

Distributions of microorganisms in foods and their impact on food safety

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Thesis

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This thesis is dedicated to my parents,
who showed me the way, the truth, and the life (John 14: 6a).

P. Jongenburger (1924-2006)

C. M. Jongenburger-Bezemer



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Abstract

The physical distributions of pathogens in foods influence the likelihood that a food product will cause illness, but knowledge about the physical distribution of microorganisms in foods and especially the heterogeneity therein is scarce. This Ph.D. research aims to increase the knowledge of microbial distributions in foods and therewith to provide better insights in their impact on public health and food safety management activities.

The research covers both theoretical investigations and practical experiments, focusing on powdered infant formula (PIF) as a suitable model product and the opportunistic pathogen *Cronobacter* spp. as a relevant microorganism in the practical experiments. The impact of spatial distributions of microorganisms, like homogeneous or more clustered distributions, on public health was investigated. Infrequent high doses were shown to mainly determine the probability of illness and also to dominate the arithmetic mean (mean of the counts) expressing the level of microorganisms present. The distribution of *Cronobacter* spp. in two industrial batches of PIF (a recalled batch and a reference batch) was quantified in detail. Additionally, batches of PIF on lab scale with well-mixed and localised contaminations of *Cronobacter sakazakii* were enumerated. In the recalled batch, the sample units were taken in the course of the filling time and the results showed that *Cronobacter* spp. were heterogeneously distributed. On local-scale, clusters of cells varying between 3 and 560 cells per cluster were present sporadically. Discrete and continuous statistical distributions were compared to model the enumeration data of the industrial and laboratory scale batches. Batches with low counts including zeros were fitted best by the Poisson-Lognormal distribution and Negative Binomial distribution. According to criteria proposed to compare the suitability of statistical distributions to model microbial distributions in foods, these two distributions had already been selected to be the most suitable candidates. Furthermore, the performances of random and systematic sampling were compared to detect a localised contamination in a batch of food. Our calculations showed that systematic sampling rather than random sampling improved the sampling performance. Moreover, taking many small sample units systematically increased the probability to detect the localised contamination. Another systematic sampling strategy evaluated was stratified random sampling. Using the enumeration data of the recalled batch, stratified random sampling appeared to improve the detection probability of *Cronobacter* spp. as compared to random sampling.

Generally, taking more and smaller sample units, while keeping the total sampling weight constant, improved the performance of the sampling plans.

The insights obtained in this thesis are considered to be relevant to a wide variety of dry products and to an extent also to other structured foods. They should be of use to food business operators to improve sampling and testing to verify control of their operation as well as to assess compliance of final products with food safety standards and guidelines before marketing. The results may equally be useful to governmental bodies setting and enforcing food safety standards (such as microbiological criteria) and conducting microbiological risk assessment.

Chapter 1

Introduction



1. Food safety

Food borne illnesses are at best unpleasant but at worst they can be fatal. People expect the food that they eat to be safe and suitable for consumption (CAC, 2003). This expectation or trust can easily be harmed. For example in the melamine case in China in 2008, powdered infant formula was contaminated with melamine, an anorganic chemical, which was added to low quality milk to falsify the protein levels. Nearly 300,000 infants were affected, more than 50,000 infants were hospitalised and six deaths have been confirmed (WHO, 2008). As a result, it will take a long time before consumers will trust this food product again in China. The food industry has an important responsibility to deliver safe food products. At the same time, the industry can easily be damaged by an outbreak of foodborne illness. In Germany in May 2011, an outbreak of *Escherichia coli* O104:H4 (EHEC) started, involving an unusual enteroaggregative strain (EAaggEC VTEC) that produced the verocytotoxin. After indications by health officials that cucumbers produced in Spain were the source of the EHEC infections, the cucumber industry across Europe was severely damaged. A couple of weeks later, not cucumbers but bean sprouts grown in Northern Germany were pointed at as the actual source, causing a major impact on this part of the industry. When the outbreak was over at the end of July, it had caused the illness of 4075 people and 50 deaths in 15 countries in Europe and North America (WHO, 2011). Besides a major impact on public health, outbreaks as these damage food industry, by loss of earnings, unemployment as well as damaged trade relationships between countries and consumer trust.

Therefore, both public and private parties need to ensure that pathogenic microorganisms are prevented, eliminated, or controlled to acceptable levels in food products. According to Codex Alimentarius (CAC, 2003), food safety assures that food will not cause harm to the consumer when it is prepared and/or eaten according to its intended use. Depending on the health hazard and the conditions in which food products are expected to be handled and consumed, precautions taken by the food industry and guidelines given by the authorities are more or less stringent (ICMSF, 2002). For instance, the production and packaging process of cooked and sliced chicken fillet, being a ready-to-eat product, has to be more stringently controlled to prevent the presence of *Salmonella* spp., than the production and packaging process of raw chicken fillet, which according to instructions will need to be baked/cooked before consumption. Obviously, consumers and food professionals, that prepare foods for final consumption, have to ensure that

the baking/cooking is adequate and have to follow relevant labelling instructions on the food. Food safety, in this regard, is a shared responsibility of all stakeholders: farmers and growers, manufacturers and processors, food handlers and consumers, as well as governmental authorities. Thus, the industry and government have established a range of food safety tools to ensure safe food products that need to be used across the farm to fork food supply chain.

Some key tools used in the industry, help it to produce wholesome food through well-controlled procedures that avoid harmful contamination whilst also avoid excess food waste. The food industry uses the food safety management system HACCP, the hazard analysis critical control points system, which identifies, evaluates, and controls all significant hazards for a particular food product. The prerequisites to HACCP are good hygienic practices (GHP) and good manufacturing practices (GMP). GHP and GMP aim to ensure that food operations implement general principles of hygiene. In HACCP, all key steps (referred to as the critical control points, CCPs) of a food production process are identified that significantly influence food safety and adequate measures are taken to monitor and control these steps (ILSI, 2004). For example, in bringing milk from the dairy farm to the consumer, *Salmonella*, *Campylobacter*, and *Mycobacterium* can be identified as significant microbial hazards to be controlled. In milk processing, the pasteurisation step by a heat exchanger is selected as a CCP. For this CCP, the temperature of the heat exchanger and the time that the milk passes through it together determine whether pasteurisation is adequate. Therefore, the heat exchanger temperature and the flow rate of the milk both need to be monitored in milk processing and corrective actions must be taken when monitoring indicates loss of control of adequate pasteurization. HACCP and GHP/GMP as well as tools based on comparable principles are explicitly used by the industry to control production, manufacturing and food service operations. Governments around the world advocate the use of these food safety management systems and can provide guidance on their implementation to the various food operations (CAC, 2003).

Microbiological risk assessment (MRA) is a tool that in principle is used by risk managers in (inter)national governmental bodies. An MRA consists of four steps: hazard identification, hazard characterisation, exposure assessment, and risk characterisation (Lammerding, 1997). An example of MRA output is a detailed description of the product and pathogen pathway for a product type or product category, which provides a clear understanding of the food safety issues related to the particular pathogen from farm

to fork by considering all relevant steps and current control measures (FAO/WHO, 2006b). Other examples are a relative risk ranking of various food products impacted by a pathogen of concern, and associated risk estimates that can be expressed in different ways like risk per serving, or risk in one or more vulnerable groups within the population (FAO/WHO, 2006b). Governmental risk managers use the output of MRA studies to make decisions on the level of stringency needed to control the pathogen and options for its mitigation in the context of public health protection. Based on the outcome of an MRA, a risk manager can decide whether expected risks are acceptable or not, given the ability of industry to implement certain mitigation options. When not, a risk manager has to choose for alternative risk management option(s) that mitigate the risk to a tolerable level; risk managers then also may inform the consumer of the measures taken.

A tool used in food safety management by both government and industry is the application of so-called microbiological criteria. A microbiological criterion (MC) provides a metric for the presence of a particular microorganism for a specific food at a specified step in the farm to fork chain, according to the nature of the microorganism, the likely exposure to consumers, and the nature of consumer group, for example a vulnerable group like children. MCs are established by competent authorities as a standard in food law or by industry as part of industry guidelines. A batch of food is sampled and tested, i.e. the presence of relevant microorganisms is determined in the samples taken. Based on the testing results, it is assessed whether the food batch complies with the particular MC. As such, MCs define the acceptability of a batch of food based on the absence/presence or level of microorganisms per batch (CAC, 1997). An MC for a particular food category states the target microorganism(s), analytical unit size, analytical reference method, and a sampling plan including microbiological limits. It also states the point(s) in the food chain where the criterion applies and any actions to be taken when the criterion is not met (CAC, 1997). The stringency of a sampling plan will depend on: 1) the type and seriousness of hazard implied by the microorganisms, and 2) the conditions under which the food is expected to be handled and consumed after sampling (CAC, 2004; ICMSF, 2002). For example, sprouted seeds have to be tested for *Salmonella* spp. by taking 5 sample units of 25 g and testing for *Salmonella* spp. according EN/ISO 6579, and the microbiological limit is absence in 25g (CEC, 2007). A batch of sprouts will not be released onto the market when one or more sample units are positive for *Salmonella* spp..

Because no practical amount of sampling and testing for harmful microorganisms

in foods on its own can assure the safety of foods, it remains important to verify that microbial hazards are controlled during the production process (CAC, 1997; CAC, 2004; ICMSF, 2002) or at other points in the chain from farm to fork. Industry uses sampling and testing against MC to verify control of an operation as well as to validate product and process designs before marketing products. The industry uses sampling and testing also to check the hygiene status of equipment and the environment of the food operation as well as for investigational studies. According to the process hygiene criteria set in European Community regulations, for example, ice cream containing milk ingredients and frozen dairy desserts have to be tested for *Enterobacteriaceae* at the end of the manufacturing process (CEC, 2007). In case the result is unsatisfactory, improvements in production hygiene have to be taken to minimise contamination. Governments use sampling and monitoring against MC to inspect batches aiming to identify batches that do not comply with the criteria and to, like industry, conduct investigational sampling and testing.

Sampling and testing are used to generate presence/absence data or enumeration data on the microbiological status of batches of food product. The enumeration data are relevant for a range of food safety activities: for governments, enumeration data are modelled and used in MRA studies or used to establish food safety standards such as MCs. It is important to validate that the data obtained, reflect the status of the investigated food batches well and that models derived on the basis of these data are appropriately accurate for their purpose. Both aspects will be influenced by the actual physical distribution of microorganisms in a particular food product. Good insight and knowledge about the physical distribution of harmful microorganisms in foods is thus key in food safety management.

2. Distribution of microorganisms in foods

Sampling and testing results from a batch of food, often referred to as a 'lot' meaning that the batch is produced and handled under uniform conditions (CEC, 2005), may vary even when microorganisms are homogeneously distributed. However, in practice, homogeneity in batches of food is rare. Due to, for instance, the structural heterogeneity of the food matrix, incomplete mixing, incidental (post-processing) contamination, and/or localised microbial growth, microorganisms are more often heterogeneously instead of homogeneously distributed. Depending on when and how contamination occurred

during production or thereafter, the spatial microbial distribution within a batch may also vary in size and concentration.

Fig. 1 shows, as a theoretical example, two batches of food in which the same amount of microorganisms is either randomly distributed throughout a batch (Fig. 1a) or is localised in a few sections of that batch (Fig. 1b).

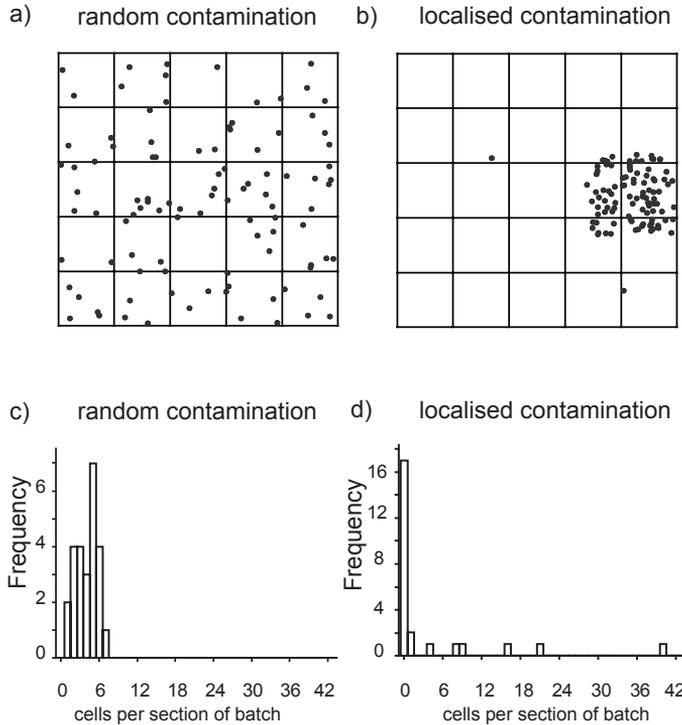


Fig. 1. Illustrative representation of food product batches divided in 25 physical sections with microorganisms distributed either (a) randomly across the batch (homogeneous distribution) and (b) being localised in a small number of sections of the batch (heterogeneous distribution); frequency distribution of the number of cells per section for the batch with (c) microorganisms randomly distributed and (d) microorganisms being localised.

In case of a random contamination, most samples from the batch will likely be positive for the microorganism and some will be negative; this would be relevant for fluids or unstructured, well mixed foods. In case of localised distribution, only some samples will likely be positive and most will be negative; this would be relevant for batches of solid, semi-solid or powdered foods. Fig. 1c and Fig. 1d show the frequency distributions

of the number of all cells per section for each individual batch. In case of the random contamination (Fig. 1c), the number of cells per section varies between 1 and 7 cells and in case of the localised contamination (Fig. 1d) most sections contain no cells and only some sections contain a high number of cells. The frequency distributions of the microbial distribution do reflect the very different physical distributions of the contaminant in the two batches and to accurately reflect the different physical distributions they should be modelled with different statistical distributions. Key is to ensure that the statistical distribution chosen to model the microbial frequency distribution provides for an optimal fit.

The ability to model microbial distributions well will depend on the properties of each statistical distribution. In practice, the Lognormal distribution is used often for establishing microbiological criteria and for assessing the performance of sampling plans. This distribution was found to describe the total viable counts in batches of frozen food products well (Kilsby and Baird-Parker, 1983). Based on this and other observations, the international commission on microbiological specification for foods (ICMSF, 2002) assumed the Lognormal distribution as the default statistical distribution in order to evaluate the performance of attribute sampling plans. Although the Lognormal distribution can model heterogeneity, it does not allow for complete absence of the microorganisms (i.e. counts/values of zero) and it also allows fractional numbers of microorganisms. These limitations are not so important in cases where microbial levels tend to be high, for example for total viable counts of microorganisms in foods. They do become important, however, when considering microbial hazards such as infectious pathogens that more likely occur at very low levels in foods, when at all. It is also important when substantial clustering of a contaminant occurs in local spots and many sections of a batch do not contain microorganisms. Indeed, the ultimate choice of the most suitable frequency distribution should depend on how well the fit would be with actual observations.

Knowledge of the spatial distribution should thus be guiding setting up a sampling strategy. If the contamination is homogeneously distributed, the probability to detect the contamination by definition is the same for each sample. In this case, the pattern or strategy that is followed for drawing samples from a batch does not influence the sampling performance (only the total sample weight). However, if the contamination is heterogeneously distributed, the sampling strategy becomes important (Battilani et al., 2006; Habraken et al., 1986; Lin et al., 1979; Rivas Casado et al., 2009) to assess the

presence and the actual level of a pathogen in a food, which may have an impact on the risk a food may represent to a consumer. Besides heterogeneity on batch-scale, also heterogeneity on local-scale is possible within a food product. One could speculate that when spilled water is present in a processing environment or after wet cleaning, bacteria may grow in the water overnight to levels of 10^9 cells/mL when sufficient nutrients are present. If a droplet of this contaminated water comes in contact with the final product, this may result in clusters of cells with high concentrations, even if 99% of the cells would die. Such clusters are not properly enumerated using an enrichment method, like the most probable number (MPN) method, but may have a major impact on public health. In practice, knowledge about the actual physical distribution and the heterogeneity of microorganisms in foods is scarce and in order to gain more specific knowledge and develop approaches to better model microbial distributions for use in food safety management activities, the current Ph.D. thesis investigation was set-up. In order to quantify in detail the batch-scale distribution of microorganisms in a batch of food, powdered infant formula (PIF) was chosen as the food product, since substantial clustering or heterogeneity of contamination may occur in dried milk products (Habraken et al., 1986). *Cronobacter* spp. was chosen as the target microorganism, since this opportunistic pathogen may occur in low numbers in PIF.

This research combines theory and practice. The theoretical part investigates properties of statistical distributions necessary to model microbial distributions in foods and studies the public health impact of different spatial microbial distributions and of specific sampling strategies. The practical part investigates homogeneous and heterogeneous distributions at laboratory scale and quantifies in detail the distribution of *Cronobacter* spp. in two industrial batches of PIF. The resulting data are used to compare various statistical distributions for optimal fit. The insights may have potential applications in a wide variety of dry products and to food testing in general.

3. Objective of the thesis

The research undertaken in this thesis aims to gain insight in the distributions of microorganisms in foods and their impact on food safety and public health. The research involves partly a theoretical study, assuming several microbial distributions and calculating their impact on MRA output like risk per serving, or on the performance of MCs. The study also aims to gain useful insights for food safety management purposes by

experimentally quantifying the microbial distribution of *Cronobacter* spp. in milk powder under laboratory conditions and industrial practice.

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4. Outline of the thesis

Next to this Introduction, the thesis consists of the following chapters devoted to specific investigations:

Chapter 2 concerns a theoretical study and discusses six mechanisms influencing the spatial distribution of microorganisms in foods. Three types of spatial distributions, i.e. regular, random, and clustered were chosen to illustrate the relationship between these spatial distribution and frequency distributions. Furthermore, it discusses the suitability of statistical distributions employed to model microbial frequency distributions in foods.

Chapter 3 continues theoretical investigations and discusses the impact of microbial clustering and different types of statistical distributions on public health, performance objectives, and operating characteristic curves of sampling plans.

Chapter 4 focuses on enumeration methodology and quantifies the influence of low numbers, microbial heterogeneity and measurement uncertainties on the accuracy of the plate count method to enumerate low numbers of viable microorganisms in milk and milk powder.

Chapter 5 is a further theoretical study that shows the impact of random versus systematic sampling on the probability to detect a localised contamination within a batch of food. A statistical model was used to compare these sampling strategies. The microbial contamination was modelled as being present in one specific localised fraction of the batch in which the cells were randomly distributed, while no cells were present in the remaining part of the batch. The probability that the entire sampling scheme contains at least one cell was calculated for various numbers of samples drawn either randomly or systematically.

Chapter 6 quantifies in detail the distribution of *Cronobacter* spp. in PIF on industrial batch-scale for both a recalled batch as well as a reference batch. Additionally, local spatial occurrence of clusters of *Cronobacter* cells was assessed. The performances of several sampling plans were both calculated and simulated according to the enumeration data. The probabilities of detection by random sampling as well as stratified random sampling were then compared.

Chapter 7 compares various statistical distributions to assess how well they fit actual

Chapter 1

observations. The actual observations were generated on laboratory scale for batches with either random or localised contamination. For laboratory scale experiments, batches of milk powder were contaminated by distributing similar numbers of cells of *C. sakazakii* either homogeneously throughout a batch of milk powder or by distributing the cells in a localised part of the batch. Each batch was then systematically sampled and the microbial distribution determined by enumerating the samples. By enumerating the remainder of the batch, a balance could be made between the total number of microorganisms added to a batch and the number retrieved from the batch on the basis of sampling. Part of the study related to the industrial scale investigation.

Chapter 8 concludes this thesis in a general discussion.

Chapter 2

Impact of microbial distributions on food safety

I. Factors influencing microbial distributions and modelling aspects

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Under revision Food Control

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Abstract

Relatively little is known about exactly how microorganisms are physically distributed in foods, yet these distributions determine the likelihood that a foodstuff will cause illness and the consequential public health burden. When a batch of food is sampled to determine the microbiological status of the batch, the effectiveness of the sampling programme is also related to the spatial distribution of the microorganisms that are being sampled for. In the absence of exact knowledge, generalising assumptions are often made as to the nature of the distributions. Better insight into the actual microbiological distributions may help to improve designing sampling plans and food safety management decision-making. This study discusses mechanisms influencing the spatial distributions of microorganisms in foods, three types of spatial distributions, i.e. regular, random, and clustered, the relationship between spatial distribution and frequency distributions, and the suitability of statistical distributions employed to model microbial distributions in foods. Commonly used statistical distributions, namely the Normal distribution, various types of the Poisson distribution, the Lognormal distribution, the Gamma distribution, the Negative Binomial distribution, and the Poisson-Lognormal distribution are examined and their strengths and weaknesses evaluated. Five specific criteria are proposed to assess the suitability of statistical distributions to model microbial distributions. These criteria require model outcomes to be non-negative, to allow zeros, to be discrete, to approximate Poisson and to approximate Lognormal. Especially the ability to model spatial clustering is investigated. It is concluded that the Poisson-Lognormal and the Negative Binomial are the most suitable statistical distributions given the suitability criteria proposed. However, the ultimate choice of the most suitable one should also depend on how well they fit actual observations.

1. Introduction

Microorganisms present in a food can be harmless or even beneficial for the food product and/or the consumer. Nevertheless, both industry and government spend considerable effort to ensure that microorganisms detrimental to food quality or consumer safety are eliminated or otherwise controlled in foods. Industry utilises food safety management systems such as good hygienic practices (GHP), good manufacturing practices (GMP) and hazard analysis critical control points (HACCP) systems to ensure this. Tools such as microbiological risk assessment (MRA) and microbiological sampling underpin the food safety management concepts utilised by both government and industry. While it is understood that no practical amount of sampling and testing for harmful microorganisms in foods can on its own assure the safety of such foods, important clues regarding food safety can be derived by assessing the likely presence of harmful microorganisms. These clues are key to making adequate decisions in food safety management.

How microorganisms are physically distributed in a food, their spatial distribution, determines the value of the data on prevalence and/or concentration obtained through sampling and testing. These data are necessary to make food safety management decisions about, for example, lot acceptance or process control. Factual insight into the actual spatial distribution of microorganisms in foods is lacking and often generalising assumptions are made that have become commonplace in day-to-day food safety management. Better insight into the microbiological distributions in food matrices may help to further improve food safety management decision-making. Understanding spatial distributions of (harmful) microorganisms is vital for establishing proper microbiological criteria and obtaining a realistic view of the performance of the associated sampling plans. An assumption often used is that microorganisms are distributed lognormally, since this distribution appears to fit actual observations in foods (Kilsby and Baird-Parker, 1983), or according to the Poisson distribution. While there is some mechanistic support for the use of these two statistical distributions, the impact of the choice of statistical distribution on food safety management decisions or the setting of public health policy needs to be examined more thoroughly. Irregular clustering of microorganisms, for example, will impact on the frequency distribution and needs to be considered as well.

This study discusses six possible mechanisms, namely contamination, microbial growth, microbial death, joining, mixing and fractionation that may lead to several spatial distributions of microorganism in foods. The impact of spatial distributions

(regular, random, or clustered) on the frequency distribution is represented and five criteria are proposed to determine the most appropriate statistical distribution(s). The study provides an appraisal of frequency distributions that may be used to describe and represent the spatial distributions of microorganisms. The compared distributions are the Normal distribution, Poisson distribution, Lognormal distribution, Gamma distribution, Negative Binomial distribution, and Poisson-Lognormal distribution. The advantages and disadvantages associated with each of these distributions are examined, especially regarding the possible impact of microbial clustering.

This paper is the first part of our study 'The impact of microbial distributions on food safety'. The companion paper 'Quantifying impacts on public health and sampling' will focus on aspects of public health burden and effectiveness of sampling.

2. Mechanisms influencing spatial distributions of microorganisms

From the initial microbial flora of raw material to consumption by the consumer, food products are exposed to a series of processes and related mechanisms that influence the level (i.e., concentration and/or prevalence) and spatial distribution of microorganisms. Contamination, microbial growth, microbial death, joining, mixing and fractionation are six of these mechanisms that can have an impact on the spatial distribution of microorganisms (Nauta, 2001; Nauta, 2005). These mechanisms, their impact on the spatial microbial distribution and an example inducing each particular mechanism are listed in Table 1.

Table 1. Mechanisms relevant for food microbiology, their impact on the spatial distribution of microorganisms (mo), and an example of an event/process inducing the mechanism

Mechanism	Impact on spatial distribution of microorganisms	Example of inducing the mechanism
Contamination	Transfer of mo from external source onto product altering the distributions of mo on the surface of the product	Contact between contaminated processing equipment and food product
Microbial growth	Clustering and uneven distribution of mo outside or inside the product	Thawing , cooling
Microbial death	Unevenly applied lethal process (e.g. thermal process) resulting in unevenly distributed mo in the product	Microwave heating
Joining	Sum of the distributions of the joined materials resulting in a different overall distribution of mo	Joining tiramisu components
Mixing	Relocation of mo throughout the product	Mincing meat
Fractionation	Relocation of mo over resulting product units	Slicing ham

The mechanisms can be described as follows:

- 1) *Contamination* transfers microorganisms onto a foodstuff from an external source. Contamination of foodstuffs generally occurs on the surface of a product, and therefore often results in an uneven spatial distribution of microorganisms.
- 2) *Microbial growth* can transform an initially homogeneous distributed contamination into a more clustered distribution on or within a foodstuff. During growth through reproduction, microbial cells may remain attached to each other and form cell clusters or micro-colonies. This may, for instance, be due to particular growth characteristics of the microorganisms or to physical constraints of the food matrix. Cells that have the ability to move actively with flagella may overcome such a clustering if the food matrix allows their movement.
- 3) *Microbial death* results from the application of lethal processes (such as thermal processing or adding lethal levels of preservatives) or from adverse effects of changing environmental conditions. Intrinsic product characteristics (e.g., water activity, pH and nutrient availability) and extrinsic product characteristics (e.g., storage temperature or storage atmosphere) both may lead to inhibition of microbial growth and when occurring at lethal levels, even complete inactivation and death of microbial cells.
- 4) *Joining* two or more materials (e.g., ingredients or food products) with different microbial distributions results in a joined product with a distribution that is different from that of the initial microbial populations of the merged materials. The overall population of the joined product will be a sum of the populations of the joined materials and the distribution of the overall population will be a function of the way in which joining occurs.
- 5) *Mixing* materials or product units relocates the original microbial population throughout the product mass. Mixing leads to a more random spatial distribution and a changing of the number of cells per unit of weight or volume (Kilsby and Pugh, 1981).
- 6) *Fractionation* relocates microorganisms over the resulting product units. For example, when a batch of milk powder that contains a localised contamination is filled into bags, some of the bags may contain clusters of the contaminating microorganism while others may be free of microorganisms. Fractionation can also involve procedures that result in the removal of contaminating microorganisms, for instance when a portion of a food product is discarded or removed by processes like peeling or rinsing.

While the six mechanisms mentioned may work alone, it is more often a combination of the six mechanisms that affects the final microbial distribution of a food product. At a particular step in a food manufacturing operation, the starting microbial distribution will be the microbial distribution resulting from the relevant mechanism(s) at the previous step. In the subsequent steps, mechanisms or sets of mechanisms may have an impact on the distribution of microorganisms in the final food product. The distribution of the pathogen *Escherichia coli* O157:H7 during the production of hamburger patties is an example in which all six mechanisms may contribute to the final distribution of the pathogen. Fig. 1 illustrates which specific mechanisms may be involved in sequential process steps for hamburger patties.

Step in production process	Mechanism impacting distribution of microorganisms	Contamination source
Rearing on farm	Contamination	Air, water, soil, other cattle, other animals
abattoir Slaughter and primary process	Fractionation, contamination	Processing equipment, viscera, hide, water
processing facilities Further process into cuts and trimmings	Fractionation, mixing, contamination	Processing equipment, workers, water
spices Mix meat and spices	Joining, mixing, contamination	Raw materials, workers, processing equipment, tools
Mince/grind	Fractionation, mixing, contamination	Trimmings from other animals, rework, grinding equipment
Form into patties	Fractionation, contamination	Moulds to form the patties, workers, processing equipment, tools
Package	Contamination	Packaging materials and equipment, other hamburger patties, workers
Freeze	Microbial death, contamination	Air, freezing equipment
consumer Thaw the patty	Microbial growth, contamination	Consumer, utensils
Cook the patty	Microbial death	Not applicable

Fig. 1. Overview of likely mechanisms and sources of contamination impacting the distribution of microorganisms for each step in the production process of hamburger patties.

E. coli O157:H7 may colonise cattle and be present in the faeces of cattle to be slaughtered. Some or all of the cattle in a given herd may be colonised, which will be influenced by environmental conditions and herd management practices. Prior to slaughtering, animals from different herds may be intermingled, which may disperse infected animals among those to be processed. At the abattoir, the cattle are slaughtered and the carcasses divided into cuts. Cross contamination of the carcass surfaces can occur due to actions during the primary process such as stunning, bleeding, de-hiding, evisceration, washing, and cutting. During further processing, the carcasses are divided into smaller cuts, which will fractionate the microbial population. Contamination may occur from cutting equipment, workers and water used in cleaning. Meat, cuts, trimmings, spices and other ingredients are combined during mixing, merging microbial populations from multiple sources. Subsequently grinding to prepare comminuted meats will further distribute the contamination from one or more sources of trimmings that are ground together. Fractionation will again occur during the preparation of patties as portions of the combined mass are made. During the packaging of the patties contamination may occur from contaminated packaging material and equipment, workers and other patties. An initial decline may occur during the freezing process, influenced by the conditions of freezing. The remaining populations may gradually decline during frozen storage, although survival of a sub-population is likely. When consumers thaw the hamburger, thawing may allow growth if the thawing temperatures are sufficiently high and the keeping times before final preparation allow. In case growth is possible, it will first occur on the surfaces of patties where the temperature is higher than inside patties. Final preparation by proper cooking or frying will result in the death of *E. coli* O157:H7. The distribution of the heat induced lethality may be influenced by the variations in density and thickness of the patty. Depending on the cooking conditions, survival may occur in cold spots in the product that do not receive sufficient heating. Variations in temperatures on a grill or within an oven could also result in undercooking of some units and, consequently, survival of the pathogen.

Each of the mechanisms described may have an impact on the spatial distribution of microorganisms in foods. The following section will discuss relationships between spatial distributions and frequency distributions. Different microbial distributions will be specified in terms of their dispersion (spatial distribution) patterns and stochastic frequency distributions will be considered that may be used for modelling those patterns.

3. Stochastic¹ distributions

3.1 Scale of analysis and types of distributions

It is unlikely that every portion of a larger bulk of food contains the same number of microorganisms. On a very small scale, comparable to the size of a microorganism (perhaps 10^{-12} cm³), there are only two kinds of portion: a portion containing a microorganism or a portion not containing a microorganism; in this case, all possible distributions are clustered. Conversely, large portions can be expected to 'average out' small scale clustering, but to reveal larger scale clustering, for example in a production run in a particular factory, or the sum of such productions within a particular country. In principle, the presence of clustering can be defined independent of scale, in terms of the probability of points (microorganisms) depending on the presence of nearby points. In practice, the exact location of microorganisms is unknown and of little interest. The distribution is deduced from, and its effect mediated by, numbers or presence in finite-sized samples.

In order to provide a more complete representation of the microbial status of a batch, distributions will be considered that might be used to model portion-to-portion variation as well as overall average. Sizes of the portions and batches of interest differ between considerations of food safety and public health on the consumer side and considerations of sampling plans and acceptance of batches on the producer side. From the perspective of food safety and public health, the portion of interest is that which is actually consumed and the size of the consumed portion (e.g. 50 g to 500 g); without actual consumption of a portion there is no risk to consumers and the size of the portion consumed, amongst others factors, definitely determines the exposure of individual consumers. The batch of interest from the consumer and public perspective may be that, which is the topic of a governmental risk assessment, or which is investigated in relation to an outbreak, or which is subject to food safety management criteria. In an industrial setting, batches are not likely much less than a tonne, but then they might be as much as hundreds of tonnes. From the producer perspective of using sampling plans to support decisions on the acceptance of a batch, the portion of interest is the analytical unit, which is actually analysed (e.g., 0.1 g to 100 g). To capture the wide range of portion sizes relevant for consumer and producer considerations, this study considers in its further work the variation between portion sizes from 0.1 g to 500 g and batches in the order of magnitude of tonnes.

¹ Adjective: having a random probability distribution or pattern that can be analysed statistically but not predicted precisely. Origin Greek *stokhastikos*, from *stokhazesthai* 'aim at, guess' (Soanes, 2003).

3.2 Spatial and frequency distribution

The differences and relationships between spatial and frequency distributions are illustrated in Fig. 2. In each panel ‘almost regular’ represents points that are quite regularly spread through-out the panel, ‘random’ represents points randomly spread and ‘one cluster’ represents points forming a single quite tight cluster against a very low density, random background. Figs. 2a shows different arrangements of 100 points among 25 panels or ‘portions’. In a food industry context, each portion could be considered a ‘unit’, with the set of 25 portions being a ‘lot’. In effect, the figures represent ‘within-a-unit’ and ‘within-a-lot’ variation. Alternatively, each portion could be considered a lot, so the figures represent ‘within lot’ and ‘between lot’ variation. To mimic real situations, the representations can be extended to three dimensions or even to four, when distribution in time is considered as well. However, the two dimensional figures presented and the accompanying discussion allow for generally applicable conclusions to be drawn, as will be discussed below.

Fig. 2a shows three different spatial distributions of 100 points over 25 portions. Fig. 2b shows the resulting number of points in each portion and Fig. 2c shows the resulting frequency distributions (i.e., representing how often each ‘points per portion’ value occurred). Fig. 2a, the spatial distributions of points, contains no numerical values as it just shows the locations of the points. Fig. 2a contains most information, the locations of the individual points. Fig. 2b as well as 2c can be deduced from Fig. 2a, but not vice versa. Fig. 2b contains values (the concentrations indicated in each portion) and locations (of portions, not of individual points). Fig. 2c represents spatial distributions as frequency distributions and contains information on values, but there is no information on locations. Different spatial distributions can produce the same frequency distribution. For example, when the high concentration portions in chart ‘random’ of Fig. 2b are clustered, a new spatial distribution of values such as shown in the chart ‘rearrangement of random’ in Fig. 2b results in an equal frequency distribution depicted in chart ‘random’ of Fig. 2c.

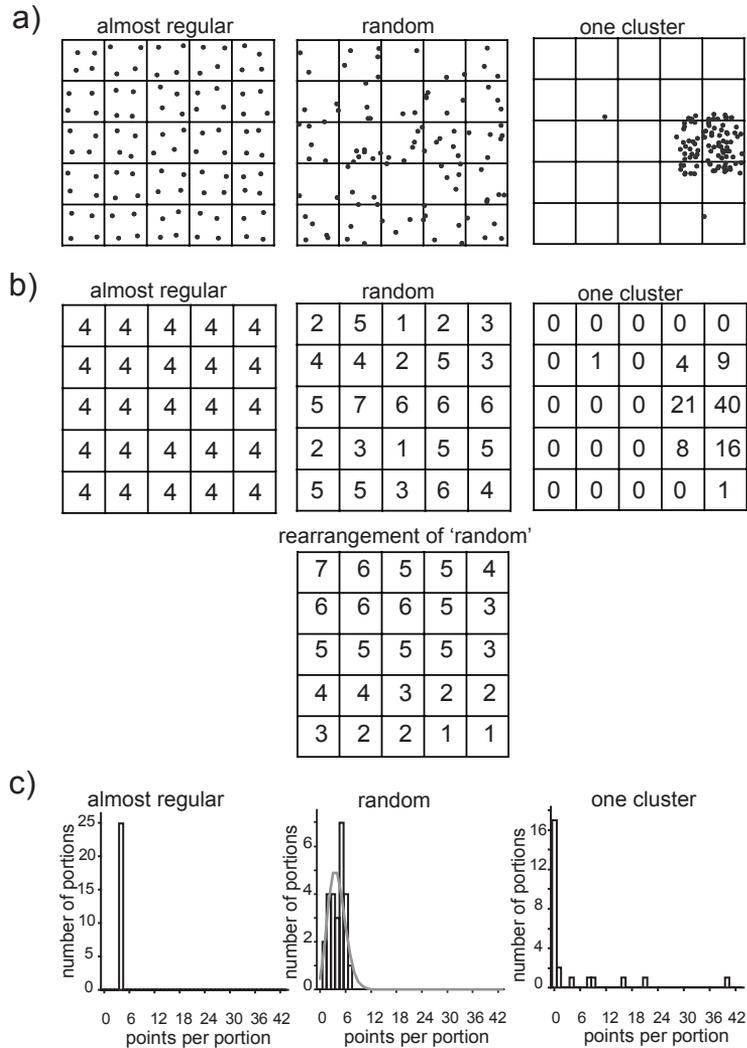


Fig. 2. The differences and relationships between spatial and frequency distributions for three different spatial distributions: 'almost regular', 'random', and 'one cluster'.

Panel a: three different spatial distributions of 100 points over 25 portions.

Panel b: Numbers of points in individual portions for the three spatial distributions depicted in panel a. The high concentration portions in 'random' are clustered in 'rearrangement of random'.

Panel c: Frequency distributions for the three spatial distributions depicted in panel a and b. Note that chart 'random' includes a Poisson distribution (grey line), which is the frequency distribution corresponding to a uniform random spatial distribution.

To describe spatial distributions in quantitative terms using the statistics of 'spatial processes', several approaches may be used. For instance, the positions of the points could be described by their X-Y coordinates or by the distances between neighbouring points. Another way of characterising spatial distributions is by stating how the chance of finding a point depends upon the closeness of other points. Discussing this approach further, leads to a more formal description of the terms 'regular', 'random', and 'clustered'.

- a) In regular distributions, points are *less likely* close to other points, so that points are relatively far apart from each other. Although such patterns are relatively unusual in food microbiology they can occur where contamination occurs following more or less regular patterns, for instance from one contaminated head of a multi-head filler.
- b) In uniform random distributions, points are *equally likely* close to or far from other points. In this case, therefore, the chance of finding a point is independent of the closeness of other points. While the points in a random pattern are equally likely everywhere (the distribution of probability is uniform), they cannot actually be everywhere (the distribution of points is not uniform). Uniform random patterns may apply for instance in the case of well-mixed liquids or powders. This type of pattern is often used to represent spatial patterns where no better, specific information or data on the 'real' spatial distribution in the food product is available, even where it may be evident from the nature of the product (e.g. structured, not well mixed food or liquid) that it might not apply.
- c) In clustered distributions, points are *more likely* close to other points, so that points are relatively close to each other. Such patterns are quite common in food microbiology, as contamination often occurs in clusters, for instance because of initial contaminants multiplying into colonies, disruption of biofilms, or localised growth of microorganisms in non-liquid foods.

Considering 'real' information or data availability, obviously, data describing actual spatial positions of individual microorganisms (e.g., as in Fig. 2a) contains most pertinent information, and can be converted into a format representing per-portion-position or frequency distribution. Such information is rarely available. Data describing spatial positions of microorganisms in portions as a whole and their concentrations per portion (e.g., as in Fig. 2b) contains some direct spatial information, which can be converted to a frequency distribution format. Such information is not common and where it is available, the concentration data is often presence/absence rather than viable counts, which limits

its value. The most commonly available data on microorganisms in foods has no spatial content at all, being simply frequency distributions (e.g., as in Fig. 2c) specifying how often particular concentrations were observed. Again, the concentration data is often presence/absence rather than counts, so that histograms such as Fig. 2c would have only two bars, one for 0 and one for >0 concentrations occurring.

The variation of values in a frequency distribution (e.g., Fig. 2c) is often called the 'dispersion' and is measured by the 'variance' (which is equal to the square of the standard deviation), whereas the average is often represented by the mean of the values. Table 2 summarizes the degree of spatial clustering by comparing the variance and the mean of the corresponding frequency distributions.

Table 2. Relationships between spatial and frequency distributions, illustrated with examples

Spatial distribution ^a	Frequency distribution characteristics ^b			Example
more spaced	more concentrated	underdispersed	variance < mean	regular contamination due to contaminated filler head
uniform random	Poisson distribution		variance = mean	perfect mixing
more clustered	more right skewed	overdispersed	variance > mean	local contamination from hand contact

^a relative to uniform random distribution

^b relative to Poisson distribution

While earlier in this section 'regular', 'random', and 'clustered' spatial distributions were defined in terms of the relative probabilities of finding points closer to and further away from other points, as illustrated in Table 2, a set of alternative descriptions can be used in terms of frequency distributions:

- A regular spatial distribution has a frequency distribution with a variance *smaller* than its mean.
- A uniform random spatial distribution has a frequency distribution with a variance *equal* to its mean.
- A clustered spatial distribution has a frequency distribution with a variance *greater* than its mean.

3.3 Mixture of distributions

The final distribution of microorganisms in a food is usually the result of multiple distinct mechanisms, having an impact individually or in combination and being active continuously or changing in a discontinuous manner. Even if individual mechanisms may result in

spatial distributions that can be represented by quite simple frequency distributions, their combination usually requires a more complicated frequency distributions, often being a mixture of the simpler distributions. Where one mechanism clearly dominates the impact on spatial distribution, it may be possible to still use a simpler frequency distribution to approximate the mixture.

To model a mixture of simple distributions, or to approximate a mixture by a single simple distribution, a number of quite 'standard' statistical distributions has been used. These will be discussed in terms of their characteristics and their ability to well represent particular spatial distributions. To judge the suitability of statistical distributions to well represent specific spatial distributions, five 'suitability criteria' may be considered as proposed here.

3.4 Proposed suitability criteria for statistical distributions

There are five criteria that statistical distributions used to model frequency distributions of microorganisms should satisfy if they are to represent or approximate spatial distributions well in real, practical situations:

1. The model outcome should not be negative, as it is not possible to have negative numbers of microorganisms in a food. This criterion can be satisfied when the statistical distribution will give zero probability to negative values.
2. The model should allow zero as an outcome, because it is possible to have no microorganisms in a portion of food. This criterion can be met when the statistical distribution gives a finite probability to zero values.
3. The model outcome should be discrete numbers only, as it is not possible to have parts of microorganisms in a portion as viable units. To satisfy this criterion, the statistical distribution should not assign probability to fractional numbers.
4. The statistical distribution should reduce to, or at least approximate, the Poisson distribution, because it can be shown that the specific case of a uniform, random, spatial distribution (as might be produced by perfect mixing) corresponds to a Poisson statistical distribution.
5. The statistical distribution should be similar to, or approximate, the Lognormal distribution at high numbers of microorganisms (i.e. when there is a negligible probability of zero microorganisms). This criterion is suggested because the Lognormal distribution has been widely and successfully used to model frequency distributions

in many circumstances. Although the frequency distribution of microorganisms must really be discrete (no fractional microorganisms), the difference between successive integers is small enough at high numbers such that continuous statistical distributions may be good approximations.

Before using these five criteria to explicitly assess the suitability of the commonly used statistical distributions, it should be stressed that any statistical distribution is only an approximation of reality. In practice, with 'real' food products, other criteria may also influence the choice of statistical distribution. Such influences may include familiarity to the user, ease of use in the context of application, and the level of agreement between the model and actual observations regarding its impact on the required level of output accuracy.

2

4. Results and Discussion

4.1 Comparing the statistical distributions with the suitability criteria

Six types of statistical distributions have been considered in this comparison: Normal distribution, Poisson distribution (with separate assessments for single-parameter Poisson, generalised Poisson, zero-inflated Poisson), Lognormal distribution, Gamma distribution, Negative Binomial distribution (also referred to as Poisson-Gamma; a specific type of generalised Poisson), Poisson-Lognormal distribution (another specific type of generalised Poisson). Table 3 shows how the various statistical distributions comply with the proposed five suitability criteria.

Table 3. Assessment of compliance of various types of frequency distribution with a set of five suitability criteria proposed. These are criteria that mathematical distributions used to model frequency distributions of microorganisms should satisfy if they are to represent or approximate spatial distributions well in real, practical situations

Distribution	Complies to suitability criteria				
	non-negative numbers	allows zeros	discrete numbers	approximates Poisson	approximates Lognormal
1 Normal	No	Yes	No	No	No
2a Poisson (single parameter)	Yes	Yes	Yes	Exact	No
2b Poisson (generalised)	Yes	Yes	Yes	Exact	No
2c Poisson (zero-inflated)	Yes	Yes	Yes	Exact	Yes/No ^a
3 Lognormal	Yes	No	No	No	Exact
4 Gamma	Yes	Yes/No ^b	No	No	Yes
5 Negative Binomial (Poisson-Gamma)	Yes	Yes	Yes	Exact	Yes
6 Poisson-Lognormal	Yes	Yes	Yes	Exact	Exact

^a Depending on the generalising distribution some generalised Poisson distributions can approximate the Lognormal distribution ('Yes' applies), others cannot ('No' applies).

^b Allows zero if shape ≤ 1

Examples of the six statistical distributions are illustrated in Fig. 3.

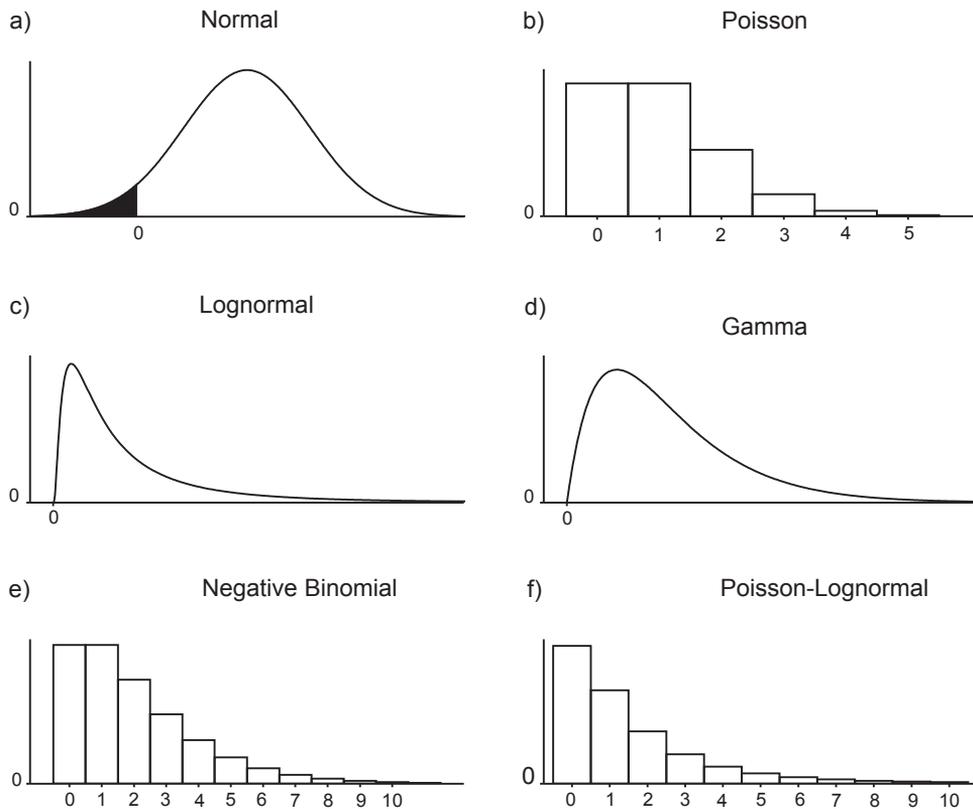


Fig. 3. Examples of a Normal, Poisson, Lognormal, Gamma, Negative Binomial, and Poisson-Lognormal distributions.

4.1.1 Normal distribution

The Normal distribution does not comply with four out of five proposed criteria. It, for instance, gives finite probabilities to negative values (as illustrated in Fig. 3a). The Normal distribution is therefore not suitable either for direct representation of microbial frequencies or to generalise the mean of a Poisson distribution.

4.1.2 Poisson distribution

Three sub-types of Poisson distribution have been assessed for suitability. The single-parameter Poisson distribution and the zero-inflated Poisson distributions both comply with the same four suitability criteria. The generalised Poisson distribution may comply with four or even all five criteria, depending on the generalising distribution.

- A *single-parameter Poisson distribution* is fully defined by its location, e.g., its mean. Its dispersion as measured by variance, is equal to the mean. Fig. 3b shows a Poisson distribution. While the Poisson distribution is the distribution of choice for well-mixed products with low concentrations of microorganisms, the single parameter Poisson distribution does not have the flexibility to model the variations in microbial concentrations seen in practice. For instance, at high concentrations (e.g., above 20 CFU (colony forming units)/portion) a Poisson distribution is essentially symmetrical, while observed distributions of microbial concentrations are often skewed to the right (i.e. indicating that the highest concentrations occur at relatively high frequencies compared to a symmetrical distribution).
- *Generalised Poisson distributions* provide more flexibility than single-parameter Poisson distributions, from which they are derived. Different terms have been used in the literature by different authors to describe such combinations of distributions. Alternatives used may include 'generalised', 'compound', 'contagious', 'aggregate', or 'mixture' distributions. In this study, the term 'generalised' is used. In a generalised distribution, the parameter of the simple distribution itself follows a distribution. The single-parameter Poisson distribution corresponds to a uniform, random spatial distribution of points, where the mean or expected number of points per portion is constant. One way to allow for clustering to be reflected in a model is to describe the number of clusters by a Poisson distribution and the number of points within each cluster by another distribution. The total number of points in a given volume then follows a 'generalised Poisson distribution'. In terms of the five proposed criteria, the generalised Poisson distribution retains the advantages of the single-parameter Poisson, but it is not restricted to having variance equal to its mean and it can model the skewness associated with for example a Lognormal distribution. If the generalising distribution allows the generalised Poisson distribution to approximate the Lognormal distribution, the generalised Poisson distribution is appropriate to fulfil also the fifth criterion.

- The *zero-inflated Poisson distribution* is a discretely generalised Poisson distribution, which generates more zero values than a single-parameter Poisson. Because the generalising distribution is discrete (i.e., Binomial or two-valued; the mean of the Poisson is either 0 or λ) the resultant generalised distribution can have more than one peak. Applying this statistical distribution may be appropriate when the overall batch of food product can be considered to be a mixture of two different groups of portions, one group having none of its portions contaminated, and the other group contaminated in a uniform random pattern. Although this distribution complies with the first four criteria, it does not approximate the Lognormal distribution.

4.1.3 Lognormal distribution

The Lognormal distribution is illustrated in Fig. 3c. The Lognormal distribution complies with only two of the suitability criteria whilst it is often used in practice to directly model frequency distributions of microbial concentrations. It does not comply with three of the criteria, and two of these pose substantial limitations when the Lognormal distribution would be deployed to reflect spatial distributions of microorganisms in foods. First, it gives zero probability for zero concentration, so it does not allow complete absence of microorganism. Second, it is continuous, thus allowing fractional numbers of microorganism. These limitations are not so important when microorganisms are present in food portions at high levels, but they are important at low concentrations. The reason for this is that in a case where the average level is, for example, 1,000,000 CFU/portion, the probability of zero may be negligible and the difference between 1,000,000 and 1,000,001 is not important. Such high numbers are often relevant for spoilage microorganism. However, when levels are low as often is the case for pathogens, the probability of zero numbers of microorganisms in a portion is more relevant and not negligible. The Lognormal distribution may be used as a generalising distribution for the Poisson distribution.

4.1.4 Gamma distribution

A Gamma distribution is usually defined by two parameters, the scale (>0) and the shape (>0). The Gamma distribution does not allow zero if the shape is >1 . In that case, the Gamma distribution is similar to the Lognormal distribution; a continuous distribution, that does not allow zeros. The Gamma distribution also fulfils two of the five suitability

criteria as illustrated in Fig. 3d. Although the Gamma distribution in principle allows any positive value for the scale, this includes values that would give a variance smaller than the mean, representing a distribution that is under-dispersed with respect to the Poisson distribution. Because the scale parameter for the Gamma distribution is equal to the variance divided by the mean, realistic distributions in this context are restricted to those with a scale parameter at least equal to 1. While, like the Lognormal distribution, the Gamma distribution is unsuitable to directly represent microbial concentrations at low numbers, it may be used as a generalising distribution for the Poisson distribution.

4.1.5 Negative Binomial

When the continuous Gamma frequency distribution is used to generalise the mean of a discrete Poisson distribution, the result is a discrete Poisson-Gamma distribution, also known as a Negative Binomial distribution. A Negative Binomial distribution is usually defined by two parameters, p (>0 , <1) and k (>0) and complies with all five criteria proposed. Fig. 3e shows an example of the Negative Binomial distribution. As a generalised Poisson distribution, the Negative Binomial may be a suitable model for a mixture of distributions. The individual distributions can be modelled as uniform random resulting in Poisson distributions, each with a constant mean contamination level. To represent clustering, the variation in mean contamination levels can be modelled by a Gamma distribution.

4.1.6 Poisson-Lognormal

When the continuous Lognormal distribution is used to generalise the mean of a discrete Poisson distribution, the result is a discrete Poisson-Lognormal distribution. An example of this distribution is presented in Fig. 3f. This distribution complies with all five suitability criteria, since the distribution is non-negative, allows zeros, is discrete, and with the appropriate parameters it converges exactly to the Poisson or exactly to the Lognormal distribution. As a generalised Poisson distribution, the Poisson-Lognormal may be a suitable model for a mixture of distributions. The individual distributions can be modelled as uniform random resulting in Poisson distributions, each with a constant mean contamination level. To represent clustering, the variation in mean contamination levels can be modelled by a Lognormal distribution.

4.1.7 Comparative assessment of suitability

Considering the overall advantages and disadvantages of the six types of statistical distributions as presented in Table 3, one can conclude that the Poisson Lognormal is the most suitable and the Negative Binomial (Poisson-Gamma) is second best choice with regard to the five proposed criteria. A disadvantage of the Poisson-Lognormal is its mathematical complexity. All other distributions considered, including the Negative Binomial, have relatively straightforward expressions for the probability mass function (the probability of a given number) and the cumulative distribution function (the probability of a given number or less). In contrast, evaluation of the Poisson-Lognormal probability mass function involves integration over the Lognormal distribution while evaluation of the cumulative distribution function involves summing the probability mass functions. Whilst the Poisson-Lognormal and the Negative Binomial (both generalised Poisson distributions) may be almost equally well-suited, the two continuous distributions (Lognormal and Gamma) fail the suitability criteria that are important for being able to model low numbers of microorganism, whereas the Poisson distribution cannot model clustering.

4.2 Comparing the impact of clustering on two continuous and two discrete distributions

For a Poisson distribution, the dispersion, as measured by variance, is equal to its mean. Accordingly, distributions whose variance is less than the mean are often called 'underdispersed' and those whose variance is greater than the mean are called 'overdispersed'. In practical terms, overdispersion of the frequency distribution reflects clustering in the spatial distribution. Underdispersion then reflects separation in the spatial distribution, meaning that it is more regular than a uniform random distribution. Underdispersion is less common than overdispersion in foods. Overdispersion or clustering can be quantified by the ratio variance/mean of the microorganisms' counts (not of the log counts). A small ratio indicates little clustering, and a high ratio indicates substantial clustering. This is illustrated in Fig. 4, where two continuous distributions (Lognormal, Gamma), and two discrete distributions (Poisson-Lognormal, Negative Binomial) are graphically compared for a number of different parameter combinations. Within each chart (Figs. 4a – 4d) all four distributions have the same values for the mean and for the standard deviation of x (microorganisms count or concentration). For the Lognormal distribution, the values for mean and standard deviation of $\log_{10}(x)$ are also

shown. Figs. 4a and 4b represent examples where the value for the mean is high, whereas Figs. 4c and 4d give examples of low means. Figs. 4a and 4c represent little clustering (small ratio variance/mean), whilst Figs. 4b and 4d represent substantial clustering (large ratio variance/mean).

At high mean values and little clustering (Fig. 4a; variance/mean = 5.59) all four distributions are very similar. With pronounced clustering (Fig. 4b; variance/mean = 204), the discrete generalised Poisson distributions are still very similar to their continuous generalising distributions, but the Gamma and Negative Binomial (Poisson-Gamma) distributions are very different from the Lognormal and Poisson-Lognormal distributions.

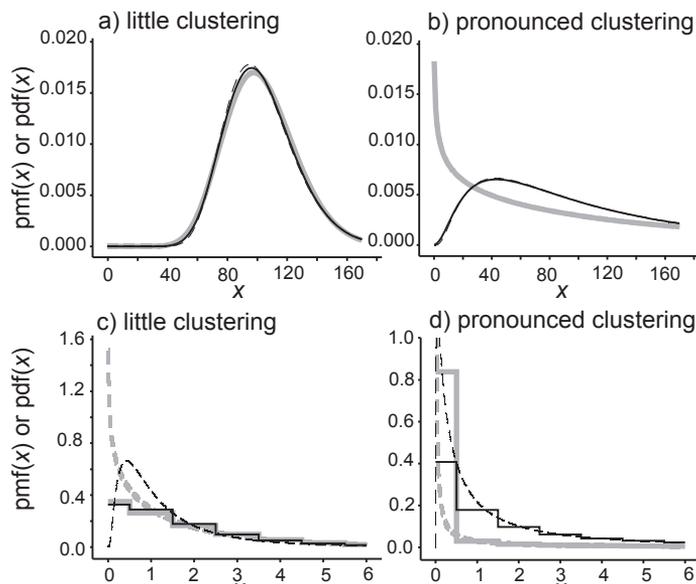


Fig. 4. Comparisons of Lognormal, Gamma, Poisson-Lognormal, and Poisson-Gamma (Negative Binomial) distributions. Dashed lines represent continuous distributions and solid lines represent discrete distributions. Thick grey lines represent gamma based distributions: Gamma (dashed line), Negative Binomial (solid line); narrow black lines represent Lognormal based distributions: Lognormal (dashed line), Poisson-Lognormal (solid line). Either x is count of microorganisms and $\text{pmf}(x)$ is probability mass function of x (discrete variable), or x is concentration of microorganisms and $\text{pdf}(x)$ is probability density function of x (continuous variable).

- a) mean = 103; sd = 24.0; var = 574; var/mean = 5.59; Lognormal: $\text{mean}(\log_{10}) = 2.0$; $\text{sd}(\log_{10}) = 0.1$
- b) mean = 153; sd = 177; var = 31197; var/mean = 204; Lognormal: $\text{mean}(\log_{10}) = 2.0$; $\text{sd}(\log_{10}) = 0.4$
- c) mean = 1.53; sd = 1.77; var = 3.12; var/mean = 2.04; Lognormal: $\text{mean}(\log_{10}) = 0.0$; $\text{sd}(\log_{10}) = 0.4$
- d) mean = 5.46; sd = 29.26; var = 856; var/mean = 157; Lognormal: $\text{mean}(\log_{10}) = 0.0$; $\text{sd}(\log_{10}) = 0.8$

With low mean values (Figs. 4c and 4d), the differences between the discrete generalised Poisson distributions and their continuous generalising distributions become clearer. Where there is little clustering (Fig. 4c; variance/mean = 2.04), the two discrete distributions are practically identical as are the two continuous distributions. The large differences between Gamma and Lognormal in Fig. 4c relate to fractional numbers, which cannot occur in practice. With pronounced clustering (Fig. 4d; variance/mean = 157), there are substantial differences between the two discrete distributions and between the two continuous distributions. There is an approximate 2-fold difference between the discrete distributions in the probability of zero, that is, in the frequency of non-contaminated portions.

The similarities between the distributions under different combinations of mean and overdispersion are illustrated in Table 4 and can be summarised as:

- 1) At high means there is little difference between a continuous distribution and its discrete generalisation of the Poisson distribution. While the discrete distribution may be more theoretically correct, the continuous distribution is easier to use and gives practically the same results.
- 2) At low means, the continuous distributions can differ substantially from their generalisations of the Poisson, and the generalised Poisson distributions should be preferred for low numbers of microorganisms.
- 3) When there is little clustering, Gamma and Lognormal distribution are very similar, as are their generalisations of the Poisson distribution.
- 4) In the presence of substantial clustering, the Gamma and Lognormal distributions are substantially different, again, as are their generalisations of the Poisson.

Table 4. Similarities of distributions for different combinations of mean (high or low) and clustering (little or pronounced) expressed as variance divided by the mean. Note that ‘=’ indicates that a pair of distributions is similar and that ‘≠’ indicates that a pair of distributions is not similar

		Clustering (overdispersion = variance/mean)					
		little (< 6)		Pronounced (>150)			
Mean	high (>100)	Gamma	=	Negative Binomial	Gamma	=	Negative Binomial
		Lognormal	=	Poisson-Lognormal	Lognormal	=	Poisson-Lognormal
	low (< 6)	Gamma	≠	Negative Binomial	Gamma	≠	Negative Binomial
		Lognormal	≠	Poisson-Lognormal	Lognormal	≠	Poisson-Lognormal

4.

Choosing an appropriate statistical distribution

To model spatial distributions of microorganisms occurring at low levels, which is generally relevant for pathogens potentially present in foods, and to consider specifically the phenomenon of (substantial) clustering, some observations can be made regarding the appropriateness of the choice of statistical distribution:

- 1) At high means and with little clustering, the choice of model statistical distribution has little effect.
- 2) The simple Poisson is inappropriate in the presence of any substantial clustering.
- 3) The continuous distributions (Lognormal, Gamma) are inappropriate when there is substantial probability of zeros, especially at low means.
- 4) The family of generalised Poisson distributions is appropriate under a wide range of circumstances.
- 5) Of the two continuously generalised Poisson distributions considered, i.e. the Poisson-Lognormal and the Negative Binomial, the Poisson-Lognormal is preferred on theoretical grounds but it is mathematically complex and may thus be difficult to apply in practice. The more often used Negative Binomial is also appropriate and probably easier to apply.

Besides theoretical grounds and ease of use, the choice between the distributions will depend on how well the different distributions will fit actual observations. At high levels of microorganisms, there is substantial positive experience supporting the use of the Lognormal distribution (e.g., Kilsby et al., 1983; Gale, 2005). At low levels of microorganisms, there is evidence of overdispersion, due to which the Negative Binomial may be superior relative to the Poisson distribution. This is in accord with findings that the Negative Binomial distribution fitted microbial data characterised by a relatively high occurrence of zero counts better than the Poisson distribution (Gonzales-Barron et al., 2010). Unfortunately, there has been very little reported use of the Poisson-Lognormal distribution, perhaps because of the practical difficulties outlined above.

The importance of model choice, especially when choosing between a Gamma basis (including Negative Binomial) and a Lognormal basis (including Poisson-Lognormal), is much greater in the presence of substantial clustering. Since clustering can be quantified by the ratio variance/mean of the numbers and not the log transformation of the numbers, real numbers are important. There are only few reports in the literature that allow this

ratio to be estimated from real data. Whether clustering is a relevant phenomenon or not in a given situation is not always apparent. In studies on microbial distributions in water (El-Shaarawi et al., 1981; Gale et al., 2002), there was evidence obtained suggesting the occasional occurrence of substantial clustering even in water, where a uniform random spatial distribution might be most likely.

5. Conclusions

In this work, six mechanisms have been reviewed that can impact the microbial distribution in foodstuffs: contamination, growth, death, joining, mixing, and fractionation. The impact of each of these mechanisms is relatively easy to predict qualitatively, in terms of the degree of clustering of microorganisms. However, the complexity is increased by the fact that it is more common to have a number of different mechanisms impacting microbial distribution simultaneously or consecutively. The level of clustering will vary depending on, for instance, materials, processes and conditions. Clustering leads to increased variation of frequency distribution, which is statistically called overdispersion. Understanding the distribution or combinations of distributions of microorganisms arising from the various mechanisms involved in the processing of food is important. However, there is a lack of objective, quantitative evidence on the nature of these distributions.

In the absence of data on actual physical distributions of microorganisms in food, this study examined six statistical distributions, that are commonly used for modelling real situations and evaluated their strengths and weaknesses on a theoretical basis next to a practical basis. Furthermore, the impact of choosing one statistical distribution over another was considered. To systematically assess suitability of different statistical distribution types to model a number of possible common spatial distributions of microorganisms, five criteria were proposed for the statistical frequency distribution. These criteria require model outcomes to be non-negative, to allow zeros, to be discrete, to approximate Poisson and to approximate Lognormal. Although Poisson distributions may be a workable approximation depending on the situation, clustering makes their use less appropriate. The continuous distributions (Lognormal, Gamma) are inappropriate when there is a substantial probability of zeros, especially at low means. When the mean is high, there is little difference between statistical distributions, regardless of clustering. While it is not possible to make firm recommendations on the most appropriate statistical distribution for each specific circumstance, a member of the family of generalised Poisson

distributions is likely to be most suitable in many cases in foods. The Poisson-Lognormal distribution is preferred on theoretical grounds but it should be considered that it may be difficult to apply in practice. The more often used Negative Binomial distribution may be easier to apply in practice and is comparatively as appropriate as the Poisson-Lognormal. Indeed, familiarity and ease of use will influence the choice of a model statistical distribution.

Summarising, the Poisson distribution is appropriate where there is good mixing; the Lognormal distribution holds well for high numbers, whereas the Poisson-Lognormal or Negative Binomial distributions generally perform well in other circumstances. Ultimately the final choice is not just a statistical one, but one that fits the data best. In order to evaluate the degree of clustering that actually happens in a food system and to be able to determine which statistical distributions may be appropriate, data are needed from multiple quantitative measurements of individual batches.

In practice, for modelling purposes generally either Poisson or the Lognormal distributions have been used. Considering the suitability assessments presented in the current study, it may be evident that these two types of distributions may not be the best choices in the presence of substantial clustering or at low numbers of microorganisms. To appreciate the impact of the choice of statistical distribution in the modelling of particular important aspects of food safety and public health burden, the effects of such choices are evaluated in detail, based on the results of the current work, in a second paper entitled 'Quantifying impacts on public health and sampling'.

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Chapter 2

Chapter 3

Impact of microbial distributions on food safety II. Quantifying impacts on public health and sampling

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Abstract

The distributions of microorganisms in foods impact the likelihood that a foodstuff will cause illness and therefore also impact the consequential public health burden. As part of food safety management systems, food is sampled and microbiologically tested. The effectiveness of the sampling programme is related to the spatial distribution of the microorganisms that are being sampled for. However, detailed information about the spatial distributions of the microorganisms in food is scarce.

*The impact of microbial clustering and different types of statistical distributions on public health, performance of sampling and performance objectives are discussed. Examples with moderate levels of *Listeria monocytogenes* and low levels of *Salmonella* spp. both distributed with various degree of clustering, show the impact of microbial clustering and the impact of different distributions of exposure on the probability of illness.*

It can be concluded that the risk to get ill can be heavily influenced by variability in doses, as caused by clustering, as well as average dose. This risk is often largely determined by infrequent high doses represented by the right hand tail of the frequency distribution. These infrequent high values are the most important contributors to the arithmetic mean in a batch and thus it is the arithmetic mean (mean of counts), which is more relevant to the assessment of risk than the geometric mean (mean of logs), which has been the most commonly used parameter to represent average microbial counts. Furthermore, a more sophisticated definition of performance objectives that includes consideration of clustering might be needed. Both clustering and the choice of statistical distributions have a substantial effect on the acceptance probability of microbiological criteria.

1. Introduction

Microorganisms in foods are often assumed to be distributed lognormally, since this distribution appears to fit the observations (Kilsby and Baird-Parker, 1983) or according to the Poisson distribution. There is some mechanistic support for the use of these distributions. The Lognormal distribution is appropriate for high numbers, and the Poisson distribution is appropriate where there is good mixing, so not when there is substantial clustering. Irregular clustering of microorganism impacts on the frequency distribution and needs to be considered as well in the choice of statistical distribution. The previous part of this paper (Factors influencing microbial distributions and modelling aspects), showed that clustering did affect the frequency distribution by impacting on both the shape and the parameters like mean and standard deviation of the frequency distribution. In theory, Poisson-Lognormal and the Negative Binomial distribution are statistical distributions which can describe clustering. However, the final choice will depend on which distribution(s) fit(s) the data best.

Although log counts are often used to indicate the concentrations of microorganisms in food, clustering of microorganisms in food can be quantified by the ratio variance/mean of the microorganisms counts and not the log counts. A small ratio indicates little clustering and a high ratio indicates substantial clustering. Different clustering of microorganisms will result in different shapes of frequency distributions and the levels of the contaminant will vary in each dose or each analytical unit. Variation in doses will impact on the probability to get ill and variation in analytical unit will impact on the sampling plan results.

Industry and government aim to protect consumers; they spend considerable effort to ensure that microorganisms detrimental to food quality or consumer safety are eliminated or otherwise controlled in foods. Industry utilises food safety management systems such as good hygienic practices (GHP), good manufacturing practices (GMP) and hazard analysis critical control point system (HACCP) to ensure this. Tools such as sampling according to microbiological criteria (MC) underpin the food safety management concepts utilised by both government and industry. Governments, via Codex Alimentarius (Codex), have been introducing several new risk-based metrics for food safety management (FAO/WHO, 2006; CAC, 2007). These metrics are the appropriate level of protection (ALOP), performance objective (PO) and food safety objective (FSO), which supplement existing management tools.

An ALOP aims to control the public health burden of a foodborne disease to an acceptable risk by setting objectives (FSO, PO) for contamination which in certain cases can be monitored or controlled by sampling plans (MC). A PO is defined as ‘the maximum frequency and/or concentration of a hazard in a food at a specified step in the food chain before the time of consumption that provides or contributes to an FSO¹ or an ALOP, as applicable’ (CAC, 2007). A PO set by a competent authority is justified in terms of public health protection as it relates to an ALOP. Accordingly, to the extent that the public health impact of microorganism depends upon their distribution in a food as well as on their number, both aspects should be considered when setting a PO. In this regard, the choice of statistical distribution is important and should best reflect important aspects of distribution, such as homogeneity or heterogeneity. If, in the relevant circumstances, clustering of the microorganism in the products concerned is likely, it may have a marked impact on the number of illnesses in the population.

Due to the variation in consumption of servings and the potential exposure to pathogenic microorganisms, the number of illnesses in a population can vary. Not all servings of a food product contain equal numbers of microorganisms and not all microorganisms are equally hazardous. The exposure distribution for batches of product manufactured over time in multiple factories will be influenced by numerous factors: the between-factory variability, the between-batch variability, the within-batch variability and the variability due to a different storage history of the products. This will result in a distribution of microorganisms throughout all products across multiple batches as well as within a product. If clustering of the microorganisms in the food products or servings occurs, it is likely that this will influence the number of illnesses in the population consuming these servings.

Understanding distributions of pathogenic microorganisms is vital to establish proper microbiological criteria, to obtain a realistic view of the performance of the associated sampling plans, and to verify risk-based metrics such as POs and FSOs.

This study investigates how microbial clustering may influence illnesses and public health. Additionally, it investigates the implications of clustering for the contamination objectives (PO, FSO) and it investigates how microbial clustering may influence the effectiveness of sampling plans. In the case of public health, we specifically focus on the impact of the degree of microbial clustering, various frequency distributions of exposure, and the ‘tails’ of a distribution on the number of illnesses.

¹ FSO is a PO set at the time of consumption. Definition FSO: The maximum frequency and/or concentration of a hazard in a food at the time of consumption that provides or contributes to the appropriate level of protection (ALOP) (CAC, 2007).

This paper is the second part of the study ‘The impact of microbial distributions on food safety’. The companion paper ‘Factors influencing microbial distributions and modelling aspects’ provides an appraisal of statistical distributions that may be used to describe and represent the spatial distributions of microorganisms, especially the capacity to represent microbial clustering.

2. Impact of microbial distributions on public health: effect of clustering and ‘tails’

2.1 Effect of microbial clustering on public health

In order to see the effect of clustering on public health and the number of illnesses, three different spatial distributions of a contaminant in a batch of servings are investigated: regular, uniform random and one cluster distribution as shown in Fig. 1.

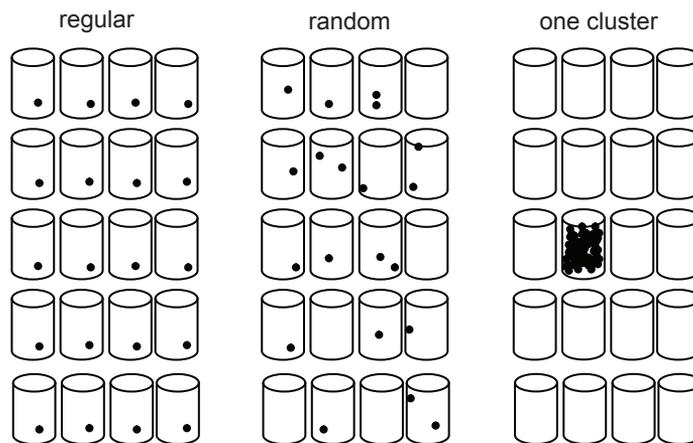


Fig. 1. Regular distribution, uniform random distribution and one cluster distribution of a contaminant in a batch of servings; 20 servings out of the complete batch are shown as examples.

The ratio variance/mean can be used to compare the degree of clustering of these spatial distributions. The mean is the same for the three distributions, however, the variance increases. The regular distribution has the smallest variance and is least clustered, the uniform random distribution is intermediately clustered and the one cluster distribution

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- is most clustered. The investigated batches consist of 10^8 servings each and each batch is contaminated with a total of 10^8 bacterial cells. Each serving will be consumed by a different consumer. Three spatial distributions of the contaminating cells are considered:
- 1) Regular distribution, in which every serving contains exactly one cell.
 - 2) Uniform random distribution, meaning that servings may contain 0 cells, or 1 cells, some 2, 3, or 4, etc. as can be described by a Poisson distribution.
 - 3) One cluster distribution, in which one serving contains all of the 10^8 cells as one cluster in a single serving and all the other servings are free of the contaminant.

While the degree of clustering of a contaminant may affect the resulting illnesses in the population upon exposure, the number of illnesses likely also depends on how virulent the contaminating microorganism is and on its ability to proliferate in the product to reach higher levels. Three examples of microorganisms are investigated: 1) a relatively low virulent microorganism represented by *Listeria* with an r value of 1×10^{-10} CFU⁻¹ (Buchanan et al., 1997) and r is a measure of the infectivity of the microorganism, 2) a relatively high virulence microorganism represented by *Salmonella* spp. with an r value of 0.002 CFU⁻¹ (FAO/WHO, 2002), and 3) a toxin producing microorganism are investigated; the toxin producing microorganism will result in illness if the serving contains more than 10^5 microorganisms. Table 1 shows the number of cells per serving for three distributions of cells after 10^x -fold growth referred to as x logs growth. In the initial situation or in case the microorganism do not grow in the product, x is zero.

Table 1. Characterisations of the distribution of cells in three cases: regular, initially uniform random, and one cluster represented as the number of cells per servings after x logs growth. At the initial situation or in case there is no growth, x is 0

Case 1: regular	Case 2: initially uniform random		Case 3: one cluster
	Cell clusters are Poisson distributed; every cluster has 10^x cells.		
	cells/serving	% servings	
Every serving contains 10^x cells	0	36.8%	One in 10^8 servings contains 10^{8+x} cells. Remaining servings have no cells.
	1×10^x	36.8%	
	2×10^x	18.4%	
	3×10^x	6.13%	
	4×10^x	1.53%	
	5×10^x	0.31%	
	6×10^x	0.051%	
	etc.		

Considering single-value outcomes and assuming an exact dose-response relationship, Table 2 shows the number of illnesses due to consumption of these 10^8 servings with the three different pathogens and with the three degrees of clustering. The considered growth stages are no growth, 4-logs growth and 5-logs growth.

Table 2. Illnesses per 10^8 servings caused by a low virulent, high virulent, and a toxin producing pathogen after different growth stages calculated with the Binomial dose-response model^{a,b}. The microorganisms are distributed regularly, initially uniform randomly, and in one cluster resulting in a degree of clustering of least, intermediate, and most clustered, respectively

Pathogen	growth	Distribution: Degree of clustering:	Case 1	Case 2	Case 3
			regular Least	uniform random Intermediate	one cluster Most
1. low virulent: $r = 1 \times 10^{-10} \text{ CFU}^{-1}$					
	No growth		0.01	0.01	0.00995
	4-logs growth		100	100	1
2. high virulent: $r = 0.002 \text{ CFU}^{-1}$					
	No growth		200,000	199,800	1
	4-logs growth		100,000,000	63,212,056	1
3. toxin producer causing illness at or above 10^5 cells					
	No growth		0	0	1
	4-logs growth		0	11.14	1
	5-logs growth		100,000,000	63,212,056	1

^a The Binomial dose-response model $\text{Pr}(\text{ill}|\text{dose}) = 1 - (1-r)^{\text{dose}}$ (Haas, 2002) is used, since the scenarios result in a specific known single dose, which is entered in the dose-response model to obtain the number of illnesses or probability of illness in one consumer.

^b If the number in the table is equal to or above one, it is the number of illnesses in the population; if the number is below 1, it is the probability that one consumer will become ill in the whole population (of 10^8 consumers consuming 10^8 servings).

In case of a low virulent pathogen and no growth, there is no significant impact of clustering on the number of illnesses. For the three degrees of clustering, the number of illnesses estimated is approximately 0.01, meaning a probability of 1 in 100 that one illness will occur in the whole population. After 4-logs growth and in the most clustered case (case 3), the number of illnesses is 1, since all the contamination is in one serving. For the initially uniform random and regular cases, many more illnesses are estimated, since in a higher proportion of the servings contamination is present and will grow to a relatively high level. Servings initially free of microorganism remain microorganism free and servings that do contain microorganisms contain much higher levels after 4-logs growth.

In case of a high virulent pathogen and both no growth and 4-logs growth, in the most clustered distribution (case 3) only the single contaminated serving results in one illness. The least and intermediate clustered distributions show much higher numbers of cases of illness. Especially high numbers are estimated after 4-logs growth. The regularly distributed contamination, it is predicted that all servings are contaminated and, thus, that all 10^8 consumers will get ill. In the initially random distribution 37% of the servings were not contaminated (due to randomness), so the number of illnesses in that case was projected as 6.3×10^7 .

In case of the toxin producing pathogen, the level necessary for toxicity is 10^5 cells/serving. In the most clustered distribution and no growth, only 1 illness will occur. In the regular and uniform random microbial distributions result in no projected illnesses, since the numbers per serving are far below the levels necessary to surpass the threshold cell concentration for toxin production. For the regular distribution and 4-logs growth, this growth will also not result in illness. For the random distribution and 4-logs growth, there are a few products that initially contain more than 10 microorganisms (although on average there is 1), and these products will result finally in levels above 10^5 cells/serving. After 5-logs growth, also in regular contaminated servings and all initially-randomly contaminated products, the threshold cell level for toxin formation is reached.

Different degrees of clustering of pathogenic microorganisms as represented in Fig. 1 showed that clustering can influence the number of illnesses.

2.2 Distribution of contaminants across multiple batches of food

The distribution of microorganisms across multiple batches of food products, which have been manufactured in a specific period in multiple factories, can show clustering within batches as well as between batches. This can be illustrated with an example of 5 litre containers of milk which represent batches of milk. Each 5 litre container holds many servings of 100 mL. Within each 5-litre container the microorganisms are randomly spread throughout the product. This means that the within-container distribution of concentration per serving (dose) is distributed according to Poisson and contains no clustering. However, the batches show between-container variation in concentrations of the contaminant, which is assumed to be lognormally distributed. In this example the within-container distribution of individual doses (Poisson) combines with the between-container distribution of means (Lognormal) to give a distribution of individual doses that

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is Poisson-Lognormal across multiple containers. Such examples are explored in 2.4 and 2.5.

2.3 Number of illnesses in a population (N_{ill})

If one consumes a serving and the serving might contain pathogenic microorganisms, there is a probability to get ill. If the serving causes illness, this means:

- a) The serving contains a number of cells, the dose D (CFU). The probability of this is the dose frequency distribution, $P_{dose}(D)$.
- b) Consumption of D (CFU) results in illness. The probability of this is the dose-response relationship, $P_{response}(D)$.

The overall probability that a serving causes illness, P_{ill} , combines these probabilities over all possible doses:

$$P_{ill} = \sum_{D=0}^{\infty} [P_{dose}(D) \cdot P_{response}(D)] \quad (1)$$

The relation between the $P_{response}(D)$ and the ingested dose can be represented with a Binomial dose-response:

$$P_{response}(D) = 1 - (1 - r)^D \quad (2)$$

where r is the dose-response parameter (CFU⁻¹), which is a measure of the infectivity of the microorganism. For a Binomial dose-response, if the dose frequency distribution is Poisson with mean \bar{D} (i.e. $P_{dose} = \bar{D}^D \exp(-\bar{D}) / D!$) then Eq. 1 becomes the exponential dose-response:

$$P_{ill}(\bar{D}) = 1 - \exp(-r\bar{D}) \quad (3)$$

While the Binomial dose-response gives the probability of illness from a specific dose, D the exponential dose-response gives the probability of illness from a random dose from a Poisson distribution with mean \bar{D} (Haas, 2002). Note that although each specific dose (D) is integer the mean dose (\bar{D}) is generally not integer.

In the examples considered in section 2.4 and 2.5 the dose frequency distributions (P_{dose}) are generalised Poisson distributions, in which doses can be considered as drawn from Poisson distributions where the mean, \bar{D} , follows a second *-generalising-* distribution,

$P_{mean}(\bar{D})$. For example, if the means follow a Lognormal distribution, $P_{mean}(\bar{D}) = \text{Lognormal}(\bar{D}; \mu, \sigma)$, then the individual doses follow a Poisson-Lognormal distribution, $P_{dose}(D) = \text{PoissonLognormal}(D; \mu, \sigma)$. In this work \bar{D} is used to denote the parameter (mean) of a Poisson dose distribution; where doses follow a generalised Poisson distribution this will not generally be the overall mean. Calculations of generalised Poisson distributions can be burdensome, making use of Eq. 1 difficult. In such circumstances it can be useful to use the exponential dose-response (Eq. 3) leading to

$$P_{ill} = \int_{\bar{D}=0}^{\infty} [P_{mean}(\bar{D}) \cdot (1 - \exp(-r\bar{D}))] d\bar{D} \quad (4)$$

Eq. 4 does not contain the distribution of individual doses (e.g. the Poisson-Lognormal), only the distribution of Poisson means (e.g. the Lognormal), which is usually much easier to work with.

In order to determine the effects on public health, the number of illnesses in a population (N_{ill}) equals the number of servings consumed in the population (S) multiplied by the probability of becoming ill by consuming a serving (P_{ill}).

$$N_{ill} = S \cdot \int_{\bar{D}=0}^{\infty} [P_{mean}(\bar{D}) \cdot (1 - \exp(-r\bar{D}))] d\bar{D} \quad (5)$$

The mean consumed dose (\bar{D}) is dependent on the mean concentration of the pathogenic microorganisms and the serving size:

$$\bar{D} = \bar{C} \cdot M \quad (6)$$

with: \bar{D} : mean dose (CFU) in a group of servings within which the dose is assumed to be Poisson distributed, \bar{C} : mean concentration (CFU/g) in that group of servings, M : serving size (g).

2.4 Examples of different Poisson-Lognormal dose frequency distributions impacting on public health

The effect of a frequency distribution of exposure will be illustrated with two examples, in which moderate levels of *Listeria monocytogenes* (section 2.4.1) and low levels of *Salmonella* spp. (section 2.4.1) are distributed within a product.

2.4.1 Example 1: *Listeria* at moderate levels

The within-batch mean concentration \bar{C} of *Listeria monocytogenes* is lognormally distributed with parameters $\log_{10}(\bar{C}) = \text{Normal}(0,2)$. The maximum level of *L. monocytogenes* is assumed to be 1×10^8 CFU/g. The serving size is 100 g, which results in a maximum dose per serving of 1×10^{10} CFU. In other words, the mean dose \bar{D} ($\log_{10}(\bar{D}) = \text{Normal}(2,2)$) is a *truncated* Lognormal, truncated at 10^{10} CFU. In Fig. 2a the black line represents the mean dose distribution $\log_{10}(\bar{D})$. The mean dose distribution $P_{mean}(\bar{D})$ can be combined with the exponential dose-response relationship with an r -value of 1×10^{-10} CFU⁻¹, representing *Listeria* (Buchanan et al.,1997) and displayed as a black line in Fig. 2b. In this example N_{ill} becomes (Eq. 7):

$$N_{ill} = S \cdot \int_{\bar{D}=0}^{10^{10}} \left[\text{Normal}(\log(\bar{D}), 2, 2) \cdot (1 - \exp(-\bar{D} \cdot 1 \cdot 10^{-10})) \right] d\bar{D} \quad (7)$$

with 10^{10} CFU is the upper limit of integration, which reflects the truncation of the Lognormal dose frequency distribution. Multiplying the mean dose distribution $P_{mean}(\bar{D})$ and the dose-response curve ($P_{ill}(\bar{D})$) results in a combined frequency of illness given a certain mean dose (Fig. 2c, black curve). After integration, the probability of illnesses, P_{ill} , is 1×10^{-4} , meaning a risk per serving of 1 in 10,000. If, for example, a million people each consume 100 servings in a year, $S = 100$ million servings would be consumed, this would result in 10,000 cases. In reality the prevalence of the contamination and the part of the population that is susceptible for the contaminant have to be taken into account. For example, if the prevalence is 10%, and the susceptible group is not the whole population but only 20% of the population, the resulting number of cases would be estimated as 200.

2.4.2 Example 2: *Salmonella* spp. at low levels

In this example, a pathogen with a higher infectivity, *Salmonella* spp. with an r -value of 0.002 CFU⁻¹ (FAO/WHO, 2002) is used to illustrate the relation between variability in dose, dose-response and ultimate level of illness. The mean dose of low levels of *Salmonella* spp. are lognormally distributed ($\log_{10}(\bar{D}) = \text{Normal}(-6,2)$) within a batch of servings. The serving size is 100 g and the maximum dose per serving is 1×10^{10} CFU. For the example of *Salmonella* spp. the grey curve in Fig. 2a shows the frequency distribution of mean doses $\log_{10}(\bar{D})$. The grey curve in Fig. 2b shows the dose-response relation using the exponential dose-response model for *Salmonella* spp..

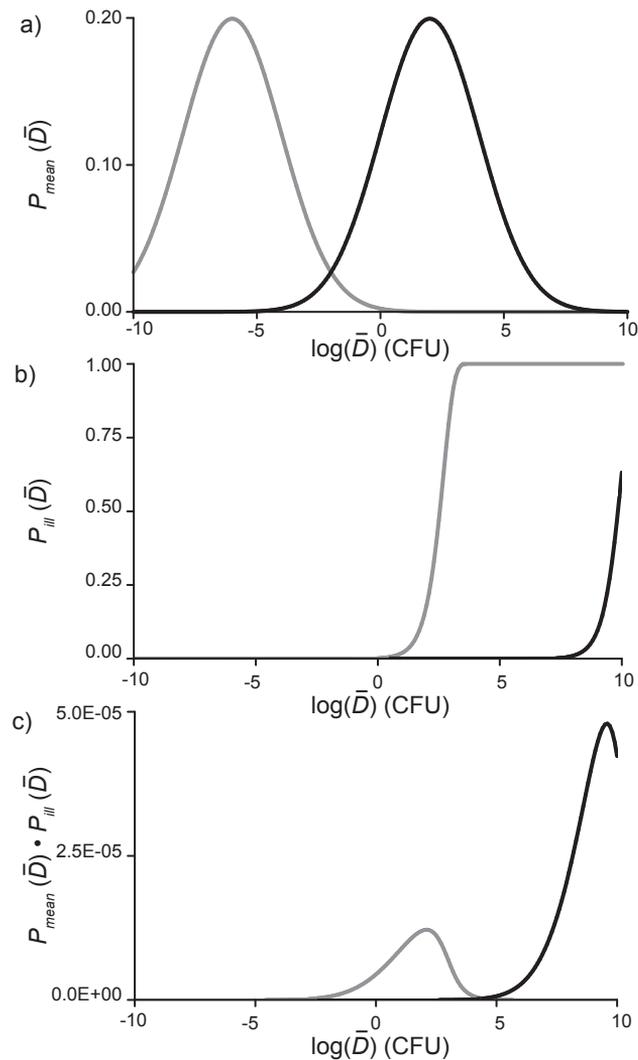


Fig. 2.

(a) Frequency distribution of lognormally distributed mean dose of *Listeria* $\log_{10}(\bar{D}) = \text{Normal}(2,2)$ (black curve) and lognormally distributed mean dose of *Salmonella* spp. $\log_{10}(\bar{D}) = \text{Normal}(-6,2)$ (grey curve) with dose on a log scale. This represents the probability that a random dose comes from a group with the given mean.

(b) Exponential dose-response relationship with $r = 1 \times 10^{-10} \text{ CFU}^{-1}$ for *Listeria* (black line) and with $r = 0.002 \text{ CFU}^{-1}$ for *Salmonella* spp. (grey line). This represents the probability that a random dose from a Poisson-distributed group of doses with the given mean causes illness.

(c) Graphs (a) and (b) combined to determine the overall frequency of illness caused by *Listeria* (black line) and by *Salmonella* spp. (grey line) in a similar approach as Stellbrink and Dahms (2004).

After integration, the probability of illnesses, P_{ill} , is 3.5×10^{-5} . If a million people each consume 100 servings in a year, this would result in a number of illnesses (N_{ill}) of 3,500.

It is striking, both examples show that the right hand tails of the mean dose distribution are responsible for the majority of illnesses. Fig. 2 shows for *Salmonella* spp. that more than 99% of the distribution of \bar{D} is smaller than 1 CFU. The doses that cause illness, however, are in the tail and mostly come from \bar{D} values above 1 CFU.

2.5 Examples of different dose frequency distributions and various degrees of clustering impacting on public health

The effect of different dose frequency distributions and of various degrees of clustering on the overall risks to consumers is illustrated with an example (similar to subsection 2.4.1). *Listeria* is assumed to be present with an arithmetic mean fixed at 500 ($\log_{10} 500 = 2.70$), but with the arithmetic standard deviation varying from 31.6 to 10^6 ($\log_{10} 31.6 = 1.5$; $\log_{10} 10^6 = 6.0$) as shown in the first two columns of Table 3. The third column shows the ratio variance/mean indicating the degree of clustering. These values are realistic for concentrations in contaminated food products with a \log_{10} standard deviation of 1.5, representing the least clustered contamination, up to a standard deviation of 6 representing the relatively most clustered contamination. The parameters of a Poisson-Lognormal distribution with those values of arithmetic mean and standard deviation are shown in the fourth and fifth columns of Table 3. Those parameters represent the mean and standard deviation of the logs of within-group Poisson means. Illness rates resulting from such Poisson-Lognormal distributions of individual doses are shown in column PL. For comparison, Table 3 also includes the illness rates calculated for Negative Binomial (column NB) distributions with the same arithmetic means and standard deviations, and for an un-clustered Poisson distribution with the same arithmetic mean dose (column Poisson). These illness rates are calculated making use of Monte-Carlo simulations. From table 3 can be concluded, that all three statistical distributions (Poisson, PL, NB) give comparable results. In most cases the risk is equal and where there are differences in the estimated risk they are marginal. For this fixed value of the arithmetic mean, neither the choice of the statistical distribution nor the standard deviation has a substantial impact on the overall level of risk.



Table 3. Probability of illness (P_{ill}) due to *Listeria* for three dose distributions with a fixed arithmetic mean dose of 500 ($\log_{10} 500 = 2.7$) and increasing standard deviations

Dose distribution parameters			Poisson-Lognormal parameters		$\log_{10}(P_{ill})$ from different dose distributions, each with the same mean(D) and sd(D) ^c		
\log_{10} (mean(D))	\log_{10} (sd(D)) ^c	\log_{10} (sd ² /mean) ^d	Mean ($\log_{10}(\bar{D})$)	sd ($\log_{10}(\bar{D})$)	PL ^a	NB ^b	Poisson
2.70	1.5	0.30	2.70	0.02	-7.30	-7.30	
2.70	2.0	1.30	2.69	0.08	-7.30	-7.30	
2.70	2.5	2.30	2.63	0.25	-7.30	-7.30	
2.70	3.0	3.30	2.35	0.55	-7.30	-7.30	
2.70	3.5	4.30	1.89	0.84	-7.30	-7.30	-7.30
2.70	4.0	5.30	1.40	1.06	-7.30	-7.30	
2.70	4.5	6.30	0.90	1.25	-7.30	-7.30	
2.70	5.0	7.30	0.40	1.41	-7.30	-7.30	
2.70	5.5	8.30	-0.10	1.56	-7.30	-7.31	
2.70	6.0	9.30	-0.60	1.69	-7.31	-7.34	

^a Poisson-Lognormal

^b Negative Binomial

^c except for Poisson for which $\log_{10}(sd(D)) = \log_{10}(\text{mean}(D))/2 = 1.35$

^d except for Poisson for which $\log_{10}(sd^2/\text{mean}) = 0$

In Table 4, the example as in Table 3 for *Listeria*, is re-calculated for *Salmonella* spp., characterised by a higher virulence with $r = 0.002 \text{ CFU}^{-1}$. The arithmetic mean is fixed at dose = 0.1 ($\log_{10} 0.1 = -1$) and the arithmetic standard deviations ranges from 3.16 to 10^5 ($\log_{10} 3.16 = 0.5$; $\log_{10} 10^5 = 5.0$).

Table 4. Probability of illness (P_{ill}) due to *Salmonella* spp. for three dose distributions with a fixed arithmetic mean dose of 0.1 and increasing standard deviations

Dose distribution parameters			Poisson-Lognormal parameters		$\log_{10}(P_{ill})$ from different dose distributions, each with the same mean(D) and sd(D) ^c		
\log_{10} (mean(D))	\log_{10} (sd(D)) ^c	\log_{10} (sd ² /mean) ^d	Mean ($\log_{10}(\bar{D})$)	sd ($\log_{10}(\bar{D})$)	PL ^a	NB ^b	Poisson
-1	0.5	2.00	-2.50	1.14	-3.72	-3.74	
-1	1.0	3.00	-3.00	1.32	-3.75	-3.96	
-1	1.5	4.00	-3.50	1.47	-3.79	-4.52	
-1	2.0	5.00	-4.00	1.61	-3.86	-5.28	
-1	2.5	6.00	-4.50	1.74	-3.93	-6.12	-3.70
-1	3.0	7.00	-5.00	1.86	-4.02	-7.00	
-1	3.5	8.00	-5.50	1.98	-4.11	-7.91	
-1	4.0	9.00	-6.00	2.08	-4.21	-8.84	
-1	4.5	10.0	-6.50	2.19	-4.32	-9.78	
-1	5.0	11.0	-7.00	2.28	-4.43	-10.76	

^a Poisson-Lognormal

^b Negative Binomial

^c except for Poisson for which $\log_{10}(sd(D)) = \log_{10}(\text{mean}(D))/2 = -0.5$

^d except for Poisson for which $\log_{10}(sd^2/\text{mean}) = 0$

Because of the higher virulence, the probability of illness for *Salmonella* spp. is estimated to be much higher than in the example with *Listeria*. However, again, the risks calculated with the Poisson and Poisson-Lognormal distributions are very similar, and markedly different from those calculated with the Negative Binomial distribution. For the Poisson-Lognormal (PL column), there is a decrease in risk with increasing standard deviation (5-fold). Considering, however, the relatively substantial uncertainties normally associated with quantitative calculations of risk, this decrease could be considered to be a minor effect. The Negative Binomial distribution gives results which are quite different from those obtained with the other two types of statistical distributions, and shows a gradual decrease in the level of risk with an increase in the standard deviation.

This example shows that, in cases where low concentrations matter, the choice of the distribution to model clustering is important. Although it is unclear which distribution is right, it is evident that the choice of the statistical distribution does influence the calculated risk outcome.

3. Impact of microbial distributions on microbiological criteria and performance objectives

3.1 Microbiological criteria

A microbiological criterion (MC) is defined (CAC, 1997; CEC, 2005) as ‘the acceptability of a product, a batch of foodstuffs or a process, based on the absence or presence or number of microorganisms per unit(s) of mass, volume, area, or batch’ and includes: 1) A sampling plan defining the number of ‘field samples’ to be taken and the size of the ‘analytical unit’, 2) Microbiological limits, 3) The number of samples units which should conform to those limits. This explicitly includes details of the size and number of sample units considered, so that the effect of the known/assumed distribution of the microorganism on the performance of the microbiological criterion can be assessed. In the context of this work, a sampling plan is defined by four numbers, n , c , m and M , where: n is the number of sample units examined to determine the acceptability of the batch; c is the maximum acceptable number of sample units with values above m and below M , but no samples are acceptable with values above M ; n and c are numbers of sample units, m and M are values, to which test results on an analytical unit may be compared. For a two-class plan, M is indefinitely large. For a presence/absence plan, m is generally zero (in an analytical unit).

The effectiveness of a sampling plan is described by its operating characteristic (OC) (ICMSF, 2002), which gives the probability of a batch meeting the criterion as a function of the quality of the batch. By convention, in graphs of operating characteristics the probability of acceptance is represented increasing upwards versus the proportion defective in a batch increasing to the right. The operating characteristic shows that there is a probability –ideally small– that a high quality batch will be rejected or a low quality batch will be accepted; these probabilities are known as Producer's (or Seller's) and Consumer's (or Buyer's) Risks, respectively. To calculate the OC as function of the concentration in the batch assumptions have to be made about the distribution of microorganisms in the batch, for example a Lognormal distribution.

Fig. 3 shows OCs for the sampling plan, $n = 30$, $c = 0$, and $m = 10$ CFU/analytical unit, against the mean of a lognormally distributed contaminant for a range of standard deviations σ (0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 \log_{10} (CFU/analytical unit)). The graph shows that changing the standard deviation of a Lognormal distribution completely changes the probability of acceptance and consequently the performance of this sampling plan.

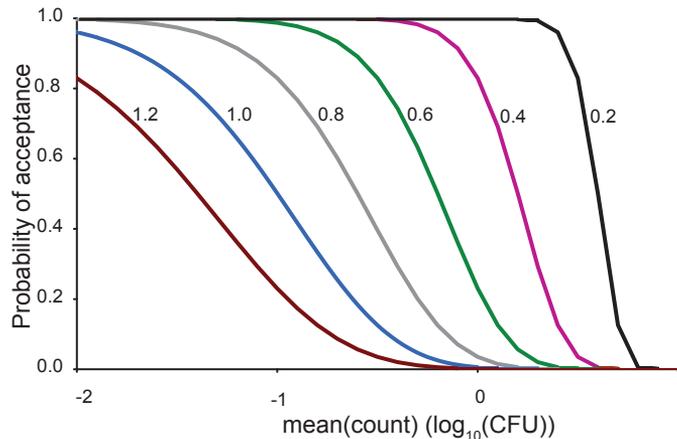


Fig. 3. Operating characteristics showing the dependence of acceptance probability on variability (Lognormal distribution). The sampling plan is characterised by $n = 30$, $c = 0$, $m = 10$ CFU. The labels show $\sigma \log_{10}(\text{count})$ (CFU). The size of the sample is an analytical unit.

For a constant high standard deviation indicating substantial clustering, Fig. 4 shows OCs for the sampling plan $n = 5$, $c = 0$, and $m = 0$ CFU/analytical unit, for five different types of statistical distributions, namely Poisson, Poisson-Lognormal, Lognormal, Gamma, and Negative Binomial. The arithmetic variances of the different distributions are equal and fixed at 1000 (except for the Poisson where the variance equals the mean by definition).

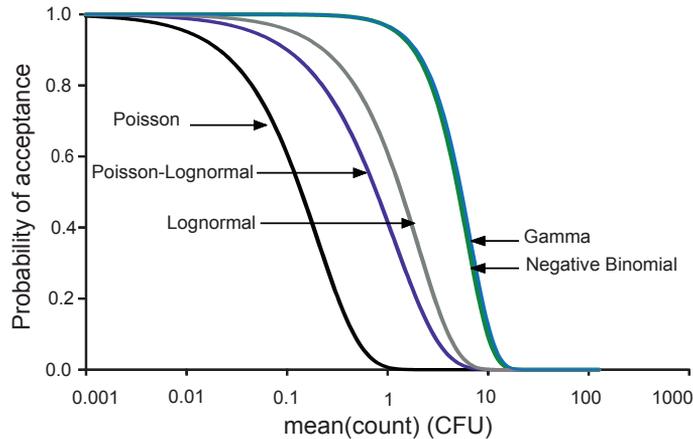


Fig. 4. Dependence of acceptance probability on types of frequency distributions in the case of substantial clustering. For all distributions $\sigma^2(\text{count}) = 1000$, except Poisson for which $\sigma^2(\text{count}) = \text{mean}(\text{count})$. The sampling plan is characterised by $n = 5$, $c = 0$, $m = 0$ CFU. The size of the sample is an analytical unit. All counts are in CFU.

The microbiological criterion in this example sampling plan is ‘absent in the analytical unit’ for any of 5 samples taken. This has a natural interpretation for the discrete distributions (i.e., the Poisson, Negative Binomial and Poisson-Lognormal distributions) but needs some elaboration for the continuous distributions (i.e., Gamma and Lognormal), where the variable is concentration in the sample (CFU/analytical unit) rather than count and where there is zero probability of 0 CFU/analytical unit. ‘Absent in the analytical unit’ has been interpreted as ‘<1 CFU/analytical unit’ for the continuous distributions. Fig. 4 shows that the OCs for different distributions are similar in shape, but are separated from each other and the differences are quite substantial. For example, the probability of acceptance at a mean of 1 CFU is for the Gamma (0.97) and the Negative Binomial distribution (0.97) much higher than Lognormal (0.61), Poisson-Lognormal (0.42), and Poisson (0.007). Even neglecting the Poisson, whose variance is considerably less than the other distributions, in the case of substantial clustering there are clear differences between the OCs based on the different distributions. The difference between the three discrete distributions is especially striking, as is the similarity between the Gamma and the Negative Binomial.

In Fig. 4, the ‘important’ part of the OCs can be thought of as those where the acceptance probability is changing rapidly, in this case the mean is less than about 10.

At this mean, the chosen variance of 1000 (except for the Poisson where the variance equals the mean) suggests substantial clustering. The variance value of 1000 was chosen arbitrarily on the basis that, for a mean of around 5 CFU, the Lognormal distribution gives a standard deviation of \log_{10} values of about 0.8, which is the standard deviation generally used (Legan et al., 2001; ICMSF, 2002; Dahms, 2004).

For a constant smaller deviation indicating less clustering an arithmetic variance of 10 is chosen. Fig. 5 shows significantly smaller differences between OCs corresponding to different statistical distributions. For example, the probability of acceptance at a mean of 1 CFU is for the Lognormal, Gamma, Poisson, Poisson-Lognormal, and the Negative Binomial distribution, 0.28, 0.38, 0.007, 0.098, and 0.28 respectively.

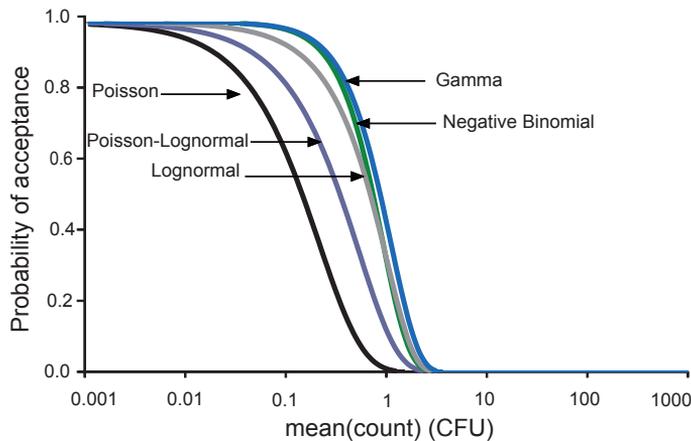


Fig. 5. Dependence of acceptance probability on types of frequency distributions in the case of little clustering. For all distributions $\sigma^2(\text{count}) = 10$, except Poisson for which $\sigma^2(\text{count}) = \text{mean}(\text{count})$. The sampling plan is characterised by $n = 5$, $c = 0$, $m = 0$ CFU. The size of the sample is an analytical unit. All counts are in CFU.

These three examples illustrate that clustering can influence the sampling plan performance. Additionally, the shape of a statistical distribution becomes important for substantial clustering.

3.2 Performance objectives

A PO and an FSO are described as a ‘maximum frequency and/or concentration’ of a hazard at a particular point in the food chain (PO) or at the time of consumption (FSO). The PO and FSO concepts are rather new in international food safety management. Some standards may appear to have features of a PO or FSO. For example, the EU Scientific Committee on Veterinary Measures Relating to Public Health (EC, 1999) recommended: ‘An objective must be to keep the concentration of *L. monocytogenes* in food below 100 CFU/g and to reduce the fraction of foods with a concentration above 100 *L. monocytogenes* per gram significantly’. This is not an FSO because 100 CFU/g is not a strict maximum valid for all packages of a food product or batch thereof, but rather a ‘target for improvement’, for which the fraction of foods above the stated level is to be reduced. Indeed, the recommendation continues ‘This objective should be expressed as a Food Safety Objective’ clarifying that, as it stands, the proposed objective is not a Food Safety Objective.

4. Discussion

4.1 Impact of microbial distributions on public health: effect of clustering and ‘tails’

Table 2 shows that clustering of pathogenic microorganism can have an impact on public health. A contamination distributed in one cluster resulted in fewer illnesses than randomly or regularly distributed contaminations.

Fig. 2 shows the relationship between exposure of *Listeria* and *Salmonella* spp. and resulting illnesses. Assuming a higher infectivity, the dose-response relationship for *Salmonella* spp. (Fig. 2b, grey curve) saturates at lower doses than for *Listeria* (Fig. 2b, black curve) and the probability of illness reaches a plateau at 1. Comparing the dose-response relationship of *Listeria* and *Salmonella* spp., the curve has moved to the left. In these examples with all the assumptions made, the overall estimated number of illness per 100 million servings is 10^4 caused by *Listeria* and is 3500 caused by *Salmonella* spp.. One can conclude that in both cases the largest number of illnesses is caused by the very infrequent but very high doses. In other words, the right hand tail of the exposure distribution largely determines the cases of illness.

Similar results were obtained by calculations (ILSI, 2010) using a Beta-Poisson dose-response model instead of the exponential dose-response model.

4.2 Impact of the microbial distributions on microbiological criteria

In the general application of acceptance sampling plans (e.g., BSI, 2005; Grant and Leavenworth, 1996), the spatial distribution is often taken as uniform random leading to a Poisson (or sometimes Binomial) distribution, defined by one parameter, so that batch quality is defined by one parameter: the mean. When considering microbiological contamination of foods and possible clustering, however, the dispersion is also important, so that other parameters as well as the mean must be considered when assessing batch quality and calculating acceptance probabilities. Fig. 3 confirms that changing the standard deviation of a Lognormal distribution may completely change the performance of a sampling plan.

The impact of microbiological frequency distributions on sampling plan performance has previously been investigated using a Lognormal distribution of microorganisms numbers (Legan et al., 2001; Dahms, 2004). Generally, both studies demonstrated the general dependence of acceptance probability on the mean with an assumed standard deviation of $0.8 \log_{10}$ units. However, the dependence of acceptance probability on standard deviation was demonstrated (Legan et al., 2001) and Dahms (2004) remarked that ‘the effect of using an attributes plan is also dependent on the validity of the underlying assumptions for the statistical distribution, especially with regard to its standard deviation’.

Fig. 4 and 5 show the choice of statistical distributions has a substantial effect on the evaluation of microbiological criteria. Also clustering has a significant impact on the acceptance probability for typical OC-curves and the effectiveness of sampling. Even if microorganisms exhibit an almost regular distribution in a batch of food, negative test results do not prove absence, but merely reflect the effect of sampling probability. This is especially true for the very low concentrations at which such microorganisms would be encountered in practice. When the spatial distribution of microorganisms is more clustered, the chance of failing to detect contamination may be even higher.

This study confirms that evaluation of the OC-curves for the proposed MC is a critical step in ensuring that the MC is able to assess whether food lots satisfy an FSO or PO (van Schothorst et al., 2009).

4.3 Impact of microbial distributions on performance objectives

Because clustering can affect the consumer risk, it is a phenomenon that also needs to be considered in the establishment of food safety management targets such as POs and FSO. However, we are not yet able to conclude, in more detail, how best to consider clustering. A strict maximum valid for all portions of the food product or batch is difficult or even impossible to achieve, especially if clustering of the microorganisms in the products is likely to occur.

A recent discussion (Rieu et al., 2007) supports the view that FSOs should be framed with due regard to dose frequency distributions, and suggests a mathematical framework within which this might be accomplished. Also the recent paper by van Schothorst et al. (2009) discusses FSOs, POs and microbiological criteria, and the relationship between them in the context of risk-based food safety management.

5. Conclusions

The risk to get ill can be heavily influenced by variability in doses, as caused by clustering, as well as 'typical' dose. Data is not available to assess the nature and degree of this effect in reality, but often this risk is largely determined by infrequent high doses, the right hand tail of the frequency distribution. These infrequent high values are the most important contributors to the arithmetic mean in a batch and thus it is the arithmetic mean (mean of counts), which is more relevant to the assessment of risk than the geometric mean (mean of logs).

A more sophisticated definition of PO that includes consideration of clustering might be needed. As it is not possible to define a true maximum and the arithmetic mean is the major determinant of risk, linking food safety management targets to the arithmetic mean may be more appropriate than setting an upper limit for concentration. Both clustering and the choice of statistical distributions have a considerable effect on the acceptance probability of OC-curves and the evaluation of microbiological criteria. Awareness of the importance of the choice of statistical distributions used to model microbial distributions is also required when evaluating microbiological criteria.

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Chapter 4

Factors influencing the accuracy of the plating method used to enumerate low numbers of viable microorganisms in foods

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Abstract

This study aims to assess several factors that influence the accuracy of the plate count technique to estimate low numbers of microorganisms in liquid and solid food. Concentrations around 10 CFU/mL or 100 CFU/g in the original sample, which can still be enumerated with the plate count technique, are considered as low numbers. The impact of low plate counts, technical errors, heterogeneity of contamination and singular versus duplicate plating were studied. Batches of liquid and powdered milk were artificially contaminated with various amounts of Cronobacter sakazakii strain ATCC 29544 to create batches with accurately known levels of contamination. After thoroughly mixing, these batches were extensively sampled and plated in duplicate. The coefficient of variation (CV) was calculated for samples from both batches of liquid and powdered product as a measure of the dispersion within the samples. The impact of technical errors and low plate counts were determined theoretically, experimentally, as well as with Monte Carlo simulations. CV-values for samples of liquid milk batches were found to be similar to their theoretical CV-values established by assuming Poisson distribution of the plate counts. However, CV-values of samples of powdered milk batches were approximately five times higher than their theoretical CV-values. In particular, powdered milk samples with low numbers of Cronobacter spp. showed much more dispersion than expected which was likely due to heterogeneity. The impact of technical errors was found to be less prominent than that of low plate counts or of heterogeneity. Considering the impact of low plate counts on accuracy, it would be advisable to keep to a lower limit for plate counts of 25 colonies/plate rather than to the currently advocated 10 colonies/plate. For a powdered product with a heterogeneous contamination, it is more accurate to use 10 plates for 10 individual samples than to use the same 10 plates for 5 samples plated in duplicate.

1. Introduction

In food microbiology, plate counting is a longstanding and widely used enumeration method to estimate the number of viable microorganisms in food samples based on the assumption that the microorganisms are homogeneously distributed within foods. Assuming that all cells are spatially separated, each viable microorganism is expected to form one colony on an agar plate provided that the medium, the temperature, the oxygen conditions and the incubation period are suitable for potential recovery and growth. The number of colony forming units (CFU) per gram or milliliter of sample is calculated from the plate counts, the dilution factor and the plated volume.

The counting range of the acceptable number of colonies per plate has been reported early on as a factor affecting the accuracy of the plate counting method and recommendations for suitable counting ranges have been published accordingly. A range of 30-500 colonies per plate has been recommended by Breed and Dotterer (1916) in their proposal to revise the standard methods of milk analysis. This original recommendation has later been amended to a range of 30-300 colonies per plate, which has found wide acceptance (Adams and Moss, 2008 ; Sutton, 2006). An optimum counting range of 25-250 colonies per plate for a 10-fold dilution series of raw milk has been recommended by Tomaszewicz et al. (1980). A range of 15-300 for non-selective plates has been prescribed in ISO standard 4833 (ISO 4833, 2003). Most recently, the lower limit of the acceptable counting range was decreased to 10 in ISO standard 7218 (ISO 7218, 2007). Over the years, the number of replicate plates advised for enumeration reduced from triplicate (Breed and Dotterer, 1916; Tomaszewicz et al., 1980), over duplicate (ISO 4833, 2003), to singular plating for at least two successive dilutions (ISO 7218, 2007). As the number of replicate plates directly affects the volume and the total number counted, this factor also impacts accuracy of the plating method.

Regarding the dilution factor and the plated volume used to calculate the number of microorganisms in a sample (expressed as CFU/g or CFU/mL), pipet volume and sample weight can both be assumed to be normally distributed and to be characterised by a mean and standard deviation. However, plate counts vary according to a Poisson distribution as Fischer et al. (1922) showed for replicate plates of soil samples and Wilson (1935) showed for plate counts of milk samples. Because the standard deviation of a Poisson distribution is equal to the square root of the mean of the distribution, the count itself is a measure of the precision of the method. Plate count data will always be more variable than the

variability resulting only from sampling homogeneously distributed microorganisms (Cowell and Morisetti, 1969). Therefore, variability in the colony count on plates enables one to calculate the limiting precision of counts. The limiting precision caused by the Poisson distribution error can be expressed by the coefficient of variation (CV). CV-values have been shown to increase for lower plate counts (Cowell and Morisetti, 1969; Jarvis, 2008). Additionally to the Poisson distribution error, the error in counting the actual colonies on plates can be assumed to be normally distributed.

Understanding the various factors that impact on accuracy of the plating method is important to confidently assess numbers of microorganisms in foods. Since the microbial distribution in foods is inherently heterogeneous (Corry et al., 2007; ICMSF, 2002), and hazardous microorganisms generally are present in low numbers, both heterogeneity and low numbers will influence the enumeration of microorganisms. Plate counts from rather homogeneous products have been studied in quite good detail. However, plate counts from heterogeneous products such as solid and powdered foods have received less attention.

Therefore, this study systematically determined the impact of three factors on the accuracy of the plating method when estimating low numbers of *Cronobacter sakazakii* strain ATCC 29544 in liquid milk as compared to powdered milk: 1) the number of colonies on plates, 2) heterogeneity of the food product and 3) technical errors caused by pipetting, weighing and counting. As the overall accuracy of the plate count technique is extensively discussed in the review of Corry et al. (2007), our study expands on this and previous investigations by also taking microbiological heterogeneity into account and determining the impact of technical errors, low numbers of microorganisms as well as singular versus duplicate plating. The accuracy of the plating was investigated theoretically, experimentally and using Monte Carlo simulations. The impact of low numbers was determined by repeating the experiment for different numbers of the *C. sakazakii* in liquid and powdered milk, taking a large series of samples in each experiment and keeping all other conditions constant.



2 Materials and methods

2.1 Defining accuracy

According to ISO standard 5725-1 (ISO 5725-1, 1994), the accuracy of measurement methods and results depends on both trueness and precision. Trueness is defined as the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. If an accepted reference value is not available, the expected measurable quantity may be used as the reference for comparison of test results. Precision is defined as the closeness of agreement between independent test results obtained under stipulated conditions. The precision of a measurement method is indicated by the reading error of a measurement or the standard deviation of a series of measurements. The accuracy in directly measured quantities such as sample weight, dilution volume, and plated volume will propagate in the final enumeration value (the number of microorganisms in a sample, expressed as CFU/g or CFU/mL).

2.2 Calculating the number of microorganisms in the original sample (N) from plate counts.

The number of microorganisms in the original sample (N) can be calculated from the plate count, the volume plated, and the dilution factor (ISO 7218, 2007):

$$N = \frac{\sum c}{V_{plate} \cdot (1.1) \cdot d} \quad (1)$$

with N : number of colony forming units per milliliter (CFU/mL) or gram (CFU/g), $\sum c$: sum of the colonies counted on two plates retained from two successive dilutions, at least one of which contains a minimum of 10 colonies, V_{plate} : plated volume (mL), 1.1: for the successive dilutions, and d : dilution factor corresponding to the first dilution retained; d is 1 when an undiluted liquid sample is plated.

For low numbers of microorganisms in a solid or powdered sample, the 10^{-1} dilution will be used instead of successive dilutions. Based on this one dilution, Equation 1 results in

$$N = \frac{C}{V_{plate} \cdot d} \quad (2)$$

with C : counted colonies on a plate.

Assuming 1 g = 1 mL for a solid or powdered sample, the dilution factor is the ratio between the sample volume and the sample volume plus the dilution volume:

$$d = \frac{S}{S + V_{dil}} \quad (3)$$

with V_{dil} : dilution volume (mL) and S : sample volume (mL) or weight (g). For low numbers of microorganisms in the original sample, combining equation 2 and 3 results in:

$$N = \frac{C}{V_{plate}} \cdot \frac{(S + V_{dil})}{S} \quad (4)$$

2.3 Using error propagation to assess the impact of technical errors on N

The precision errors in the directly measured quantities C , V_{plate} , V_{dil} and S , will propagate to an error in the resulting N . For each measured quantity, the precision error is expressed in the standard deviation: σ_C , $\sigma_{V_{plate}}$, $\sigma_{V_{dil}}$ and σ_S . The standard deviation in the plated volume ($\sigma_{V_{plate}}$) has been determined by weighing 30 plated volumes with an analytical balance (Sartorius, Göttingen, Germany). The standard deviations in the dilution volume ($\sigma_{V_{dil}}$) and in the sample S from liquid milk ($\sigma_{S_{liquid}}$) or powdered milk ($\sigma_{S_{powder}}$) were determined in the same way. If the error in C is only determined by counting, the standard deviation σ_C can be derived from a count error of 5% (Peeler, 1982). Assuming normally distributed count data, and given a mean value of μ , a maximal count error of 5% results in $\sigma_C = 5/3\%$ of μ as 99% of normally distributed data are within the interval $\mu \pm 3\sigma$.

For independent random errors, the propagation of the precision error was calculated using two rules (Taylor, 1982): the error (δq) in the result of an addition or subtraction (Eq. 5) and the relative error ($\frac{\delta q}{|q|}$) in the result of a multiplication or division (Eq. 6).

$$\text{Rule 1: If } q = x + y \text{ or } q = x - y \text{ then } \delta q = \sqrt{\delta x^2 + \delta y^2} \quad (5)$$

$$\text{Rule 2: If } q = x \cdot y \text{ or } q = \frac{x}{y} \text{ then } \frac{\delta q}{|q|} = \sqrt{\left(\frac{\delta x}{x}\right)^2 + \left(\frac{\delta y}{y}\right)^2} \quad (6)$$

Using these two rules and N from Eq. 3, the relative error of N can be described as:

$$\frac{\sigma_N}{N} = \sqrt{\left(\frac{\sigma_C}{C}\right)^2 + \left(\frac{\sigma_{V_{plate}}}{V_{plate}}\right)^2 + \left(\frac{\sigma_S}{S}\right)^2 + \left(\frac{\sqrt{(\sigma_{V_{dil}})^2 + (\sigma_S)^2}}{V_{dil} + S}\right)^2} \quad (7)$$

2.4 Simulating the error in N with Monte Carlo analysis

The distribution of N was simulated using Monte Carlo analysis using @Risk 5.0 (Palisade Corporation) performing 10,000 iterations by Latin Hypercube sampling with random seed generation. N was simulated in three different distribution scenarios for C using Eq. 4, in which V_{plate} , V_{dil} and S were assumed to be normally distributed with standard deviations as determined experimentally. The error in C varied in the three scenarios as follows: 1) C normally distributed with a count error of 5%, 2) C Poisson distributed, and 3) C Poisson distributed and having an additional normally distributed count error of 5%. The sensitivity of the output variable N to the input variables C , V_{plate} , V_{dil} and S was analysed with a tornado chart.

2.5 Enumerating the microorganism in liquid milk

2.5.1 Preparing the bacterial suspension to inoculate the milk

A full grown culture of *Cronobacter sakazakii* strain ATCC 29544 in 100 mL brain heart infusion (BHI) broth (Beckton Dickinson and Co., Le Point du Claix, France) was stored frozen (-80°C) with 30% glycerol (87%, Fluka-Analytical GmbH, Buchs, Switzerland). A loopful (1 µL) of this culture was inoculated into 100 mL BHI and grown for 22 hours at 37°C. From the resulting BHI suspension containing 1.1×10^{10} CFU/mL, 10^{-2} , 10^{-3} and 10^{-4} dilutions were made using peptone physiological salt (PPS; 8.5 g NaCl/L and 1 g peptone/L; Oxoid, Basingstoke, England).

2.5.2 Inoculating, sampling, and plating

Commercially sterilised milk obtained from local retail was inoculated with different volumes to obtain 1 liter batches of milk with different numbers of *C. sakazakii* aiming at 4×10^2 , 7×10^2 , 1×10^3 , 3×10^3 , 5×10^3 , 1×10^4 , 2×10^4 CFU/mL. While each batch was being thoroughly stirred, 30 samples of 0.5 mL were taken with a pipette. Each sample was diluted in 4.5 mL PPS and 0.1 mL was plated in duplicate on Trypton Soy Agar plates

(TSA; Oxoid, Basingstoke, England) with a spiral plater (Eddy Jet; IUL Instruments, I.K.S., Leerdam, The Netherlands). The TSA plates were incubated overnight at 37°C and the numbers of colonies on each plate counted manually. The detection limit of the enumeration method was 1.7 log CFU/mL (50 CFU/mL). A concentration of 50 CFU/mL in a sample can be detected by plating 0.2 mL of a 10⁻¹ dilution.

2.6 Enumerating the microorganism in powdered milk

2.6.1 Preparing the bacterial suspension to spike the powder

A loopful (1 µL) of the *Cronobacter sakazakii* strain ATCC 29544 culture stored frozen was inoculated into 100 mL BHI and grown for 22 hours at 37°C. To harvest the cells, the BHI suspension was centrifuged 10 minutes at 20°C at 1725 g (Eppendorf AG, Hamburg, Germany). *C. sakazakii* cells were washed in 40 mL PPS and centrifuged 10 minutes at 20°C at 1725 g twice and subsequently suspended in 10 mL PPS.

2.6.2 Spiking the powdered milk

Powdered infant formula (PIF) obtained from local retail was artificially contaminated as follows. *C. sakazakii* cells suspended in PPS were sprayed three times with a perfume sprayer (designed by Gérard Brinard, DA Drogisterij, Leusden, The Netherlands) over a flat layer of 20 g PIF. The powder was stirred well and again sprayed three times. The contaminated powder was stored in a desiccator with saturated lithium chloride (VWR international, Fontenay sous Bois, France) at 20°C to maintain a water activity of 0.11. After 3 days, the contaminated powder contained between 10⁶ and 10⁷ CFU/g (data not shown).

2.6.3 Mixing, sampling and plating

Small amounts (0.15, 0.3, 1, 2 and 3 g) of the contaminated powder (1.93x10⁶ CFU/g, measured at the day of mixing and sampling) were mixed into batches of 1 kg PIF for 1 hour with a 3-dimensional powder mixer (Willy A. Bachofen AG Maschinenfabrik, Basel, Switzerland) with a rotational speed of 56 rpm. After thorough mixing, each batch of PIF was separately poured into a stainless steel box (60 cm x 30 cm x 10 cm). A plasticised grid (Gamma, Leusden, The Netherlands) was placed on top of the box to visually divide the box into 72 square sections of 5 x 5 cm allowing for systematic sampling of the powder. Two samples of 0.5 g were drawn from each section, resulting in 144 samples.

Each sample was suspended in 4.5 mL PPS and 0.1 mL of the suspension was plated in duplicate onto TSA plates. After overnight incubation at 37°C, the number of colonies per plate was counted. The lower detection limit was 1.7 log CFU/g.

2.7 Assessing the expected number of microorganisms in a batch of powdered or liquid milk as the reference number.

Since the amount of spiked powder (with a *C. sakazakii* concentration of 1.93×10^6 CFU/g) mixed into the batch of PIF is known, the expected number of microorganisms in a batch can be calculated. For instance, mixing 3 g of spiked powder into 1 kg PIF will result in an expected concentration of 3.76 log CFU/g. This expected number can be used as a reference. In the same way, the expected number of microorganisms in milk can be calculated as the number of microorganisms in the suspension (with a *C. sakazakii* concentration of 1.1×10^{10} CFU/mL), the dilution factor and the volume mixed into 1 L milk are known. The expected concentration for the highest level of contaminant in liquid milk is 4.34 log CFU/mL.

If the microorganisms are log-normally distributed within a batch, the log counts of the samples and the variance between the log counts will also give an estimation of the number of microorganisms in the batch. According to Rahman (1968), the arithmetic mean \bar{C} is related to the geometric mean $\overline{\log C}$ as follows:

$$\log(\bar{C}) = \overline{\log C} + 0.5 \cdot \ln 10 \cdot \sigma_{\log C}^2 \quad (8)$$

with: $\overline{\log C}$ the mean of the log counts of the samples, and $\sigma_{\log C}^2$ the variance of the log counts of the samples.

2.8 Preparing representations of variability between sample results

Since the location in the box of the samples drawn from the powdered milk was known, the sampling data for the powdered milk can be represented as a function of the sampling location using MATLAB® 7.8.0, R2009a (The MathWorks™, Natick, Massachusetts). The sampling data for both liquid and powdered milk were displayed as an empirical cumulative distribution function (ECDF). Calculations were performed in Microsoft Excel 2003.

2.9 Using the coefficient of variation (CV) to assess the Poisson distribution error

The dispersion of data points around the mean in data series is commonly quantified by variance, standard deviation, or coefficient of variation (CV). Since the CV is the standard deviation divided by the mean, this scaled measure compares the degree of variation in situations where means differ. For plate counts, CV is:

$$CV = \frac{\sigma_C}{\bar{C}} \cdot 100\% \quad (9)$$

with \bar{C} being the mean colony count per plate of a sample. If the number of colonies on a plate follows a Poisson distribution, the standard deviation will be equal to the square root of the mean of the counts ($\sigma_c = \sqrt{\bar{C}}$), which leads to:

$$CV = \frac{1}{\sqrt{\bar{C}}} \cdot 100\% \quad (10)$$

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3. Results

3.1 The relative error $\frac{\sigma_N}{N}$ calculated with error propagation

The various measured quantities (i.e. plated volume, dilution volume, and sample weight/volume) that affect the error in the final enumeration value N (the number of microorganisms in a sample, expressed as CFU/g or CFU/mL) were determined individually and are shown in Table 1 in terms of mean (\bar{x}) measure values, standard deviations (s) and precision errors (s/\bar{x}).

Table 1. Estimators for the mean (\bar{x}), standard deviation (s) and relative standard deviation (s/\bar{x}) in various measured quantities determined with an analytical balance from 30 measurements

Measured quantity	Target quantity	\bar{x}	s	s/\bar{x} (%)
Plated volume (V_{plate} (mL))	0.10	0.1001	0.001769	1.77%
Dilution volume (V_{dil} (mL))	4.5	4.4390	0.04445	1.00%
Sample milk (S (mL))	0.50	0.4874	0.007573	1.55%
Sample milk powder (S (g))	0.50	0.4973	0.01408	2.83%

The theoretical relative error $\frac{\sigma_N}{N}$ for liquid and powdered milk can then be calculated with Eq. 7 using the individual standard deviations $\sigma_{V_{plate}}$, $\sigma_{V_{dil}}$ and σ_S from Table 1 and assuming a normally distributed count error (scenario 1) with $\sigma_C = 5/3\%$. From this it follows that the relative error $\frac{\sigma_N}{N}$ for liquid milk is:

$$\frac{\sigma_N}{N} = \sqrt{(1.67\%)^2 + (1.77\%)^2 + (1.55\%)^2 + (0.915\%)^2} = 3.03\% \quad (11)$$

For powdered milk the relative error is:

$$\frac{\sigma_N}{N} = \sqrt{(1.67\%)^2 + (1.77\%)^2 + (2.83\%)^2 + (0.944\%)^2} = 3.85\% \quad (12)$$

In these equations, every precision error contributes to the relative error $\frac{\sigma_N}{N}$. Since the precision errors are squared, the larger precision errors have a proportionally large impact on the relative error in the final enumeration value. As proposed by Taylor (Taylor, 1982), if one of the errors is 5 times any of the other errors, then its square is 25 times that of the others and the other errors can be ignored. Assuming that the counts on plates are Poisson distributed (scenario 2), the relative error in the counted number of colonies on plates $\frac{\sigma_C}{C}$ will increase for lower counts. For example, for a colony count of 300, the relative error is 5.77% ($\sqrt{300}/300$); for liquid milk, this will result in:

$$\frac{\sigma_N}{N} = \sqrt{(5.77\%)^2 + (1.77\%)^2 + (1.55\%)^2 + (0.915\%)^2} = 6.30\% \quad (13)$$

If the count is 25, the relative error $\frac{\sigma_C}{C}$ is 20.0%, which will result in:

$$\frac{\sigma_N}{N} = \sqrt{(20.0\%)^2 + (1.77\%)^2 + (1.55\%)^2 + (0.915\%)^2} = 20.2\% \quad (14)$$

If the count is 10, the relative error $\frac{\sigma_C}{C}$ is 31.6%, which will result in:

$$\frac{\sigma_N}{N} = \sqrt{(31.6\%)^2 + (1.77\%)^2 + (1.55\%)^2 + (0.915\%)^2} = 31.7\% \quad (15)$$

The relative errors $\frac{\sigma_{V_{plate}}}{V_{plate}}$, $\frac{\sigma_{V_{dil}}}{V_{dil}}$ and $\frac{\sigma_S}{S}$ are independent of the colony counts on plates, but the relative error $\frac{\sigma_C}{C}$ increases greatly for lower colony counts. Using the error propagation approach therefore shows that the Poisson distributed count error greatly determines $\frac{\sigma_N}{N}$. Even for high plate counts (Eq. 13), precision errors contribute little to the error in the enumeration value and thus the precision errors do not need to be considered in establishing the higher limit of the counting range. Comparing equations 14 and 15 shows that changing from a lower limit of the counting range of 10 to 25 colonies/plate, would reduce the Poisson distribution error from 32% to 20% and thus improves accuracy of the plating method.

3.2 The relative error $\frac{\sigma_N}{N}$ simulated with Monte Carlo

The relative error $\frac{\sigma_N}{N}$ was simulated using Monte Carlo analysis for colony counts between 5 and 300 for three different scenarios as compared to the theoretical CV, shown as the solid line in Fig. 1. From this it is evident that the dispersion of the plate count data (also called Poisson distribution error) increases very significantly for the lower counts. The colony counts 10, 15, 25, and 30 were chosen because they were previously advocated as possible lower plate count boundaries. For both liquid and powdered milk, the relative errors $\frac{\sigma_N}{N}$ are presented as CV-values in Table 2. For liquid milk, the relative errors are presented as CV-values in Fig. 1.

In scenario 1, all input variables V_{plate} , V_{dil} , S , and C were assumed to be normally distributed. For all colony counts, this resulted in a normally distributed N with a CV-value of 2.9 for liquid milk. For powdered milk, the CV-value was 3.6. These CV-values correspond well to the relative errors in $\frac{\sigma_N}{N}$ (liquid milk 3.03, powdered milk 3.85) calculated with the error propagation. According to sensitivity analysis, the input variables ranked as V_{plate} , C , S and V_{dil} determined N (data not shown).

In scenario 2, the input variables V_{plate} , V_{dil} and S were assumed to be normally distributed while C was Poisson distributed. The input variable C significantly determined N as shown in Table 2 and according to the sensitivity analysis (data not shown). The relative error $\frac{\sigma_N}{N}$ was slightly higher than the theoretical Poisson distribution error.

In scenario 3, C was assumed to be Poisson distributed with an additional count error of 5%, which also resulted in a strong relationship between N and C . The error in N was slightly higher than if C was only Poisson distributed.

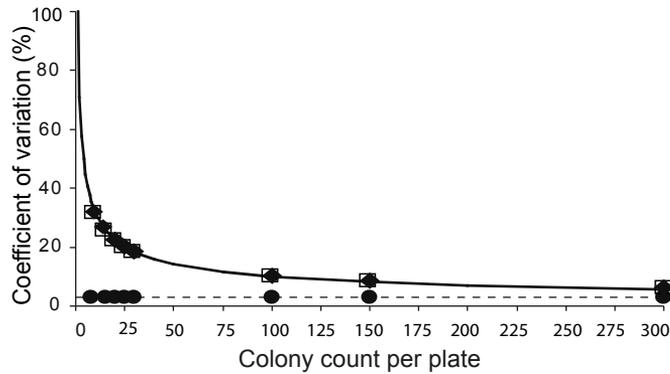


Fig. 1. The coefficient of variation (CV) as a function of the number of colonies on a plate. The dark line represents the theoretical CV assuming that the colonies per plate are Poisson distributed. The relative error $\frac{\sigma_N}{N}$ samples of liquid milk was simulated for three scenarios regarding the error in colony count on plate (C) namely: 1) normally distributed with a count error of 5%, (\bullet), 2) Poisson distributed (\blacklozenge), and 3) Poisson distributed and having an additional normally distributed count error of 5% (\square).

Table 2. The relative error $\frac{\sigma_N}{N}$, expressed as coefficient of variation (CV) for samples drawn of liquid or powdered milk simulated for three scenarios^{a,b} regarding the error in the colony count on plate (C).

Colony count	Theoretical CV	Liquid milk			Powdered milk		
	C (Poisson) and no error in V_{plate}, V_{dil}, S	Scenario 1	Scenario 2	Scenario 3	Scenario 1	Scenario 2	Scenario 3
5	44.7	2.9	44.8	44.9	3.6	44.9	45.0
10	31.6	2.9	31.7	31.8	3.6	31.8	31.9
15	25.8	2.9	25.9	26.0	3.6	26.1	26.1
20	22.4	2.9	22.5	22.5	3.6	22.6	22.7
25	20.0	2.9	20.2	20.2	3.6	20.2	20.3
30	18.3	2.9	18.4	18.5	3.6	18.5	18.6
100	10.0	2.9	10.3	10.4	3.6	10.5	10.6
150	8.16	2.9	8.5	8.6	3.6	8.8	8.9
300	5.77	2.9	6.2	6.4	3.6	6.6	6.8

^a Scenario 1: C normally distributed with a count error of 5%; scenario 2: C Poisson distributed; scenario 3: C Poisson distributed and having an additional normally distributed count error of 5%.

^b All scenarios: V_{plate}, V_{dil} and S normally distributed with a standard deviation as mentioned in Table 1.

3.3 The sampling data of liquid milk

Using the experimental ECDF-curve established at the highest inoculum level (2×10^4 CFU/mL) as the reference and assuming an identical variability at lower inoculum levels, predictions were made of the ECDF-curves for the lower inoculum levels evaluated (i.e. 4×10^2 , 7×10^2 , 1×10^3 , 3×10^3 , 5×10^3 , and 1×10^4 CFU/mL). Predicted ECDF-curves are displayed as lines in Figure 2a and can be compared with the experimental ECDF-curves for the individual batches which are displayed as symbols. Although for low concentrations the variability is slightly higher than the predicted lines, experimental and predicted ECDF-curves match well.

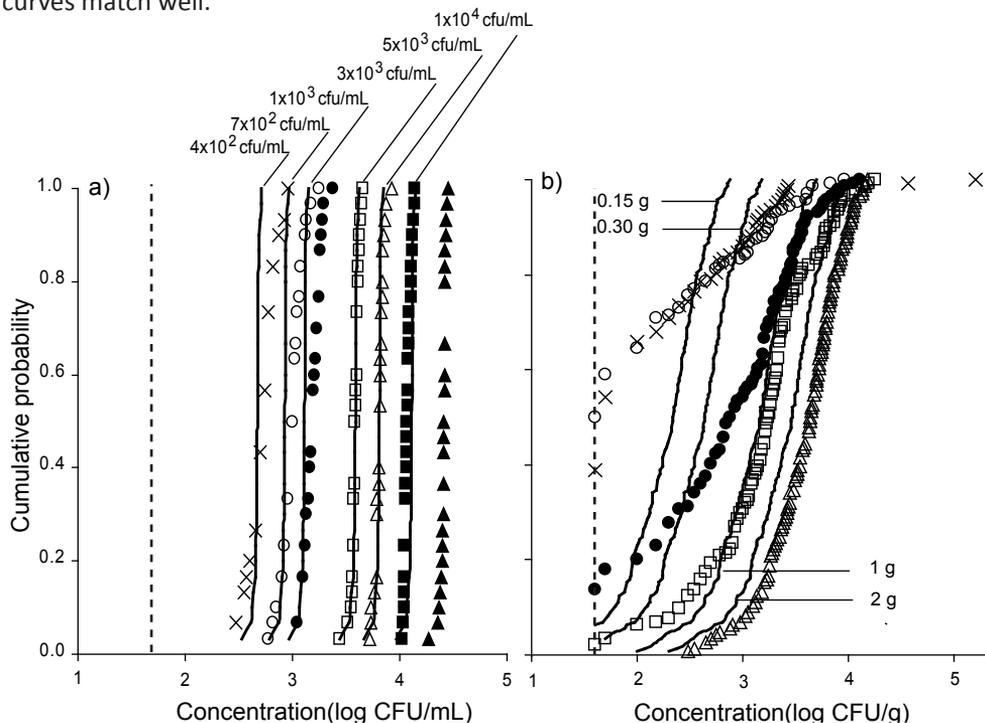


Fig. 2. Comparison between predicted and experimental ECDF-curves for (a) liquid milk and (b) powdered milk. The broken vertical line represents the detection limit of 1.7 (log CFU/ml or log CFU/g). For liquid milk, six predicted ECDF-curves are shown as lines with an indication of the *Cronobacter sakazakii* contamination level they were derived for from the reference (the experimental ECDF of 2×10^4 CFU/mL); the symbols depict the experimental ECDF-curves for the following contamination levels: (x) 4×10^2 , (o) 7×10^2 , (●) 1×10^3 , (□) 3×10^3 , (Δ) 5×10^3 , (■) 1×10^4 , and (▲) 2×10^4 CFU/mL. For powdered milk, the reference experimental ECDF was established for a contamination level of 3g spiked powder per 1 batch of 1 kg (Δ); the lines show ECDF-curves derived for the various contamination levels indicated in the figure; experimental ECDF (symbols) were generated with the amount of spiked powder being: (x) 0.15 g, (o) 0.3 g, (●) 1 g, (□) 2 g, or (Δ) 3 g.

3.4 The sampling data of powdered milk

Also for the contaminated milk powder, ECDF-curves were predicted for various levels of the microorganism evaluated using the ECDF-curve derived from experimental data for the most highly contaminated batch as the reference and assuming the same variability for all levels. The reference batch contained 3 g of spiked powder, while the other four batches contained 0.15, 0.30, 1, and 2 g of spiked powder. Fig. 2b shows the various predicted ECDF-curves as lines, while the experimental ECDF-curves are displayed as symbols. Because all batches were very thoroughly mixed using 3-D mixing equipment, it was expected that the contaminant would have been well distributed throughout the sample and that even for low contamination levels samples would mostly be above the detection limit (1.7 log CFU/g). However, as can be seen from Fig. 2b, for the lowest three contamination levels there were rather many samples below detection limit. The percentages of samples below the detection limit were 39%, 50%, 14% and 2% for the batches mixed with 0.15 g, 0.30 g, 1 g and 2 g, respectively.

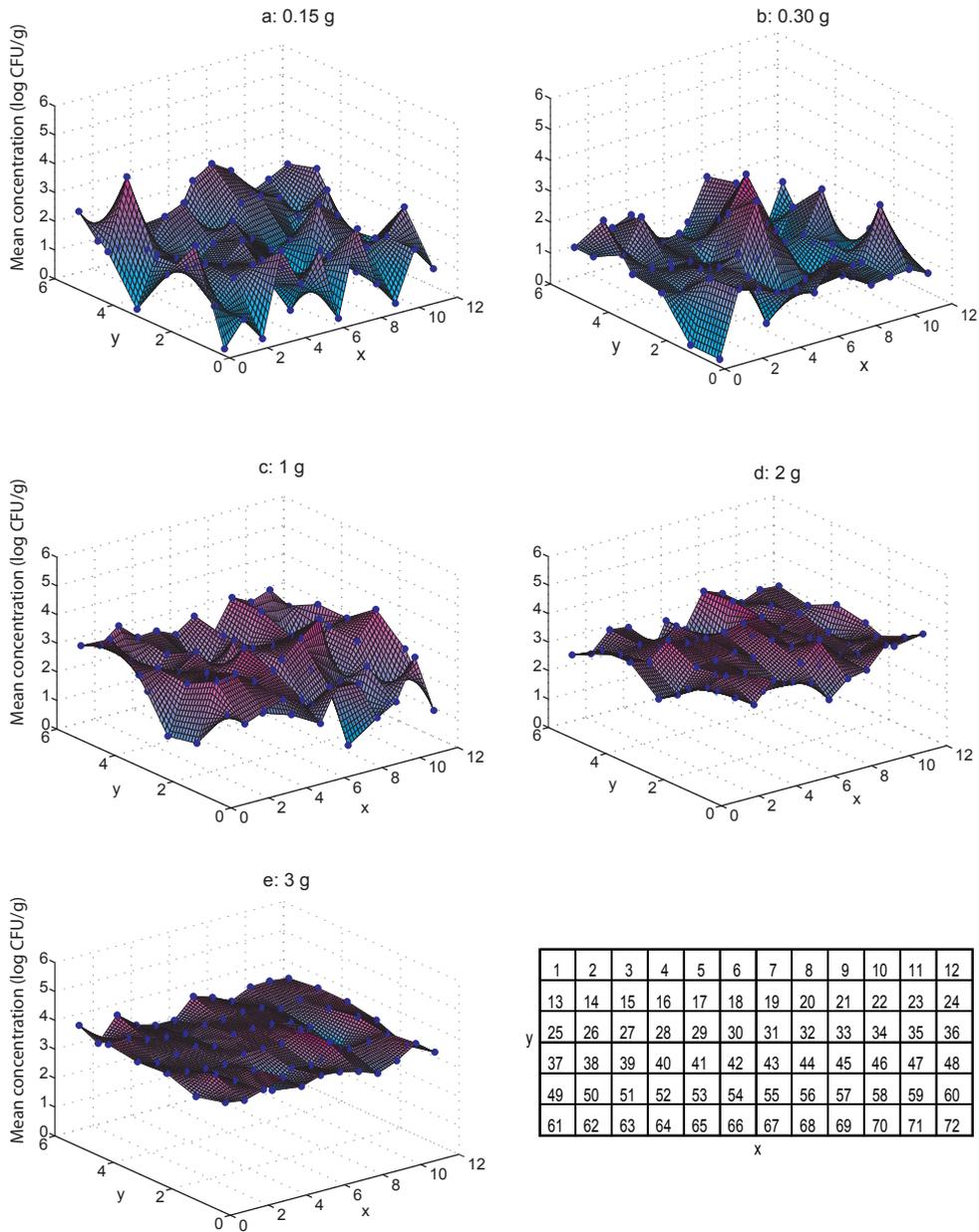
The ECDF-curves derived from the reference at the highest concentration level run comparably steep, but less steep than the ECDF-curves found for liquid milk. It can be clearly seen that experimental ECDF data deviate considerably from the predicted ECDF-curves for all contamination levels and mostly so for the lowest levels of contamination.

The experimental ECDF-curve for the batch spiked with 0.15 g contaminated milk powder showed two outliers, namely at 4.6 and 5.2 log CFU/g. For both outliers, one of the plate counts was above 100 colonies whereas the other had a colony count of zero. Such a large difference in colony count may have been caused by clumping of cells in the 10^{-1} dilution, with clumps not dissolving after vortexing. These two outliers have not been taken into account in further calculations.

The samples of the batch mixed with 3 g of spiked powder had a mean ($\overline{\log C}$) of 3.57 log CFU/g and a standard deviation ($s_{\log C}$) of 0.36 log CFU/g. Assuming log-normally distributed microorganisms and using Eq. 8, this resulted in an arithmetic mean ($\log(\bar{C})$) of 3.73 log CFU/g, which is close to the reference concentration of 3.76 log CFU/g.

In Fig. 3 the sampling data of powdered milk for the 5 levels of contamination investigated are displayed as 3-dimensional graphs. The mean concentration of the duplicate samples drawn from each section in the box with milk powder is displayed.

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Fig. 3. The mean concentration of *C. sakazakii* in two samples (log CFU/g) powdered milk as a function of their location in the box (x and y axis). 1 kg batches of powdered milk were thoroughly mixed with (a) 0.15, (b) 0.30, (c) 1, (d) 2, or (e) 3 g of spiked powder.

Comparing the graphs, it can be seen that the surface plot is positioned higher in terms of mean concentration with increasing contamination level but also that there is an apparent relationship between the level of contamination of the powdered milk batch and the smoothness of the surface plot. The higher the contamination level (going from Graph 3a to 3d) the smoother the surface plot, which indicates that there is increasingly less variability between the samples. The experimental data for batches spiked with 0.15 g and 0.30 g contaminated powder in particular resulted in very erratic surface plots, with some sections characterised by very high counts, whereas in others no contamination could be detected at all.

3.5 The Poisson distribution error of liquid and powdered milk samples

Fig. 4 shows the Poisson distribution error of the liquid and powdered milk samples expressed as the coefficient of variation and its relationship to the mean colony count of the samples per batch.

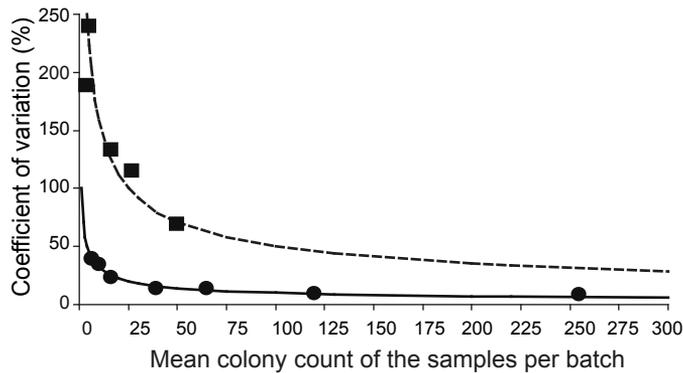


Fig. 4. Coefficient of variation (CV) as a function of the mean number of colonies of the samples per batch. The symbols represent the CV-values based on experimental values from batches of liquid milk (●) and powdered milk powder (■). The solid line represents the curve of theoretical CV-values assuming that the mean colony count of the samples per batch are Poisson distributed. The broken line represent the curve of theoretical CV-values times 5.

The CV-values of the samples from liquid milk are very well in line with the curve of theoretical CV-value that has been established assuming a Poisson distribution. Moreover, fitting the plate counts of the samples per batch to a Poisson distribution with χ^2 as a criterion, also confirms that plate counts are Poisson distributed. As compared to the

curve of theoretical CV-values for liquid milk, CV-values of samples from powdered milk were always much higher. They coincided relatively well with a curve of theoretical CV-values established by multiplying values five times.

For both liquid and powdered milk samples the coefficient of variation increases for low plate counts. Increasing the lower limit of the counting range from 10 to 25 will reduce the CV for liquid milk from 32% to 20% (reduction of the Poisson distribution error) and for powdered milk from 160% to 100% (reduction of the Poisson distribution error times five).

3.6 The difference in concentration based on singular or duplicate plating

Two methods, singular and duplicate plating, to enumerate the contaminating microorganisms were evaluated. Fig. 5 shows the concentration of the same sample singular plated versus duplicate plated assessed for liquid milk (Fig. 5a) and powdered milk (Fig. 5b).

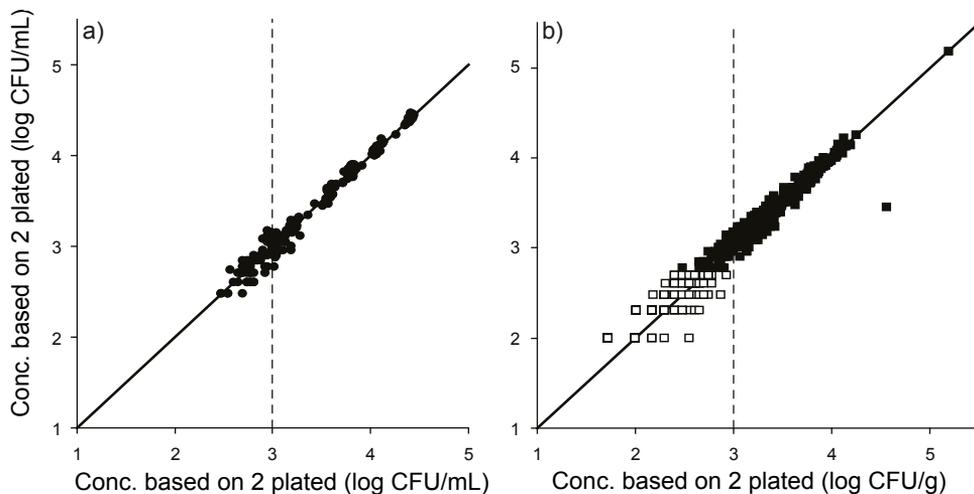


Fig. 5. Relationship between the concentration (log CFU/mL or log CFU/g) in the samples of (a) liquid milk and (b) powdered milk, based on enumeration using one plate per sample versus two plates per sample. Solid line: $y = x$. The vertical broken line indicates the concentration of 3 log CFU/mL or 3 log CFU/g, which equates to the currently advocated lower limit of the enumeration range (10 colonies per plate).

All plate counts of liquid milk contained more than 1 colony per plate. For powdered milk, at the lowest contamination levels one of the duplicate plates contained zero colonies, resulting in series of data points laying in horizontal lines. In both figures, the vertical line at a reference concentration of 3 log CFU/mL (or 3 log CFU/g) corresponds to 10 colonies per plate, which is the currently advocated lower limit of the plate counting range (ISO 7218, 2007). From the reference level upward, for both liquid and powdered milk, concentrations determined by both methods coincided well; the data points were close to the line of equality ($y = x$), which is according to Bland and Altman (1986) the criterion for a perfect agreement between two methods. Below the reference concentration, however, the distance of data points to the line of equality increased, which resulted in a clear difference between the two methods especially in the case of powdered milk.

3.7 The impact of samples taken and singular or duplicate plating related to heterogeneity

The impact of samples taken and singular or duplicate plating in relation to heterogeneity was investigated. Using Monte Carlo simulations, it was evaluated whether it would be better to take 10 samples and plate them singularly, or to take 5 samples and plate them in duplicate. Two powdered milk batches characterised by a different level of heterogeneous distribution of the contaminant were investigated. The levels of the contaminant were either 0.15 or 3 g of spiked milk powder per 1 kg batch of milk powder. The spiked powder was mixed into each batch, with the lower contamination level representing the more heterogeneous distribution (Fig. 3a) and the higher contamination level representing the more homogeneous distribution (Fig. 3e). The data of the homogeneous and heterogeneous powder were re-sampled in silico (Bootstrap @Risk, 10.000 simulations) by drawing 5 samples plated in duplicate and 10 samples plated singularly. Fig. 6 represents the distribution of the mean concentrations of the log counts calculated from 5 samples (duplicate) and 10 samples (singular) drawn from homogeneous data (Fig. 6a) and heterogeneous data (Fig. 6b). Re-sampling the data of the homogeneous powder resulted in no significant difference between the means of the log counts from 5 samples plated in duplicate or 10 samples plated singularly. The mean values as well as the standard deviation values matched closely. However, re-sampling the data of the heterogeneous powder resulted for 5 samples plated in duplicate in a significantly smaller mean and a larger standard deviation, than for 10 samples plated singularly.

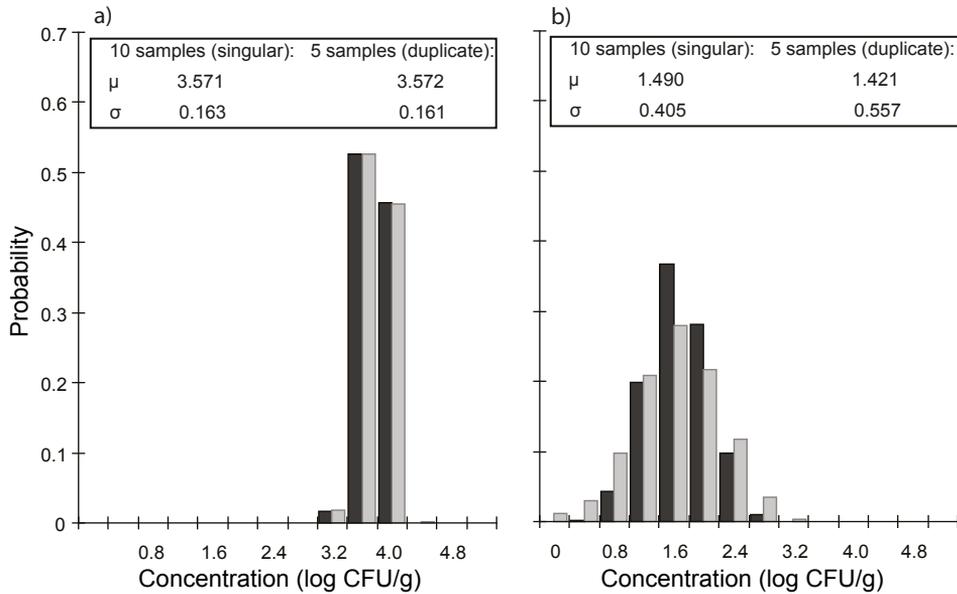


Fig. 6. Comparison of two sampling strategies by re-sampling using the bootstrap method the powdered milk sampling data (a) homogeneously distributed *C. sakazakii* (3 g spiked powder/kg powdered milk) and (b) heterogeneously distributed *C. sakazakii* (0.15 g of spiked powder/kg powdered milk). Probability distributions of the mean concentration (log CFU/g) were established by a scenario of taking 10 samples plated singularly (black bars) or the mean of 5 samples plated in duplicate (grey bars). Parameters μ and σ represent mean and standard deviation of the 10,000 simulations drawing 5 (duplicate) or 10 samples (singular).

4. Discussion

This study set out to determine the relative importance of low plate counts, technical errors, heterogeneity in the distribution of microorganisms, and singular or duplicate plating as factors influencing accuracy of the plating method for microbiological contaminants in liquid and solid food.

Using an error propagation approach, Monte Carlo analysis simulation, as well as generation of experimental data, it was consistently found that low plate counts largely determine the plate count accuracy for samples of liquid and powdered milk. It was furthermore observed that, as compared to the Poisson distributed error in the number of colonies counted on plates, technical errors can be neglected as factors influencing accuracy of the plating method when technical practices are under control. The experimentally determined technical errors were found to be comparable with the

errors (1.1% for pipetting sample or diluent fluid) as quantified by Voss et al. (2000), who concluded that counting errors had a much larger effect than pipetting errors. The impact of colony counts has also been indicated by Augustin and Carlier (2006), whereas Forster (2009) has emphasised that low plate counts (i.e. counts < 20) are a major contributor to uncertainty.

The impact of heterogeneity in the distribution of a contaminant on accuracy of the plate count technique has not been studied before and forms a specific aspect of the current work. Heterogeneity was investigated by comparing this accuracy for known contamination levels in liquid (with microorganisms assumed to be rather homogeneously distributed and Poisson distributed) and in powdered milk (with microorganisms being rather heterogeneously distributed). By comparing the data obtained for liquid and powdered milk, it was observed that heterogeneity greatly impacts the accuracy of the plating method. That microorganisms are indeed homogeneously distributed in liquid milk, was confirmed experimentally by the steep ECDF-curves obtained. These showed only a small variation between the samples and the CV-values for mean colony counts of the samples per batch. The CV-values found through sampling furthermore matched the theoretical CV-values assuming a Poisson distribution. Since the plate count of the samples from liquid milk fitted the Poisson distribution, and CV-values were consistent with Poisson distribution, distribution of the contaminant was homogeneous in liquid milk. However, the investigations with powdered milk showed a much larger variation in enumeration outcomes due to heterogeneity. It was found that CV-values generated experimentally aligned well to a theoretical CV-values curve positioned five times higher than the theoretical CV-values curve that has been established assuming a Poisson distribution.

As the number of replicate plates affects the total number of colonies counted, this factor may also impact accuracy of the plating method. Therefore, the difference between singular and duplicate plating was investigated experimentally. Since the concentration in each sample was calculated using both methods, the difference between singular and duplicate plating could be visualised. Above 10 colonies per plate, both methods showed a strong agreement. These findings are in line with the ISO 7218 (2007), which prescribes to count plates with at least 10 colonies per plate of two successive dilutions that are singularly plated. This was also supported by Wille et al. (1996), who showed that duplicate or triplicate plating is not more accurate than singular plating provided that there are 10-50 colonies per plate. By doubling the plated volume,

however, duplicate plating will increase the detection limit. By doubling the total number of colonies duplicate plating will lower the Poisson distribution error. As Wille et al. (1996) concluded, duplicate plating will heighten the confidence in the reliability of bacterial counts from single plates.

The impact of heterogeneity on the possible benefits of duplicate plating over singular plating was investigated by drawing 5 samples plated in duplicate or 10 samples plated singular. In both approaches, the same sample volume was plated. The experimental data generated for the most homogeneously contaminated milk powder (that with the highest level of spiked powder) and the most heterogeneous powder (with the lowest level of spiked powder) were re-sampled using Monte Carlo simulations. Re-sampling the homogeneous powder showed no significant difference between the means of the 5 or 10 samples. However, re-sampling the heterogeneous powder showed a significantly smaller mean and a larger standard deviation between the means. Drawing 5 samples plated in duplicate resulted in a probability of 1.1% that in all 5 samples no *C. sakazakii* was detected. Although a relatively small probability, such an incorrect enumeration could have hazardous consequences for consumers in case of severe pathogens. In case of 10 samples plated singularly, *C. sakazakii* was detected in all cases, even though the same amounts of plates and dilution fluid was used.

Since the plate count technique is a simple, fast method to quantify levels of microorganisms, it is an important tool to estimate numbers of microorganisms in food samples to establish the microbiological quality and or safety of these food. Many generalising assumptions are made in the process of establishing what enumeration results would comply with quality or safe foods. A key assumption is that microorganisms are homogeneously distributed even for foods where this is quite improbably such as structured, semi-solid, solid and powdered foods. It is often acknowledged that the distribution of microorganisms in food products is inherently heterogeneous (Corry et al., 2007). Nevertheless, the impact of heterogeneity between the samples on accuracy of plating method has not been systematically quantified to the degree as in the current study. To evaluate the accuracy of the plating method, sample taking is important. If the samples do not represent the microbial status of the batch of food, although the plate counts may be accurate, these plate counts will give insufficient information about the microbial status of the batch. As the experiments reported on here have confirmed, low plate counts as well as microbial heterogeneity both have an important influence on the

accuracy of the plating method, and are much more prominent than technical errors. For low plate counts, increasing the lower limit of the counting range will notably increase the accuracy of the plate count technique. Because plate counts below 25 are highly dominated by the Poisson distribution error, as shown here, increasing the currently advised lower limit from 10 to at least 25 would reduce the Poisson distribution error from 32% to 20% for liquid milk and from 160% to 100% for powdered milk. For the powdered product with a heterogeneously distributed contamination, taking 10 samples plated singularly provides more accurate information about the product than 5 samples plated in duplicate.

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Chapter 5

Random or systematic sampling to detect a localised microbial contamination within a batch of food

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Abstract

Pathogenic microorganisms are known to be distributed heterogeneously in food products that are solid, semi solid or powdered, like for instance peanut butter, cereals, or powdered milk. This complicates effective detection of the pathogens by sampling. Two-class sampling plans, which are deployed when the health hazard is severe and direct, specify how many sample units have to be drawn. In order to take a representative sample, the sampling strategy is important, especially when the microorganisms are distributed heterogeneously or localised.

This theoretical study shows the impact of random versus systematic sampling on the probability to detect localised microbial contamination in a batch of food. A statistical model was used to compare these sampling strategies. The microbial contamination was modelled as being present in one specific localised fraction of the batch in which the cells were randomly distributed, while no cells were present in the remaining part of the batch. The probability that the entire sampling scheme contains at least one cell was calculated for various numbers of sample units drawn either randomly or systematically and was shown to depend on the size of the contaminated fraction, the microbial concentrations, and the number of sample units drawn. The probability of detection was either equal or higher for systematic sampling as compared to random sampling. The maximal improvement in probability of detection was 0.37, when the sampling interval was equal to the size of the contaminated fraction, meaning that exactly one systematic sample hits the contaminated fraction. In those cases where the size of the contaminated fraction can be estimated, this study may assist in selecting the sampling strategy that is most optimal regarding probability of detection.

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1. Introduction

Good hygienic practice (GHP) and hazard analysis and critical control points (HACCP) constitute important parts of effective food safety management systems (Legan et al., 2001). As part of such food safety management systems, batches of food products may be sampled to assess whether they comply to particular microbiological criteria (MC) set for these foods in law or industry guidelines. In the European Union, two types of MC for finished products are regulated: one that indicates that the food products are safe to eat and another that indicates that the foods are produced under adequate hygienic conditions (CEC, 2007). Within a food manufacturing operation, sampling and microbiological testing at different points in the production process (e.g. targeting ingredients, the environment, intermediate and finished products) is also an important undertaking within food safety management systems, since the results obtained are essential to verify ongoing control of microorganisms by the production process. An underlying assumption for sampling against MC, and many other sampling schemes, is that the sensitivity and the selectivity of the microbiological test used in the sampling protocol are perfect. However, in practice, microbiological methods may lack precision due to which not all target microorganisms will be detected. Also, microorganisms may not be distributed in and recovered from foods as single and discrete units. Conglomerates or clusters of cells may form when cells multiply locally and the food matrix constraints distribution. Thus, whilst in most sampling protocols the microbial concentration is derived from the number of colony forming units (CFU) per sample unit, CFU may not always originate from a single cell but may also be the result of a cluster of cells.

The attribute sampling plans for MC as proposed by the International Commission on Microbiological Specifications for Foods (ICMSF, 1974, 2002) have been widely adopted by public and private parties. These sampling plans have been incorporated into specifications in commercial trading contracts and have been enshrined in food law in different countries (Legan et al., 2001). Attribute sampling plans consist of two types: a two-class plan places results into the two classes acceptable or defective; a three-class plan segregates results into the three classes acceptable, marginally acceptable, and defective. Evaluating the compliance of a batch to an MC depends on the criteria of the sampling plans. In general, two-class sampling plans are used when the health hazard is severe and direct (ICMSF, 1974, 2002; Legan et al., 2001) and its plan stringency depends on the number of sample units tested (n) and the upper limit (m). Plans become more

stringent as n increases and/or m decreases.

There is relatively little knowledge about how microorganisms are actually physically distributed in foods (i.e. their spatial distribution in a batch), while the physical distribution in a food determines the value of the data on prevalence and/or concentration obtained through sampling and testing for informing food safety management decision-making and, ultimately, their value for determining the associated public health burden (ILSI, 2010). In many cases, generalising or default assumptions are made regarding the physical distribution and appropriate sampling strategies. In theory, a batch of food is produced and handled under uniform conditions which will result in a batch in which the microorganisms present are homogeneously distributed. When drawn from a perfectly homogeneously contaminated batch, the levels of microorganisms found in the samples will be Poisson distributed and will only depend on the microbial concentration within the batch. In practice, however, microorganisms are rarely homogeneously distributed within batches of food. Due to for instance the heterogeneity of the food matrix, incidental contamination, localised microbial growth or incomplete mixing, microorganisms are heterogeneously distributed, which results in an unequal probability to detect microorganisms in equal amounts of sample drawn throughout different parts of the whole batch. Depending on when and how the contamination has occurred during the production or thereafter, the spatial microbial distribution within the batch may also vary in size and concentration. During a continuous production process, the microbial level may vary along the daily production. Under certain circumstances, it has been shown that logarithms of counts from a batch of food are likely to be normally distributed (Kilsby and Baird-Parker, 1983). According to Habracken et al. (1986), it had been established that substantial stratification of contamination occurs in dried milk products. This heterogeneity makes the interpretation of the sampling results difficult, especially when the sampling strategy is not tailored to insight in the actual spatial distribution of target microorganisms. This may be illustrated by those documented cases in which heterogeneous distribution led to discrepancies between initial test results and results of extensive retesting (ICMSF, 2002).

To draw a representative sample in which the characteristics of the batch are maintained and to avoid biased results, random sampling is prescribed. In a random sampling scheme, each part of batch has the same probability to enter the sample (CEC, 2005). It is easier to select a representative sample from a moving stream of product

than from a static lot such as trucks or rail cars (Whitaker, 2003). If the contamination is homogeneously distributed, the probability to detect the contamination by definition is the same for each sample unit. In this case, the pattern or strategy in which the sample units are drawn from the batch does not influence the sampling performance. However, if the contamination is heterogeneously distributed or clustered in local spots, the sampling strategy becomes important (Lin et al., 1979; Battilani et al., 2006; Rivas Casado, et al., 2009). Systematic sampling was reported to be more effective to detect a localised contamination (Habraken et al., 1986).

This theoretical study uses a statistical model to compare random and systematic two-class sampling with respect to their ability to detect a localised contamination of a pathogen. For illustrative purposes, this statistical model was established based upon powdered infant formula (PIF) as the food product; pathogens such as *Cronobacter* spp. and *Salmonella* spp. may be relevant for PIF (CAC, 2008). The objective was to gain more quantitative insight in the impact of different sampling strategies. More knowledge about sampling strategies will further improve our ability to accurately assess the risk to consumers of pathogens that occur heterogeneously distributed in foods.

2. Calculation

2.1 Modelling localised microbial contamination in a batch.

The microbial contamination within a food product was statistically modelled such that contamination by a pathogen was present only in a specific part of the batch (referred to as the 'localised contaminated fraction') with the contaminated fraction of the batch indicated as c (dimensionless with a minimum value of 0 and a maximum value of 1). Microorganisms present in the localised contaminated fraction were assumed to be randomly distributed; this assumption is a simplification which is considered to be appropriate in many circumstances. The remainder part of the batch was assumed to be completely free of any microbial contamination, with the uncontaminated part of the batch indicated as $1 - c$. This modelling approach was similar to that utilised by Habraken et al. (1986). Considering the extremes for the contaminated fraction of the batch, $c = 1$ would be relevant in the case of for instance a fluid or a very well mixed food and $c = 0$ would be relevant for sterilised foods. Furthermore, a contaminated fraction of the batch, $c < 1$, could occur in solid, semi solid or powdered foods. Fig. 1 illustrates two spatial distributions $c = 1$ and $c = 0.05$. The microbial contamination was assumed to

contain viable cells that are present as single cells or as conglomerates of cells. When such conglomerates are not fully dispersed by maceration or dilution as part of sample preparation, they will, like single cells, lead to one colony on agar plate, constituting one CFU in plate counts, or one positive tube in MPN determinations. In the statistical modelling it was assumed that all microorganisms present will be detected, i.e. that the microbiological method to detect the viable cells works perfectly and is 100% sensitive and 100% selective.

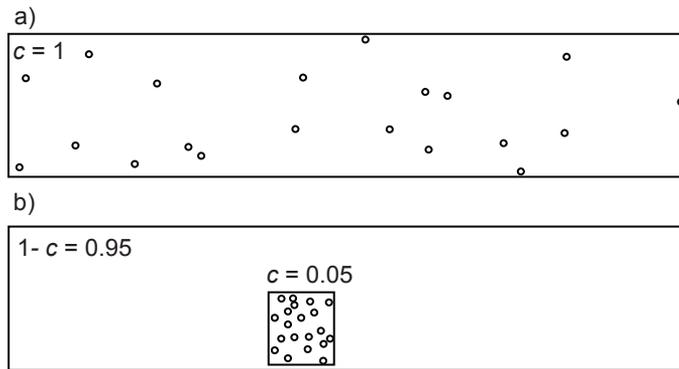


Fig. 1. Schematic representation of contaminated batches in which the microorganisms are randomly distributed in the contaminated fraction (c); (a) homogeneously contaminated batch ($c = 1$); (b) heterogeneously contaminated batch ($c = 0.05$).



Compared to the size of the batch (which can be several thousands kg in the case of PIF) and size of the contaminated fraction of the batch, the size of a sample is small (generally an analytical unit for enumeration is 10 g up to 25 g) and will be drawn either from the non-contaminated part or from the localised contaminated fraction.

2.2 Sampling strategy

In random sampling each sample unit drawn from the batch is independent of previous draws. Every draw has the same probability to hit the contaminated fraction. In systematic sampling investigated here, however, sample units are taken at fixed intervals (w_{int}), e.g. every 100 kg. Systematic sampling is based on weight. Assuming ($w_{batch} > w_{int}$) the number of sample units (n) is given by:

$$n = \text{int} \left(\frac{w_{batch}}{w_{int}} \right) \quad [\text{where } \text{int}(x) = \text{largest integer } \leq x] \tag{1}$$

with n : number of sample units; w_{batch} : the weight of the batch (kg); w_{int} : weight of the fixed interval (kg).

How often a sample unit hits the contaminated fraction depends on the fixed interval (w_{int}) and the size of the contaminated fraction (w_c). For example, from a batch of 20,000 kg every 100 kg a sample unit is drawn ($w_{int} = 100$ kg). The size of the contaminated fraction is 200 kg. In this case, exactly two sample units will be drawn from the contaminated fraction (Fig. 2a). In case the size of the contaminated fraction is 240 kg, at least two and possibly three sample units will be drawn from the contaminated fraction (Fig. 2b). This depends on the location of the first sample unit hitting the contaminated fraction.

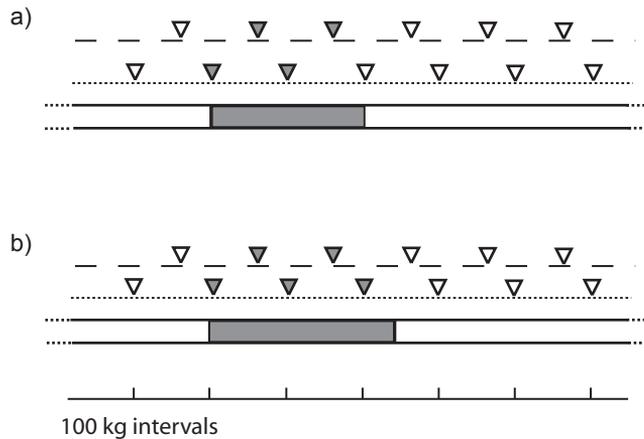


Fig. 2. Systematic sampling with a sampling interval of 100 kg. Part of a batch of 20,000 kg is indicated by the horizontal bars. The batch contains one localised contaminated fraction (c). Grey indicates contamination and white indicates absence of contamination. The black line shows the sampling interval of 100 kg. Dashed lines and dotted lines show the sampling frames offset at different positions in the batch. (a) $w_c = 200$ kg systematic sampling always results in drawing twice from c . (b) $w_c = 240$ kg; if the first sample unit to hit c is located within the first 40 kg of c , this results in drawing three sample units from c , else this results in drawing two sample units from c .

2.3 Detection of the contamination

In order to detect the contamination in the batch, the sample drawn from the batch must contain at least one viable cell and this cell must be detected with the applied detection method. In this theoretical study, the performance of the detection method is considered to be perfect, although in reality this will depend greatly on the sensitivity and selectivity of the detection method. To compare random and systematic sampling, the focus is not

on detection probability related to the detection method but on the probability that the entire sampling scheme (all sample units) contains one or more viable cells, $\Pr(K_{samples} > 0)$ with $K_{samples}$ the number of cells in the entire sampling scheme.

2.4 Probability that sampling scheme includes one or more cells $\Pr(K_{samples} > 0)$

The detection of a localised contaminated fraction within a batch depends on the contaminated fraction (c), the number of cells therein, the number of sample units drawn, and the weight of a sample unit. The probability that the entire sampling scheme includes one or more cells ($\Pr(K_{samples} > 0)$) depends on the probability that at least one sample unit is drawn from the contaminated fraction and the probability that this sample unit contains at least one cell. The following two sections provide the statistical details to calculate $\Pr(K_{samples} > 0)$ for a homogeneously contaminated batch (section 2.4.1) and for a heterogeneously contaminated batch (section 2.4.2). All calculations were performed both in Microsoft Excel 2003 and in MATLAB® 7.8.0, R2009a (The MathWorks™, Natick, Massachusetts, USA).

2.4.1 Statistical calculations to detect a homogeneous contamination throughout the batch

When cells are randomly distributed throughout the batch, the way the sample units are drawn does not affect the detection. The total weight of sample units drawn, which is the number of sample units (n) times the weight of a sample unit (w_{sample}) will determine $\Pr(K_{samples} > 0)$. The expected number of cells in each sample unit is:

$$k^* = K_{batch} \cdot \frac{w_{sample}}{w_{batch}} \quad (2)$$

with k^* : expected number of cells in each sample unit, K_{batch} : number of cells in batch, and w_{sample} : weight of a sample unit.

The probability of any sample unit containing a given number of cells is given by the Poisson distribution:

$$\Pr(k = i) = \text{Poisson}(i; k^*) = \frac{(k^*)^i \cdot e^{-k^*}}{i!} \quad (3)$$

The probability that a given number of sample units contains no cells (n_{-ve}) is given by the Binomial distribution:

$$\Pr(n_{-ve} = i) = \text{Binomial}(i; n; p_{-ve}) = \binom{n}{i} \cdot p_{-ve}^i \cdot (1 - p_{-ve})^{n-i} \quad (4)$$

with n_{-ve} is the number of sample units containing no cells. The probability that in all n sample units no sample unit contains a cell is given by

$$\Pr(K_{samples} = 0) = \Pr(n_{-ve} = n) = p_{-ve}^n \quad (5)$$

and the probability that at least one sample unit contains at least one cell is

$$\Pr(K_{samples} > 0) = 1 - p_{-ve}^n \quad (6)$$

If n sample units are drawn from the batch, the expected number of cells in the entire sample ($n \cdot w_{sample}$) is the number of cells in the batch multiplied by the proportion of the batch in the entire sample. The probability that at least one sample unit contains at least one cell can be expressed as

$$\Pr(K_{samples} > 0) = 1 - (e^{-k^*})^n = \text{Poisson}(0; n k^*) = \text{Poisson}\left(0; K_{batch} \cdot \frac{n w_{sample}}{w_{batch}}\right) \quad (7)$$

2.4.2 Statistical calculations to detect a localised contamination within the batch

When the cells are randomly distributed within the contaminated fraction (c),

$\Pr(K_{samples} > 0)$ depends on two probabilities:

- 1) $\Pr(\text{sample in } c)$, the probability that any given sample unit is drawn from the localised contaminated fraction.
- 2) $\Pr(k > 0 \mid \text{sample in } c)$, the probability that any given sample unit drawn from the contaminated fraction contains at least one cell.

$\Pr(\text{sample in } c)$ depends on the way the sample units are drawn, randomly or systematically.

$\Pr(k > 0 \mid \text{sample in } c)$, however, is equal for both sampling strategies.

$\Pr(k > 0 \mid \text{sample in } c)$ depends on the expected number of cells in the sample unit, which is related to the size of the contaminated fraction and the weight of a sample unit.

The probability to detect no cells in a sample unit is the same as Eq. 3. In this case, however, the expected number of cells in the sample unit is:

$$k_c^* = K_{batch} \cdot \frac{w_{sample}}{w_{batch}} \cdot \frac{1}{c} \quad (8)$$

with k_c^* : expected (mean) number of cells in each sample unit drawn from the contaminated fraction.

2.4.2.1 Random sampling

In random sampling each sample unit has an equal probability ($= c$) of being drawn from the contaminated fraction. The number of sample units drawn from the contaminated fraction (n_c) is distributed according to a binomial distribution

$$\Pr(n_c = i) = \text{Binomial}(i; n; c) = \binom{n}{i} \cdot c^i \cdot (1-c)^{n-i} \quad (9)$$

The probability that at least one sample unit has at least one cell ($\Pr(K_{samples} > 0)$) is equal to 1 minus the probability that in all sample units (n) no cells are detected.

$$\Pr_{\text{rand}}(K_{samples} > 0) = 1 - \sum_{n_c=0}^n \left[\text{Binomial}(n_c; n; c) \cdot \text{Poisson}(0; k_c^*)^{n_c} \right] \quad (10)$$

In case c is 1, all sample units will be drawn from the contaminated fraction and Eq. 10 equals Eq. 7.

2.4.2.2 Systematic sampling

In systematic sampling the number of sample units drawn from c depends on the size of the contaminated fraction ($w_c = c \cdot w_{batch}$) and the sampling interval (w_{int}). The smallest number ($n_{c:\min}$) that can be drawn from the contaminated fraction is:

$$n_{c:\min} = \text{int}\left(\frac{w_c}{w_{int}}\right) \quad (11)$$

Depending on the position of the regularly spaced sample units with respect to the contaminated fraction, a single additional sample unit may be drawn from the contaminated fraction. Assuming that all relative positions of the regular sample grid with

respect to the contaminated fraction are equally likely, the probability of this occurring is proportional to the difference between the size of the contaminated fraction and that part of it spanned by the $n_{c:\min}$ sample units.

$$\Pr(n_c = n_{c:\min} + 1) = \frac{w_c}{w_{int}} - \text{int}\left(\frac{w_c}{w_{int}}\right) \quad (12)$$

The probability that at least one sample unit has at least one cell ($\Pr(K_{samples} > 0)$) is equal to 1 minus the probability that in all sample units (n) no cells are detected. There are two possibilities: either $n_{c:\min}$ or $n_{c:\min} + 1$ sample units are drawn from the contaminated fraction:

$$\Pr_{\text{sys}}(K_{samples} > 0) = \quad (13)$$

$$1 - \left[\Pr(n_c = n_{c:\min}) \cdot \Pr(k = 0 | \text{sample in } c)^{n_{c:\min}} + \Pr(n_c = n_{c:\min} + 1) \cdot \Pr(k = 0 | \text{sample in } c)^{n_{c:\min} + 1} \right]$$

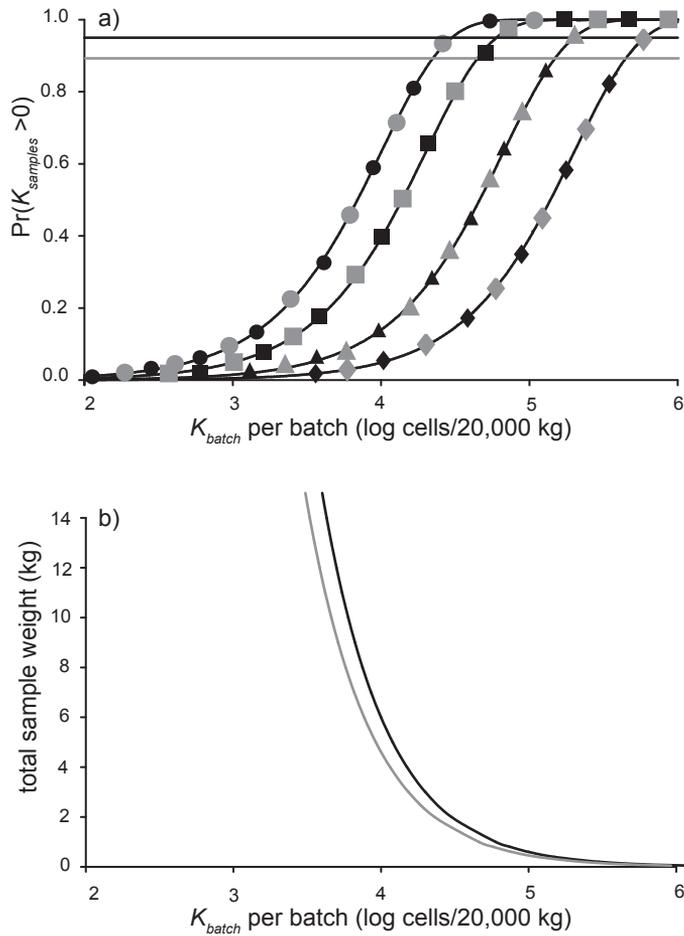
In case c is 1, Eq. 13 (systematic sampling) equals Eq. 7.

3. Results

This theoretical study investigated the difference between random and systematic sampling of a batch of food in which low numbers of target microorganisms are heterogeneously distributed. It investigated how $\Pr(K_{samples} > 0)$, the probability that the entire sampling scheme includes one or more cells, is affected by the sampling strategy, the number of sample units drawn, the size of the food product part that is locally contaminated, and the microbial concentration. Powdered infant formula (PIF) was chosen as the food product. *Cronobacter* spp. and *Salmonella* spp. are relevant pathogens of concern for PIF and they potentially contaminate the product at very low levels. The size of a single sample unit was chosen to be 10 g, since the relevant MC established in the European Union is based on testing for *Cronobacter* spp. by drawing 30 sample units of 10 g (CEC, 2007). The contamination was modelled as one localised contaminated fraction within the batch and it was assumed that if the microorganism is present it is also detected.

3.1 Sampling a homogeneously contaminated batch ($c = 1$)

Fig. 3a illustrates $\Pr(K_{samples} > 0)$, the probability that the sampling scheme includes one or more cells, by drawing 10, 30, 100, and 200 sample units of 10 g assuming that the cells are homogeneously distributed within the whole batch.



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Fig. 3. Homogeneously contaminated batch of 20,000 kg ($c = 1$). (a) $\Pr(K_{samples} > 0)$, the probability that the sampling scheme includes one or more cells by drawing 10 (◆), 30 (▲), 100 (■), and 200 (●) sample units of 10 g as a function of K_{batch} , the number of cells per batch. The cells are distributed over the whole batch ($c = 1$). The grey symbols indicate random sampling and the black symbols indicate systematic sampling. The horizontal lines show $\Pr(K_{samples} > 0)$ at 0.90 (grey line) and at 0.95 (black line). (b) The total weight of the sample units drawn as a function of K_{batch} , the number of cells per batch with $\Pr(K_{samples} > 0)$ of 0.90 (grey line) or 0.95 (black line).

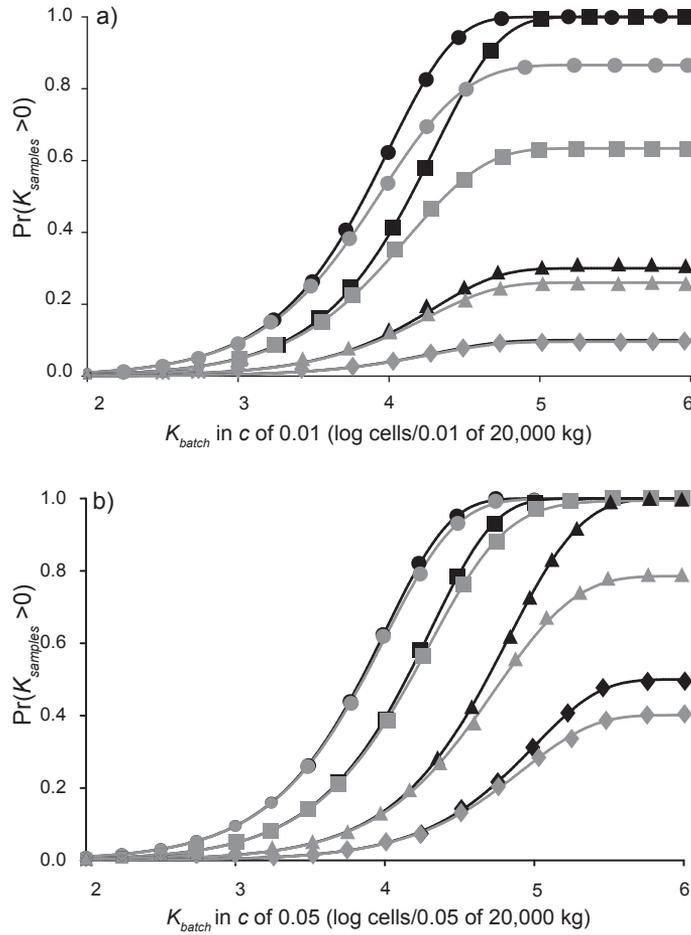
Evidently, $\Pr(K_{\text{samples}} > 0)$ increases with an increasing number of cells per batch. For example, drawing 30 sample units from a batch in which 10^4 cells occur per 20,000 kg (i.e. 1 cell per 2 kg) results in $\Pr(K_{\text{samples}} > 0) = 0.14$, whereas the probability is 0.78 when 10^5 cells are present per 20,000 kg (i.e. 5 cells per kg). $\Pr(K_{\text{samples}} > 0)$ also increases by drawing more sample units. Drawing 200 sample units instead of 30 sample units at a level of 10^4 cells per 20,000 kg results in $\Pr(K_{\text{samples}} > 0) = 0.63$.

Since the contamination is homogeneously distributed within the batch, the type of sampling strategy (random or systematic) will not influence $\Pr(K_{\text{samples}} > 0)$. Only the total sample weight, which is the product of number of sample units and the weight of a single sample unit, determines the detection probability. Independent of the sampling strategy, drawing 10 sample units of 50 g or 50 sample units of 10 g will result in the same $\Pr(K_{\text{samples}} > 0)$. Fig. 3b shows the total sample weight (kg) at $\Pr(K_{\text{samples}} > 0)$ values of 0.90 or 0.95 as a function of the number of cells per batch (expressed as log cells/20,000 kg). When, for example, a total sample weight of 0.3 kg is drawn, the probability that this sample will include one or more cells ($\Pr(K_{\text{samples}} > 0) = 0.95$) at a concentration of $10^{5.3}$ cells per batch, while a total sample weight of 1 kg is drawn, $\Pr(K_{\text{samples}} > 0) = 0.95$ at a concentration of $10^{4.8}$ cells per batch. Increasing the total sample weight to 10 kg, $\Pr(K_{\text{samples}} > 0) = 0.95$ at a concentration of $10^{3.8}$ cells per batch.

3.2 Sampling a batch with single localised contaminated fraction

Data from a recent FAO/WHO risk assessment (FAO/WHO, 2006) were used to get the order of magnitude of the size of the contaminated fraction. This risk assessment estimated the concentration of *Cronobacter* spp. in batches of powdered infant formula from prevalence data in published literature and from unpublished studies provided to FAO/WHO. In 62% of the cases, the number of positive samples was reported to be between 0% and 5% of the total number of samples tested. Considering these data, for the statistical modelling in this study values for the contaminated fraction ($c = w_c/w_{\text{batch}}$) of 0.01 and 0.05 were chosen in illustrated examples.

Fig. 4 depicts $\Pr(K_{\text{samples}} > 0)$ as a function of the number of cells in the localised contaminated fraction (expressed as log cells/c of 20,000 kg), assuming that the microorganisms are randomly distributed within 0.01 (Fig. 4a) or 0.05 (Fig. 4b) of a batch.

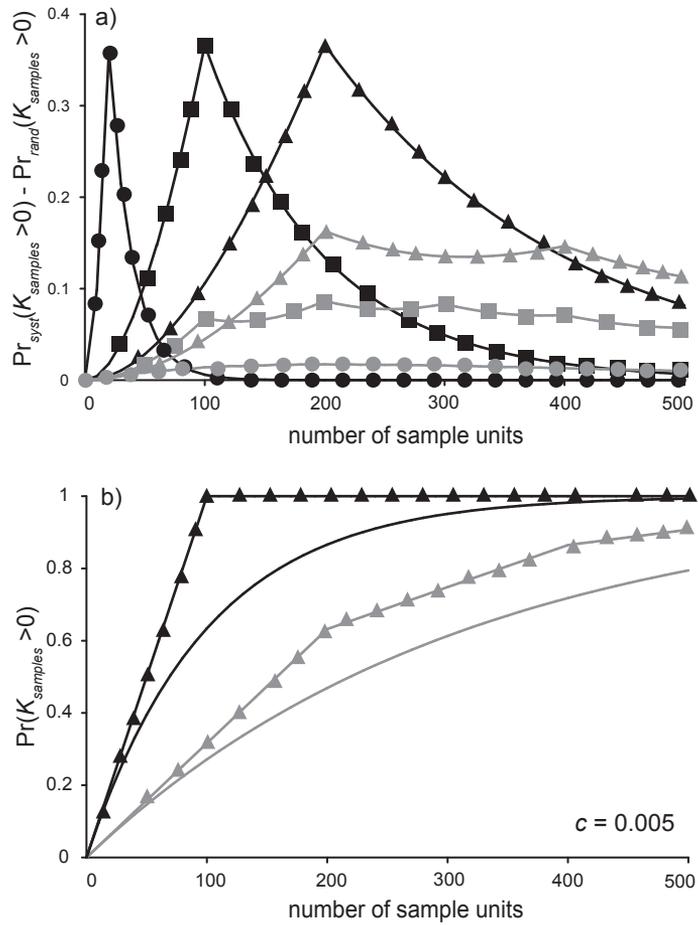


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Fig. 4. Heterogeneously contaminated batch of 20,000 kg with a contaminated fraction (c) of 0.01 (4a) and 0.05 (4b). $\Pr(K_{\text{samples}} > 0)$, the probability that the sampling scheme includes one or more cells by drawing 10 (◆), 30 (▲), 100 (■), and 200 (●) sample units of 10 g, as a function of K_{batch} , the number of cells in the contaminated fraction of a batch. The grey lines and symbols indicate random sampling whereas the black lines and symbols indicate systematic sampling.

In these examples, random or systematic sampling was modelled for an increasing number of sample units, ranging from 10, 30, 100, to 200 sample units, and for a weight of a sample unit of 10 g. For low levels of contamination, i.e. below $\sim 10^3$ cells in c of 0.01 or 0.05, the type of sampling did not show a visible difference. For higher levels of contamination, however, both figures show that the sampling strategy impacts on $\Pr(K_{\text{samples}} > 0)$. Systematic sampling results in a probability of detection ($\Pr_{\text{syst}}(K_{\text{samples}} > 0)$) that is either equal or higher than the probability of detection with random sampling ($\Pr_{\text{rand}}(K_{\text{samples}} > 0)$). For example, Fig. 4a ($c = 0.01$) shows that for 10 sample units being drawn, both random and systematic sampling have the same small probability to hit the contaminated fraction independently of the number of cells therein. Above $\sim 10^4$ cells, however, the difference between systematic and random sampling increases markedly as the number of sample units drawn increases from 10 to 100 sample units. Comparing drawing 100 with 200 sample units, systematic sampling still has a higher detection probability in the case that 200 sample units are drawn, but the difference is less than when 100 sample units are drawn. This phenomenon is also shown in Fig. 4b ($c = 0.05$): drawing 30 instead of 10 increases the difference, drawing more sample units results in less of a difference between random and systematic sampling. These graphs show that, the difference in detection probability depends on both the size of the contaminated fraction and the number of sample units, which will be further illustrated in Fig. 5 and 6.

Fig. 5a presents the difference between detection probabilities for systematic and random sampling, indicated as $\Pr_{\text{syst}}(K_{\text{samples}} > 0) - \Pr_{\text{rand}}(K_{\text{samples}} > 0)$, as a function of the number of sample units in the case contaminated fractions are 0.005, 0.01, and 0.05 with either 10^4 or 10^6 cells present in the contaminated fraction. At 10^6 cells per contaminated fraction, $\Pr_{\text{syst}}(K_{\text{samples}} > 0) - \Pr_{\text{rand}}(K_{\text{samples}} > 0)$ smoothly increases until it reaches a maximal difference (global maximum) whereupon it smoothly decreases as the number of sample units drawn further increases. The improvement of $\Pr_{\text{syst}}(K_{\text{samples}} > 0)$ over $\Pr_{\text{rand}}(K_{\text{samples}} > 0)$ goes up to 0.37 when drawing 20, 100, and 200 sample units of 10 g. Graphs for a situation with 10^5 cells per contaminated fraction (not displayed in Fig. 5) are similar to the graphs for 10^6 cells per contaminated fraction. For a contaminated fraction of 0.005 or 0.01 and 10^4 cells present per contaminated fraction, the graphs show a less pronounced difference and a waving behaviour with global maximum at 200 sample units.



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Fig. 5. Random or systematic sampling of a heterogeneously contaminated batch as a function of the number of sample units drawn. The batch size is 20,000 kg and the weight of a sample unit is 10 g. (a) Difference in probability between systematic sampling ($\Pr_{\text{syst}}(K_{\text{samples}} > 0)$) and random sampling ($\Pr_{\text{rand}}(K_{\text{samples}} > 0)$), which both represent the probability that the sampling scheme includes one or more cells. The contaminated fraction is 0.005 ($\blacktriangle, \blacktriangle$), 0.01 ($\blacksquare, \blacksquare$), and 0.05 (\bullet, \bullet). The black lines and symbols indicate 10^6 cells in the contaminated fraction and the grey lines and symbols indicate 10^4 cells in the contaminated fraction. (b) $\Pr_{\text{syst}}(K_{\text{samples}} > 0)$ (lines with $\blacktriangle, \blacktriangle$) or $\Pr_{\text{rand}}(K_{\text{samples}} > 0)$ (lines with no symbols), for a batch with 10^6 cells (black) or with 10^4 cells (grey) in a contaminated fraction of 0.005.

To consider the difference $\Pr_{\text{syst}}(K_{\text{samples}} > 0) - \Pr_{\text{rand}}(K_{\text{samples}} > 0)$ in more detail, Fig. 5b shows $\Pr_{\text{syst}}(K_{\text{samples}} > 0)$ and $\Pr_{\text{rand}}(K_{\text{samples}} > 0)$ with 10^4 or 10^6 cells in a contaminated fraction of 0.005. For random sampling, increasing the number of sample units gradually increases $\Pr_{\text{rand}}(K_{\text{samples}} > 0)$. For systematic sampling, however, increasing the number of sample units increases $\Pr_{\text{syst}}(K_{\text{samples}} > 0)$ with a linear increase and change of the slope. For 10^6 cells the slope changes at 100 sample units and for 10^4 cells the slope changes at 200 and 400 sample units. Nevertheless, the $\Pr_{\text{syst}}(K_{\text{samples}} > 0)$ increases when more sample units are taken. Table 1 provides combinations of sampling interval (w_{int}), number of sample units (n), and contaminated fraction (c) with the maximal differences ($\Pr_{\text{syst}}(K_{\text{samples}} > 0) - \Pr_{\text{rand}}(K_{\text{samples}} > 0)$) as shown in Fig. 5a. The related variables expected number of cells in a sample unit of 10 g (k^*), and the probability that the sample unit drawn from the contaminated fraction contains at least one cell, $\Pr(k > 0 \mid \text{sample in } c)$, are shown. At these combinations exactly one of the systematic sample units will be drawn from the contaminated fraction ($w_c = w_{\text{int}}$). If the concentration of cells in the contaminated fraction is high enough such that this sample unit will always contain at least one cell, then $\Pr_{\text{syst}}(K_{\text{samples}} > 0)$ will be 1. This will result in a maximal difference between $\Pr_{\text{syst}}(K_{\text{samples}} > 0)$ and $\Pr_{\text{rand}}(K_{\text{samples}} > 0)$, displayed as a global maximum in Fig. 5a.

Table 1. Overview of the combinations sampling interval (w_{int}), number of sample units (n) and contaminated fraction (c) of a batch for which the differences between $\Pr_{\text{syst}}(K_{\text{samples}} > 0)$ and $\Pr_{\text{rand}}(K_{\text{samples}} > 0)$ are maximal (global maxima in Fig. 5a and Fig. 6a). If $w_c = w_{\text{int}}$, exactly one systematic sample unit will hit the contaminated fraction. Shown are the sampling interval (w_{int}) in a batch of 20,000 kg, the number of sample units (n), contaminated fraction (c), the expected number of cells in a sample unit of 10 g (k^*) drawn from the contaminated part, and the probability that the sample unit contains at least one cell ($\Pr(k > 0 \mid \text{sample in } c)$) and assuming that this cell is detected. The contaminated parts contain either 10^6 or 10^4 cells

w_{int} (kg)	n	c	10 ⁶ cells in c		10 ⁴ cells in c	
			k^* ^a	$\Pr(k > 0 \mid \text{sample in } c)$	k^* ^a	$\Pr(k > 0 \mid \text{sample in } c)$
1000	20	0.05	10	1	0.10	0.10
400	50	0.02	25	1	0.25	0.22
200	100	0.01	50	1	0.50	0.39
100	200	0.005	100	1	1.0	0.63

^a k^* = expected number of cells in 10 g

Fig. 6 presents the difference between systematic and random sampling, indicated as $\Pr_{\text{syst}}(K_{\text{samples}} > 0) - \Pr_{\text{rand}}(K_{\text{samples}} > 0)$, as a function of the contaminated fraction taking 50, 100 or 200 sample units of 10 g, but with the number of cells present being 10^4 , $10^{4.5}$, or 10^6 per contaminated fraction.

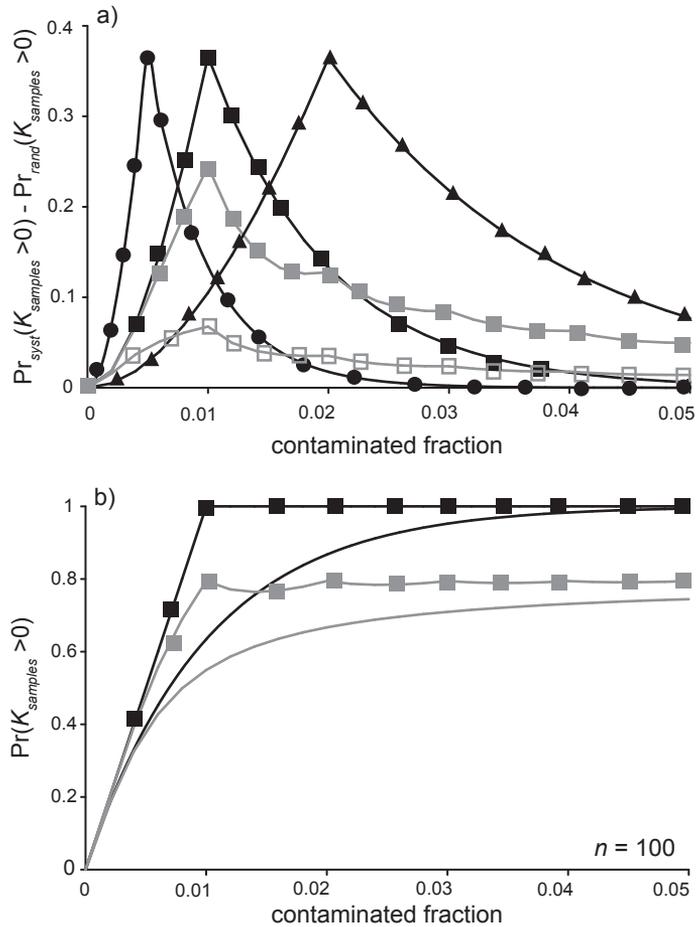


Fig. 6. Random or systematic sampling of a heterogeneously contaminated batch as a function of the contaminated fraction. The batch size is 20,000 kg and the weight of a sample unit is 10 g. (a) Difference in probability between systematic sampling ($\Pr_{\text{syst}}(K_{\text{samples}} > 0)$) and random sampling ($\Pr_{\text{rand}}(K_{\text{samples}} > 0)$), which both represent the probability that the sampling scheme includes one or more cells while drawing 50 (\blacktriangle), 100 (\blacksquare , \square), or 200 (\bullet) sample units. The number of cells in the contaminated fraction is indicated by the symbols: 10^4 (\square), $10^{4.5}$ (\blacksquare), and 10^6 (\bullet , \blacksquare , \blacktriangle) cells. (b) $\Pr_{\text{syst}}(K_{\text{samples}} > 0)$ (lines with \blacksquare , \square) or $\Pr_{\text{rand}}(K_{\text{samples}} > 0)$ (lines with no symbols)), for a batch with 10^6 cells (black) or with $10^{4.5}$ cells (grey). The number of sample units is 100.

At 10^6 cells per contaminated fraction, the difference $\Pr_{\text{syst}}(K_{\text{samples}} > 0) - \Pr_{\text{rand}}(K_{\text{samples}} > 0)$ smoothly increases until it reaches a maximum and smoothly decreases. The maximal differences in detection probability are at a contaminated fraction of 0.005, 0.01, and 0.02 when drawing 200, 100, or 50 sample units respectively. In these cases, the improvement of $\Pr_{\text{syst}}(K_{\text{samples}} > 0)$ over $\Pr_{\text{rand}}(K_{\text{samples}} > 0)$ increases to 0.37. At a lower number of cells (10^4 ; $10^{4.5}$) the graphs show less pronounced differences between systematic and random sampling and a wavy curve after the maximum. To illustrate this difference, Fig. 6b shows $\Pr_{\text{syst}}(K_{\text{samples}} > 0)$ and $\Pr_{\text{rand}}(K_{\text{samples}} > 0)$ with $10^{4.5}$ and 10^6 cells when drawing 100 sample units. For random sampling and for both $10^{4.5}$ and 10^6 cells in the contaminated fraction, increasing the contaminated fraction gradually increases $\Pr_{\text{rand}}(K_{\text{samples}} > 0)$. For systematic sampling and $10^{4.5}$ or 10^6 cells in the contaminated fraction, increasing the contaminated fraction linearly increases $\Pr_{\text{syst}}(K_{\text{samples}} > 0)$ until a maximum at a contaminated fraction of 0.01. For 10^6 cells, $\Pr_{\text{syst}}(K_{\text{samples}} > 0)$ remains 1, if the size of the contaminated fraction increases from 0.01 to 0.05. For $10^{4.5}$ cells, however, further increase of the contaminated fraction causes a decrease of $\Pr_{\text{syst}}(K_{\text{samples}} > 0)$, followed again by an increase. The graph shows a wavy line after the first maximum, which can be explained as follows. For $10^{4.5}$ cells in the contaminated fraction, the expected number of cells in a sample unit of 10 g (k^*) drawn from the contaminated fraction will become smaller when the contaminated fraction increases. This lowers the probability that the sample unit contains at least 1 cell ($\Pr(k > 0 \mid \text{sample in } c)$). However, as the contaminated fraction increases, the probability to draw an additional sample unit, $\Pr(n_c = n_{c,\text{min}} + 1)$ increases. The balance between those two probabilities causes the wavy curves.

4. Discussion

When food is sampled in an effort to assess the presence and concentration of microorganisms, the effectiveness of the sampling scheme is amongst others related to the spatial distribution of the target microorganisms. Microorganisms that potentially cause illness generally occur in foods at very low levels, when at all, and their actual spatial distribution in different foods is very difficult to assess with a high level of precision. In the absence of exact knowledge, generalising assumptions are often made as to the nature of microbial distributions. Better insight into the actual microbiological distributions may help to improve food safety management decision-making (ILSI, 2010). The current study confirms that systematic sampling increases probability to detect a

localised contaminated fraction as compared to random sampling under a variety of conditions. This is in line with the publication of Habraken et al. (1986), who estimated the probability of detecting *Salmonella* spp. in powdered milk products. Casado et al. (2009) modelled a two-dimensional spatial distribution of mycotoxins in bulk commodities to design effective sample strategies, and also concluded that systematic ('regular') sampling strategies should be preferred over random sampling. Since systematic sampling improves the probability of detection, the results presented in the current study show that this improvement depends on the contaminated fraction and the number of sample units taken. The improvement reaches a maximum, when exactly one systematic sample unit will be drawn from the contaminated fraction. In this case, the sampling interval equals the size of the contaminated fraction. Estimating the size of the contaminated fraction or the optimal sampling interval is a 'chicken and egg' dilemma. However, if one can estimate the size of the contaminated fraction, the optimal number of systematic sample units may be derived from that. In this study, using PIF as the model food product, the contaminated fraction was estimated by using data of an FAO/WHO report (FAO/WHO, 2006). When the batch size of a daily production is known, one can likewise calculate how many sample units ($n = \text{int}(w_{\text{batch}}/w_{\text{int}})$) have to be taken to hit the contaminated fraction ($w_c = w_{\text{int}}$).

In order to detect a cell in a sample unit ($\text{Pr}(k > 0 \mid \text{sample in } c)$), both the weight of a sample unit and the concentration of cells within the contaminated fraction influence this probability. A low concentration of cells or a small weight of a sample unit will lower the probability that the sample unit contains at least one cell. Accordingly, the calculations showed that the difference between random and systematic reduced with low numbers of cells present in the contaminated fraction of the batch.

Systematically taking 400 sample units of 2.5 g, 200 of 5 g, or 100 of 10 g will have the same $\text{Pr}(K_{\text{samples}} > 0)$ for a localised contaminated fraction of 0.01 (data not shown). Taking 50 sample units of 20 g, however, reduces $\text{Pr}(K_{\text{samples}} > 0)$ from 1 to 0.5 at 10^5 cells in the contaminated fraction (data not shown). A practical way of realising the benefits of systematic sampling is using an auto-sampler to collect the necessary large number of small sample units per batch. A disadvantage of auto-sampling is that it does not test the packed product, but samples from the line. Testing so many packages of end product, will be very time consuming, may still require additional human resources and may give significant loss of product. Auto-sampling could be deployed best just before

filling, though any contamination arising at the filling stage would not be covered in the assessment. Auto-sampling may be a good way to monitor the microbiological status of a batch of food during production and to determine ongoing control over the production process. It can be combined with verification of testing the end product, according to a suitable microbiological criterion.

In this study the modelling of the heterogeneous contamination was focused on a single localised contaminated fraction. It does not take into account situations where there are multiple localised contaminated fractions in a batch which may be spread over different physical locations. Also, the concentration of cells within the contaminated fraction was assumed to be constant. If a contamination occurs systematically, for example when one of a series of filler heads is contaminated, it is possible to miss the contamination systematically. In this case, stratified random sampling would be more appropriate. Although our model simplifies reality, it provides an elegant way to calculate the detection probability for systematic and random sampling. It is applicable to detect a localised contamination in food products that are solid, semi-solid or powdered, like for instance *Salmonella* spp. in peanut butter, mycotoxins in grain, or *Cronobacter* spp. in powdered infant formula. For a single localised contaminated fraction, certainly, systematic sampling is found to be preferred to random sampling to detect such a contamination within a batch.

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Chapter 6

Actual distribution of *Cronobacter* spp. in industrial batches of powdered infant formula and consequences for performance of sampling strategies

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Abstract

The actual spatial distribution of microorganisms within a batch of food influences the results of sampling for microbiological testing when this distribution is non-homogeneous. In the case of pathogens being non-homogeneously distributed, it markedly influences public health risk. This study investigated the spatial distribution of Cronobacter spp. in powdered infant formula (PIF) on industrial batch-scale for both a recalled batch as well a reference batch. Additionally, local spatial occurrence of clusters of Cronobacter cells was assessed, as well as the performance of typical sampling strategies to determine the presence of the microorganisms. The concentration of Cronobacter spp. was assessed in the course of the filling time of each batch, by taking samples of 333 g using the most probable number (MPN) enrichment technique. The occurrence of clusters of Cronobacter spp. cells was investigated by plate counting.

From the recalled batch, 415 MPN samples of 333 g were drawn. The expected heterogeneous distribution of Cronobacter spp. could be quantified from these samples, which showed no detectable level (detection limit of $-2.52 \log \text{CFU/g}$) in 58% of samples, while in the remainder concentrations were found to be between -2.52 and $2.75 \log \text{CFU/g}$. The estimated average concentration in the recalled batch was $-2.78 \log \text{CFU/g}$ and a standard deviation of $1.10 \log \text{CFU/g}$. The estimated average concentration in the reference batch was $-4.41 \log \text{CFU/g}$, with 99% of the 93 samples being below the detection limit. In the recalled batch, clusters of cells occurred sporadically in 8 out of 2290 samples of 1g taken. The two largest clusters contained 123 ($2.09 \log \text{CFU/g}$) and 560 ($2.75 \log \text{CFU/g}$) cells. Various sampling strategies were evaluated for the recalled batch. Taking more and smaller sample units and keeping the total sampling weight constant, considerably improved the performance of the sampling plans to detect such a type of contaminated batch. Compared to random sampling, stratified random sampling improved the probability to detect the heterogeneous contamination.

6

1. Introduction

A batch of food is defined as a definite quantity of some commodity manufactured or produced under presumed uniform conditions (CAC, 2004). Such a batch produced and handled under uniform conditions (CEC, 2005; ICMSF, 2002) will result, in theory, in homogeneously distributed levels of microorganisms. Sampling and testing results from this batch may vary even when microorganisms are homogeneously or uniform randomly distributed. In practice, homogeneity in batches of food is rare. Due to, for instance, the structural heterogeneity of the food matrix, incomplete mixing, incidental (post-processing) contamination, and/or localised microbial growth, microorganisms are most often heterogeneously distributed. This results in an unequal probability to detect microorganisms in equal amounts of sample drawn from different sections of a batch. Depending on when and how contamination occurred during production or thereafter, the spatial microbial distribution within the batch may also vary in size and concentration (Jongenburger et al., 2011a).

The spatial distribution of microorganisms determines our ability to quantify prevalence and concentration of microorganisms through sampling or testing. Knowledge about how microorganisms are actually spatially distributed in foods is relatively scarce. In many cases, generalising or default assumptions are made regarding the spatial distribution. The currently employed sampling strategies generally are based on these assumptions. According to Kilsby and Baird-Parker (1983), total viable counts data from batches of frozen meat, frozen vegetable, frozen dairy, and powdered products appeared to be lognormally distributed in 92% of the batches; in 8% of the batches, the total viable count appeared to be not lognormally, with a maximum of 13% for powdered products. Based on studies such as that of Kilsby and Baird-Parker, the International Commission on Microbiological Specification for Foods (ICMSF, 2002) assumed a Lognormal distribution in order to evaluate the performance of attribute sampling plans. Assuming a lognormally distributed contamination, the size of the standard deviation will affect the performance of an attributes sampling plan (Dahms, 2004; Legan et al., 2001). For the performance of attribute sampling, ICMSF (2002) has chosen a standard deviation of 0.8 log CFU/g based on data derived from the meat industry (Greenberg et al., 1966) and similar observations in other food products. When the underlying distribution of microorganisms within the batch is known, however, variable sampling plans may be an option (CAC, 2004; ICMSF, 2002; van Schothorst et al., 2009).

Habraken et al. (1986) established that substantial clustering or heterogeneity of contamination occurs in dried milk products, with sections of a batch containing microorganisms and other sections containing no microorganisms at all. Besides heterogeneity on batch-scale, heterogeneity on local-scale is possible within a food product. One could speculate that, if water remains in the processing environment of the plant due to spilling or wet cleaning, bacteria may grow overnight to levels of 10^9 cells/mL. If a droplet of this contaminated water comes in contact with the final product, this may result in clusters of cells with high concentrations, even in case 99% of the cells would die upon dehydration. A cluster of cells resulting from such droplet may not be properly assessed using current sampling strategies but can have a significant public health impact.

In order to investigate batch-scale and local-scale heterogeneity in detail in a batch of food and their impact on several possible sampling approaches, powdered infant formula (PIF) was chosen as the food product and *Cronobacter* spp. as target microorganism.

PIF can be milk-based or soy-based. The major components are skimmed milk powder, demineralised whey powder, soy proteins, sucrose, lactose, starch or other thickeners like carob, oils, fruit powder, and lecithin (Proudy et al., 2008). PIF can be manufactured in a wet-mix process, a dry-mix process or a combined process (Cordier, 2008). In the wet-mix process, all unprocessed material, such as raw milk or liquid whey, as well as separately processed ingredients are handled in a liquid phase, heat treated by pasteurisation or sterilisation, and dried to obtain the final product. In the dry-mix process, individual ingredients are prepared, heat-treated if appropriate, dried, and then dry-blended. The process may include various mixing steps to obtain the final product. In the combined process, part of the raw material and part of the ingredients are processed and mixed as liquids and dried to obtain a base powder. This base powder and the other ingredients are consequently dry blended. For these three types of processes, the final product is further handled to the filling stage.

PIF given to infants during the first months of life needs to be manufactured according to very stringent hygiene measures, since PIF has been linked to outbreaks related to the presence of *Cronobacter* spp. (CAC, 2008; Cordier, 2008; FAO/WHO, 2006a; Weir, 2002). *Cronobacter* spp. have caused serious diseases in newborn and premature babies (Gurtler et al., 2005; Muytjens et al., 1983). According to the current

microbiological criteria (MC) for PIF (CAC, 2008; CEC, 2007), every batch has to be tested, apart from *Salmonella* spp., for *Cronobacter* spp. by testing 30 sample units of 10 g. Viable *Cronobacter* spp. cells show no particular heat resistance and are easily killed in liquids at temperatures ranging from 60 to 70°C (Breeuwer et al., 2003; Iversen et al., 2004). Pasteurisation is a critical control point (CCP), but recontamination might occur afterwards. Since *Cronobacter* spp. is a widespread microorganism and can be present in the dry-processing environment (Kandhai et al., 2004; Reich et al. 2010), the contamination may occur, for example, via air, presence of water, niches, and filler heads. The high tolerance of *Cronobacter* spp. to desiccation increases the risk of post-pasteurisation contamination of the finished product (Breeuwer et al., 2003). The concentration of *Cronobacter* spp. in batches of PIF has been estimated from prevalence data in published literature and from unpublished studies provided to FAO/WHO (2006). The minimum and maximum concentrations estimated were respectively -5.3 and -2.8 log CFU/g, and the mean concentration being -3.84 log CFU/g and the standard deviation being 0.696 log CFU/g.

This study quantified in detail the batch-scale and local-scale heterogeneity of *Cronobacter* spp. within a batch of PIF that was recalled after *Cronobacter* spp. was detected. For comparison, a reference batch produced in the same factory was investigated. The low microbial concentration levels required using the most probable number (MPN) technique by enrichment for enumeration. Since the MPN does not distinguish between single cells or clusters of cells, for investigation of local-scale heterogeneity, clusters of *Cronobacter* spp. cells were enumerated by plate counting numerous small samples. The performances of several sampling plans were both calculated and simulated according to the enumeration data. The probabilities of detection by random sampling as well as stratified random sampling were then compared.

2. Methods

2.1 Describing the investigated batches of powdered infant formula

2.1.1 Recalled batch

The batch of approximately 22,000 kg was produced in 3 shifts between 04:00 and 24:00 hrs in January 2007 according a dry-mix process. Each package contained two bags of 400 g of PIF. At that time, PIF had to be tested for *Enterobacteriaceae* by drawing 10 sample units of 10 g. If *Enterobacteriaceae* were detected in any of these sample units, apart

from *Salmonella* spp., 30 sample units of 10 g had to be tested for *Cronobacter* spp. (CEC, 2005). According to the extended procedure of the manufacturer, 10 sample units of 10 g were drawn from the batch and tested for *Enterobacteriaceae* and *Cronobacter* spp.. The samples were found to be negative and the batch was released. Several months later, competent authorities found one package produced around 22:00 hrs to be positive for *Cronobacter* spp.. The product was then recalled immediately and the retrieved product was securely stored for further investigation. To assess the distribution of *Cronobacter* spp. in this batch, 415 MPN of 333 g were investigated; 266 by the manufacturer's laboratory (in the course of 2007-2008) and 149 by a university laboratory in 2009. For investigating the occurrence of clusters, portions of 67 g of several bags of powder remaining after MPN were stored in a desiccator with saturated lithium chloride at 20°C to maintain a water activity of 0.11.

2.1.2 Reference batch

The reference batch, produced in 2009, was tested for *Cronobacter* spp. (30 sample units of 10 g (CEC, 2007)) and accepted. However, the batch was not released to the market because an ingredient was under-dosed. Each package contained 600 g of powder (2 bags of 300 g). The batch included 495 packages divided over 5 pallets; 4 pallets with 120 packages (8 layers with 15 packages) and 1 pallet with 1 layer. In order to sample systematically, 2-3 packages were taken from each layer. In total, 93 packages (19% of the batch) were taken and from each package 333 g of powder was investigated to estimate the concentration of *Cronobacter* spp. therein.

2.2 Assessing the concentration of *Cronobacter* spp. in PIF

The concentration of *Cronobacter* spp. was estimated in samples of 333 g using the most probable number (MPN) technique (3 x 100 g, 3 x 10 g, and 3 x 1 g). The ISO/TS 22964 method (ISO 22964, 2006) was used by the manufacturer in the course of 2007-2008 and an extended version of the screening method as published by Iversen et al. (2008) was used by the university in 2009. Since the tested product was very rich in nutrients and many samples had to be investigated, in both methods buffered pepton water (BPW) was replaced by sterilised demineralised water in the pre-enrichment step. This change in buffering capacity did not lead to a drop in pH during the pre-enrichment (data not shown). From each bag, 3 samples of 100 g, 10 g, and 1 g were suspended in sterilised

demineralised water (1:10) and incubated overnight at 37°C.

As the manufacturer used the ISO/TS 22964 method, 0.1 mL of the enrichment was suspended in 10 mL modified lauryl sulphate tryptose broth (mLST) with 10 mg/L vancomycin and incubated for 24 hrs at 44°C. A loopful of the culture was streaked on *Enterobacter sakazakii* isolation agar (ESIA; AES Chemunex, France) plates and incubated for 24 hrs at 44°C.

As the university used the screening method (Iversen et al., 2008), 0.1 mL of the enrichment was suspended in 10 mL of *Cronobacter* screening broth (CSB, CM1121; Oxoid, Basingstoke, UK) with 10 mg/L vancomycin hydrochloride (Duchefa, Haarlem, The Netherlands) and incubated for 24 hrs at 42°C. The method was extended by streaking both positive and negative tubes on ESIA (*Cronobacter* Isolation Agar, CM1134; Oxoid, Basingstoke, UK) and Chromogenic *Cronobacter* Isolation agar (CCI, CM1122; Oxoid, Basingstoke, UK) plates to test for metabolism of α -glucopyranoside (Iversen et al., 2008). The plates were incubated for 24 hrs, the ESIA plates at 44°C and the CCI agar plates at 42°C. From each positive ESIA plate, a colony was cultured overnight in brain heart infusion (BHI) broth (Beckton Dickinson and Co., Le Point du Claix, France) and was stored frozen (-80°C) with 30% glycerol (87%, Fluka-Analytical GmbH, Buchs, Switzerland). Isolates were confirmed by 16S rRNA gene sequencing. The university confirmed from every positive package (two bags per package) one isolate (in total 37 isolates) and the manufacturer confirmed 7 isolates out of 105 positives bags. The resulting MPN codes were used to estimate the concentrations using an MPN table (ISO 7218, 2007).

2.3 Confirming the isolates by 16S rRNA gene sequencing

Each isolate was cultured overnight in BHI and the DNA was isolated using the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, USA) according to the instructions. The DNA extracts were directly used to amplify the 16S rRNA gene using forward and reverse primer as designed by Edwards et al. (1989). PCR was performed in 50 μ L reaction mixture (Fermentas, ThermoFisher Scientific, Waltham, Massachusetts, USA) containing 1 μ L (50 ng) of DNA, 1 x PCR buffer, 200 μ mol/L dNTPs, 0.04 μ U/L Taq polymerase, 1.5 mmol/L MgCl₂, and 0.125 μ mol/L of each primer. The thermal profile started at 94°C for 5 min, 35 amplification cycles with denaturation at 94°C for 20 s, primer annealing at 56°C for 20 s, and extension at 72°C for 1 min, final extension at 72°C for 30 min. The thermocycler GeneAmp® PCR System 9700 (Perkin Elmer Applied Biosystems,

Norwalk CT., USA) was used. The PCR products were sequenced with the same set of primers by GATC Biotech (Germany). The resulting sequences were assembled in SeqMan (Lasergene v5.08, Dnastar Inc.). The contigs were compared to similar sequences (Altschul et al., 1990) in the GenBank database at the National Center for Biotechnology Information (NCBI) (<http://blast.ncbi.nlm.nih.gov/>). The isolates were assumed to belong to a given species if the similarity between the query 16S rRNA sequence and the sequences in the databases was higher than 97% (Stackebrandt and Goebel, 1994).

2.4 Optimising the recovery of stressed *Cronobacter* spp. cells during direct plating

As injured *Cronobacter* spp. cells may require resuscitation, enumeration by plating might miss a considerable portion of viable, but injured cells. According to Gurtler and Beuchat (2005), the resuscitation of stressed *Cronobacter* spp. cells during spiral plating can be enhanced by adding sodium pyruvate to Trypton Soy Agar at a concentration of 0.1% (wt/vol) (TSAP). *Cronobacter sakazakii* cells, strain ATCC 29544, were spiked on PIF as described in Jongenburger et al. (2010), and were stored in this PIF for two years in a desiccator with saturated lithium chloride at 20°C. One gram of this powder was diluted in 9 mL of PPS at room temperature and after 1 hour, volumes of 1 mL were plated using the following combination of media and techniques: 1) Trypton Soy Agar spread plate (TSA; Oxoid, Basingstoke, UK), 2) CCI agar spread plate, 3) TSA pour plate with top layer of TSA, 4) TSAP pour plate with top layer of TSAP, 5) TSAP pour plate with top layer of TSAP with 1% vancomycin hydrochloride. A top layer was added to prevent the spread of disturbing flora like bacilli. The mean count of *Cronobacter* colonies per plate and the presence of disturbing flora were assessed. Four replicates were made.

2.5 Assessing the presence of single or locally clustered cells in PIF

In order to investigate the presence of clusters of cells, 28 bags were chosen both from those found with high levels and levels below the detection limit. The remaining 67 g of powder of these bags as well as 3 new bags containing 400 g of powder were investigated in detail. Samples of 1 gram from the remaining powder and samples of 2 gram from the new bags were diluted in 9 mL of PPS and 3 mL of the suspension was pour plated in TSAP and a top layer of TSAP. This -1 dilution was then stored at 10°C. All suspected colonies in the agar, presumptive *Cronobacter* spp., were picked and streaked onto Violet Red Bile Glucose Agar (VRBGA; CM0485; Oxoid, Basingstoke, UK) to detect *Enterobacteriaceae* and

on CCI agar plates. Both VRBGA and CCI need to be positive. For plates with numerous similar colonies, a minimum of 5 colonies were tested. If the plate counts on TSAP were higher than 300 colonies, the -1 dilution could be used to make higher dilutions. Since 3 mL of the -1 dilution was plated, the lower detection limit was 3.3 CFU/g for a sample size of 1 g, and 1.7 CFU/g for a sample size of 2 g. The upper detection limit depended on the dilution factor.

2.6 Presenting the sampling data

The experimental sampling data were displayed as the concentrations in the samples versus the filling time. The data were also displayed in a frequency histogram and as an empirical cumulative distribution function (ECDF). The maximum likelihood estimates (MLE) were estimated using the MLE function of MATLAB® 7.8.0, R2009a (The MathWorks™, Natick, Massachusetts).

2.7 Assessing the fraction positive samples in the recalled and reference batch

With each MPN of 333 g containing 9 samples (3 x 100 g, 3 x 10 g, and 3 x 1 g), the number of positive samples of 100, 10, 1, 300, 30, and 3g can be calculated from the MPN codes. The fraction of positive samples is the ratio between the number of positive samples and the total number of samples.

2.8 Assessing the probability that the sampling scheme includes one or more positive sample units $Pr(n_+ > 0)$ for different sampling schemes and sampling strategies

2.8.1 Random sampling

By randomly drawing a number of sample units (n) with a specific sample size from the data set, the probability that the sampling scheme includes one or more positive sample units $Pr_{\text{rand}}(n_+ > 0)$ can be calculated as follows:

$$Pr_{\text{rand}}(n_+ > 0) = 1 - (1 - s_+)^n \quad (1)$$

with n : number of sample units; n_+ : number of positive sample units; s_+ : fraction of positive samples of a specific sample size (Note: 'sample unit' is used when it is part of sampling scheme or sampling plan). Since the data set contained information on triplicate samples of 100, 10, and 1 g, it was also possible to assess fractions of positive samples for sample sizes of 300, 30, and 3 g. In addition, random sampling was simulated using

MATLAB® 7.8.0, R2009a (The MathWorks™, Natick, Massachusetts) and re-sampling the dataset (bootstrap, 10,000 times).

In every sampling simulation, n sample units were drawn and the total number of positive sample units was assessed. Based on the MPN code corresponding to each sample unit, the sample unit was either positive or negative (MPN code = 3: positive, MPN code = 0: negative, MPN code = 1: probability of 0.33 to be positive, MPN code 2: probability of 0.67 to be positive). $\Pr_{\text{rand}}(n_+ > 0)$ is the fraction of sampling simulations with at least one, positive sample unit. Using this method sample sizes of 2, 20 and 200 g were also evaluated.

2.8.2 Stratified random sampling

Stratified sampling is a systematic sampling strategy that draws a number of random sample units from every time interval (= stratum). By drawing stratified random sample units with a specific sample size (g), the probability, $\Pr_{\text{st.rand}}(n_+ > 0)$, that the sampling scheme includes one or more positive sample units, can be calculated as follows:

$$\Pr_{\text{st.rand}}(n_+ > 0) = 1 - \prod_{i=1}^L [(1 - s_{+i})^{n_{\text{int}}}] \quad (2)$$

with L : number of time intervals (strata), n_{int} : number of sample units in each interval (equal to n/L), s_{+i} : fraction of positive sample units of a specific sample size in the i^{th} stratum. The fractions of positive sample units are assessed in ten time intervals ($L=10$) of two hours.

6

3. Results

3.1 Optimising the recovery of stressed *Cronobacter* spp. cells during direct plating

Stressed *Cronobacter* cells were plated on different media to optimise the recovery: 1) TSA spread plate, 2) CCI agar spread plate, 3) TSA pour plate with top layer of TSA, 4) TSAP pour plate with top layer of TSAP, and 5) TSAP pour plate with top layer of TSAP with 1% vancomycin hydrochloride. The mean and standard deviation (between brackets) were 19.8 (2.1), 45.5 (1.7), 36.5 (2.5), 41 (5.9), and 36.8 (6.2) CFU/plate, respectively. Both CCI agar spread plate and TSAP pour plate with top layer of TSAP had a high mean count and less disturbing flora as compared to the other methods. For this reason, for ease of use as well as for economical reasons, TSAP pour plate with top layer of TSAP was chosen to investigate the presence of single or clustered cells.

3.2 Presenting concentrations of *Cronobacter* spp. in the course of the filling time

The distribution of *Cronobacter* spp. cells throughout the recalled and the reference batch was investigated by relating concentrations to the time that a bag was actually filled as indicated by the time stamp on the bag. This was believed to give more specific information about the microbial distribution than converting the data to a frequency distribution. With the microbial concentrations versus the filling time, it is also possible to compare different sampling strategies like random or stratified random sampling.

3.2.1 Recalled batch

Fig. 1a shows the distribution of concentrations *Cronobacter* spp. in the course of the filling time assessed by 415 MPNs of 333 g for the recalled batch. Fifty-eight percent of the MPNs had an MPN code of 0,0,0 and the concentration was thus below the detection limit of $-2.52 \log \text{CFU/g}$ (0.003 CFU/g). At four time intervals, between 05:00 and 06:00 hrs, around 09:30 hrs, between 14:00 and 15:00 hrs, and between 17:30 and 23:30 hrs concentrations above the lower detection limit were measured. The highest concentrations were measured between 14:00 and 15:00 hrs, with two samples having a concentration above the upper limit of $0.041 \log \text{CFU/g}$ (1.1 CFU/g) for an MPN of 333 g. One bag produced at 14:00 hrs, with a concentration estimated of 0.15 CFU/g based on an MPN of 333 g, was further investigated by assessing an MPN of 33.3 g. The concentration was determined as $0.66 \log \text{CFU/g}$ (4.6 CFU/g), which is indeed over the detection limit of $0.041 \log \text{CFU/g}$. The remaining powder of this bag was further investigated by plating the remaining contents of this bag in 1 g quantities.

Fig. 1b shows the distribution of concentrations of *Cronobacter* spp. in the recalled batch in the course of the filling time, which was assessed by plating 2290 samples of 1 gram taken from 31 bags with various levels of *Cronobacter* spp.. In 8 out of the 2290 samples, plate counts were found above the detection limit of $0.52 \log \text{CFU/g}$. These samples originated from bags filled in the time interval 14:00 and 15:00 hrs, with the highest MPN concentrations (see Fig. 1a). Table 1 shows the filling time of each bag, the number of samples of 1 g in each bag, the number of positive samples in each bag, and the concentration per positive sample of 1 g. The mean concentration in each bag based on the plate counts and the MPN concentrations assessed previously are presented in the last two columns. The concentration in samples of 1 g from the bags produced at 13:58, 14:00, and 14:04 hrs, varied between 3.3 and 560 CFU/g and two concentrations

peaked at 123 and 560 CFU/g. The cluster of 560 cells originated from the bag produced at 14:00 hrs with a concentration estimated of 0.15 CFU/g based on an MPN of 333 g and 4.6 CFU/g based on an MPN of 33.3 g.

The ESIA and CCI plates showed similar results. The 7 isolates tested by the manufacturer and the 37 isolates tested by the university were confirmed by 16S rRNA gene sequencing to be *Cronobacter sakazakii*.

Table 1. The concentration *Cronobacter* spp. (CFU/g) as estimated in 31 bags of the recalled batch by direct plating of 0.3 g in TSAP. The detection limit was 3.3 CFU/g

Filling time bag (hrs)	Number of samples of 1 g	Number of positive samples	Concentration per sample (CFU/g)	Concentration in bag (CFU/g)	
				based on plate counts	based on MPN of 333 g
3:52	63	0	0	<3.3	<0.003
3:52	61	0	0	<3.3	<0.003
4:07	70	0	0	<3.3	<0.003
4:07	54	0	0	<3.3	<0.003
4:08	59	0	0	<3.3	<0.003
4:11	62	0	0	<3.3	0.0036
5:29	59	0	0	<3.3	0.043
5:29	59	0	0	<3.3	0.023
5:30	67	0	0	<3.3	0.0092
5:30	64	0	0	<3.3	0.023
5:30	63	0	0	<3.3	0.043
5:30	65	0	0	<3.3	0.043
9:28	68	0	0	<3.3	<0.003
9:28	66	0	0	<3.3	<0.003
10:56	65	0	0	<3.3	<0.003
10:56	64	0	0	<3.3	<0.003
13:58	63	0	0	<3.3	0.093
13:58	65	3	3.3	0.8	0.46
			10.0		
			40.0		
14:00	26 ^a	1	560.0	21.5	0.15 (4.6 ^b)
14:00	69	3	10.0	2.1	>1.1
			13.3		
			123.3		
14:04	66	0	0	<3.3	0.24
14:04	68	1	20.0	0.3	0.15
14:07	61	0	0	<3.3	0.043
14:07	63	0	0	<3.3	0.15
14:58	68	0	0	<3.3	0.21
14:58	63	0	0	<3.3	0.043
17:21	62	0	0	<3.3	0.0036
17:21	65	0	0	<3.3	<0.003
4:11	177 ^c	0	0	<1.7	₋ ^d
5:29	184 ^c	0	0	<1.7	₋ ^d
5:29	181 ^c	0	0	<1.7	₋ ^d
Total	2290	8	779.9		

^a Only 26 samples were remaining, since an MPN of 33.3 g had been taken.

^b Concentration based on MPN of 33.3 g.

^c Samples of 2 g were drawn from a new bag.

^d MPN of 333 g of this new bag was not determined.

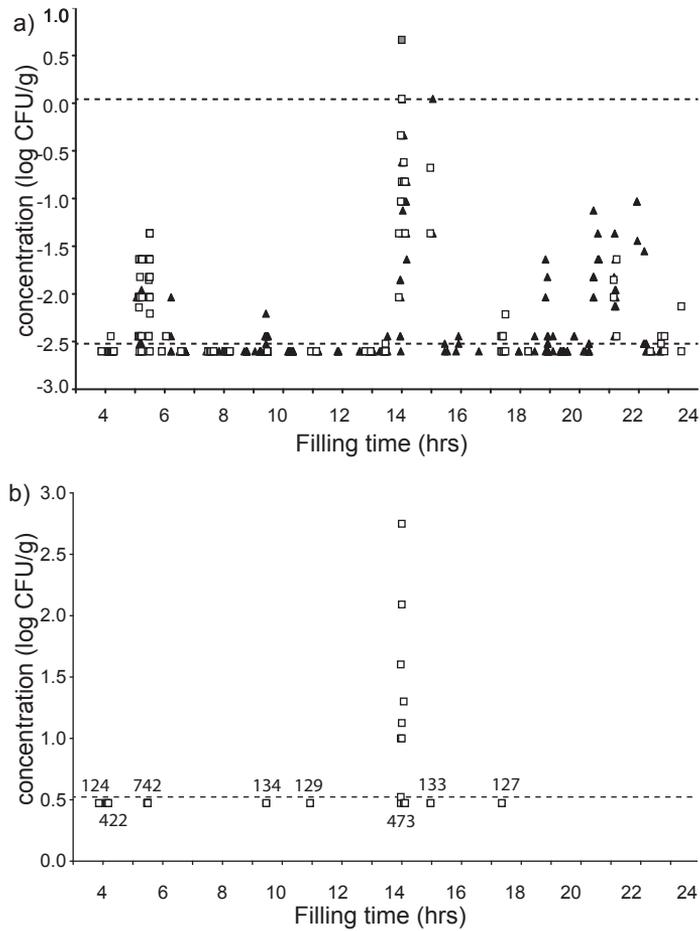


Fig. 1. Overview of the concentration of *Cronobacter* spp. in a recalled batch of PIF. (a) The concentration of *Cronobacter* spp. (log CFU/g) in the course of the filling time (hrs) estimated by taking 415 samples of 333 g. The data are assessed by (▲) the manufacturer's lab and (□) a university lab. The dotted horizontal lines indicate the lower (-2.52 log CFU/g) and upper (0.041 log CFU/g) detection limits of an MPN of 333 g. From the package with a bag with a concentration above the detection limit of the MPN of 333 g, (■) the concentration in the corresponding bag was estimated by taking also an MPN of 33.3 g. (b) *Cronobacter* spp. concentration (log CFU/g) in the course of the filling time (hrs) determined by plate counting 2290 samples of 1 g of PIF. The dotted horizontal line indicates the lower detection limit (0.52 log CFU/g). Labels indicate the number of negative samples.

3.2.2 Reference batch

Fig. 2 shows the distribution of *Cronobacter* spp. in course of the filling time assessed by 93 MPNs of 333 g for the reference batch. All but one sample had an MPN code of 0,0,0, thus below the detection limit of $-2.52 \log \text{CFU/g}$. The one positive sample had an MPN code of 1,0,0 and the concentration was estimated to be $-2.44 \log \text{CFU/g}$, which is 0.0036CFU/g or 2 CFU per package of 600 g. The isolate of the positive sample was confirmed by 16S rRNA gene sequencing to be *Cronobacter sakazakii*. The positive package was thoroughly investigated by plating the residual content separately in samples of 1 g. In none of the 267 samples were colonies detected on the TSAP plates.

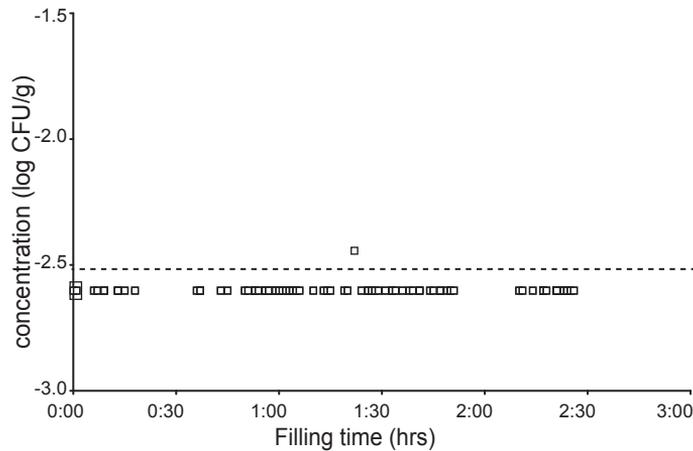


Fig. 2. Overview of the concentration of *Cronobacter* spp. (log CFU/g) in the reference batch of PIF in the course of the filling time (hrs). The dotted horizontal line indicates the lower detection limit ($-2.52 \log \text{CFU/g}$).

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3.3 Presenting concentrations of *Cronobacter* spp. as a histogram and an ECDF

Fig. 3 displays the concentrations of the recalled batch (415 samples) as a relative frequency histogram. Fig. 4 displays the empirical cumulative distribution function (ECDF) of the concentrations found in the recalled and the reference batch.

Since the concentrations are left-censored (Lorimer and Kiermeier, 2007), the mean and standard variation can be estimated with the method of maximum likelihood estimation (MLE), assuming an underlying Normal distribution for the \log_{10} concentrations. Using this method, the mean and the standard variation were estimated to be -2.76 and $1.10 \log \text{CFU/g}$, respectively (Fig. 4, black curve).

Using only the positive samples (42% of the samples), the mean calculated was $-1.78 \log \text{CFU/g}$, whilst the standard deviation was $0.68 \log \text{CFU/g}$ (Fig. 4, dotted curve).

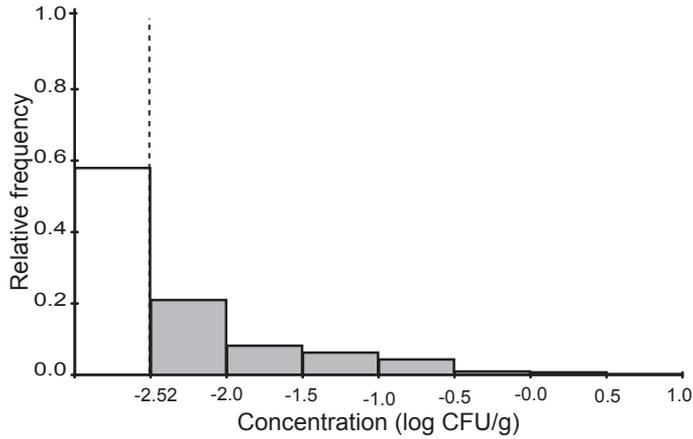


Fig. 3. Histogram of frequencies of *Cronobacter* spp. in the recalled batch (415 samples). The dotted line indicates the detection limit of $-2.52 \log \text{CFU/g}$. The first bar (white) indicates the samples below the detection limit and the grey bars indicate the positive samples.

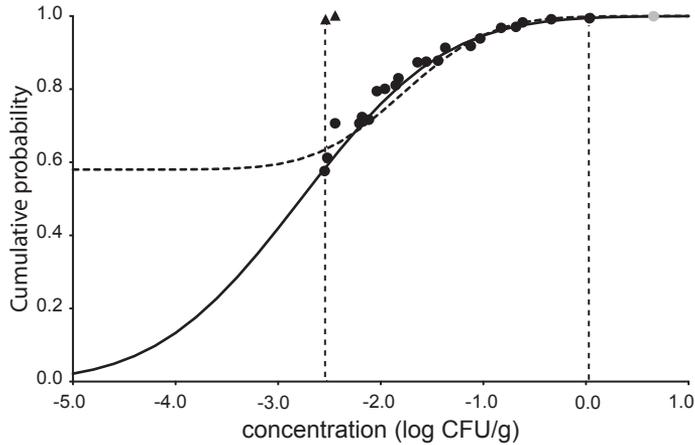


Fig. 4. Empirical cumulative distribution functions of the concentrations of *Cronobacter* spp. ($\log \text{CFU/g}$) in MPNs of 333 g drawn from the (\blacktriangle) reference and (\bullet, \bullet) recalled batch; (\bullet) concentration estimates by taking an MPN of 33.3 g. The black curve represents the Normal distribution with a mean of $-2.76 \log \text{CFU/g}$ and a standard deviation of $1.10 \log \text{CFU/g}$, estimated with Maximum Likelihood Estimation. The dotted curve represents a Normal distribution with a mean $-1.78 \log \text{CFU/g}$ and standard deviation $0.68 \log \text{CFU/g}$ of the positive samples ($y = 0.42 \times \text{Normal}(-1.78, 0.68) + 0.58$). The dotted vertical lines indicate the lower ($-2.52 \log \text{CFU/g}$) and the upper ($0.041 \log \text{CFU/g}$) detection limits.

3.4 Fraction of positive samples in recalled and reference batch

Table 2 shows the fraction of positive samples of 1, 10, 100, 3, 30, and 300 g drawn from the recalled and reference batch over the entire batch.

Table 2. Fractions of positive samples in the recalled and the reference batch as determined by enriching 1245 samples (3 x 415 for recalled batch) and 279 samples (93 x 3 for reference batch) of 1, 10, and 100 g

Sample Size (g)	Fraction positive samples (s_+)	
	Recalled batch	Reference batch
1	0.0297	0
10	0.109	0
100	0.271	0.0036
3	0.075	0
30	0.202	0
300	0.378	0.0108

Dividing the samples originating from the recalled batch in ten time intervals of two hours, Fig. 5a shows that the samples enumerated by MPN were more or less equally distributed over the course of the day.

To enable stratified random sampling from the data set of the recalled batch, the fractions of positive samples were assessed in each of the ten time intervals of two hours as shown in Fig. 5b. In the time intervals 06:00 -12:00 hrs and 18:00 – 20:00 hrs, the positive fractions of 1 g samples were 0. The interval 14:00 -16:00 hrs contained the largest fractions of positive samples.

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3.5 The probability that the sampling scheme includes one or more positive sample units by random ($Pr_{\text{rand}}(n_+ > 0)$) or stratified random ($Pr_{\text{st.rand}}(n_+ > 0)$) sampling

3.5.1 Random sampling

Table 3 shows $Pr_{\text{rand}}(n_+ > 0)$, the calculated probability that the sampling scheme includes one or more positive sample units, by drawing random sample units from the recalled and reference batch. Eq. 1 and the fractions of positive samples from Table 2 were used to calculate $Pr_{\text{rand}}(n_+ > 0)$. $Pr_{\text{rand}}(n_+ > 0)$ was also simulated by re-sampling the data sets by bootstrapping. The calculated and simulated values correspond well with each other.

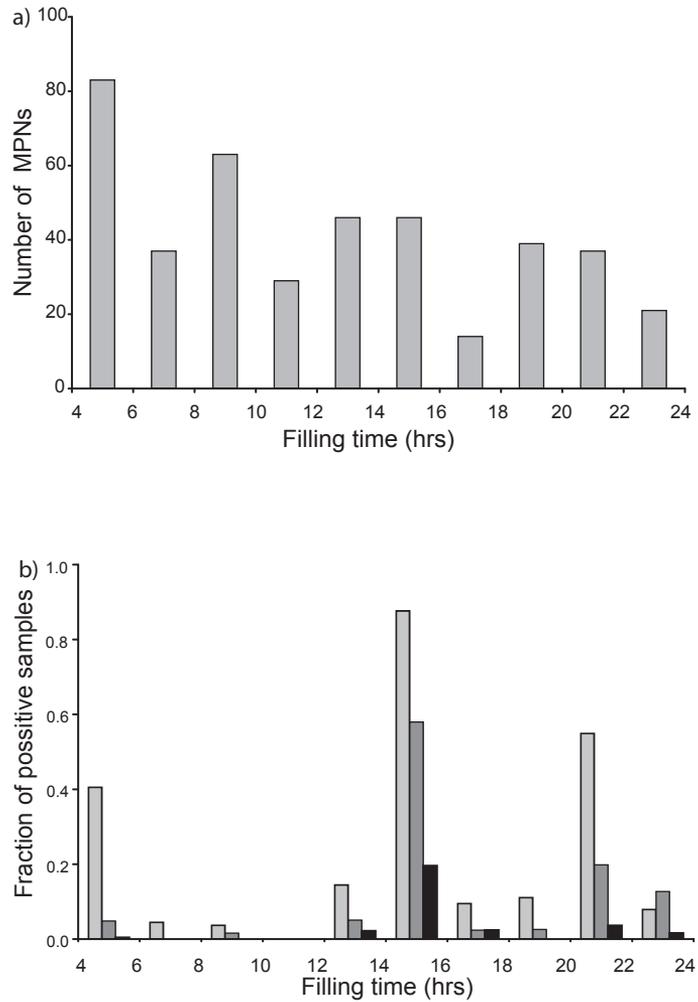


Fig. 5. Overview of the time intervals evaluated for the recalled batch
 (a) The number of MPN's of 333 g in ten time intervals of two hours. In total 415 MPNs were assessed.
 (b) The fraction of positive samples in each of the ten time intervals of two hours. The sample sizes are 100 g (light grey), 10 g (dark grey), and 1 g (black).

Table 3 shows that keeping the total sample weight constant at 300 g and increasing the number of sample units from 1 to 300, increases $Pr_{rand}(n_+>0)$ from 0.3783 for 1 sample to 0.9999 for 300 sample units. The probability that the contamination would have been detected by drawing 10 sample units of 10 g was 0.6855. However, when 30 sample units of 10 g would have been drawn, the probability to detect the contamination was 0.9689. Increasing the sample number from 30 to 60, further increases $Pr_{rand}(n_+>0)$ from 0.9689 to 0.9990. The sampling schemes of 60 sample units of 20 and 30 g simulate the more stringent sampling for *Salmonella*. In the case of 60 sample units of 30 g each, the detection probability is virtually 1.00.

Table 3. The probability ($Pr_{rand}(n_+>0)$) that the entire sampling scheme contains one or more positive sample units by sampling randomly with various numbers of sample units and sample sizes from the recalled and the reference batch. $Pr_{rand}(n_+>0)$ was calculated with Eq. 1 using the fractions of positive samples in Table 2 and simulated (bootstrap, 10,000 times)

Total sample weight (g)	Number of sample units	Sample size (g)	Recalled batch		Reference batch	
			$Pr_{rand}(n_+>0)$ calculated	$Pr_{rand}(n_+>0)$ simulated	$Pr_{rand}(n_+>0)$ calculated	$Pr_{rand}(n_+>0)$ simulated
300	1	300	0.3783	0.3810	0.0108	0.0107
300	3	100	0.6121	0.6115	0.0107	0.0107
300	10	30	0.8958	0.8958	._a	._a
300	30	10	0.9689	0.9687	._a	._a
300	100	3	0.9996	0.9996	._a	._a
300	300	1	0.9999	0.9998	._a	._a
100	10	10	0.6855	0.6887	._a	._a
600	30	20	._b	0.9946	._a	._a
900	30	30	0.9989	0.9991	._a	._a
600	60	10	0.9990	0.9991	._a	._a
1200	60	20	._b	1.0000	._a	._a
1800	60	30	1.0000	1.0000	._a	._a

^a No positive sample available with this sample size

^b Cannot be determined using this method



3.5.2 Stratified random sampling

From each time interval, as shown in Fig. 5, 1, 2, or 3 samples of 1 and 10 g were drawn to calculate sampling schemes of 10, 20, or 30 samples of either 1 or 10 g.

$\Pr_{st.rand}(n_+>0)$ was calculated by Eq. 2 using the fractions of positive samples per time interval as in Fig. 5b. Table 4 shows the probability of detection by sampling both random ($\Pr_{rand}(n_+>0)$) and stratified random ($\Pr_{st.rand}(n_+>0)$). The differences between the two sampling strategies are also displayed. In all cases $\Pr_{st.rand}(n_+>0)$ was larger than $\Pr_{rand}(n_+>0)$ and the differences between $\Pr_{st.rand}(n_+>0)$ and $\Pr_{rand}(n_+>0)$ varied between 0.008 and 0.066.

Table 4. The probability that the entire sampling scheme contains one or more positive samples by sampling randomly ($\Pr_{rand}(n_+>0)$) or stratified randomly ($\Pr_{st.rand}(n_+>0)$) from the recalled batch. The stratification was ten periods of two hours as displayed in Fig. 5a and 5b. $\Pr_{rand}(n_+>0)$ was calculated with Eq. 1 using the fractions positive samples in Table 2. $\Pr_{st.rand}(n_+>0)$ was calculated with Eq. 2 using the fractions positive samples as displayed in Fig. 5b

Number of samples	Sample size (g)	$\Pr_{rand}(n_+>0)$	$\Pr_{st.rand}(n_+>0)$	$\Pr_{st.rand}(n_+>0) - \Pr_{rand}(n_+>0)$
		random	stratified random	
10	1	0.2604	0.2742	0.014
10	10	0.6855	0.7512	0.066
20	1	0.4530	0.4733	0.020
20	10	0.9011	0.9381	0.037
30	1	0.5955	0.6177	0.022
30	10	0.9689	0.9846	0.016
100	1	0.9510	0.9595	0.008

4. Discussion and conclusions

4.1 'Batch-scale' heterogeneity: distribution of concentrations of *Cronobacter* spp. in PIF

In the recalled batch the concentration of *Cronobacter* spp. in the course of the filling time showed parts having no detectable contamination and parts with concentrations varying between -2.52 and 2.75 log CFU/g. Evidently, *Cronobacter* spp. cells were distributed heterogeneously within this batch; 58% of the samples were below the detection limit. Using the positive samples only, the mean and standard deviation were respectively -1.78 log CFU/g and 0.68 log CFU/g. Using all data and the maximum likelihood approach, assuming an underlying Normal distribution of the \log_{10} concentrations, the mean was

-2.76 log CFU/g and the standard deviation 1.10 log CFU/g. The Normal distribution with these estimated parameters clearly fitted the data better than the fit based on the positive samples ($y = 0.42 \times \text{Normal}(-1.78, 0.68) + 0.58$). The MLE of the standard deviation (1.10 log CFU/g) for this largely contaminated batch is considerably larger than the default standard deviation of 0.8 log CFU/g chosen by the ICMSF (2002).

Since the *Cronobacter* spp. levels in the course of the production time clearly showed a heterogeneous distribution, we assumed that in the packages below the detection limit no *Cronobacter* spp. cells were present and the concentration in these samples can be set to 0 CFU/g. Based on the 415 MPN samples, the arithmetic mean concentration in the recalled batch was 3.75×10^{-2} CFU/g.

In the reference batch, 19% of the total batch was investigated by systematically sampling 93 samples out of 495 packages. This resulted in 99% of the samples found to be below the detection limit. Based on only 1 sample found positive, with a concentration of -2.44 log CFU/g (0.0036 CFU/g), the average concentration in the reference batch was estimated as 3.87×10^{-5} CFU/g (-4.41 log CFU/g or 40 CFU per 1000 kg). This is a factor 1000 lower than the concentration in the recalled batch.

These results can be compared with the concentrations of *Cronobacter* spp. in PIF estimated based on a range of industry data in the recent FAO/WHO risk assessment (FAO/WHO, 2006); in this risk assessment the mean concentration was -3.84 log CFU/g and the standard deviation was 0.696 log CFU/g. This would result in an arithmetic mean of 5.21×10^{-4} CFU/g. Compared to the average concentration estimate from the FAO/WHO data, that in the recalled batch was 72 times higher and in the reference batch 13 times lower.

4.2 'Local-scale' heterogeneity: single cells or clusters of *Cronobacter* spp. in PIF

The presence of clusters of *Cronobacter* spp. cells occurred with a low frequency of 8 out of 2290 samples varying between 3 and 560 cells per cluster. The two largest clusters contained 123 and 560 cells. It was striking that these two clusters, which together contained 88% of all the cells, originated from 2 bags only. Finding such clusters is like looking for a needle in a haystack. The levels of cells are not as high as expected by speculating that a droplet of contaminated water could have fallen into the final product, Nevertheless, when these clusters end up in one or a limited number of servings of an individual consumer, they may significantly impact public health.



The heterogeneity of cells was expected to influence concentrations assessed by the MPN technique, since this enrichment method can not distinguish between single and cluster of cells. Comparing the concentrations based on MPN and plating, however, resulted in comparable concentrations. Only in case of the largest cluster, the MPN concentration was $2 \log_{10}$ units lower than the concentration based on the plating. Additionally, comparing the concentrations based on MPN and plating also showed no apparent underestimation by the plate counting.

4.3 Comparing the sampling performance of different sampling schemes

The probability that at least one of the 30 random samples of 10 g (CEC, 2007) drawn from the recalled batch contained a positive sample ($\Pr_{\text{rand}}(n_+ > 0)$) is 0.9689. This is a big improvement compared to $\Pr_{\text{rand}}(n_+ > 0)$ of 0.6855 when 10 samples of 10 g are drawn as prescribed by the former guidelines to test for *Enterobacteriaceae* (CEC, 2005). If, instead of taking 30 random sample units of 10 g per day, during 3 shifts in each shift 10 packages in a row were taken for testing (in this case 10 sample units of 10 g is comparable to 1 sample unit of 100 g), $\Pr_{\text{rand}}(n_+ > 0)$ appears to be also much lower, at 0.6121 (3 sample units of 100 g). On the other hand, $\Pr_{\text{rand}}(n_+ > 0)$ increases substantially when many smaller sample units would have been taken (100 sample units of 3 g or 300 sample units of 1 g). A practical way to collect the necessary large number of small sample units per batch is by auto-sampling. A disadvantage of auto-sampling is that it does not test the packed product after the filling stage, but samples from the line. Auto-sampling could be deployed best just before filling in combination with a thorough control and monitoring of the filling area, since any contamination arising at the filling stage would not be covered by the auto-sampler.

Assuming that the recalled batch (Normal(-2.76, 1.10)), had been ten times less contaminated, this would result in an hypothetical less-contaminated batch (Normal(-3.76, 1.10)). This hypothetical batch would be in the same order of magnitude as the concentration estimated by the FAO/WHO (2006) of Normal(-3.84, 0.696). In the case that the contamination is 10 times less, we can assume that the fractions of positive samples (s_+) of 1, 10, and 100 g would have been ten times smaller. If 30 random sample units of 10 g would have been taken, $\Pr_{\text{rand}}(n_+ > 0)$ would be 0.2807 and 300 sample units of 1 g would result in $\Pr_{\text{rand}}(n_+ > 0)$ of 0.5905. These probabilities are clearly smaller than the probabilities calculated for the recalled batch. Nevertheless, this hypothetical example shows that many smaller sample units increase the performance of the sampling plan.

4.4 Comparing random or stratified random sampling

The comparison of random and stratified random sampling using real sampling data showed that stratified random sampling improved the probability that the sampling scheme does include one or more positive sample units. Thus, the choice of sampling strategy is important when contamination is heterogeneously distributed or clustered in local spots. This in line with the finding of other authors (Battilani et al., 2006; Habraken et al., 1986; Jongenburger et al., 2011a; Kiermeier et al., 2011; Lin et al., 1979; Rivas Casado et al., 2009).

4.5 Conclusions

This report quantifies in detail the microbial distribution of *Cronobacter* spp. levels during the filling stage of a batch of PIF on an industrial scale. The thorough investigation of the recalled batch showed that *Cronobacter* spp. cells were heterogeneously distributed throughout the batch, sections of which contained no detectable contamination and sections of which were found to be contaminated at levels between -2.52 and 2.75 log CFU/g. Clusters of cells occurred sporadically in 8 out of 2290 samples of 1 g. The two largest clusters contained 123 (2.10 log CFU/g) and 560 (2.75 log CFU/g) cells.

Using random sampling, the probability to detect *Cronobacter* spp. by taking 30 sample units of 10 g from the recalled batch was determined to be 0.9689, which is much higher than the probability of 0.6855 when taking 10 sample units of 10 g. Compared to random sampling, stratified random sampling improved the probability to detect the heterogeneous contamination. This sampling strategy is therefore recommended, also because it will detect systematic contaminations, caused, for example, by a contaminated filler head. Taking more and smaller sample units and keeping the total sampling weight constant, clearly improved the performance of the sampling plans. Therefore to improve the probability of detection, auto-sampling employed just before filling will be a practical way to collect the necessary large number of small sample units per batch.

Acknowledgements

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Chapter 7

Modelling homogeneous and heterogeneous microbial contaminations within a powdered food product

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Abstract

The actual physical distribution of microorganisms within a batch of food influences quantification of microorganisms in the batch, resulting from sampling and enumeration by microbiological tests. Quantification may be most accurate for batches in which microorganisms are distributed homogeneously. However, when the distribution is non-homogeneous, quantification may result in an under-, or overestimation. In the case of pathogens being non-homogeneously distributed, this heterogeneity will impact on public health. Enumeration data are commonly modelled by the Lognormal distribution. Although the Lognormal distribution can model heterogeneity, it does not allow for complete absence of microorganisms. Studies that validate the appropriateness of using Lognormal or other statistical distributions are scarce. This study systematically investigated laboratory and industrial scale batches of powdered infant formula, modelled the enumeration data using a range of statistical distributions, and assessed the appropriateness of individual models. For laboratory scale experiments, batches of milk powder were contaminated by distributing similar numbers of cells of Cronobacter sakazakii either homogeneously throughout a batch of milk powder or by distributing the cells in a localised part of the batch. Each batch was then systematically sampled and the distribution determined by enumerating the samples. By also enumerating the remainder of the batch, a balance could be made of the total number of microorganisms added and of the number retrieved from a batch. Discrete, as well as continuous statistical distributions, were fitted to enumeration data and the parameters estimated by Maximum Likelihood. The data were fitted both as censored and uncensored data. Enumeration data obtained for an industrial batch of powdered infant formula were investigated in this way as well. It was found that Normal, Poisson and Zero-Inflated Poisson distributions fitted the data sets very poorly. In case of homogeneous contamination, there was not a notable difference between the ability of Negative Binomial, Poisson-Lognormal, Weibull, Gamma, and Lognormal distributions to model the data. Overall, either the Negative Binomial distribution or the Poisson-Lognormal distribution fitted the data best in the 10 batches studied, especially when part of a data set contained zeros and/or the numbers were low. The Negative Binomial fitted the laboratory batches best and the Poisson-Lognormal fitted the industrial batch best.

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1. Introduction

Sampling and testing are used to generate enumeration or presence/absence data to evaluate the microbiological status of batches of food product. The data are relevant for a range of food safety management activities, such as verifying operational control in a food manufacturing operation or checking compliance with governmental food standards in an import situation. Where data on contamination by microbial pathogens are concerned, the data may be directly related to a potential impact on the health of consumers, i.e. to the safety of the food batch. Especially regarding food safety, enumeration data are often modelled and used in other food safety management activities such as microbiological risk assessments (MRAs) or establishing food safety standards such as microbiological criteria (MC). It is important to validate that the enumeration data obtained reflect the status of the investigated food batches well and that models derived on the basis of these data are appropriately accurate for their purpose. Both aspects will be influenced by the actual physical distribution of microorganisms in a particular food product.

The physical distribution of microorganisms within a batch of food will influence quantification of the number of microorganisms in the batch obtained from sampling and microbiological testing. In principle, a batch of food is produced and handled under uniform conditions (CEC, 2005) and microorganisms are supposed to be homogeneously distributed throughout the batch. In practice, however, microorganisms may actually be heterogeneously distributed due to, for instance, the structural heterogeneity of the food matrix, incomplete mixing, incidental (post-processing) contamination, and/or localised microbial growth. As a consequence, individual sections of a batch do contain microorganisms, whilst in other sections, microorganisms are absent. Unlike a homogeneously contaminated batch, samples taken from a heterogeneously contaminated batch are likely to show both high variability and zero counts.

These extremes in physical microbial distribution will influence the quantification results for a batch and therefore the interpretation of the safety status of the food (Buchanan, 2000). The observations and frequency distributions of the microbial distribution have to reflect the very different physical distributions of contaminants occurring in practice and can be modelled with different statistical distributions. Key is then to ensure that the statistical distribution chosen to model the frequency distribution provides for an optimal fit. Five criteria have previously been proposed to assess the suitability of statistical distributions to model frequency distributions from a number

of possible common spatial distributions of microorganisms (ILSI, 2010): the model 1) should be non-negative, 2) should allow for zero values, 3) should be discrete, 4) should reduce to (or approximate) the Poisson distribution and 5) should approximate the Lognormal distribution at high numbers of microorganisms. The ability to model localised contamination (or 'clustering') was investigated in the ILSI study. The authors concluded, that determining suitable statistical distributions would require fitting actual observations rather than relying on theoretical considerations.

Fitting a frequency distribution to observed data, two properties of the frequency distribution impact the fitting procedure, namely whether the distribution is either continuous or discrete and whether it is able to model zeros. Observed plate counts and observed MPN enrichments are discrete variables. The concentration in a sample (CFU/g), however, is deduced from an actual observation, i.e. estimated from the observed plate count of the sample or from the observed enrichments of the MPN samples. Concentrations are continuous variables, as they can take any value. Although MPN/g values can only take a finite number of values (discrete values), confidence limits are broad and overlapping between adjacent MPN values and can be treated as continuous estimates. Concentration values are suitable for fitting with continuous frequency distributions. The Normal, Lognormal, Gamma, and Weibull distribution are continuous distributions, whereas Poisson, Zero-Inflated Poisson, Negative Binomial, and the Poisson-Lognormal distribution are discrete distributions. The Lognormal, Gamma, and Weibull distribution are not able to model values of zero.

In order to investigate how well these statistical distributions fit actual observations, the current study was conducted, using powdered infant formula (PIF) as a model food in which both homogeneous and heterogeneous contamination could be simulated and using *Cronobacter sakazakii* as the contaminant, as it is a relevant opportunistic pathogen that can occur in this food product at low levels. Enumeration data were systematically generated on laboratory scale for batches with either homogeneous or localised contamination. By enumerating the remainder of each batch, a balance could be made for the total number of microorganisms added to a batch and the number retrieved from the batch on the basis of sampling. The various statistical distributions were assessed for their ability to fit the actual observations. In addition to the data from laboratory batches, enumeration data from an earlier industrial scale batch of contaminated PIF (Jongenburger et al., 2011b) were used and fitted to various

statistical distributions.

The current study provides the necessary practical investigations to support the conclusions of the earlier theoretical study. Thus, it provides a better basis to choose models in food safety management activities for different microbial distributions that may be encountered.

2. Methods

2.1 Studying laboratory scale batches

Nine different batches were investigated at laboratory scale, six of which were characterised by the contaminant being homogeneously distributed and three with the contaminant being heterogeneously distributed.

2.1.1 Preparing spiked milk powder

PIF obtained from local retail was artificially contaminated with *Cronobacter sakazakii* strain ATCC 29544 as described in Jongenburger et al. (2010). In short, cells were cultured in brain heart infusion (BHI) broth (Beckton Dickinson and Co., Le Point du Claix, France), washed and sprayed over a flat layer of PIF. The contaminated powder was stored in a desiccator with saturated lithium chloride (VWR international, Fontenay sous Bois, France) at 20°C to maintain a water activity of 0.11. After 3 days, the contaminated powder contained 10^6 - 10^7 CFU/g of *C. sakazakii* cells (data not shown).

2.1.2 Enumerating C. sakazakii in spiked powder

The exact concentration of the contaminant was measured on the day a spiked powder preparation was used. The concentration of *C. sakazakii* cells in the spiked powder was measured in 5 samples of 1 g by suspending in 9 mL peptone physiological salt (PPS; 8.5 g NaCl/L and 1 g peptone/L; Oxoid, Basingstoke, England), well mixing, and plating appropriate dilutions in duplicate onto Trypton Soy Agar plates (TSA; Oxoid, Basingstoke, England) with a spiral plater (Eddy Jet; IUL Instruments, I.K.S., Leerdam, The Netherlands). After overnight incubation at 37°C, the number of colonies per plate was counted manually. The average concentration was calculated from the plate count data obtained from all 5 samples analysed in duplicate.

2.1.3 Preparing batches differing in the distribution of spiked powder

Small amounts (0.3 - 5 g) of the spiked powder were distributed within batches of 1 kg PIF in two ways: a) homogeneously by thorough mechanical mixing or by thorough manual mixing, and b) heterogeneously by localising the contaminant in part of the batch and not mixing. Thorough mechanical mixing was achieved by mixing the spiked powder together with a 1 kg batch of PIF for 1 hour using a 3-dimensional powder mixer (Willy A. Bachofen AG Maschinenfabrik, Basel, Switzerland) at a rotational speed of 56 rpm. Thorough manual mixing was achieved by adding the small amount of spiked powder to the same amount of PIF, manually mixing for 1 minute, and repeating the one to one mixing until 1 kg contaminated milk powder was obtained. After either thorough mechanical or manual mixing, the homogenous 1 kg batch of contaminated milk powder was poured into a stainless steel box (60 cm x 30 cm x 10 cm). Localised contamination was achieved by placing the spiked powder on top of the 1 kg PIF and pouring all powder through a funnel at constant rate, while the funnel was slowly moved over a stainless steel box with the same dimensions. This method was also evaluated by replacing the spiked powder by blue chalk in order to visualise the distribution of the local contamination.

2.1.4 Enumerating *C. sakazakii* for laboratory scale batches

A plasticised grid (Gamma, Leusden, The Netherlands) was placed on top of the layer of contaminated milk powder to visually divide the box into 72 sections of 5 x 5 cm allowing for systematic sampling of the powder. Two samples of 0.5 g were drawn from each section, resulting in 144 samples representing 72 g of product removed from the batch. Each sample was then suspended in 4.5 mL PPS and 0.1 mL of the suspension was plated onto TSA in duplicate. The number of *C. sakazakii* in the powder remaining after removing 72 g of the batch was also enumerated. Eight portions of 100 g and one portion of approximately 125 g were each diluted 10-fold in PPS, dissolved, well mixed, and 0.1 mL of the suspensions was plated onto TSA in duplicate. After overnight incubation of the TSA plates at 37°C, the number of colonies per plate was counted manually.

2.1.5 Determining the balance of contamination added (N_{in}) and retrieved (N_{out})

The number of microorganisms added to every batch (N_{in} (CFU)) was the concentration of microorganisms in the spiked powder multiplied by the weight of the added spiked powder. The total number of microorganisms retrieved by sampling the complete batch

(N_{out} (CFU)) was:

$$N_{out} = N_{samples} + N_{remainder} = \sum_{i=1}^{144} (c_i \cdot w_i) + \sum_{k=1}^9 (C_k \cdot W_k) \quad (1)$$

with $N_{samples}$ being the number of microorganisms retrieved by sampling, $N_{remainder}$ being the number of microorganisms in the remainder of the batch, c_i : concentration of cells (CFU/g) in a sample, w_i : weight of a sample (0.5 g), C_k : concentration of cells (CFU/g) in the remainder parts of the batch, W_k : weight of the remainder parts (either 100 g or approximately 125 g). For each batch the ratio C_{out}/C_{in} (%) and $C_{samples}/C_{in}$ (%) was calculated, where c_{in} (CFU/g) is $N_{in}/1000$ g, c_{out} (CFU/g) is $N_{out}/1000$ g, and $c_{samples}$ (CFU/g) is $N_{samples}/72$ g.

2.2 Studying the enumeration data of an industrial batch of PIF

To investigate the fit of statistical distributions to enumeration data, also use was made of enumeration data of *Cronobacter* spp., which occurred in an full scale industrial batch of PIF withdrawn from the market. Details regarding this industrial batch can be found in Jongenburger et al. (2011b). In short, the batch was produced in January 2007 at a size of approximately 22,000 kg; according to the extended procedure of the manufacturer compared to the legislation prevailing at that time (CEC, 2005), the batch could be released onto the market; however, competent authorities found one package to be positive for *Cronobacter* spp., whereupon the remainder of the batch was recalled and the retrieved product securely stored for further investigation; to assess the distribution of *Cronobacter* spp., 415 samples of 333 g as well as 1 sample of 33.3 g were investigated and the concentration of *Cronobacter* spp. in each sample estimated using the Most Probable Number (MPN) technique. Furthermore, 2290 samples of 1 g PIF were enumerated by plating; 8 samples were positive. In the current study, the concentrations in these 8 clusters were added to the data obtained by MPN.

2.3 Presenting the enumeration data

Enumeration data for individual batches were displayed as an empirical cumulative distribution function (ECDF). Enumeration data of the laboratory scale batches were also presented as a function of the sampling location using MATLAB® 7.8.0, R2009a (The MathWorksTM, Natick, Massachusetts, USA).

2.4 Fitting statistical distributions to the data

The Normal, Lognormal, Gamma, Weibull, Poisson, Zero-Inflated Poisson, Negative Binomial, and the Poisson-Lognormal distributions were fitted to the data. The fitting procedure depended on two properties of the fitted distribution: 1) whether a distribution was continuous or discrete and 2) whether a distribution was able to model values of zero. The fits of various continuous and discrete distributions to the observed data were compared. Since discrete distributions described counts or numbers rather than concentrations, plate counts were used instead of concentrations deduced from plate counts, and the estimated MPN concentrations were converted to counts by rounding them to integers. All distributions were fitted to the observed plate counts of the laboratory scale batches and to the estimated MPN concentrations of the industrial scale batch of PIF. The fit procedure was performed in two ways, i.e. treating the data as uncensored data and as censored data.

Treating the original discrete plate counts obtained for 0.02 g samples as uncensored data means that a plate count of zero was considered to be equivalent to zero cells in a sample of 0.02 g; this assumed that the microbiological method to detect viable cells is perfect. The fit to uncensored data was evaluated for the Normal, Lognormal, Gamma, Weibull, Poisson, Zero-Inflated Poisson, Negative Binomial, and the Poisson-Lognormal distribution. These distributions were fitted to the data with statistical software R (R Development Core Team, 2011) by maximum likelihood using the 'fitdist' function from the R package 'MASS' (Venables and Ripley, 2002). The inability of distributions to model zeros was taken into account for the Lognormal, Gamma, and Weibull distribution by replacing zero values by $\frac{L}{\sqrt{2}}$ (Hornung and Reed, 1990), in which L is the limit of detection, i.e. 1 CFU/0.02 g for the plate counts and 3 CFU/kg for the MPNs.

Treating the plate counts of 0.02 g samples as censored data means that a count value of zero was considered as an unknown value equal or larger than 0 and smaller than 1 CFU/0.02 g, i.e. ≥ 0 and < 50 CFU/g. The continuous distributions: Normal, Lognormal, Gamma, and Weibull were evaluated for the fit to censored data by fitting them with the statistical software R (R Development Core Team, 2011) optimising maximum likelihood using the 'fitdistcens' function from the R package 'fitdistrplus' (Delignette-Muller et al., 2010), which has been described in Pouillot and Delignette-Muller (2010).

2.5 Comparing the fits of statistical distributions to the observed microbial distributions

In order to judge the suitability of the various statistical distributions to reflect the microbial frequency distributions observed, visual observation was used next to determining the goodness of fit. There are several common test statistics like the Kolmogorov-Smirnov (K-S) test and the Anderson-Darling (A-D) test (Stephens, 1974) to investigate whether a sample comes from a population with a specific distribution. Although these two goodness-of-fit tests are able to rank the fitted distributions, they are restricted to continuous distributions. To assess the goodness-of-fit, the Pearson's Chi-square (χ^2) test was used, because it can be applied to any univariate distribution for which a cumulative distribution function can be calculated (Snedecor and Cochran, 1989).

The χ^2 -test can be calculated after dividing the data into k bins:

$$\chi^2 = \sum_{i=1}^k \left[\frac{(O_i - E_i)^2}{E_i} \right] \quad (2)$$

for which O_i is the observed frequency for bin i and E_i the expected frequency for bin i . The hypothesis that the sample comes from a specific distribution is rejected if

$$\chi^2 > \chi^2_{(\alpha, k-1-c)} \quad (3)$$

where significance level α is chosen to be 0.05, $(k - 1 - c)$ is the number of degrees of freedom, k is the number of bins, and c is the number of estimated parameters. The χ^2 -test is able to rank the fits of the accepted distributions.

In the fitting process the (Log) likelihood values were maximised; the likelihood of the data given a particular distribution. These Log-likelihood values (LL) were compared for the various types of statistical distributions for each individual data set.

3. Results

3.1 Balance of contamination added to and retrieved from each laboratory batch

Nine laboratory scale batches of powdered infant formula were contaminated with *C. sakazakii*, 6 had been homogeneously contaminated with the microorganisms (batches 1 – 6) and 3 had been heterogeneously contaminated (batches 7 – 9). Three different spiked powders were used to produce these 9 batches.

Table 1 shows the balance of the number of cells added (N_{in}) to the individual 1 kg batches with the spiked powder, the number of cells retrieved after drawing 144 samples ($N_{samples}$), the number of cells remaining in the batch after sampling ($N_{remainder}$), and the total number of cells retrieved from the complete batch (N_{out}) derived from eq. 1. Table 1 also provides information on weight of spiked powder added (w_{added}) and the mixing characteristics of the various laboratory batches, indicating which homogeneously contaminated batches were mechanically or manually mixed.

Table 1. Balance of the number of cells (N_{in}) added to 1 kg batch of milk powder, the number of cells ($N_{samples}$) retrieved by drawing 144 samples, the remaining number of cells in the batch ($N_{remainder}$) and the number of cells (N_{out}) retrieved after sampling the complete batch (Eq. 1). The contaminations were distributed homogeneously (H) throughout the batch or localised (L) in a section of the batch. The $C_{samples}/C_{in}$ and C_{out}/C_{in} indicate the ratio of the concentration estimated by drawing 144 samples ($c_{samples}$) and the concentration estimated by sampling the complete batch (c_{out}) compared to c_{in}

Batch number	w_{added} ^a (g)	distributed	Balance				$C_{samples}/C_{in}$ (%)	C_{out}/C_{in} (%)
			N_{in} (CFU)	$N_{samples}$ (CFU)	$N_{remainder}$ (CFU)	N_{out} (CFU)		
1	0.30 ^b	H ^e	5.78×10^5	3.89×10^4	8.36×10^5	8.75×10^5	94	151
2	1 ^b	H ^e	1.93×10^5	1.16×10^5	1.58×10^5	1.69×10^5	84	88
3	2 ^c	H ^e	3.24×10^5	1.92×10^5	2.05×10^5	2.24×10^5	82	69
4	3 ^c	H ^e	4.87×10^5	3.57×10^5	3.87×10^5	4.23×10^5	102	87
5	1 ^d	H ^f	1.26×10^7	6.81×10^5	1.21×10^7	1.28×10^7	75	101
6	2 ^d	H ^f	2.53×10^7	2.25×10^6	2.72×10^7	2.95×10^7	123	117
7	3 ^d	L	3.79×10^7	1.32×10^6	3.12×10^7	3.25×10^7	48	86
8	3 ^d	L	3.79×10^7	1.93×10^6	3.25×10^7	3.44×10^7	71	91
9	5 ^d	L	6.32×10^7	2.75×10^6	5.16×10^7	5.43×10^7	60	86

^a w_{added} = weight of spiked powder (g) added to 1 kg powder

^b $\mu_{sp} = 1.93 \times 10^6$ CFU/g, $\sigma_{sp} = 3.38 \times 10^5$ CFU/g based on 5 samples of 1 g

^c $\mu_{sp} = 1.62 \times 10^6$ CFU/g, $\sigma_{sp} = 2.58 \times 10^5$ CFU/g based on 5 samples of 1 g

^d $\mu_{sp} = 1.26 \times 10^7$ CFU/g, $\sigma_{sp} = 2.04 \times 10^6$ CFU/g based on 5 samples of 1 g

^e Mechanical mixing

^f Manual mixing

On average, 97% of the cells that had been added to the batches could be accounted for based on sampling the complete batch and calculating the mean of the ratios

C_{out}/C_{in} ($\mu_{Cout/Cin} = 97\%$; $\sigma_{Cout/Cin} = 23\%$). The extent to which the microorganisms in the 144 samples from all 72 sections of a batch were representative for the mean concentration

in a batch could be derived from the ratios $C_{samples}/C_{in}$ for relevant batches. A mean ratio of 93% ($\sigma_{C_{samples}/C_{in}} = 18\%$) was found for the 6 batches with homogeneously distributed contamination, whereas a mean ratio of 60% ($\sigma_{C_{samples}/C_{in}} = 11\%$) was found for the three batches with localised contamination. A *t*-test showed a significant difference ($p = 0.006$) between the 6 observations of homogeneous batches (1 – 6) and the 3 observations of heterogeneous batches (7 – 9), indicating that the estimated concentration based on samples is lower in heterogeneous batches than in homogenous batches.

3.2 Laboratory scale and industry scale enumeration data

Enumeration data were obtained for 9 laboratory scale batches of PIF contaminated with *Cronobacter sakazakii* and for one industrial scale batch recalled from the market due to the presence of *Cronobacter* spp. in this batch.

Fig. 1 uses a 3-dimensional surface plot to illustrate the distribution profiles observed in selected laboratory scale batches. The graphs were based on the mean concentration of the duplicate samples drawn from each section in the box.

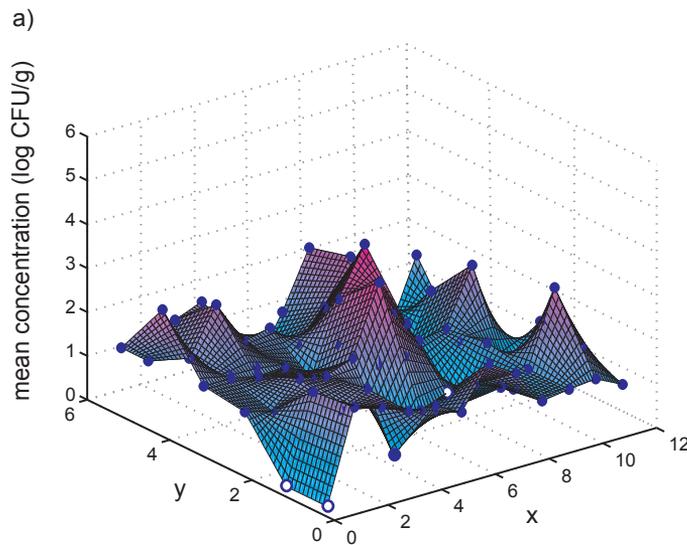


Fig. 1a. Surface plot of the mean concentration of *C. sakazakii*, based on duplicate enumeration of milk powder samples versus location in the box (*x* and *y* axes), representing different sections in the batch. Different amounts of spiked powder were added to batches of 1 kg of milk powder: (a) batch 1, illustrating homogeneous contamination achieved by mechanical mixing 0.3 g of spiked powder; the open dots represent mean concentrations below the detection limit of 1.7 log CFU/g.

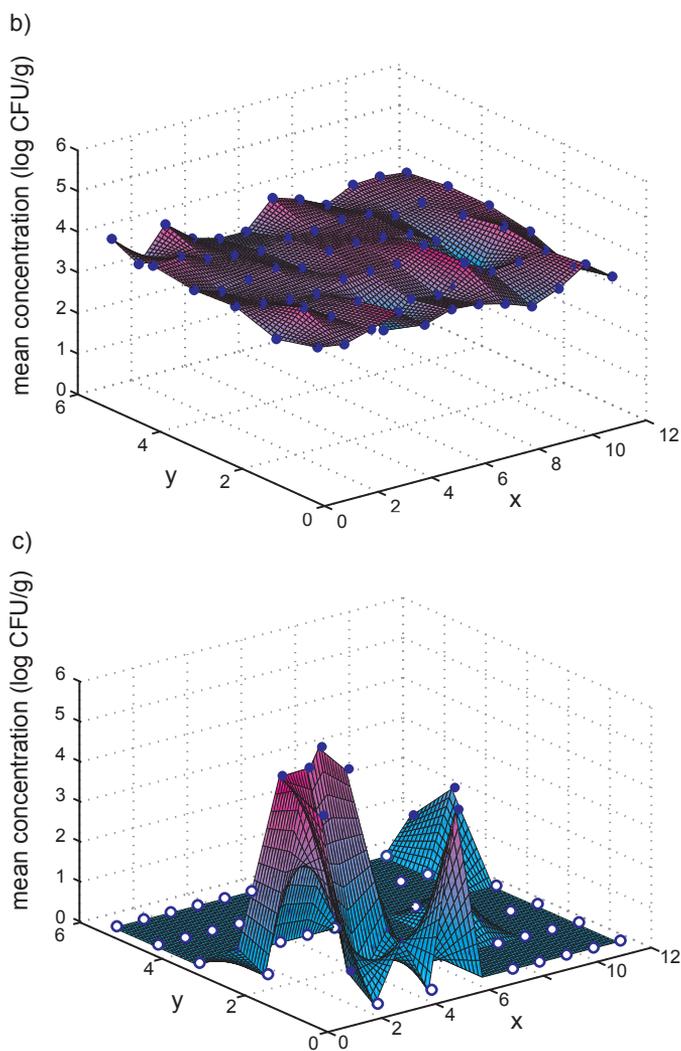


Fig. 1b,c. Surface plot of the mean concentration of *C. sakazakii*, based on duplicate enumeration of milk powder samples versus location in the box (x and y axes), representing different sections in the batch. Different amounts of spiked powder were added to batches of 1 kg of milk powder: (b) batch 4, homogeneous contamination achieved by mechanical mixing 3 g of spiked powder, and (c) batch 7, heterogeneous contamination by pouring 3 g of spiked powder instead of mixing; the open dots represent mean concentrations below the detection limit of 1.7 log CFU/g. Graph 1a and 1b are adapted from Jongenburger et al. (2010).

For illustration, three of the nine batches were selected, namely two with homogeneously distributed contamination (Fig. 1a, batch 1 with 0.3 g spiked powder mixed in; Fig. 1b, batch 4 with 3 g spiked powder mixed in) and one with a localised contamination (Fig. 1c, batch 7 with 3 g spiked powder not mixed in). The open dots represent mean concentration below the detection limit of 1.7 log CFU/g. Although a lot of the samples from the homogeneous distribution with the lowest contamination (Fig. 1a) were found to be below the detection limit, the mean concentration in each section was in most cases above the detection limit. Comparing the two mechanically mixed batches to illustrate a homogeneous distribution, the results for the lowest contamination level showed an erratic plot (Fig. 1a), indicating a large variability in concentrations observed across batch sections. For the higher contamination level, the plot was much more smooth indicating limited variability around the mean concentration data (Fig. 1b). For the batch chosen to illustrate heterogeneous distribution of contamination, the plot clearly showed isolated sections to be characterised by very high counts, whereas the majority of sections showed contamination below the detection limit (Fig. 1c).

Fig. 2 shows the empirical cumulative distribution functions (ECDF) of the samples from the various laboratory scale batches. The ECDF curve of the thoroughly mixed batch with 3 g spiked powder/kg batch shows the steepest slope, indicating the least variation in concentration across samples (Fig. 2a). In the batches with the localised contamination, the ECDF plots show a gentle slope indicating large variability in concentration (Fig. 2b); the concentration in more than half of the samples was below the detection limit, 85% and 67% for the batches with 3 g spiked powder/kg batch and 51% for those with 5 g spiked powder/kg batch, respectively, whereas the measurable concentrations varied between 1.7 log CFU/g and 5.90 log CFU/g (Fig. 2b). The ECDF curve of the thoroughly mixed batch with 0.3 g spiked powder/kg (batch 1) was far less steep than the other ECDF curves of thoroughly mixed batches (Fig. 2a) and thus quite comparable to the ECDF plots obtained with data from the batches with localised contaminations; 50% of samples in batch 1 were found to be below the detection limit, with measurable concentrations varying between 1.7 log CFU/g and 4.0 log CFU/g.

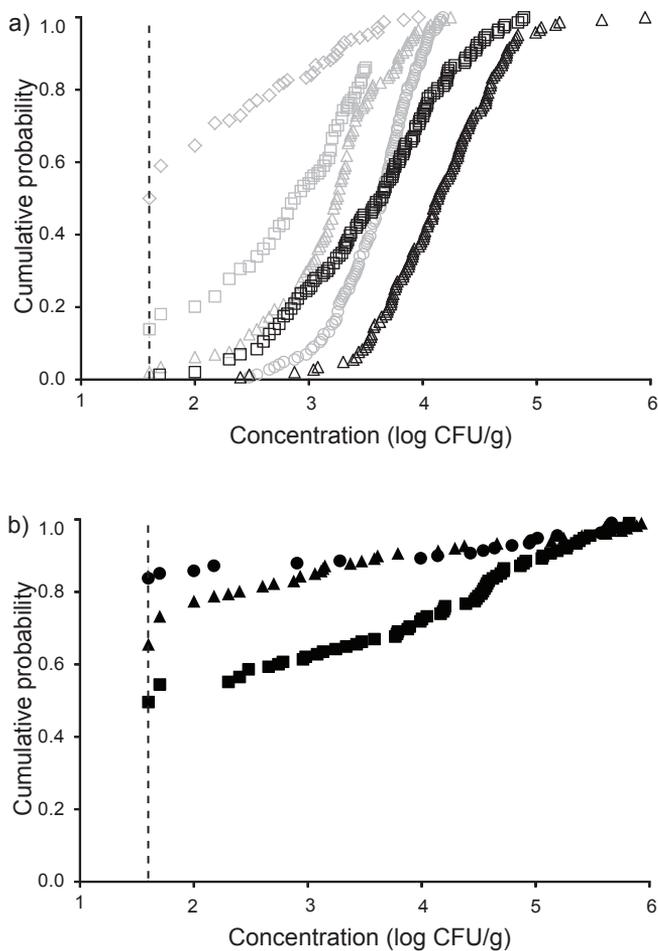


Fig. 2. Empirical cumulative distribution functions of the concentrations of *C. sakazakii* (log CFU/g) in 144 samples of 0.5 g drawn from the mechanically mixed (open grey symbols), manually mixed (open black symbols) and not mixed (black closed symbols) batches of 1 kg milk powder. The dotted vertical line indicates the detection limit of 1.7 log CFU/g.

a) (◇) 0.30 g, (□) 1 g, (Δ) 2 g, (○) 3 g of spiked powder mechanically mixed; (□) 1 g, (Δ) 2 g of spiked powder manually mixed;

b) (●, ▲) 3 g and (■) 5 g of the spiked powder not mixed.



Fig. 3 displays two ECDF curves of the concentration values derived from MPNs of 333 g and plate counts of 1 g from the industrial batch.

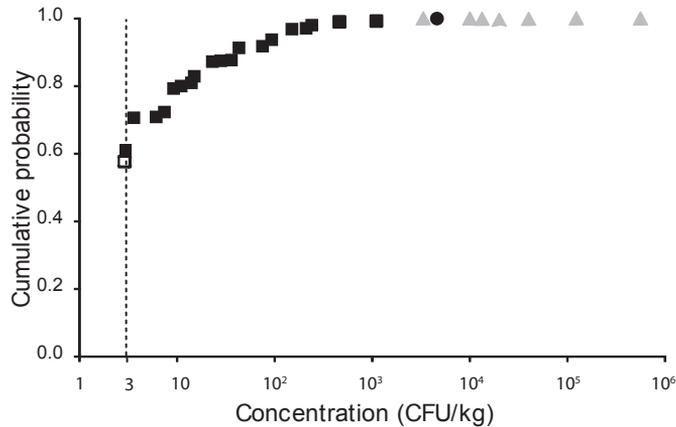


Fig. 3. Two empirical cumulative distribution functions of *Cronobacter* spp. concentration in the industrial batch of PIF. The first ECDF is based on (■) 415 MPNs of 333 g, and (●) 1 MPN of 33.3 g. The second ECDF is based on (▲) 2290 plate counts of 1 g. The dotted vertical line indicate the lower detection limit (3 CFU/kg) of an MPN of 333 g. The open square indicates the number of samples (MPNs) below this detection limit. The graph is adapted from Jongenburger et al. (2011b).

In the ECDF curve of the 416 MPNs, 57.7% of the samples were found to be below the lower detection limit of 3 CFU/kg. In the ECDF curve of the 2290 plate counts, 99.7% of the samples were below the detection limit and only 8 samples (0.3%) were positive. In order to fit the statistical distributions to the data, these two ECDF curves have been combined to one ECDF curve by adding the 8 positive samples to the 416 MPNs. In the combined ECDF curve 56.6% of the samples were below the detection limit. In this way, a more realistic picture is obtained of all possible concentrations that may occur in the batch. The gentle slope of the plot again indicates a large variability between the concentrations determined for individual samples.

3.3 Results of fitting enumeration data to statistical distributions

The Normal, Lognormal, Gamma, Weibull, Poisson, Zero-Inflated Poisson, Negative Binomial, and the Poisson-Lognormal distribution were fitted to the sampling data obtained for the laboratory scale batches and the industrial batch as uncensored data (Table 2), whereas the Normal, Lognormal, Gamma, Weibull were fitted to the sampling

data as censored data (Table 3). The Normal, Poisson, and Zero-Inflated Poisson distribution fitted the data of all batches very poorly (not shown).

Table 2. The χ^2 test statistics (a), Log-likelihood values (b) of the Lognormal, Gamma, Weibull, Negative Binomial (NB), and Poisson-Lognormal (PL) distributions (continuous (C) and discrete (D) distributions) fitted to enumeration data of 9 laboratory scale batches of milk powder with different contaminations and to enumeration data of the industrial batch of PIF. The bold χ^2 test statistics are smaller than $\chi^2_{(0.05,k-1-c)}$ and the bold LL-values are maxima. The data were treated as uncensored data

Table 2a. uncensored data

		χ^2 test statistics										
Batch		1	2	3	4	5	6	7	8	9	industrial (clusters excluded)	industrial (clusters included)
Bins (k)		9	9	9	9	10	10	10	10	10	9	10
Distribution	$\chi^2_{(0.05,k-1-c)}$ ^b	12.6	12.6	12.6	12.6	14.1	14.1	14.1	14.1	14.1	12.6	14.1
Lognormal	C	47.3	21.3	20.4	16.9	11.5	6.2	>100	>100	50.8	>100	>100
Gamma	C	41.8	10.1	8.5	9.2	13.2	_a	_a	_a	_a	_a	_a
Weibull	C	35.3	10.6	8.1	4.9	13.1	15.6	>100	>100	32.3	>100	>100
NB	D	9.2	5.4	7.4	5.4	19.3	19.6	12.3	27.9	9.7	83.1	>100
PL	D	19.4	17.0	9.4	13.3	13.3	5.0	_a	7.7	40.7	41.0	50.8

^a No convergence

^b $\chi^2_{(0.05,k-1-c)}$ with $k-1-c$ is the number of degrees of freedom, k is the number of bins, c is the number of parameters

Table 2b. uncensored data

		Log-likelihood values										
Batch		1	2	3	4	5	6	7	8	9	industrial (clusters excluded)	industrial (clusters included)
Distribution												
Lognormal	C	-391	-636	-723	-799	-875	-1041	-414	-457	-720	-1415	-1619
Gamma	C	-436	-632	-717	-792	-883	_a	_a	_a	_a	_a	_a
Weibull	C	-419	-631	-717	-790	-875	-1057	-467	-506	-739	-1544	-1802
NB	D	-370	-629	-718	-791	-879	-1065	-237	-363	-631	-1133	-1342
PL	D	-376	-643	-722	-798	-876	-1041	_a	-358	-649	-1093	-1272

^a No convergence



Table 3. The χ^2 test statistics (a), Log-likelihood values (b) of the continuous distributions: Lognormal, Gamma, and Weibull fitted to enumeration data of 9 laboratory scale batches of milk powder with different contaminations and to enumeration data of the industrial batch of PIF. The bold χ^2 test statistics are smaller than $\chi^2_{(0.05,k-1-c)}$ and the bold LL-values are maxima. The data were treated as censored data

Table 3a. censored data

Distribution	Batch	χ^2 test statistics										industrial (clusters excluded)	industrial (clusters included)
		1	2	3	4	5	6	7	8	9			
	Bins (<i>k</i>)	9	9	9	9	10	10	10	10	10	9	10	
	$\chi^2_{(0.05,k-1-c)}$ ^b	12.6	12.6	12.6	12.6	14.1	14.1	14.1	14.1	14.1	12.6	14.1	
Lognormal		23.7	23.0	20.1	16.9	11.3	6.2	16.3	12.4	37.1	16.3	27.3	
Gamma		25.2	8.9	8.2	5.5	16.1	.. ^a	.. ^a	.. ^a	.. ^a	63.7	.. ^a	
Weibull		21.6	10.3	8.0	4.9	12.9	15.8	.. ^a	16.6	24.0	19.3	51.4	

^a No convergence

^b $\chi^2_{(0.05,k-1-c)}$ with *k*-1-*c* is the number of degrees of freedom, *k* is the number of bins, *c* is the number of parameters

Table 3b. censored data

Distribution	Batch	Log-likelihood values										industrial (clusters excluded)	industrial (clusters included)
		1	2	3	4	5	6	7	8	9			
Lognormal		-364	-639	-725	-799	-876	-1041	-242	-352	-643	-1051	-1196	
Gamma		-363	-624	-717	-791	-878	.. ^a	.. ^a	.. ^a	.. ^a	-1090	.. ^a	
Weibull		-362	-626	-717	-790	-875	-1057	.. ^a	-354	-637	-1058	-1219	

^a No convergence

The fits of the selected statistical distributions to the experimental data were first compared visually. To illustrate the typical results observed, Fig. 4 and 5 show cumulative probability plots of selected statistical distributions, namely Negative Binomial, Poisson-Lognormal and Lognormal, fitted to the data. In Fig. 4, the plots have been fitted to the plate count data of two laboratory scale batches: batch 2, with homogeneously distributed contamination (Fig. 4a), and batch 9 with localised contamination (Fig. 4b). In Figure 5, the plots have been fitted to the data of the industrial batch: 416 measurements (Fig. 5a), and 424 measurements: 416 MPNs plus the 8 clusters (Fig. 5b). In each figure, panel 'I' is for uncensored data and 'II' for censored data.

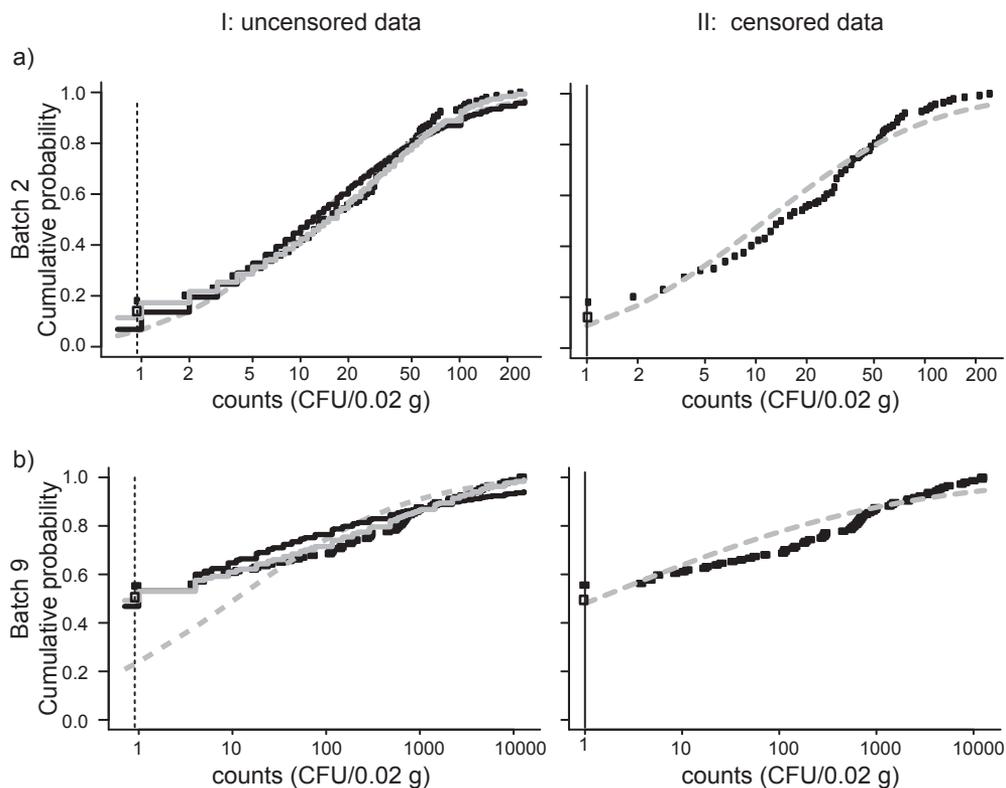


Fig. 4. Fit of the Lognormal (dotted grey curve), the Negative Binomial (solid grey curve), and the Poisson-Lognormal (solid black curve) distributions to enumeration data from laboratory scale batches: a) batch 2: homogeneous contamination, b) batch 9: heterogeneous contamination.

The fits in panel 'I' and panel 'II' are based on uncensored data and censored data, respectively. In panel I, the dotted vertical line indicates the detection limit of 0 CFU/0.02 g. In panel II, the vertical line indicates 1 CFU/0.02 g below which the data are left censored. The open square indicates the percentage of samples below the detection limit.

For uncensored data in the case of sampling data from a batch with homogenous contamination (Fig. 4a, panel 'I'), it was observed that the discrete distributions such as Negative Binomial and Poisson-Lognormal fitted better at low counts than their continuous equivalent, i.e. the Lognormal. At intermediate and high counts, the Negative Binomial distribution performed better than the Poisson-Lognormal distribution. Since only 14% of the data points were censored data and the rest actual observations, the graph for censored data (Fig. 4a, panel 'II') showed a similar result. For uncensored data

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from batches with localised contamination (Fig. 4b, panel 'I'), the Negative Binomial and Poisson-Lognormal distributions fitted better than the Lognormal distribution. Censoring the data, Fig. 4b (panel 'II') improved the fit by the Lognormal distribution as compared to the uncensored data.

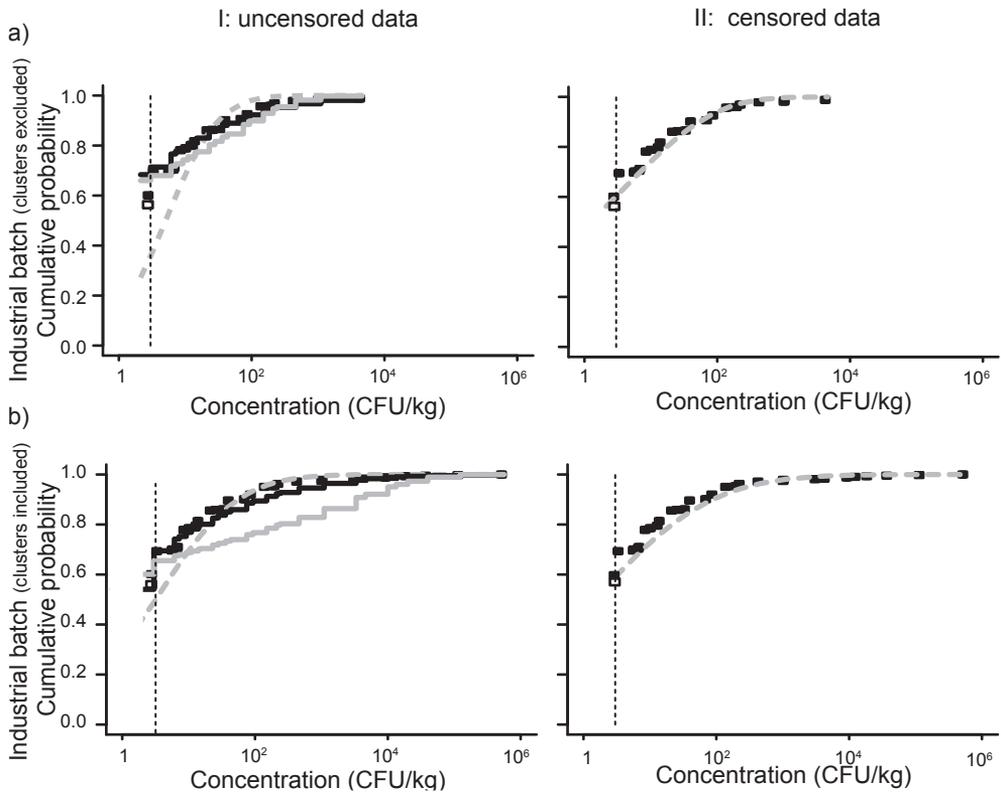


Fig. 5. Fit of the Lognormal (dotted grey curve), the Negative Binomial (solid grey curve), and the Poisson-Lognormal (solid black curve) distributions to enumeration data from an industrial scale batch of PIF: a) industrial batch with clusters excluded, b) industrial batch with 8 clusters included. The fits in panel 'I' and panel 'II' are based on uncensored data and censored data, respectively. The dotted vertical line indicates the detection limit of 3 CFU/kg. The open square indicates the percentage of samples (MPNs) below the detection limit.

For the industrial batch with clusters excluded (Fig. 5a, panel 'I') and with 8 clusters included (Fig. 5b, panel 'I'), the Poisson-Lognormal distribution performed better than the Negative Binomial and Lognormal distributions.

Fig. 5b, panel 'I', which includes the 8 high numbers, resulted in a profound better fit of the Poisson-Lognormal distribution as compared to the Negative Binomial distribution. Censoring the data (panel 'II'), the Lognormal distribution fitted better as compared to the Poisson-Lognormal distribution in Panel 'I'.

Besides visual observations of the fits, the ability of the continuous and discrete distributions to fit the data obtained for the laboratory scale batches and the industrial batch were compared using the Pearson's Chi-square (χ^2) test and on the basis of Log-likelihood (LL) values. The fits were ranked for each specific data set. Table 2 and Table 3 show the χ^2 and the LL values for the uncensored data and for the censored data, respectively; χ^2 and LL values roughly put the statistical distributions in the same order per data set. In all those cases that batches were characterised by having all counts larger than zero (batch 2-6), the χ^2 for the uncensored and censored data were comparable and also LL values for the uncensored and censored data were comparable. In 6 out of 9 laboratory batches, the χ^2 value of the Negative Binomial was smaller than $\chi^2_{(0.05, k-1-c)}$. The χ^2 values in the industrial batch with clusters excluded were larger than $\chi^2_{(0.05, k-1-c)}$; the high values are caused by the first bin (59.3 and 22.1 for the Poisson-Lognormal and the Negative Binomial respectively). Nevertheless, the χ^2 value for the Poisson-Lognormal was smaller than the χ^2 value of the Negative Binomial. The LL values also showed that the Poisson-Lognormal distribution fitted the uncensored enumeration data best, which is in line with the plots in Fig. 5a. The Lognormal and Weibull distributions fitted the censored data best.

Table 4 shows the estimated parameters of the fits to various statistical distributions for the industrial batch (416 MPNs and 416 MPNs plus 8 clusters). In case of the Lognormal distribution, currently widely used to model enumeration data, the meanlog_e estimate was 0.546 CFU/kg and the sdlog_e was 2.51 CFU/kg for the industrial batch with 416 MPNs. As it is more common to use Normal distribution of the \log_{10} concentrations (\log CFU/g), these estimates can be converted to a mean of -2.76 \log_{10} CFU/g and a sd. of 1.09 \log_{10} CFU/g. The Poisson-Lognormal fitted in both cases (excluding and including clusters) the industrial data best. For the industrial data with clusters excluded, the sdlog_e of the Poisson-Lognormal distribution was 3.67 CFU/kg and the sdlog_e was higher, 4.46 CFU/kg, when the clusters were included.

Table 4. The parameter estimate and error between brackets of the Lognormal, Gamma, Weibull, Negative Binomial (NB), and Poisson-Lognormal (PL) distributions fitted to enumeration data of the industrial batch of PIF (with clusters excluded and with clusters included) as uncensored and censored data (CFU/kg)

Distribution	Parameters	Batch with clusters excluded		Batch with clusters included	
		Uncensored	Censored	Uncensored	Censored
Lognormal	Meanlog _e	1.62 (0.07)	0.546 (0.167)	1.79 (0.09)	0.37 (0.21)
	Sdlog _e	1.43 (0.05)	2.506 (0.15)	1.85 (0.06)	3.20 (0.19)
Gamma	shape	._ ^a	0.120 (0.01)	._ ^a	
	rate	._ ^a	0.003 (5.0x10 ⁻⁴)	._ ^a	
Weibull	shape	0.512 (0.016)	0.318 (0.017)	0.334 (0.0095)	0.22 (0.011)
	scale	11.5 (1.17)	3.869 (0.728)	17.9 (2.77)	3.477 (0.92)
NB	mu	37.5 (6.12)	._ ^b	1.88x10 ³ (415)	._ ^b
	size	0.091 (0.008)	._ ^b	0.00482 (0.0038)	._ ^b
PL	Meanlog _e	-0.932 (0.253)	._ ^b	-1.03 (0.30)	._ ^b
	Sdlog _e	3.67 (0.228)	._ ^b	4.46 (0.27)	._ ^b

^a No convergence

^b Not determined

4. Discussion

To follow-up from the conclusions of a theoretical study conducted preciously (ILSI, 2010), the current practical study investigated how well laboratory and full scale enumeration results reflect the microbial status of certain food batches and which statistical distributions best fit the microbial frequency distributions. Nine different batches of PIF were contaminated with different levels and distributions of *C. sakazakii* to simulate either homogeneous or heterogeneous distributions of microbial contamination. The enumeration data of laboratory scale batches and one industrial batch of PIF were then modelled with a range of continuous and discrete statistical distributions.

4.1 Comparing the enumeration results for various laboratory batches

The laboratory scale batches were thoroughly investigated by taking duplicate samples systematically across 72 sections of each batch. Comparison of the enumeration results of the homogeneous batches showed that the results were most consistent for batches with a relatively high level of contaminant, whereas a low level of contaminant

considerably increased the variability in concentration determined. For batches with a heterogeneous contamination, large variations in concentrations were noted. In practice, a limited number of samples will be taken from a batch. Samples from homogeneously contaminated batches will likely represent the actual mean level of contamination in a batch. In contrast, samples from batches with localised contamination may be expected to over- or underestimate the actual mean level of contamination in the batch, depending on which combination of sections have been sampled and the total number of samples taken and analysed per section.

The balance of the microorganisms added to and retrieved from the laboratory scale batches (Table 1) was calculated as a metric to judge how representative samples of a batch were to the actual contamination level of the batch. The ratio ($C_{samples}/C_{in}$) of the concentrations based on the samples ($C_{samples}$) and added to the batch (C_{in}) were for the heterogeneous batches considerable lower than for the homogeneous batches. The spatial distribution of the microorganisms clearly influenced the actual level of the contamination in the batch. When the complete batches were investigated, nearly the whole contamination was retrieved for both the homogeneous and heterogeneous batches. In case of the 3 heterogeneous batches, at least 7 of the 144 samples of each batch contained between 10^5 and 10^6 CFU/g. Nevertheless, on average, the estimated concentration was clearly underestimating the actual level of contamination in the batch. However, if a cluster with a higher number of microorganisms (e.g. 5×10^6 CFU/g) would have been present in one of the samples, the concentration might have been overestimated.

4.2 Comparing ECDFs characteristics for different enumeration data

To analyse variability in the enumeration data of the samples, ECDF curves were established for various laboratory batches and the industrial batch. For laboratory batches with localised contaminations, the laboratory batch with low-level homogeneous contamination, and the industrial batch, cumulative probabilities started at values over 0.5, indicating that a large part of the samples contained zero counts, or was below the detection limit. Although these ECDF curves or frequency distributions of sampling data provide information about the number of counts equal or below zero, they do not provide a rationale for the zero counts. The rationale can be either, that the level of contamination is below the detection limit (Lorimer and Kiermeier, 2007) or that contamination is

distributed heterogeneously with some sections of a batch containing measurable contamination levels and other sections containing no contamination (Habraken et al., 1986; Jongenburger et al., 2011a; Kiermeier et al., 2011). Using ECDFs for the two distinct types of distributions investigated, it has become apparent that 1) when the slope of the ECDF curve is steep and most samples are above the detection limit, the enumeration data most likely correspond to a homogeneous distribution, and that 2) when the slope of the ECDF curve is gentle and part of the samples is below the detection limit, the data may either correspond to a homogeneous distribution with a low level contamination or to a heterogeneous distribution. The ECDF established for the enumeration data from the full-scale industrial batch showed the ECDF characteristics of a heterogeneous distribution. Since these enumeration data were obtained from samples taken in the course of filling of the batch into bags and knowing the filling times (Jongenburger et al., 2011b), it can be confirmed that *Cronobacter* spp. was actually heterogeneously distributed in the industrial batch of PIF.

4.3 Comparing statistical distributions to model the ECDFs

It is essential to represent microbial distributions accurately, when levels of microorganisms are modelled in food safety management activities as to conduct a MRA or to establish MC. The ability to model microbial distributions well, will depend on the properties of each statistical distribution and these show marked differences (ILSI, 2010). In practice, the Lognormal distribution has been used to establish MC and to assess the performance of sampling plans (ICMSF, 2002). Although the Lognormal distribution can model heterogeneity, it does not allow for complete absence of the microorganisms, which does occur, and it allows for fractional numbers of microorganisms, which is physically impossible. These limitations are not so important when levels of a microbiological contaminant are relatively high, but they become important at low contaminant levels or when contamination is localised in parts of the batch (Habraken et al., 1986).

Previously, five criteria have been proposed to judge the suitability of different statistical distributions to fit microbial distributions (ILSI, 2010). Based on these criteria, in theory, the Poisson Lognormal and the Negative Binomial distributions would be the most suitable distributions, but this conclusion remained to be validated. The current investigation provided for the actual observations needed in this regard. It was found that the Normal, Poisson, and Zero-Inflated Poisson distributions fitted all data sets

very poorly. In case the contamination was homogeneously distributed, there was not a noticeable difference between the ability of the Negative Binomial, Poisson-Lognormal, Weibull, Gamma, and Lognormal distribution to model the data. Overall, either the Negative Binomial distribution or the Poisson-Lognormal distribution fitted the data best (including the industrial batch), especially when the data contain zero counts, which is in line with the two most suitable distributions deduced on theoretical grounds (ILSI, 2010).

Apart from modelling low numbers correctly, a statistical distribution should be able to model high numbers as well. Based on visual observations and χ^2 values and *LL* values, the Poisson-Lognormal distribution fitted the industrial (uncensored) observations best. Although more than 50% of the samples were below the detection limit, the observations covered the entire range between 3.0×10^0 and 5.6×10^5 CFU/kg. Especially, catching the tail of a distribution with high infrequent numbers is important for an accurate assessment of the food safety status of a batch and the potential public health impact.

Our results are partly in line with a recent study, which compared the Negative-Binomial (Poisson-Gamma) and Poisson-Lognormal distributions to characterise microbial counts in foods. It was concluded that the Poisson-Lognormal distribution fitted better to high counts data sets, and the Negative Binomial distribution represented the low counts data sets better than the Poisson-Lognormal (Gonzales-Barron and Butler, 2011).

4.4 Censored and uncensored data

The data were treated as censored and uncensored data. In the case that data were censored, an underlying distribution is assumed and the data points below the detection limit will influence the parameter estimates. One might question the appropriateness of this procedure for situations in which microorganisms are present only in a localised part of the batch and all other parts of the batch contain no microorganisms. The Lognormal distribution fitted the censored data of the industrial batch better than the Poisson-Lognormal for uncensored data. Although, the Lognormal fitted the data best in the positive samples (above detection limit), one may question whether this estimate reflects the status of the batch correctly for samples below the detection limit. Especially, since *Cronobacter* spp. cells were heterogeneously distributed throughout the batch. The batch contained sections with no detectable contamination and sections with contamination levels between -2.52 and 2.75 log CFU/g.

5. Conclusions

The Lognormal distribution is generally, and often by default, used for quantitative modelling of microorganisms in food. This systematic study compared in detail the performance of several other statistical distribution(s) that may be used to model different microbial frequency distributions for data obtained at both laboratory and industrial scale regarding the pathogen/food product combination *Cronobacter* spp. in PIF. In the laboratory scale batches, the contamination was arranged to be either homogeneous or heterogeneous (i.e. localised in a section of the batch). The full scale industrial batch investigated showed clear features of heterogeneous distribution. It was found, that due to differences in the actual distribution of the pathogen in the food product, the concentration in heterogeneous batches was underestimated as compared to that in homogeneous batches.

In the homogeneously contaminated batches with few or no zero counts, both continuous distributions (i.e. Lognormal, Gamma, and Weibull distributions) as well as discrete distributions (i.e. Negative Binomial and Poisson-Lognormal distributions) fitted the enumeration data well. When part of a data set contained zeros and/or the numbers were low, either the Negative Binomial or the Poisson-Lognormal distribution fitted the data best. For the laboratory batches, the Negative Binomial fitted the uncensored and censored data best. For the industrial batch, the Poisson-Lognormal distribution fitted the uncensored data best, whilst the Lognormal distribution fitted the censored data best.

It can be concluded that the physical distribution of the microbial contamination indeed impacts the observed microbial frequency distribution. Thus, to model the observed frequency distributions of the microorganisms in a food, the appropriate statistical distribution should be selected. This would require case-by-case validation of fit, which will depend on the enumeration data. Based on the data investigated in this study, the Negative Binomial and the Poisson-Lognormal distributions are good candidates.

Acknowledgements

The authors are very grateful to Keith Jewell for his valuable advice and assistance.

Chapter 8

General discussion



1. Distributions of pathogens in batches of food

Pathogens need to be prevented, eliminated, or controlled to acceptable levels in foods in order to assure that the food product will cause no harm to the consumer when it is prepared and/or eaten according to its intended use (CAC, 2003). For primary production, good agricultural practice (GAP) and for food production, good hygienic practice (GHP), good manufacturing practice (GMP), and HACCP are systems to prevent, eliminate or control pathogens. In order to better be able to manage food safety, knowledge about the distributions of pathogenic microorganisms in batches of food is important for both the food industry and the government. To investigate in detail the microbial distribution within a batch of food, powdered infant formula (PIF) has been chosen in this thesis study as the food product and *Cronobacter* spp. as the target microorganism. During the production process of PIF, mechanisms such as contamination, microbial growth, microbial death, joining, mixing, and fractionation (Chapter 2, Table 1) may contribute to the final distribution of *Cronobacter* spp. in a batch of PIF. PIF can be manufactured in a wet-mix process, a dry-mix process or a combined process (Cordier, 2008). Fig. 1 shows a flow chart of the manufacture process of powdered infant formula according to a dry-mix process (Cordier, 2008).

The manufacturing process starts with a dry base product, which is an intermediate product packed in big bags. This intermediate product results from a wet-mix process in which all unprocessed material, like raw milk or liquid whey, are handled in a liquid phase, heat treated by pasteurisation or sterilisation and dried to obtain the base powder. Viable *Cronobacter* spp. cells show no particular heat resistance and are easily killed in liquids at temperatures ranging from 60 to 70°C (Breeuwer et al., 2003; Iversen et al., 2004).

The base powder enters the factory in big bags on wooden pallets. First, the big bags are transferred to specific pallets that are used only in the factory and then the base powder is stored in silos. In the next step, the base powder is blended with ingredients like vitamins and minerals. Next, the powder is distributed over two different silos by a separator. The product is subsequently sieved and transported to the filling line, where it is packed as finished product as part of the daily produced batch of PIF.

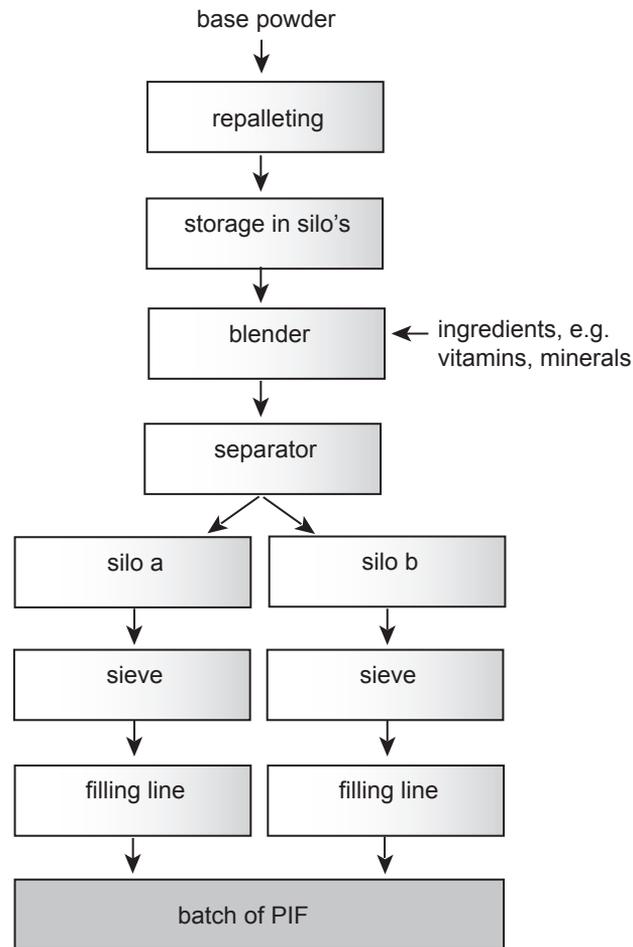


Fig. 1. Flow chart of a dry-mix production process of powdered infant formula.

The microbiological quality of the base powder and the mixed ingredients are critical because no further inactivation step is applied in the manufacturing process. The mechanisms of joining and mixing may influence the microbial distributions during blending. Fractionation may impact the distributions during the separation of the powder into two silos and the filling of the packages at the filling line. Microbial growth in the powder will be unlikely, since the product is dry and *Cronobacter* spp. will not be able to multiply although it may well be able to remain viable. *Cronobacter* spp. cells, in fact, have a high tolerance to desiccation (Breeuwer et al., 2003) and our investigations showed that

Cronobacter sakazakii survives in PIF over more than 2 years (Fig. 2). *Cronobacter* spp. are ubiquitous microorganisms, occurring rather widespread in nature as well as in dry-processing environments (Kandhai et al., 2004; Reich et al., 2010). Therefore, in the latter, contamination may occur, for example, via air, presence of water, harbouring niches, and filler heads. Notably, all of the mechanisms mentioned above for PIF production may have an impact on the final distribution of *Cronobacter* spp. in the product.

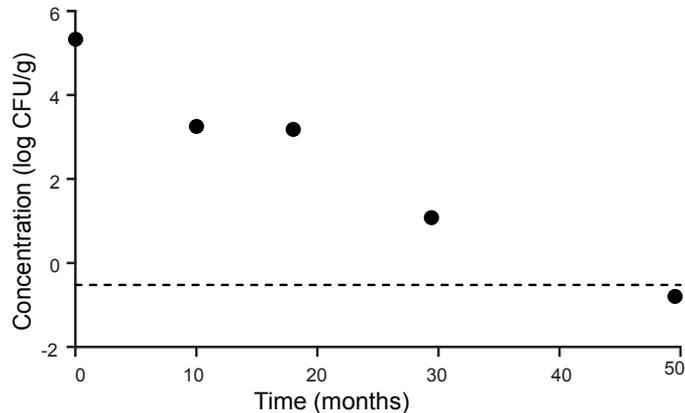


Fig. 2. Survival of *Cronobacter sakazakii* strain ATCC 29544 in powdered infant formula. The dotted line indicates the detection limit of an MPN of 33.3 g (-0.52 log CFU/g). The point below the detection was negative in this MPN.

In order to map the microbial distribution within a batch of food, the location as well as the numbers of microorganisms will determine the outcome as illustrated in Fig. 3. Three types of locations within a batch are possible: microorganisms localised in the whole batch, microorganisms localised in one part of the batch, and microorganisms localised in systematic or random parts of the batch. As indicated, the microbial number can be below the detection limit, low but still measurable, here defined between 0.003 and 100 CFU/g, and moderate to high, being defined as larger than 100 CFU/g.

The actual level of the microorganism present in a sample can be estimated with quantitative methods. To interpret the enumeration results, the characteristics of the methods have to be taken into account, particularly the lower detection level (Buchanan, 2000). Table 1 provides some rule-of-thumb values for various detection methods that are commonly employed to the analysis of foods (Buchanan, 2000).

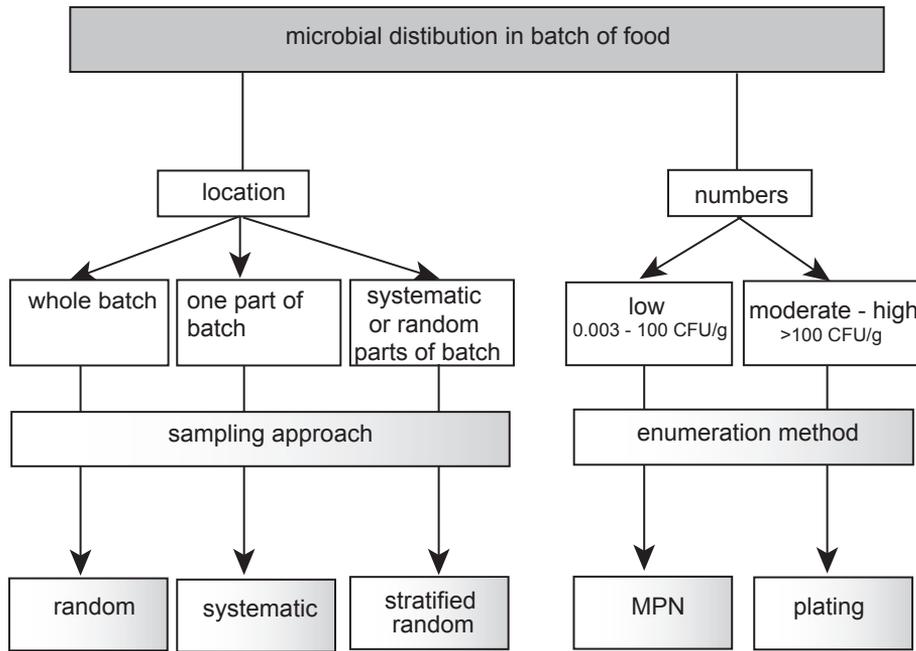


Fig. 3. Microbial distribution in a batch of food represented by 3 types of possible location and ranges of numbers present and their influence on both the sampling approach and the enumeration method.

Table 1. Rule of thumb for the lower limit of detection for microbiological methods commonly used to quantify foodborne microbial contaminants (Buchanan, 2000)

Method	Typical lower limit of detection (CFU/g)
Most probable number	< 10 – 100
Viable counts	> 10 – 100
DEFT ^a	10 ³ –10 ⁴
ELISA ^b , flow cytometry, quantitative PCR ^c	10 ⁴ –10 ⁵
Direct microscopy, spectrophotometry	10 ⁵ –10 ⁶

^a DEFT: direct epifluorescent filter technique

^b ELISA: enzyme linked immunosorbent assay

^c PCR: polymerase chain reaction



The type of food product greatly impacts the actual physical distribution of microorganisms in a batch of food. Pathogens in fluids are more likely to be more equally distributed throughout the whole batch. Pathogens in solid, semi-solid, or powdered products are more likely to be localised in one or, more probably, several parts of the batch. Assumptions on the location have to be made in order to investigate the microbial distribution within a batch of food by an optimal search strategy and enumeration method. For *Cronobacter* spp. in PIF, used as a model for powdered and other types of structured foods, both low numbers of the pathogen and a localised or heterogeneous distribution are expected to occur in practice.

In this discussion, search and sampling strategies as well as enumeration methods will be evaluated to find the optimal approach regarding *Cronobacter* spp. in PIF. First, the search behaviour of marine predators and their optimal sampling strategy will be discussed and compared to the sampling approaches used in this thesis. Next, enumeration by the plating method and the MPN method will be discussed. Then, the impact of sampling approaches and enumeration results will be discussed in the perspective of public health and food safety management.

2. Sampling approaches

2.1 Comparing the sampling approach of marine predators to stratified random sampling

In nature, many free-ranging predators have to make foraging decisions with little, if any knowledge of the actual prey distribution (Stephens and Krebs; 1986). An electronic tagging study of over a million movement displacements of individual marine predators, including sharks, bony fish, sea turtles, and penguins, provided data needed to analyse predator search patterns (Sims et al. , 2008). What emerged was that the search pattern of a predator with little prior knowledge of prey distribution is in line with the 'Lévy-walk' model. Lévy-walk, a specialised random walk, is characterised by super-diffusive 'walk clusters' of short move steps length (distance moved per unit time) with longer reorientation jumps. Subsequent simulations showed that the foraging predators that adopt this random walk maximised the encounter rates in nature-like prey fields. Based on these results, the authors explained the predator-prey interaction according to their hypothesis that animal search patterns are adapted stochastically to their prey field structures, because their environment is so heterogeneous. Predators feeding on patchy,

heterogeneous prey should adapt the best probabilistic search strategy given that they are essentially 'blind' hunters at the spatial and temporal scales over which they typically forage. Although the search pattern of predators differs from stratified random sampling to detect microorganisms in a batch of food, they have several components in common. Both are basically 'blind' hunting, and aim to maximise the sampling performance when prior knowledge of the distributions is lacking. Furthermore, the rare long steps can be compared with the strata and the many short steps with the random samples taken from each stratum. Thus, looking at nature provides inspiration and might help to learn how to optimise our search strategy.

2.2 Designing a sampling approach to detect *Cronobacter* spp. in PIF

Before starting a detailed investigation of the distribution of *Cronobacter* spp. within a batch of PIF, several questions had to be answered: which kind of distribution is to be expected, which sampling strategy would optimise the detection and how many samples need to be positive to give a clear picture of the microbial distribution in the batch. In order to prepare the research, several of these questions were first answered in a theoretical part of the study.

2.3 Random or systematic sampling to detect a localised contamination

Random and systematic sampling approaches were compared to detect a localised contamination within a batch of food (chapter 5). The microbial contamination within a food product was statistically modelled for a case where a microbial contamination was present only in a specific part of the batch, called the contaminated fraction of the batch and indicated as c (referred to as the 'localised contaminated fraction'). Microorganisms present in the localised contaminated fraction were assumed to be randomly distributed. Contamination throughout the whole batch, $c = 1$, could be relevant for fluids or very well mixed foods; $c < 1$ could occur in solid, semi-solid or powdered foods such as for instance *Salmonella* spp. in peanut butter, mycotoxins in grain, or *Cronobacter* spp. in PIF. It was concluded that in the case of a single localised contaminated fraction, systematic sampling should be preferred over random sampling in order to detect such a contamination. This is also in line with other studies (Habraken et al., 1986; Casado et al., 2009).

In the case of systematic contaminations, like a contaminated filler head, it might be possible to miss the contamination when using systematic sampling. In this

case, stratified random sampling, another type of systematic sampling, would be more appropriate as shown in Fig. 4. This figure illustrates three sampling strategies to draw 30 sample units: random, systematic and stratified random sampling. In stratified random sampling, 3 random sample units are drawn from each interval or stratum. In a moving stream of product, these intervals could be a specific weight or a time interval that the product passes a specific point. In the investigation of the industrial batch of PIF, the intervals were based on the filling time of the packages. Since stratified random sampling combines the qualities of systematic and random sampling, this strategy is preferred and is most appropriate to use in case of a systematic contamination.

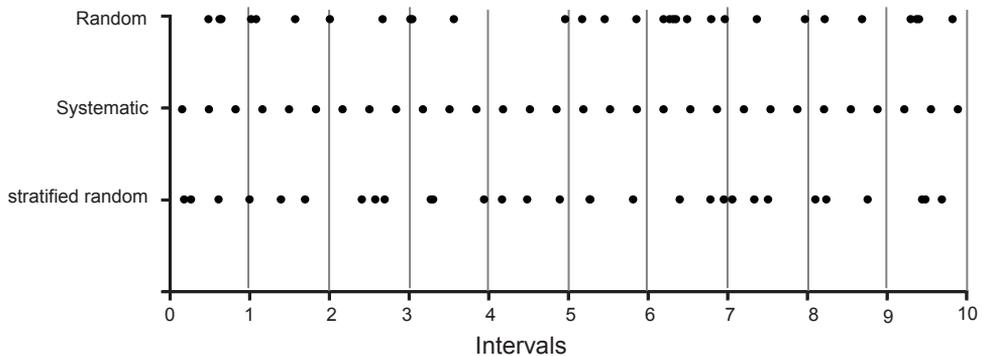


Fig. 4. Illustration of random, systematic, and stratified random sampling of 30 samples units.

Although this was a theoretical study (chapter 5), it showed that systematically taking many small sample units increased the probability to detect a localised contamination and it furthermore introduced a new way of looking at the relationship between the sampling approach and the number of sample units relative to the size of the contaminated fraction. If we assume that *Cronobacter* cells are only present in the contaminated fraction of the batch, the size of the contaminated fraction needs to be estimated in order to derive the optimal number of systematic samples. In the microbiological risk assessment of *Cronobacter* spp. in PIF (FAO/WHO, 2006a), prevalence data from published and unpublished studies were available to the experts. These have been very well documented and at the time used to estimate a range of concentrations of *Cronobacter* spp. in PIF. This assessment confirmed that the microorganism occurs at low levels only, when at all, with mean levels of the microorganisms in contaminated product being of the order of 10^{-3} CFU/g. Based on the fraction positive samples, we assume that the contaminated

fraction in PIF is 1% or smaller. To detect such heterogeneity and concentration range with a probability of 95%, 1500 sample units of 50 grams have to be taken systematically from a batch of PIF. In order to map the microbial distribution from positive packages of product found, the concentration of *Cronobacter* has to be estimated using the MPN method.

2.4 Sampling industrial batches of PIF

Overall, an enormous amount of samples has to be enumerated in operational practice, with very significant costs for materials and human resources. In case positives are found, a batch will not be released onto the market, representing a further loss of resources for the manufacturer, often a day's production. In the current study, a PIF batch was included that had been cleared for the market on the basis of the sampling data obtained for the final product at the manufacturing stage, but was later recalled due to the fact that positives were found in the marketed product. Besides this recalled batch, a reference batch produced in the same factory was included. This research has been described in chapter 6. The thorough investigation of the recalled batch showed that *Cronobacter* spp. cells were heterogeneously distributed throughout the batch, with sections that contained no detectable contamination and sections that contained levels between -2.52 and 2.75 log CFU/g. Clusters of cells occurred sporadically in 8 out of 2290 samples of 1 g. Although the root cause of the contamination that resulted in the batch being heterogeneously contaminated is unknown, the fact remains that a fraction of products on the market may be relatively heavily contaminated. Because the levels and number of positive samples were determined in the course of the filling time, it was possible to calculate the difference in performance of random or stratified random sampling. Compared to random sampling, stratified random sampling improved the probability to detect this heterogeneous contamination. Therefore, this sampling strategy is recommended, also because it will detect systematic contaminations. This study agreed well with the previous theoretical study (chapter 5), that taking more and smaller sample units while keeping the total sampling weight constant, clearly improved the performance of the sampling plans.

Evidently, the choice of sampling strategy is important when contamination is heterogeneously distributed or clustered in local spots. In the theoretical study as well in the study of the recalled batch, the performance of a systematic sampling approach (i.e.

systematic or stratified random sampling) appeared to be equal or better than random sampling. Therefore, in case the microbial distribution is unknown, stratified random sampling can be recommended.

2.5 Indirect sampling strategies

Apart from sampling the batch itself to detect the target microorganism (direct sampling), indirect sampling may also deliver information about the microbial status of the batch. Indirect sampling can be performed by sampling the environment for the target microorganism, for instance, sampling the dust filters of the filling room for *Cronobacter* spp., or the rinsing water of chicken breast filet for *Salmonella* spp. (Straver et al., 2007), the drip of a meat package, or the water used to soak and spout beans.

Indirect sampling can also be performed by sampling for an index microorganism. An index organism is a microorganism or group of microorganisms that is indicative of a specific pathogen (Buchanan, 2000). Obviously, for an index microorganism to be a valuable indicator for a pathogen there should ideally be a systematic quantitative relationship between the index organism and the target microorganism. The environment as well as the product can be sampled for an index microorganism.

Index microorganisms in the environment are, for example, species of the family *Enterobacteriaceae*, such as *E. coli* which may appear in food manufacturing environments and are harmless most of the time. However, pathogens (such as *E. coli* O157:H7 and *Salmonella* spp.) are also members of this family. By testing specifically for *Enterobacteriaceae*, positive samples may contain both immaculate species as well as harmful species, the latter assumed to be present generally only at low levels. Therefore, the family *Enterobacteriaceae* is often used for routine monitoring of the environment and if they are found to be present at relatively high numbers, testing for specific pathogens should be started (CEC, 2005).

Index microorganisms in the product are, for example, *Enterobacteriaceae* included in the aforementioned EU standard (CEC, 2005). According to the former EU standard, *Enterobacteriaceae* were suitable index microorganisms for PIF and product had to be tested for *Enterobacteriaceae* by drawing 10 sample units of 10 g. If *Enterobacteriaceae* were detected in any of these sample units, 30 sample units of 10 g had to be tested for *Cronobacter* spp. and also *Salmonella* spp. had to be tested for. Since there was no solidly proven correlation between the presence of *Enterobacteriaceae* and

Cronobacter spp., the standard was amended (CEC, 2007) such that *Enterobacteriaceae* and *Cronobacter* spp. standards are effectively two different standards, like they are in the relevant Codex guidelines (CAC, 2008).

In many cases it is important to measure physico-chemical parameters like for instance water activity or the pH of the product. These parameters are directly measurable during the production process and might provide information about the microbial status of the batch.

3. Enumeration methods

Several methods to enumerate the contamination are available and the choice of method will impact on the mapping of unknown microbial distributions in food batches.

3.1 Plate count method

In order to detect and enumerate clusters of cells in the recalled batch of PIF, the plate method was used (chapter 6). Because it is of interest to know the accuracy of these counts, in chapter 4 the relative error was assessed as a measure for the accuracy of the plating method. The relative error was expressed as $\frac{\sigma_N}{N}$, in which N is the number of cells per sample. This relative error can also be calculated for the clusters in the recalled batch. Table 2 presents for each cluster: the number of cells per sample (N), the colony count per plate (C), and the relative error ($\frac{\sigma_N}{N}$).

Table 2. The clusters *Cronobacter* spp. cells detected in the recalled batch of PIF, and per cluster: the colony count per plate (C), the number (N) of cells per gram (CFU/g), the relative error ($\frac{\sigma_N}{N}$), and the error (σ_N)

Cluster	C (CFU/plate)	N (CFU/g)	$\frac{\sigma_N}{N}$ (%)	σ_N (CFU/g)
1	1	3.3	100.0	3.3
2	3	10	57.8	5.8
3	3	10	57.8	5.8
4	4	13	50.1	6.5
5	6	20	40.9	8.2
6	12	40.0	29.0	11.6
7	37	123	16.6	20.5
8	168	560	8.1	45.5

$$N_{total} = \sum_{i=1}^8 N = 779 \quad \frac{\sigma_{N_{total}}}{N_{total}} = 6.8 \quad \sigma_{N_{total}} = 53.0$$



As shown in Table 2, a higher colony count per plate, results in a lower relative error $\frac{\sigma_N}{N}$. The smaller clusters with a colony count between 1 and 12 had a relative error $\frac{\sigma_N}{N}$ between 100.1% and 29.1%. The two largest clusters were quantified considerably more accurate. These large clusters are the most important ones considering their potential impact on public health (88% of N_{total}).

From the above it is evident that the low colony counts increase the relative error $\frac{\sigma_N}{N}$ and thus the accuracy of the plating method. In this regard, it is noteworthy that the plate count range advised for optimum plate counting has changed over the last century and especially over the last decades (Table 3). A range of 30-500 colonies per plate has been recommended by Breed and Dotterer (1916). This original recommendation has later been amended to a range of 30-300 colonies per plate, which has found wide acceptance (Adams and Moss, 2008; Sutton, 2006). An optimum counting range of 25-250 colonies per plate for a 10-fold dilution series has been recommended by Tomasiewicz et al. (1980). ASTM advises countable ranges of 20-200 colonies per plate for spread plates and 30-300 colonies per plate for pour plates (ASTM, 2004) and the FDA bacterial analytical manual (BAM) recommends 25-250 colonies per plate as a countable range (FDA, 2001).

Table 3. Overview of the plate count range and the number of plates advised in the past century

Range	Type	Reference	year
30-500	Non-selective	Breed and Dotterer	1916
30-300	Non- selective	General rule based on the work of Breed and Dotterer	1916 - present
25-250	Non- selective	Tomasiewicz et al.	1980
25-250	Non- selective	General rule based on the work of Tomasiewicz et al.	1980 - present
20-200 or 30-300	Spread plate/pour plate	ASTM international	1998, 2004
25-250	Non- selective	BAM (FDA)	2001
15-300	Non- selective	ISO 4833	2003
10-300	Non- selective	ISO 7218	2007

For decades, the general accepted ranges for countable number of colonies per plate are 30 - 300 and 25 – 250. However, the international standards organisation (ISO) decreased the lower limit in 2003 and again in 2007 in their standards. A range of 15-300 for non-selective plates has been prescribed in ISO standard 4833 (ISO 4833, 2003) and most recently, the lower limit was decreased to 10 in ISO standard 7218 (ISO 7218, 2007). In this regard, it is good to mention that the limit of detection (LOD) is 1 CFU. However,

also the estimated concentration and the limit of quantification (LOQ) are important in reporting. In the FDA BAM method, all counts are recorded as the raw data, but the estimated concentrations are reported as <LOQ. For example, a 1:10 dilution yielding counts of 9 and 12 would be reported as <250 CFU/g.

Our research documented in chapter 4, clearly showed that technical errors in weighing and diluting of samples hardly influence the plate count accuracy and that the colony count per plate almost entirely determine the plate count accuracy. Increasing the lower limit may lead to an increase of the work load in laboratories. Based on our results, we advise to increase the lower limit to 25 colonies (Chapter 4), which is similar to the recommendations in FDA's BAM (FDA, 2001).

3.2 Most Probable Number technique

In order to enumerate *Cronobacter* spp. in the recalled batch of PIF, the most probable number (MPN) technique was used (Chapter 6). The MPN method enumerates viable microorganisms and is used when low numbers are expected (< 100 CFU/g). The principle of the MPN technique is to dilute a sample to such a degree that inocula will sometimes but not always contain viable microorganisms (ISO 7218, 2007). Fig. 5 shows a schematic overview of the MPN method for a solid product, which first is diluted (1:10) and then serially diluted followed by inoculation of the pre-enrichment media.

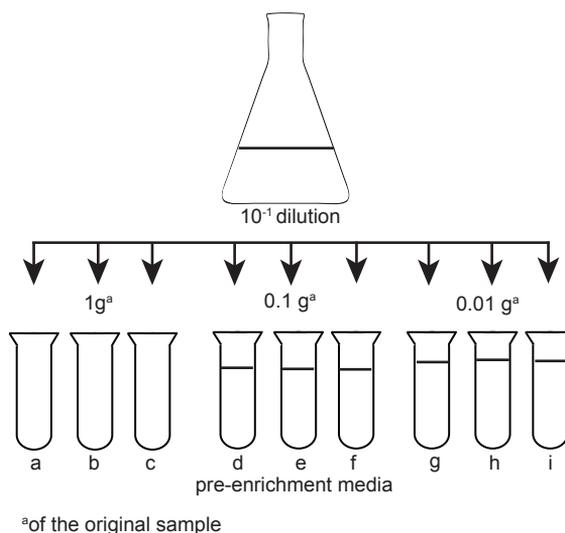


Fig. 5. Scheme for an MPN method for a solid product, which is diluted (1:10) and the test portions are divided over pre-enrichment media (10 mL of 10⁻¹ ≈ 1 g, 1 mL of 10⁻¹ ≈ 0.1 g, 0.1 mL of 10⁻¹ ≈ 0.01 g).

After incubation, the 'outcome', i.e. the number of inocula producing growth at each dilution, will give an estimate of the initial concentration of microorganisms in the sample. The MPN is the number that makes the observed outcome most probable. The outcome can be calculated or found in MPN tables. The following assumptions are necessary to support the MPN method: 1) the microorganisms are distributed randomly within the sample, 2) the microorganisms are separate, not clustered together and they do not repel each other, and 3) every tube of which the inoculum contains one or more viable organism will produce detectable growth or change, 4) the individual tubes of the sample are independent (Taylor, 1962; FDA, 2010). The latter assumption means that in order to make a higher dilution, the original -1 dilution must be used. Thus, instead of the tubes d, e, and f, the original -1 dilution must be used to make the dilutions in tubes g, h, and i. In order to meet the first assumption, that the microorganisms are randomly distributed in the sample, the samples need to be mixed very well. In case a sample is non-liquid, this mixing is even more important. It will be more likely to reach a random distribution, when the sample is diluted (1:10 dilution). By diluting the sample, clustered cells are also more likely to be separated.

An MPN of 333 g (3 x 100 g, 3 x 10 g, 3 x 1 g) can be taken to estimate concentrations between 0.003 and 1.1 CFU/g. For instance, in the presumptive test to detect and quantify *Cronobacter* spp. in PIF (FDA, 2002), a three-tube MPN method has been used with a sample size of 333 g. In triplicate scoops of 100 g, 10 g, and 1 g are weighed and suspended in sterilised demineralised water (1:10 dilution). Adding the test portions of powder to water or buffered pepton water (BPW), like in the ISO/TS 22964 standard (ISO 22964, 2006), is commonly used in certified laboratories and is in accordance to the standards.

In principle, the MPN estimate obtained with this procedure is incorrect. In order to meet the requirement that the microorganisms are distributed randomly in the original sample, it is important to dilute or mix the original sample thoroughly before deploying an MPN. In case the original sample contains clusters of cells, which are not separated after mixing, the MPN will be lower than the original number of cells in the sample. In the following hypothetical example, one cluster is present in 333 g powder. This cluster can end up either in the sample unit of 100, 10, or 1 g. Table 4 shows the MPN code, the estimated concentration and the probability that the cluster ends up in the sample unit for each of these three cases. Evidently, the probability is highest that the cluster is

contained in the sample unit of 100 g. The estimated concentration is either 0.003 MPN/g or 0.0036 MPN/g and, as 1 cluster is present in one enrichment, the number of cells in the cluster has no impact on the estimated concentration. The impact of the inaccurate quantification is depended on the numbers of cells that make up the cluster.

Table 4. The probability (Pr(cluster in sample), that the cluster ends up in a sample unit of 1, 10, or 100 g, the corresponding MPN code and estimated concentration (MPN/g). One cluster (more than 1 CFU) is present in a sample of 333 g powder and the sample is directly divided (3 x 100 g, 3 x 10 g, 3 x 1 g)

Sample unit (g)	MPN code	Concentration MPN/g	Pr(cluster in sample unit)
1	0 0 1	0.0030	0.009
10	0 1 0	0.0030	0.0901
100	1 0 0	0.0036	0.901

However, when the original sample is first diluted, the number of cells present in the cluster will markedly influence the estimated concentration. In the following hypothetical example, one cluster is present in 333 g of powder and the sample is first diluted, due to which the cells are better randomly distributed, and then the sample is divided in the corresponding volumes. Table 5 shows the number of cells in the cluster, the minimal MPN code (i.e. the minimal number of positive tubes), the estimated concentration, 95% confidence intervals, and the true concentration.

Table 5. Number of cells per cluster (CFU), the minimal MPN code and estimated concentration (MPN/g), 95% C.I., true concentration in the original sample; one cluster is present in a sample of 333 g powder; first, the sample is diluted (1:10) and then divided in corresponding volumes (3 x 1000 mL, 3 x 100 mL, and 3 x 10 mL)

One cluster (CFU/cluster)	MPN code minimal	Estimated concentration (MPN/g)	95% C.I.	True concentration (CFU/g)
10 ⁵	3 3 3	> 1.1	> 0.42	300
10 ⁴	3 3 0	0.24	0.042 - 1	30
560	3 3 0	0.24	0.042 - 1	1.7
123	3 0 0	0.023	0.0046 - 0.094	0.37

In this example, the estimated concentration for a cluster of 123 cells was at least 0.023 MPN/g (minimal MPN code). This estimated concentration (MPN/g) is lower than the true concentration in the original sample (number of cells in one cluster per 333 g sample).

The distribution of *Cronobacter* spp was investigated by taking more than 500 MPNs: 416 drawn from the recalled batch and 93 drawn from the reference batch. Before starting this, we investigated the possibility to dilute the 333 g powder first before performing the MPN. A homogenate was made by suspending 333 g powder in 666 mL pre-enrichment medium. The final volume verified was approximately 900 mL ($333 \text{ g} / 900 \text{ mL} = 0.37 \text{ g PIF/ mL}$). The 100 g, 10 g, and 1 g sample units were reached by adding 270 mL, 27 mL, and 2.7 mL homogenate in the right volumes pre-enrichment medium. In principle, the procedure could be done; however, this method appeared to be unpractical when processing hundreds of MPNs. Therefore, it was decided to use the common method and dissolve directly the 100 g, 10 g and 1 g of powder into the demineralised water. It was assumed, that the powder was thoroughly mixed during the production process. There was then the possibility that we underestimated the concentrations.

Next to assessing concentrations in samples from the recalled batch of PIF based on MPNs of 333g, 31 bags of PIF were investigated in detail. The remainder of the powder in a bag was investigated by plating samples of 1 g each. Although clustering was expected to influence concentrations assessed by the MPN technique, the concentrations based on MPN and plating were in the same order of magnitude (Chapter 6, Table 1). Only in case of the largest cluster of 560 CFU/g, the MPN concentration was 2 log units lower than the concentration based on plating.

It is recommended to further study the impact of adding the powder directly to the enrichment media or diluting the original sample first on the estimated MPN concentration, for conditions in which this is practically possible.

4. Public health and Food Safety management

Up to now, sampling strategies and enumeration methods have been discussed to determine the microbial distribution of a batch. The next sections discuss relevant subjects in the perspective of public health and food safety management. Successively, arithmetic mean, infrequent occurrence of high numbers, and an example of pathogens in a dry product, mung beans for sprout production are discussed.

4.1 Arithmetic mean

The risk of illness is often largely determined by infrequent high doses represented by the right hand tail of the frequency distribution (Chapter 3). These infrequent high

values are also the most important contributors to the arithmetic mean in a batch. Thus, the arithmetic mean (mean of counts) is more relevant to the risk assessment than the geometric mean (mean of logs), which has been the most commonly used parameter to represent average microbial counts.

The arithmetic mean (μ_A) can be calculated directly from count data ($\mu_{Accounts} = \text{mean}(x)$). After fitting a statistical distribution to the enumeration data, however, the μ_A can also be calculated from the estimated parameters of the fitted distributions. The arithmetic mean of the samples from the industrial batch, investigated in Chapter 6, was calculated directly from the counts and in Chapter 7, different statistical distributions were compared to model the ECDF of the industrial batch (with clusters excluded or included). Table 6 shows the estimated parameters, the arithmetic means for the enumeration data (with clusters excluded or included) calculated for various distributions. The μ_A based on the Lognormal is:

$$\mu_{A_L} = e^{\xi_L + (\sigma_L^2 / 2)} \quad (1)$$

with $\xi_L = \text{mean}(\ln(x))$ and $\sigma_L = \text{sd}(\ln(x))$

The μ_A based on the Negative Binomial is:

$$\mu_{A_{NB}} = \alpha \cdot \beta = mu \quad (2)$$

with $\alpha = \text{size}$ and $\beta = mu/\text{size}$

The μ_A based on the Poisson-Lognormal is:

$$\mu_{A_{pL}} = e^{\xi_{pL} + (\sigma_{pL}^2 / 2)} \quad (3)$$

with $\xi_{pL} = \text{mean}(\ln(x))$ and $\sigma_{pL} = \text{sd}(\ln(x))$

Based on 416 MPNs, the arithmetic mean ($\mu_{Accounts}$) was 3.75×10^{-2} CFU/g. By additionally including the 8 clusters found, the arithmetic mean increases to 1.88 CFU/g. Thus, the arithmetic mean increased 50-fold by these particular infrequent high counts. Comparing the arithmetic mean based on the counts ($\mu_{Accounts}$) with that based on each of the three distributions, the Lognormal was found to be lower and the Poisson-Lognormal higher.

Table 6. The arithmetic means of the industrial batch of PIF with clusters excluded or included; the arithmetic mean (μ_A) based on counts and based on the parameter estimates for the Lognormal, Negative Binomial, and Poisson-Lognormal distributions fitted to the enumeration data (CFU/g) as uncensored and censored data

Based on	μ_A			μ_A		
	Batch with clusters excluded (CFU/g)			Batch with clusters included (CFU/g)		
	counts	fitted		counts	fitted	
uncensored		censored	uncensored		censored	
Counts	0.0375			1.88		
Lognormal		0.0141	0.0399		0.0329	0.246
Negative Binomial		0.0375	0.0377		1.88	1.88
Poisson-Lognormal		0.336	- ^a		7.36	- ^a

^a Not determined

The size of arithmetic mean based on the Negative binomial (μ_{Anb}) was in line with $\mu_{Accounts}$ and is equal for fitting the data as censored and uncensored data. This is because the MLE fit for a Negative Binomial sets the fitted mean (mu) equal to the mean of the data or counts and iteration is needed to estimate the second parameter ($size$) (Lord and Park, 2008). Conceivably, μ_{Anb} by definition is equal to the mean of the counts $\mu_{Accounts}$.

For comparison, arithmetic means were also calculated for the laboratory batches, which are described and investigated in Chapter 7 (Table 7).

Table 7. The arithmetic mean of the laboratory batches of PIF; the contaminations were distributed homogeneously (H) throughout the batch or localised (L) in a section of the batch; the arithmetic mean ($\mu_{Accounts}$) based on plate counts (CFU/g) and based on the parameter estimates for the Lognormal, Negative Binomial (NB), and Poisson-Lognormal (PL) distributions fitted to enumeration data (CFU/g) as uncensored data and as censored for the Lognormal distribution; arithmetic mean based the number of cells added to 1 kg batch of PIF (μ_{in}), and the ratio between $\mu_{Accounts}$ and μ_{in} (%)

Based on	Arithmetic mean (CFU/g)								
	Batch 1	2	3	4	5	6	7	8	9
	H	H	H	H	H	H	L	L	L
Counts	5.41x10 ²	1.61x10 ³	2.67x10 ³	4.96x10 ³	9.53x10 ³	3.12x10 ⁴	1.89x10 ⁴	2.68x10 ⁴	3.82x10 ⁴
Lognormal	1.23x10 ³	2.82x10 ³	3.26x10 ³	5.29x10 ³	1.26x10 ⁴	2.88x10 ⁴	1.80x10 ¹⁸	5.90x10 ⁶	8.32x10 ⁸
NB	5.41x10 ²	1.61x10 ³	2.67x10 ³	4.97x10 ³	9.53x10 ³	3.12x10 ⁴	1.89x10 ⁴	2.68x10 ⁴	3.82x10 ⁴
PL	2.08x10 ³	2.55x10 ³	2.98x10 ³	5.22x10 ³	1.21x10 ⁴	2.85x10 ⁴	- ^a	6.73x10 ⁷	6.83x10 ⁹
μ_{in}	5.78x10 ²	1.93x10 ³	3.24x10 ³	4.87x10 ³	1.26x10 ⁴	2.53x10 ⁴	3.79x10 ⁴	3.79x10 ⁴	6.32x10 ⁴
$\mu_{Accounts}/\mu_{in}$ (%)	94	84	82	102	76	123	50	71	60

^a No convergence

In case the contamination was distributed homogeneously, the values of all arithmetic means ($\mu_{Accounts}$, μ_{Al} , μ_{Anb} , and μ_{Apl}) were comparable. When the contamination was localised and the data set contained a lot of zeros, μ_{Al} and μ_{Apl} deviated from $\mu_{Accounts}$. This is because the mean of the data set ($\text{mean}(x)$) underestimated the mean of the population ($\text{mean}(X)$). As the number of cells added to the batch was known, it was possible to compare μ_{in} and $\mu_{Accounts}$. It appeared that when the contamination was localised in a section of the batch, the arithmetic mean based on the sample counts ($\mu_{Accounts} = \text{mean}(x)$) underestimated the concentration in the batch. This can be seen in the ratio between $\mu_{Accounts}$ and μ_{in} (%). For the localised distributions, this ratio was remarkably lower than for the homogeneous distributions (see also Chapter 7, Table 1: Balance of the number of cells (N_{in}) added to 1 kg batch of milk powder and the number of cells ($N_{samples}$) retrieved).

Since the arithmetic mean is relevant to risk assessment, this parameter can best be used to represent average microbial counts (and not the log counts). However, our laboratory experiments showed that this parameter is influenced by the distribution of the contamination and may underestimate the mean of the concentration of microorganisms.

4.2 Infrequent high numbers of *Listeria monocytogenes* in ready-to-eat foods

Listeria monocytogenes has been known as a human pathogen for more than 70 years, and only the past 3 decades *L. monocytogenes* has been associated with food and classified as a food born pathogen (Gombas et al., 2003). *L. monocytogenes* is widespread in nature and can be found in, for example, raw fish, meat, milk, poultry, and vegetables. A prevalence of 5% of *L. monocytogenes* in ready-to-eat (RTE) foods has been estimated (Levine et al., 2001). According to Gombas et al. (2003), such a prevalence of this organism in these frequently consumed products implies that consumers are exposed to detectable levels of *L. monocytogenes* millions of times each year. *L. monocytogenes* affects people in risk groups, namely, infants and young children, the elderly, pregnant women, and immune-compromised people. In November 2011, the Centers for Disease Control and Prevention (CDC) reported a *Listeria* outbreak causing 139 illnesses and 29 deaths in 28 states in the USA (2-11-2011; CDC, 2011). Cantaloupes grown in Colorado were the vehicle transporting the pathogen. The outbreak of the cantaloupe was the deadliest in the last decades. However, relatively few listeriosis cases have been reported by the CDC. One of the possible explanations for the discrepancy between exposure and

low number of listeriosis cases is that only exposure to high levels of *L. monocytogenes* causes listeriosis (Gombas et al., 2003). Also earlier studies concluded that high numbers of *L. monocytogenes* may pose an unacceptable risk even to healthy individuals, and that the risk group may be at risk even after ingesting small amounts (van Schothorst, 1996). The microbiological criterion for RTE food sets a limit of 100 CFU/g for RTE products placed on the market during their shelf-life and is more stringent for RTE foods intended for infants or medical purposes, i.e. absence in 25 g (van Schothorst, 1996; CEC, 2007).

In order to develop data on the risk of listeriosis to support a science-based strategy for addressing *L. monocytogenes* in foods in the United States, the prevalence of *L. monocytogenes* in RTE foods has been extensively investigated (Gombas et al., 2003); 30,705 samples were collected from relevant food categories, for example, deli salads, luncheon meats, and smoked seafood. The samples were collected over 14 to 23 months from retail market at Maryland and northern California Foodnet sites. The samples were enumerated by both direct plating and MPN enumeration. 577 samples were positive for *L. monocytogenes*, which is a percentage of 1.82% of the total number of samples tested. Table 8 shows the number of positive samples per measured levels of *L. monocytogenes*, prevalence (%) and exposure (%) for each level based on the upper boundary of the levels and the percentage of the total exposure to which each level contributed.

Table 8. Classification of levels of *L. monocytogenes* contaminations (CFU/g) detected in 577 positive samples of 31,705 samples of various RTE products collected, prevalence and exposure (Gombas et al., 2003)

	No. of positive samples in concentration range (CFU/g)								Total
	0.04-0.01	>0.1-1	>1-10	>10-10 ²	>10 ² -10 ³	>10 ³ -10 ⁴	>10 ⁴ -10 ⁵	>10 ⁵ -10 ⁶	
Samples	402	82	52	20	16	3	0	2	577
Prevalence (%)	1.3	0.26	0.16	0.063	0.051	0.0095	0	0.0063	1.82
Exposure (CFU/g)	40.2	82	520	2.0 x10 ³	1.6 x10 ⁴	3.0 x10 ⁴	0	2.0 x10 ⁶	2.05 x10 ⁶
Percentage total exposure (%)	0.0020	0.004	0.025	0.098	0.78	1.46	0	97.6	100

The exposure based on the lower boundary results in similar numbers. The majority of the positive samples were contaminated at levels < 10 CFU/g (1.72%). Only in 41 samples (0.13%), levels larger than 10 CFU/g were measured. The highest levels were detected in 2 samples of smoked sea food. Although the prevalence of these 2 samples is the lowest

(0.0063%), the total exposure is highly determined by these two samples since it is 97.6% of the total exposure of all positive samples. This shows that the infrequently occurring high numbers, the right hand tail of a distribution, determine largely the exposure. Deriving a prevalence of positive samples of 1.82% based on the findings of Gombas et al., is lower than the earlier estimated number of 5% (Levine et al., 2001) and is also in line with the findings of an extended risk assessment of *L. monocytogenes* in RTE foods (USDA, 2003). In this USDA study, contamination data from published and unpublished international sources were collected mostly from food samples collected at retail. These data were assembled into a database of contamination levels in food samples; the data base included 387 studies with over 336,000 samples. The foods were divided into 5 main food categories, namely seafood, produce, dairy, meat, and combination foods. Out of 336,228 samples, 6549 samples were positive for *L. monocytogenes*, which is 1.92% of the total number of samples tested. Table 9 shows the number of samples per contamination level (CFU/g), prevalence (%) and exposure (%). This study also showed that the number of positive samples with high numbers ($> 1 \times 10^4$ – 10^6 CFU/g) is only 42 (prevalence of 0.013%), yet these high numbers contribute to the exposure for more than 94%. Notably, the samples were collected at retail and growth is still possible, meaning that the numbers at consumption may be higher.

Table 9. Classification of levels of *L. monocytogenes* contaminations (CFU/g) detected in 336,228 samples of various RTE products, prevalence and exposure (USDA, 2003)

	No. of positive samples in concentration range (CFU/g)								Total
	0.04-0.01	>0.1-1	>1-10	>10-10 ²	>10 ² -10 ³	>10 ³ -10 ⁴	>10 ⁴ -10 ⁵	>10 ⁵ -10 ⁶	
Samples	5,219	100	300	533	173	92	25	17	6549
Prevalence (%)	1.55	0.029	0.089	0.16	0.052	0.027	0.0074	0.0051	1.92
Exposure (CFU/g)	522	100	3.0 x10 ³	5.3 x10 ⁴	1.7 x10 ⁵	9.2 x10 ⁵	2.5 x10 ⁶	1.7 x10 ⁷	2.1 x10 ⁷
Percentage total exposure (%)	0.0025	0.0005	0.015	0.26	0.84	4.6	12.1	82.3	100

According to the risk assessment of *L. monocytogenes* in RTE foods performed by FAO/WHO (2004), one of the key findings is that the food matrix, virulence of the strain and susceptibility of the consumer are important factors. It also states, that the models developed predicted that nearly all cases of listeriosis result from ingesting high numbers of *L. monocytogenes*. Another key finding was that control measures that specifically

prevent the occurrence of high levels of contamination at consumption would be expected to have the greatest impact in reducing rates of listeriosis. Control measures are, for example, the storage temperature and duration for foods that permit growth during storage.

In a similar way as the *L. monocytogenes* studies, Table 10 shows the classification of the levels of *Cronobacter* spp. in the recalled batch of PIF samples.

Table 10. Classification of levels of *Cronobacter* spp. contaminations (CFU/g) detected in the recalled batch PIF: a) based on 416 MPNs, and b) based on 2290 plate counts

Table 10a. MPNs

	No. of positive samples in concentration range (CFU/g)			Total
	$\geq 0.003-0.1$	$\geq 0.1-1$	$\geq 1-10$	
Total	150	22	4	176
Prevalence (%)	35.4	5.19	0.94	42
Exposure (CFU/g)	15	22	40	77
Percentage of total exposure (%)	19.5	28.6	52.0	100

Table 10b. plate counts

	No. of positive samples in concentration range (CFU/g)			Total
	$\geq 1-10$	$\geq 10-10^2$	$\geq 10^2-10^3$	
Total	1	5	2	8
Prevalence (%)	0.24	1.18	0.47	1.9
Exposure (CFU/g)	10	500	2000	2510
Percentage of total exposure (%)	0.40	19.9	79.7	100

Since the enumerations were based on MPNs and plate counts, positive samples are presented in Table 10a (416 MPNs) and 10b (2290 plate counts). The total exposure based on the MPNs is calculated to be 77 CFU/g or 2510 CFU/g based on plate counts. It can be concluded that the level of exposure estimated is clearly influenced by the detection range of the enumeration method. The 4 MPNs with high numbers ($\geq 1-10$ CFU/g) contributed to 52% of the total exposure, whereas the 2 plate count with high numbers ($10^2 - 10^3$ CFU/g) contributed to 80% of the total exposure. Like in the *L. monocytogenes* risk assessments, control measures to prevent these high numbers are obviously important for risk mitigation.

4.3 An example of sampling mung bean seeds for sprout production

This section discusses another example of sampling a dry food product in which pathogens are likely to be distributed heterogeneously, namely mung bean seeds before they are used for the production of mung bean sprouts.

In the production of mung bean sprouts, the germination of the seeds involves keeping the seeds warm and moist for four to seven days. Under these conditions, low levels of microbial contaminants possibly present on seeds can quickly reach levels high enough to cause illness. Outbreak investigations have indicated that microorganisms found on sprouts most likely originate from the seeds (CFIA, 2007). Pathogens well known to be associated with sprouted seeds are *Salmonella* spp. and *E. coli* O517:H7. Recently also *E. coli* O104:H4 has become of concern. Several members of *Enterobacteriaceae* survive under dry conditions. Inami et al. (2001) showed that *Salmonella* spp. remained viable on naturally contaminated seeds that have been stored for 2 years at room temperature in the dark. Thus, seed age does not necessarily diminish the risk to the public. The key aspect of sprouted seeds that increases the risk to foodborne disease compared to other fresh produce is the sprouting conditions. As sprouting conditions benefit hazardous microorganisms present on the seeds to multiply, complete absence of these is necessary. Consequently, the mung bean sprout producer has to rely heavily on the seed producer being able to provide pathogen free seeds.

The safety of mung bean sprouts is highly influenced by the degree of preventive measures used on farms to avoid contamination of seeds (CFIA, 2007) as well as adequate verification of control of the seed production process. Preventative measures generally include the seed producers to adopt good agricultural practices (GAP) and to take measures to avoid microbial contamination during cultivation and harvesting of seeds in fields or during storage and transportation. For verification of control, sampling the seeds or beans delivered to the mung bean sprout producer is important. Based on the consideration that the beans will be contaminated with a low level of pathogens, when at all, and most likely that the contamination occurs in a localised fashion, a producer may ask the following questions: 1) according to which sampling scheme is a batch of beans best to be sampled in order to detect a heterogeneous contamination of pathogens?; 2) what is the probability to detect such a contamination? The following production and evaluation procedure is assumed: the mung beans arrive in batches of 22,000 kg in containers and are repacked in big bags of 1000 kg in approximately 50 min. During repacking, 50 times a

sample unit of 100 g is taken from the main stream. The total sample is then 5 kg beans. One part of 2.5 kg will be sprouted and the leak water will be investigated for pathogens over a period of 48-72 hrs. The other 2.5 kg will be used to investigate the sprouting capacity of the beans. Based on the results the batch will be released to start the mung bean sprout production.

The sample units are taken from a moving stream of product, which enables taking a representative sample (Whitaker, 2003). This method also enables sampling systematically. Since half of the sample (2.5 kg) is used to detect the presence of pathogens, the weight of the sample unit is 50 g, and the number of sample units is 50. In chapter 5, systematic sampling to detect a heterogeneous contamination has been studied. The focus here is on the probability that the entire sampling scheme (or entire sample) contains one or more viable cells of a pathogen, $\Pr(K_{\text{samples}} > 0)$ with K_{samples} being the number of cells in all sample units. A localised heterogeneous contamination of 1% of the batch (220 kg) is assumed, which is a contaminated fraction of 0.01. $\Pr(K_{\text{samples}} > 0)$ will then depend on the concentration of cells in the contaminated fraction of the batch. Fig. 6a depicts $\Pr(K_{\text{samples}} > 0)$ as a function of the number of cells in the localised contaminated fraction (expressed as log cells in the 0.01 contaminated fraction of the 22,000 kg), assuming that the microorganisms are randomly distributed within this 0.01 fraction of the batch. In case 10^3 cells are distributed randomly in the 220 kg, $\Pr(K_{\text{samples}} > 0)$ is 0.10 and in case 10^4 cells $\Pr(K_{\text{samples}} > 0)$ is 0.45. When taking 1 sample unit of 25 g every 30 s (100 systematic sample units), resulting in the same sample weight in the 50 min interval (50 sample units of 50 g and 100 sample units of 25 g respectively), the probability increases remarkably at higher contamination levels. Fig. 6b assumes a contaminated fraction of 0.02 of the batch. In this case, 50 sample units of 50 g result in the same probability as drawing 100 sample units of 25 g.

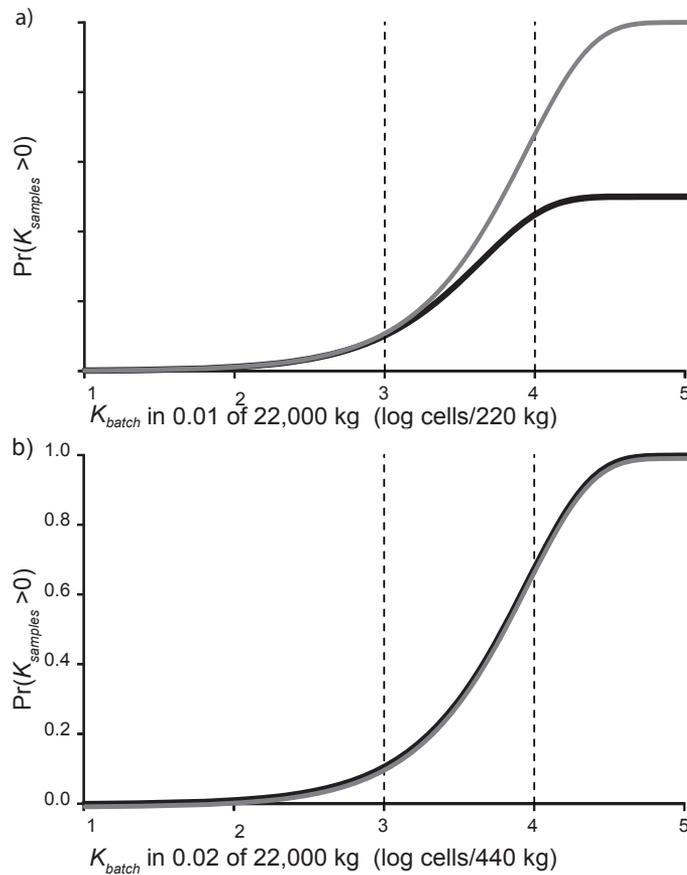


Fig. 6. Heterogeneously contaminated batch of 22,000 kg with a contaminated fraction of 0.01 (a) and 0.02 (b). $\Pr(K_{\text{samples}} > 0)$, the probability that the entire sample scheme contains one or more cells by drawing 50 sample units of 50 g (black curve) or 100 sample units of 25 g (grey curve).

Evidently, when sampling systematically, the sampling performance is influenced by the number of sample units relative to the size of the contaminated fraction of the batch. It is therefore important in many situations to be able to estimate the contaminated fraction in the batch well. In the example, a composite sample has been made of the 50 sample units of 100 g. Investigating a composite sample will lower the workload. On the other hand, information about the microbial status of the batch will be lost. There is also a risk for the producer that the complete batch needs to be rejected when one or more positive

samples are found. A better but more laborious option is to investigate the sample units separately. If each sample unit is divided in two parts, one part can be used to investigate the presence of pathogens, and the other part to investigate the sprouting capacities of the beans. When the time point of each sample unit is registered and sample units are found to be positive, this will give the necessary important information about the size of the contaminated fraction in the batch. It may also give an opportunity to release part of the big bags and reject the big bags that correspond to the positive samples. This example furthermore shows, that the research in this thesis is also relevant to other food products.

5. Overall conclusions

Knowledge about the physical distribution and the heterogeneity in the presence of harmful microorganisms in foods is important to food safety management. The research undertaken in this Ph.D. thesis therefore combined theoretical studies and experimentation in practice to investigate the distribution of microorganisms in foods.

The theoretical part investigated properties of statistical distributions necessary to model frequency distributions of microorganisms in foods. Five criteria were proposed to compare the suitability of the statistical distributions. Based on these criteria, it was concluded that the Poisson-Lognormal distribution and Negative Binomial distribution are the most suitable statistical distributions to model microbial distributions in foods. However, the ultimate choice will depend on the fit of the model to actual observations. The theoretical part also investigated microbial distributions in the perspective of public health, microbiological criteria (MC) and sampling strategies. Examples showed that clustering and the choice of statistical distributions affected the acceptance probability against a set MC. It was concluded that infrequent high doses dominated the arithmetic mean and determined the risk of illness. Furthermore, the sampling strategies to detect a localised contamination in a batch of food were compared. It was concluded that systematic sampling is preferred over random sampling. On top of that, it was concluded that taking many small sample units systematically increased the probability to detect a localised contamination.

The practical part quantified in detail the distribution of *Cronobacter* spp. in two industrial batches of PIF, namely a recalled and a reference batch. The results showed that *Cronobacter* spp. was heterogeneously distributed in the recalled batch. On local-

scale, clusters of cells were present with a low frequency (8 out of 2290 samples). Using the enumeration data of the recalled batch, calculations and simulations also showed that taking more and smaller sample units, while keeping the total sampling weight constant, improved the performance of the sampling plans. It was concluded that, compared to random sampling, stratified random sampling improved the detection probability of *Cronobacter* spp. in this recalled batch. Besides industrial scale batches of PIF, this study investigated microbial distributions in powder at laboratory scale. In the laboratory scale batches, well-mixed and localised contaminations of *Cronobacter sakazakii* were enumerated and discrete and continuous statistical distributions were compared to model the enumeration data or cell counts. Most statistical distributions fitted the enumeration data of homogenous batches with no zeros. It was concluded that low counts including zeros were fitted best by the Poisson-Lognormal distribution and by the Negative Binomial distribution.

This study showed the impact of microbial distributions in batches of food on public health and food safety management activities. The insights and results are useful to governmental bodies to improve food safety management tools like microbiological criteria and microbiological risk assessment. The results can also be of use to the manufacturers of food to improve sampling and testing to verify control of the food operation as well as to verify before marketing that the final product meets relevant standards. This research can be further extended by investigating the variability between batches manufactured by the same factory or multiple factories and by investigating the spatial distributions of pathogenic microorganisms in other food commodities.

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Summary

Consumers expect food to be safe and suitable for consumption. Food containing pathogenic microorganisms may cause food borne illnesses, which may vary in severity from unpleasant to fatal. The physical distribution of pathogens in foods influences the likelihood that a food product will cause illness and also the possibility that a microbial contaminant is detected using a specific sampling plan. But, knowledge about the distribution and the heterogeneity of microorganisms in foods is scarce. This Ph.D. research combines theoretical aspects and practical investigations aiming to increase knowledge of the microbial distributions in foods and their impact on public health and food safety management activities.

In a theoretical study, the properties of statistical distributions necessary to model microbial distributions in foods were determined. Five criteria were proposed in this study to compare the suitability of statistical distributions. Based on these criteria, two discrete distributions, namely, the Poisson-Lognormal distribution and the Negative Binomial distribution were found to be the most suitable statistical distributions to model microbial distributions in foods. Notably, rather than these two types of statistical distributions, the Lognormal distribution is most often used in modelling, but this type cannot model complete absence of microorganisms and thus it is not optimal when enumeration data contain zeros, which does generally occur when microorganisms occur at low levels and/or are distributed heterogeneously.

Also in theoretical investigations, the impact of spatial distributions of microorganisms, like homogeneous or more clustered distributions, on public health was investigated. Infrequent high doses were shown to mainly determine the risk of illness and also to dominate the arithmetic mean (mean of the counts). Furthermore, the performance of random sampling was compared with systematic sampling to detect a localised contamination in a batch of food. Calculations showed that systematic sampling improved the sampling performance. On top of that, taking systematically many small sample units increased the probability to detect a localised contamination.

To experimentally quantify in detail the distribution of microorganisms in a batch of food, the distribution of *Cronobacter* spp. in two industrial batches of powdered infant formula (PIF) was investigated: a recalled batch and a reference batch. The sampling results were clearly different for these two batches. Taking samples (333 g) in the course

of the filling time, *Cronobacter* spp. was found to be heterogeneously distributed in the recalled batch. On local-scale, clusters of cells were present with a low frequency (8 out of 2290 samples of 1 g). The two largest clusters observed apparently contained 123 and 560 cells. Using the enumeration data of the recalled batch, our calculations and simulations showed that taking more and smaller sample units, while keeping the total sampling weight constant, improved the performance of the sampling plans. Stratified random sampling, another systematic sampling strategy was also evaluated. It was concluded that, as compared to random sampling, stratified random sampling would improve the detection probability of *Cronobacter* spp. in this recalled batch. The results obtained investigating the industrial scale batches will be relevant to a wide variety of dry products and to food testing more generally.

Besides industrial scale batches of PIF, research conducted in this Ph.D. thesis investigated distributions of PIF at laboratory scale. Using laboratory scale batches, well-mixed and localised contaminations of *Cronobacter sakazakii* in PIF were enumerated. The data obtained were then used to model the enumeration data or cell counts with several discrete and continuous statistical distributions. Most statistical distributions fitted the enumeration data of homogenous batches as long as the enumeration data did not contain zeros. However, low counts including zeros were fitted best by the Poisson-Lognormal distribution and by the Negative Binomial distribution. Low numbers are generally relevant regarding the occurrence of pathogens in foods and zero counts may occur when the microorganisms are distributed heterogeneously throughout a batch, with some sections containing detectable numbers and other sections containing no detectable level of microorganisms. Overall, the Negative Binomial fitted the enumeration data obtained for the various laboratory batches investigated best. The Poisson-Lognormal distribution fitted the recalled batch best, also when clusters with high numbers of the contaminant were included in the data set. It is important that the statistical distribution used to model the distributions of microorganisms in foods can also fit these infrequent high numbers, since these numbers have a high impact on the probability of illness, thus on the impact to public health.

The insights and results obtained in this thesis may be useful to governmental bodies to further improve their food safety management tools such as microbiological criteria used in food safety standards; they also offer new data, modelling approaches and knowledge to be considered in future microbiological risk assessment. Also for suppliers

of ingredients and raw materials as well as for manufacturers of food, our findings may support better sampling and testing approaches for instance used for verifying control of food operations as well as to validate compliance of product safety before marketing.

Samenvatting

De consument verwacht dat het voedsel dat hij of zij nuttigt geschikt en veilig is voor consumptie. Ziekteverwekkende micro-organismen in levensmiddelen kunnen voor voedselvergiftiging of voedselinfecties zorgen. Deze vergiftigingen of infecties kunnen symptomen zoals overgeven en diarree veroorzaken en in het ergste geval zelfs de dood tot gevolg hebben. De fysieke distributie van pathogene micro-organismen in levensmiddelen beïnvloedt zowel de volksgezondheid als de kans op detectie door het bemonsteren van een partij levensmiddelen. Echter, kennis over de fysieke distributie van micro-organismen in levensmiddelen is beperkt. Dit promotieonderzoek omvat zowel theoretisch als ook experimenteel onderzoek om deze kennis te vergroten.

In het theoretische gedeelte werden de eigenschappen van statistische verdelingen onderzocht om frequentiedistributies van micro-organismen in voedsel te modelleren. Om de geschiktheid van deze statistische verdelingen te beoordelen, werden vijf criteria voorgesteld. Op basis van deze criteria bleken twee discrete verdelingen, te weten de poisson lognormale en de negatieve-binomiale verdeling, geschikte kandidaten te zijn met name omdat zij data-sets met nul-waarde waarnemingen kunnen modelleren. Hoewel de lognormale verdeling vaak wordt gebruikt voor het modelleren van microbiële distributies, is de lognormale verdeling beperkt tot het modelleren van positieve kiemgetallen of tellingen. Hierdoor is de lognormale verdeling een minder geschikte kandidaat bij lage concentraties, een situatie waarbij het voorkomen van nul-waarden wordt verwacht, of wanneer de micro-organismen slechts lokaal aanwezig zijn, wat juist kan leiden tot veel nul-waarden.

In het theoretische deel van het onderzoek werd ook nagegaan wat de invloed is van de fysieke distributie van pathogene micro-organismen in een partij levensmiddelen op de volksgezondheid en op de kans om ziek te worden. Juist hoge, infrequent voorkomende kiemgetallen bleken deze kans te bepalen en zij bepaalden ook het rekenkundig gemiddelde van de concentratie van micro-organismen op basis van de waargenomen tellingen. Verder zijn verschillende bemonsteringsstrategieën vergeleken. Onderzocht werd of aselct dan wel systematisch bemonsteren meer kans heeft om een lokale besmetting te detecteren. Uit berekeningen bleek dat bij het systematisch nemen van monsters de effectiviteit van het bemonsteringsschema werd verhoogd. Ook bleek het dat het systematisch nemen van veel kleine deelmonsters de kans verhoogt om een

lokale besmetting te detecteren.

In het experimentele deel van het onderzoek werd de verdeling van *Cronobacter* spp. in detail onderzocht en gekwantificeerd in twee industriële partijen poedervormige zuigelingenvoeding. Eén partij zuigelingenvoeding was uit de handel gehaald en ter vergelijking werd een referentiepartij onderzocht, die niet was vrijgegeven door de producent omdat de dosis van een bepaald ingrediënt te laag was. De bemonsteringsresultaten lieten een duidelijk verschil zien tussen de uit de handel gehaalde partij en de referentiepartij. De monsters waren gedurende een dagproductie genomen, waarbij de afvultijd van elk monster bekend was. De resultaten lieten zien dat *Cronobacter* spp. heterogeen verdeeld was in deze partij. Op detailniveau werd de aanwezigheid van celclusters onderzocht. In 2290 deelmonsters bleken 8 celclusters aanwezig te zijn, waarvan de twee grootste clusters bleken te bestaan uit 123 en 560 cellen per cluster, respectievelijk. De gevonden kiemgetallen van *Cronobacter* spp. in de uit de handel genomen partij werden gebruikt om verschillende bemonsteringsschema's nader te onderzoeken. Zowel berekeningen als simulaties toonden aan dat door het nemen van een groter aantal kleinere deelmonsters, bij een gelijkblijvend gewicht van het totale monster, de effectiviteit van de bemonstering toeneemt. Daarnaast bleek de effectiviteit ook toe te nemen door aselekt deelmonsters binnen vaste tijdintervallen of strata te trekken, in plaats van aselekte trekking van deelmonsters uit de gehele partij.

Deze inzichten zijn relevant voor uiteenlopende soorten droge poedervormige en gestructureerde producten zoals granen, bonen en cacao, en voor het bemonsteren van partijen grondstoffen en levensmiddelen meer in het algemeen.

Naast het onderzoeken van industriële partijen poedervormige zuigelingenvoeding, werden ook experimenten op kleine schaal in het microbiologische laboratorium uitgevoerd. Verschillende distributies van *Cronobacter sakazakii* in melkpoeder werden gecreëerd en bemonsterd, namelijk homogene distributies alsook distributies met een lokale besmetting. De frequentiedistributies van *Cronobacter sakazakii* werden gemodelleerd met discrete en continue statistische verdelingen. De meeste statistische verdelingen konden de frequentiedistributies van homogene verdelingen van *Cronobacter sakazakii* goed modelleren. Distributies met lage kiemgetallen of veel nul-waarden konden het beste gemodelleerd worden door de poisson lognormale en de negatieve-binomiale verdelingen. Lage kiemgetallen zijn relevant voor pathogene micro-organismen in levensmiddelen en bij lage aantallen zijn

nul-waarnemingen bij bemonstering te verwachten. Nul-waarden kunnen met name voorkomen wanneer lage aantallen micro-organismen tevens heterogeen verdeeld zijn in een partij levensmiddelen, waarbij in een gedeelte van de partij micro-organismen aanwezig zijn en in een ander gedeelte geen micro-organismen aanwezig zijn. De datasets van experimenten op laboratoriumschaal werden het beste gemodelleerd door de negatieve-binomiale verdeling. De dataset van de uit de handel gehaalde partij werd het beste gemodelleerd door de poisson-lognormale verdeling; wanneer de clusters aan deze dataset werden toegevoegd, bleek de poisson-lognormale ook het beste te modelleren. Het is belangrijk dat een statistische verdeling ook in-frequent voorkomende hoge kiemgetallen kan modelleren, omdat juist deze kiemgetallen het risico op ziekte grotendeels bepalen.

De resultaten en verworven inzichten van dit onderzoek kunnen een bijdrage leveren ter verdere verbetering van voedselveiligheid en voedselveiligheidsystemen, zoals bijvoorbeeld de microbiologische risico bepaling (MRA) en het gebruik van microbiologische criteria (MCs) in standaarden en richtlijnen voor voedselveiligheid. De resultaten kunnen nuttig zijn voor toeleveranciers van grondstoffen en ingrediënten, maar ook voor levensmiddelenproducenten, met name bij de verificatie van HACCP ('Hazard Analysis and Critical Control Points') procedures en onderliggende hygiënemaatregelen en het vaststellen, dat een voedselproduct aan geldende voedselveiligheids standaarden voldoet voordat het op de markt wordt gebracht.

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Ida

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- Zwietering, M. H., Jongenburger, I., Rombouts, F.M., van 't Riet, K., 1990. Modeling of the bacterial growth curve. *Applied and environmental microbiology*, 56, 1875-1881.
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Overview of completed training activities

Discipline specific activities

Courses:

Management of microbiological hazards in foods, 2006, VLAG, Wageningen

Reaction kinetics in food science, 2006, VLAG, Wageningen

Basic & advanced statistics, 2007, PE&RC, Wageningen

Meetings:

Expert group ILSI Europe (International Life Science Institute), 'Impact of microorganisms on Food safety management', 2006 - 2010, ILSI, Brussels, Belgium.

Work Group 2 'Statistics' of ISO/TC 34/SC 9 'Food products microbiology', 2008 – 2011, AFNOR, Paris, Paris, France.

Symposia:

NVvM conference, Papendal (2006; 2007, poster presentation)

Symposium Methoden in de Levensmiddelenmicrobiologie, FIMM, Ede (2007, oral presentation; 2010)

Food Micro 2008, 2008, Aberdeen, Ireland (poster presentation)

Int. Conference Rapid Methods Europe 2009, 2009, Noordwijkerhout, (oral presentation)

Int. Conference Predictive Modelling in Foods, 2007, Athens, Greece (poster presentation)

Int. Conference Predictive Modelling in Foods, 2009, Washington, USA, (2 presentations)

Int. Conference Predictive Modelling in Foods, 2011, Dublin, Ireland (oral presentation)

General courses

Vlag Ph.D. week, 2006, VLAG, Bilthoven

Ph.D. Competence assessment, 2007, WGS, Wageningen

Project and time management, 2008, WGS, Wageningen

Scientific writing, 2008, Centa, Wageningen

Writing for publication, 2009, McPhee, Utrecht

Training and supervising thesis students, 2010, OWU, Wageningen

Advanced course guide to scientific artwork, 2010, library WUR, Wageningen

Other activities

Preparing of Ph.D. research proposal / literature research, 2006

Participating seminars Laboratory of Food Microbiology, 2006-2011

Supervising of MSc thesis students (2006-2010)

Representing Ph.D. students at department meetings, 2007-2010

Participating annual progress meetings of European Chair in Food Safety Microbiology, 2007-2011

Assisting practical courses Food Microbiology and Advanced Food Microbiology, 2007-2008

Coordinating group work Risk Assessments of Foods, 2008-2010

Ph.D. trip of Laboratory of Food Microbiology to Canada, 2008

Ph.D. trip of Laboratory of Food Microbiology to Switzerland, 2010

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