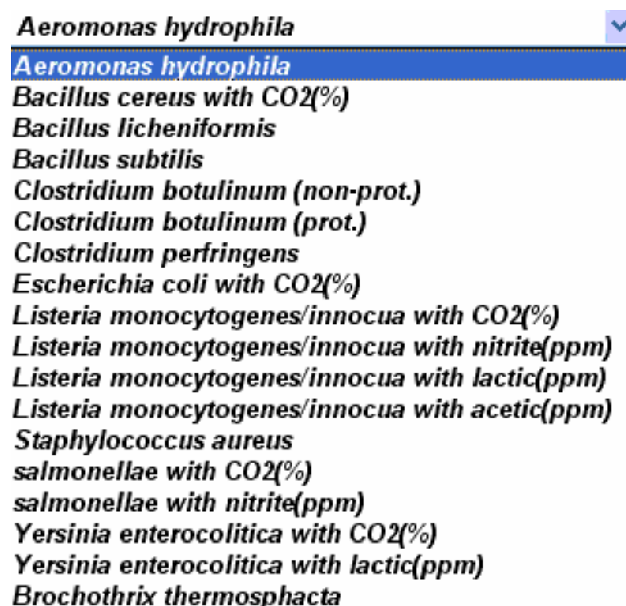
Generating a single prediction at static temperature

The first step in producing a prediction is to select ‘static temperature’ in the temperature input field.



Select the model category: select 'growth model' for prediction of bacterial growth, 'thermal death model' for prediction of thermal inactivation, or ‘non thermal inactivation’.

Select the required model: The next step is to select a model. A drop-down menu of models included in *ComBase Predictor* can be viewed in the ‘select a model’ list box. Click on the arrow to view the full list. The model required for the organism of interest can then be highlighted and selected from the menu.



Many of the models encompass three environmental factors (temperature, pH and water activity). Some models also have an additional fourth environmental factor e.g. CO_2 or lactic acid. One basic principle of empirical modelling is that one should not extrapolate: predictions should not be made outside the region of observations. The limits of the selected model for each environmental factor appear below the input fields.

Input environmental factor values: Default values for the environmental factors (temperature, pH, water activity, etc) automatically appear in each of the input fields. Water activity can be expressed in terms of sodium chloride (%NaCl) or water activity. Water activity can be calculated from NaCl concentration by toggling between NaCl and Aw buttons (it is assumed that the salt is dissolved in water).

Use this box to express water activity in terms of sodium chloride or water activity.

Default values should be replaced by values of interest to you. The range within which input values must fall is indicated beside the input field for each of the factors. If values outside the range are selected, an error message will be generated.

Listeria monocytogenes/innocua with CO2(%)

Initial level < =7	Phys.state [0-1]		T (ºC) [1-35]	pH [4.4-7.5]	NaCl (%) [0.0-10.2]	CO2(%) [0-100]
3	0.019255	Help	5	5	1.5	0

Enter here the values of interest for temperature, pH and a_w. The range of the model is indicated above the textboxes.

A value for the 4th factor (e.g CO_2 or nitrite) may be entered here if a 4-factor model is selected.

When a four-factor model has been selected, values are also required for the 'Factor 4' field. If preferred, the fields ‘Initial level’ (initial cell count expressed as $\log_{10}$ cfu/ml), ‘Phys. state’ (the physiological state of the cells expressed in terms of a value between 0 and 1) and ‘Time (h)’ (desired duration of observation) may be left empty; default values will then be inserted automatically by the program. The default value for the initial count is $\log_{10}$cell concentration = 3 (i.e. 10^3 cells/ml). For the physiological state, the default value is the value which was typical for the curves providing the base for the models. It is not possible to select ‘initial level’ values for thermal death models as results are provided as a relative decrease in log concentration.

To get a prediction: When all required values for the environmental factors have been satisfactorily selected, click ‘Predict’.

The prediction output: The right-hand output panel now shows a graphical representation of the prediction. A statement of maximum growth rate and doubling time (or maximum death rate and D-value) are presented next to the input field.

Max.rate (log.conc/h)	Dbl.time (h)
0.01	34.99

Additionally, each of the time vs. cell concentration data points for the prediction are listed below the graph and may be selected and copied for use in other application (e.g. Excel).

Predictions

time (h)	conc. (Log10 cells/g)
0.00	3.00
0.00	3.00
23.22	3.00
46.44	3.01
69.66	3.02
92.88	3.04
116.10	3.07
139.32	3.11
162.54	3.17
185.76	3.24

Generating a single prediction under fluctuating temperature

ComBase Predictor not only allows prediction to be made for static temperature conditions but also predictions under certain fluctuating (changing) temperature conditions. Note that this facility is available only for growth and thermal death models but not for non thermal inactivation model. Predictions could be made for example from data representing the fluctuating time vs. temperature profile expected or measured for the following processes in sequence: the later stages of cooling, storage within the factory, storage during transportation, storage at retail premises, possible purchaser temperature abuse followed by domestic storage for the organisms *Listeria monocytogenes* and nonproteolytic *Clostridium botulinum* using the appropriate growth models. This 'fluctuating temperature' feature may also be useful to demonstrate the effect of a thermal process using logged data which fall within the relevant temperature range of the thermal death model.

Select 'changing temperature': Click the 'changing temperature' button. This will deselect 'static temperature' and generate the 'changing temperature' page.

Select the required model: Select the required model as described for static temperature predictions.

Input environmental factor values: Values for environmental factors for ‘changing temperature’ predictions should be entered as described for ‘static temperature’ predictions except for the values pertaining to temperature. To make predictions for ‘changing temperature’ situations, a time vs. temperature profile must be entered as the temperature parameter.

The time/temperature profile should be entered in the textbox on the 'changing temperature' page in the format illustrated and described below:

Format requirements:

1. Only the numeric characters (0-9), tabulation, space, and return carriage are allowed in the textbox.
2. The records must be recorded in chronological order.
3. The first time-point must be '0'.
4. The last time point must be less than or equal to ‘5000' hours.

For each time temperature record of your profile, time is to be entered first (in hours) then temperature (in °C). The 'point' symbol "." must be used as the decimal separator. The profile can be typed directly into the ‘changing temperature' textbox as follows: value, space, value, return, (repeat for each line). Alternatively, the required data may be copied from another application (e.g. Excel spreadsheet, text file) and pasted directly into the text box. Note also that all temperature values must be within the range for the models selected and that there must be a minimum of 4 and a maximum of 100 (time vs. temperature) records in the profile input.

To get a prediction: When all the required values and the temperature profile have been satisfactorily selected, click ‘Predict’. If some input data are incorrect an error message will appear. To continue with the prediction replace unacceptable data with data within the accepted range.

The prediction output: The right-hand output panel now shows time vs. cell concentration data for the given prediction (white) alongside the selected time vs. temperature profile (green). No statements of maximum growth rate or doubling time are provided as these fluctuate according to the time vs. temperature input. However, cell concentration data points are provided over time in a graph that may be selected and copied for use in other applications.

Generating multiple predictions at fluctuating temperature

This process is performed in a similar manner to that already described. This facility can be used to compare organisms or key parameters for a single fluctuating temperature profile. It is not possible to make predictions when the temperature ranges of the models do not overlap.

4. *Sym'Previus* decision making tool

The *Sym'Previus* network started in 1999 with the aim to propose an assistance in food safety management for agrofood industries. *Sym'Previus* decision making tool is composed of 2 complementary softwares. Firstly, a database gathers, in a structured way, information on the behaviour of microorganisms in/on foods as well as natural food contamination. Specific interrogation of this database for given food and microorganisms is possible via MIEL query system which has been specifically developed for *Sym'Previus* database. Secondly, a user-friendly simulation software estimates microorganism growth in food matrix and then provide simple access to predictive microbiology for food companies. Even though growth, inactivation, and heat destruction simulation are available in *Sym'Previus*, only growth simulation was studied in Truefood. Moreover, within WP3 of TrueFood project, the influence of texture and bacterial interactions on microorganism growth was studied. Implementation of *Sym'Previus* decision-making tool with characteristic parameters of food texture will then enable microorganisms' growth simulation in food.

The main advantage of models used in *Sym'Previus* is to be suited to food products, i.e. from a growth kinetic determined in food, *Sym'Previus* estimates μ_{opt}, characteristic parameter linked to a given association of microorganism/food. It is then possible to simulate growth for other static or dynamic conditions of temperature, pH or water activity without the need of more challenge tests in food. Furthermore, up to 12 bacterial strains are registered for the main pathogens such as *L. monocytogenes*, *Salmonella*, *B. cereus* and *E. coli* yielding simulation taking into account biological variability for a same species.

Sym'Previus database contains growth, survival, and thermal destruction kinetics obtained for different foodstuff categories that could be contaminated by major pathogens and spoilage bacteria. Data are mainly bibliographic and are progressively enriched by data from national and international research programmes. This database can be accessed using an interrogation module that is used to make structured requests on the foodstuff and micro-organism (Figure 4). The data collected are used to estimate foodstuff's or micro-organism's characteristics in terms of the potential for growth. The different calculation modules can then be used to simulate the development or destruction of a given microorganism in a particular foodstuff.

Figure 4. Example of results shown as graphs or tables that can directly be exported to MS Excel sheet

Several moduli are already proposed in *Sym'Previus* decision making tool (Table 2) to simulate pathogenic and spoilage bacterial growth considering environmental parameters as well as biological factors. It needs to be noted that the probabilistic approach includes the latest knowledge on bacterial physiological state including the influence of lag phase and food process stress (Guillier and Augustin 2006).

Table 2. List of the different moduli available in *Sym'Previus* simulation tool

Sym'Previus existing moduli	Example of application
Probabilistic approach	Simulation of the evolution of a microbial contamination throughout food product shelf-life, yielding the probability of exceeding a critical threshold at different stages of its shelf-life.

Sym’Previus existing moduli	Example of application
HACCP assistance	Quantification of the effect of each process step on growth or destruction of specific bacterial species allowing the identification of critical stages.
Thermal destruction simulation	Bacterial thermal destruction kinetics simulation for given bacterial species taking into account bacterial thermal resistance and the influence of various environmental factors (pH, temperature, a_w).
Non thermal inactivation	Destruction kinetics simulation when environmental conditions are not favourable for given bacterial species, for instance in the case of fermented or salted food products.
Growth/no growth boundaries	Determination of the combinations of environmental factors (temperature, pH, a_w, lactic acid) that represent the limits of growth/no growth for a given bacterial species.
Growth curve fitting	Growth model fitting using a given set of experimental data (challenge test) that allow the determination of growth rate, lag time, initial and maximum population size.
Growth simulation	Growth simulation taking into account bacterial cardinal values, food matrix and the main environmental factors encountered in a given food sample.

Concept and Models

Predictive microbiology can be divided into two groups of models, namely primary and secondary models. Primary models describe the evolution of the bacterial population with time. In the 1990’s, new models appeared in predictive microbiology. The empiric models were progressively re-parameterised or replaced by new models in which all parameters had a physical or biological significance. Baranyi *et al.* (1993), and Rosso (1995, equation 1) introduced new models to describe growth curves. The results were more accurate and all parameters had a physical meaning. The primary model proposed by Rosso is described below:

Equation 1:

$$\begin{cases} \dfrac{dx}{dt} = 0 \ ; \ x = x_0 & \text{if } t \le lag \\ \dfrac{dx}{dt} = \mu_{\max}\left(1 - \dfrac{x}{x_{\max}}\right) & \text{if } t > lag \end{cases}$$

where x_0 and x_{max} are respectively the initial and the maximum population in the medium, lag is the time before initiation of growth, and μ_{max} is the standard growth rate.

Secondary models describe how primary model parameters vary with environmental factors. A family of secondary models was proposed by Rosso *et al.* (1993, 1995) who introduced the « cardinal » models:

$$g(X,\Theta_2) = \sqrt{\mu_{opt}(X)\cdot CM_n(X)}$$

$$CM_n(X) = \begin{cases} 0 & , X \leq X_{min} \\ \dfrac{(X - X_{max})\cdot(X - X_{min})^n}{(X_{opt} - X_{min})^{n-1}\cdot\left[(X_{opt} - X_{min})\cdot(X - X_{opt}) - (X_{opt} - X_{max})\cdot((n-1)\cdot X_{opt} + X_{min} - n\cdot X)\right]} & , X_{min} < X < X_{max} \\ 0 & , X \geq X_{max} \end{cases}$$

Where $\Theta_2 = \left(\mu_{opt}(X),\, X_{min},\, X_{opt},\, X_{max}\right)$

And X : the environmental factor

X_{min} : lower limit for growth for X factor

X_{opt} : value at which growth is maximal for X factor

X_{max} : upper limit for growth for X factor

n : shape parameter

Applied to the gamma concept proposed by Zwietering *et al.* (1992), these models describe with a satisfactory accuracy the growth rate versus several environmental factors such as temperature, pH and water activity.

Augustin *et al*. (2005) proposed a model (a cardinal model including interactions) to predict the growth probability in any food products (dairy, meat and seafood products). The growth probability includes on the one hand, the effect of food matrix (i.e., all physico-chemical factors which are not taken into account in the model) and on the other hand, the microbiological variability. This model was fitted and validated with *Listeria monocytogenes* data.

Similarly, *Sym'Previus* extends this model to other microorganisms (*Salmonella*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Clostridium perfringens*, *Clostridium botulinum*), for 4 environmental factors (temperature, pH, water activity and organic acids).

$$\sqrt{\mu_{max}} = \sqrt{\mu_{opt}\cdot\gamma_T\cdot\gamma_{pH}\cdot\gamma_{aw}\cdot\gamma_{interaction}}$$

Because cardinal values are independent from growth medium, these physiological parameters are estimated in broth. To appreciate the intraspecific variability, the behaviour of many strains needs to be studied. For instance, parameters of twelve *Listeria monocytogenes* have been acquired by *Sym'Previus* labs to calculate the associated confidence band which includes biological variability.

Including food matrix in growth simulation

The main advantage of models used in *Sym'Previus* is the applicability to specific food products, i.e. from a growth kinetic determined in food, *Sym'Previus* estimates μ_{opt}, a characteristic parameter associated with a given association of microorganism/food. It is then possible to simulate growth for other conditions of temperature, pH or water activity without the need of more challenge tests in food (Figure 5).

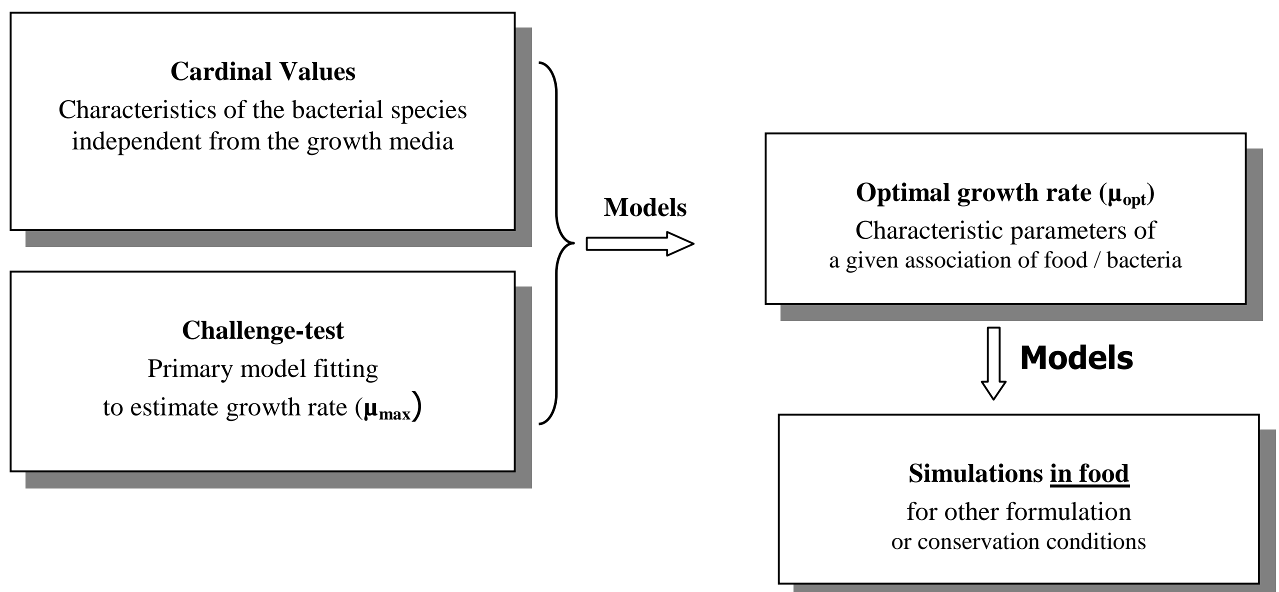


Figure 5. Microbial growth simulations according to *Sym'Previus* models taking food matrix into account.

Data acquired during TrueFood project contributed to the enrichment of the database. New models and new microbiological parameters, proposed and validated during the project allowed for a development of this simulation software, in particular prediction of the impact of texture on *L. monocytogenes* growth.

5. Application of the *Combase Predictor* in determining the growth profile of *Bacillus cereus* in pasteurized vanilla cream.

Materials and Methods

Pasteurized vanilla cream samples were collected from the manufacturer and stored in isothermal conditions (5, 10, 15, and 20°C in high precision temperature incubators). Throughout preservation, samples were taken at appropriate time intervals to allow for an efficient kinetic analysis of *Bacillus cereus* which was determined as the specific spoilage organism for this commodity.

Microbiological analyses

Samples (25g) of the cream were aseptically weighed, added to ¼ strength Ringer's solution (225ml), and homogenized in a stomacher (Lab Blender 400, Seward Medical, London) for 60s at room temperature. Decimal dilutions in quarter strength Ringer's solution were prepared and duplicate 0.1 ml samples of appropriate dilutions were poured or spread on Baird-Parker-agar (BP, MERCK code1.05406 supplemented with egg yolk code 1.03785) incubated at 37°C for 2 days. All plates were examined visually for typical colony types and morphological characteristics that were associated with each growth medium. In addition, the selectivity of each medium was checked routinely by Gram staining and microscopic examination of smears prepared from randomly selected colonies obtained from all media.

Results

The output of *Combase Predictor* for the growth profile of *B. cereus* at 15 and 20°C is presented in Figures 6 and 7. At 5 and 10°C slow or no growth was observed throughout storage, so no modelling approach was followed at these two temperatures.

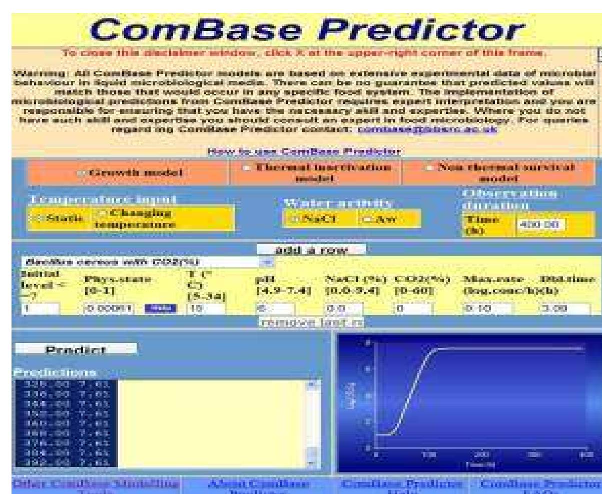


Figure 6. *Combase Predictor* output for the growth of *B. cereus* in vanilla cream stored at 15°C.

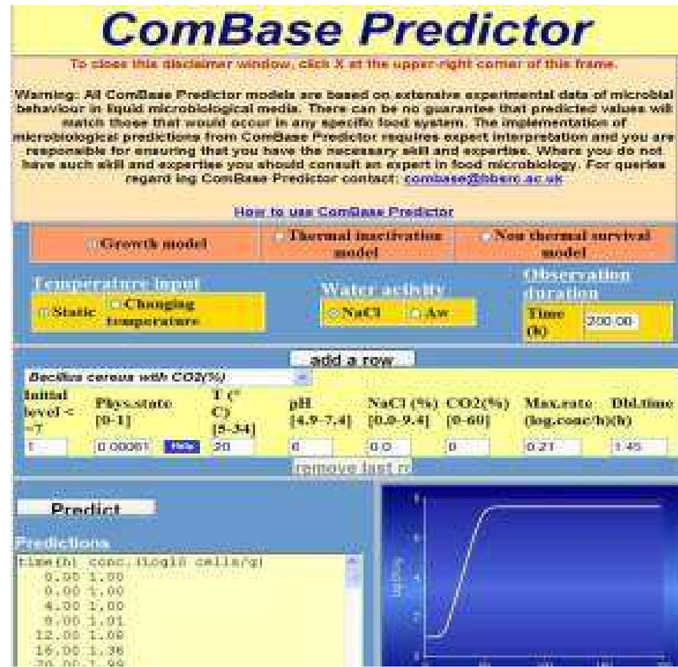


Figure 7. *Combase Predictor* output for the growth profile of *B. cereus* in vanilla cream stored at 20°C.

The obtained profile from *Combase Predictor* was compared with the profile obtained by the equation of Baranyi. An explicit version of the model is the following:

$$y(t) = y_0 + \mu_{\max} A(t) - \frac{1}{m} \ln\left(1 + \frac{e^{m\mu_{\max} A(t)} - 1}{e^{m(y_{\max} - y_0)}}\right) \tag{1}$$

$$A(t) = t + \frac{1}{\nu} \ln\left(\frac{e^{-\nu t} + q_o}{1 + q_o}\right) \tag{2}$$

where μ_{max} is the maximum specific growth/inactivation rate of cell population, y_0 and $y_{\max}$ are the initial and final cell populations, respectively, $A(t)$ is a delayed time variable (lag phase), ν is the rate of increase of the so-called 'limiting substrate', m is a curvature parameter, and q_o is a measure of the initial physiological state of the cells. The results of the simulation are presented in Figure 8.

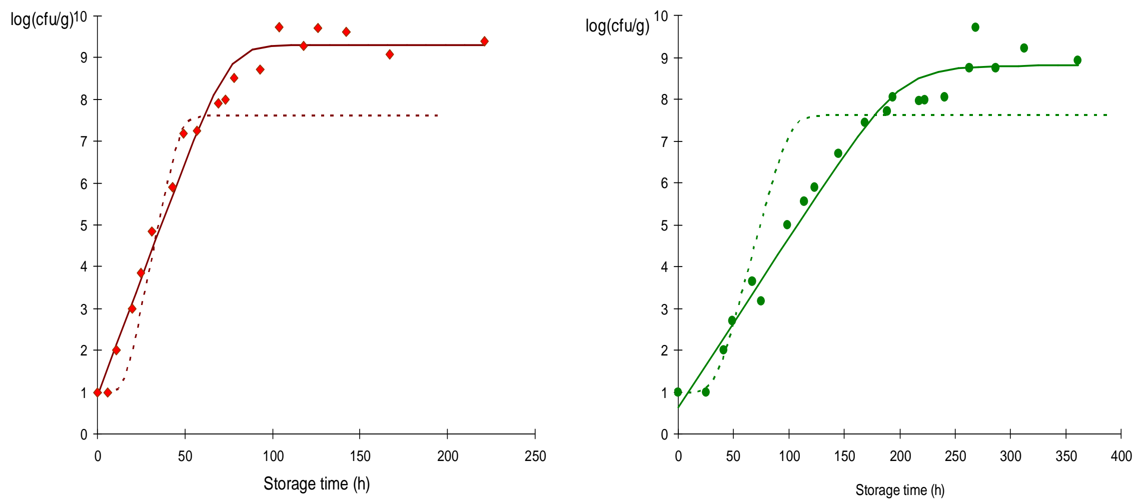


Figure 8. Growth profile of *B. cereus* at 20°C (red lines) and 15°C (green lines) simulated by the Baranyi equation (solid line) and *Combase Predictor* (dashed line).

In both cases, *Growth Predictor* could not provide realistic responses of the pathogen as it under-estimated the maximum population at the stationary phase. In addition, at 15°C there was an over-estimation of the pathogen's counts at the exponential phase after 50 hours of storage. It is also characteristic that at both temperatures *Growth Predictor* could not provide an estimation of the lag phase of the microorganism that was evident from the experimental data.

6. Comparison of on-line predictive modelling platforms of *Combase Predictor* and *Sym'Previus* to determine the responses of *Listeria monocytogenes* in pasteurized vanilla cream under dynamic (fluctuating) temperature profiles.

Materials and methods

Packages (170 g) of commercially prepared vanilla cream, obtained directly from the manufacturer, were inoculated with a four-strain cocktail of *L. monocytogenes.* The cocktail was prepared by combining individual cultures in sterile tubes followed by centrifugation at 6000 rpm for 30 min at 4°C. The resulting pellet was washed with sterile Ringer's solution, re-centrifuged and finally re-suspended in the same diluent to a final volume of 5 mL. The cell concentration in the resulting composite inoculum was *ca.* 8 log cfu ml^{-1}, assessed with a Neubauer counting chamber (Brand, Wertheim, Germany) and served as the inoculum for the experiments. A volume of 200 μL of appropriately diluted culture was added on the surface of the cream in each package to obtain a target initial inoculum of *ca.* 2 log cfu g^{-1} as determined by preliminary trials. To ensure uniform distribution, the inoculum was spread over the surface of the cream by means of a sterile spatula. Packages were stored under two fluctuating temperature scenarios, namely (a) 12 h at 4°C, 6 h at 8°C and 12 h at 15°C, and (b) 12 h at 4°C and 12 h at 12°C. Data loggers were placed in each chamber to record the temperature every 30 min.

At appropriate time intervals, duplicate samples were analyzed to allow for efficient kinetic analysis of microbial growth. For enumeration of *L. monocytogenes,* 25-g vanilla cream samples were transferred aseptically in a stomacher bag and 225 mL of sterile quarter-strength Ringer's solution were added. The mixture was homogenized for 60 s at room temperature (*ca.* 20°C) in a stomacher. Further decimal dilutions were prepared with the same diluent, and duplicate 0.1-mL samples of three appropriate dilutions were spread in triplicate on the following agar media: Plate Count Agar for total viable counts, incubated at 25°C for 48h, and *Listeria* PALCAM agar base for enumeration of *Listeriae*, incubated at 30°C for 48 h. Growth data from plate counts were log transformed.

The observed growth of the pathogen under dynamic temperature conditions was compared with the simulation provided by the *Growth Predictor* of *Combase* (www.combase.cc) and the *Sym'Previus* simulation tool (www.symprevius.org). Both softwares have the ability to provide predictions for microbial growth under fluctuating temperature conditions. For factors other than temperature, the pH was set to 6.7, based on initial values measured in the cream. The values of other factors such as sodium nitrite or CO_2 concentration included in the *Growth Predictor* were set equal to zero. Comparison of prediction was based on the bias factor (B_f), the accuracy factor (A_f) and the goodness-of-fit index (GoF) (Mataragas *et al.* 2006, Ross 1996). The performance of

simulation was also graphically illustrated by the percent relative error (%RE) index (Xanthiakos *et al.* 2006).

Results

The simulation of the growth of the pathogen on vanilla cream under dynamic temperature conditions, based on *Growth Predictor* and *Sym'Previus* is presented in Figures 9 and 10, whereas the values of the relevant performance indices are summarized in Table 3.

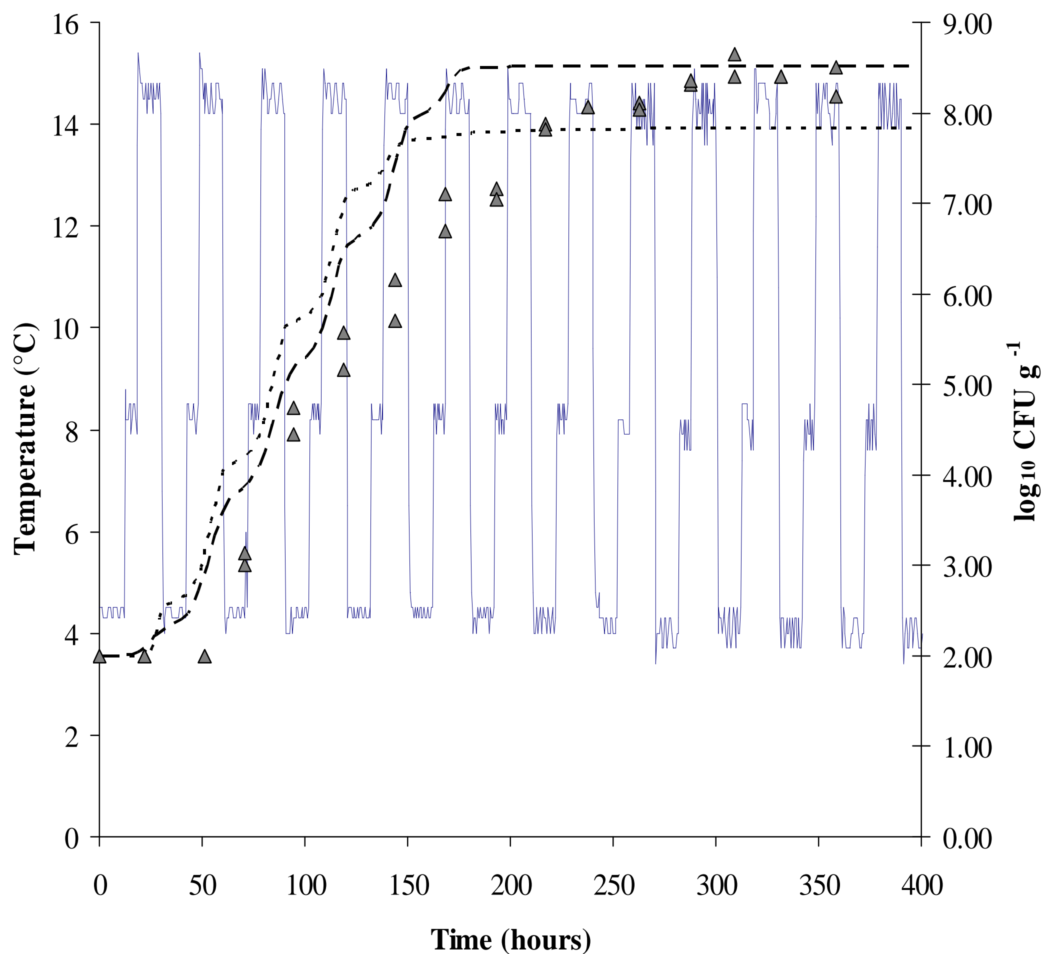


Figure 9. Comparison between observed (points) and predicted (lines) growth of *Listeria monocytogenes* in vanilla cream stored under periodically changing temperature profile (12 h at 4°C, 6 h at 8°C and 12 h at 15°C). Lines correspond to *Growth Predictor* (dashed line) and *Sym'Previus* (dotted line) software.

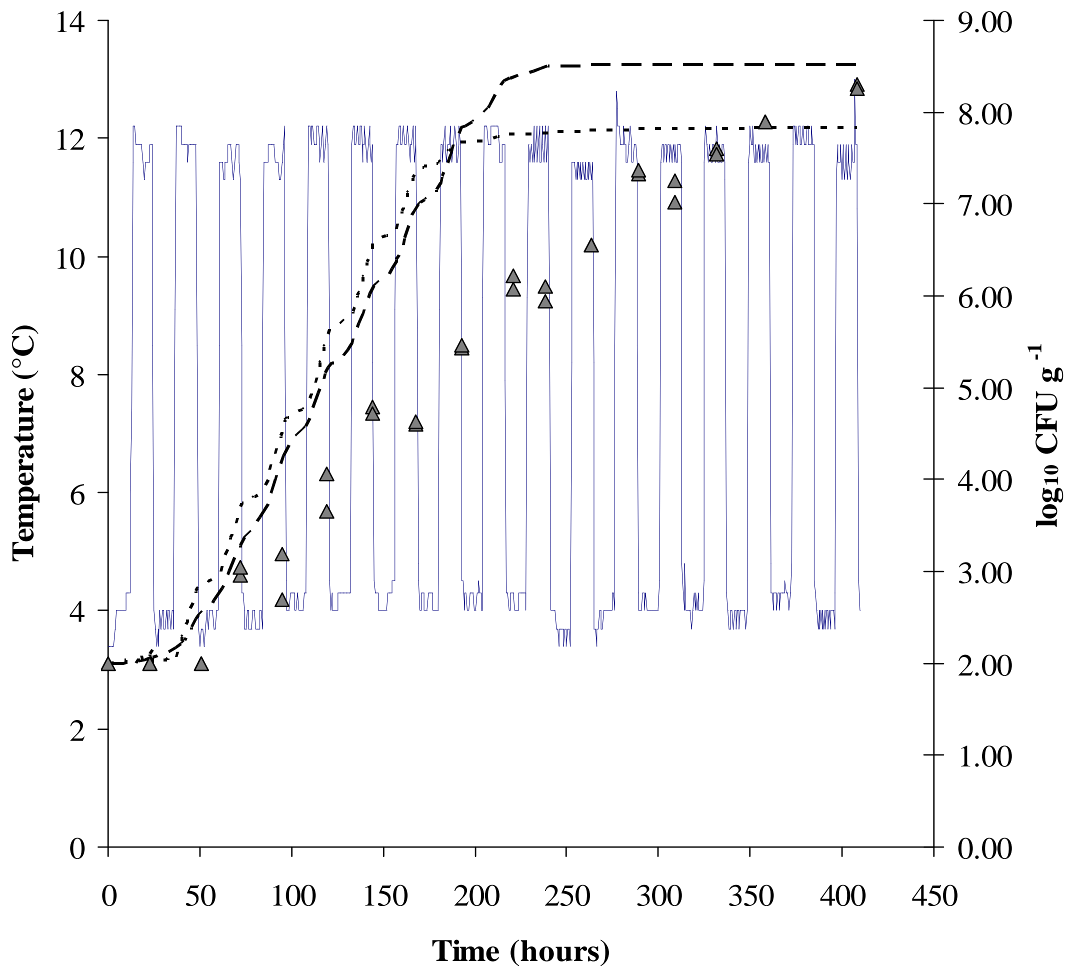


Figure 10. Comparison between observed (points) and predicted (lines) growth of *Listeria monocytogenes* in vanilla cream stored under periodically changing temperature profile (12 h at 4°C and 12 h at 12°C). Lines correspond to *Growth Predictor* (dashed line) and *Sym'Previus* (dotted line).

The average difference between predictions and observations for the *Growth Predictor* was 11.9 and 22.8% for the three and two step dynamic temperature shift, whereas for *Sym'Previus* the values were 8.9 and 21.5%, respectively, as indicated by the values of the accuracy factor (A_f). The performance of both models is also graphically presented in Figure 11. The percent relative error values fall within the ± 20% zone for 73 and 40.6% of predictions made by *Growth Predictor*, and 66.7 and 43.7% of predictions made by *Sym'Previus*, for the three and two step dynamic temperature shift, respectively.

Table 3. Comparison of validation indices (B_f, A_f, *GoF*) between *Growth Predictor* and *Sym'Previus* for the growth of *Listeria monocytogenes* under periodically changing temperature profiles.

Model	B_f	A_f	*GoF*
Growth Predictor			
12 h at 4°C, 6 h at 8°C and 12 h at 15°C	1.118	1.119	0.821
12 h at 4°C and 12 h at 12°C	1.228	1.228	1.503
Sym'Previus			
12 h at 4°C, 6 h at 8°C and 12 h at 15°C	1.089	1.137	0.864
12 h at 4°C and 12 h at 12°C	1.215	1.224	1.335

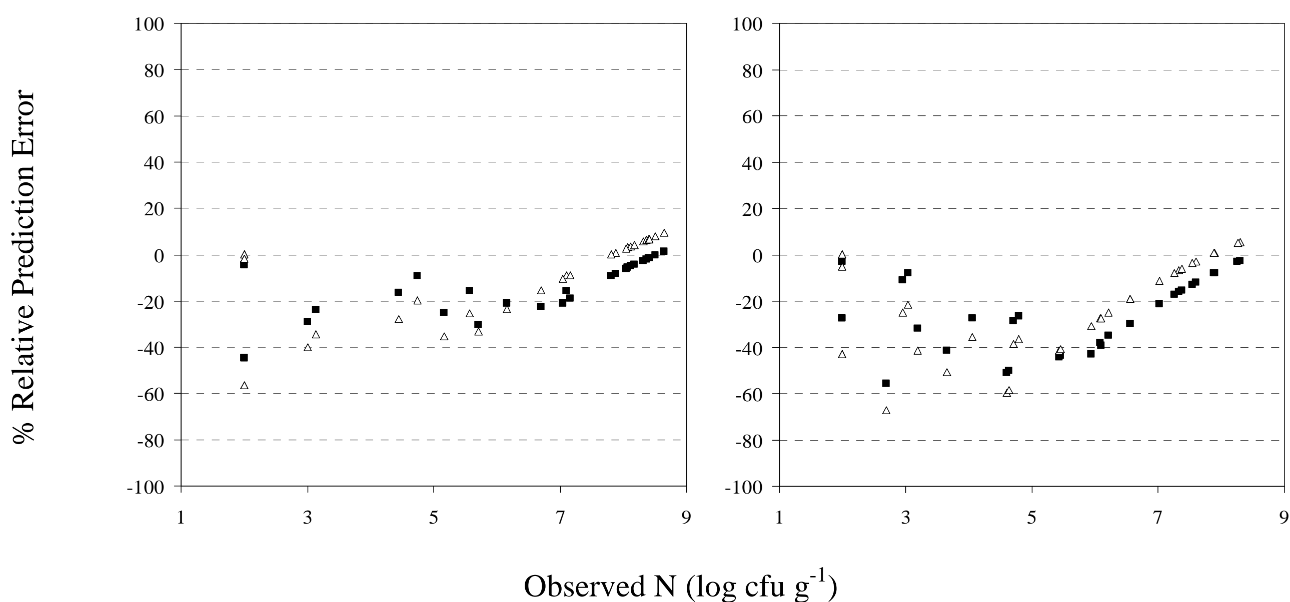


Figure 11. Percent relative prediction errors between observed and predicted growth of *Listeria monocytogenes* in pasteurized vanilla cream during storage at different periodically changing temperature profiles (a: 12 h at 4°C, 6 h at 8°C and 12 h at 15°C; b: 12 h at 4°C and 12 h at 12°C). Data points correspond to *Growth Predictor* (■) and *Sym'Previus* (△).

7. Improvement of performance of *Combase Predictor*

It can be concluded from Figures 9 and 10 that *Combase Predictor* overestimated the growth of the pathogen in the samples of pasteurized vanilla cream. Ross (1996) has proposed a simple index of the performance of models in predictive microbiology by evaluating the level of confidence one can have in the predictions of the model and whether the model displays any bias which could lead to fail dangerous predictions. The index used in this work to improve the performance of *Combase Predictor* and produce more realistic predictions is the bias factor (B_f) index. This index indicates whether, on average, the observed values lie above the line of equivalence ($y = x$) and, if so, by how much. It provides the structural deviation of the model and it is calculated by the following formula:

$$Bf = 10^{\left(\frac{\sum \log\left(\frac{\log N(t)_{predicted}}{\log N(t)_{observed}}\right)}{n}\right)} \qquad (3)$$

where n is the number of observations. A bias factor <1 indicates a “fail-safe” model, i.e., observed values are larger than predicted ones, such that predicted values provide a margin of safety. Therefore growth rate predictions were corrected using the following formula:

$$\mu_{\max}(vanilla) = \frac{\mu_{\max}(CP)}{B_f} \qquad (4)$$

where μ_{max}(*vanilla*) is the growth rate predicted in vanilla cream specific model, μ_{max} (CP) is the growth rate predicted by the *Combase Predictor,* and B_f is the bias factor. To facilitate calculations, a spreadsheet was developed on Excel in which the user must input the initial pH and water activity of the cream, the initial concentration, and the initial physiological state (α_0) of the bacterial population. The time/temperature profile should also be inserted in the appropriate field. The user can also choose between performing predictions in broth (initial model) and applying the corrective factor proposed above. The system of equations of Baranyi and Roberts was solved numerically by programming a fourth order Runge-Kutta method.

Results indicated that in the case of the three-step dynamic temperature shift (Figure 12), the corrected model was able to improve the prediction performance, however in the two step temperature shift (Figure 13), there was still over-estimation of the pathogen’s counts although the degree of over-estimation has been reduced.

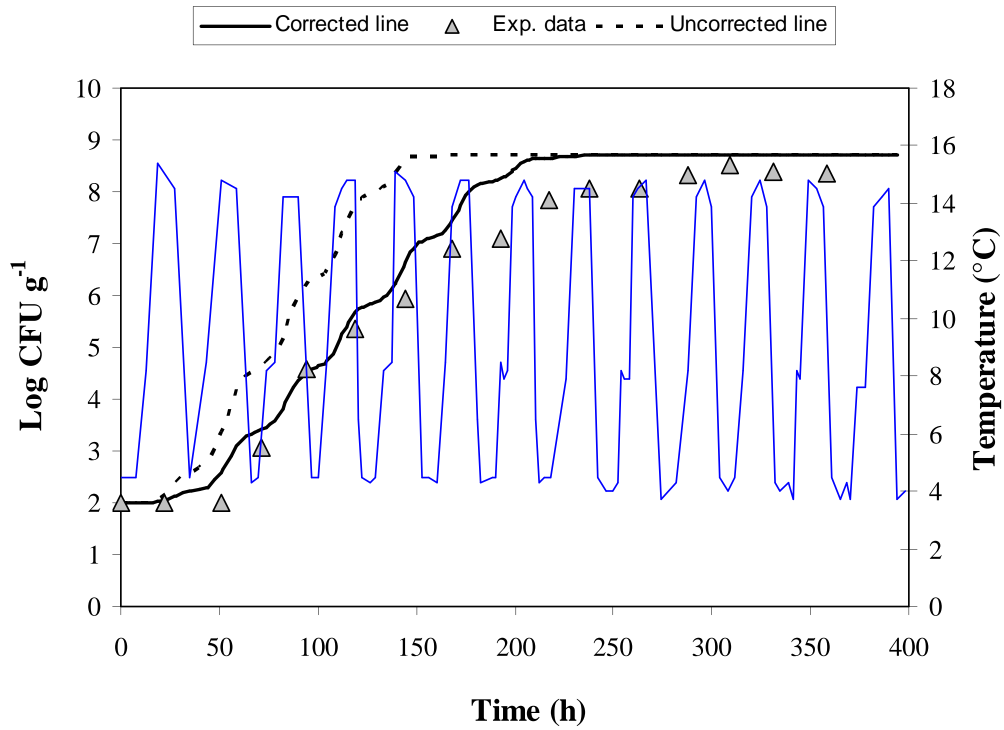


Figure 12. Comparison between observed (points) and predicted (lines) growth of *Listeria monocytogenes* in vanilla cream stored under periodically changing temperature profile (12 h at 4°C, 6 h at 8°C and 12 h at 15°C). Lines correspond to *Combase Predictor* with correction (solid line) and without correction (dotted line).

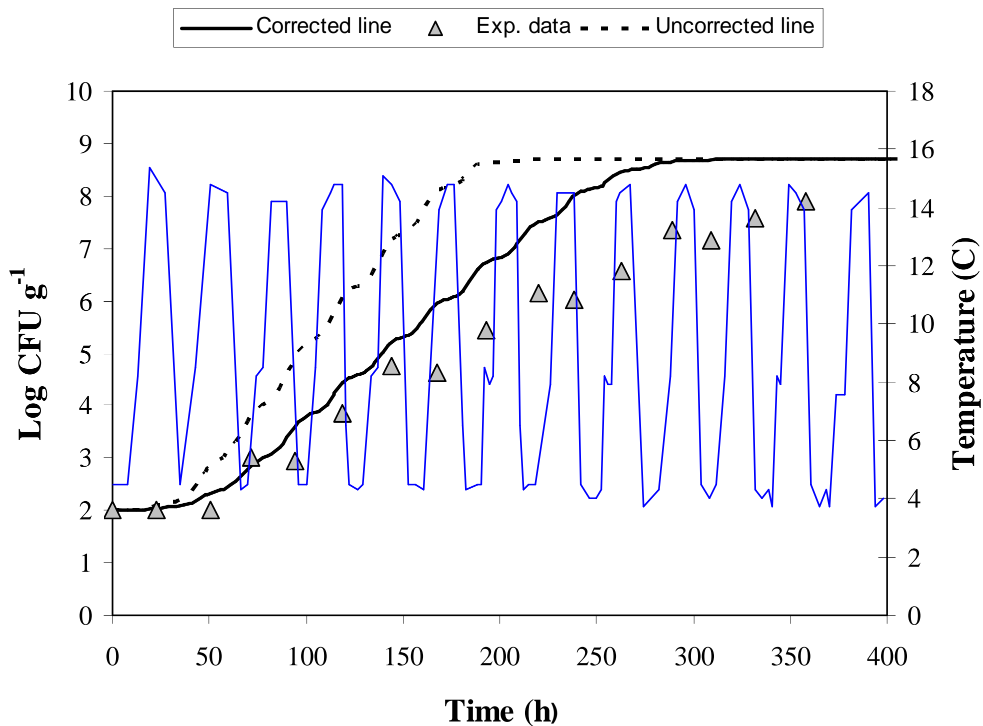


Figure 13. Comparison between observed (points) and predicted (lines) growth of *Listeria monocytogenes* in vanilla cream stored under periodically changing temperature profile (12 h at 4°C and 12 h at 12°C). Lines correspond to *Combase Predictor* with correction (solid line) and without correction (dotted line).

8. On-line *Sym'Previus* predictive modelling platforms to simulate *L. monocytogenes* growth in meat-based products under dynamic temperature profiles: example of cooked sausages

Materials and Methods

Smoked and cooked sausage samples were collected from the manufacturer and stored at isothermal conditions (4° C) until artificial inoculation with a strain of *L. monocytogenes* adapted to growth at 8°C. Surface inoculation has been done with *L. monocytogenes* AOOC015 that has been previously isolated from meat. Samples of 20g sausage were aseptically weighed, inoculated at 200 CFU/g and vacuum packaged.

Two temperature storage scenarios have been selected for food shelf-life, i.e. static storage condition at 8°C and dynamic conditions of storage with a cold chain break of 2h at 15°C. Samples incubation has been carried out in high precision temperature incubators. Throughout storage, samples were taken at appropriate time intervals to allow efficient kinetic analysis of *L. monocytogenes,* specific pathogenic organism for this commodity.

L. monocytogenes counts have been performed on ALOA chromogenic agar medium after 1/10 food sample dilution in peptone water and homogenisation in a stomacher (Lab Blender 400, Seward Medical, London) for 60s at room temperature. Values of pH have been determined using HI8418 pH meter (HANNA Instruments, Laboratoires Humeau La Chappelle S/Erdre) while water activity has been measured with AquaLab Series 3 a_w meter (Biotrace SA).

Results

Along a 15-day storage, pH and water activity remained stable with mean values of 6.4 and 0.96, respectively. *L. monocytogenes* kinetics obtained during a 8°C static temperature storage, enabled the determination of optimal growth rate (μ_{opt}) and characteristic parameters such as lag time, initial and final bacterial population. Figure 14 illustrates the output of the online *Sym'Previus* decision-making tool. Growth kinetics obtained from a food sample which has been artificially inoculated with a known microorganism strain enables the determination of optimal growth rate (µopt) in static condition of storage. Using Sym'Previus software, the impact of pH, aw and temperature on growth is evaluated as well as the µopt which is independent from these environmental parameters and allow comparison between various growth rates

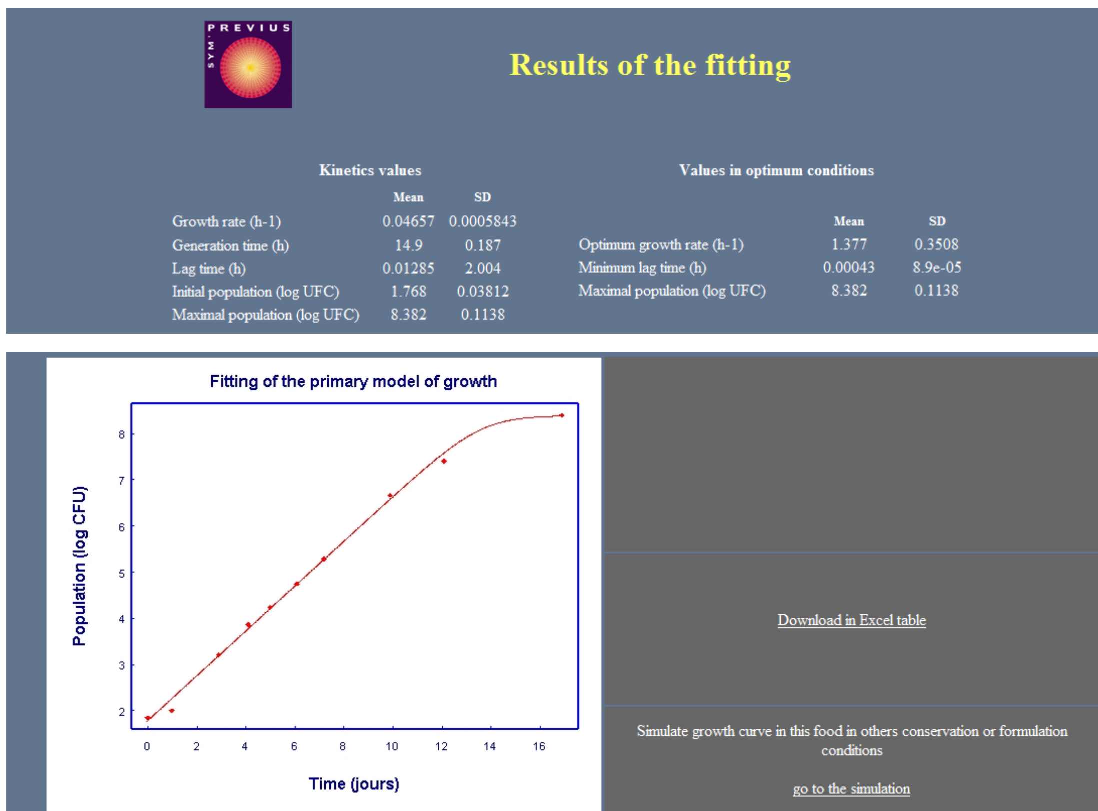


Figure 14. *Sym'Previus* growth fitting output based on *L. monocytogenes* kinetics obtained from challenge test performed on sausage under isothermal conditions (8°C).

Food specific optimal growth rate, as well as kinetics of only one challenge test performed for a given food allowed growth simulation under dynamic temperature conditions. Growth simulation has been performed on a predefined temperature profile, i.e. 1/3 of food shelf life at 4°C and 2/3 of shelf life at 8°C with a cold chain break. Figure 15 shows *L. monocytogenes* growth simulation (blue line) and 90% confidence intervals (red lines). Experimental counts have been reported as green dots after dynamic storage of 5 days at 4°C, 2 hours at 15°C and 10 days at 8°C. Comparison of growth simulation and experimental *L. monocytogenes* counts highlights the adequacy of software simulation.

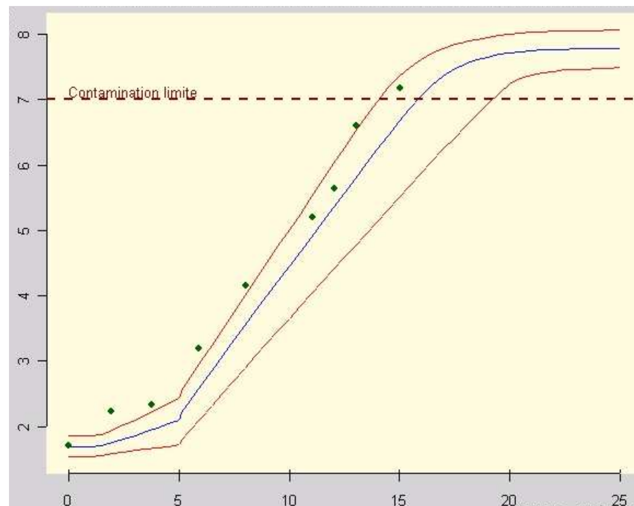


Figure 15. *L. monocytogenes* growth kinetics expressed in population (log CFU/g) as a function of time (days) for dynamic temperature storage. *Sym'Previus* growth kinetic simulation (blue line) with 90% confidence intervals as compared to experimental *L. monocytogenes* counts represented as green dots.

9. Potential of *Sym'Previus* on-line predictive modelling platform to simulate *L. monocytogenes* growth in dairy products during ripening: example of WP2A experimental data of several surface microbial consortia.

An attempt to use *Sym'Previus* simulation software was made based on a set of experimental *L. monocytogenes* kinetics performed to select raw milk cheese microbial consortia with anti-listeria activity (WP2A). These experiments concerned complex consortia isolated from 18 days ripened surface of Saint Nectaire raw milk cheeses. *L. monocytogenes* kinetics and dynamic pH profiles were recorded along 28 days for a total of 6 consortia (CTR1009, CTR1109, CTR1509, CTR1909, CTR2409, CTR2609). Experimental data have been provided and analyzed using *Sym'Previus* software.

Results

Experiment "LmCcontrol09" consisted of *L. monocytogenes* kinetics recorded along ripening with fluctuating pH conditions without the presence of tested surface microbial consortium. "LmCcontrol09" was used as control treatment for the determination of optimal growth rate after data generation with linear interpolation. Table 4 reports the characteristic parameters determined for this treatment.

Table 4. *L. monocytogenes* kinetics and pH profile obtained for control treatment "LmCcontrol09"

Time (days)	Log CFU/cm^2	pH
1	1.0	5.26
8	1.0	5.35
11	3.1	5.81
14	3.8	7.44
18	5.2	7.81
21	5.5	7.74
25	6.0	8.12
28	6.3	8.01

Characteristic parameters	
μ_{opt}	0.49 h^{-1}
Lag (min)	3 days
N_o	0.9 Log CFU/cm^2
N_{max}	6.1 Log CFU/cm^2

Based on growth parameters determined for this control treatment, several kinetics have been simulated for dynamic pH conditions recorded with tested microbial consortia. Figure 16 shows *Sym'Previus* simulation (red line) as compared to experimental counts (green dots). Simulation has been done considering environmental factors which could affect *L. monocytogenes* such as pH, a_w and temperature. Differences observed between simulations and experimental bacterial counts could be attributed to the presence of surface cheese consortium, microbial interaction or gradients of bioactive products. Surface consortia, isolated from raw milk cheeses, have complex composition resulting in various responses of *L. monocytogenes* on cheese surface. It is worth noting that three different responses have been reported in Figure 16. Based on experimental counts, succession of events may be envisaged for consortium "CTR2609" and "CTR2409". Consortium CTR1509 seems to have a stronger inhibitory effect on the growth of *L. monocytogenes* in tested conditions.

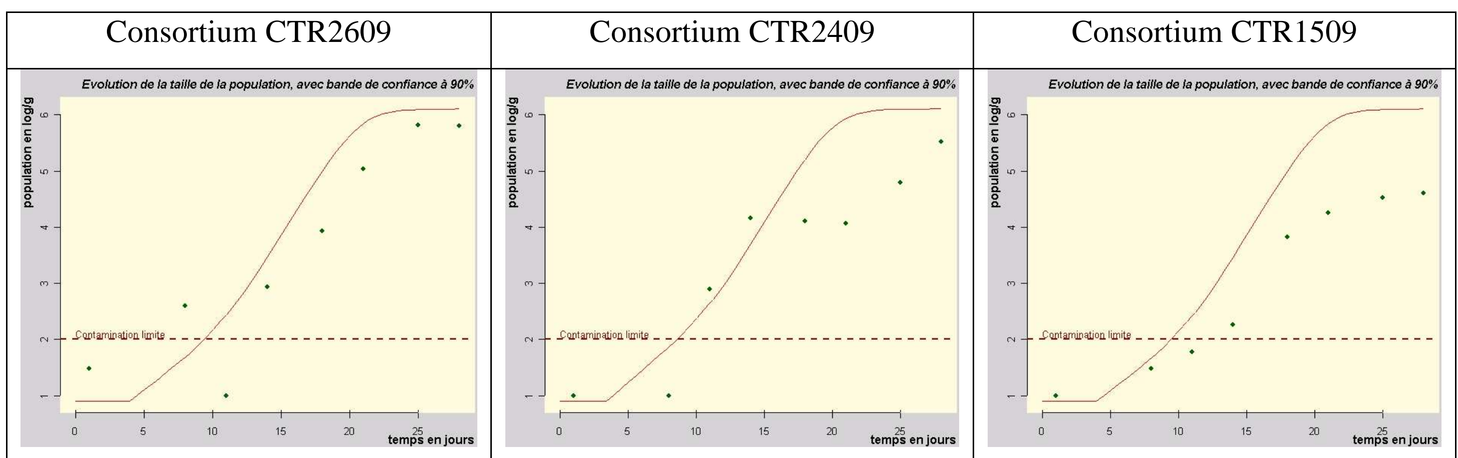


Figure 16. *Sym'Previus* growth kinetic simulation (red line) as compared to experimental *L. monocytogenes* counts represented as green dots in the presence of 3 cheese surface consortia.

Similarly to control conditions, the determination of μ_{opt} has been performed for each surface consortium tested, after data generation with linear interpolation. Comparison between µopt obtained for each condition, allows the selection of consortia showing an effect on *L. monocytogenes* growth as compared to control treatment “LmCcontrol09”. Indeed the growth rate of consortium “CTR1509” is lower as compared to the μ_{opt} of 0.49 h^{-1}.obtained for the control condition, which could comfort the fact of an impact of the consortium on *L. monocytogenes*. However, the expression of global differences between experimental counts and simulations with statistical indices (Table 5) was not suited for a succession of complex and not elucidated interactions on raw milk cheese surface.

Table 5. Calculated parameters for the performance of simulation and correlation with the interaction of microbial consortia on *L. monocytogenes* growth on cheese surface.

Tested Surface Consortia	μ_{opt} (h^{-1})	Bias factor	Accuracy factor	GOF*
CTR1109	0.46	1.031	1.065	0.0038
CTR1009	0.45	1.103	1.103	0.0040
CTR1909	0.45	1.033	1.059	0.0038
CTR2609	0.40	1.202	1.202	0.0038
CTR2409	0.57	0.831	1.204	0.0045
CTR1509	0.44	1.100	1.101	0.0039

* GOF: Goodness of fit

Even though the impact of microbial interactions on growth is not yet integrated as a gamma function in *Sym’Previus* simulation tool, the cardinal approach enabled to take into account the impact of food on pathogen growth via the determination of food specific μ_{opt}. The definition of the experimental set-up is crucial in order to follow and quantify factors that could then be simulated via the definition of appropriated parameters.

10. Probabilistic approach developed in *Sym'Previus* decision making tool: example of *L. monocytogenes* contamination in smoked salmon.

The probabilistic approach developed in *Sym'Previus* decision-making tool is available online since May 2008. Based on physico-chemical characterisation and microbiological counts obtained from industrial controls, the probabilistic modulus enable the determination of distribution of contamination in a given food product at the end of the targeted shelf-life considering product batch and bacterial intra-species variability.

A stochastic approach of bacterial growth is essential as food contamination with pathogens generally occurs with very few cells. Bacterial behaviour must then be studied at the level of cell to improve predictions of the growth of these microorganisms in food. Guillier and Augustin (2006) modelled lag time distributions of individual *Listeria monocytogenes* cells for 22 initial physiological states, 18 growth conditions, and 11 strains yielding 54 combinations in total. The proposed models allow the prediction of individual cell lag time distribution of *L. monocytogenes* for different growth conditions. In practise, population lag time is estimated from one challenge-test to evaluate the distribution of individual cell lag times for the tested temperature, pH and water activity conditions. From this distribution of individual lag time, it also possible to predict the distribution of individual cell lag time and population lag time in another conditions, or for another inoculum size (Figure 17).

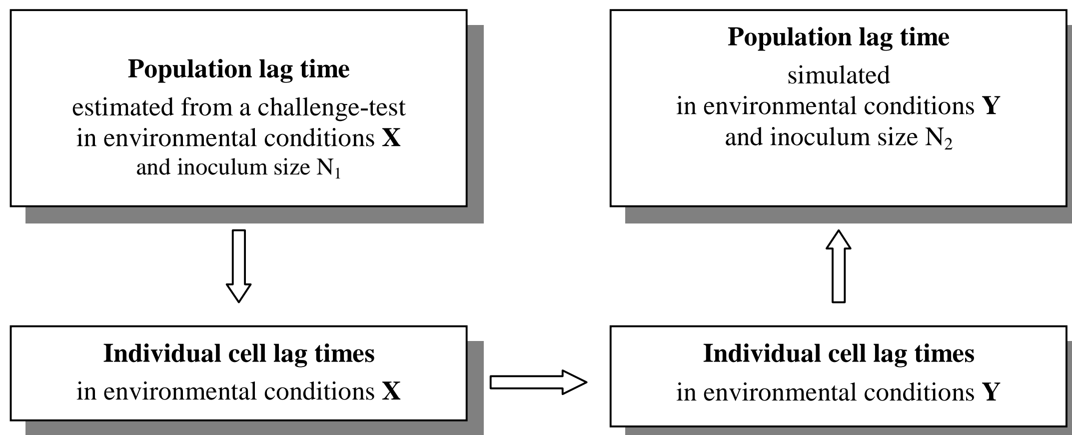


Figure 17. Lag time simulation concept.

This approach allows the prediction of the lag phase duration based on a single growth experiment performed with high initial concentration for a given physiological state, whatever growth conditions and initial bacterial concentration.

The models are applied in the software, taking into account the variability observed in product, process and microbes population. Industrial initial parameter input is not limited to a single mean value, but all physico-chemical or microbiological analyses realized on the product. *Sym'Previus* software calculates the growth curve and contamination distribution during shelf-life using Monte Carlo based simulations.

Results

Based on one challenge test of *L. monocytogenes* in salmon and published contamination levels within 7 factories, *Sym'Previus* probabilistic approach has been used to predict contamination distribution at the end of shelf-life. Available and targeted information is presented in Table 6.

Table 6. Information used to fill the query boxes in the relevant pages of *Sym'Previus* probabilistic tool.

Information needed	Information filled for probabilistic simulation
Targeted microorganism	13 strains of *L. monocytogenes* isolated from various foods are available in *Sym'Previus* database.
One kinetic of *L. monocytogenes* in smoked salmon	Challenge test obtained from artificial contamination of *L. monocytogenes* (10 CFU/g) for given physico-chemical conditions at 8°C, pH 6.03 and a_w 0.959. Kinetics obtained for a shelf-life of 30 days.
Initial contamination	Mean industrial controls of 41/626 positive samples of 25g.
Targeted parameters for simulation	Targeted shelf-life of 21 days for food product of 100 ± 3g during storage at 6°C for a pH of 5.92 ± 0.09 and a_w of 0.963 ± 0.006.

Simulations based on risk analysis approach taking into account the initial contamination distributions estimated from industrial monitoring data sets, individual cell lag time distributions and growth simulations enable the prediction of the probability to exceed critical limits of *L. monocytogenes* concentration in food. Simulation of *L. monocytogenes* growth for the 41 contaminated samples during storage yields the contamination distribution during shelf-life and the probability to exceed the contamination criterion of 2 log/g for *L. monocytogenes* as shown in Figure 18.

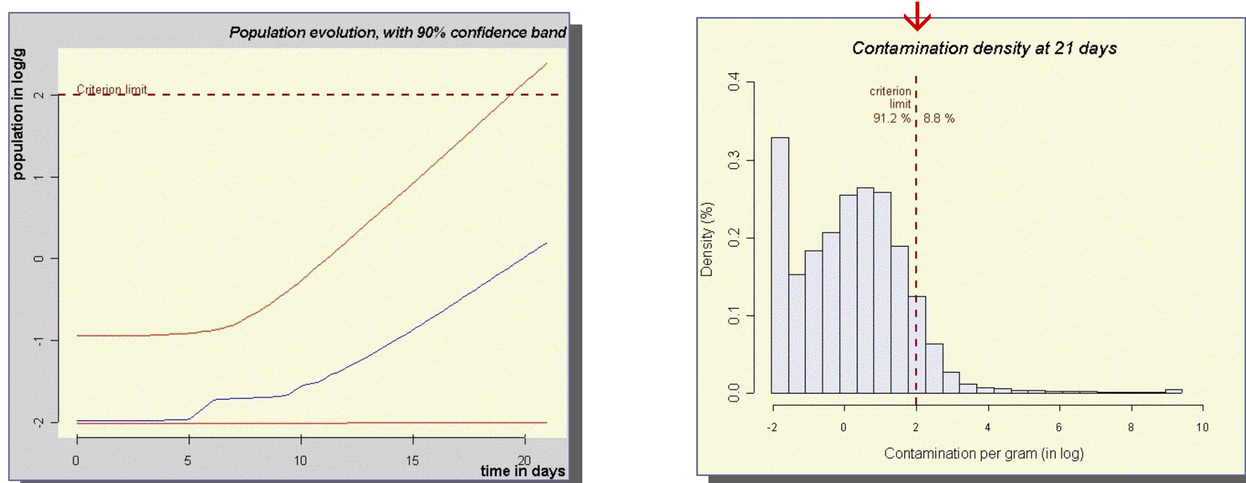


Figure 18. *L. monocytogenes* growth simulation during shelf-life with associated contamination distribution and a probability of 8.8% to exceed contamination criterion of 2 logs at the end of shelf-life for contaminated product.

Sym'Previus probabilistic modulus estimates that 8.8% of contaminated product will exceed the targeted contamination criterion of 2 log/g at the end of shelf-life, yielding 3.4% of end-product in the proposed example. Software simulation evaluates the impact of each factor to decrease the probability of exceeding targeted contamination criteria. Moreover, *Sym'Previus* probabilistic approach takes into account biological and physico-chemical variability and evaluates the weight of each factor affecting final contamination in order to give a tailor-made answer in an industrial environment and improve food safety.

11 Concluding remarks

Predictive microbiology knowledge is nowadays available in operational softwares for industrial applications which allow the simulation of microbial behaviour for more and more species in order to identify and control microbial hazards. Both Combase and Sym'Previus have been successfully used to provide realistic simulations and predictions of microbial responses in food. Finally, the new probabilistic module developed in the existing Sym'Previus software could provide better predictions suited to the needs of SMEs. As the databases of both softwares are continuously enriched with experimental data from the literature to quantify microbial responses

not only on synthetic laboratory media but also on real food commodities, better predictions are anticipated in the future and hence the application by the industry is expected to increase.

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