The problem of detection and enumeration at low numbers in food: the examples of *Listeria monocytogenes* and *Cronobacter spp.*

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L. monocytogenes and Cronobacter spp.: 2 major foodborne pathogens present at low levels in food.

L. monocytogenes is a Gram-positive bacterium responsible for listeriosis, a severe foodborne illness which can result in gastroenteritis, meningitis, septicemia, spontaneous abortion, and perinatal infections. Despite its low incidence, listeriosis is associated with a high rate of mortality up to 30%, particularly in the elderly and immuno-compromised individuals. Worldwide estimates for 2010 indicate that this pathogen was responsible for 23150 illnesses, 5463 deaths, and the loss of 172 823 disability-adjusted life-years. Moreover, since 2000, the number of listeriosis cases has increased in several European countries. Not only does this pathogen have a significant impact on the costs of public health, but its detection in a food product has economic consequences for the food manufacturer including recall and withdrawal of contaminated products, and lost sales for incriminated food products. *L. monocytogenes* is controlled using standard processing procedures such as pasteurization, but post processing contamination of food remains a critical concern, as *L. monocytogenes* is able to grow at refrigeration temperatures. Problems are predominantly associated with ready-to-eat (RTE) foods where pH may be permissive for growth and where there may be insufficient levels of salt or preservatives to restrict its growth. Outbreaks have been associated with processed meat, seafood, dairy products and produce. Risk assessments from recent years have concluded that nearly all cases of listeriosis occur due to a very high dose of *L. monocytogenes* such as 1 000 to 1 000 000 colonies forming units (CFU) after consumption of food that has been stored for a long time and/or at temperature abuse conditions. Due to growth of the bacterium during storage, initial concentration of *L. monocytogenes* in such products may be low. A European Union-wide baseline survey was carried out in 2010 and 2011 with the aim of estimating the European Union level prevalence of *L. monocytogenes* in certain ready-to-eat foods at retail. It concerned packaged hot or cold smoked or gravad fish, packaged heat-treated meat products and soft or semi-soft cheeses. The prevalence in fish samples at the end of shelf-life was 10.3 %, while for meat and cheese samples these prevalences were 2.07 % and 0.47 %, respectively. The proportion of samples with a *L. monocytogenes* count exceeding the level of 100 CFU/g at the end of shelf-life was 1.7 %, 0.43 % and 0.06 % for fish, meat and cheese samples respectively.

Cronobacter species are Gram-negative, rod-shaped bacteria within the *Enterobacteriaceae* family that have been identified as the causal agent of severe neonatal infections causing sepsis, meningitis, necrotising enterocolitis as a result of the consumption of contaminated powdered infant formula (PIF). It can also affect immunocompromised adults. Currently, the genus includes 7 species (*C. sakazakii, C. malonaticus, C. dublinensis, C. turicensis, C. muytjensii, C. universalis* and *C. condimenti*) which can be differentiated through a multilocus sequence typing (MLST) scheme based on seven alleles (www.pubMLST. org/cronobacter). Presently, only *C. sakazakii, C. malonaticus* and *C. turicensis* are associated with serious human illnesses, while contamination problems in the PIF industry are mainly due to *C. sakazakii* and *C. malonaticus*. *Cronobacter*'s high resistance to osmotic and dry stresses explains its presence and survival in PIF factory products and environments. Since pasteurisation kills the bacteria, most contaminations result from the environment, after processing. In PIF, contamination levels in most cases are lower than 1 CFU per 100 g. Errors in feeding bottle-preparation practices, such as improper holding temperatures, may lead to a critical cell level and the occurrence of the infection. Compared with other *Enterobacteriaceae*, *Cronobacter* is remarkably osmotolerant, surviving for at least 2 years in PIF which typically has a water activity of 0,2. In a study with 7 *Cronobacter* strains representing four different species (*C. sakazakii, C.malonaticus, C. muytjensii, C. turicensis*) we showed a high level of persistence of cultivable cells during extended periods of more than 3 months, in PIF at room temperature. The survivor curves showed significant, but moderate, variability regarding the level of resistance between species, and as already reported, suggest the coexistence of two distinct subpopulations in desiccated cultures, with the minority subpopulation being very resistant to prolonged desiccated storage.

Context of the studies:

From a regulatory point of view, criteria for *L. monocytogenes* in ready-to-eat foods take into account that the pathogen can grow in many products under cold storage conditions. The European Regulation 2073/2005 on microbiological criteria for foodstuffs amended by the recent EC Regulation 1441/2007 set the maximum level in ready-to-eat products at 100 CFU/g in products which do not support growth, and at 100 CFU/g at the last day of shelf life in products that do support growth. For products intended for medical purposes, the criterion is the absence of *L. monocytogenes* in 25 g. Food producers have to predict the level in the products at the last day of shelf life based on the *L. monocytogenes* levels in the products at processing and on shelf life studies, and can develop performance objectives. These are limit values at an early process stage based on the criterion that the maximum limit of *L. monocytogenes* should not be exceeded on the last day of shelf life. However, this is a challenge to precisely estimate these limits, as the detection level in the quantitative EN ISO 11290-2 standard method prescribed in the EU Regulation is 10 CFU/g.

Regarding *Cronobacter spp.*, the European Regulation 2073/2005 amended by Regulation N°. 365/2010, requires its absence in 30 units of 10 g test portions of dried infant formulae and dried dietary foods for special medical purposes intended for infants below six months of age. No further identification of *Cronobacter* to the level of species is necessary. European Regulation prescribes the revised Standard horizontal method ISO 22964:2017 for the detection of *Cronobacter spp.* to address the potential risk to infants and neonates who may consume dried infant formulae and dried dietary foods.

The European Committee for Standardization (CEN), Technical Committee TC 275, Food Analysis, Working Group WG 6, Microbiology of the Food Chain CEN/TC 275/WG 6, in charge of standardization in microbiology of the food chain at European level, has received a mandate from the European Commission (Mandate M381 signed in December 2010) to validate by inter-laboratory studies (ILS) and standardize a set of reference methods in food chain microbiology. These methods are the ones cited as the reference methods in the Regulation 2073/2005 on microbiological criteria for foodstuffs, in particular the reference methods for the detection and enumeration of *L. monocytogenes* in food (Standards EN ISO 11290-1&2) and the revised Standard horizontal method EN ISO 22964:2017 for the detection of *Cronobacter spp.* The ILS had to be performed before the end of 2013, and Standards to be published by the end of June 2017. The determined performance characteristics have now been published in the corresponding CEN ISO standard methods.

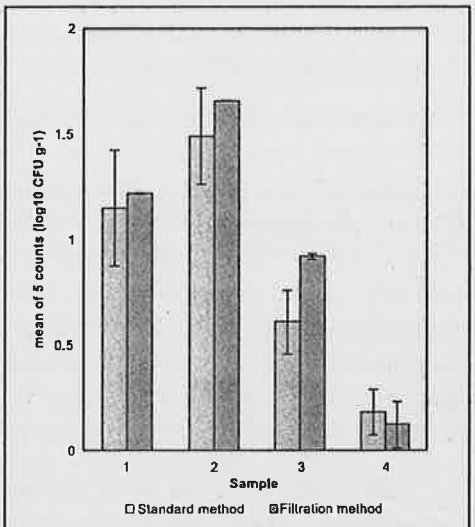
In conditions of very low contamination levels, individual cell variability can have an important impact on pathogen bacteria growth. The behavior of food-borne pathogens is increasingly studied at the cell level to improve predictions of their growth in food. This is also important for evaluating growth performance in detection methods involving enrichment steps. Additionally, in naturally contaminated products, foodborne pathogens are generally minor components of the total microflora and their growth may be then limited by specific interactions and by the so-called Jameson effect. This term designates the simultaneous ceasing of all microflora growth in food, when the dominant bacterial population reaches its stationary phase, because of unspecific competition for a common nutritional resource. The recent revision and validation of EN ISO Standards for detection of pathogens in food was the occasion to include these aspects when evaluating the performances of the methods. Sensitive enumeration methods were also needed to reach low contamination numbers and to demonstrate compliance with microbiological criteria.

Studies on enumeration

For the enumeration of *L. monocytogenes* in food, a sensitive enumeration method based on membrane filtration followed by transfer of the filter to a selective medium has been developed. This study was carried out with cold-smoked salmon, a product likely to be contaminated with *L. monocytogenes*. The operating protocol utilizes three filtration runs in parallel (5, 15 and 30 ml) of a 1 in 10 dilution of the salmon suspension through 0.45µm pore-size cellulose ester membranes, and then culture of the filters on agar *Listeria* according to Ottaviani and Agosti. The results obtained with the technique were compared with those from the reference EN ISO 11290-2 method and found to provide more precise results in the enumeration of *L. monocytogenes* from both artificially and naturally contaminated cold-smoked salmon. Its protocol is presented below:

X g sample (not crushed)
+
9X ml TS diluent
↓
Homogenize for 1 min using a stomacher apparatus in a 280 µm pore-size filter bag
↓
Pour the suspension in a second filter bag, then remove the following volumes to be analysed :
↓

5 + 15 + 30 ml		1 or 0.1 ml
↓		↓
Add 0.83% tween 80 + 0.83% trypsin1/250 from bovin origin (add 1 ml of each 10% stock solutions per 10 ml suspension)		EN ISO 11290-2 reference method
↓		
Incubate for 20-25 min at 37°C in a water bath shaker		
↓		
Filter 5, 15 and 30 ml of treated suspensions (corresponding to final volumes of 6, 18 and 36 ml)		
↓		
Lay filters on ALOA agar		
↓		
Incubate for 48 h at 37°C		
↓		
Spot inoculate all blue colonies on ALOA agar		
↓		
Incubate for 8-24 h at 37°C		
↓		
Confirm 5 typical L. monocytogenes colonies (blue colonies with a halo)		

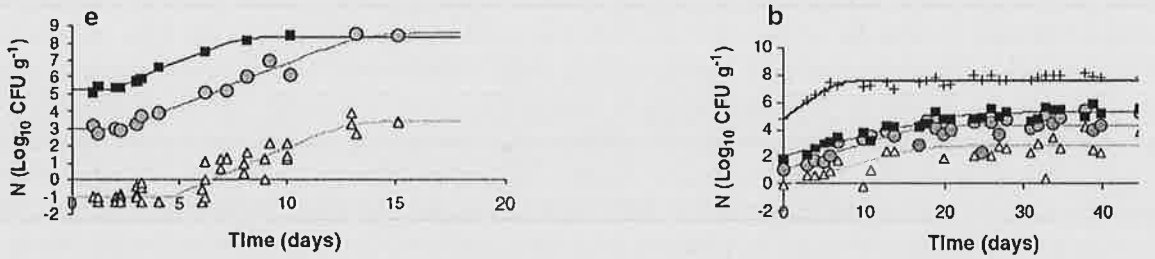


Comparison of enumeration results obtained by the modified standard method and filtration method with naturally contaminated samples: mean of five counts obtained by each method on the same sample and standard deviation.

The performance of this enumeration method was assessed through an inter-laboratory study, using cold-smoked salmon artificially contaminated at 2 different levels (approximately 0.6 and 1.6 $\log_{10}$ CFU/g). Reproducibility standard deviations of 0.23 $\log_{10}$ CFU/g and 0.15 $\log_{10}$ CFU/g were obtained for the method respectively at the lower level and the higher level. Under certain conditions, the uncertainty of measurement can be derived from the method reproducibility standard deviation and was calculated to be 0.46 $\log_{10}$ CFU/g for the lower contamination level and 0.30 $\log_{10}$ CFU/g for the higher contamination level. These values can be considered as satisfactory for such low contamination levels.

The method has been standardized in France as a sectorial vertical method (NF V 45-008, Poissons transformés — Méthode pour le dénombrement de *Listeria monocytogenes* aux faibles niveaux de contamination dans le saumon fumé et la truite fumée. 2009).

The developed method allowed us to investigate the effect of the initial *L. monocytogenes* density on the growth parameters of the pathogen in cold-smoked salmon. Depending on the experimental conditions, we found a significant effect of the inoculum size, both on lag phase duration, and on the maximal population attained. Moreover, this effect was dependent on a complex set of interactions. Impacting factors included the cells physiological state, the background microflora, the texture of the media and the packaging system.

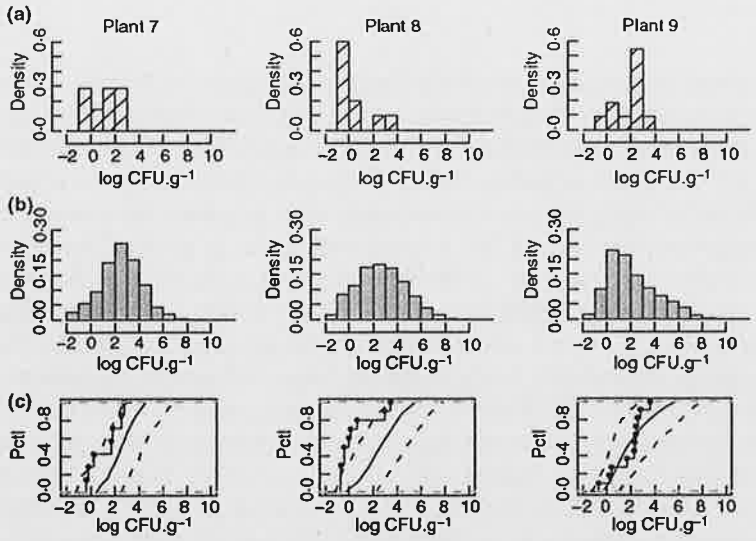


Growth curves of L. monocytogenes and background microflora (+) at 8 °C, under various conditions: aerobic conditions, gelatine medium, injured inoculum (e), cold-smoked salmon, uninjured inoculum (b). For each condition, growth curves were performed at various initial L. monocytogenes population density: low inoculum (Δ), average inoculum (☺), high inoculum (•). In each figure, the curves are fitted using the logistic model with delay.

The method was then used to investigate *L. monocytogenes* contamination and behaviour in naturally contaminated French cold-smoked salmon (CSS) to include data in a quantitative risk assessment. Between 2001 and 2004, *L. monocytogenes* was detected in CSS produced by nine French plants, with different prevalence (from 0% to 41%). The initial contamination, was low (92% of contaminated products below 1 CFU/ g) Most enumeration results were negative, < 0.2 CFU/g. Other values were relatively low, as only 8% of the contaminated products reached or exceeded 1 CFU /g. The highest observed level was 7 CFU/ g. Each of the samples testing positive for *L. monocytogenes*, was again examined after the product had been stored in resealed vacuum packs and kept first at 4°C during 8–15 days and then for the last 7 days at 8°C. In the second examination, 17% of the contaminated products exceeded 100 CFU/g, and the highest contamination level detected was 2800 CFU/ g.

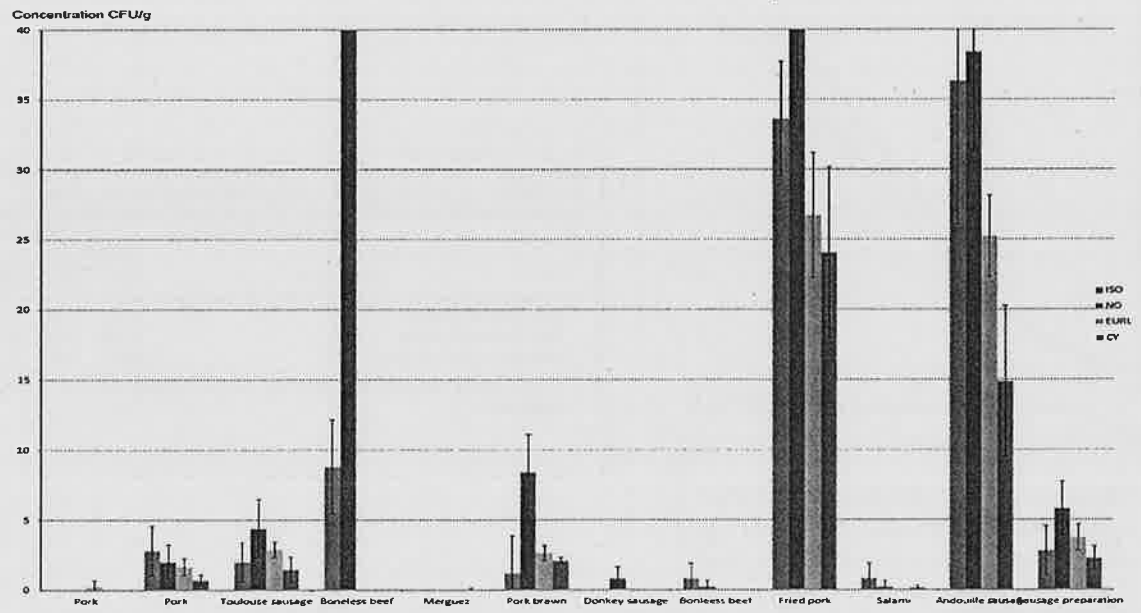
For each flora and each plant separately, a log normal distribution was adjusted on the initial contamination levels, using a maximum likelihood method for censored data. From these initial distributions, the competitive evolution of *L. monocytogenes* concentration and mesophilic aerobic flora concentration in contaminated products during the storage trial was predicted using exponential primary growth model (without lag), and square root secondary model. Parameters were estimated by Bayesian inference. The Jameson effect was described by the stopping of the growth of both populations as soon as one of them has reached the maximum population density in the product. For each parameter, variability and uncertainty were modelled by two-dimensional Monte Carlo simulation.

Growth was consistent with results of a predictive model including microbial competition. However, the model appeared partly fail-safe, particularly for plant 8. Indeed, the predictions overestimated growth. This may be explained by the fail-safe hypothesis of null lag times in the growth model. In storage trials, the naturally occurring stressed cells, in low numbers, may have longer lag times than high inoculums of cells in challenge tests.



Predicted and observed final contamination levels of *Listeria monocytogenes* in naturally contaminated CSS for each plant (7, 8 and 9). (a) Histogram of observations (dashed bars), see Table 2, negative enumeration results being excluded. (b) Histogram of predictions (grey bars), i.e. the output of the growth model with Jameson effect (Delignette-Muller *et al.* 2006), variability and uncertainty confounded. (c) Cumulative distributions of observations and predictions. *Reading key:* 50% of the observations for plant 7 are below 1·7 log CFU g^{-1} (49 CFU g^{-1}), which is then the observed 50th percentile (Pctl), or median. Using the model, the point-estimate of this 50th Pctl is 2·5 log CFU g^{-1} (330 CFU g^{-1}), with a 95% uncertainty interval of (0·8–4·3) log CFU g^{-1}.

Finally, the filtration method was evaluated with several other categories of foods naturally contaminated with *L. monocytogenes* and found to provide more precise results than modified reference EN ISO 11290-2. In most cases, it enabled to examine a greater quantity of food thus greatly improving the sensitivity of the enumeration. However, it was hardly applicable to some food categories because of filtration problems and background microbiota interference. Therefore, we evaluated EN ISO 11290-2 method and three modifications of it with lower threshold levels for enumeration in terms of performances and practical limitations for use. Two of the methods, the filtration method previously described and the Cyprus protocol, use membrane filtration to obtain a more concentrated test suspension, and the third, called the Norway protocol, uses less diluent spreading 2 ml of a 1:2 food suspension. This study included foods naturally contaminated with *L. monocytogenes* at concentrations of 0.2-80 CFU/g. All methods yielded valid results with good repeatability. The Norway protocol, though a (not significant) tendency for overestimation, was the least laborious method and gave good results even for samples that could not be filtered.

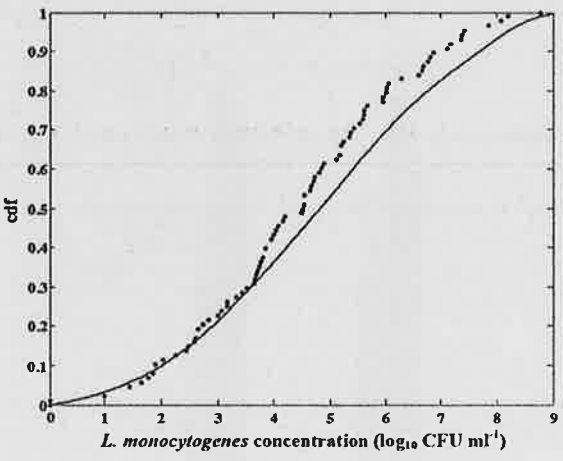


Comparison of enumeration results for Listeria monocytogenes obtained using the alternative methods filtration (EURL), Cyprus (CY) and Norway (NO)) and the modified reference method (ISO) on naturally contaminated meat products (mean and standard deviation).

Studies on detection

Manufacturers are faced with two problems in complying with the European Regulation. Firstly, since foods contaminated with *L. monocytogenes* typically contain low levels of cells and contamination can be sporadic during the production run, it necessitates the testing of multiple samples from single batches. Secondly, comprehensive environmental and product testing for *L. monocytogenes* is expensive, and represents a significant additional cost particularly for small enterprises. One approach to reduce the economic burden associated with comprehensive testing for *L. monocytogenes* is pooling multiple samples following the pre-enrichment step. "Wet pooling" is the combining of multiple pre-enriched samples into a single sample which is then used in a rapid detection method or for the completion of the standard method. The advantage of wet pooling is that it can significantly reduce the costs per sample in a sampling program, but little information is currently available on the impact of sample preparation and subsequent handling. The current standard EN ISO 6887-1 for the preparation of test samples for microbiological analyses now includes a general approach and experimental design for sample pooling (Annex D verification protocol for pooling samples). Specifically this allows either pooling of test sample portions or enrichment broths, in order to reduce analytical costs. However, it must be verified that either practice has no impact on method performance. Unfortunately, this type of study is not only laborious but it must also be conducted for each pathogen within each specific food matrix.

A stochastic model describing the growth of *L. monocytogenes* during enrichment in half Fraser was developed for the purpose of estimating the effects of modifications to the first enrichment step of the EN ISO 11290-1 detection method. Information pertaining to the variability of growth rates, physiological state of the cell, and the behavior of individual cells contaminating the food were obtained from previously published studies. The model was validated by comparing its predictions to numbers of *L. monocytogenes* occurring in 88 naturally contaminated foods following pre-enrichment. Then, we used this model to investigate the impact of wet pooling on the performance of the Standard method. Simulations representative of the natural contamination observed for smoked salmon were based on the results of European baseline survey of 2010-2011. The model-estimated *L. monocytogenes* levels following individual enrichment or following the pooling of five preenrichment broths where only one would be contaminated were compared. The model indicated a 10% loss of method sensitivity resulting from wet pooling. The model also predicted a 5% decrease in the sensitivity of the method when the duration of the enrichment was reduced from 24 to 22 h.



Observed (.) and simulated (solid line) cumulative distributions (cdf) of L. monocytogenes concentrations in half Fraser broth after an enrichment period of 24 h at 30 °C.

Probability to exceed the detection threshold in half Fraser broth according to the enrichment step duration or when pooling five smoked salmon samples.

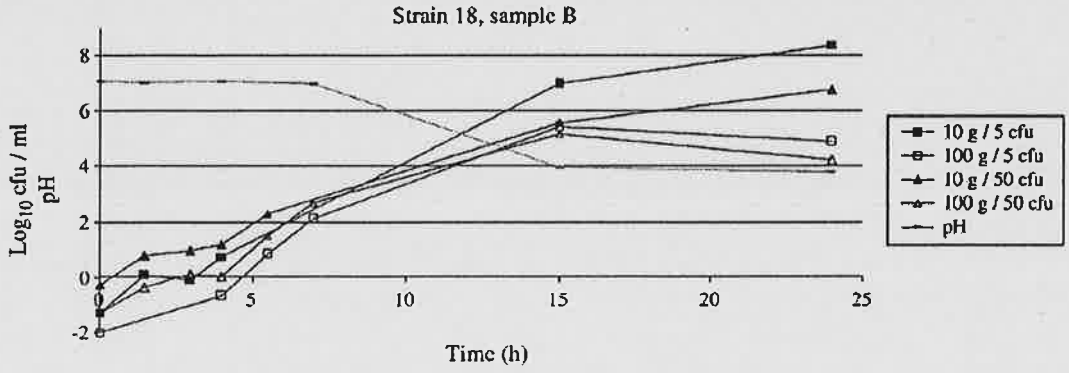
	Enrichment duration (h)	Detection threshold (CFU mL^{-1})		
		10^2	10^3	10^4
Individual enrichment	24	0.704	0.567	0.414
Individual enrichment	22	0.652	0.497	0.343
Pooled enriched portions[a]	24	0.612	0.458	0.318

[a] Five pooled samples and only one sample among the five is assumed contaminated.

Therefore, with this limited loss of 10%, validation of the performance of EN ISO 11290-1 with or without pooling through the trial design recommended in Annex D cannot be applied with an acceptable level of statistical confidence. In fact, the statistical power of this approach is quite limited. For example, at a probability approaching 0.8 for an observed 10% reduction in sensitivity, approximately 300 trials would be required (https://www.stat.ubc.ca/~rollin/stats/ssize/b2.html). On a practical basis, this means that these trial requirements are only adequate under conditions where wet pooling has large effects result. It is important for quality assurance managers and regulators to understand these limitations of the validation protocol. Together, these findings raise an important question regarding what is an acceptable level of risk. In fact, there are clear advantages in terms of labor and costs associated with wet pooling. The decrease in method sensitivity can be counterbalanced to some extent by increasing the number of samples analyzed and thus the probability to sample a positive unit in a batch. Additionally, these findings also helped fixing the minimum duration of pre-enrichment to 24h, in EN ISO 11290-1 detection method.

The problem is similar for the detection of *Cronobacter* : the food safety criteria is absence in 10 g, for PIF and dried dietary foods for special medical purposes, intended for infants below 6 months of age. The sampling plan requires 30 sample units to be tested in a batch. For greater convenience and in order to reduce analysis cost, a common practice in the PIF industry is to pool samples at a constant dilution rate, in order to perform a single first pre-enrichment and subsequent analysis ("dry pooling"). For example the analysis of one 100 g pooled sample diluted in 900 g of buffered peptone water (BPW), instead of ten samples of 10 g diluted each in 90 g of BPW (which represents the first enrichment step of the Standard detection method) is quite common. However, the consequences on the sensitivity of *Cronobacter* detection are not evident.

We evaluated the impact of pooling on the growth of *Cronobacter* and PIF background microflora in samples undergoing pre-enrichment culturing in BPW. Growth of the pathogen was monitored by direct plating onto selective agar or by using a membrane filtration method. The evolution of the total bacterial population of the PIF was monitored from a qualitative and quantitative point, using molecular (temporal temperature gradient gel electrophoresis : TTGE and denaturing gradient gel electrophoresis: DGGE) or classical microbiological methods. Results showed that pooling had a negative impact on the maximum population of *Cronobacter* attained. This suggests strong bacterial interactions with the PIF background microflora. From our assays, we concluded that *Bacillus spp.* represented the most abundant competitors in the PIF samples undergoing pre-enrichment, responsible for *Cronobacter* overgrowth. The 100 g samples generally allowed a more rapid and greater evolution of total microflora. In the PIF samples, bacteria are submitted to dry stress, and this physiological state is likely to enhance the distribution of individual cell growth parameters within each strain, notably lag time. Given the very low contamination levels, we assumed that the 100 g samples facilitated a selection of more rapid and abundant cells of background microflora initially present in the sample. Since the initial *Cronobacter* concentration is lower in 100 g than in 10 g samples, its level attained when growth ceases is likely to be lower. These findings suggest that, in some cases, the practice of pooling samples may affect the performance of the detection method.

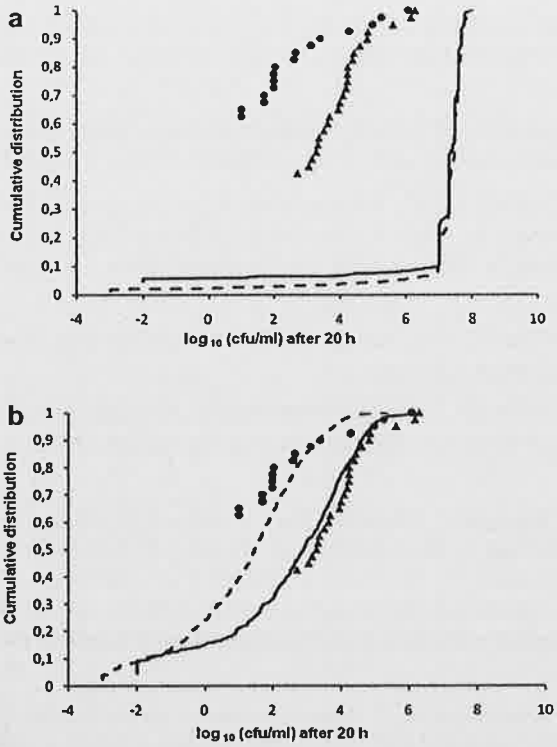


Cronobacter evolution in PIF samples undergoing non-selective enrichment culturing: impact of samples pooling.

Knowing how their long-term presence in PIF, and subsequent stress, affect the variability of single-cell lag times is important in assessing growth of low levels of *Cronobacter* in the pre-enrichment step of the standard detection method. Recently, a model for characterizing individual cell lag time distribution from population growth parameters was developed for other food-borne pathogenic bacteria such as *L. monocytogenes*. We confirmed this model's applicability to *Cronobacter* by comparing the mean and the standard deviation of individual cell lag times to populational lag times observed with high initial concentration experiments.

We also validated the model in realistic conditions by studying growth in powdered infant formula decimally diluted in BPW: 40 bags of 10 g and 40 bags of 100 g (mimicking a pooling of 10 samples of 10 g) of product were prepared and homogenized in sterile BPW diluent pre-warmed to 37 °C (1 in 10 dilution). Each sample was inoculated with a BPW suspension of freeze-dried *C. sakazakii* type strain ATCC 29544, at an expected contamination level of 4 cells per bag. All bags were incubated at 37 °C in a water bath and enumerated after 8 h and 20 h by plating onto selective agar. Observed and simulated *Cronobacter* distributions for preenriched 10 g samples and 100 g samples showed good agreement for 8 h incubation at 37 °C. After 20 h, observed vertical distributions were much lower than the predicted values of log counts, and this phenomenon was more emphasized for the 100 g samples. We attributed this difference to bacterial interactions. Indeed, background flora reached its stationary phase after approximately 9 h of enrichment. To better explore this phenomenon, we used the same previous simulations but with *Cronobacter* growth ceasing at 9h, when the total microflora attained its maximum concentration, in the hypothesis of a Jameson effect. Taking into account bacterial competition improved the prediction for both for 10 g bags and for 100 g bags. Furthermore, for the 100 g samples, the initial pathogen concentration was smaller than for the 10 g samples, which emphasized the negative impact of the early ceasing of *Cronobacter* growth, due to bacterial interactions. Indeed, after 20 h of preenrichment, which is the duration recommended in the Standard method (18 +/-2 h), 80% of the 100 g samples contained less than 100 CFU/ml, whereas 80% of the 10 g samples contained less than 10000 CFU/ml. This stressed the negative impact of pooling on *Cronobacter* growth and detection.

The EN ISO 22964 method is based on pre-enrichment in non-selective BPW, followed by a selective enrichment procedure and plating on a chromogenic selective isolation agar. The revision introduced the use of the Cronobacter Screening Broth (CSB) as selective broth. CSB allows presumptive *Cronobacter*-positive samples to be identified before the selective plating step, by the inclusion of sucrose and the pH indicator bromocresol purple. Whilst *Cronobacter spp.* can ferment sucrose, most other *Enterobacteriaceae*, which grow as false positives on chromogenic agars, cannot. Consequently, a yellow/orange change in coloration of the broth after incubation (due to sucrose fermentation) indicates presumptive *Cronobacter*. One must note that for a slow strain, a minimum of 10^4 CFU/ml in BPW may be required after pre-enrichment to ferment CSB. We calculated the probabilities of transferring an effective concentration of 10^2 CFU/ml bacteria in CSB. Though the impact of pooling on colour change was negative, even without pooling, we observed significant probabilities of false negatives. The revised validated Standard now prescribes a plating of all CSB broths, even if no change of colour occurred.



Observed distributions of *C. sakazakii* for 10 g (▲) and 100 g (●) samples at 20 h, Simulated distributions (a) without taking into account the interaction with background microflora for 10 g (—) and for 100 g (- - -); (b) with the interaction for 10 g (—) and for 100 g (- - -).

Conclusions

The development of more sensitive enumeration methods, and the study of individual cell variability and impact of background microflora allowed us to better understand the behavior of *L. monocytogenes* and *Cronobacter* in food and in enrichment procedures. The low and uneven distribution of the pathogens between samples indicates that easy-to use and efficient sampling and pooling procedures should be developped to better estimate the contamination.

Bibliography

Anonymous (2000). Rapport de la commission d'étude des risques liés à Listeria monocytogenes, juillet 2000, AFSSA. Maisons Alfort : Agence française de sécurité sanitaire des aliments, 143 p.

Anonymous (2005). Commission regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Off. J. Eur. Union L338 (1).

Anonymous (2006) Enterobacter sakazakii and Salmonella in Powdered Infant Formula: Meeting Report. Microbiological risk assessment series 10. Geneva and Rome: World Health Organisation-Food and Agriculture Organisation of the United Nations. Available at: http://www.who.int/foodsafety/publications/mra10/en/.

Anonymous (2007a). The commissions of the European communities, Commission Regulation (EC) No 1441/2007 of 5 December 2007 amending Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. Official Journal of the European Union, L322, 12-29.

Anonymous (2007b). European Food Safety Authority (EFSA) Scientific opinion of the panel on biological hazards on a request from the European Commission on Request for updating the former SCVPH opinion on Listeria monocytogenes risk related to ready-to-eat foods and scientific advice on different levels of Listeria monocytogenes in ready-to-eat foods and the related risk for human illness. EFSA Journal 59:1-42.

Anonymous (2010). Commission regulation (EU) No. 365/2010 of 28 April 2010 amending regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs as regards Enterobacteriaceae in pasteurised milk and other pasteurized liquid dairy products and Listeria monocytogenes in food grade salt. Off. J. Eur. Comm. L107, 9–11.

Anonymous (2012). European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC). The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2010. EFSA Journal 10(3):2597.

Anonymous (2013). Analysis of the baseline survey on the prevalence of Listeria monocytogenes in certain ready-to-eat (RTE) foods in the EU, 2010-2011. Part A: Listeria monocytogenes prevalence estimates. EFSA J. 11, 3241.

Anonymous (2016). ISO 6887-1 Microbiology of Food and Animal Feed – Preparation of Test Samples, Initial Suspension and Decimal Dilutions for Microbiological Examination - Part 1: General Rules for the Preparation of the Initial Suspension and Decimal Dilutions. International Organisation for Standardisation, Geneva, Switzerland.

Anonymous (2017a). EN ISO 22964:2017 Microbiology of the Food Chain — Horizontal Method for the Detection of Cronobacter spp. International Organization for Standardization, Geneva.

Anonymous (2017b). EN ISO 11290, Microbiology of the Food Chain — Horizontal Method for the Detection and Enumeration of Listeria monocytogenes and of Listeria spp. International Organization for Standardization, Geneva.

Anonymous (2018). EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), Ricci A, Allende A, Bolton D, Chemaly M, Davies R, Fernández Escámez PS, Girones R, Herman L, Koutsoumanis K, Nørrung B, Robertson L, Ru G, Sanaa M, Simmons M, Skandamis P, Snary E, Speybroeck N, Ter Kuile B, Threlfall J, Wahlström H, Takkinen J, Wagner M, Arcella D, Da Silva Felicio MT, Georgiadis M, Messens W, Lindqvist R. 2018. Scientific Opinion on the Listeria monocytogenes contamination of ready-to-eat foods and the risk for human health in the EU. EFSA Journal 16(1):5134, 173 pp.

Augustin JC, Kalmokoff M, Ells T, Favret S, Desreumaux J, Decourseulles Brasseur E, Gnanou Besse N. (2016) Modeling the behavior of Listeria monocytogenes during enrichment in half Fraser broth; impact of pooling and the duration of enrichment on the detection of L. monocytogenes in food. Food Microbiology, 60:131-6.

Barre L., Brasseur E., Doux C., Lombard B., Gnanou-Besse N (2015). Sensitive enumeration of Listeria monocytogenes and other Listeria species in various naturally contaminated matrices using a membrane filtration method. Food Microbiology, 48 (2015) 171-177.

Barre L., T. Skjerdal, E. Brasseur, G. T. Papageorgiou, E. M. Odiatou, H. Bulawova, A. Auvolat, S. Favret, B. Lombard and N. Gnanou Besse. (2020). Comparison of the ISO method and three modifications of it for the enumeration of low concentrations of Listeria monocytogenes in naturally contaminated foods. Euroreference 4, Juin 2020.

Beaufort A., S. Rudelle, N. Gnanou-Besse, M.T. Toquin, A. Kerouanton, H. Bergis, G. Salvat, M. Cornu (2007). Prevalence and growth of Listeria monocytogenes in naturally contaminated cold smoked salmon. Letters in Applied Microbiology,44, 406-411.

Bennour Hennekinne R., L. Guillier, L. Fazeuilh, T. Ells, S. Forsythe, E. Jackson, T. Meheut, N. Gnanou Besse (2018). Survival of Cronobacter in powdered infant formula and their variation in biofilm formation. Letters in Applied Microbiology Jun;66(6):496-505.

Bennour Miled R., L. Guillier, S. Neves, J.-C. Augustin, P. Colin, N. Gnanou Besse (2010). Individual cell lag time distributions of Cronobacter (Enterobacter sakazakii) and impact of pooling samples on its detection in powdered infant formula. Food Microbiology, 28, 648-655.

Bennour Miled R., S. Neves, N. Baudouin, B. Lombard, V. Deperrois, P. Colin and N. Gnanou Besse (2010). Impact of pooling powdered infant formula samples on bacterial evolution and Cronobacter detection. International Journal of Food Microbiology,138, 250-259.

Buchanan RL, Gorris LGM, Hayman MM, Jackson TC, Whiting RC. 2017. A review of Listeria monocytogenes: An update on outbreaks, virulence, dose-response, ecology, and risk assessments. Food Control, 75:1-13, 10.1016/j.foodcont.2016.12.016

Caubilla-Barron, J. and Forsythe, S. (2007) Dry stress and survival time of Enterobacter sakazakii and other Enterobacteriaceae. J Food Protect 70, 2111–2117.

Cornu, M. (2000) Modelling the competitive growth of Listeria monocytogenes and food flora in situ. Acta Hortic 566, 151– 157.

De Benito A., N. Gnanou Besse, I. Desforges, B. Gerten, B. Ruiz, D. Tomás (2019). Validation of standard method EN ISO 22964:2017 - Microbiology of the food chain - Horizontal method for the detection of Cronobacter spp. International Journal of Food Microbiology. 288, 47-52.

De Noordhout, M.C., Devleesschauwer, B., Angulo, F.J., Verbeke, G., Haagsma, J., Kirk, M., Havelaar, A., Speybroeck, N., 2014. The global burden of listeriosis: a systematic review and meta-analysis. Lancet Infect. Dis. 14, 1073e1082.

Delignette-Muller, M.L., Cornu, M., Pouillot, R. and Denis, J.B. (2006) Use of Bayesian modelling in risk assessment: application to growth of Listeria monocytogenes and food flora in cold-smoked salmon. Int J Food Microbiol 106, 195–208.

Forsythe, S.J., Dickins, B. and Jolley, K.A. (2014) Cronobacter, the emergent bacterial pathogen Enterobacter sakazakii comes of age; MLST and whole genome sequence analysis. BMC Genom 15, 1121.

Gnanou Besse N., A. Beaufort, S. Rudelle, C. Denis and B. Lombard (2008). Evaluation of an enumeration method for Listeria monocytogenes at low contamination levels in cold-smoked salmon. International Journal of Food Microbiology, 124, 271-274.

Gnanou Besse N., B. Lombard, L. Guillier, D. François, K. Romero, S. Pierru, L. Bouhier, P. Rollier (2019). Validation of standard method EN ISO 11290 - Part 1 - Detection of Listeria monocytogenes in food. International Journal of Food Microbiology. 288, 13-21.

Gnanou Besse N., N. Audinet, A. Beaufort, P. Colin, M. Cornu and B. Lombard (2004). A contribution to the improvement of Listeria monocytogenes enumeration in cold-smoked salmon. International Journal of Food Microbiology, 91, 119-127.

Gnanou Besse N., N. Audinet, A. Kérouanton, P. Colin and M. Kalmokoff (2005). Evolution of Listeria populations in food samples undergoing enrichment culturing. International Journal of Food Microbiology, 104, 123-134.

Gnanou Besse N., N. Audinet, L. Barre, A. Cauquil, M. Cornu and P. Colin (2006). Effect of the inoculum size on Listeria monocytogenes growth in solid media. International Journal of Food Microbiology, 110, 43-51.

Gnanou Besse N., S. Favret, J. Desreumaux, E. Brasseur and Kalmokoff M. (2016). Evaluation of reduction of Fraser incubation by 24h in the EN ISO 11290-1 standard on detection and diversity of Listeria species. International Journal of Food Microbiology, 2 ;224:16-21.

Guillier, L., Augustin, J.C., 2006, 2008. Modelling the individual cell lag time distributions of Listeria monocytogenes as a function of the physiological state and the growth conditions. International Journal of Food Microbiology 111, 241-251 and its erratum

Iversen, C., Mullane, N., McCardell, B., Tall, B.D., Lehner, A., Fanning, S., Stephan, R. and Joosten, H. (2008) Cronobacter gen., a new genus to accommodate the biogroups of Enterobacter sakazakii, and proposal of Cronobacter sakazakii gen. nov., comb. nov., Cronobacter malonaticus sp. nov., Cronobacter turicensis sp. nov., Cronobacter muytjensii sp. nov., Cronobacter dublinensis sp. nov., Cronobacter genomospecies 1, and of three subspecies, Cronobacter dublinensis subsp. dublinensis subsp. nov., Cronobacter dublinensis subsp. lausannensis subsp. nov. and Cronobacter dublinensis subsp. lactaridi subsp. nov. Int J Syst Evol Microbiol 58, 1442–1447.

Joosten, H., Iversen, C., 2009. Development of a CEN-ISO horizontal standard method for detection of Cronobacter. Cronobacter 2009: First international meeting on Cronobacter, Dublin, Ireland, January, 22e23th.

Osaili, T. and Forsythe, S. (2009) Desiccation resistance and persistence of Cronobacter species in infant formula. Int J Food Microbiol 136, 214–220.

Pouillot, R., Miconnet, N., Afchain, A.L., Delignette-Muller, M.L., Beaufort, A., Rosso, L., Denis, J.B. and Cornu, M. (2007) Quantitative risk assessment of Listeria monocytogenes in French cold-smoked salmon. I. Quantitative exposure assessment. Risk Anal

Rollier P., B. Lombard, L. Guillier, D. François, K. Romero, S. Pierru, L. Bouhier, N. Gnanou Besse (2019). Validation of standard method EN ISO 11290-part 2 for the enumeration of Listeria monocytogenes in food. International Journal of Food Microbiology. 288, 22-31.

Ross, T., Dalgaard, P. and Tienungoon, S. (2000) Predictive modelling of the growth and survival of Listeria in fishery products. Int J Food Microbiol 62, 231–245.