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## Next generation of microbiological risk assessment: Potential of omics data for exposure assessment

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### ABSTRACT

In food safety and public health risk evaluations, microbiological exposure assessment plays a central role as it provides an estimation of both the likelihood and the level of the microbial hazard in a specified consumer portion of food and takes microbial behaviour into account. While until now mostly phenotypic data have been used in exposure assessment, mechanistic cellular information, obtained using omics techniques, will enable the fine tuning of exposure assessments to move towards the next generation of microbiological risk assessment. In particular, metagenomics can help in characterizing the food and factory environment microbiota (endogenous microbiota and potentially pathogens) and the changes over time under the environmental conditions associated with processing, preservation and storage. The difficulty lies in moving up to a quantitative exposure assessment, because the development of models that enable the prediction of dynamics of pathogens in a complex food ecosystem is still in its infancy in the food safety domain. In addition, collecting and storing the environmental data (metadata) required to inform the models has not yet been organised at a large scale. In contrast, progress in biomarker identification and characterization has already opened the possibility of making qualitative or even quantitative connection between process and formulation conditions and microbial responses at the strain level. In term of modelling approaches, without changing radically the usual model structure, changes in model inputs are expected: instead of (or as well as) building models upon phenotypic characteristics such as for example minimal temperature where growth is expected, exposure assessment models could use biomarker response intensity as inputs. These new generations of strain-level models will bring an added value in predicting the variability in pathogen behaviour. Altogether, these insights based upon omics techniques will increase our (quantitative) knowledge on pathogenic strains and consequently will reduce our uncertainty; the exposure assessment of a specific combination of pathogen and food will be then more accurate. This progress will benefit the whole community of safety assessors and research scientists from academia, regulatory agencies and industry.

### 1. Introduction

In the food safety arena, exposure assessment (EA) is one of the four steps of risk assessment, itself belonging to the broader risk analysis

paradigm, along with risk management and risk communication (glossary provided in Table S1). This paradigm was set initially for chemical risks (National Research Council, 1983) and afterwards adopted for microbiological risks. The objective of EA is to evaluate

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qualitatively and/or quantitatively the likely intake of biological, chemical, and physical agents *via* food, as well as exposures from other sources if relevant (Codex Alimentarius Commission, 1999). In microbiology, EA includes assessment of both (i) the level (prevalence and concentration) of microbial pathogens and/or microbial toxins in food and (ii) food consumption patterns. Factors affecting the level in foods are numerous. Among them, it is important to highlight the characteristics of the pathogenic agent, the microbial ecology of the food, the initial contamination of the raw material, the processing, packaging, distribution and storage of the foods (Codex Alimentarius Commission, 1999).

In 2009, although the separation of risk assessment and risk management was not challenged, a more interactive and comprehensive risk analysis framework was introduced (Levy, 2009). In this framework, risk assessment was preceded by an initial step of problem formulation and scoping by risk managers. It was also recommended to view risk assessment as a method for evaluating the relative merits of various options for managing risk. The importance of EA was then reaffirmed as within this step the influence of various operational options such as factors related to factory environment contamination, raw material quality, process and formulation design, are quantitatively evaluated (Lammerding and Fazil, 2000).

Technological developments in the field of microbiology captured under the term omics have significantly enhanced our understanding of the behaviour of microorganisms and particularly their physiological state. Omics technologies include genome-wide sequencing tools, genome-wide transcript and protein analysis and assessment of the metabolic profile of microorganisms (Zhang et al., 2010). Most of the omics studies performed so far have either not been designed for the purpose of food safety risk assessment (Brul et al., 2012; Pielaat et al., 2013), or have not been quantitative enough to be of direct use in exposure or risk assessment (Membré and Guillou, 2016). However, omics approaches could reveal patterns of responses that cannot be detected by classical methods and have the potential to ultimately uncover new and powerful methods to control hazards in food and feed (Pielaat et al., 2013). This may potentially bring more insight than just the usual ‘snapshot’ in the farm-to-fork contamination process analysis and therefore contribute to the next generation of EA. In particular, omics approaches could help in progressing towards the characterization of biological variability, which has a crucial importance in EA (Delignette-Muller and Rosso, 2000). Variability provides a mechanism for an organism to increase its range of responses to changing environmental conditions (Altschuler and Wu, 2010). Indeed the relationship between differences in gene expression and phenotypic variability gives an increasingly detailed insight into cellular responses to changing environments (MacNeil and Walhout, 2011).

The objectives of this paper are to illustrate how omics could make a difference in understanding the dynamics of pathogens in a complex food ecosystem, and in predicting pathogen behaviour variability. Advancements in the research activities of these two domains will be presented and discussed, through examples, with a special focus on industrial applications.

## 2. Microbial dynamics along the food chain

### 2.1. Ecology of food-associated microbial community

The conditions encountered during food processing and storage shape the composition of the food-associated microbial community and have a governing influence on the growth, persistence, and inactivation of a pathogen in the food (Boddy and Wimpenny, 1992). This was demonstrated for the microbiota of Italian Grana like cheese during manufacture and ripening (Alessandria et al., 2016), for seafood storage until spoilage (Chaillou et al., 2015), for meat (Chaillou et al., 2015; Ferrocino et al., 2016), beefsteak (De Filippis et al., 2013), beef carpaccio (Luquin et al., 2012) and broiler chicken (Nieminen et al.,

2012). The microbial communities interact in different ways. These interactions were investigated as (i) a win-win relationship or mutualism, where two species would exchange metabolic products to the benefit of both, (ii) loss-win or predator-prey relationship, such as host-parasite relationship, or (iii) loss-loss relationship where the species would produce antagonistic metabolites (Faust and Raes, 2012). Interactions between pathogenic species and other ecosystem microbiota are worth investigating to determine if some specific bacterial ecosystem patterns would favour or, in contrast, prevent the growth, survival and/or inactivation of pathogens. Indeed, it has been shown that some bacterial communities in cheese had a significant reducing effect on the growth of *Listeria monocytogenes* whereas, on the contrary, *L. monocytogenes* did not affect the growth of the other bacteria (Imran et al., 2013). The presence of some lactic acid bacteria (LAB, see Table S2 for acronyms) has also been shown to influence the growth or survival of pathogens (Portella et al., 2009; Szala et al., 2012) by exerting for instance an antagonistic effect through the production of bacteriocins (Arqués et al., 2015). In contrast, another group of spoilage organisms, namely pseudomonads, enhanced the growth of *L. monocytogenes* in the case of meat stored under different storage conditions (Tsigarida et al., 2000). It appears thus relevant to characterize the influence of the bacterial ecosystem on both the pathogen survival and growth. Bioprotective bacteria may exert an antagonist action against pathogens by limiting for example their growth through various inhibitory effects (e.g. substrate competition, production of antimicrobial compounds such as bacteriocins, organic acids, hydrogen peroxide), quorum sensing, or yet unknown mechanisms (Blana et al., 2011; Blana et al., 2015; Saraoui et al., 2016). More general studies on interactions between bacteria in food and along the food chain have just been initiated by assessing co-occurrence and co-exclusion relationships between predominant species from similarity-based network inference (Alessandria et al., 2016; Chaillou et al., 2015). In addition to co-occurrence and co-exclusion analysis, functional metagenomics techniques could be deployed to characterize the metabolic potential of the microbial communities and their possible capability to produce antagonistic or protective effects against pathogens (Illegheems et al., 2015; Jung et al., 2011).

For further inclusion in a farm-to-fork EA it is crucial to understand the link between the factory environment microbial community and pathogen growth, survival or decline. There are still too few studies in this research area. Fox et al. (2014) demonstrated the link between the microbial community of different sections of a food factory and the persistence/presence of *Listeria*. Four drains were examined in a meat manufacturing facility, with two classified as *Listeria*-positive and two classified as *Listeria*-negative. A 16S rRNA gene analysis revealed that 21 bacterial families were found only in *Listeria* positive drains; in contrast, *Janthinobacterium*, *Prevotella*, and *Pseudomonas* were more abundant in *Listeria*-negative drains. Co-culturing experiments with specific species supported this effect by demonstrating increased and decreased biofilm formation of *L. monocytogenes* in the presence of identified protagonists and antagonists, respectively. Fig. 1 illustrates possible pathogen interactions with other microbiota in food and factory environments, and their role in final exposure levels taking into account the processing steps. Microbial contaminants can originate from raw material, airborne contamination or contact surfaces that were improperly cleaned and/or disinfected. These contaminants can be commensal bacteria, spoilers or pathogens, and metagenetic analysis can help to characterize the composition of the food ecosystem throughout the manufacture and storage steps and to elucidate transmission routes (Fig. 1). The behaviour of each of these contaminants is known to be influenced by the process and storage conditions such as pH,  $a_w$  and temperature. When assessing the compliance with a Food Safety Objective (FSO), so far the pathogen behaviour (growth or inactivation) has been often quantified on its own, *i.e.* independently of the ecosystem (Fig. 1, “A-pathogen alone”). The next step would be a more comprehensive assessment of FSO compliance with various

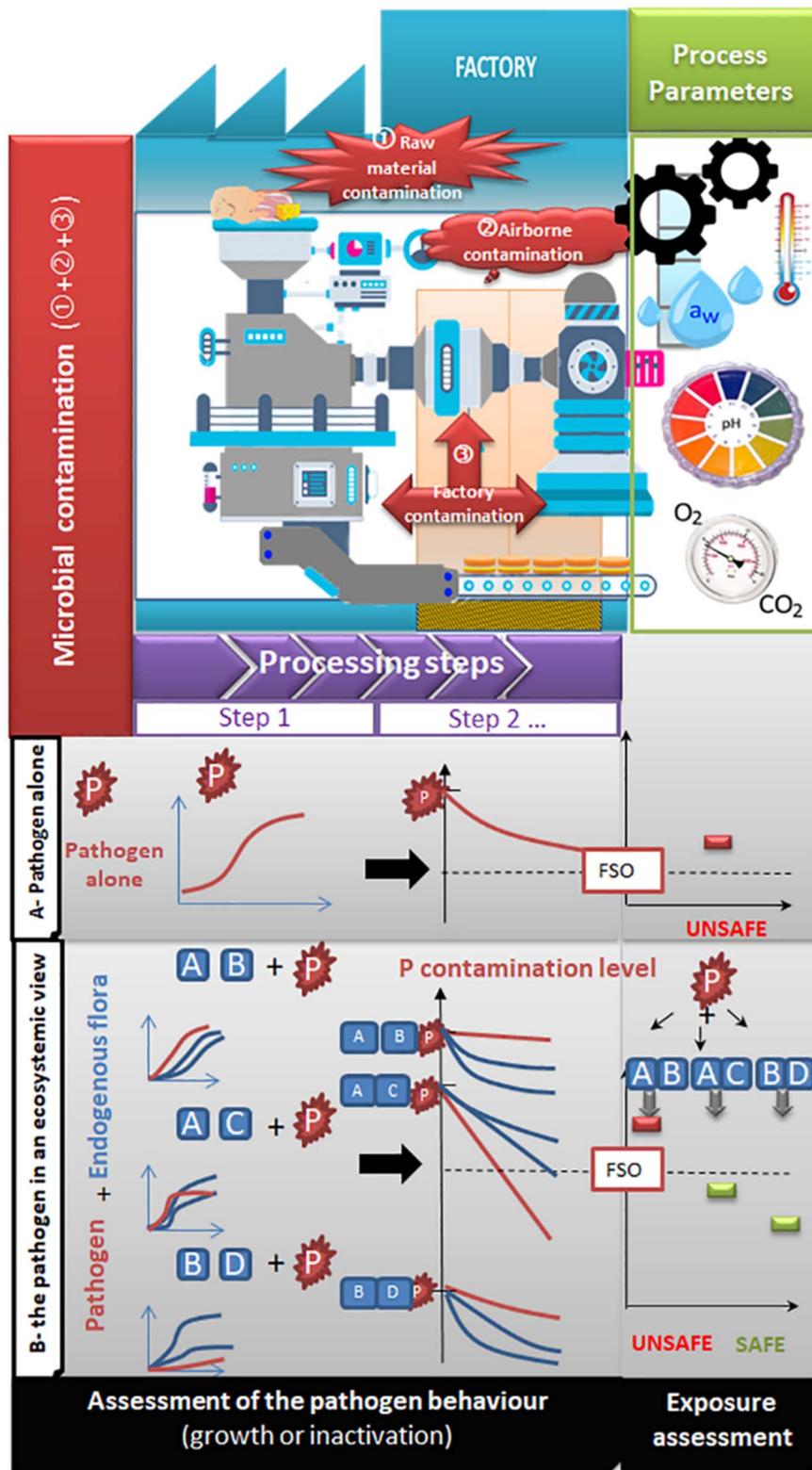


Fig. 1. Top of the figure. Schematic illustration of a food process, pointing out various sources of microbial contamination: ① Contamination from raw materials ② Airborne contamination and ③ Contamination from equipment and surfaces in factory. Process parameters such as temperature, food  $a_w$ , and pH, and concentration of oxygen and carbon dioxide in food packaging influence microbial growth and survival.

Bottom of the figure. Illustration of growth during the 1st step of the process and inactivation following the 2nd step of the process. A - The pathogen is considered alone and not affected by the presence of other bacteria. Studies performed on pure pathogen culture predict a FSO exceedance. B - The pathogen is considered among other bacterial flora A and B, A and C, or B and D. Growth and survival of the pathogen are differently influenced by these flora. In some cases (Pathogen + (A,C) or (B,D)), the presence of the flora are detrimental to growth and/or survival of the pathogen, which leads to FSO compliance. In other cases (Pathogen + (A,B)) the presence of the flora favours growth and survival of the pathogen, which leads to FSO exceedance.

scenarios depending on the microbiota (Fig. 1, “B-pathogen in an ecosystemic view”).

The food matrix structure should also be considered in the assessment because the physical structure and the chemical composition in time and space are heterogeneous. There are environments where various compounds, e.g. metabolites and/or molecules, are released but not at the same concentrations over the time and space, meaning that microbial adaptation to environmental stress in space and in time is a

continuous process (Boddy and Wimpenny, 1992; Skandamis et al., 2000). The spatial heterogeneity of the food matrix has not often been taken into account in EA: models have been generally built assuming that the cells are exposed to the same factor levels, e.g. the same concentration of signal molecules, in the whole solid food (Wilson et al., 2002). Studies on food matrix structure effects that take nutrient diffusion into account as well as individual (local) cell level behaviour will be definitively beneficial to EA (Bridson and Gould, 2000;

Koutsoumanis and Aspridou, 2017; Abee et al., 2016). However, including these extra pieces of information in a quantitative manner might be complex and difficult to achieve.

## 2.2. Modelling pathogen dynamics in food microbial ecosystems: Towards the next generation of predictive models

The first generation of predictive models was based on a simple principle. It was stated that microbial responses to given environmental conditions were reproducible and could therefore be further used to predict microbial growth or inactivation (McKellar and Lu, 2004). In predictive microbiology terminology, primary models describe microbial evolution with time, based on specific parameters such as the (growth or inactivation) rate, the lag time and the maximum population density. Secondary models describe the effects of the environmental conditions (e.g. temperature, pH,  $a_{wv}$ , organic acids) on the parameters of the primary models. Based on this approach, numerous models were developed for several pathogens to describe their growth and/or inactivation (Brul et al., 2007; Membré and Valdramidis, 2016). Similar models were also developed for spoilers, however, models predicting microbial interactions between pathogens and background food microorganisms are less common. These models considered the competition between *L. monocytogenes* and LAB in several matrices such as cottage cheese (Østergaard et al., 2015), pork meat products (Cornu et al., 2011) and lightly preserved seafood (Mejlholm and Dalgaard, 2007). Other studies modelled *L. monocytogenes* with other competitive and less characterized microorganisms such as biofilm microorganisms from wooden shelves of smear cheese (Guillier et al., 2008). Also, models addressing other pathogens such as *Yersinia enterocolitica* in co-culture with LAB (Janssen et al., 2006) and *Staphylococcus aureus* with starter culture in milk (Le Marc et al., 2009) were published. Some scientists modelled the effects of yeasts and moulds in competition with LAB in different matrices such as olives (Echevarria et al., 2010). These studies showed that, if the conditions were favourable for growth, the growth rate and lag time of the pathogen did not change in the presence of the competitive microorganisms. The maximum population density, however, was different. This was called the Jameson effect, and it could be summarized as a race between different microbial populations: when the environmental resources are depleted, the race is over, and the growth of each species in the population stops (Mellefont et al., 2008). The Jameson effect was reported by several authors (Le Marc et al., 2009; Mejlholm and Dalgaard, 2015). On the other hand, the effect of competitive microorganisms on pathogen inactivation is usually modelled indirectly. For example, the effect of starter cultures on the inactivation of *Salmonella* and *L. monocytogenes* in fermented dried sausages is taken into account through the pH drop and lactic acid concentration increase caused by LAB (Coroller et al., 2015; Mataragas et al., 2015a).

The next generation of predictive microbiology models is likely to include predictions of the behaviour of the ecosystem as a whole. From a modelling perspective, there are a number of questions to be answered, chief among these are: can a profile of the microbiota of a food matrix or other environment be used to predict the presence or absence of a pathogen or spoilage microorganism? Or given a specific microbial ecology, what growth dynamics are we likely to observe and can we predict this? An immediate follow-on question is then whether or not the microbiota can be modified in order to control the presence of an undesirable microorganism and can we propose efficient intervention strategies? More generally, which model adaptation or which new models are necessary to incorporate the ecosystem information in the EA? Predictions based on modelling of microbial communities have been reported for cheese fermentation (Mounier et al., 2008) and marine phage abundance (Hoffmann et al., 2007) using Lotka-Volterra equations (corresponding to a nonlinear system of differential equations) to model the predator-prey relationship of a simple ecosystem). Studying microbial dynamics with generalized Lotka-Volterra equations

requires the cell concentrations of the different taxa, the growth rates of each taxon and its abundance at different times, and the interaction strengths of the different community members, which can be estimated by using network inference (Faust and Raes, 2012). Overall, that means that realistically a prediction based Lotka-Volterra equation system cannot be applied to an ecosystem where many species are involved.

Incorporation of abiotic factors in network inference is possible and it enables the prediction of bacterial community patterns as a function of both biotic and abiotic factors. However, to be valuable, this means that database containing genomic, metagenomic or marker gene sequence data have to be populated with metadata (e.g. temperature and other environmental factor values), according to a specific standard (Yilmaz et al., 2011). Elaborating a format for contextual additional information has been initiated in the Minimum Information about any (X) Sequence - Genomic Standards Consortium (MixS-GSC) project (<http://gensc.org/projects/mixs-gsc-project/>) in which data describing 15 environments, including one dedicated to microbial material and biofilm, are available online. In a food safety context, for future use in EA, gathering metadata describing environmental conditions will require the definition of a standard for collecting the information and a very clear definition of the model inputs and outputs to build the database. Next, populating the database will involve a tremendous amount of work, which will require collaborations between different stakeholders including academia, industry and governments.

One project exploring this route is the Sequencing Alliance for Food Environments (SAFE) project recently commenced in Ireland. Here, foods and food environments provided by five contributing companies will be monitored over two years, examining both the dynamics of the microbiota (analysed using 16S rRNA metagenetics) and the traits of specific isolates (analysed using whole genome sequencing (WGS)). As both the microbiota and the specific isolates will be analysed from the same samples, there will be an opportunity to try and quantify or model the link between the two. 16S rRNA metagenetics will be used to quantify the relative abundance of the different species or genera of bacteria as a characterization of the microbiome of a particular environment within a manufacturing facility. WGS will be used to identify the specific strains of concern that exist in the same environment and their genotypes, and statistical modelling techniques will be used to link the two.

## 3. Predicting behaviour: towards building quantitative models based on biological insights

### 3.1. Microbial behaviour in a variable world

Within the biological and life related sciences, the term “behaviour” can be used to refer to the aggregate of acts, reactions and/or functioning that an organism, as an individual or as part of a system, produced in response to a particular circumstance and induced by stimuli or inputs from its internal or external environment (e.g. food characteristics, storage conditions, interaction with other organisms, the succession of the microbial community in a continuously modified food matrix). This response can be innate and/or acquired based on previous exposure. Such general definitions therefore include a wide variety of events that can be located at transcriptomic, proteomic, metabolomics and/or fluxomic levels within the cellular compartment (i.e. system biology). Furthermore, and particularly relevant for microbiological risk assessment, it also refers to the final outcome of all these cellular events in relation to phenotypic characteristics, such as resistance, survival, adaptation, growth, toxin production or virulence. Even if any of these responses are determined by the genomic potential of the organism, the environmental influence is crucial to trigger a given behaviour. These responses can be considered as input data for risk assessment models. They can be qualitative (e.g. growth is observed; resistance is acquired) or quantitative (e.g. the probability of observing growth is 0.01, the enhanced survival is 3 log CFU/g) and depending on

these, quantitative or qualitative microbiological risk assessments are performed.

Variability has an essential place in microbiological risk assessments and variability associated with microbial responses in foods can be classified into two types. The first type corresponds to environmental variability, which is a consequence of the natural diversity in food-related environments (pH, composition, preservative) and differences in the technological parameters of industrial processes (pasteurization temperatures, pressure level in HHP, etc.). Regarding variability in key product and process parameters, these can be measured at the research and development (R & D) phase to characterize variability and thus be incorporated into safe design before rolling out a new product to a manufacturing facility. However, in many cases direct measurement of certain key parameters is not always possible and other approaches such as mathematical modelling and biological indicators must be used (e.g. internal temperature profiles in aseptically processed foods containing particulates). Data on consumer practices and habits is often not widely available for many regions of the world. Therefore food companies must commission such studies privately. However, where the consumer phase plays a key role in food safety assurance (e.g. chilled or frozen microwavable foods containing raw ingredients), it is the responsibility of the manufacturer to validate the cooking instructions that will go on the pack during the R & D phase.

The second type of variability associated with microbial responses in food is the pathogen-related biological variability, which is associated with the natural variation existing between microbial sub-populations and between individuals inside the same microbial population. There are many sources of biological variability which must be considered, including pathogen strain or cell variability, variability in sources of contamination, and variability in a pathogen's behaviour, *i.e.* its ability to resist or adapt to food environment (Membré and Guillou, 2016). This multiplicity of sources of variability affects pathogen contamination level at the time of consumption and therefore has an impact on EA (Koutsoumanis et al., 2016). Extreme individual responses of single cells behaving as “outliers” (noise) of a larger homogeneous population and masked by adjacent cells showing an “average” behaviour may be revealed when cells are studied individually (Skandamis and Nychas, 2012; Koutsoumanis and Aspridou, 2017). Genomic similarities do not necessarily imply similarities in behaviour. Additionally, small genetic changes may result in large phenotypic differences (Metselaar et al., 2015), which pose a considerable challenge when it comes to quantitatively associating genomics and cellular behaviour with the extent of growth, survival or death of a microorganism, taking into account the biological variability. This has recently been illustrated by Abee et al. (2016), who simulated the fate of *L. monocytogenes* in a model food chain. They found that the population composition of this pathogen was significantly affected by the stresses encountered along the chain and the variant types initially present. Currently, it is common industry practice to use cocktails of microorganisms for challenge testing or surrogates for process validation. The choice of such organisms is often on the basis of historical knowledge and aims to represent the “expected variability” of resilient sub-populations that have been associated with incidents of contamination (IFT/FDA, 2003). This safe-side practice does not allow a straightforward re-use of challenge-test data in quantitative risk assessment studies which aims at estimating the actual risk, *i.e.* avoiding over or under estimation of the risk.

There is a need to understand and quantify the relative importance of variability factors across the food chain to determine which ones take higher relevance and have to be considered in EA (Den Besten et al., 2017). If strain variability was ultimately shown to be decisive among the other variability factors, the identification of “high risk” bacterial subpopulations should be first evidenced by omics techniques, to determine their distribution in the whole bacterial population. The assumption is that specific genetic material that renders a strain as “high-risk” is shared by other subpopulations deemed to be “high-risk” as well

(Berendsen et al., 2016). In this context, one option is to consider that strains or bacterial subtypes involved in outbreaks are implicitly “high risk”. When grouping of species in subgroups is justifiable based on mechanistic insights, then one could fine tune EAs taking into account the characteristics of these different subgroups following the example of Afchain et al. (2008). They proposed mean cardinal temperatures for six genetic groups of *Bacillus cereus*, and Carlin et al. (2013) reported the variability in cardinal growth parameters for these genetic groups. This quantitative information on behaviour of subgroups of species can be used to estimate the growth performance of the different subgroups, and when combined with information on robustness to lethal stresses, this will provide more precision in quantitative EA than when taking the species as a whole.

### 3.2. Biomarkers to predict variable behaviour

The drive to use more mechanism-based approaches for predicting microbial behaviour gave rise to the search for molecular biomarkers. In the context of food microbiology, a biomarker could be defined as a cellular compound or a structure of cellular compounds that can be measured in a food-borne pathogen, which will enable the prediction of the phenotypic behaviour of this pathogen. A biomarker has to be relevant, *i.e.* provide appropriate information on questions of interest and importance to food safety decision-makers; it has also to be valid, knowing that the use of invalid biomarkers can lead to invalid inferences and generalizations and ultimately to erroneous risk assessments (WHO, 2001).

#### 3.2.1. Biomarkers: promises and expectations

Prediction of phenotypic behaviour using cellular indicators is a key area of research (Brul et al., 2006; Kort et al., 2008; Greppi and Rantsiou, 2016). Quantitatively correlating microbial responses at the molecular level to observed phenotypes can provide mechanistic understanding of the behaviour and can suggest means for identifying cellular indicators for bacterial performance. Robustness to lethal stresses is known to vary highly between pathogenic species, and also between strains belonging to the same species. Recent work of Berendsen et al. (2015) demonstrated large differences in the heat resistance of spores of *Bacillus subtilis*, and they could group *B. subtilis* strains in two distinct groups based on spore heat resistance. They provided evidence that this was due to the presence of a mobile genetic element that was demonstrated to confer high-level heat resistance to spores. Genetic elements could function as absence/presence biomarkers for robustness to stresses, and a next step of interest would be to evaluate whether such genetic fingerprints could also function as genetic biomarkers for heat resistance in pathogenic sporeformers like *Bacillus cereus*. Interestingly, the genetic element that was demonstrated to be present in *B. subtilis* strains that produced high-level heat resistant spores was also found in *B. cereus* strains, but it needs to be investigated whether this has an influence on the heat resistance of these spores (Berendsen et al., 2016).

Adaptation to changing environments encountered along the food chain is known to induce a lag phase allowing the adaptation required for bacterial cells to begin to exploit new environmental conditions. This lag phase is known to be highly variable. The physiology of bacterial adaptation during the lag phase to repair damage and to prepare for growth initiation has not been well characterized (Rolfe et al., 2012), and metabolic processes involved in getting out of lag phase are fairly unknown. There is a lack of knowledge on recovery promoting factors, synthesis of cellular components necessary for growth initiation, and biomarkers that predict ‘coming-out of lag’. These biomarkers could give us useful mechanistic information on realistically predicting lag phase duration. However, direct application of this biological knowledge in practice would be fairly challenging because pathogens are present in very low concentrations, with high variability among the cells. Nevertheless, biological insights in lag phase duration might aid

in the further development and optimization of methods to detect pathogens in foods and environments.

Pathogens are exposed to suboptimal conditions along the food chain, and adaptation to suboptimal or stressful growth conditions has received much attention in the last few years, often with molecular mechanisms underlying stress adaptation during growth being identified. Mild stress conditions do not inactivate pathogens, but can instead trigger adaptation mechanisms in the pathogenic cells, which can confer protection to subsequent exposure to more unfavourable stress conditions in the environment or in the food (Alvarez-Ordóñez et al., 2015; Begley and Hill, 2015). The combination of omics technologies, such as transcriptomics, proteomics and metabolomics, have provided insight in critical adaptation mechanisms involved in stress adaptation, and the advantages and disadvantages of various methodologies were reviewed recently by Greppi and Rantsiou (2016). Alternative sigma factors such as SigB in Gram-positive pathogens and RpoS in Gram-negative pathogens play an important role in stress adaptation, and bacteria defective in *sigB* or *rpoS* are highly sensitive to food processing conditions (Abee and Wouters, 1999). Van Schaik and Abee (2005) pointed therefore to a potential role for sigma factors as biomarkers for stress resistance. Indeed, studies that aimed at finding potential stress response biomarkers demonstrated that the transcript of *sigB*, the corresponding SigB protein, or  $\sigma^B$  regulon members could function as biomarkers for stress response in *B. cereus* (Den Besten et al., 2010), *Bacillus weihenstephanensis* (Desriac et al., 2015) and *L. monocytogenes* (Mataragas et al., 2015b). Alvarez-Ordóñez et al. (2015) also pointed to the possible role of *rpoS* activity as an indicator for bacterial resistance, among others, in *Enterobacteriaceae*. Despite the clear role of this regulator in stress response, several studies described truncated RpoS in isolates of different pathogenic species of *Enterobacteriaceae* (Alvarez-Ordóñez et al., 2012; Robbe-Saule et al., 2003). Mutations in the *rpoS* gene are linked to reduced stress tolerance, but on the other hand, have been demonstrated to provide a growth benefit (King et al., 2004). Trade-off mechanisms affecting resistance to environmental stresses can be at the cost of slower growth and thus lower fitness (Ferenci, 2016). Indeed, these trade-off mechanisms shape intra- and interspecies diversity (Ferenci, 2016). Predictive determinants of robustness have been identified on various cellular levels, namely, transcripts, proteins, and enzymes (Den Besten et al., 2010; Den Besten et al., 2013; Desriac et al., 2013; Desriac et al., 2015; Mataragas et al., 2015b). Gene-expression is the first bacterial response to changing environments, but the often transient nature of mRNA expression might complicate direct correlation between expression of biomarkers and gained robustness (Desriac et al., 2013).

### 3.2.2. Current attempts of building predictive models based upon biomarkers

The ultimate goal of moving towards quantitative biomarkers to predict microbial behaviour is challenged by the inherent complexity of the regulation of stress response mechanisms and the corresponding expression of indicators and candidate biomarkers in the cell. Moreover, it questions the possibility of extrapolation of results between strains and species. Biomarkers that are quantitatively correlated to phenotypes of interest in EAs, like fitness and robustness, will be of indisputable significance for developing tools to screen for resistant or sensitive cells and will complement our quantitative empirical approach of predicting microbial behaviour. In terms of modelling approaches, without changing radically the model structure, this could lead to changes in the model inputs: instead of (or as well as) building models upon phenotypic characteristics such as for example minimal temperature where growth is expected, EA models could use biomarker response intensity as inputs. This is not yet done in the food microbiology domain, but in the medical health domain already numerous multigene signatures have been identified that aim to outperform traditional clinical prognostic markers to facilitate decision-making processes (Weigel and Dowsett, 2010).

Nonetheless, when different traits or phenotypes are related to different biomarkers, the risk modelling process can become more complex. For example, the study by Den Besten et al. (2010) identified different biomarkers relating to the adaptive behaviour and the robustness of *B. cereus* to lethal treatments. How to combine information of different biomarkers into EA is an issue that greatly depends on the level of knowledge that is available in relation to the microbial genome, metabolic reactions and the interrelation between them. If we assume independence (*i.e.* the presence of one does not imply the presence of the other and *vice versa*) between two biomarkers and 100% prediction capacity of biomarkers to determine the probability of finding a bacterial population showing two specific phenotypes; *e.g.*, a food contaminated with *B. cereus* from Group VI (low  $T_{min}$  according classification of Guinebretiere et al., 2010) and biomarkers related to adaptive stress conferring resistance to heat treatments (SigB, ClpC, *etc.*), the following generic equation could be applied.

$$P(A_j \cap B_i) = P(A_j) \cdot P(B_i) \quad (1)$$

where  $A_j$  stands for  $j^{th}$  phylogenetic group of *B. cereus* with  $j = 1, 2, \dots, 7$ , corresponding with the seven *sensu lato* groups of *B. cereus* (Guinebretiere et al., 2010);  $B_i$  corresponds to  $i^{th}$  biomarker associated with adaptive behaviour, with  $i = 1, 2, \dots, m$  representing each biomarker (SigB, ClpC, *etc.*).

Nonetheless, biomarkers seldom have a 100% prediction capacity and therefore, the attendant uncertainty should be considered in EA. In such cases a Bayesian approach can be a suitable mathematical technique to consider the level of uncertainty (*i.e.* belief) in biomarker prediction capacity (Hernández et al., 2015). Eq. (2) conceptually summarizes the Bayesian (inference) approach that could be applied in an EA model to determine the probability (or prevalence) of one phenotype ( $F_p$ ) knowing both the probability (or prevalence) of  $k$  biomarkers ( $B_k$ , with  $k = 1, 2, \dots, m$ ) and the probability of finding the biomarker  $B_k$  for the phenotype  $F_p$ . The former probability (*i.e.*  $P(B_k)$ ) would be determined by “field experiments”, while the latter probability ( $P(B_k | F_p)$ ) would be derived from “*in-vitro*” experiments as explained previously. Bayesian inference derives the posterior probability,  $P(F_p | B_k)$ , as the result from a prior probability and a “likelihood function” estimated from observations (second term in Eq. (2)).

$$P(F_p | B_k) = \frac{P(B_k \cap F_p)}{P(B_k)} = \frac{P(F_p) \cdot P(B_k | F_p)}{P(B_k)} \quad (2)$$

Models can become even more complicated, if dependencies between biomarkers exist, *i.e.* a biomarker is linked to the presence of other. For example, following our example, a recent study proposed the hypothesis of divergence of the generalized stress response (SigB) across the *B. cereus sensu lato* group (Scott and Dyer, 2012; Toby et al., 2014). Results from the study suggest that regulon SigB structure differs between Group 1–7, and strains from these seven groups could be assigned to four clades A through D. These differences in SigB are determinant in the stress response. Clades A and B showed an enhanced stress response to deleterious environmental conditions in comparison to clades C and D. When these traits are translated into a multi-biomarker dimension to take into account the whole system of genes involved, the network becomes more intricate and complex (Qiu et al., 2007), making a quantitative prediction of the phenotype difficult.

## 4. Exposure assessment and omics: a double challenge for the food industry

Several publications demonstrate that major food companies have adopted the microbiological risk assessment methodology in their safety assessments (Pujol et al., 2013; Tenenhaus-Aziza et al., 2014; Membré et al., 2015). Among the microbiological risk assessment elements, EA is particularly relevant since it allows transparent decision-making based on information directly related to operational metrics.

However, accurate EA usually requires a considerable amount of data, not only regarding intrinsic and extrinsic factors of the food product, but also related to the prevalence and the behaviour of microbial hazards. With the availability of new miniaturized sequencing equipment, pathogen surveillance and indicator trend analysis can be facilitated. That is why, besides academia and regulators, the food industry is also interested in omics tools, to gain more insight in the microbial ecology of the factory and how it relates to the product's ecology. This can help to assess the presence or the emergence of highly resistant micro-organisms to current preservative systems, and can also give a clear understanding and preferably, a quantitative estimate of the dominant species and their changes over time under the environmental conditions associated with processing, preservation and storage. It is also relevant to mention that spoilers are also a concern for the industry since their growth will lead to food that is unfit for consumption, and thus classified as unsafe according to the EU regulation framework (*i.e.* Regulation (EC) 178/2002, Art14).

The mechanistic approach for applying WGS in EA will require translating multidimensional genotypic data into reduced information on the phenotype to ultimately generate a metric that matches the stakeholders' requirements (Franz et al., 2016). This will probably need several future developments. However, an empirical approach can be foreseen to combine metagenomic outputs and predictive microbiology models. This can be achieved in two steps (Fig. 2). First, knowledge building is necessary. For a specific food product, several analyses would be performed at different stages within the factory then throughout the food chain. These analyses would include metagenomics and their corresponding bioinformatic analysis along with plate counting (or any other enumeration technique), but also food characterization (*e.g.* pH,  $a_w$ ) and temperature monitoring. The latter is fundamental metadata that must be collected alongside the metagenomic data for meaningful assessments. The metagenomic data can be used to extract relevant information, *e.g.* 16S rRNA sequences, to describe the microbial diversity and the relative abundance of species in the food product at several time points. When coupled with microbial enumerations, kinetics will be generated for the most prevalent species. Predictive microbiology models will then be used to fit the data and assess the growth or inactivation parameters. Those quantitative data will be stored in a database as well as some qualitative data to identify among the initial species of the ecosystem the ones that will be dominant over time given the ecosystem composition and the environmental conditions. Once this knowledge is built it will be possible to proceed with the predictions. To do so, the first step will be to run metagenomics experiments as well as plate counts (or any other enumeration technique) on the studied sample at a given point of time. Combining

the relative abundance information with the enumerations, taking into account the food and environmental characteristics and based on previous knowledge captured in the database, it will be possible to predict the dominant species and to simulate their behaviour (growth, inactivation) over time. However, pathogens, when present, are generally much less abundant than spoilers, starter or protective cultures, so this approach may be challenging for food safety assessments.

Delhalle et al. (2013) combined the use of predictive models and metagenetics to predict bacterial population changes in white pudding. To do so, the ecology of the product was studied at several times during the storage at both constant and dynamic temperatures, in the presence or in the absence of lactic acid and under modified atmosphere. Classical microbiological plate counting was performed followed by meta-genetic analysis. The bio-informatic pipeline using Mothur, BLAST and STAMP was used to assign a taxonomical identity to the sequences and to obtain the bacterial population proportions of the samples (Schloss et al., 2009). Then, with the plate count estimates, the proportions were transformed into quantitative estimates for the dominant populations. These observations were fitted to predictive microbiological models. Thus, the growth parameters obtained were used to simulate microbial behaviour in dynamic temperature conditions, and these simulations were compared to observations generated by the metagenetic analysis. Predictions at static temperatures were in line with observations, whereas predictions obtained at dynamic conditions slightly differed from observations. The study of Benson et al. (2014) provides another example of the application of metagenetics to understand the ecology of complex microbial communities in real food matrices. Analysing the relative abundances of individual taxa based on 16S rRNA gene sequences, this study found reproducible ecological successions over an 80-day shelf life study in fresh pork sausage. Data analyses identified strong effects of the spice blend on population dynamics and enabled source-tracking of a single species (*Lactobacillus graminis*) as the main causative spoilage organism. Next generation sequencing data was also able to reconcile an existing gap between culture-based microbial data and perceivable sensory traits, providing a powerful tool for establishing causal relationships between the sausage microbiota and relevant physico-chemical properties for the manufacturer. The authors hypothesized that, with enough data, it would be possible to develop predictive models based on machine learning algorithms capable of articulating species-specific signatures associated with batch-to-batch variability.

These case studies show that metagenomics provide valuable information to perform EA, taking into account the initial bacterial contamination in terms of abundance and diversity as well as several factors influencing microbial behaviour. This approach will help the food

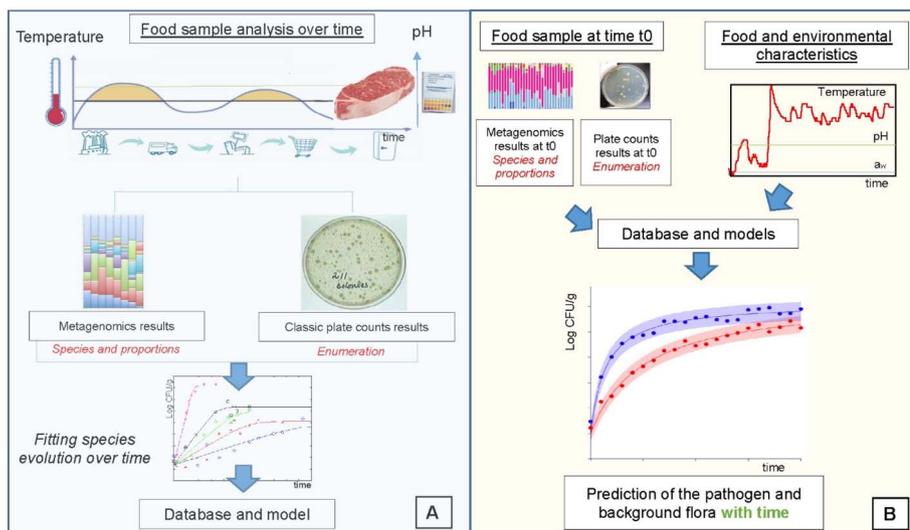


Fig. 2. Proof of the concept: using both metagenomics and plate count tools to facilitate exposure assessment. A. Building the knowledge. For a specific food product, metagenomics analysis, plate counting, food characterization and temperature monitoring will be performed. Then, kinetics will be generated for the most prevalent species and predictive microbiology models will be built. Quantitative data will be stored in a database. B. Predicting. Combining metagenomics, plate count and food characteristic information collected at a given point of time with knowledge and model stored in the database, prediction of dominant species behaviour will be possible.

industry to evaluate the effects of interventions such as shortening the distribution chain, considering a supplementary technological step (e.g. pasteurization, freezing) or adapting the packaging and gas composition of the products and thus act early in the production chain to control microbial populations.

## 5. Conclusion

Microbiological EA plays a central role within public health risk evaluations, as it provides an estimation of both the likelihood and the level of the microbial hazard in a specified consumer portion of food, taking microbial behaviour (e.g. death, survival and growth in the food) into account. So far, EA has been mainly based on phenotypic data and predictive models built at the population level. The next generation of EA will be fine-tuned with mechanistic cellular information that can be obtained now through omics techniques. For instance, the dynamics of a pathogen in a complex food eco-system could be better understood with omics data (e.g. obtained by metagenomics) than with phenotypic data. Also, omics technologies could make a difference in EA by going a step further in the prediction of pathogen behaviour variability due to heterogeneity in physiological states and stress responses. Indeed, progress in biomarker identification and characterization opens the possibility of making the connection between process and formulation conditions and microbial response at the strain level. Nevertheless, using omics technology to decipher complex food ecosystem dynamics or strain variability will generate huge sets of data (big data), which have to be properly analysed, summarized and stored. That could be both time consuming and resource demanding, as new database structures have to be conceived, and techniques not yet routinely used in EA (e.g. ontology) have to be encouraged. Another drawback of omics is the difficulty of applying these technologies in food and more generally in real situations (e.g. low concentrations of pathogens).

Nevertheless, despite these limitations, we are confident that omics will be part of the next generation of microbiological EA since it provides valuable tools (among others, metagenomics and biomarkers) which enable better understanding of microbial behaviour and dynamics, brings additional knowledge on pathogenic strains and then, altogether, reduces the uncertainty when conducting an EA for a specific combination of pathogen and food, and with that, the associated risk.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijfoodmicro.2017.10.006>.

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