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Modelling microbial growth in structured foods: towards a unified approach

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Abstract

Historically, the ability of foods to support the growth of spoilage organisms and food-borne pathogens has been assessed by inoculating a food with an organism of interest, and following its growth over a period of time. Information gained from such *challenge tests*, together with knowledge of the organoleptic stability of the product, can then be used to determine an appropriate shelf-life for the food. Whilst this approach may be seen as the "gold-standard" of microbiological assessment of food, it is both time-consuming and costly. A major advance to complement challenge testing was the development of predictive modelling, when it was demonstrated that the growth of a wide range of organisms of interest could be quite accurately modelled as a function of only a few environmental parameters—primarily temperature, pH and water activity (a_w), with perhaps other factors such as nitrite, organic acids and oxygen. This approach to predictive microbiology is embodied in software tools such as the UK Food MicroModel and the Pathogen Modeling Program from the USA. Whilst modelling of this form yields accurate predictions of the growth of organisms in the majority of foods, there are occasions when there are discrepancies between the model and the observed growth. These discrepancies are most often described as "fail-safe", i.e. the observed growth is slower than predicted by the model. This paper examines the role of food structure in the development of microbial populations and communities, and describes the methodologies we propose to begin to tackle some of these complex and interlinked issues.

Keywords: Food structure; *Listeria*; *Bacillus cereus*; *Staphylococcus aureus*; Immobilisation; Predictive modelling

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1. Introduction and background

The survival and growth of microorganisms in food is affected not just by the chemical composition of the food and its storage conditions, but also by its structure (Wilson, 2000). The effects of food structure include constraints on the mechanical distribution of water (Hills et al., 1996, 1997), the chemical redistribution of organic acids, including food preservatives (Brocklehurst et al., 1993; Brocklehurst and Wilson, 2000), and physical constraints on mobility of microorganisms (Mattila and Frost, 1988a,b; Dodd, 1990; Dodd and Waites, 1991; Robins et al., 1994; Robins and Wilson, 1994; Wimpenny et al., 1995). It is this latter effect that is discussed further here.

The site of microbial growth in food is the aqueous phase, and the structural features of this phase are relevant to the length scale of microorganisms that influence microbial growth. This we define here as a micro-architecture, which might be uniform throughout the food, or particular regions may have a specific structure (Table 1).

Growth of microorganisms in a liquid aqueous phase in foods is typically planktonic and can be accompanied by motility allowing taxis to preferred regions of the food. Transport of nutrients to the bacteria and of metabolites away from them results in a locally uniform environment until considerable accumulation of microbial biomass and metabolites cause bulk chemical changes (such as a decline in pH). It is this micro-architecture in food that is mimicked in microbiological experiments by the use of broth culture medium.

Table 1
Examples of food micro-architecture, and representative foods

Micro-architecture	Food examples
Liquid	soups, juices: these are predominantly uniform liquids, although with some suspended material
Gel	pâté, jellies, cheeses such as cottage cheeses which are made from skimmed milk and hence are protein gels
Oil-in-water emulsion	dairy cream, milk, salad cream, mayonnaise
Water-in-oil emulsion	butter, margarine, low-fat spread
Gelled emulsion	whole-milk cheese, sausage
Surface	vegetable tissues, meat tissues

More usually, the aqueous phase of food is structured. In gelled regions of food, microorganisms are immobilised and are constrained to grow as colonies (Dodd, 1990; Dodd and Waites, 1991; Katsaras and Leistner, 1991; Wimpenny et al., 1995; Johnson et al., 1996; Parker et al., 1998). Colonial growth has been studied in model experimental systems in agar (Mitchell and Wimpenny, 1997) and in gelatin in a specifically designed Gel Cassette System (Brocklehurst et al., 1995a), where local depletion of oxygen (Wimpenny et al., 1981, 1995) and local accumulation of end-products of metabolism have been measured (Wimpenny et al., 1995; Walker et al., 1997; Malakar et al., 2000). It is also apparent that bacteria immobilised in gel differ from those in planktonic culture in their susceptibility to antimicrobial compounds, in their energy metabolism and in their metabolic end-products (Walker et al., 1998; Skandamis et al., 2000). Accordingly, where microorganisms grow in gelled regions of foods, it can be assumed that local changes in the concentration of their growth requirements and metabolites occur. Additionally, because of the local accumulation of metabolic end-products, interaction between colonies can occur. Such competition between bacterial species resulting from close spatial distribution (propinquity) of colonies was shown to occur up to a separation distance of between 1400 and 2000 μm (Wimpenny et al., 1995; Thomas et al., 1997).

Oil-in-water food emulsions differ widely in their concentration of oil (Brocklehurst and Lund, 1984; Tunaley et al., 1985; Ranken, 1988; Macrae et al., 1993; Buehheim and Dejmek, 1997; Brocklehurst and Wilson, 2000). Milk is typically between 3% and 5% v/v lipid, and mayonnaise can be between 26% and 85% v/v lipid. The lipid is in the form of droplets (typically between 0.15 and 8 μm in diameter) dispersed throughout the aqueous phase. Studies in model experimental systems have established a relationship between the concentration of oil within the emulsion and the form of growth of microorganisms (Parker et al., 1995). Where the oil concentration remains below 80% v/v the oil droplets are freely dispersed throughout the aqueous phase and microbial growth is typically planktonic. At greater concentrations of oil phase, the oil droplets are close-packed, mobility of bacteria is prevented and growth is con-

strained as colonies which form between the oil droplets, and displace the droplets as the colonies expand (Brocklehurst et al., 1995b; Parker et al., 1995). Restriction of growth in this way can result in a decline in growth rate and yield.

Many foods are emulsions that have been gelled. This might be due to the deliberate addition of gums and thickeners to increase the bulk viscosity (such as in sausages) or the denaturation of protein to form protein micelles (such as in cheese). Microorganisms in such foods are immobilised, and constrained to form colonies (Dodd, 1990; Dodd and Waites, 1991; Parker et al., 1998).

Water-in-oil food emulsions have an internal water phase dispersed as droplets within a continuous lipid phase. Growth of microorganisms is confined to within the droplets (Verrips and Zaalberg, 1980; Charteris, 1995), and classical theories to describe microbial growth rely on a micro-architecture of discrete compartmentalised droplets which limit the availability of water, space and nutrients for growth. Based on these assumptions, the models of Verrips and Zaalberg (1980) and Verrips et al. (1980) can be used mechanistically to predict the potential for growth of bacteria within discrete droplets related to the dimensions of the occupied droplets (Verrips and Zaalberg, 1980; Charteris, 1995) and the energy demands of inoculated bacteria (ter Steeg et al., 1995). These models show that bacterial growth and survival is restricted when the micro-architecture remains intact, and when coalescence of the droplets does not occur. This was confirmed in model experimental systems were an increased in numbers of bacteria in water-in-oil emulsions was always accompanied by coalescence of the droplets of aqueous phase (Brocklehurst et al., 1993).

Finally, the simplest form of micro-architecture affecting the growth of microorganisms is the surface. Growth of bacteria on food surfaces has been measured on Canadian wieners (McKellar et al., 1994), pâté (Farber et al., 1995) and vegetable tissues (Brocklehurst, 1994). Model experimental systems have included agar gels in petri dishes (Cooper et al., 1968; Wimpenny and Lewis, 1977; Wimpenny, 1979; Thomas et al., 1991; McKay and Peters, 1995), an agar film coating a microscope slide (McKay and Peters, 1995), two-dimensional gradient plates (Wimpenny and Waters, 1984, 1987; Thomas et al., 1991, 1992, 1993) and a modification of the Gel Cassette System mentioned above (Brocklehurst et al., 1997). Surface growth typically results in the formation of colonies, and hence constraints on the growth of microorganisms are similar to those described above in the case of growth in gels. However, diffusion limitations are greater at a surface than within a gel, and depletion of oxygen and accumulation of protons immediately beneath the colony and extending into the substratum have been measured by Wimpenny and Coombs (1983), Peters et al. (1987) and Robinson et al. (1991). This can result in decreased growth rates,

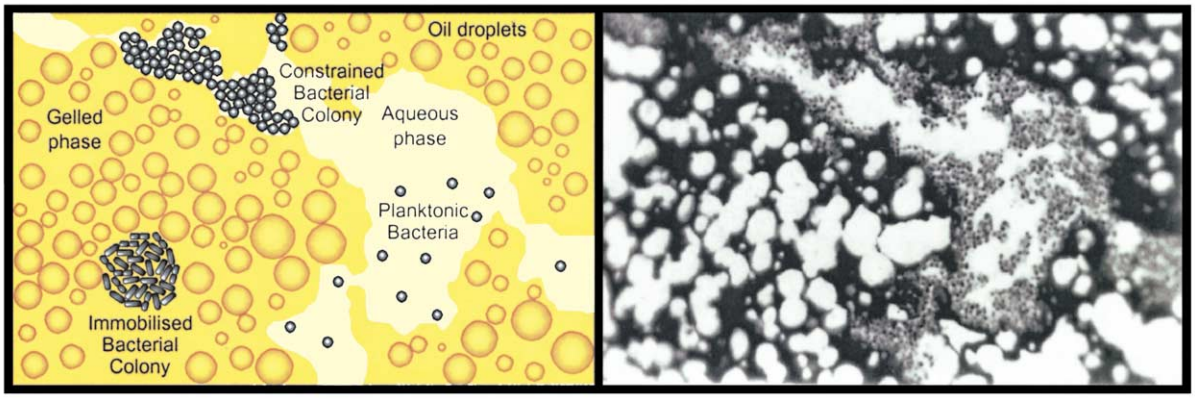

Fig. 1. Schematic diagram (left) and light micrograph (right) showing different environments for microbial growth in a sample of cheese. See Parker et al. (1998) for details.

and comparisons of the growth rates of surface colonies of *Salmonella* Typhimurium with the growth rate of cells immobilised in gel and with those growing planktonically in liquid medium followed the order: broth > immersed colonies > surface colonies (Brocklehurst et al., 1997). Interaction between colonies resulting from spatial distribution on surfaces has also been measured (Thomas and Wimpenny, 1993).

In practice, many foods may contain a number of micro-architectures, and the behaviour of microorganisms within the food will be a composite of those behaviours described above. This is illustrated here by a light micrograph of a portion of Serra cheese (Parker et al., 1998) (Fig. 1). This food is a gelled emulsion, and the accompanying schematic illustrates bacteria in coexisting micro-architectures. Hence, some occurred as planktonic cells, while others formed colonies on surfaces within the cheese, and as colonies immobilised adjacent to oil droplets within the protein gel of the curd.

2. How does food structure affect the behaviour of microorganisms?

In many cases, predictions based on data obtained from broth systems are applicable to the growth of organisms growing in structured foods. However, there are a number of cases where the structure of the food appears to result in a different behaviour. This behaviour is often what is referred to as "fail-safe". In other words, the organisms grow more slowly in structured systems than they do in broths. This may explain the differences which food manufacturers sometimes observe, where challenge testing indicates growth at a slower rate than suggested from predictive models.

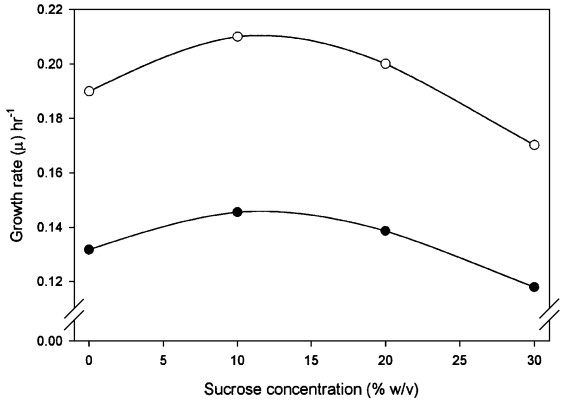

Fig. 2. Growth rate of *L. monocytogenes* Scott A as a function of sucrose concentration in broth culture (open circles) or immobilised with gelatin (closed circles) in an IFR Gel Cassette. See text for culture details.

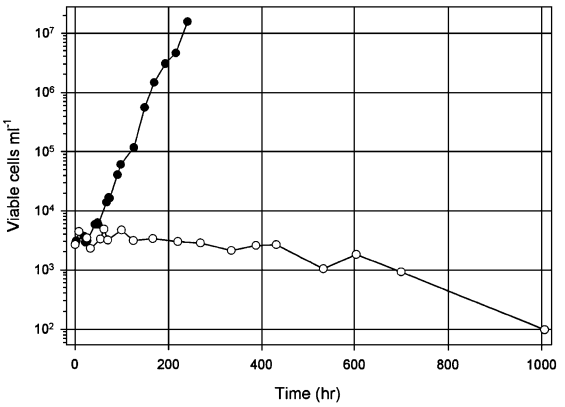

Fig. 3. Growth of *L. innocua* in broth culture (closed circles) or immobilised with gelatin (open circles) in an IFR Gel Cassette. See text for culture details.

To illustrate this point, we present some data comparing the growth of a number of food-borne pathogens (or models thereof) in either broth systems or in Gel Cassettes. Fig. 2 shows the growth rates of *Listeria monocytogenes* growing either in broth [3.0% (w/v) Trypticase Soy Broth (BBL); 0.3% (w/v) Yeast Extract (Oxoid); 0.75% (w/v) Glucose, denoted TSBYG] or in the same medium gelled with 10% (w/v) Bovine skin gelatin, Type B, ca. 225 bloom (Sigma) containing a range of concentrations of sucrose, at pH 6.0 and 20 °C (Meldrum et al., in preparation). It can be clearly seen that growth in a gelled system appears to exert an additional stress on the organisms, which grow more slowly in all circumstances. The divergence of the curves in each graph is indicative that the additional stress resulting from the structured environment is greater under less hospitable conditions.

Fig. 3 shows how food structure can also shrink the habitat domain of an organism. *Listeria innocua* was

grown on 3.7% (w/v) Brain Heart Infusion broth (Oxiod); 0.6% (w/v) Yeast Extract (Oxoid); 0.4% (w/v) Glucose total (BDH) (denoted PREMIUM BHI), with or without 10% (w/v) Bovine skin gelatin. It can be seen that in the broth system, growth of the organism occurs up to a cell density exceeding 10^7 cfu/ml, with a growth rate of 0.05 h^{-1} ($t_d = 15$ h) whereas no growth occurs in those cells immobilised by gelatin in the same medium. Indeed, the viability of the cells is progressively lost over time.

Figs. 4 and 5 illustrate the effect of food structure on both the growth rate and habitat domain of *Bacillus cereus* (Brocklehurst et al., in preparation b). In this case, the organism was grown in TSBYG at 20 °C and pH 6, either in broth in shaken flasks or in Gel Cassettes, immobilised with 10% (w/v) gelatin as above. Growth was studied at a range of water activities, adjusted either with sucrose (Fig. 4) or with NaCl (Fig. 5). It can be seen that in all cases, growth is slower in the immobilised Gel Cassette cultures than in the broths. It is interesting to note as well that for the same water activity, growth is more strongly suppressed when NaCl is used as the humectant, rather than sucrose. This indicates the additional influence of sodium toxicity over the a_w effects. Furthermore, it can be seen that at a water activity of 0.969, adjusted with either NaCl or sucrose, no growth occurs (up to 600 h) in the gelled system in contrast to growth in the broth. So, as well as a reduction in growth rate, we also see shrinkage of the habitat domain for *B. cereus*.

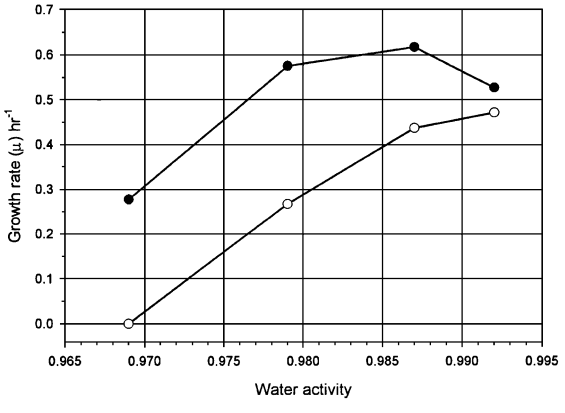

Fig. 4. Growth rate of *B. cereus* as a function of water activity, adjusted with sucrose, in broth culture (closed circles) or immobilised with gelatin (open circles) in an IFR Gel Cassette. See text for culture details.

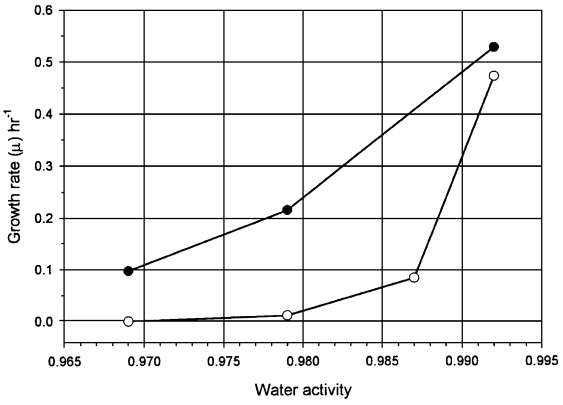

Fig. 5. Growth rate of *B. cereus* as a function of water activity, adjusted with NaCl in broth culture (closed circles) or immobilized with gelatin (open circle) in an IFR Gel Cassette. See text for culture details.

In all the above cases, use of a predictive model based on data from the broth experiments would lead to a "fail-safe" prediction in the gelled system. However, there are occasions where the effect of structure can act in the opposite way. Fig. 6 illustrates the growth rate of *Staphylococcus aureus* as a function of sucrose concentration, as a humectant (*Staphylococcus* cannot use sucrose as a carbon source) (Brocklehurst et al., in preparation a). The experiment was performed at pH 6 in TSBYG medium (see above) at 20 °C either as a broth in shaken flasks, or gelled with gelatin in Gel Cassettes (as the sucrose concentration was varied, the gelatin concentration was adjusted to maintain a constant gel strength).

In the absence of sucrose, growth is slower in the immobilised system than in the broth cultures. However, as the sucrose concentration is increased, growth in the broth progressively slows (as would be expected), but is essentially unaffected in the immobilised cultures. Thus, for sucrose concentrations above ca. 15% (w/v) at pH 6, growth proceeds faster in immobilised cultures than it does in broths. So, in this case, a predictive model based upon data from the broth cultures would "fail-dangerous". The mechanism behind this phenomenon is not currently

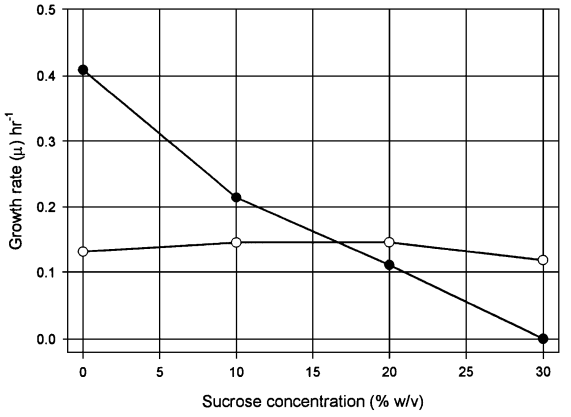

Fig. 6. Growth rate of *Staphylococcus aureus* as a function of sucrose concentration in broth culture (closed circles) or immobilised with gelatin (open circles) in an IFR Gel Cassette. See text for culture details.

understood, and is the subject of further investigation.

3. Effects of food structure on robustness and virulence

It is clear from the above that one of the key effects of food structure is the immobilisation of microorganisms, and their resultant growth as colonies. This results in local changes in the concentration of substrates (Wimpenny et al., 1995) and, particularly, a local accumulation of acidic metabolic end-products leading to a decline in pH within and around the colony (Walker et al., 1997; Malakar et al., 2000) with a pH gradient extending into the surrounding menstrum (Wimpenny et al., 1995; Walker et al., 1997). In the case of *S.* Typhimurium, the pH gradient extended from the original pH 7.0 in the surrounding medium to pH 4.3 inside the colony (Walker et al., 1997).

Such a local decline in pH within the colony is greater than the change required to stimulate an acid tolerance response (ATR) in *Salmonella* and other Gram-negative enteric bacteria (Lee et al., 1994) and in *L. monocytogenes* (Kroll and Patchett, 1992). ATR has been defined as the resistance of cells to low pH when they have been grown at moderately low pH or when exposed to a low pH for some time (Dilworth et al., 1999), and is typically demonstrated in broth culture, where pH 4.8–5.0 is reported to give an optimum ATR (Davis et al., 1996).

It is conceivable, therefore, that cells of food-borne pathogenic bacteria immobilised as colonies embedded in a food matrix may undergo a self-induced ATR stimulated by a localised pH which has declined by virtue of the colony's own metabolic processes. It is known that acid shock proteins are synthesised and exported from cells experiencing adaptation in broths. Should this also be the case in colonies, then it would result in all cells within the colony becoming acid tolerant. Hitherto, the ATR response has been demonstrated at 30 or 37 °C (Davis et al., 1996), which is clearly irrelevant in the food context. We have recently demonstrated ATR in *L. monocytogenes* at 20 °C, but not at 10 °C, confirming that immobilised growth of food-borne pathogenic bacteria at or near room temperature could result in an adaptive response.

In *L. monocytogenes*, the ATR has been attributed to the de novo synthesis of proteins (sometimes referred to as shock proteins) when exposed to ATR induction procedures, which is normally the deliberate alteration of extracellular pH (O'Driscoll et al., 1996). Such biochemical changes clearly confer acid resistance on the organisms, but O'Driscoll et al. (1996) also noted that *L. monocytogenes* that had been induced to show the ATR also had an increased resistance to thermal, osmotic and cold stresses. In addition, O'Driscoll et al. (1996) also reported that an acid tolerant mutant of *L. monocytogenes* also showed increased virulence in mice compared to a wild-type strain, which could possibly be attributed to enhanced in vivo survival, rather than a biochemical or genetic response. Indeed, once acid tolerant cells are ingested the death rate of cells as they pass through the digestive system is decreased (due to the ATR) compared to non-adapted thereby leading to a potential increase in virulence.

It is suggested here, therefore, that the immobilisation of microorganisms in foods might result in sufficient local changes in pH to stimulate an ATR, which has been shown to increase the resistance of adapted cells to a number of the typical inimical systems used to preserve food. In addition, an enhanced virulence of these adapted bacteria has been demonstrated. Studies are in place at IFR to explore

these possibilities in model systems that mimic the food environment, and at temperatures pertinent to food. Single-cell studies (e.g. Siegumfeldt et al., 1999, 2000; Budde and Jakobsen, 2000) are also a very valuable tool in understanding bacterial responses to such local pH effects at the cellular level.

4. A unified approach to modelling in structured systems

In 1997, the European Commission funded a 3-year research project (PREMIUM-FAIR CT97-3129) to develop a unifying modelling and data collection framework for predictive microbiology in structured foods. The framework was to be developed by considering both the axenic growth of *Listeria* in food structures and its interaction with *Lactococcus*. This paper describes some of the conclusions of the development of modelling methodologies.

The overall philosophy of our modelling approach is summarised in Fig. 7. It is assumed that the local environmental conditions define the instantaneous local growth rate of the organism of concern. This relationship is described by the use of a *Secondary Growth Model*. (The selection of appropriate model building blocks is described in more detail below.) The resulting growth rate describes the local increase in biomass, though the application of a *Primary Growth Model*. The increase in biomass causes local changes to the environment, such as a depletion of carbon source, or accumulation of the excreted products of metabolism. This may be described by a stoichiometric or physiological model. The local environmental changes may then propagate through the food structure by, e.g., diffusion. The metabolic products may also interact with the local chemical environment of the food — for example, changes in lactic acid concentration will result in changes in local pH, the magnitude of which will depend on the buffering capacity of the system. These processes finally lead to a modification of the local environment of the organisms, and the cycle begins again. In reality, of course, these events are not cyclic, but rather simultaneous, and the models need to be solved simultaneously.

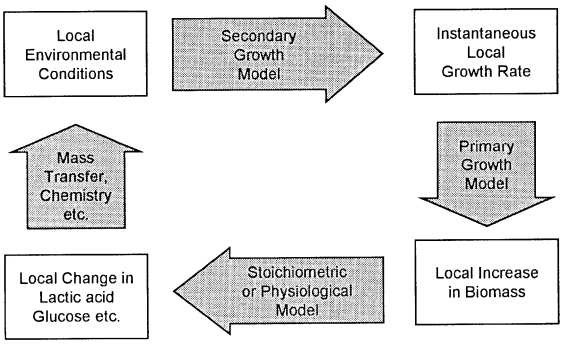

Fig. 7. Schematic diagram illustrating integrated modelling methodology proposed for modelling growth in structured systems.

5. Selection of model building blocks

5.1. Secondary models

Secondary growth models describe the dependence of primary model parameters on environmental factors such as temperature, water activity, pH and organic acids. A number of different secondary model types exist in the field of predictive microbiology, and can be broadly classified as: Arrhenius-type models, Bělehrádek-type models, polynomial models, cardinal models and artificial neural networks. Vereecken et al. (in preparation) have evaluated these model classes on the basis of criteria inspired by McMeekin et al. (1993) and Rosso et al. (1995), as follows:

(i) biological interpretability of the model parameters,
(ii) number of model parameters,
(iii) initial parameter estimation,
(iv) correlation between model parameters,
(v) extendibility of the model to more factors,
(vi) general applicability, and
(vii) quality of fit.

Based on this assessment of available secondary models, we have chosen to use cardinal models (Rosso et al., 1995) when the number of environmental conditions included in the model is small, and recognize that artificial neural networks (Geeraerd et al., 1998) may be of great use when taking into account more than two influencing factors.

5.2. Primary models

Primary models describe the evolution (i.e. growth, inactivation or survival) of microorganisms as a function of time. In the current context, we are concerned primarily with primary growth models. It is important to distinguish between static models, which are only valid under static (i.e. invariant) environmental conditions, and dynamic models, which can deal with time-varying environmental factors. Vereecken et al. (in preparation) have also assessed the applicability of a wide range of primary growth models for use in modelling growth of microorganism in structured foods. Using this assessment, we have decided to use a slight modification (see below) of the model of Baranyi and Roberts (1994) as the primary growth model. This model is well suited to describing microbial evolution in homogeneous systems. In structured foods, microorganisms grow as colonies, and the model can be applied locally to describe spatial variations. In these cases, we need to use sophisticated mathematical techniques such as cellular automata to solve the equations in both space and time. This is discussed further below.

6. Modification to Baranyi model

One aspect that we wish to include in the model output is the possibility of variation in the final concentration of cells in response to the environmental conditions. In most primary growth models, including the Baranyi model, the ultimate asymptotic cell concentration is usually included as a parameter such as $N_{\max}$. Under some circumstances, particularly in the situation of mixed culture with an antagonistic organism, $N_{\max}$ could be affected by the environmental conditions. In principle, this could itself be described by a secondary growth model, but we have taken the option of developing a variant of the Baranyi model which excludes specific stationary phase behaviour.

The original Baranyi growth model can be expressed as

$$\frac{dy}{dt} = \frac{\mu}{1 + e^{-q}}\left(1 - e^{m(y-y_{\max})}\right) \quad (1)$$

with

$$\frac{dq}{dt} = \mu \quad (2)$$

where y is the logarithm of the cell numbers and q is a parameter which relates to the initial physiology of the cells. μ, $y_{\max}$ and m are the growth rate, maximum cell numbers and a curvature parameter, respectively. We have simplified the equation to

$$\frac{dy}{dt} = \frac{\mu}{1 + e^{-q}} \quad (3)$$

thus eliminating the stationary phase from the model definition. Instead, the growth parameter μ is now time-dependent, i.e.

$$\mu = f(t) \quad (4)$$

The declining and stationary phases of the growth curve now emerge naturally by changes in the instantaneous growth rate, μ, being driven by the secondary model, in response to changes in the (local) environment.

The lag time for growth is still embodied in the model, by virtue of the terms in the denominator of the right hand side of Eq. (3), and can be determined from the initial physiological state (q_0) and the growth rate by the following relationship

$$t_{\text{lag}}\mu = \left(1 + \frac{1}{e^{q_0}}\right) \quad (5)$$

7. Stoichiometric models

In order to relate the increase in biomass to local changes in the chemical environment, it is necessary to know the stoichiometry of the conversion of carbon source to cells and metabolic products. This information is described in the physical science arena as the *reaction scheme*. The biochemistry of bacterial metabolism is, of course, both complex and dependant on the environmental conditions. One of the philosophies of modelling is, however, to choose the simplest representation of a system that embodies the behav-

iour of interest. In our studies of the behaviour of *Listeria* and *Lactococcus*, we proposed a simple reaction scheme as follows

$$N + G + O \rightarrow 2N + P$$

In other words, increase in cell numbers, N, consume the carbon source (in our case glucose, G) and oxygen, and produce metabolic products, P. We have chosen to focus on the acidic metabolic products, due to their direct influence on the local pH, known to strongly affect the growth rate. Under the conditions used in our studies, *Listeria* produces primarily acetic acid, and *Lactococcus* produces primarily lactic acid. Using bioreactor studies we have measured the consumption of glucose and oxygen, as well as the production of weak acid associated with the birth of each new cell. These are used in the stoichiometric model depicted in Fig. 7.

8. Local chemistry and mass transfer

Using the stoichiometric model, we can predict the local changes in weak acid concentration resulting from the microbial growth. The next task is to use this to predict changes in local pH. Although this is a common problem in many biological sciences, it is not straightforward in any mechanistic way, due to the poorly characterised, but central role of the buffering capacity of the microbiological growth medium (or food). It is possible to use an empirical characterisation, merely by using a titration of the growth environment with the acid of interest, and fitting a curve to these data. Alternatively a quasi-mechanistically based approach may be taken (Nicolaï et al., 1993). We have chosen to use the Buffering Theory of Wilson et al. (2000), which uses an empirical characterisation of the buffering capacity of the system together with a mechanistic knowledge of the dissociation behaviour of the weak acids of concern. An advantage of this approach is that the model may be easily applied to systems of differing buffering capacity, and can combine the effects of mixtures of weak acids.

To predict the dissipation of local metabolites and influx of consumed nutrients, we use a standard model of Fickian diffusion, currently using published diffusion coefficients in aqueous solution. It is not clear whether this description is entirely appropriate for

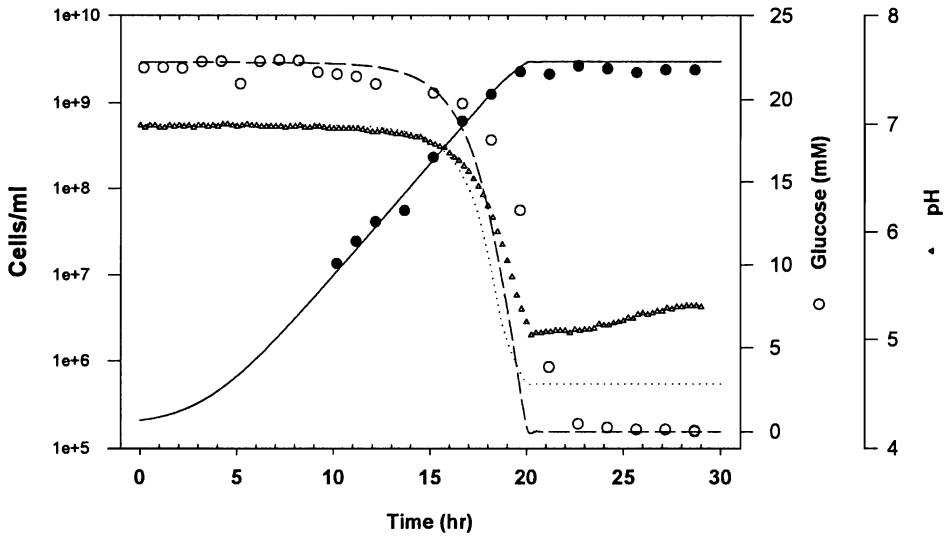

Fig. 8. Experimental data (points) and model prediction (lines) of growth of *L. lactis* SL05 (closed circles and solid line) in PREMIUM BHI broth (see text) showing evolution of pH (closed triangles and dotted line) and glucose concentration (open circles and dashed line). Cell counts by microscopic counting.

colonial growth in structured systems, and will be discussed further below.

9. Model solution

To solve the overall modelling scheme depicted in Fig. 7, a number of approaches are possible. For homogeneous systems such as broths, we may solve the differential, algebraic and equilibrium relationship in each sub-model (Cardinal pH model, modified Baranyi equation, Stoichiometric model and Buffering Theory) by using any convenient numerical integration algorithm. For the following examples, the equations were solved using SimuSolv (Dow Chemical). For immobilised colonies, the spatial dimension is important, and diffusion must be introduced. In order to have the ability to solve the equations in systems with complex boundaries, we have employed a cellular automaton (CA) solution to the equation, based on the original automaton of Barker and Grimson (1993). In principle, the automaton operates on discrete spatial lattice, each lattice point being home to a number of "particles" representing the local concentration of biomass, cell numbers, glucose, oxygen and product. There can only be a whole number of these particles at any one time, (and in fact for O, G and P particles there can only be a maximum of six such particles at each lattice point) and so the instantaneous local concentration is a discrete quantity. Particles may be generated, annihilated or they may move to a new lattice position at each discrete time step in the simulation. The time-averaging of particle numbers gives the means by which the solution maps onto the continuous scale of real-world concentration being simulated.

10. Performance of the modelling methodology

10.1. Homogeneous culture

Fig. 8 shows results from a bioreactor experiment following the growth of *Lactococcus lactis*. Samples were taken for glucose analysis, and the pH of the culture was continuously monitored. The lines on the graph show predictions of the model. Parameters of the model were as follows: For the growth model, a cardinal pH model was employed using data obtained by Bioscreen measurements (le Marc et al., 2000). Instantaneous growth rates from this model were used in the modified Baranyi model above, together with

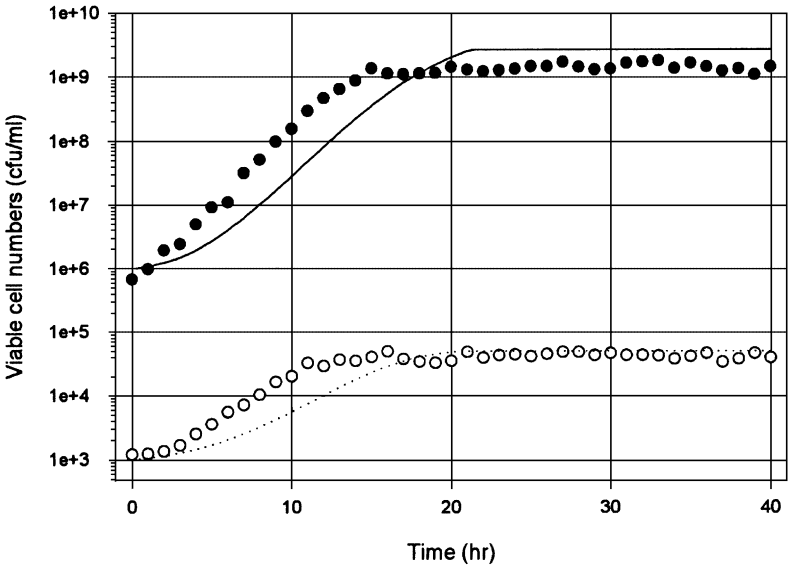

Fig. 9. Experimental data (points) and model prediction (lines) of growth of a mixed culture of *L. lactis* SL05 (closed circles and solid line) and *L. innocua* (open circles and dotted line) in PREMIUM BHI broth (see text). Cell determinations by plating onto selective agar.

stoichiometric parameters determined from bioreactor experiments (Wilson et al., IFR, unpublished data). The change in pH from production of lactic acid was determined by use of the Buffering Theory of Wilson et al. (2000). It can be seen that there is a very close agreement between the model and the data. In particular, the predicted stationary phase of growth is a result of exhaustion of glucose, a necessary carbon source in the model. The decay of glucose is predicted well by the model. The change in pH is again quite well predicted, except for one feature. The measured in pH increased from around 20 h, but remains static in the model prediction. This rise is likely to be caused by either production of ammonia from the degradation of proteinaceous material in the medium, or by consumption of lactic acid. Neither of these two features is described in the model, and the importance of the discrepancy would have to be assessed in light of the proposed application.

11. Mixed culture

Fig. 9 shows the growth of a mixed culture of *L. lactis* and *L. innocua* in a bioreactor at pH 4.5, 20 °C, on PREMIUM BHI medium. Also shown are predictions from the above modelling scheme, again using cardinal model parameters determined by le Marc et al. (ADRIA), and stoichiometric parameters from IFR. The Buffering Theory of Wilson et al. (2000) is again used to predict changes in pH (not shown). It can be seen that the modelling approach provides good prediction of both the rate and extent of growth of the two organisms. It should be noted that the stationary phase concentration of the *Listeria* is approximately four orders of magnitude smaller than that observed in axenic culture. This phenomenon would not be predictable with approaches incorporating a stationary phase into the primary growth model.

12. Immobilised growth in gel cassette

Fig. 10 shows the cellular automaton solution to the modelling scheme for growth of *L. innocua* at pH 4.5, together with experimental data gathered under the same conditions (these data are also presented in Fig. 4). It can be seen that the solution to the modelling scheme completely fails to predict the

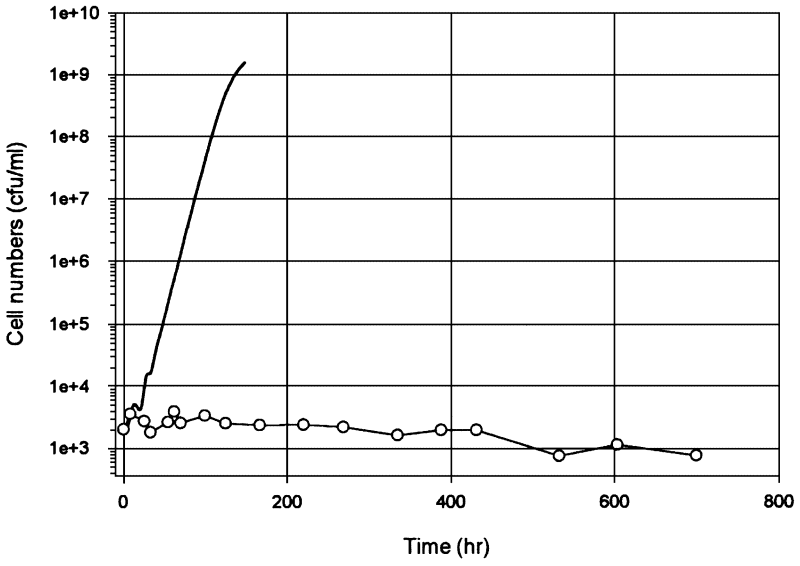

Fig. 10. Experimental data (open circles) and cellular automaton solution to model (line) for the growth of *L. innocua* in PREMIUM BHI (see text) at pH 4.5, and 20 °C in an IFR Gel Cassette.

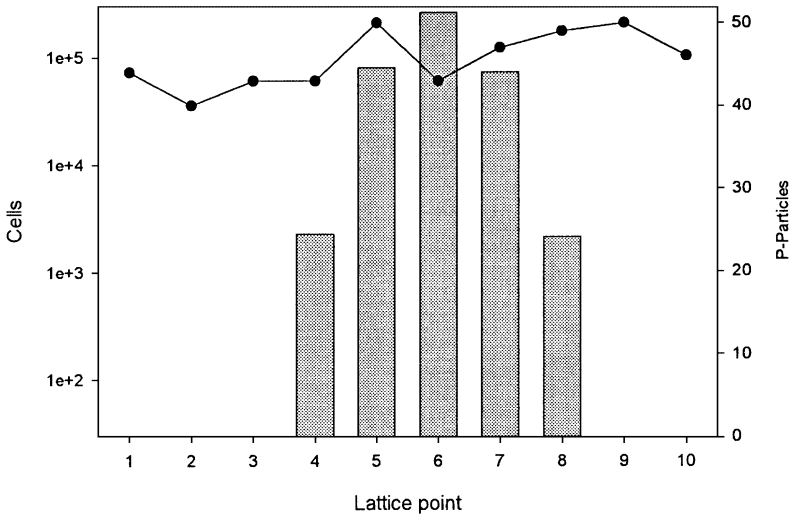

Fig. 11. Spatial distribution of cells (bars) and P-particles (closed circles) on a section through the centre of a $10 \times 10 \times 10$ cellular automaton lattice simulating growth of *L. innocua* in a gel at pH 4.5 (conditions as in Fig. 10).

observed effect of structure. There is evidence from both pH microelectrode studies (Walker et al., 1997) and pH mapping by fluorescence ratio imaging (Malakar et al., 2000) that strong pH gradients can develop within growing colonies. We hypothesised that this was one of the factors producing a reduced growth rate in immobilised cultures. Fig. 11 shows the spatial distribution of both cells and P-particles on a line through the centre of the simulated colony on the automaton lattice. No gradient of P-particles are evident either within or around the growing colony. It is clear from these observations that our current model of growth in structured systems is far from complete. The lack of pH gradients in the model solution is indicative of either a change in the metabolic product production rate or a diffusion rate greatly different from that observed in aqueous solution. Both these possibilities are under current investigation. Should these issues fail to explain the observations, more complex hypotheses will need to be put forward, and eventually incorporated into the modelling methodology.

It is just this type of failure of models to predict observed behaviour that highlights our lack of knowledge. We look forward to the field of predictive microbiology developing to understand these phenomena and provide perhaps new opportunities for the food industry to meet the needs of consumer safety.

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