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The fate of *Bacillus cereus* in the gastro-intestinal tract

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Het rapport in het kort

Dit rapport presenteert een wiskundig dynamisch model waarmee het gedrag van *Bacillus cereus* in het maag-darmkanaal beschreven wordt. Microbiologische processen en processen in het maag/darmkanaal vormen samen de basis voor dit mechanistische model. Variabiliteit in groeikarakteristieken en fysieke eigenschappen van *B. cereus* stammen komen tot uitdrukking in de parameterwaarden verkregen uit experimenten. Met het model zijn verschillende hypothesen getest betreffende initiële inname van *B. cereus* microben en daaropvolgende "*in vivo*" processen welke tot een potentiële infectie kunnen leiden. Modeluitkomsten laten het lot van vegetatieve cellen en/of sporen in de maag en dunne darm tijdens de vertering van een maaltijd met *B. cereus* microben zien. Hieruit blijkt dat de maag weinig invloed heeft op het uiteindelijke aantal vegetatieve cellen in de dunne darm. Een "milde" blootstelling [10^3 kolonievormende eenheden (kve) g^{-1}] geeft nog steeds een verhoogde kans op een toxico-infectie wanneer 100 g voedsel wordt geconsumeerd met daarin, tenminste, licht mesofiele *B. cereus* stammen. Blootstellingsnivo's juist boven de Nederlandse gestelde norm van < 10^5 kve g^{-1} vormen volgens dit model altijd een potentieel gevaar. Verder geeft dit model inzicht in de onzekerheid van bepaalde parameterwaarden welke nader experimenteel onderzocht zouden moeten worden om tot een betere risicobeoordeling te kunnen komen. Integratie van experimentele data in een dynamisch model met daarin de belangrijkste componenten voor voedselinfectie zal uiteindelijk leiden tot verbeterde suggesties voor voedselmicrobiologische criteria.

Trefwoorden: maag-darm passage, risicoschatting, voedsel microbiologie, enterotoxinen, wiskundig model

Abstract

This report presents a mathematical dynamical model for the behaviour of *Bacillus cereus* in the gastro-intestinal tract. Biological processes and system dynamics are simultaneously incorporated in this mechanistic model. Variability in growth characteristics and physical traits of different *B. cereus* strains are expressed through the incorporation of a range of reasonable parameter values obtained from experiments. Different hypotheses concerning initial ingestion of *B. cereus* microbes and subsequent *in vivo* processes leading to a potential infection are tested. Model outputs show the course of (attached) vegetative cells and/or spores in the stomach and small intestine during the digestion of food containing *B. cereus* microbes. Results show the minor influence of the stomach on the ultimate number of vegetative cells in the small intestine. A "mild" exposure (10^3 cfu g^{-1}) still causes an increased probability on food intoxication when 100 g of food containing, at least, slightly mesophilic *B. cereus* strains is consumed. Exposure to levels just above the Dutch standard (set at < 10^5 cfu g^{-1}) will, according to this model, always form a food hazard problem. Furthermore, this model gives insight in the uncertainty of some parameter values that need elaborated experimental investigation to come to an improved hazard characterisation and, with that, to improved suggestions for food microbiological criteria.

Key words: gastro-intestinal passage, hazard characterisation, food microbiology, enterotoxins, mathematical model

Contents

Samenvatting

Algemeen: *Bacillus cereus* is een voedselgerelateerde bacterie die zowel emetische als enterotoxines kan produceren. Ondanks dat er weinig bekend is over de virulentie eigenschappen van *B. cereus* is de maximaal toegestane hoeveelheid in voedsel gereed voor consumptie gesteld op $< 10^5$ kve g^{-1}.
Dit rapport: Dit onderzoek is gericht op het inzichtelijk maken van de mogelijke gevaren voor de volksgezondheid als gevolg van deze toegestane hoeveelheden. Deze studie geeft een eerste risico-inschatting voor een eventuele toxico-infectie na de consumptie van een maaltijd met enterotoxinen producerende stammen van *B. cereus*.
Methode: Deze risicoinschatting is gebaseerd op de groei van vegetatieve cellen in de dunne darm. Hiertoe is een dosis respons model ontwikkeld waarin het biofysische gedrag van *B. cereus*, verkregen uit gesimuleerde "*in vivo*" experimenten, in een dynamisch maag-darmkanaal beschreven wordt. Inactivatie van vegetatieve cellen onder invloed van pH is het belangrijkste mechanisme representatief voor de maagpassage. Vanuit de maag vindt een continue doorstroom plaats van sporen en vegetatieve cellen naar de dunne darm. Biofysische mechanismen in de dunne darm bevatten: 1- Ontkieming van sporen tot vegetatieve cellen, 2- Groei van vegetatieve cellen, 3- Adhesie/Loslaten van bacteriën aan/van de darmwand, 4- Inactivatie van bacteriën onder invloed van galsappen en 5- Uitstroom van bacteriën naar de dikke darm. Biodiversiteit in *B. cereus* komt tot uiting in de parameterwaarden van het model.
Resultaten en Aanbevelingen: Dit model laat zien dat de maag relatief weinig invloed heeft op de uiteindelijke aantallen vegetatieve cellen in de darm, 24 uur na de consumptie van 100 gram voedsel met in totaal 10^5 *B. cereus* cellen. Deze "milde" (10^3 kve g^{-1}) blootstelling zal vervolgens niet leiden tot een toxico-infectie in de dunne darm wanneer het gaat om voedselinname met psychrotolerante *B. cereus* stammen. Voor een potentiële toxico-infectie zal men, in dit blootstellingsscenario, in ieder geval blootgesteld moeten worden aan "super" mesofiele *B. cereus* stammen, dat wil zeggen, met optimaal werkende biofysische eigenschappen. Echter, blootstelling aan de Nederlandse maximum gestelde limiet van 10^5 kve g^{-1} vormt volgens dit model altijd een potentieel gevaar. Deze resultaten laten zien dat de huidige limiet voor *B. cereus* in voedsel te hoog is en dat de maximaal toegestane hoeveelheid in voedsel meer richting 10^3 kve g^{-1} zou moeten liggen. Modelresultaten geven ook inzicht in welke mechanismen nader onderzocht moeten worden om tot een minder onzekere risico-inschatting te komen. Dit zijn: 1- Aantasting van het darmepitheelmembraam onder invloed van toxineproduktie, 2- Biofysische mechanismen achter toxineproduktie, en 3- De rol van in het darm lumen vrij voorkomende bacteriën en aan de darmwand geadheerde bacteriën met betrekking tot toxineproduktie.

Summary

General: *Bacillus cereus* is a food borne pathogen and can produce both emetic and enterotoxins. Little is known about the virulence of different *Bacillus cereus* strains. Yet, the maximum limit for *Bacillus cereus* in food at consumption is set to < 10^5 cfu g^{-1}.
This report: This research gives insight in the possible public health risk associated with these limits. A first hazard characterisation for possible food intoxication is based on the consumption of a meal with enterotoxin producing *B. cereus* strains.
Methods: The hazard characterisation is based on the development of vegetative cells in the small intestine following the consumption of a meal. A biophysical dose response model has been developed for this purpose. Insights in the behaviour of *B. cereus* from simulated "*in vivo*" experiments are integrated in a mechanistic model describing the gastro-intestinal tract dynamics. Inactivation of vegetative cells, influenced by gastric pH, represent stomach dynamics. Both spores and vegetative cells flow continuously to the small intestine. Biophysical mechanisms in the small intestine include: 1- Germination of spores to vegetative cells, 2- Growth of vegetative cell , 3-Adhesion/Release of bacteria to/from the epithelial cell membrane, 4-Inactivation of bacteria by bile juices and 5-Outflow of bacteria to the large intestine. Biodiversity in *B. cereus* is expressed in the model's parameter values.
Results and Recommendations: This model shows that the stomach has relatively little influence on the ultimate numbers of vegetative cells, 24 hours after the consumption of 100 g of food containing 10^5 *Bacillus cereus* cells. This "mild" (10^3 cfu g^{-1}) exposure will, subsequently, not lead to intoxication when the food was contaminated with psychrotolerant *B. cereus* strains. A potential food intoxication could, in this scenario, only result upon the exposure to "super" *B. cereus* strains, *i.e* strains growing under optimal biophysical conditions. However, exposure to levels just above the Dutch standard (set at <10^5 cfu g^{-1}) will, according to this model, always form a food hazard problem. These results suggests that the current allowed limit is too high and a limit towards 10^3 cfu g^{-1} would be more appropriate. Furthermore, model output gives insight in which mechanisms need extensive investigation to come to a comprehensive hazard characterisation, *i.e.* 1- Epithelial cell membrane turnover in relation to toxin production, 2-Biophysics behind toxin production, and 3- The role of in the lumen "free floating'' bacteria compared to bacteria adhered to the epithelium with respect to toxin production.

1. Introduction

Bacillus cereus is a foodborne bacterium which can produce both emetic and enterotoxins and thus potentially cause emesis or diarrhoea (Granum, 1997; Kotiranta et al., 2000; Wijnands et al., 2002a). So far, it has not been possible to decide which level of *B. cereus* is acceptable in food at consumption. The maximum limit for *B. cereus* in some foods in the Netherlands is fairly high (10^5 cfu g^{-1}). This may be sufficient for innocuous and mildly virulent strains but will not protect the consumer in case of hazardous strains. A hazard characterisation accounting for the variability in virulent properties of *B. cereus* strains, therefore, needs further attention.

This research aims at improving the hazard characterisation for diarrhoeal strains of *B. cereus* focussing on the fate of *B. cereus* in the gastro-intestinal tract and the potential production of enterotoxins in the small intestine during the multiplication of vegetative cells. In addition, *B. cereus* can produce spores to survive harsh conditions. Spores can survive acidic stomach conditions, germinate and grow in the small intestine and, maybe, then contribute to toxin production. Still, little is known about the survival of *B. cereus* in the stomach and subsequent behaviour of both spores and vegetative cells in the small intestine. Many experiments are being performed to obtain insight in the behaviour of *B. cereus* under simulated *in vivo* conditions. Growth patterns of different *B. cereus* strains have been assessed at 37 °C in combination with different pH values (Andersen Borge et al., 2001). Clavel et al. (2004) assessed the survival of *B. cereus* in acid media. The influence of gastric pH and subsequent exposure to bile juices on the survival of microbes has been studied by Marteau et al. (1997) and Gänzle et al. (1999) for several bacteria and by Wijnands et al. (submitted) for *B. cereus* in particular. Furthermore, germination of spores under different conditions has been assessed by Wijnands et al. (submitted). The ability to produce the enterotoxins HBL, NHE and cytK has been investigated by Andersen Borge et al. (2001), Christiansson et al. (1989), Prüss et al. (1999) and Duport et al. (2004).

Enterotoxins are known to cause diarrhoea resulting from an unbalanced transport of solutes and water in the epithelium of the small intestine. Yet, research on toxin production has revealed that enterotoxins are highly instable proteins which are readily being decomposed in the environment of the small intestine (De Bruin, 2004). The remaining question is; “how can enterotoxins form a hazard to human when actually they are probably instantly degraded in the lumen of the small intestine?” This raised the hypothesis that adhesion of *B. cereus* to the epithelium cell membrane might be necessary to result in a toxic infection. If a significant amount of vegetative cells would adhere to the cell membrane, then little time would be needed for the produced toxins to reach their target and cause an effect. Therefore, the adhesion of both vegetative cells and spores to cells simulating the epithelium cell membrane

in the small intestine (differentiated Caco-2 cells) has been assessed (Andersson et al., 1998; and Wijnands et al., submitted).

Although these investigations have a joint goal of getting more insight in the *in vivo* behaviour of *B. cereus*, laboratory experiments alone will never be sufficient to come to an integrated hazard characterisation based on bacteria/system interactions. A modelling approach is needed to integrate bacterial behaviour with gastro-intestinal system dynamics in order to find ways to assess the potential hazard of foodborne *B. cereus*. Biodiversity in *B. cereus* strains can be expressed in the parameter values to account for the variable impact of this pathogen in the small intestine.
Several models have been developed to describe the transport of bacteria through the stomach as well as through the small intestine of human and other mammals. Minekus et al. (1995), for example, used a power exponential equation to describe the delivery of a meal marker from simulated gastric and ileal compartments. This model has subsequently been applied by several researchers (Marteau et al., 1997; Gänzle et al., 1999). Takumi et al. (2000) developed a dynamical stomach model to describe the inactivation of *Escherichia coli*. Furthermore, Rivest et al. (2000) give a mechanistic representation for the digestion of proteins in the small intestine of pigs. More recently, Bayesian belief networks have been applied for risk assessment purposes. Barker et al. (2002), for example, used this technique to analyse the hazard associated with foodborne botulism.

This report outlines a mechanistic approach to model the fate of *B. cereus* in the gastro-intestinal tract. The interaction of biological processes with system dynamics is described based on experimental results. Biodiversity concerning growth characteristics and physical traits of *B. cereus* is expressed through the incorporation of a range of parameter values. In addition, different hypothesis concerning initial exposure and subsequent processes leading to a potential infection are tested. Model outputs show the course of (adhered) vegetative cells and/or spores in the stomach and small intestine during the digestion of *B. cereus* for different exposure scenarios.

The model will give insight in:

- the most important *B. cereus* growth characteristics (*e.g.* slow/fast growers) influencing potential toxic infection,
- the relative impact of different gastro-intestinal system dynamics on the potential hazard of *B. cereus* food poisoning and
- the impact of different hypotheses concerning initial exposure (*e.g.* spores and/or vegetative cells) and subsequent physical processes of *B. cereus* in the stomach and small intestine (*e.g.* with/without adhesion) on the ultimate amount of *B. cereus* units which can potentially cause an infection.

Simulation of different process scenarios will result in conditional suggestions for microbiological criteria to identify potentially hazardous food products.

2. Dose response dynamics of *B. cereus*

A mechanistic model should represent the most important microbe/system interaction dynamics to be able to predict potential food intoxication scenarios. This section will discuss the most important biophysical dynamics and accompanying assumptions resulting in an explorative model representing the fate of *B. cereus* microbes in the gastro-intestinal tract.

Figure 1 gives a schematic representation of the infection route of *B.cereus* spores and vegetative cells when contaminated food is consumed. The model will focus on the *in vivo* development of spores and vegetative cells assuming different exposure scenarios.

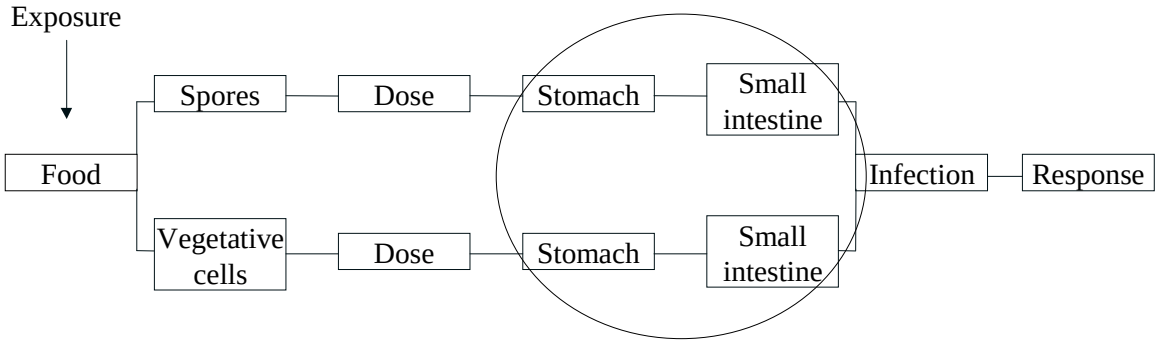

Figure 1 Infection route of spores and vegetative cells upon the consumption of food infected with B. cereus microbes. The circle points out which part of the infection route has been modeled.

2.1 Exposure to B. cereus

There are three hypotheses about possible exposure to *B. cereus*:

1. *Consuming food contaminated with spores.*
   This food type will contain no vegetative cells since they died during the heating process. Spores, however, can survive a heat treatment and, on top of that, will be triggered to germinate relatively fast after being exposed to relatively high temperatures. Instant consumption of this type of food can, therefore, lead to more rapid toxin production.

2. *Consuming food contaminated with only vegetative cells.*
   Spores are being triggered to germinate relatively fast after heating (see hypothesis 1). If food is subsequently stored instead of being consumed, spores will readily germinate and so the food is left with vegetative cells (Pielaat et al., submitted).

3. *Consuming food contaminated with both spores and vegetative cells.*
   Long term stored food might contain both vegetative cells and spores. The vegetative cells, as presented in hypothesis 2, have now had enough time to form spores.

Once vegetative cells and/or spores are consumed they have to be transported to the small intestine in order to become a hazard. As this study focuses on the dynamics of *B cereus* in the stomach and small intestine, these microbe/gastro-intestinal tract interactions will be discussed separately in the following subsections.

2.2 Stomach dynamics

The fate of *B. cereus* microbes in the stomach is modelled according to the work of Takumi et al. (2000). A schematic representation of their model applied to *B.cereus* microbes is given in Figure 2.

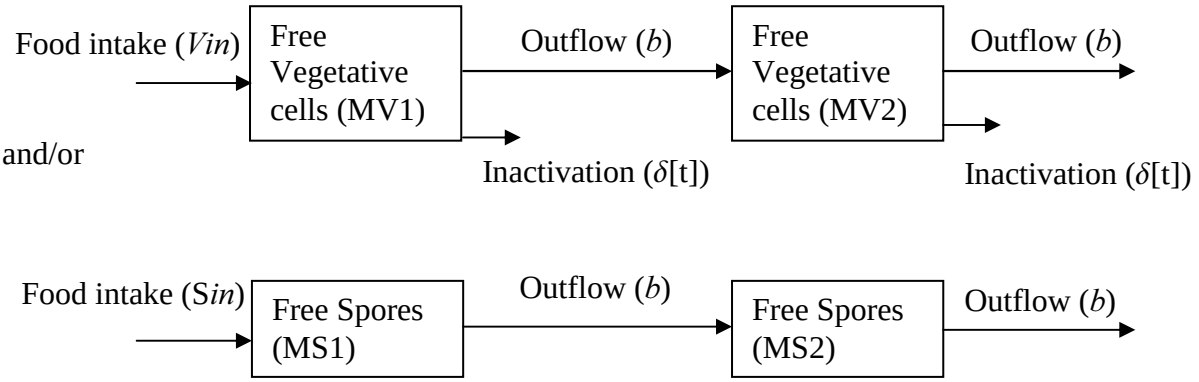

Figure 2 Flow diagram of the most important B.cereus dynamics in the stomach. Inactivation of vegetative cells is described by a time dependent function δ[t]. *The two compartments represent the passage of food parcels through the stomach in 2 steps and incorporate the parameter b (see text for explanation).*

Takumi et al. (2000) estimated that a meal is transported through the stomach in, on average, two steps. To be able to model both stomach and small intestine dynamics in a consistent way (see also Section 2.3), the stomach is represented by a two-compartment system in Figure 2. Food with microbes is assumed to be homogeneously mixed during digestion. Furthermore, vegetative cells will pass the stomach proportional to food transport (Outflow). In addition, at each point in time, a part of the present vegetative cells will be inactivated due to the time dependent stomach acidic pH. Spores, on the other hand, will not be inactivated by the

stomach and flow to the small intestine according to food transport. And so the change in time [t] in the number of vegetative cells and spores in the stomach can be described by:

$$V[t] = -inactivation[t]V[t] - outflow[t]V[t] \quad (1a)$$

and

$$S[t] = -ouflow[t]S[t], \quad (1b)$$

where

$V[t]$ = change in the number of "free floating" vegetative cells at time t=T.

$S[t]$ = change in the number of "free floating" spores at time t=T.

$V[t]$ = absolute number of "free floating" vegetative cells at time t=T.

$S[t]$ = absolute number of "free floating" spores at time t=T.

Inactivation, δ[t], of vegetative cells in time is described as a function of the pH course following Takumi et al. (2000). That is,

$$\delta[t] = e^{-d\ Ln(10)\,pH[t]} \quad (2)$$

and

$$pH[t] = (pH_{\max} - pH_{\min})e^{-k\,t} + pH_{\min}, \quad (3)$$

where

δ[t] = inactivation of vegetative cells as a function of time,

d = parameter describing inactivation as a function of external hydrogen ion concentration (Takumi et al., 2000),

$pH[t]$ = stomach acidic pH course,

pH_{max} =maximum pH upon food consumption,

pH_{min} =minimum pH after the consumption of a meal,

k = rate with which the pH decreases from pH_{max} to pH_{min}.

Eq. 3 shows that at t=0, pH[t]=pH_{max} and as t $\rightarrow\infty$, pH[t] $\rightarrow$ pH_{min} following an exponentially decreasing function. Substituting these values in eq.2 shows that inactivation is smallest just after food intake and increases as the pH decreases. Figure 3 gives a qualitative representation of the inactivation curve (eq.2). This figure implies that the fraction of the initially consumed vegetative cells that survive in the stomach up until time t=T is a decreasing function with time as shown in Figure 4.

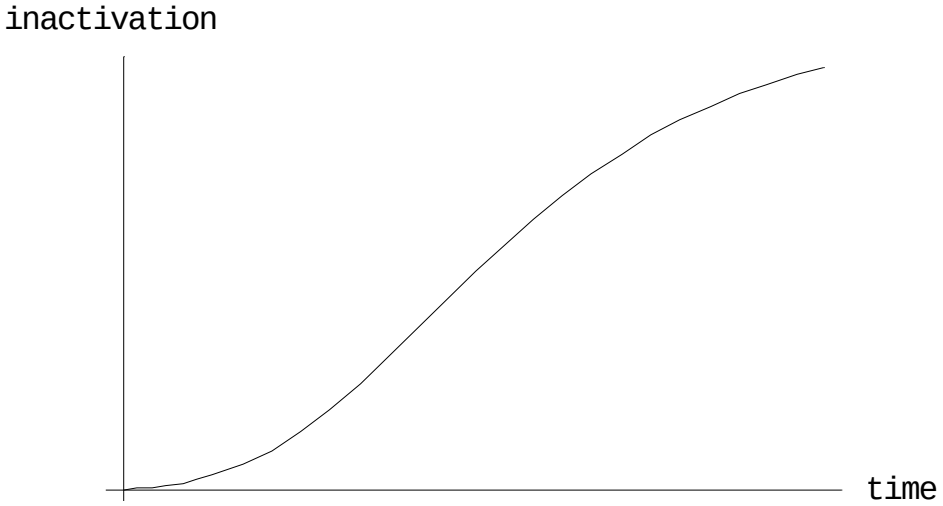

Figure 3 *Qualtitative representation of the inactivation of vegetative cells in the stomach with time.*

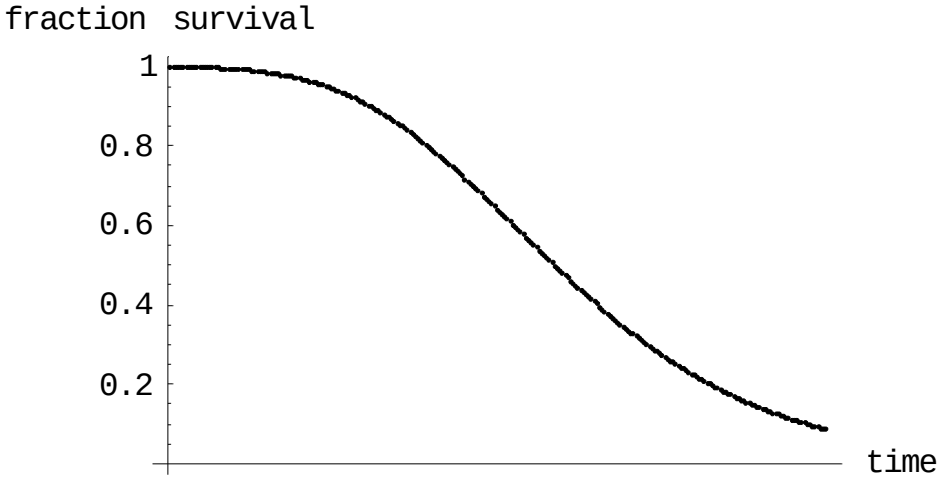

Figure 4 *Fraction of the initially consumed vegetative cells surviving the stomach with time.*

Outflow of microbes from the stomach is modelled by a gamma distribution that describes the transport of food through a series of compartments (in this case two). A second parameter (*b*) in the gamma distribution represents the average time it takes to flow through one compartment (on average 82 minutes according to Takumi et al. (2000)). Overall, the gamma distribution represents the fraction of the initially consumed food volume that leaves the stomach per time interval. As vegetative cells and spores are assumed to be homogeneously distributed in the food, this distribution also represents the fraction of microbes that leave the stomach per time unit. Figure 5 visualises the passage of microbes enclosed in food parcels

through the stomach as proposed by Takumi et al. (2000). Microbes are homogeneously distributed over the initially consumed food. Little food parcels are transported at the start of digestion, followed by bigger pieces as digestion progresses and, again small pieces are transported towards the end of the stomach passage. Note, however, that Figure 5 implies that particles are being transported in discrete units over some time span Δt, whereas the model, described by differential equations, represents a continuous system, *i.e.* $\Delta t \rightarrow 0$.

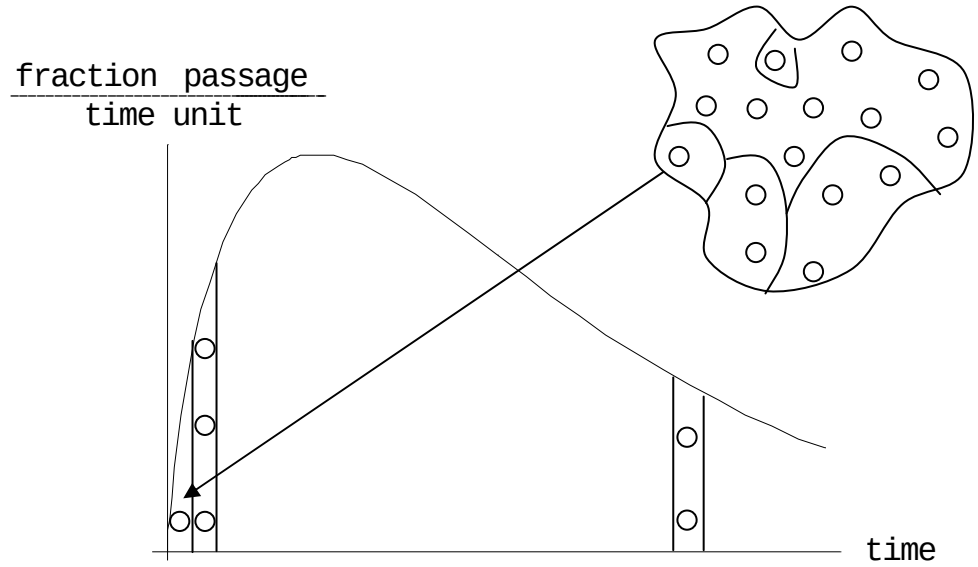

Figure 5 Visualisation of the fraction of initially consumed microbes (enclosed in the food parcel, top right corner) being transported to the small intestine in time.

Overall stomach passage is a combination of inactivation and outflow (eq. 1a) for the vegetative cells and, as spores are not inactivated during stomach passage, the gamma distribution represents spore transport. Figure 6 shows the total number of *B. cereus* units that passed the stomach in time for vegetative cells and spores as described by eq.1a and 1b respectively.

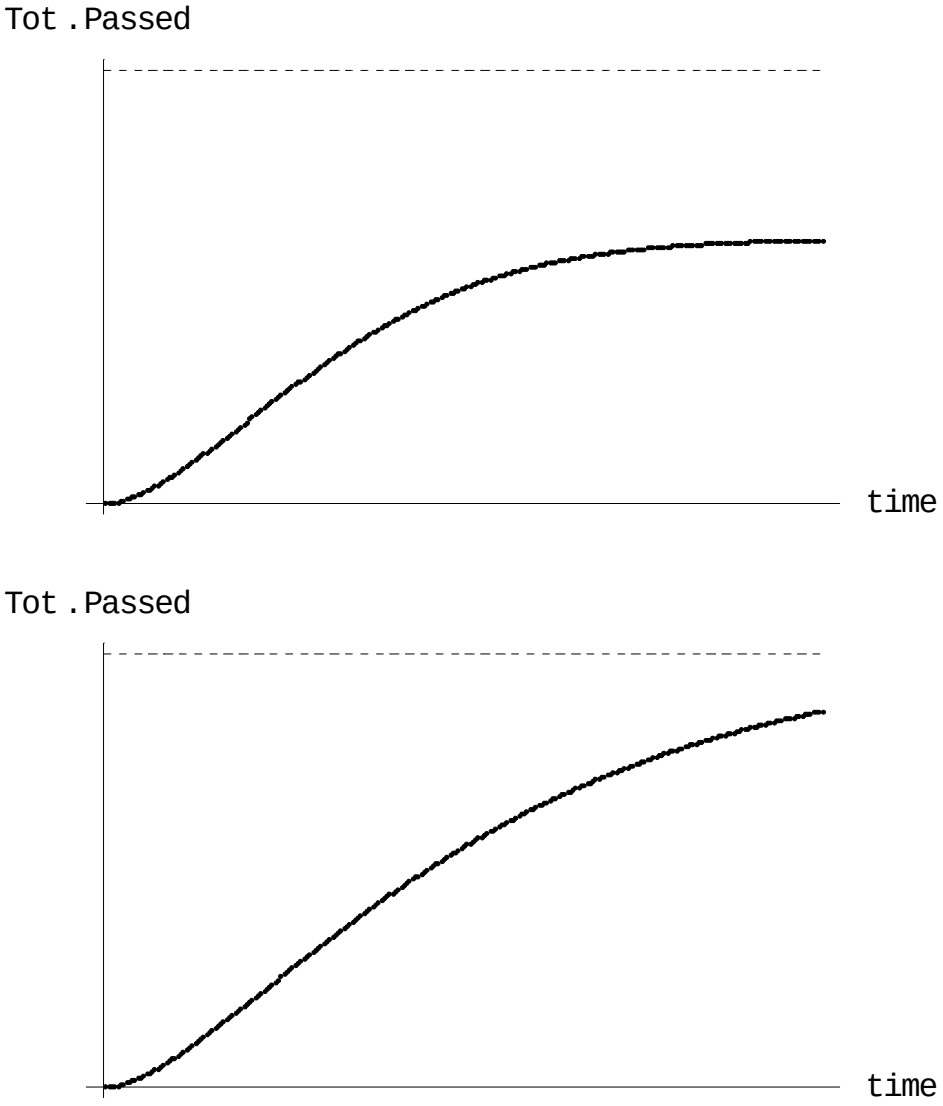

Figure 6 Qualitative representation of the total amount of vegetative cells (top) and spores (bottom) that have passed the stomach in time. (dotted horizontal line represents the initially consumed amount of vegetative cells and spores).

2.3 Small intestine dynamics

Vegetative cells and spores are assumed to be transported continuously from the stomach into the small intestine until all of the initially consumed food has been transported. Depending on the initially consumed potentially infectious units (vegetative cells and/or spores), the following processes can play a role influencing the ultimate production of enterotoxins:

1. Consuming food contaminated with spores.

Spores flowing into the small intestine are assumed to adjust to their new environment relatively fast and so start germinating once they passed their relatively short lag-phase (Clements and Moir, 1998). Multiplication of the then formed vegetative cells is assumed not to start instantly after germination. Instead, growth is assumed to be initiated after a lag-phase too. An additional process in the small intestine concerns the adhesion of microbes to the epithelial cell membrane. Although the relevance of adhesion to ultimate toxin production is point of present discussion (see Introduction), it is important with respect to the residence time of microbes. That is, being adhered to the epithelial cell membrane gives microbes more time to contribute to enterotoxin production circumventing the continuous flush through the small intestine that would result in a relatively fast outflow of microbes to the large intestine. Adherence of microbes to the cell membrane of the small intestine is incorporated in Figure 7 which gives a schematic representation of the routes through which spores can contribute to the potential production of enterotoxines.

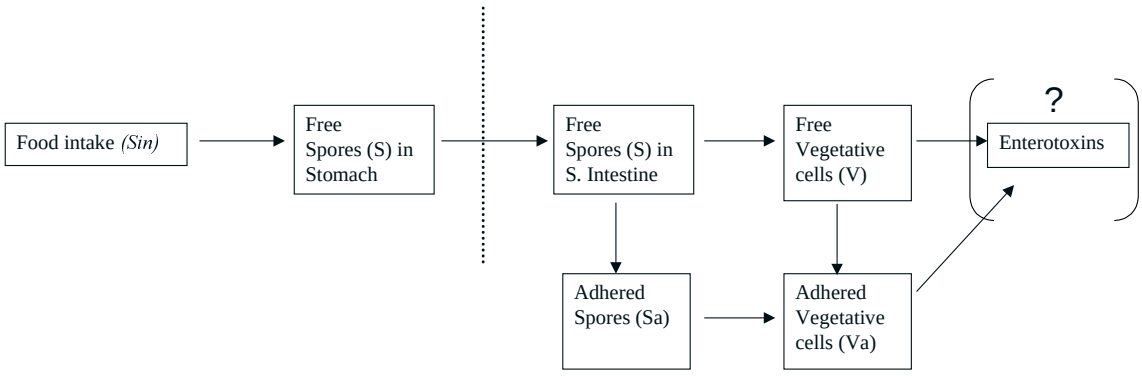

Figure 7 Microbe dynamics upon the consumption of spores.

As can be seen from Figure 7, adhered spores are assumed to germinate to become adhered vegetative cells. Release of adhered microbes together with inactivation and outflow processes will have a negative impact on toxin production and will be discussed later in this section. The enterotoxin box has been put between brackets in Figure 7, because little is known about the ultimate production process of toxins. Model output stops with the development of spores and vegetative cells in the small intestine. Suggestions for potential food intoxication will be based on these outputs.

2. *Consuming food contaminated with only vegetative cells.*

Just like spores,vegetative cells entering the small intestine will have to adjust to their new environment, which is expressed in a lag-phase induced growth process. As with spores, vegetative cells can also adhere to the epithelial cell membrane (Wijnands et al., submitted). Figure 8 gives a qualitative representation of possible processes through which vegetative cells can contribute to enterotoxin production in the small intestine.

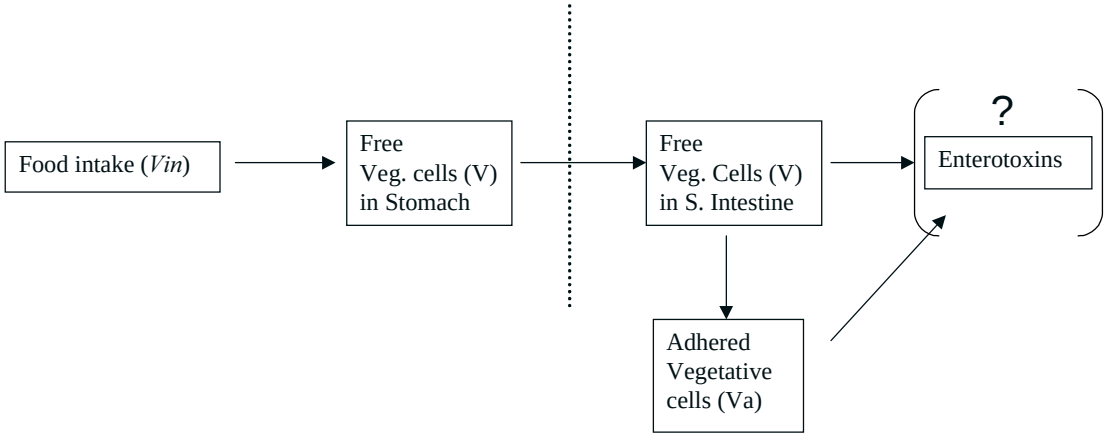

Figure 8 Intoxication process upon the consumption of vegetative cells

Again, release of adhered *B. cereus* cells, together with inactivation and outflow, will have a negative impact on toxin production and will be discussed in the next section.

3. *Consuming food contaminated with both spores and vegetative cells*.
Figure 9 shows the overall assumed major *B. cereus* / small intestine interaction dynamics that influence potential enterotoxin production when food is consumed in which both spores and vegetative cells are present.

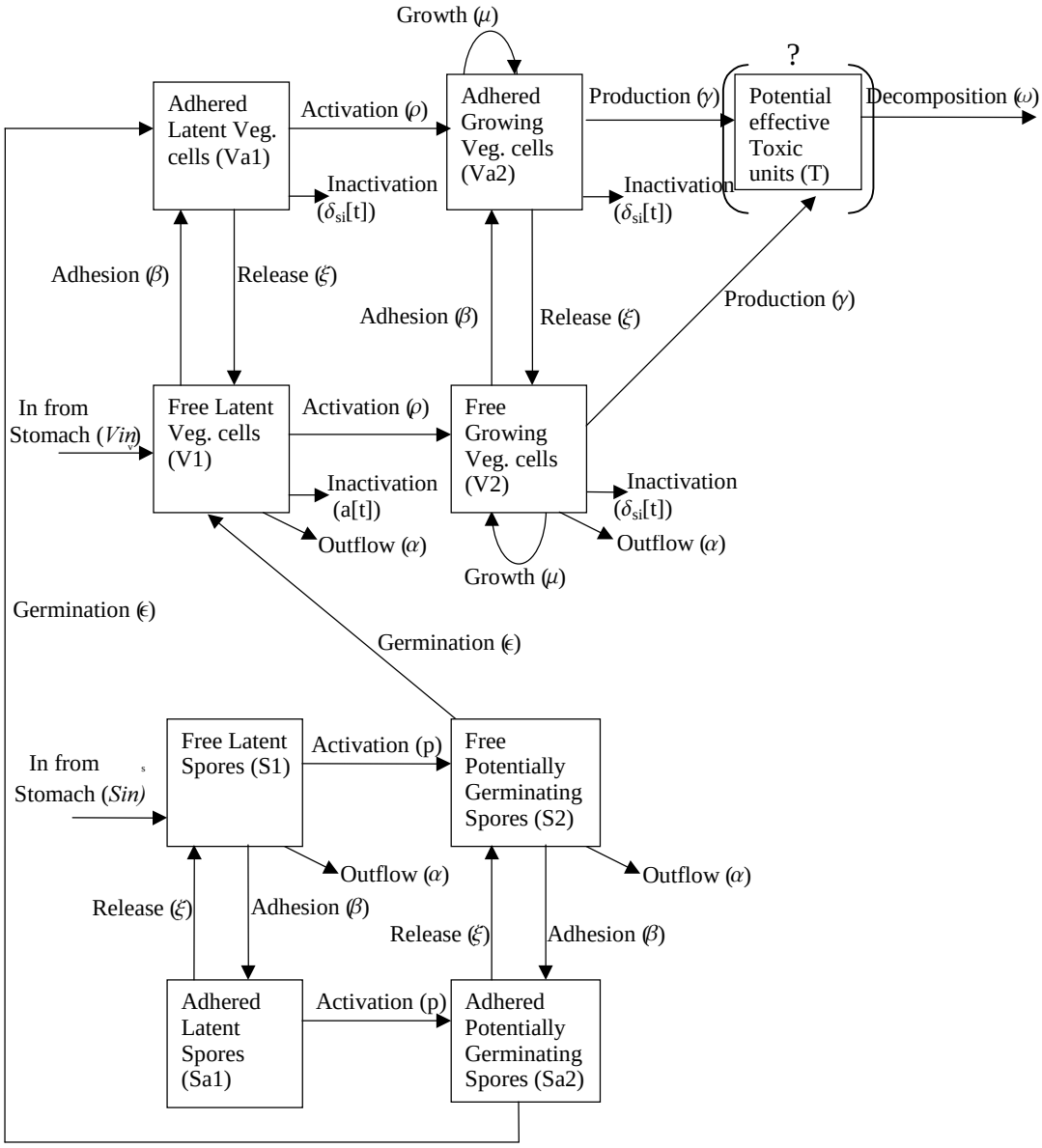

Figure 9 Flow diagram of the most important B. cereus dynamics in the small intestine. Inactivation of vegetative cells is described by a time dependent function $\delta_{si}[t]$ and is explained, as all other variables, in the text.

Although this might look like a complicated schedule, it basically just combines Figures 7 and 8, now including the following quantitative variables:

2.3.1 Activation *(ρ, p)*

Once vegetative cells enter the small intestine they are assumed to need some time τ to adapt to their new environment before they are "activated" and start multiplying (Figure 9, top).

Traditionally, this so-called lag-phase is assumed to be a fixed time period after which bacteria start multiplying. However, due to the diversity in *B. cereus* microbes, the lag-phase is more likely to be variable (*i.e.* some of the vegetative cells need a longer time to adapt than others do). The deterministic (fixed lag times) and stochastic (variable lag times) approach of modelling the bacterial lag phase has thoroughly been explained by Baranyi (1998 and 2002). McMeekin et al. (1993) give a review on modelling the microbial lag phase.
We assume a stochastic process where vegetative cells are being activated independently and, therefore, the number of activated vegetative cells within time T=t is poisson distributed. Instead of the traditional "all-or-nothing" principle, this distribution results in variable lag times for a population of *B. cereus* cells entering the small intestine. More explicitly, given the probability of being activated per time unit is denoted by ρ, the mean number of activations within time T=t equals ρ t. Under these conditions, the probability of a vegetative cell being activated within some time span has an exponential probability distribution. In general:

$$F(t) = 1 - e^{-\lambda t}, \qquad (4)$$

where
$F(t)$ cumulative probability of events within time t,
λ probability of an event to occur per time unit.

For our purposes, F(t) can be interpreted as the fraction of the total number of vegetative cells being activated within some time t.

Now let t=τ, the median latent period, *i.e.* the time within which at least 50% of the latent vegetative cells are activated and start growing. Then let $\lambda = \rho$, the fraction of latent vegetative cells being activated per time unit, which can be calculated from (4) following

$$\rho = \frac{-\ln[1 - F(t)]}{\tau} \quad | F(\tau) = 0.5 \qquad (5)$$
$$= \frac{\ln[2]}{\tau}.$$

Figures 10 and 11 visualise the activation process of vegetative cells when the median lag-time before the onset of multiplication is set to 60 minutes. Figure 10 shows that the probability of a cell being activated per time unit, $f(t) = \frac{d}{dt}F(t)$, decreases exponentially with time. It shows that at least 50 % of the vegetative cells have started multiplying after 60 minutes. It also indicates that, in this case, 1 % of the vegetative cells started growing after 1 minute upon arrival in the small intestine. As time progresses more and more vegetative cells will have passed their individual lag phase and started growing (Figure 11).

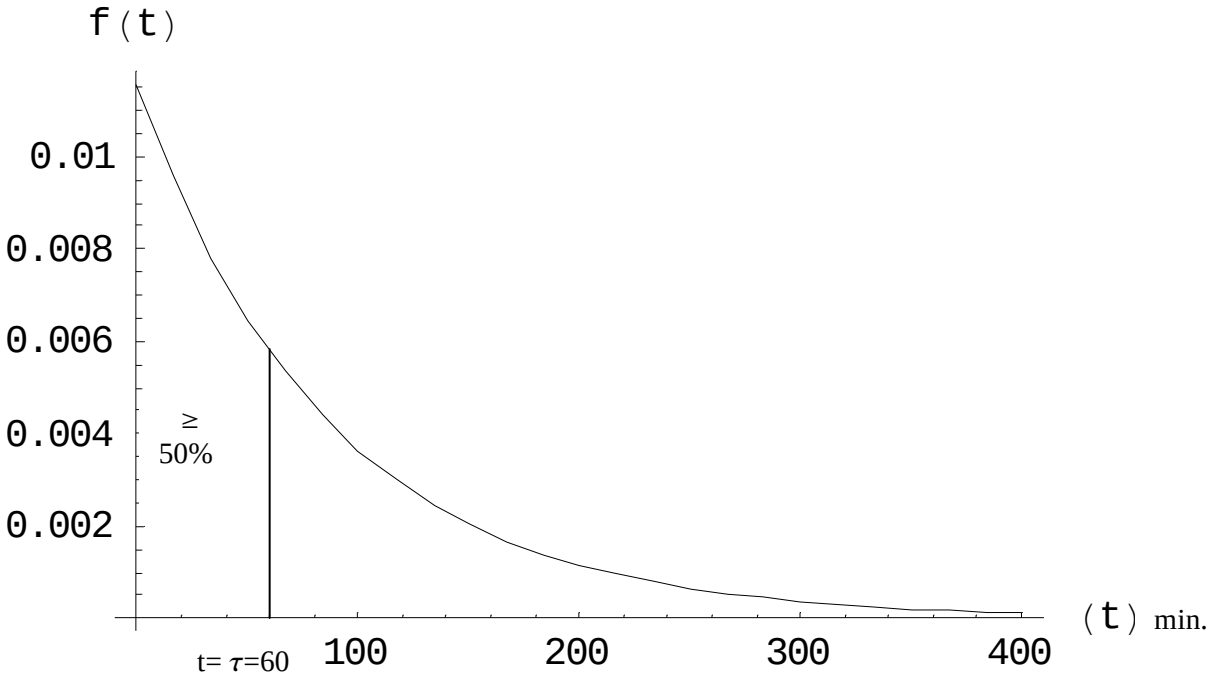

Figure 10 Activation of vegetative cells following an exponential curve.

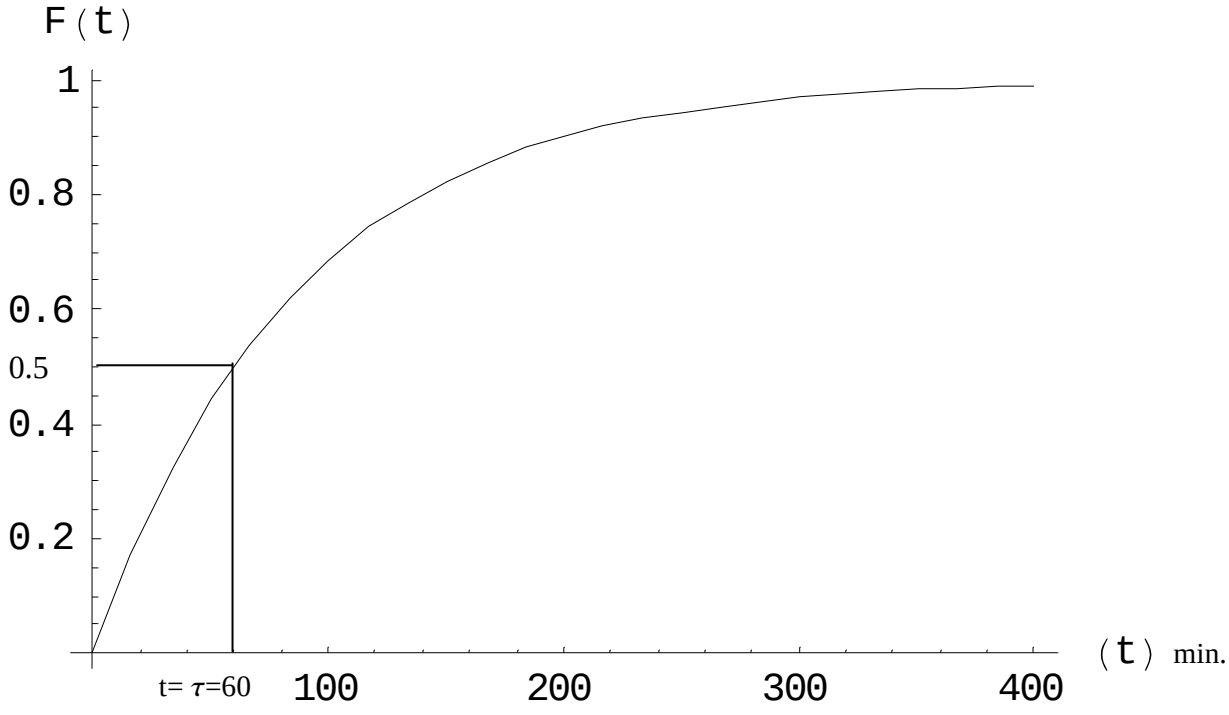

Figure 11 Total fraction of vegetative cells being activated in time upon arrival in the small intestine.

Free spores entering the small intestine experience a lag-phase too upon which they can start germinating (Figure 9, bottom). The fraction of latent spores being activated per time unit, p, can be calculated following the reasoning for vegetative cells. If the median latent period for spores is denoted by Γ, then $p = -\ln(0.5)/\Gamma = \ln(2)/\Gamma$.

Similar calculations apply to adhered latent vegetative cells and spores.

2.3.2 Growth *(μ)*

Once activated, vegetative cells are assumed to start growing exponentially (Van Gerwen and Zwietering, 1998) with growth rate μ following:

$$\ln(V) = \ln(V_0) + \mu\, t\,, \qquad (6)$$

in which

V number of (adhered, Va2) vegetative (V2) cells (Figure 9, top)
μ average change in $\ln(V)$ per time unit,
t time.

The implementation of a lag time based on stochastic principles gives, on average, a development of bacteria in time that is similar to using the traditional lag-exponential growth curve (Van Gerwen and Zwietering, 1998) following:

$$\ln(V) = \ln(V_0), \qquad for \;\; t < \tau$$
$$\ln(V) = \ln(V_0) + \mu(t - \tau), \qquad for \;\; t \geq \tau.$$

Figure 12 shows the similar growth curves using a realistic lag-time, τ=28 minutes (see section 3.3.1), which corresponds to ρ=0.025. In addition, a growth rate, μ=0.016 min^{-1}, has been used.

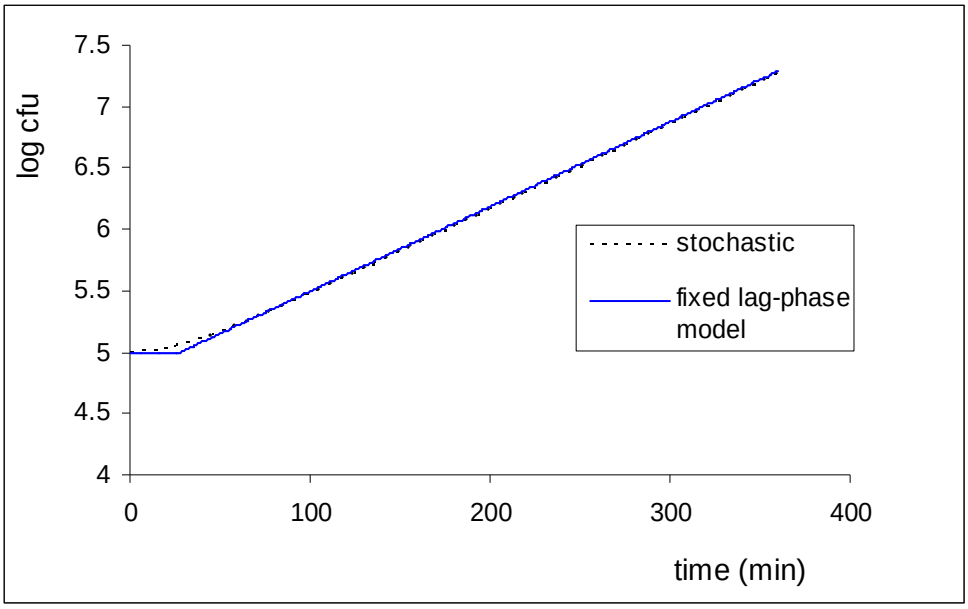

Figure 12 Difference between vegetative cell development based on the implementation of a traditional lag-exponential growth curve (solid line) or using stochastic processes (dotted line)

2.3.3 Germination *(ϵ)*

Spores germinate with rate ϵ. However, it is difficult to assess this parameter as lag- and germination time is always measured jointly during experiments. If, for example, experimental results show that 99 % of the spores have germinated within 30 minutes, then which part of this 30 minutes can be attributed to lag-time and after what time period did the spores actually start germinating? Variability in germination and growth of microbes has been previously discussed by Barker et al. (2003). The separation of a lag-phase from the actual germination of spores has been included in this model. Results will show the impact of varying lag- and germination times (within the constant time slot as found in experimental assessments) on the growth of vegetative cells in the small intestine.

Spores are assumed to germinate independently. The germination rate can therefore (following section 2.3.1), be calculated using an exponential probability distribution with ϵ being the probability of a spore germinating per time unit. Coming back to the example of 99 % of the spores having germinated within 30 minutes then:

$$\varepsilon = \frac{-\ln(0.01)}{30}.$$

2.3.4 Adhesion *(β)*

The number of vegetative cells and spores that adhere to Caco-2 cells within some time span can be assessed experimentally (Wijnands et al., submitted). From that, the fraction adhered

vegetative cells (spores) of the initial number can be calculated. If the duration of the experiment is known and microbes are assumed to adhere independently then, as explained above, the adhesion rate can be calculated following an exponential probability distribution and so,

$$\beta = \frac{-\ln[1 - F(t)]}{t}. \qquad (7)$$

To this end it is assumed that the adhesion process is density independent and that Caco-2 cells are not saturated within the time of the assessment.

2.3.5 Release *(ξ)*

Microbe adhesion is assumed to mainly occur in the first part of the small intestine (duodenum) where they are assumed to being spread homogeneously over the cell membrane surface. Release is assumed to only occur during epithelial cell membrane turnover and considered proportional to this cell renewal. Subsequently, the release rate can be calculated based on the speed with which the cells of the duodenum are renewed.

2.3.6 Inactivation *(δ_{si})*

Bile juices are assumed to have a major effect on vegetative cell inactivation in the small intestine. Bile concentration is high when food first arrives in the small intestine and decreases with time (Minekus et al., 1995). Therefore, the bile course is described with an exponentially decreasing function with time analogous to the pH course in the stomach (eq.3). So,

$$Bile[t] = (Bile_{\max} - Bile_{\min})e^{-k_{bile}t} + Bile_{\min}, \qquad (8)$$

where
$Bile[t]$ = small intestine bile concentration course,
$Bile_{max}$ =maximum bile concentration upon food consumption,
$Bile_{min}$ =minimum bile concentration after the consumption of a meal,
k_{bile} = rate with which the bile concentration decreases from $Bile_{max}$ to $Bile_{min}$.

Contrary to the function describing the pH course in the stomach, inactivation of vegetative cells in the small intestine is proportional to the bile concentration. Therefore, the inactivation rate (δ_{si}) of vegetative cells is introduced as

$$\delta_{si}[t] = c\ Bile[t], \qquad (9)$$

where

c = constant describing the linear inactivation of vegetative cells with bile concentration.

2.3.7 Outflow *(α)*

The speed with which microbes are transported from the duodenum to the jejunum is assumed to be proportional to the average small intestinal flow through. If the average flow through time (time until, say, 99% of the food has been transported to the jejunum) is known, then the outflow rate of microbes (α) can be calculated following

$$\alpha = \frac{-\ln[1-F(t)]}{t}. \qquad (10)$$

2.3.8 Production *(γ)*

Although several studies exist on the expression of enterotoxins, little is known about the actual production process of these toxins by vegetative cells in the small intestine. McKillip (2000), for example, provides a literature review on enterotoxin production by *Bacillus cereus*. Wijnands et al. (2002b) describe the pathogenic mechanism of the diarrheal syndrome. Duport et al. (2004) found an increased production of the enterotoxin HBL for *B. cereus* microbes having low growth rates under anaerobic conditions. In addition, preliminary *in vitro* experiments (not published) showed that vegetative cells are supposed to have a threshold value above which toxin production starts after which toxin production was found to be linear with bacterial growth (*i.e.* duplication of microbes means duplication of toxin content). This would indicate that the change in the number of toxic units ($\dot{T}$) equals the change in the number of growing (free / adhered) vegetative cells ($\dot{V}2/\dot{V}a2$) once a certain threshold value of vegetative cells in the small intestine has been reached. Or,

$$\dot{T} = \dot{V}2 + \dot{Va}2, \quad | \quad V2 + Va2 \geq threshold\,. \qquad (11)$$

This threshold level seems to be quite arbitrary and closely related to a detectable level of enterotoxins in experiments. The practical implication of this lack of knowledge will be discussed further in section 3.3.9.

2.3.9 Decomposition *(ω)*

The preliminary experiments on toxin production of vegetative cells (not published) also showed a drastic decrease in enterotoxic activity during the stationary phase of *B. cereus* growth. Little information is available about the decomposition process during toxin production, only that toxins are probably highly unstable.

Above described processes with accompanying assumptions have lead to the mathematical model formulation describing the fate of *B. cereus* in the gastro-intestinal tract and will be discussed in the next section.

2.3.10 The model

The processes described so far are considered to be of major importance when analysing the fate of *B. cereus* in the gastro-intestinal tract to ultimately be able to predict the amount of enterotoxin production in the small intestine. Taking into account the assumptions under which the *B. cereus* / gastro-intestinal biophysical dynamics are described, the following mathematical model was formulated for stomach dynamics:

$$\dot{MV}1 = -bMV1(t) - \delta(t)MV1(t), \quad | MV1(0) = Vin$$

$$\dot{MV}2 = bMV1(t) - bMV2(t) - \delta(t)MV2(t), \quad | MV2(0) = 0$$

$$\dot{MS}1 = -bMS1(t), \quad | MS1(0) = Sin$$

$$\dot{MS}2 = bMS1(t) - bMS2(t), \quad | MS2(0) = 0,$$

and, for small intestine dynamics:

$$\dot{V}1 = bMV2(t) + \varepsilon S2(t) + \xi Va1(t) - \beta V1(t) - \rho V1(t) - \delta_{si}(t)V1(t) - \alpha V1(t), \ | V1(0) = 0$$

$$\dot{V}2 = \rho V1(t) + \mu V2(t) + \xi Va2(t) - \beta V2(t) - \delta_{si}(t)V2(t) - \alpha V2(t), \ | V2(0) = 0$$

$$\dot{S}1 = bMS2(t) + \xi Sa1(t) - \beta S1(t) - pS1(t) - \alpha S1(t), \ | S1(0) = 0$$

$$\dot{S}2 = pS1(t) + \xi Sa2(t) - \beta S2(t) - \varepsilon S2(t) - \alpha S2(t), \ | S2(0) = 0$$

$$\dot{Va}1 = \varepsilon Sa2(t) + \beta V1(t) - \xi Va1(t) - \rho Va1(t) - \delta_{si}(t)Va1(t), \ | Va1(0) = 0$$

$$\dot{Va}2 = \rho Va1(t) + \mu Va2(t) + \beta V2(t) - \xi Va2(t) - \delta_{si}(t)Va2(t), \ | Va2(0) = 0$$

$$\dot{Sa}1 = \beta S1(t) - \xi Sa1(t) - pSa1(t), \ | Sa1(0) = 0$$

$$\dot{Sa}2 = pSa1(t) + \beta S2(t) - \xi Sa2(t) - \varepsilon Sa2(t), \ | Sa2(0) = 0$$

$$\dot{T} = \gamma \dot{V}2 + \gamma \dot{Va}2 - \omega T(t), \ | \ T(0) = 0, \quad and \ V2 + Va2 \geq threshold.$$

These differential equations show the *change* in the different *B. cereus* units and toxic compounds per time unit for both the stomach and small intestine. Solving this set of linear equations will give the number of (adhered) vegetative cells, (adhered) spores and toxin units in time present in the stomach and small intestine during the digestion of a meal containing an initial number of vegetative cells (*Vin*) and/or spores (*Sin*).
Model output will reveal the relative impact of included processes and accompanying variables on the potential production of enterotoxins. To give an example, not only the effect of including adhesion on toxin production can be assessed, but with that the impact of the rate with which this process occurs.

Still, before any model outputs can be shown, reasonable parameter values have to be obtained. The source of parameter values used for model simulations will be discussed in the next section.

3. Implementation of reasonable parameter values for model simulation

3.1 Exposure to B. cereus

An initial exposure to an *absolute total number* of 10^5 vegetative cells and/or spores has been used for all model simulations. And so $Vin = 10^5$ and/or $Sin = 10^5$. This number is comparable with the consumption of a meal in which at most $2 \cdot 10^5$ cells are present.
An extra set of model runs has been performed in which exposure to the maximum limit (10^5 cfu g^{-1}, see Introduction) *i.e.* 10^7 *Bacillus cereus* cells per meal of 100 g, was simulated. This was done to put the proposed criterion in the light of potential food intoxication.

3.2 Stomach dynamics

Average results as reported by Takumi et al. (2000) have been used as default parameter values to model transport and inactivation of *B. cereus* microbes in the stomach (Table 1).

Table 1 Default parameter values representing stomach dynamics following Takumi et al. (2000).

Parameter	Value
pH_{max}	5
pH_{min}	2
k	$1.6 \cdot 10^{-2}$ min^{-1}
d	0.83 min^{-1}
b	$5.6 \cdot 10^{-2}$ min^{-1}

Although all parameters have been discussed in earlier sections, the outflow rate parameter (*b*) still needs some more explanation. Takumi et al. (2000) estimated an average time of 82 minutes for a meal to pass a stomach compartment. Following the reasoning as given for the outflow of microbes from the small intestine (Section 2.3.7), the outflow rate, *b*, for microbes in the stomach can then be calculated as:

$$\frac{-\ln[1-0.99]}{82} = 0.056 \text{ min}^{-1}.$$

3.3 Small intestine dynamics

Table 2 shows the default parameter values to model the biophysical small intestine dynamics.

Table 2 Default parameter values representing small intestine dynamics

Parameter	Value
ρ	$2.5 \cdot 10^{-2}$ min^{-1}
p	$9.6 \cdot 10^{-3}$ min^{-1}
μ	$1.6 \cdot 10^{-2}$ min^{-1}
ϵ	$1.5 \cdot 10^{-1}$ min^{-1}
β	$2.9 \cdot 10^{-4}$ min^{-1}
ξ	$6.4 \cdot 10^{-4}$ min^{-1}
Bile_{max}	15.2
Bile_{min}	4
k_{bile}	$0.7 \cdot 10^{-2}$ min^{-1}
c	$0.1 \cdot 10^{-2}$ min^{-1}
α	$2.6 \cdot 10^{-2}$ min^{-1}
γ	1
ω	?

As the origin of these values is more ambiguous, the derivation of these values needs an elaborated explanation.

From a hazard characterisation perspective it would be interesting to see if specific subgroups of *B. cereus* strains can potentially produce more enterotoxins than others under the same circumstances. The hypothesis is that mesophilic strains (having a minimum growth temperature at 10 °C and able to grow at temperatures > 37 °C (Pielaat et al., submitted)) should have high enterotoxin production capacity in the small intestine where growing conditions are optimal. On the other hand, psychrotolerant strains (able to grow at temperatures below 10 °C and have a maximum growth temperature at 37 °C (Pielaat et al., submitted)) are assumed to be less hazardous in the small intestine because low growth rates in these conditions might prevent high toxin production. Therefore, separate parameter values

were calculated/estimated, and shown in the tables below, for mesophylic and psychrotolerant strains whenever these data were available.

3.3.1 Activation *(ρ, p)*

The activation rate, ρ, of vegetative *B.cereus* cells was calculated using eq. 5. To this end lag-times have been estimated from experiments in which growth curves of a set of 100 *B. cereus* strains were assessed at 37 °C and pH 7 following Pielaat et al. (submitted). To be able to distinguish slow from fast growers in later simulations, lag-times were estimated subdividing the 100 strain set in psychrotolerant and mesophylic strains respectively. Table 3 shows a median estimated lag time of 28 min for both psychrotolerant and mesophylic strains. With this result the default activation rate was calculated to be $2.5 \cdot 10^{-2}$ min^{-1}.

Table 3 *Estimated lag times, τ (min), of vegetative cells following (Pielaat et al. submitted)*

	minimum	average / median	maximum
Overall	0	42 / 28	873
Psychrotolerant	0	38 / 28	113 (= largest no.2)[1]
Mesophylic	0	46 / 28	273 (= largest no.4)

[1] In this case the second highest estimated value was used as the maximum as all higher values were assumed to be outliers (*i.e.* > 1.5 ·inter quartile range away from the closest quartile).

Lag- and generation (or doubling) times inclusive were assessed experimentally for spores of 10 *B. cereus* strains kept in broth at 37 °C (Wijnands et al., submitted). This means the lag time of spores extended with the time upon which first vegetative cell doubling was observed during experiments. Table 4 shows the general results of these experiments. As the median lag time for vegetative cells was estimated to be 28 min irrespective of *B. cereus* subgroup in the 100 tested strains, the lag time, Γ, for spores was calculated by substracting 28 min from the median values in Table 4. Subsequently, activation rates of spores, p, could be calculated. This resulted in an average activation rate of $9.6 \cdot 10^{-2}$ min^{-1} for spores in general, of $6.1 \cdot 10^{-2}$ min^{-1} for psychrotolerant spores and of $1.1 \cdot 10^{-2}$ min^{-1} for mesophylic spores.

Table 4 *Lag- and generation time inclusive, $\Gamma+\tau$ (min), of spores*

	minimum	average / median	maximum
Overall	93	114 / 100	159
Psychrotolerant	105	135 / 141	159 (= largest no.2) [1]
Mesophylic	87	92 / 93	96 (= largest no.4)

[1] In this case the second highest estimated value was used as the maximum as all higher values were assumed to be outliers.

As no specific data were found for adhered microbes with respect to activation rates, above values were also used in the model for adhered latent vegetative cells and spores.

3.3.2 Growth *(μ)*

The average growth rate μ was calculated using eq. 6 with data on generation times of 10 *B. cereus* strains. Generation times were assessed for vegetative cells both grown in broth and adhered to Caco-2 cells at 37 °C (Wijnands et al., submitted). Table 5 shows the resulting average, minimum and maximum growth rate for the 10 *B. cereus* strains.

Table 5 *Growth rate, μ min^{-1} (minimum – average – maximum) for:*

	1.Vegetative cells in broth at 37 °C		
Overall	$1.0 \cdot 10^{-2}$	$1.6 \cdot 10^{-2}$	$2.3 \cdot 10^{-2}$
Psychrotolerant	$1.0 \cdot 10^{-2}$	$1.3 \cdot 10^{-2}$	$1.8 \cdot 10^{-2}$
Mesophylic	$1.6 \cdot 10^{-2}$	$2.0 \cdot 10^{-2}$	$2.3 \cdot 10^{-2}$
	2.Adhered vegetative cells to Caco-2 cells at 37 °C		
Overall	$0.3 \cdot 10^{-2}$	$1.7 \cdot 10^{-2}$	$3.5 \cdot 10^{-2}$
Psychrotolerant	$0.3 \cdot 10^{-2}$	$0.9 \cdot 10^{-2}$	$1.6 \cdot 10^{-2}$
Mesophylic	$0.9 \cdot 10^{-2}$	$2.4 \cdot 10^{-2}$	$3.5 \cdot 10^{-2}$

3.3.3 Germination *(ϵ)*

In vivo studies show that *B. cereus* spores are able to germinate in the small intestine (Casula and Cutting, 2002). Andersson et al. (1998) show that *B. cereus* spores adhering to epithelial cells were also able to germinate and Le Duc et al. (2003) showed that spores of *B. subtilis* are poorly affected during germination by bile salts. Based on literature information we assume that 99 % of *B. cereus* spores have usually germinated within 30 minutes. A default

germination value was therefore set to 0.15 min^{-1} (see section 2.3.3 for further explanation). Model results will show the impact of using different germination rates on the ultimate potential enterotoxin production.

3.3.4 Adhesion *(β)*

Percentages of vegetative cells and spores that adhere to Caco-2 cells within one hour were assessed experimentally using the same 10 *B. cereus* strains as in section 3.3.1. and 3.3.2 (Wijnands et al., submitted). Resulting adhesion rates (calculated using eq. 7) are shown in Table 6.

Table 6 *Adhesion rate, β min^{-1} (minimum – average - maximum) for:*

	1.Vegetative cells and spores		
Overall	$1.0 \cdot 10^{-7}$	$2.9 \cdot 10^{-4}$	$1.3 \cdot 10^{-3}$
Psychrotolerant	$8.3 \cdot 10^{-6}$	$3.9 \cdot 10^{-4}$	$1.3 \cdot 10^{-3}$
Mesophylic	$1 \cdot 10^{-7}$	$1.7 \cdot 10^{-4}$	$5.9 \cdot 10^{-4}$
	2.Vegetative cells		
Overall	$1.0 \cdot 10^{-7}$	$4.6 \cdot 10^{-4}$	$1.3 \cdot 10^{-3}$
Psychrotolerant	$3.3 \cdot 10^{-5}$	$6.3 \cdot 10^{-4}$	$1.3 \cdot 10^{-3}$
Mesophylic	$1.0 \cdot 10^{-7}$	$2.3 \cdot 10^{-4}$	$5.9 \cdot 10^{-4}$
	3.Spores		
Overall	$5.0 \cdot 10^{-7}$	$1.3 \cdot 10^{-4}$	$3.7 \cdot 10^{-4}$
Psychrotolerant	$8.3 \cdot 10^{-6}$	$1.6 \cdot 10^{-4}$	$3.6 \cdot 10^{-4}$
Mesophylic	$5.0 \cdot 10^{-7}$	$1.0 \cdot 10^{-4}$	$3.0 \cdot 10^{-4}$

3.3.5 Release *(ξ)*

Experiments at our laboratory showed that $1.6 \cdot 10^{5}$ Caco-2 cells occupy a total surface area of 0.0035 m^2. The surface area of the small intestine is approximately 200 m^2 with a length of 6 m (Marieb, 1998). This would correspond to a total of approximately $9 \cdot 10^{10}$ epithelial cells forming the small intestine. It is assumed that the main toxic effect of the *B. cereus* microbes occurs in the first meter (duodenum) which occupies $1.5 \cdot 10^{10}$ epithelial cells. These cells, say 99%, are assumed to be renewed approximately every 5 days (Moffett et al., 1993). That means, a turn over rate of

$$\frac{-\ln[1-0.99]}{5 \cdot 24 \cdot 60} = 0.00064 \text{ cells min}^{-1}.$$

As vegetative cells and spores are assumed to homogeneously adhere to the duodenal cell surface and microbe release is assumed to be proportional to epithelial cell turn over, a default release rate was set to $6.4 \cdot 10^{-4}$ min^{-1}.

3.3.6 Inactivation *(δ_{si})*

Parameter values describing the course of the bile concentration (eq. 8) have been estimated using kinetics of bile salt concentrations in the duodenum from Fig. 7 in Minekus et al. (1995). The rate with which the bile concentration decreases from $Bile_{max}$ to $Bile_{min}$ (k_{bile}) has been estimated fitting eq. 8 to the duodenal data of Fig. 7 in Minekus et al. (1995).

As for the inactivation of vegetative cells in the presence of bile salts, the report of Wijnands et al. (submitted) suggests that inactivation of *B. cereus* can be assumed to be very small. Tables 4 and 5 in Appendix 3 of their report show no growth (so, inactivation might occur) of psychrotolerant strains and slow growth of mesophilic strains. As no further data were available describing the inactivation process of vegetative cells under the influence of different bile concentrations, c was set to a small inactivation value of 0.001. Although this is a rough estimate, still, the sensitivity of the model output becomes smaller as $c \rightarrow 0$.

The resulting parameter estimates are shown in Table 2.

3.3.7 Outflow *(α)*

The average total flow through time (time until, say 99% of the food has been transported to the jejunum) is assumed to be 3 h (Fig 2 in Minekus et al. (1995)). Therefore, the outflow rate of microbes (α) was calculated from eq. 10 to be 0.026 min^{-1}.

3.3.8 Production *(γ)*

Preliminary in vitro experiments (not published) showed (through ELISA techniques) that the production of HBL and NHE toxins can be detected at bacterial counts of approximately $5 \cdot 10^6$ to $1 \cdot 10^7$ colony forming units ml^{-1}. However, preliminary cytotoxicity tests showed the onset of cell destruction at cell counts of approximately 10^5 after 3 hours upon the initial adhesion of 10^3 spores to Caco-2 cells (unpublished).
As the toxin production rate was subsequently found to be proportional to germ growth (eq. 11), γ was set to be 1. This indicates that duplication of microbes and duplication of toxin content has a 1:1 relationship once a threshold of bacteria has been formed.

3.3.9 Decomposition *(ω)*

As stated before, little is known about the decomposition rate of enterotoxins in the small intestine. Therefore, no default parameter value could be filled out for this variable.

Up until now no information is available about the exact production process nor about the behaviour of the different enterotoxins HBL, NHE and cytK in the small intestine leading to the toxic effects upon exposure to *B. cereus*. Preliminary experiments raise questions like; "do *B. cereus* strains actually not produce any toxins until bacterial counts in the order of 10^6 - 10^7 ml^{-1} or can toxins only then be detected?" In other words, do these bacterial counts actually represent the onset of toxin production or are they intrinsic to a measuring bias? Other questions concern the circumstances under which bacteria produce toxins. That is, does *B. cereus* always produce enterotoxins or, maybe, only in stress situations? In addition, particular food components could possibly influence toxin production, etc.

Further research is needed on the production of the separate toxin compounds by different *B. cereus* strains, their hazardousness and potential toxin interactions, before valuable model outputs can be obtained on enterotoxin production. To this end model outputs will only show the fate of both spores and vegetative cells in the small intestine under different biophysical conditions. Subsequently, potential hazardous food poisoning scenario's can be identified without making insinuations about corresponding toxin concentrations.

4. Results and interpretation

The impact of different food consumption scenarios on the ultimate development of microbes in the duodenal part of the small intestine was tested under different biophysical conditions. For example, the difference between eating preheated food, containing only spores, and chilled foods with vegetative cells and/or spores with respect to the ultimate microbe development in the duodenum was assessed for different *B. cereus* growing conditions. However, before testing different interesting potential food poisoning scenarios in combination with different system dynamics, model output will first be explained under default conditions (Figure 13 with Tables 1 and 2).

Kramer and Gilbert (1989) reported that *B. cereus* microbes can initiate symptoms between approximately 8 and 16 hours after food consumption. Granum et al. (1995), however, reported symptoms after a day. Based on these references all simulations were run for 24 hours. However, for a transparent view of microbe development (numbers in time), figures only show the first 12 hours (760 minutes on the x-axis) of the output. The ultimate number of microbes present in the duodenum after 24 hours will be mentioned and further discussed in the text where relevant.

Figures 13a and b show the fate of *B. cereus* microbes in the gastro-intestinal tract under default biophysical conditions after the consumption of a meal containing 10^5 spores and 10^5 vegetative cells. Subfigures show the dynamics of microbes in their different stages represented by the model compartments in Figures 2 and 9. That is, Figure 13a shows stomach dynamics; Figure 13b shows the development of (adhered) vegetative cells and spores in the duodenum.

Figure 13a shows the quantitative behaviour of microbes in the stomach with an average flow through time of close on 3 hours. The inactivation rate of vegetative cells seems to be small relative to the initially consumed amount of microbes (compare MV2 with MS2). A continuous food flow through results in the influx of latent vegetative cells (MV1→V1) and latent spores (S1→MS1) in the small intestine.

After a median lag time of 28 min (Section 3.3.1), vegetative cells enter their active stage, V2, where further growth dynamics determine the ultimate number of *B. cereus* cells in the small intestine. Recall, however, that this median lag-time is associated with an exponential distribution (Figure 10), resulting in a continuous activation of V1 cells to V2 cells upon entering the duodenum. In addition, latent vegetative cells can adhere to the epithelial cell membrane and so become Va1 cells from where they can go into the active growing stage (Va2). The adhered, growing, vegetative cells can themselves also contribute to potential

toxin production. Furthermore, there is an exchange between adhered and “free” vegetative cells, *i.e.* V1⇆ Va1 and V2 ⇆ Va2.

In addition to the activation process that causes the number of latent vegetative cells ultimately to decline, there are other processes contributing to this decline. That is, outflow to the jejunum of V1 cells, turn over of the epithelial cell membrane followed by the outflow of Va1 cells and inactivation of both V1 and Va1 cells influenced by bile juices.

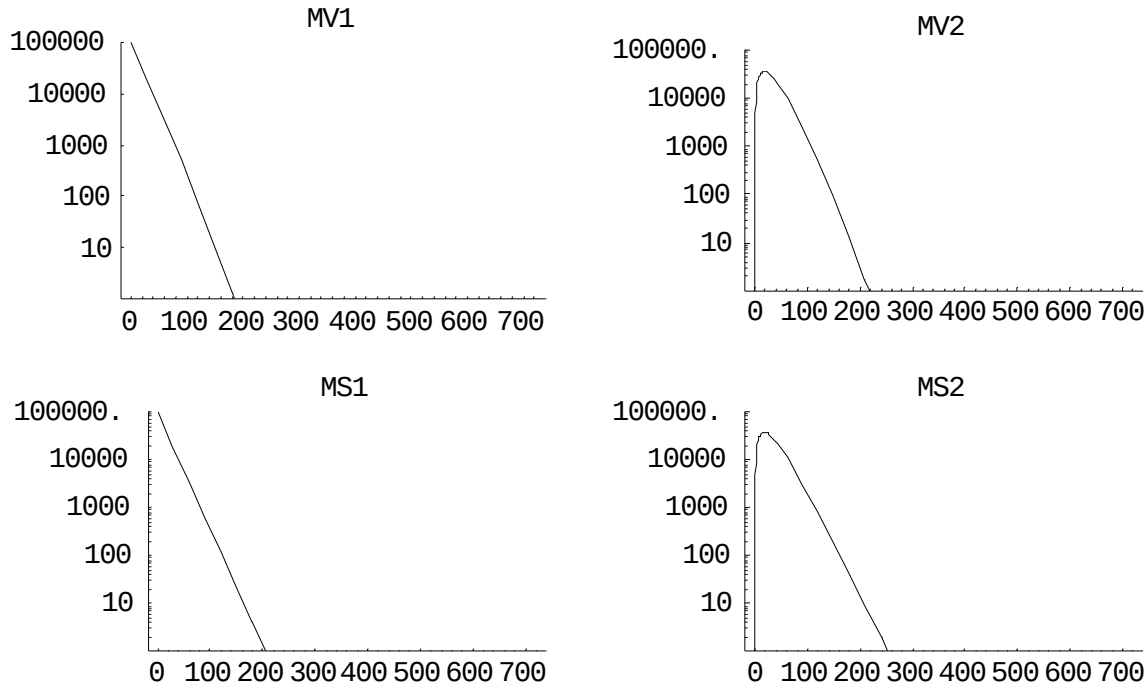

Figure 13a Model output for stomach dynamics using default parameter values, i.e. b=0.056, ρ=0.025, p=0.0096, ϵ=0.15, β=0.00029, ξ=0.00064, α=0.026, μ=0.016. (x-axis values are in minutes after food intake; y-axis represents microbe numbers. The separate figure headings MV1 to MS2 are explained in Figure 2.

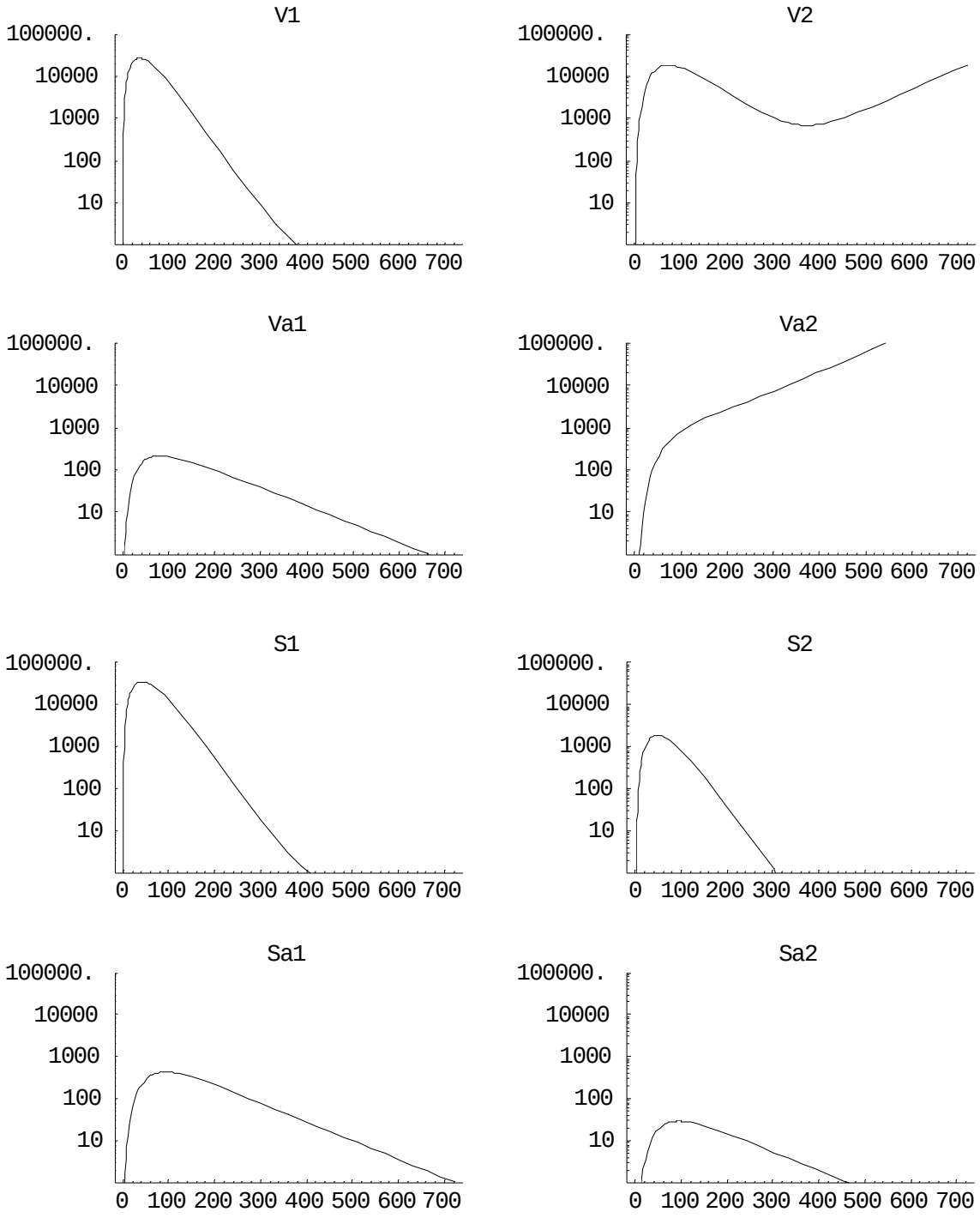

Figure 13b Model output for duodenal dynamics using default parameter values, i.e. b=0.056, ρ=0.025, p=0.0096, ϵ=0.15, β=0.00029, ξ=0.00064, α=0.026, μ=0.016. (x-axis values are in minutes after food intake; y-axis represents microbe numbers). The separate figure headings V1 to Sa2 are explained in Figure 9.

The activated, multiplying, vegetative cells (V2 and Va2) determine the ultimate potential toxin concentrations in the small intestine. Free floating vegetative cells grow to a maximum

of $1.8 \cdot 10^4$ after having spent 73 minutes in the small intestine. Numbers then decrease for, about, 4 hours after which growth continues. The number of free floating vegetative cells decreases when outflow starts dominating the activation of V1 cells. However, after 5 hours, another process starts to contribute significantly to the growth of V1 cells, namely, the release of adhered vegetative cells due to epithelial cell membrane turn over. Figure 13b shows that Va2 cells need some time to develop, a process influenced by the adhesion rate and lag-phase of vegetative cells. During this time the dynamics of free floating V2 cells is mainly influenced by the activation of V1 cells and the outflow of particles from the duodenum. At some point, Va2 cells have grown to such numbers that they can start influencing V2 dynamics. If the release rate of adhered vegetative cells is low enough (at least lower than the outflow rate in the duodenum), then adhered cells will continue to be released over a long time span and thus continuously contribute to the outgrowth of vegetative cells. In other words, the rate at which the epithelial cell membrane is being renewed has a significant impact on the potential toxin production. Figure 13b shows that this model will predict a continuous growth of (adhered) vegetative cells under default conditions and, with that, a continuous production of enterotoxins.

This model output raises the question whether the model actually improves the insight in the microbe/system interaction dynamics when a continuous production of enterotoxins is inevitable under the proposed model assumptions. Of course, an infinit production of enterotoxins is inconceivable from a physical perspective and the model could be improved on this point. Still, this relatively simple model helps us to reveal those biophysical dynamics having a major impact on the potential toxin production even when this continuous toxin production is taken into account. Although a continuous toxin production might be unrealistic, the time of onset of this second bloom of vegetative cells might be an important intoxication mechanism. Furthermore, in depth research on the mechanisms behind the most important biophysical factors can help to develop a more profound model and, with that, improve the prediction of hazardous food consumption scenarios.

Figure 13b also shows the importance of a better insight in the mechanisms behind the renewal of the epithelial cell membrane. The current model includes a release rate proportional to the present number of adhered cells at some point in time. This is inherent to assuming that adhered cells are being released independent of their location. This assumption does not represent actual microbe/small intestine interaction dynamics where colonies of vegetative cells are being formed during growth. These colonies form a cluster of many vegetative cells which are more likely to be released at the same time instead of having homogeneously adhered cells which all have equal probability of being released per time unit. In addition, the release rate is probably not constant in time. The rate at which the epithelial cell membrane is being destroyed increases as intoxication progresses. This means that the release rate of adhered cells should actually be a function of enterotoxin concentration. Further research is needed to get insight in the feed-back mechanism of epithelial cell membrane turn-over and enterotoxin production. Still, using the present model

this argumentation would indicate that the continuous growth of vegetative cells after a period of 6 hours in the small intestine is unrealistic. Subsequently, this continuous bloom of vegetative cells could be ignored when assessing the potential toxin production that could result from the consumption of a meal containing 10^5 spores and 10^5 vegetative cells under default conditions. At least, if sufficient toxins have been produced within 6 hours to induce an accelerated turn-over rate.

In contrast to the dynamics of vegetative cells, spores show a straightforward behaviour (Figures 13a and 13b).
After a median lag time of 72 min (see section 3.3.1), spores become active and start germinating with rate 0.15 S2(t) min^{-1}in the small intestine. In other words, spores enter the small intestine in the S1 stage, are then transformed to a germinating stage (S2) from which a fraction 0.15 min^{-1} become V2 cells. Like vegetative cells, spores can also adhere to the epithelial cell membrane. Therefore, an exchange exists between adhered and "free" spores following S1⇆ Sa1 and S2 ⇆ Sa2. Furthermore, adhered active spores can germinate to become adhered vegetative cells and so contribute to the ultimate toxin production.
Spore numbers ultimately decline due to a combination of activation (S1→S2 and Sa1→Sa2), germination (S2→V2 and Sa2→Va2), release due to epithelial cell membrane turn over (Sa1 and Sa2) and regular flow through processes (S1 and S2). And, as vegetative cells are not assumed to sporulate in the small intestine, spore numbers will ultimately decrease to zero.

The main conclusion from Figures 13a and b is that the consumption of food containing 10^5 spores and 10^5 vegetative cells apparently cannot lead to a toxic effect under average conditions, unless adhered vegetative cells play an active role in the toxin production. An initial maximum absolute number of $1.8 \cdot 10^4$ free floating vegetative cells is not enough if bacterial counts of $5 \cdot 10^6 – 1 \cdot 10^7$ ml^{-1} are considered to be a threshold number for toxin production. According to cytotoxicity tests, $1.8 \cdot 10^4$ cells does not seem sufficient for the onset of cell damage (visible at 10^5 cell counts). Moreover, enterotoxins are very unstable compounds which are readily degraded in the lumen of the small intestine. This supports the need for toxins produced by adhered vegetative cells as these can act directly on the cell membrane. At least $8.2 \cdot 10^4$ adhered vegetative cells would be needed in addition to the $1.8 \cdot 10^4$ V2 cells to induce the onset of cell destruction. This number is only reached after approximately 9 hours (Figure 13b, Va2). At that time, V2 cells have, however, already drastically decreased. Intoxication seems, therefore, unlikely to result under default conditions if a threshold of $5 \cdot 10^6 – 1 \cdot 10^7$ ml^{-1} vegetative cells is assumed to be necessary for toxin production.
However, if this threshold is considered to be inherent to a detection limit, then intoxication could still result under these conditions if an additive effect of relatively low concentrations of toxins is considered. The question is then whether the enterotoxins that have been produced so far by V2 cells have not yet been decomposed and are thus still additive to the onset of significant toxin production by the Va2 cells. Furthermore, once adhered vegetative

cells start producing significant amounts of toxins to initiate cell destruction (after 9 hours), the destruction of the cell membrane will probably be accelerated. This indicates that the second continuous outgrow of free floating vegetative cells will probably not occur and, thus, a possible intoxication after 9 hours is not likely to occur. In other words, the question remains whether the consumption of a meal containing 10^5 vegetative cells and 10^5 spores under default conditions can lead to intoxication.

Above argumentation shows that, within its limitations, this model can still reveal important biophysical dynamics influencing the potential toxin production. Moreover, considering the model framework, preliminary insights in the potential toxin production under different food consumption scenarios can be obtained. Further simulations will be explained considering model assumptions and, with that, its limitations.
Furthermore, stomach dynamics are kept constant during further simulations. In addition, only vegetative cells can directly influence the potential toxin production. Therefore, only the development of (adhered) vegetative cells (V2 and Va2) during the digestion of food in the duodenum will be shown in further model output.

4.1 Influence of the release rate of adhered vegetative cells on potential toxin production

Section 4. suggests that the turn-over rate of the epithelial cell membrane is directly related to enterotoxin production. This premise results from the following considered mechanisms.
The release rate of adhered vegetative cells influences the amount of free floating vegetative cells. If, under natural conditions, the release rate is equal to, or higher than, the outflow rate of cells, adhered cells cannot contribute to an accelerated outgrowth of free floating vegetative cells after 6 hours anymore (as can be seen in Figure 13). Here, the free floating vegetative cells can only reach their initial maximum of $1.8 \cdot 10^4$ after 73 min in the small intestine, whereas accelerated growth is an inherent result of the default system (compare Figures 13 and 14). A second outgrowth of vegetative cells as shown in Figure 13 is not possible under these conditions, because there are no adhered cells left to be released and contribute to this “renewed” growth.

Moreover, if the turn over rate of the epithelial cell membrane increases with toxin production a negative feed-back mechanism will be put into action where the toxic effect of vegetative cells becomes self destructive. A second bloom of bacterial cells is then practically impossible. The upper lines in Figure 14 will then gradually change to the lower lines in time.

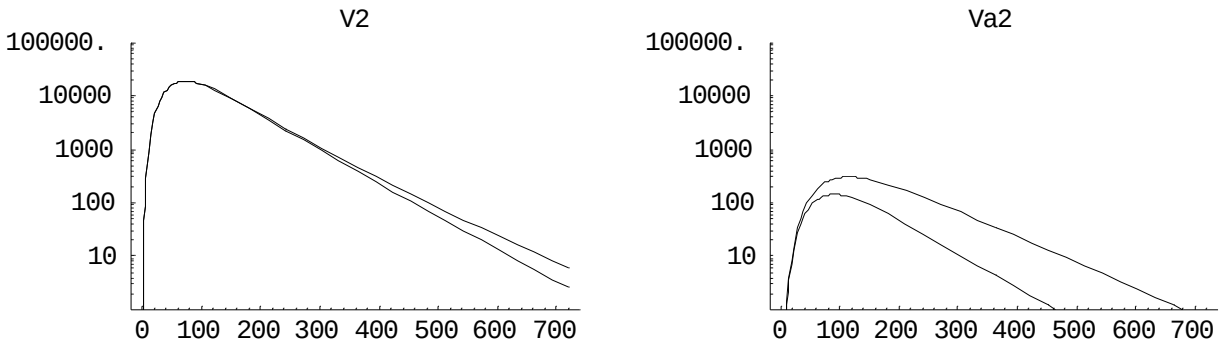

Figure 14 Model output with a release rate equal to the outflow rate, i.e. $\xi=\alpha=0.026$ (upper lines) and output with a release rate being twice the outflow rate, i.e. $\xi= 2\alpha=0.052$ (lower lines). Rest of parameter values is default (Tables 1 and 2).

4.2 Initial exposure to only spores or vegetative cells

As explained in Section 2.1, exposure to both vegetative cells and spores at the same time is only one of the possible scenarios in consuming a meal. The two other realistic infection scenarios were simulated too and results are shown below.

Figure 15 shows the results of microbe development in the small intestine when consuming pre-heated food contaminated with *B. cereus* spores. Vegetative cell growth can now be attributed to spore germination only which results in an initial maximum of $3.3 \cdot 10^3$ free floating vegetative cells after 105 minutes upon arrival of the food in the duodenum.

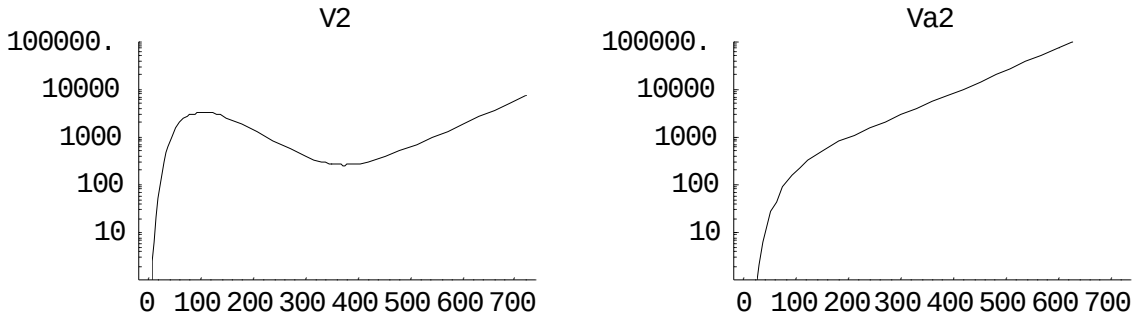

Figure 15 Model output when Vin=0, Sin=10^5, rest of parameter values is default (Tables 1 and 2).

A presumed onset of toxin production at a threshold of $5 \cdot 10^6$ - $1 \cdot 10^7$ bacteria ml^{-1} suggests that eating food containing only spores cannot lead to intoxication if an additional toxin production of adhered cells was to be omitted.
Considering the high toxin instability when produced in the lumen of the small intestine, an onset of cell destruction could probably only occur after approximately 10 hours when adhered cells have grown to 10^5 cells in the duodenum under these circumstances. An increased epithelial cell membrane turn-over following toxin production would, again, decrease the quantitative development of adhered cells and, with that, the probability of a second bloom of free floating vegetative cells within 24 hours. (a detailed argumentation behind the presumed mechanisms can be found in section 4)

Consuming chilled food contaminated with only vegetative cells can result in a *B. cereus* pathogen development as shown in Figure 16.

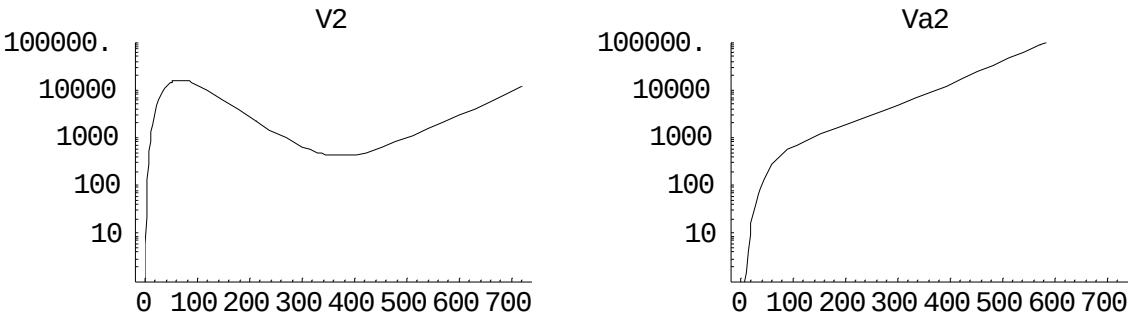

Figure 16 Model output using Vin=10^5, Sin=0 and rest of parameter values is default (Tables 1 and 2).

The growth of vegetative cells is only slightly lower than microbe development when both spores and vegetative cells are consumed in equal amounts under default conditions. Conclusions on the potential of intoxication from the consumption of a meal only containing vegetative cells is, therefore, comparable to the effects when both vegetative cells and spores are present. (see section 4 for a detailed discussion)

4.3 Biodiversity and cell growth

Since *B. cereus* bacteria show a large biodiversity in growth characteristics, consuming food under different conditions can lead to different potential toxin production scenario's. Parameter values have been changed in the following simulations to incorporate this biophysical variability.

Figure 17 shows model output when simulating a virtual worst case scenario in the form of a, say, super mesophylic *B. cereus* strain. Spores of these strains are supposed to have a very short lag phase. According to Tables 3 and 4, still realistic super mesophylic spores could have a lag-time as low as 87-28 min. This results in an activation rate of p=0.012 min^{-1} (eq.5) upon which 99 % of the spores germinate, say, twice as fast as under normal conditions (*i.e.*within 15 min) and so ϵ=0.31. In addition, the lag-phase of vegetative cells is nil (*e.g.* 5 min with ρ=0.14) followed by fast growth (*e.g.* μ=0.035, Table 5).

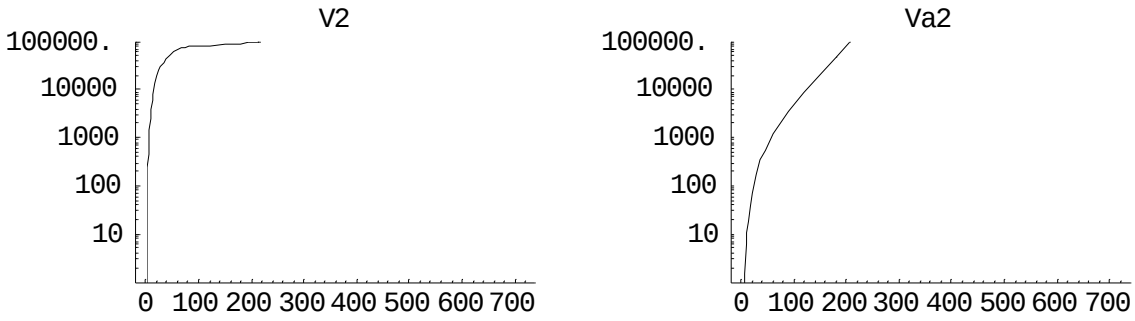

Figure 17 Development of a super Mesophylic strain (see text for explanation) in the small entestine when Vin = 10^5 and Sin = 10^5. Rest of parameter values is default (Tables 1 and 2).

Explosive growth of both free floating and adhered vegetative cells occurs within approximately 6 hours under these conditions. It is, therefore, reasonable to conclude that the consumption of a meal containing these *B. cereus* strains would inevitably lead to intoxication independent of system dynamics or the possible unequal effect of toxin production by adhered or free floating cells.

Figure 18 shows that even if the adhesion mechanism was to be omitted, vegetative cells can rapidly multiply under these conditions (growth up to 3.2 $\cdot 10^7$ units at 24 hours, not shown) and thus probably still cause effects. The free floating vegetative cells have grown up to 10^5 microbes after approximately 4 hours in the small intestine. If cell destruction was to be as effective for V2 cells as for Va2 cells then the onset of intoxication at cell level could start after 4 hours.

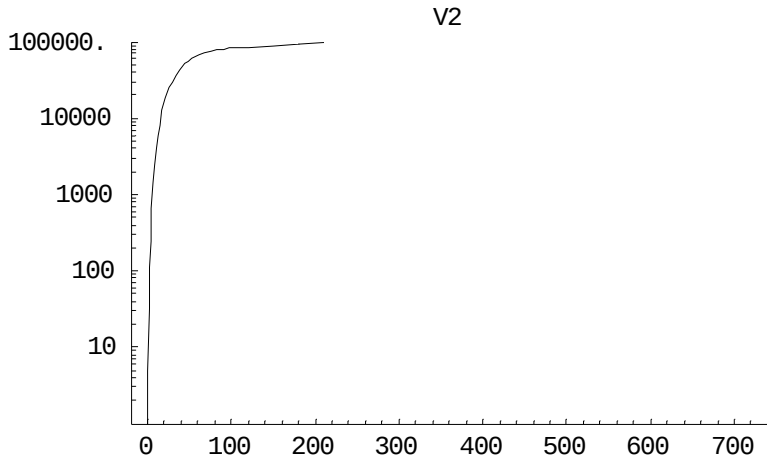

Figure 18: (see Figure 17, but using β=0).

This form of explosive growth persists (results not shown) when

1. only spores of super mesophylic *B. cereus* strains are consumed (up to 10^8 V2 and 10^9 Va2 cells within 12 hours). Recall, however, that this kind of explosive growth is an inherent consequence of the assumed system dynamics, *i.e.* incorporating exponential growth (eq. 6)

1a. only spores are consumed and adhesion is set to zero (up to $8 \cdot 10^6$ V2 cells at 24 hours).

2. only vegetative cells of super mesophylic *B. cereus* strains are consumed (up to 10^8 V2 and 10^9 Va2 cells within 10 hours).

2a. only vegetative cells are consumed and adhesion is set to zero (up to $2.5 \cdot 10^7$ V2 cells at 24 hours).

These results indicate that the intake of vegetative cells and/or spores of a super mesophylic strain will very likely always lead to the onset of cell destruction if V2 cells alone were to be as effective as Va2 cells in their mechanistic mode of action (referring to point 1a and 2a). Moreover, under the assumption that cell adhesion is inevitable, the consumption of a meal containing only vegetative cells or spores will probably lead to intoxication within 12 hours upon arrival of the food in the small intestine.

Another interesting scenario from a hazard characterisation perspective is to simulate the fate of a relatively slow growing *B. cereus* strain under *in vivo* conditions.
This, so called, super psychrotrolerant strain has been assigned all biophysical properties of a "slow" grower as presented in Tables 3 to 5, *i.e.* the lag phase of vegetative cells is relatively long (113 min) followed by a low multiplication rate (μ=0.009 min^{-1}). In addition, spores have a relatively long lag phase (131 min) after which a germination process starts that last approximately 1 hour (*i.e.* ϵ=0.077).

Figure 19 shows that under these conditions free floating vegetative cells grow to a maximum of "only" $4.8 \cdot 10^3$ cells within 72 minutes, whereas adhered vegetative cells continue their slow growth resulting in a maximum of $1.1 \cdot 10^5$ cells at 24 hours. The question arises whether enough toxins can be produced leading to intoxication of the small intestine from this vegetative cell progression in time. These values are far below the presumed threshold for a measurable amount of toxin production of $5 \cdot 10^6 - 1 \cdot 10^7$ bacterial counts ml^{-1}. Even the onset of cell membrane destruction is unlikely to occur under these circumstances.

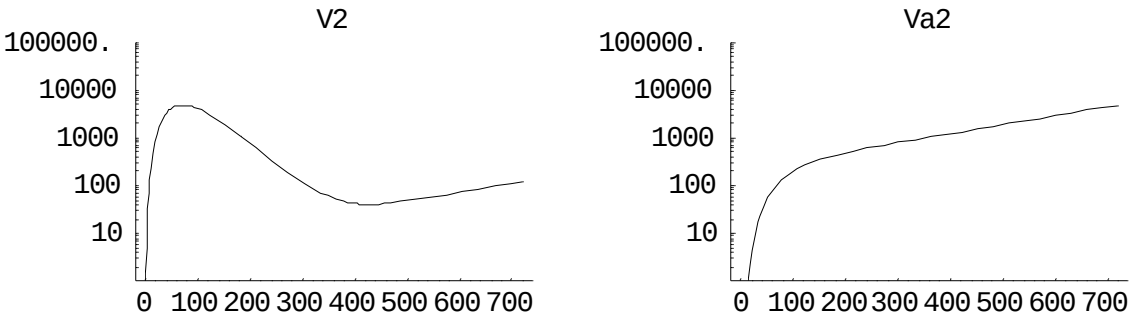

Figure 19 Development of a super Psychrotolerant strain (see text for explanation) in the small intestine when Vin = 10^5 and Sin = 10^5. Rest of parameter values is default (Tables 1 and 2).

Consuming only *B. cereus* spores under these conditions shows a vegetative cell development course as shown in Figure 20, where a maximum number V2 cells of $1.3 \cdot 10^3$ is reached and Va2 cells continue to increase up to $5.3 \cdot 10^4$ at 24 hours. Evidently it would be unlikely for *B. cereus* to affect the small intestine here, even under conditions where a second bloom of free floating vegetative cells would occur.

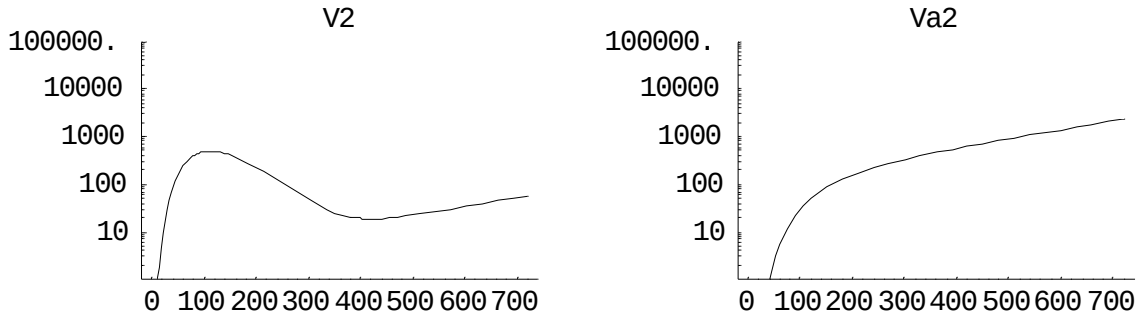

Figure 20: Vin=0 and Sin=10^5. Rest as Figure 18.

Finally, consuming only psychrotolerant vegetative cells results in a vegetative cell course similar to the one presented in Figure 19. Vegetative cells now reaching a maximum of $4.4 \cdot 10^3$ cells at 69 minutes and adhered cells now continuing to progress reaching a maximum of $5.8 \cdot 10^4$ cells at 24 hours (results not shown).

Above simulations show that super psychrotolerant *B. cereus* strains are not likely to lead to intoxication of the small intestine.

4.4 Exposure to B. cereus in the light of microbiological food criteria

As stated in the Introduction, the criterion for *B. cereus* in foods is set to 10^5 cfu g^{-1}. This would correspond with exposure to 10^7 *absolute number* of *B. cereus* cells in a meal containing 100 g of some contaminated food product. Running the model with an initial exposure to 10^7 cells, instead of the *Vin* and/or *Sin* = 10^5 under default conditions, results in qualitatively the same development of vegetative cells and spores in the stomach and small intestine as shown in the previous paragraphs. The difference is only quantitative and, in fact, it is linear. This means that multiplying the cell numbers on the y-axis with 10^2, for example in Figure 13, results in cell development when exposed to 10^7 instead of 10^5 *B. cereus* cells. Under default parameter values (as in Figure 13), free floating vegetative cells will now grow to a maximum of $1.8\ 10^6$ after having spent 73 minutes in the small intestine (instead of the $1.8\ 10^4$ cells when *Vin* = 10^5 and *Sin* = 10^5, Figure 13b).

5. General conclusions and discussion

The objective of this study was to improve the hazard characterisation for *B. cereus* using a dynamic modelling approach. The proposed model predicts the fate of *B. cereus* microbes in the gastro-intestinal tract under different biophysical conditions. The gastro-intestinal tract system dynamics are incorporated in the model development. Specific *B. cereus* characteristics are expressed in the model parameters. Model output showed the development of *absolute numbers* of vegetative cells in the duodenal part of the small intestine for different food consumption scenarios in combination with different biophysical conditions. As little is known about the toxin production process in the duodenum, preliminary conclusions on potential food intoxication were based on vegetative cell progression within approximately 24 hours after food consumption.

5.1 *Initial exposure to 10^5 Bacillus cereus cells*

A summary of exposure scenarios and their potential of leading to food intoxication when initially exposed to 10^5 vegetative cells (*Vin*) and/or 10^5 spores (*Sin*) is given in Tables 7 and 8.

Table 7 shows at what time an initial peak of free floating vegetative cells (V2) occurred upon arrival in the duodenum for different consumption scenarios. The time at which vegetative cells have grown to 10^5 and 10^6 cells has been included in Tables 7 and 8 to identify potential food intoxication scenarios. That is, the onset of cell destruction is supposed to start when adhered vegetative cells have developed to approximately 10^5 cells in the duodenum (not published). In addition, the onset of enterotoxin production could be detected at bacterial counts of approximately $5 \cdot 10^6$ to $1 \cdot 10^7$ cfu's ml^{-1} (Wijnands et al., in preparation).

The question is whether the combination of free floating vegetative cells and adhered vegetative cells can grow to concentrations in the duodenum that could potentially lead to intoxication within 24 hours, the time until which effects can be expected.

Table 7 Development of vegetative cells upon arrival in the duodenal. ('-' indicates an initial peak did not occur, or cell numbers of 10^5 or 10^6 have not been reached within 24 hours).

Exposure scenario	Time after which an initial peak of V2 cells occurs (min., number)	Time (h) within which the number of V2 cells have developed to	
		10^5	10^6
Default:			
Vin=10^5, Sin=10^5	73, $1.8 \cdot 10^4$	15	18
Vin= 0 , Sin=10^5	105, $3.3 \cdot 10^3$	16	20
Vin=10^5, Sin= 0	67, $1.6 \cdot 10^4$	16	19
Super mesophylic:			
Vin=10^5, Sin=10^5	-	4	7
Vin= 0 , Sin=10^5	-	7	8
Vin=10^5, Sin= 0	-	5	7
Super psychrotolerant:			
Vin=10^5, Sin=10^5	72, $4.8 \cdot 10^3$	-	-
Vin= 0 , Sin=10^5	113, $4.8 \cdot 10^2$	-	-
Vin=10^5, Sin= 0	69, $4.4 \cdot 10^3$	-	-

Table 8 Development of adhered vegetative cells upon arrival in the duodenal. ('-' indicates cell numbers of 10^5 or 10^6 have not been reached within 24 hours)

Exposure scenario	Time (h) within which the number of Va2 cells have developed to	
	10^5	10^6
Default:		
Vin=10^5, Sin=10^5	10	13
Vin= 0 , Sin=10^5	11	14
Vin=10^5, Sin= 0	10	14
Super mesophylic:		
Vin=10^5, Sin=10^5	4	5
Vin= 0 , Sin=10^5	5	6
Vin=10^5, Sin= 0	4	5
Super psychrotolerant:		
Vin=10^5, Sin=10^5	24	-
Vin= 0 , Sin=10^5	-	-
Vin=10^5, Sin= 0	-	-

Considering the predefined intoxication thresholds and assumed system dynamics, this model predicts potential food intoxication resulting from the consumption of a meal containing, at least slightly, mesophylic strains. Consuming 10^5 *B. cereus* microbes (*i.e.* vegetative cells and/or spores) with default characteristics will less likely lead to toxic effects. That is, the threshold for toxic effects is only reached after approximately 10 to 20 hours upon arrival in the duodenum (Tables 7 and 8). The question is whether a combination of V2 and Va2 cells can lead to toxic effects considering this relatively slow growth of microbes and the additional effect of an accelerated cell membrane destruction (increasing cell outflow) once toxin production has been initiated.
The more psychrotolerant strains will even never reach the presumed threshold for toxin production within 24 hours. More mesophylic strains, however, can grow to threshold levels within 4 to 8 hours upon the consumption of a meal containing vegetative cells and/or spores. *B. cereus* strains with mesophylic characteristics are, therefore, most likely to cause food intoxication.

5.2 *Initial exposure to 10^7 Bacillus cereus cells*

Model simulations were run to check whether the microbiological food criterion of 10^5 cfu g^{-1} is sufficient to prevent food intoxication. Hereto, different scenario's for exposure to 10^7 *B. cereus* microbes, corresponding to consuming 100 g of a food product with 10^5 cfu g^{-1}, were simulated. A summary of exposure scenarios and their potential of leading to food intoxication when initially exposed to 10^7 vegetative cells (*Vin*) and/or 10^7 spores (*Sin*) is given in Tables 9 and 10.

Table 9 Development of vegetative cells upon arrival in the duodenal. ('-' indicates an initial peak did not occur, or cell numbers of 10^5 or 10^6 have not been reached within 24 hours).

Exposure scenario	Time after which an initial peak of V2 cells occurs (min., number)	Time (min) within which the number of V2 cells have developed to	
		10^5	10^6
Default:			
Vin=10^7, Sin=10^7	73, $1.8 \cdot 10^6$	11	34
Vin= 0 , Sin=10^7	105, $3.3 \cdot 10^5$	45	756 (13 h)
Vin=10^7, Sin= 0	67, $1.6 \cdot 10^6$	12	36
Super mesophylic:			
Vin=10^7, Sin=10^7	-	6	16
Vin= 0 , Sin=10^7	-	25	71
Vin=10^7, Sin= 0	-	6	16
Super psychrotolerant:			
Vin=10^7, Sin=10^7	72, $4.8 \cdot 10^5$	21	-
Vin= 0 , Sin=10^7	113, $4.8 \cdot 10^4$	1375 (23 h)	-
Vin=10^7, Sin= 0	69, $4.4 \cdot 10^5$	21	-

Table 10 Development of adhered vegetative cells upon arrival in the duodenal. ('-' indicates cell numbers of 10^5 or 10^6 have not been reached within 24 hours)

Exposure scenario	Time (h) within which the number of Va2 cells have developed to	
	10^5	10^6
Default:		
Vin=10^7, Sin=10^7	2	6
Vin= 0 , Sin=10^7	3	7
Vin=10^7, Sin= 0	2	6
Super mesophylic:		
Vin=10^7, Sin=10^7	1	2
Vin= 0 , Sin=10^7	2	3
Vin=10^7, Sin= 0	1	2
Super psychrotolerant:		
Vin=10^7, Sin=10^7	6	15
Vin= 0 , Sin=10^7	9	19
Vin=10^7, Sin= 0	8	17

Considering the predefined intoxication thresholds and assumed system dynamics, this model predicts potential food intoxication resulting from the consumption of 100 g of a food product containing the *Bacillus cereus* microbiological criterion of 10^5 cfu g^{-1}. This result suggests that the current allowed limit is too high and a limit towards 10^3 cfu g^{-1} would be more appropriate (corresponding to the results in Section 5.1).

Of course, more research would be necessary to test the presented results. This model is a good first approach to describe the hazard associated with *B. cereus* in order to come to profound microbiological criteria in food products. In addition, model output gave insight in which mechanisms need to be investigated more thoroughly to come to an improved model. That is:

- epithelial cell membrane turn over in relation to toxin production. This would improve insight in the potential second bloom of free floating vegetative cells.
- an improved insight in mechanisms behind toxin production would lead to a model in which the development of toxin concentrations in the duodenum would be the model output.
- the role of free floating vegetative cells compared to adhered cells with respect to toxin production.

Furthermore, most of the parameter values are based on the outcome of one measurement in time. For example, a germination rate is estimated based on the assumption that 99 % of the spores germinate within 30 minutes. Parameter values would become more reliable if they were estimated from data including at least more than one assessment in time. A sensitivity analysis would be useful for those parameters which values will not (or cannot) be assessed more precise during experiments and, thus, remain uncertain in the future. Such a sensitivity analysis will then give insight in the predictive value of this model.
Finally, adding a spatial component to this temporal model will attribute to an even more realistic hazard characterisation, where the influence of clustering dynamics of *B. cereus* microbes on the ultimate toxin production could be incorporated.

When the present model were to be extended with these suggestions, predictions on toxin production in the duodenum could be made under different biophysical conditions. This would lead to improved suggestions for microbiological criteria.

References

Andersen Borge G.I., Skeie M., Sorhaug T., Langsrud T. and Granum, P.E. (2001) Growth and toxin profiles of *Bacillus cereus* isolated from different food sources. International Journal of Food Microbiology 69 , 237-246.

Andersson A., Granum P.E. and Rönner U. (1998) The adhesion of *Bacillus cereus* spores to epithelial cells might be a additional virulence mechanism. International Journal of food Microbiology 39, 93-99.

Baranyi J. (1998) Comparison of stochastic and deterministic concepts of bacterial lag. Journal of Theoretical Biology 192, 403-408.

Baranyi, J. (2002) Stochastic modelling of bacterial lag phase. International Journal of Food Microbiology 73, 203-206.

Barker G.C., Malakar P.K. and Peck M.W. (2003) Germination and growth from spores: variability and uncertainty in the assessment of food borne hazards. In: Proceedings of the 4th International conference on Predictive Modelling in Foods, Eds. Van Impe J.F.M., Geeraerd A.H., Leguérinel I. and Mafart P., KU Leuven/BioTeC, Belgium, pp. 206-208.

Barker G.C., Talbot N.L.C. and Peck M.W. (2002) Risk assessment for *Clostridium botulinum*: a network approach. International Biodeterioration and Biodegradation 50, 167-175.

Bruin de, M.M. (2004) The production of enterotoxins by *Bacillus cereus* under simulated intestinal conditions. MSc Thesis, Wageningen University, The Netherlands.

Casula, G. and Cutting, S.M. (2002) *Bacillus* Probiotics: Spore germination in the gastro-intestinal tract. Applied and Environmental Microbiology 68, 2344-2352.

Christiansson, A., Naidu, A.S., Nilsson, I., Wadstrom, T. and Pettersson, H.E. (1989) Toxin production by *Bacillus cereus* diary isolates in milk at low temperatures. Applied and Environmental Microbiology 55, 2595-2600.

Clavel, T., Carlin, F., Lairon, D., Nguyen-The, C. and Schmitt, P. (2004) Survival of *Bacillus cereus* spores and vegetative cells in acid media simulating human stomach. Journal of Applied Microbiology 97, 214-219.

Clements, M.O. and Moir, A. (1998) Role of gerI operon of *Bacillus cereus* 569 in the response of spores to germinants. Journal of Bacteriology 180, 6729-6735.

Duc le, H., Hong, H.A. and Cutting, S.M. (2003) Germination of the spore in the gastro-intestinal tract provides a novel route for heterologous antigen delivery. Vaccine 21, 4215-4224.

Duport, C., Thomassin, S. and Schmitt, G.B.P. (2004) Anaerobiosis and low specific growth rates enhance hemolysin BL production by *Bacillus cereus* F4430/73. Archives of Microbiology 182, 90-95.

Gerwen van S.J.C. and Zwietering M.H. (1998) Growth and inactivation models to be used in quantitative risk assessments. Journal of Food Protection 61(11), 1541-1549.

Granum P.E. (1997) Bacillus cereus. In: Food Microbiology: Fundamentals and Frontiers, (Ed.) pp. 327-336. Washington, D.C.: ASM Press

Granum, P.E., Naestvold, A. and Nordi Gundersby, K. (1995) An outbreak of *Bacillus cereus* food poisoning during the Norwegian ski championships for juniors. Norsk Vet. Tidskr 107, 945-948.

Gänzle M.G., Hertel C., van der Vossen J.M.B.M. and Hammes W.P. (1999) Effect of bacteriocin-producing lactobacilli on the survival of *Escherichia coli* and *Listeria* in a dynamic model of the stomach and the small intestine. International Journal of Food Microbiology 48, 21-35.

Kotiranta A., Lounatmaa K. and Haapasalo M. (2000) Epidemiology and pathogenesis of *Bacillus cereus* infection. Microbes and Infection 2, 189-198.

Kuipers F.F. (1998) Voor de Variatie, Wageningen: Wageningen Pers, The Netherlands.

Marieb, E. (1998) Human anatomy and Physiology, (Ed.) 4th edn. pp. 875-876. Menlo Park, CA: Addison Wesley Longman inc.

Marteau P., Minekus M., Havenaar R. and Huis in 't Veld J.H.J. (1997) Survival of lactic acid bacteria in a dynamic model of stomach and small intestine: validation and the effects of bile. Journal of Dairy Sciences 80, 1031-1037.

McKillip J.L. (2000) Prevalence and expression of enterotoxins in *Bacillus cereus* and other *Bacillus* spp., a literature review. Antonie van Leeuwenhoek 77, 393-399.

McMeekin, T.A., Olley, J.N., Ross, T. and Ratkowsky, D.A. (1993) Optical density methods. In: Predictive Microbiology and theory and application, (Eds.) pp. 31-34. Great Britain: Research Studies press ltd. and John Wiley & Sons inc.

Minekus M., Marteau P., Havenaar R. and Huis in 't Veld J.H.J (1995) A multicompartmental dynamic computer-controlled model simulating the stomach and small intestine. ATLA 23, 197-209.

Moffett, D.E., Moffett, S.B. and Schauf. C.L. (1993) Human physiology, f.a.f., (Ed.) 2nd edn. pp. 601 St. Louis: Mosby

Notermans S., Dufrenne J., Teunis P., Beumer R., Te Giffel M. and Peeters Weem P. (1997) A risk assessment study of *Bacillus cereus* present in pasteurized milk. Food Microbiology 14, 143-151.

Pielaat, A., Fricker, M., Nauta, M.J. and Van Leusden, F.M. (submitted) Biodiversity in *Bacillus cereus*. RIVM report 250912004, Bilthoven: RIVM. Institute for Public Health and the Environment, The Netherlands.

Prüss, B.M., Dietrich, R., Nibler, B., Martlbauer, E. and Scherer, S. (1999) The hemolytic enterotoxin HBL is broadly distributed among species of the Bacillus cereus group. Applied and Environmental Microbiology 65 (12):5436-42. 0099-2240.

Rivest J., Bernier J.F. and Pomar C. (2000) A dynamic model of protein digestion in the small intestine of pigs. Journal of Animal Sciences 78, 328-340.

Takumi K., de Jonge R. and Havelaar A. (2000) Modelling inactivation of *Escherichia coli* by low pH: application to passage through the stomach of young and elderly people. Journal of Applied Microbiology 89, 935-943.

Wijnands, L.M., Dufrenne, F.M. and Van Leusden, F.M. (2002a) Characterization of *Bacillus cereus*. RIVM report 250912002, Bilthoven: RIVM. Institute for Public Health and the Environment, The Netherlands.

Wijnands, L.M., Dufrenne, J.B. and Van Leusden, F.M. (2002b) The pathogenis mechanism of the diarrheal syndrome caused by *Bacillus cereus*. RIVM report 250912001, Bilthoven: RIVM. Institute for Public Health and the Environment, The Netherlands.

Wijnands, L.M., Dufrenne, J.B. and Van Leusden, F.M. (submitted) Bacillus cereus, various aspects concerning the diarrhoeal syndrome. RIVM report 250912003, Bilthoven: RIVM . Institute for Public Health and the Environment, The Netherlands.

Wijnands, L.M., Abee, T., Zwietering, M.H. and Van Leusden, F.M. (in preparation) Investigations into adherence, enterotoxin production and enterotoxin activity in relation to the diarrhoeal syndrome by Bacillus cereus.

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