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Next generation microbiological risk assessment—Potential of omics data for hazard characterisation

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ABSTRACT

According to the World Health Organization estimates in 2015, 600 million people fall ill every year from contaminated food and 420,000 die. Microbial risk assessment (MRA) was developed as a tool to reduce and prevent risks presented by pathogens and/or their toxins. MRA is organized in four steps to analyse information and assist in both designing appropriate control options and implementation of regulatory decisions and programs. Among the four steps, hazard characterisation is performed to establish the probability and severity of a disease outcome, which is determined as function of the dose of toxin and/or pathogen ingested. This dose-response relationship is subject to both variability and uncertainty. The purpose of this review/opinion article is to discuss how Next Generation Omics can impact hazard characterisation and, more precisely, how it can improve our understanding of variability and limit the uncertainty in the dose-response relation. The expansion of omics tools (*e.g.* genomics, transcriptomics, proteomics and metabolomics) allows for a better understanding of pathogenicity mechanisms and virulence levels of bacterial strains. Detection and identification of virulence genes, comparative genomics, analyses of mRNA and protein levels and the development of biomarkers can help in building a mechanistic dose-response model to predict disease severity. In this respect, systems biology can help to identify critical system characteristics that confer virulence and explain variability between strains. Despite challenges in the integration of omics into risk assessment, some omics methods have already been used by regulatory agencies for hazard identification. Standardized methods, reproducibility and datasets obtained from realistic conditions remain a challenge, and are needed to improve accuracy of hazard characterisation. When these improvements are realized, they will allow the health authorities and government policy makers to prioritize hazards more accurately and thus refine surveillance programs with the collaboration of all stakeholders of the food chain.

1. Introduction

1.1. Scope

The rapid developments in whole genome sequencing (WGS), next generation sequencing (NGS) and other omics tools, called Next Generation Omics (NG Omics) in this paper, have led to an increase in information about foodborne microbes, improving our understanding of how they survive in foods, how they cause disease, and why some strains are more virulent than others. These new insights are useful to better understand the dose-response relationship for various pathogens and the impact of pathogen-food combinations on risk. Furthermore,

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NG Omics tools are powerful in foodborne outbreak investigations, but can also be used to identify and assess hazards, including those associated with emerging pathogens and novel pathogen-food combinations (Ronholm et al., 2016). All of this information is now available for microbial risk assessment (MRA). However, researchers, regulatory authorities and industry stakeholders alike are currently struggling with how best to use these new datasets to reduce the uncertainty in all areas of risk assessment, as it is difficult to quantify the new NG Omics-based insights and determine how to best implement these in MRA. The focus here will be on how NG omics impacts on hazard characterisation and the ways in which it may provide more insight in variability and help to limit the uncertainty in this stage of MRA. The impact of NG omics on the other aspects (Hazard Identification, Exposure Assessment, and Risk Characterisation) of MRA are considered in accompanying papers. Together, these papers provide insight into the potential role of NG omics in MRA and how it can be applied by those working in the field of quantitative risk assessment across academia, industry and government.

1.2. Risk assessment

The formal process of MRA consists of four stages, *i.e.* Hazard Identification, Exposure Assessment, Hazard Characterisation and Risk Characterisation. In Hazard Identification, agents with potentially adverse health effects are identified and defined. In Exposure Assessment, the dose at the moment of exposure is determined. In Hazard Characterisation the probability and severity of a disease outcome is determined as a function of the dose. Lastly, in Risk Characterisation the overall probability and severity of the illness is determined, including variability and uncertainty. All four activities are functionally separated, however there are relations between the four stages. All four stages are prone to uncertainty and variability. Variability results from variation in the many relevant factors determining the levels and physiological state of microbes and their effects. Uncertainty results from imperfect knowledge of relevant phenomena including those affecting the microbe, the food matrix and the susceptibility of the human population. The advantage of quantitative risk assessment is that it can provide insight into this uncertainty and variability, but, even more importantly, into the factors controlling the risks. In this manner one can base decisions on the best information available.

1.3. Hazard characterisation

In Hazard Characterisation, the hazard is characterised with regard to its various aspects and an important part of hazard characterisation is the determination of the dose-response relation, *i.e.* the probability and severity of a disease as function of the dose. The Codex defines hazard characterisation as “The qualitative and/or quantitative evaluation of the nature of the adverse health effects associated with biological, chemical and physical agents which may be present in food. For chemical agents, a dose–response assessment should be performed. For biological or physical agents, a dose–response assessment should be performed if the data are obtainable.” (CAC, 2016). However, for a real quantitative risk assessment a dose-response relation may be considered essential for biological agents as well.

Illness caused by microbial pathogens can manifest as either foodborne intoxication or infection. In the case of intoxication, a toxin produced by a microbial agent in the food is ingested, causing illness and being the hazard. In the case of foodborne infection, with the organism being the hazard, a pathogen present in food is ingested, and disease is caused by toxins and other bacterial products produced *in vivo* (toxin-mediated illness) and/or following invasion of host tissues. Overall, the severity of the outcome of intoxications or infections depends on i) the effect of the toxin or the pathogen, ii) the level of exposure to a pathogen and/or its toxin, iii) the physiological state of the pathogen and iv) the susceptibility of the individual (*e.g.* infant/elderly/pregnant/healthy adult). The uncertainty and variability associated with each of these factors in the dose-response relationship is discussed below.

1.4. Uncertainty and variability in dose-response

Although all aspects of risk assessment are prone to large variability and uncertainty, this is particularly a challenge at the stage of hazard characterisation. This stems partially from the fact that it is typically impossible to carry out relevant experiments to determine the dose-response relation. It is ethically unacceptable to expose large groups of people to food that is deliberately contaminated with various doses of a hazardous agent and measure the probability and severity of illness. Therefore, experiments are typically conducted in either animal or cell culture model systems. While data from these experiments can provide insights into the relative pathogenicity and virulence potential of the pathogens, the results require extrapolation to identify the human dose-response, and such extrapolation can be difficult. For instance, effects may be host-specific. Moreover, the human infectious process typically involves multiple steps which cannot easily be simulated in cell culture-based experiments, such as pathogen survival through the stomach, the presence of competitive intestinal microbiota and variation in immune system attributes and thus host-specific susceptibility. Alternatively, epidemiological data obtained from foodborne outbreaks can be used to develop dose-response relations (*e.g.* as reported by the FAO/WHO, 2002), as these constitute unintentional, real-life experiments. However, large uncertainty about dose-response relations also exists in an outbreak, as it is difficult and often impossible to determine the exact doses at consumption with reasonable accuracy. Even in cases in which the dose can be reasonably well estimated, large uncertainty in other aspects still exists (Pouillot et al., 2016). Finally, dose-responses can be estimated using more generic epidemiological data of attack rates, together with estimates of concentrations in the implicated food products and serving sizes (Buchanan et al., 1997), but the same large uncertainties exist as for outbreaks. Apart from uncertainty, large variability in dose-response can be encountered. There can be huge variability in virulence between strains of the same species. Similarly, enormous variability in vulnerability and disease severity exists within the human population. The latter is aptly demonstrated by the acronym YOPIs (Young, Old, Pregnant and Immunocompromised), referring to individuals more vulnerable than the general population.

1.5. Effect of agent status and history, food, and host

Another driver of variability in the dose-response is the environmental history of the pathogen which is typically identified in the exposure assessment stage. This can account for the population but also individual cell variability may affect the dose-response relationship. Although exposure assessment and hazard characterisation are two different parts of the risk assessment, they are inter-related as the probability of illness (in the dose-response equation) depends on the dose, which is the output of the exposure assessment process. However, the physiological state of the microorganism is also relevant to its virulence potential; as a result of its history (*e.g.* acid or low a_w exposure), an organism may have become more sensitive or more resistant to stress. Additionally, food products can vary in their buffering activity, or impact the residence time of the microorganism in the stomach.

2. Is a strain pathogenic?

2.1. Black and white effects: some organisms are pathogenic while others are not

To qualify an organism as pathogenic or not pathogenic, the context needs to be specified. If sufficient toxins are formed in a food by a

Table 1
Example of derivation of a descriptor of pathogenicity.

Bacillus cereus is presumptively pathogenic if it harbours [(*hblA* AND *hblC* AND *hblD*) OR (*nheA* AND *nheB* AND *nheC*) OR *cytK* OR *bceT* OR *entFM* OR *entS* OR *ces*]
B. cereus is presumably not pathogenic if it does NOT contain (*hblA* AND *hblC* AND *hblD*) AND NOT(*nheA* AND *nheB* AND *nheC*) AND NOT(*cytK*) AND NOT(*bceT*) AND NOT(*entFM*) AND NOT(*entS*) AND NOT(*ces*)

hblA and *hblC* and *hblD* are needed for Haemolysisn BL (HBL); *nheA*, *nheB*, *nheC* are needed for non haemolytic enterotoxin (NHE), *cytK* codes for Cytotoxin K; *entFM* encodes enterotoxin FM; *entS* encodes enterotoxin S; *bceT* encodes enterotoxin T; ces encodes cereulide. In addition hemolysin A (*hlyA*), hemolysin II (*hlyII*), hemolysin III (*hlyIII*), phosphatidylinositol-specific phospholipase C (*plcA*), cereolysin A or phospholipase C (*cerA*), cereolysin B or sphingomyelinase (*cerB*), cereolysin O (*cerO*), and their pleiotropic transcriptional activator (*plcR*), are all involved in pathogenesis of *B. cereus* (Kim et al., 2015).

toxinogenic organism, all individuals will be susceptible. For infectious agents, the probability of illness can vary and symptoms can also vary in severity among patients belonging to different categories of susceptibility. Certain pathogens mainly cause illness in specific susceptible populations, *e.g.* the YOPIS. An extreme example of opportunistic pathogenesis would be illnesses caused by lactobacilli (Abgrall et al., 1997; Antonie et al., 1996). Thus, it is virtually impossible to have a clear separation between pathogenic and non-pathogenic microorganisms.

Furthermore, generally multiple genetic or phenotypic determinants need to be considered in the definition of pathogenicity (Table 1).

If the classification is too strict (Table 2), one might unexpectedly be confronted with the emergence of a microbial hazard with previously unknown virulence gene combinations, as in the *Escherichia coli* O104 outbreak in Germany (Yan et al., 2015). On the other hand, if the classification is too generic, safe product might be declared as unfit.

In the Dutch guidelines of 2014 (NVWA, 2014) a rating based on genetic characteristics was combined with the risk profile of the food product: for high-risk, ready-to-eat (RTE) foods, all STEC with (*stx1* OR *stx2*) are considered unacceptable, while for low risk food products expected to be cooked, only STEC's that have (*stx1* OR *stx2*) AND [(*eae*) OR (*aaiC* AND *aggR*)] AND belonging to serotypes (O26, O103, O111, O145, O157, O104, O45, O121 or 0174) are considered unacceptable.

2.2. Emergence of new virulence gene combinations

Classifications should not be static, but need to be adaptable with progressing insights, or with newly emerging biological attributes. For instance, previously it was considered that a STEC either contained the *stx1* gene and/or the *stx2* gene, as well as the *eae* (Intimin) gene, which was thought to be the only way for the organism to attach to the intestinal barrier. The *E. coli* strain implicated in the O104 outbreak in Germany in 2011 (Yan et al., 2015), however, was a member of a different pathovar, enteroaggregative *E. coli*, which had acquired the ability to produce Shiga toxin and lacked *eae* but possessed alternative factors (encoded by *aaiC* and *aggR*).

3. How virulent is a strain?

Once the status of the microorganism has been established and its potential pathogenicity confirmed, the “virulence level” of the bacterium should be explored. Below, uses of NG Omics to specify the virulence level of pathogenic strains will be discussed.

3.1. Sequencing analyses

In contrast to the recognized value of WGS for outbreak investigation (McGann et al., 2016), its application in MRA is largely unexplored and faces important challenges. A first approach in methodology development is described by Pielaat et al. (2015). Genetic data of *E. coli* (single nucleotide polymorphisms; SNPs) were combined with epidemiological and phenotypic analysis (*in vitro* attachment to epithelial cells as a proxy for virulence) to inform hazard identification and hazard characterisation. This application, further elaborated in Section 5, revealed practical implications when using SNP data for MRA. In order for WGS to be incorporated into risk assessment in a useful manner, priority setting of high-risk phenotypes is necessary. More importantly, high levels of genome similarity do not imply similar behaviour in the food chain or similar levels of virulence since small genetic changes, *e.g.* a single substitution in a virulence gene, may result in large phenotypic differences. It is therefore of high importance to link genome sequences with phenotypic attributes related to persistence in the food chain and *in vitro* or *in vivo* virulence assessments (Franz et al., 2016).

WGS and comparative genomic analysis of bacterial isolates that show distinct virulence or toxicity can be used to assess virulence and/or toxicity properties of a strain, *e.g.* to detect virulence factors.

However, genome sequence analyses have some limitations. The complexity in gene regulation and post-translation modifications may lead to a high level of diversity in strain phenotypes depending on their location (*e.g.* in food, the gut, intracellularly). Therefore, investigation at the mRNA and protein levels requires expression studies using relevant conditions, under which putative virulence factors or toxins are produced.

Sequencing-based microbial community analysis has been used extensively to study microbial community composition in foods and the gut microbiota, especially associated with the development of various diseases related to the digestive system such as inflammatory bowel disease and irritable bowel syndrome. In addition, such tools are used to follow pathogens in a complex microbial environment. Such complex ecological communities include interactions among hundreds of bacterial species but also with the host cells and evolves over time, which make the interpretation of results difficult (Mandal et al., 2015). This is, however, a promising technique that is discussed further in the article “Next generation Microbiological Risk Assessment - Meta-Omics: the next need for integration” (Cocolin and collegues, 2018, this issue).

3.2. Transcriptomics and proteomics studies

Transcriptomics and proteomics approaches have the potential to allow for characterisation of the physiological state of pathogens, which may lead to paradigm shifts in approaches to hazard characterisation. Certain conditions present in food prior to consumption may for instance allow for physiological adaptation of a pathogen, rendering it more likely to survive upon passage through the stomach (Kim et al., 2016). The work on stressosomes in *Vibrio* spp., *Bacillus subtilis* and *Listeria monocytogenes* provides a good example of how expression and physiological response studies upon exposure to stress within different food and host environments can help unravel how cell history exposure to hurdles may collectively determine survival and virulence (Jia et al., 2016; Utratna et al., 2014; Williams et al., 2014; Young et al., 2013). Such information can be integrated into the hazard characterisation process and will be addressed below.

Monitoring gene expression and bacterial behaviour in conditions that simulate the human gastro-intestinal tract may provide more relevant insights than studying the pathogen under optimal growth condition. Virulence properties of foodborne pathogens are frequently evaluated in cell culture models or in laboratory media, but may need to be validated in an animal model to account for the complexities of the host environment. As proposed by Greppi and Rantsiou (2016), the effect of food processing and preservation conditions on a pathogen's virulence and toxin production might be eventually predicted if the “food chain-human gastrointestinal tract continuum” is considered. Such *in vitro* and *in vivo* approaches are commonly used to assess pathogenicity, even if host specificity may play such an important role that the quantitative level of infection risk is almost impossible to deduct. Nonetheless, relative ratios of virulence gene expression remain of

Table 2
Example of a sequence of increasingly strict criteria for definition of pathogenic potential.

STEC = (*stx1* OR *stx2*)
STEC = (*stx1* OR *stx2*) AND an attachment factor like genetic element
STEC = (*stx1* OR *stx2*) AND known attachment factor
STEC = (*stx1* OR *stx2*) AND (*Eae* OR (*aaiC* and *aggR*))
STEC = (*stx1* OR *stx2*) AND (*Eae*)

interest to enhance risk estimations by improving dose-response models and the conditions in which the pathogen expresses its virulence factors. To integrate these data into hazard characterisation, they can be quantified using quantitative methods as detailed below.

Quantitative transcriptomic and/or proteomics coupled to virulence and toxicity assays (and/or clinical data) can be beneficial to define potential biomarkers of virulence or illness.

Dual transcriptomics for example, which involves simultaneously measurement of gene expression during infection in both the host and the pathogen (Westermann et al., 2012) would define or refine host-related factors coupled to pathogen virulence determinants, and thus selection of biomarkers related to illness, and more precisely to the disease process or clinical signs. In this case, such biomarkers may help predict clinical outcomes, and the link between dose-response and virulence factors can be determined using omics data. The use of omics tools to define potential "virulence biomarkers" can limit the need for animal experiments. If pathogenicity results from a few well-characterised virulence factors which are correlated to symptoms, as for *B. cereus* or *E. coli* O157:H7, it may be possible to adjust a dose-response model to the expression of these factors. However, the ingested dose (number of cells) is still of relevance. The main challenges here will be to establish and quantify the correlation between the amplitude of the biomarker response and illness.

Transcriptional biomarkers are promising tools because of their pathogenesis relevance in niches relevant for disease. In addition, transcriptomics tools (*e.g.* RT-qPCR, RNA-seq) are well-established, fast and cost-effective. Transcriptomics coupled with *in vivo* and *ex vivo* experiments may allow for more in-depth investigation of the role played by regulators in virulence, including the small non-coding regulatory RNAs; indeed, in some cases, expression of these regulators depends on the site of infection within the host (Toledo-Arana et al., 2009).

Although transcriptional biomarkers can improve the diagnosis and prognosis of infectious diseases, proteins represent the functional level of gene expression, and may thus better reflect the bacterial phenotype. Therefore, proteins are a preferred target for biomarker studies, despite their more challenging analysis, especially at the site of infection. As limited examples can be found in food microbiology, we employ an example with the pathogen *Acinetobater baumannii*, for which identification of diagnostic biomarkers for pathogenesis was aided by a quantitative proteomics approach to identify virulence factors in *ex vivo* models (Mendez et al., 2015). Numerous proteomic applications with foodborne pathogens are available in laboratory culture media. For instance, two-dimensional electrophoresis coupled with mass spectrometry were employed to investigate virulence properties of a *Cronobacter* strain panel (Du et al., 2015; Ye et al., 2016).

Insight from the use of such tools helps to elucidate pathogenisis mechanisms and can be used to characterise the hazard, but only a fraction will be quantitative enough to be linked directly to dose-response relations. Therefore, despite the identification of several virulence factors, their quantification remains insufficient and until now no mathematical models were employed to validate biomarkers identified *via* such transcriptomic or proteomic approaches.

3.3. Perspectives: quantitative proteogenomics and metabolomics towards systems biology

More recently, a quantitative proteogenomics strategy with *Streptococcus pyogenes* was introduced to explore the consequences of genome adaptation at the proteome level (Malmström et al., 2015). This integrated analysis of SNPs and proteomic differences between non-invasive and invasive isolates, identified proteins that may play a role in disease development, and clearly this methodology can be applied to foodborne pathogens.

In addition to transcriptomics and proteomics techniques, metabolomic approaches are among the newest class of diagnostic approaches in infectious disease, and employ analysis of metabolite signals in biological samples to identify or characterise infectious agents. Näsström et al. (2014) used gas chromatography with time-of-flight mass spectrometry on plasma samples and found that a combination of six metabolites could accurately distinguish between samples from patients with *Salmonella* Typhi, *S.* Paratyphi A or uninfected patients. However, the cost, equipment, and analytic requirements of these approaches are currently too high for their use in routine diagnosis.

Several virulence factors may be promising candidates for biomarkers (Table 3), provided that the omics data are coupled to statistical and probabilistic analysis. After defining the level of virulence of a pathogen, it is also necessary to specify the likely severity of the disease. This part will be discussed in the next section.

4. The severity of the outcome

4.1. Factors affecting severity

As there is overlap between virulence and the severity of the outcome, omics tools can contribute to predicting the severity of foodborne intoxications and infections. For foodborne pathogens, such tools can facilitate the determination of the presence or absence of specific genes for determinants which contribute to the likelihood, severity and outcome of the illness. These include toxins and confirmed virulence factors, and in addition, attributes that determine the severity and outcome of diseases, such as the ability to survive in the gastrointestinal tract and other sites (*e.g.* acid tolerance, substrate utilization, genes involved with growth at body temperature). Antibiotic resistance may be a factor to consider as well, as it may determine the efficacy of treatment of invasive foodborne pathogens.

In the case of foodborne intoxications, the severity depends on the type of toxin pre-formed in food and its effect, the level of exposure, and the susceptibility of the individual. Considerable differences in toxic doses and disease outcomes exist for different toxins. Some basic characteristics of toxins produced by foodborne pathogens are presented in Table 4. For certain toxins, *e.g.* botulinum neurotoxins and staphylococcal enterotoxin (SE), toxin type is already known to be a predictor for the severity of the outcome.

In the case of foodborne infection, many of the factors that determine the severity of the outcome are similar to those described above for foodborne intoxication. The major difference is that toxin

Table 3
Examples of potential biomarkers associated with strain virulence[a].

Omic methods used	Type of biomarker	Biomarker candidate	Type of response measured	Remarks and experimental reproducibility	References
Genomics	Gene (CDS)	*stx* of *E. coli*	Qualitative	/	Lindsey et al., 2016
	SNP	*stx* of *E. coli*	Qualitative	/	Pielaat et al., 2015
	Type of toxin gene (CDS)	Gene encoding neurotoxin of *Clostridium botulinum*	Qualitative	/	Peck and van Vliet, 2016
Transcriptomic	mRNA	*SPI-1 genes or hil1A* of *Salmonella enterica*	Quantitative	Comparison between two different serotypes. 2 biological replicates	Elhadad et al., 2016
Proteomic	Protein	*typA* of *Cronobacter sakazakii*	Quantitative	Comparison between virulent and non-virulent strains. 3 technical replicates, but no biological replicate	Du et al., 2015
Metabolomic	Metabolite	Cereulide toxin of *B. cereus*	Quantitative	/	Biesta-Peters et al., 2010; Marxen et al., 2015

[a] Abbreviations: CDS = Coding DNA sequence, SNP = Single-nucleotide polymorphism.

production in these cases does not take place in the food, but in the infected host, and that a multitude of virulence factors may play important roles.

Examples of foodborne bacteria that cause toxin-mediated infection, or toxico-infections, are given in Table 4 and include *Clostridium perfringens*, *Bacillus cereus*, *Shigella* species and Shiga toxin-producing *E. coli*; some of the most severe outcomes with *E. coli* are seen with Shiga toxin leading to hemolytic uremic syndrome (HUS) (Majowicz et al., 2014). Other bacteria causing foodborne infections may actively invade the gut tissue and some can cause severe damage to the gut tissue. Examples include enteroinvasive *E. coli* (*EIEC*), *Shigella*, *S. enterica*, and *Campylobacter jejuni* (Backert et al., 2013; Kaakoush et al., 2015). The outcomes may be especially severe in highly vulnerable individuals in whom the organism can cause systemic infections (De Cock et al., 2012; Kobayashi et al., 2014; Rani et al., 2011).

4.2. Intoxication

Overall, omics technologies provide powerful tools to assess the presence, diversity and expression of genes that encode toxins, toxin-assembling complexes, and virulence factors. For instance, botulinum neurotoxins (BoNTs) have been classified into seven serotypes, A to G. Types A, B, E, F and G are toxic to humans (but types C and D not), and type A is known to have the lowest lethal dose. This information is relevant for clinical and diagnostic purposes and when treating botulism *via* administration of neutralizing antibody (Montecucco and Rasotto, 2015). Substantial variation between toxins of a certain type has also been reported for the emetic SEs and SE-like toxins produced by *S. aureus* (Argudín et al., 2010; Omoe et al., 2013; Johler et al., 2015b). In a recent study, sequences of the major enterotoxins SEB, SEC, and SED were studied for a well-characterised set of enterotoxigenic *S. aureus* strains originating from foodborne outbreaks, human infections, human nasal colonization, rabbits, and cattle (Johler et al., 2016). This study exemplifies a further step towards improved understanding of strain-specific differences in enterotoxin expression and potential source-tracking tools. Such information is important for hazard characterisation and prediction of the severity of the outcome. Different toxin variants with different levels of toxicity also exist for the *B. cereus* toxin cereulide, with up to 8-fold differences in levels of toxicity (Marxen et al., 2015), and for *B. cereus* toxins Nhe and Hbl (Böhm et al., 2016). While for the Nhe and Hbl toxins a nucleotide sequence-based omics approach can be directly predictive of the level of toxicity, this is not the case for cereulide, which is a non-ribosomally synthesized cyclic peptide that is assembled by gene products encoded by the *ces* gene cluster (Ehling-Schulz and Messelhäusser, 2013). As it is not transcribed from mRNA, analysis of the toxin itself (*e.g.* by GC-MS-MS) is required to establish the type (Marxen et al., 2015). Based on knowledge on the composition of known toxins and relevant domains therein, omics tools can help establish risk factors in unexpected species (*e.g.* detection of botulinum toxin genes in *C. baratii* or *C. butyricum* (Fach et al., 2009)), and can help identify modified or hybrid toxins, *e.g.* the novel hybrid botulinum neurotoxin FA (Pellett et al., 2016). Moreover, omics tools can aid the identification of novel toxins from unique sources, as demonstrated for SEs (Johler et al., 2015a).

4.3. Foodborne infection

In the case of foodborne infections, in the first instance, the dose of bacteria that reach the gut may depend on the ability of an organism to survive acid exposure in the stomach (Ceuppens et al., 2012) and bile acid in the upper intestinal tract (Crawford et al., 2012). For sporeformers such as *C. perfringens* and *B. cereus* which may be ingested as spores, the ability of spores to germinate and grow in the gut is another determining factor (Berthold-Pluta et al., 2015). In addition, the ability of a bacterium to thrive in the gut where it may produce toxins or virulence factors (adhesins, invasins) may play a critical role. This includes the optimal growth temperature of a pathogen. Psychrotrophic *Bacillus weihenstephanensis* strains, for example, carry the same toxin genes as *B. cereus* strains, but have not been associated with foodborne illness, which might be due to the fact that these strains do not thrive at 37 °C (Stenfors Arnesen et al., 2011).

Other factors that may determine the severity of the illness relate to the ability of the bacterium to utilize nutrients present in the gut, compete adequately with other gut microbiota, sometimes even impacting the microbial community composition in the gut, and to grow at low redox potential. For example, the anaerobic *C. perfringens* lacks many genes related to amino acid biosynthesis and, thus, its ability to upregulate the production of toxins and enzymes is important to its growth. Toxin production, concomitant with sporulation, is upregulated by contact with Caco-2 cells (Chen et al., 2014).

The overall microbiota in the gut may also be a factor that determines the outcome of disease. There is evidence that the composition of the gut microbiota is associated with infant botulism (Shirey et al., 2015). Other examples include reports that the gut bacterium *Bacteroides thetaiotaomicron* influences the virulence potential of enterohemorrhagic *E. coli* (Iversen et al., 2015; Cordonnier et al., 2016). Furthermore, in orally infected mice, the ability of certain strains of *L. monocytogenes* to produce the bacteriocin listeriolysin S in the intestine was found to alter microbial community composition in the gut, increasing intestinal populations of the pathogen and deeper organ infection (Quereda et al., 2016).

4.4. Omics and predicting severity

WGS (or other omics)-based analysis of a foodborne pathogen's population structure in an epidemiological context has clear potential to identify clonal groups that may differ in their contribution to illness, types of illness outcomes or their propensity for certain populations. For

Table 4
Examples of toxins produced by foodborne pathogens that contribute to the severity of the outcome of disease.

Toxin formed in food	Effect	Action	Estimated dose	Reference
Toxins playing a role in foodborne intoxication (toxin produced in the food)				
Botulinum neurotoxin (BoNT) by *C. botulinum*	Neuroparalysis, prolonged effect	Preventing release of acetylcholine from axon endings at the neuromuscular junction	20–100 ng lethal	Carter and Peck, 2015; Sobel, 2005
Cereulide toxin by *B. cereus*	Nausea, vomiting, diarrhoea and cramps; in severe cases fulminant liver failure	K+ ionophore, disrupting mitochondrial membrane potential; interacts with serotonin 5-HT3 receptor and stimulates vagus afferent nerve	Emetic syndrome, 8 μg/kg bodyweight adults, lower in children; lethal dose 8 mg/kg	Agata et al., 1995; Delbrassinne et al., 2015; Ehling-Schulz et al., 2015; Jääskeläinen et al., 2003; Mahler et al., 1997; Mikkola et al., 1999; Naranjo et al., 2011
Staphylococcus enterotoxin (SE) by *S. aureus*	Nausea, vomiting, possibly diarrhoea	Electrolyte imbalances and dehydration	Total few μg in adults; 100 ng in children	Argudín et al., 2010; Asao et al., 2003; Johler et al., 2015a
Toxins playing a role in foodborne infection				
Clostridium perfringens enterotoxin (CPE)	Diarrhoea, generally self-limiting	Claudin binding, tight junctions affected		Shinoda et al., 2016; Chen et al., 2014
B. cereus 3—component Non-haemolytic (Nhe) toxins	Diarrhoea, generally self-limiting	Three proteins with enterotoxin activity; NheA pore formation		Granum and Lund, 1997; Sastalla et al., 2013
B. cereus 3—component haemolysin (HBL) toxin	Diarrhoea, generally self-limiting	Three proteins B, L1 and L2 with enterotoxin activity, poreforming		Granum and Lund, 1997; Sastalla et al., 2013
cytotoxin K (cytK) by *B. cereus*	Diarrhoea, possibly fatal	Poreforming, cytotoxic and haemolytic		Hardy et al., 2001
Shiga toxin	Hemorrhagic colitis or hemolytic uremic syndrome (HUS); severe	Binding GB3 receptors predominantly present in renal tissue		Lee et al., 2010; Majowicz et al., 2014; Ergonul et al., 2003

instance, such analysis of *L. monocytogenes* from clinical cases revealed "hypervirulent" clones consisting of strains with propensity for invasive illness in individuals with few or no evident co-morbidities (Maury et al., 2016). Strains of these hypervirulent clonal groups were more prone to breach the blood-placenta and blood-brain barriers, thus resulting in Central Nervous System (CNS) infections and perinatal listeriosis. In contrast, other strains with reduced virulence (hypovirulent clones) tended to cause listeriosis in highly compromised individuals with multiple co-morbidities and often led to septicemia without further invasion of CNS or placenta (Maury et al., 2016).

When a foodborne pathogen causes systemic infection, certain host biomarkers are used in clinical microbiology as indicator for outcomes such as sepsis, *e.g.* procalcitonin level as host-specific marker (Lee, 2013), or to predict the severity of the outcome, *e.g.* procalcitonin as a prognostic biomarker for severe sepsis and septic shock (Poddar et al., 2015). Thus, exploration of host biomarkers can also be a way to predict the severity of clinical signs or relapses, and can complement risk assessments based on the pathogen, including efforts utilizing omics tools.

Host responses may significantly contribute to the disease burden. For instance in the case of *Campylobacter* infections, some patients develop the debilitating Guillain-Barré syndrome (GBS). The development of GBS is an autoimmune complication mediated by antibodies raised in the course of infection against specific antigens of *C. jejuni* that mimic antigens on the human myelin sheath. Only certain strains of *C. jejuni* possess relevant antigens, hence WGS and other omics tools have the capacity to readily determine whether the infecting strain is likely to result in post-infection sequelae such as GBS. Coupling high-throughput data omics analyses, clinical data and mathematical modelling would improve or refine systems biology to understand complex biological systems as a whole, like dose-response and disease severity (Dix et al., 2016). The application of systems biology for hazard characterisation will be elaborated in Section 5.

Lastly, the occurrence of antibiotic resistance poses a great challenge to therapeutic treatment options (*e.g.* Zhang et al., 2014). With a global increase in antibiotic resistance in the food chain (Doyle, 2015), antibiotic resistance of foodborne pathogens is a major risk factor in the outcome or severity of disease. Antimicrobial resistance may be correlated with illness that has enhanced severity and duration (Angulo et al., 2004; Mølbak, 2005) or with treatment failure when antibiotics are used to combat the illness (Lammie and Hughes, 2016). The emergence of antibiotic resistance in foodborne pathogens can be readily identified and monitored using omics approaches.

5. Implications for the dose-response

5.1. Salmonella as a case study

In this case study, we will demonstrate how omics technologies described above may impact MRA by re-examining the dose-response outlined in the 2002 WHO-FAO hazard characterisation of *Salmonella*.

Salmonellosis is caused by *Salmonella enterica*, a pathogen which can be found in the digestive tract of humans and animals, such as birds, cattle and reptiles. More than 2500 *S. enterica* serotypes are known, and epidemiological data suggest that virulence is variable within and among serotypes (Lianou and Koutsoumanis, 2013). Virulence in pathogenic strains has been linked to the acquisition by horizontal gene transfer of pathogenicity islands (SPIs), which encode secretion systems for virulence proteins (Mills et al., 1995). Besides the five SPIs present in strains which cause gastroenteritis, some serovars harbour virulence plasmids which encode genes involved in the intra-macrophage survival of *Salmonella*. While some *Salmonella* serovars are restricted to one or few hosts, others have a broad host spectrum. Furthermore, to survive the hurdles of the immune system and internalisation in host cells, *Salmonella* needs to be able to scavenge scarce ions and resist reactive oxygen species. Taken together, these factors constitute a complex

Box 1

The main mode of regulation in bacteria is the binding of transcription factors (TF) in the promotor regions of operons to either activate or inhibit the transcription of genes. The binding sites in the promotor region of orthologous genes of *Salmonella* are strain-specific (Métris et al., 2017b). As a consequence, not only are genes that confer virulence or resistance in *Salmonella* strain-specific but also they are interacting. Information about regulatory networks is scarce for organisms other than model organisms such as *E. coli* or *B. subtilis* but has recently been gathered for 10 strains of *Salmonella* (www.SalmoNet.org). Based on a list of 233 genes associated to virulence as described in a virulence factor database (VFDB) (www.mgc.ac.cn/VFs/main.htm), a regulatory network of a particularly virulent strain of *Salmonella* Typhimurium was constructed which is shown in Fig. 1. As no data were available for the binding sites of that specific strain, we assumed that if links were found for orthologous genes in any other strain (www.salmoNet.org), they might also be present in this particular strain. Note that for about 25% of the genes selected the regulation was completely unknown, because *Salmonella* has TFs for which the binding sites have not yet been determined (Métris et al., 2017b). Some of the links determined by bio-informatics are spurious as factors other than the binding site such DNA topology influence regulation. Moreover, regulation is condition-specific so the genes that are linked are not necessarily all expressed during infection.

system regulated by a network of transcription factors, which co-ordinate the expression of virulence and stress response genes (Rhen and Dorman, 2005). Fig. 1 shows the regulatory network of a particularly virulent strain of *S.* Typhimurium.

The complexity of the *Salmonella* infection system outlined above impacts the dose-response relationship for the organism. In 2002, the WHO-FAO reported on the hazard characterisation of *Salmonella* as part of a larger risk assessment framework for this zoonosis in eggs and broiler chickens. It describes both pathogen and host characteristics, as well as food-related factors as contributing factors to the dose-response relationship. An overarching beta-Poisson dose-response model was derived from available outbreak data and an attempt was made to parameterize the model depending on the underlying pathogen (different *Salmonella* serovars) and host characteristics (age-dependent susceptibility).

One of the drawbacks of fitting a dose-response model to outbreak data is the uncertainty in the data; for instance the actual ingested dose and/or true number of exposed humans may not be accurately known.

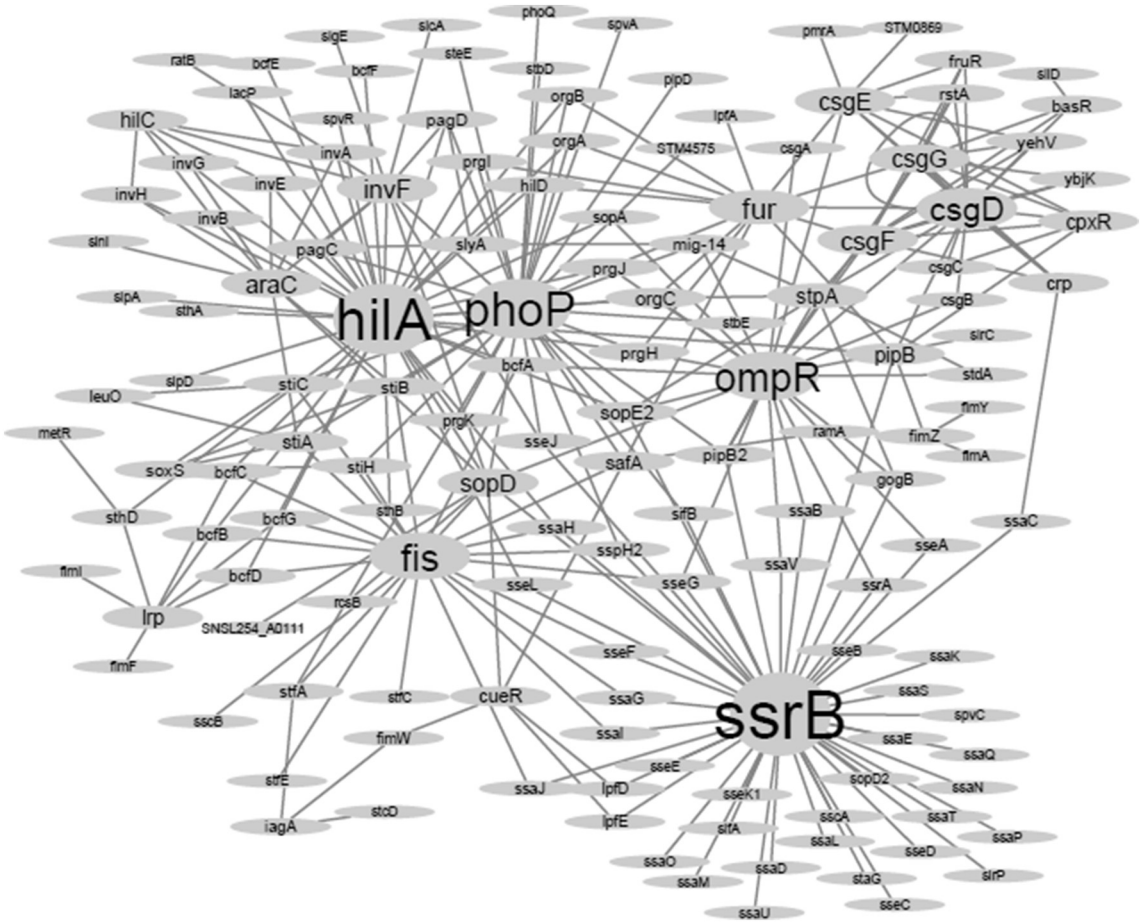

Fig. 1. Regulatory network of virulence genes of *Salmonella* serovar Typhimurium which has a high probability of illness as shown in a model and by epidemiological data (Zomer et al., 2014). As in clinical studies, network analysis could lead to the discovery of biomarkers or emerging properties which characterise virulence in different strains. The nodes represent genes or transcription factors (for details, see Box 1), the size of the nodes is proportional to the number of its connections; the main regulators from the data available are SsrB, hilA, PhoP, Fis, OmpR and CsgD. They are known to contribute to virulence but are usually considered in isolation. The regulatory links are undirected for clarity of presentation. They were inferred from SalmoNet (www.SalmoNet.org), see Box 1.

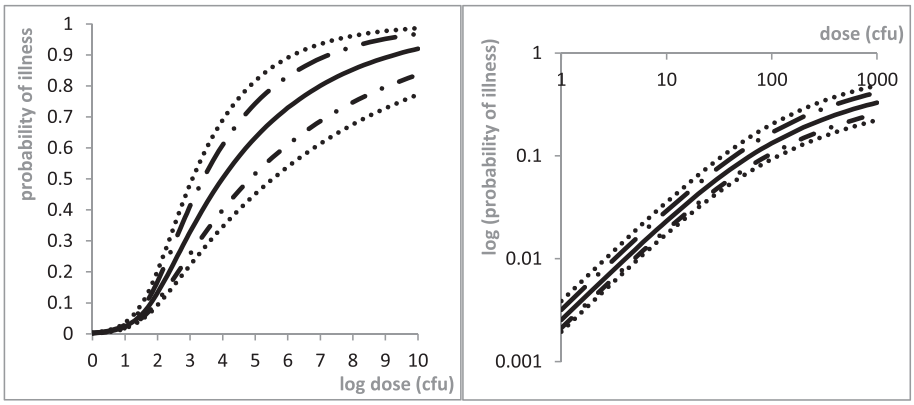

Fig. 2. Uncertainty in the dose-response parameters for *Salmonella* of the beta-Poisson model used in the WHO-FAO report (WHO-FAO, 2002); the thick continuous line is the expected value, the dashed lines represent the 2.5 and 97.5th percentiles and the thin dotted line the upper and lower bounds. The uncertainty is also large at low infectious doses (in cfu; right panel). See the WHO-FAO report (2002 Table 3.16 therein) for summary statistics of the 5000 model fits.

Retrospective studies, in general, make it difficult to assess the contribution of different biological factors to the overall response. All 5000 data sets used in this study resulted in a wide distribution of beta-Poisson dose-response parameters as illustrated in Fig. 2.

For risk assessment to support food safety management decisions, the question remains: "what dose-response curve should be used to come to risk assessments that can be used in decision support regarding interventions for *Salmonella* in different food chains when uncertainty and variability within and between serovars captured by applying a beta-Poisson model are as large as those found in the study? (WHO-FAO, 2002)".

Moreover, in contrast to the retrospective WHO-FAO (2002) study, the basis of risk assessment lies in a bottom-up, farm-to-fork, approach developed to predict microbial risk based on a low-dose region causing sporadic cases rather than explaining outbreaks. From the outbreak data used in the WHO-FAO study to investigate the dose-response relationship for *Salmonella*, there was also no evidence that the likelihood of poultry-related *S.* Enteritidis to produce illness differed from other serovars.

To summarize, an important outcome of the study was that serovar alone may not be a good predictor for probability of illness; the information at that time was insufficient to quantify the contribution of the different biological factors, both from the human and microbial side, affecting the dose-response relationship.

5.2. Towards improved understanding of dose-response relations

5.2.1. Linking phenotypic with 'omics' data

Experimental (*in vitro*) research under controlled conditions can help to unravel the relationship between exposure and virulence as an explanation for the difference in number of illness cases associated with different strains. Moreover, combining experimental results with new information from omics data will be a step forward in understanding the biological dynamics underlying the complex system of expression of virulence and stress resistance genes for different strains independent of serovar. Although it is evident that the true biological dose-response for *Salmonella* will not change with the generation of omics data, the utilization of the latter does open opportunities to build a mechanistic dose-response model instead of applying an empirical model fitted to outbreak data (*e.g.* beta-Poisson). A mechanistic model, in which parameters have biological meaning, provides a basis to include different strain-dependent characteristics and create opportunities for less uncertain risk estimates independent of the dose-region.

Recent work shows large variability in infectivity for different *Salmonella* strains (in the range of 10^{-5} for *S.* Kedougou to 10^{-1} for S1 1,4,[5],12:i:-) obtained from "*in vitro* gastro intestinal tract" (GIT) experiments (Kuijpers et al., 2018). The survival of 60 strains of different *Salmonella* serovars was followed through the GIT system and the fraction of cells invading intestinal cells compared to the initial overnight culture was calculated as proxy for infectivity. In accordance to the WHO-FAO report of 2002, consistent testing of different strains from 32 different serovars underpins the suggestion that serovar alone may not be a good predictor for human illness. Yet, independent of serovar, current investigations show an association between certain virulence genes in the strains under investigation and their *in vitro* virulence when categorized into high – mid high – mid low and low infectivity strains (Kuijpers et al., 2018). Further analysis, *e.g.* with gene knock-out experiments, is needed to assess how much of the variability in infectivity in the *in vitro* GIT system can be explained by one or multiple virulence genes. Subsequent transcriptomics or proteomics studies can help in establishing a true biological dose-response relationship.

5.2.2. Immediate new possibilities with NGS sequencing of pathogens

Since the 2002 WHO-FAO report there has been a vast increase in the amount of information from diverse data sources on the genetics and molecular biology of foodborne pathogens. Whether more genomic information about strain variability will allow us to significantly decrease the uncertainty in the dose-response is an open question because of other sources of variability and uncertainty such as those associated with host susceptibility. The challenge with these new data is to link quantitatively genomic information with the probability of illness. First of all it is not clear which genomic information (*e.g.* SNPs, virulence factors *etc.*) will best serve as indicator of virulence. Secondly, metrics for virulence will need to be integrated into the dose-response in a quantitative manner.

Two possible approaches are to (i) reduce the complex network of virulence factors to one or a few markers that are correlated to virulence or (ii) determine the probability of illness as an emergent property of a complex system with systems biology methods.

The first approach, *i.e.* the reduction of complexity has been investigated by Pielaat et al. (2015), where the association between phenotypic and genotypic data was determined with a Genome Wide Association Studies (GWAS) to reveal marker genes for 'virulence' of Shiga-toxin-producing *E. coli* O157.

Once virulence markers have been identified, the next question to be addressed is how to integrate them into the dose-response equation. The "mapping problem" of translating information on 10^4 SNPs through 10^3 genes and 10^1 biologically relevant effects to 1 measure of response (number of ill people) has been described for *E. coli* by Pielaat et al. (2015). Besides practical implications, this study suggests statistical elements and biological confirmation that need to be considered before an association can be applied to MRA. While markers may be a solution for toxin producers like *E. coli*, an alternative strategy may be needed when the virulence network is more complex and multifactorial, as in the case of *Salmonella*.

The second systems biology-based approach, could help identify critical system characteristics that confer stress resistance and virulence.

Modelling regulatory interactions may explain for instance how different strains of *Salmonella* have different virulence and resistance to stress (Baranyi et al., 2015). Which particular properties of the network are relevant to virulence is yet to be determined; for example, should the network be divided into elementary subgraphs (Alon, 2007) or analysed as a whole with information theory methods (Jia et al., 2013)? How can we best integrate metabolism (Kim et al., 2013) or small metabolites, which seem to play a prominent role in response to environmental stress (Métris et al., 2017a) with regulation?

As shown in the *Salmonella* case study, the use of biomarkers and/or systems biology approaches have the potential of determining virulence factors *in silico* provided that:

- biomarkers that reliably correlate to virulence are identified,
- different data types (genome sequence and condition/time specific data such as transcriptomics, proteomics and/or metabolomics) can be reliably integrated into a model to predict virulence.

To decipher the sources of uncertainty and variability in the dose-response however, still requires a quantitative relationship between *in silico* predictions and results from epidemiological studies. We have focused here on NGS data obtained from the foodborne pathogens, yet, biomarkers may equally be used in humans *(in vivo*) or *in vitro* (cell lines, models of organs) as in toxicological assessments (Krewski et al., 2014). Results are encouraging to provide crucial insight in the area of hazard characterisation (Pielaat et al., 2015, Kuijpers et al., in prep., Abdo et al., 2015) but require further research before the results can be linked into a full quantitative MRA.

6. Implications NG omics for industry, academia and regulatory agencies

6.1. Omics-based risk assessment models

An enduring challenge for hazard characterisation is to be able to rigorously convert multiple factors, from genomics, transcriptomics, and proteomics data *etc.*, into parameters that define risk (Pielaat et al., 2015; Membré and Guillou, 2016) without adding new sources of uncertainty. Den Besten and colleagues (2018, this issue) make suggestions for some analytical tools to tackle this difficult issue. In Sections 3 and 4 we also expanded on this with an example on how this could be addressed using aspects of this new technology.

The importance of understanding aspects, such as stressosome mechanisms, by applying NG omics is an interesting venue for risk assessment development, where impacts from different hurdles in the food chain may interact with the infection pathways and, thereby, affect the ultimate probability of illness. The sequential nature of the farm-to-fork approach in risk assessment, provides an opportunity to implement NG omics studies to fully integrate the history between these exposure assessment and hazard characterisation components. This suggests that although 'the quantity as a dose' is very important in evaluating risk from an identified hazard, the 'state of the dose' can also contribute significantly.

Understanding strain variability in virulence profiles, and the mechanistic interaction between pathogen and host are topics which NG omics could address and improve upon. Improvements in this field are expected to lead to more accurate mechanistic and quantitative, predictive models of the dose-response and probability of illness (Membré and Guillou, 2016). Indeed, by combining NG omics-derived virulence data with risk assessment it is expected to more accurately establish the identity of hazards, to reduce the uncertainty and improve accuracy in calculating the dose-response and probability of illness.

Additionally, NG omics case studies suggest that previously identified hazard groups, at the level of species or serogroup, may in the future be improved by further disaggregation into more numerous subgroupings based on distinct risk categories (Pielaat et al., 2015). This use of *E. coli* O157:H7 strains and clustering the strains based on SNP analysis demonstrates how an understanding of omics-based virulence profiles could broaden our appreciation of risk. For example, clustering strains based on various pertinent epidemiological factors could delineate specific geographies and commercial markets. This example also shows that many different aspects need to be combined and validated in order to have sufficient certainty in the outcome of such disaggregation. WGS data analysis can thus be used in risk assessments to prioritise specific phenotypes using risk-ranking (Franz et al., 2016). This insight and approach could have significant impact on the management of risk by food authorities and food companies, changing intervention strategies, determining how risk assessments are integrated across food chains and what hazards are identified.

A number of additional reservations need consideration when applying novel technologies, such as NG omics. Firstly, most, if not all, current studies that use omics data use *in vitro* measurements, targeting marker expression for prediction of virulence *in vivo*. Beyond their use as ranking tools, translation from genotypic to phenotypic data, with direct relevant risk outcome to the host, must be established with careful validation if these NG omics-based models are to have wider quantitative use in risk assessment.

Secondly, several statistical concerns are highlighted by Lay Jr. et al. (2006), emphasizing the issues with lack of standardization of current methods and reproducibility. The design of experiments in omics-based studies (due to *e.g.* overparameterization and defining the population for sampling) also can lead to bias towards, or higher probability of, false positives outnumbering true positives.

Lastly, as mentioned above, hazard characterisation using omics-based approaches will be difficult to be constructed accurately because of a natural paucity of relevant datasets. At the moment, generating or obtaining omics datasets under realistic conditions will continue to be a hurdle. However, the historical and current focus of these databases has been on clinical and outbreak isolates, which has led to an unintentional bias in the datasets composition. For example, the majority of NCBI data collected on *Salmonella enterica* from the UK (accessed on 31.01.2017) corresponds to 12,204 isolates, of which 94.7% were derived from human sources. Thus, the current bioinformatics analyses do not give a balanced perspective of the ecology of the hazard groups, and engenders a bias when attempting to use this information for virulence profiling and predicting evolutionary emergence in such groups. For industry it is equally important to understand how these pathogens survive in both the product and process.

As a consequence of new NG omics understanding, more pertinent (different and new) hazards can be prioritized by health authorities and government policy to implement new microbiological criteria, import controls and surveillance. Such changes should benefit the general public health of the markets implicated. Food regulators and health authorities may need to refine existing hazards, or identify new hazards to target in monitoring programmes. If NG omics technologies are applied to paired host-pathogen relationships and, as such, lead to better understanding of virulence and risk, then significant effort will be required by policy makers and risk managers to communicate to the public the ever more complicated and ethically sensitive risk outcomes. One aspect of this is the reductionist tendency when applying a practical policy agenda, which is a challenge for NG omics-driven quantitative MRA, and reflected by the so-called "mapping" problem, discussed earlier (Pielaat et al., 2015), where multiplicity of data is summarised as single risk outcomes.

6.2. Concluding remarks

The impact of NG omics technology use will likely require changes in food product specifications, surveillance programmes and detection methodology by food companies. In addition, companies distributing across many markets may encounter increased regulatory diversity in risk management strategies, as information on virulence and dose-

response in relationship to specific hazard-food-host combinations becomes increasingly available using NG omics.

To turn the potential future health benefits presented by NG omics and 'big data' technologies into a reality, for the benefit of consumers and food safety, it will require the collaborative and combined responsibility of food companies, regulatory bodies, academia, healthcare professionals and institutions and governmental policy makers. For MRA in particular, the application of NG omics technologies offers tantalizing opportunities to improve both the quality and accuracy of current hazard characterisation efforts. However, it is in the integration of these technologies across all the domains of risk assessment and management (Bergholz et al., 2014; Ringus et al., 2013) where most advancement and improvement will be made.

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Supplementary data

The glossary and abbreviations list are presented in Supplementary Tables S1 and S2, respectively, and are reproduced in the four joint papers on "Next generation Microbiological Risk Assessment". Supplementary data to this article can be found online at doi:https://doi.org/10.1016/j.ijfoodmicro.2018.04.015.

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