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PROBABILISTIC MODELLING IN FOOD SAFETY
A science-based approach for policy decisions

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Probabilistic Modelling in Food Safety:

A science-based approach for policy decision

Abstract. This thesis deals with use of qualitative and quantitative probabilistic models for the animal-derived food safety management. Four unrelated models are presented: three quantitative and one qualitative. Two of the quantitative models concern the risk posed by pathogens in raw milk, in the first study, a probabilistic approach for the inclusion of the variability and the uncertainty in the consumers' habits and the bacterial pathogenic potential is proposed while the second study, demonstrate how the overlook of the relationship between the storage time and temperature has led to overestimated results in raw milk-related models published so far and an equation to address the issue is provided. In the third study, quantitative modelling techniques are used to simulate the dynamics underlying the spread of *Campylobacter* in broiler flocks and quantify the potential effects that different on-farm mitigation strategies or management measures have on the microbial load in the intestine of infected birds at the end of the rearing period. In the qualitative study, a general approach for the estimation of the likelihoods of introduction of live parasites in aquaculture implants and the commercialization of infested product is outlined by using the example of Anisakids in farmed Atlantic salmon.

Abstract. Questa tesi si concentra sull'utilizzo della modellazione probabilistica quantitativa e qualitativa per fornire informazioni in supporto alla gestione della sicurezza alimentare dei prodotti di origine animale. Quattro lavori indipendenti vengono presentati: tre quantitativi e uno qualitativo. Due dei tre studi quantitativi hanno riguardato la modellazione del rischio legato alla presenza di microrganismi patogeni nel latte crudo; nel primo si propone un approccio probabilistico per l'inclusione della variabilità e l'incertezza relativa ai fattori di patogenicità a livello batterico ed il comportamento dei consumatori a livello domestico; nel secondo si è dimostrato come i modelli di analisi del rischio legati al latte crudo sviluppati e pubblicati negli ultimi anni riportino risultati probabilmente sovrastimati a causa del non aver considerato la relazione che intercorre tra le variabili tempo e temperatura di conservazione con la probabilità che il prodotto venga realmente consumato e una equazione ad-hoc viene proposta. Nel terzo studio, le tecniche di modellazione quantitativa sono state utilizzate per riprodurre le dinamiche biologiche relative alla diffusione di *Campylobacter* negli allevamenti di polli da carne e quantificare l'effetto che diverse strategie di contenimento o gestionali possono avere sulla carica microbica a livello intestinale alla fine del ciclo di allevamento. Lo studio qualitativo ha riguardato la formulazione di un approccio generale per la stima delle probabilità che parassiti vivi si introducano negli allevamenti di acquacultura e che il prodotto infestato da larve vitali venga commercializzato, l'esempio di Anisakis negli allevamenti di Salmone Atlantico viene presentato.

Preface

The works of this thesis have been conducted at the Veterinary Science and Public Health Department at the University of Milan (IT) in collaboration with the Zooprophylactic Institute of Lombardy and Emilia Romagna (IT) and at the Veterinary Epidemiology, Economics and Pubic Health Group at the Royal Veterinary College (UK).

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Chapter 1.

General Introduction

GENERAL INTRODUCTION

The food safety is an essential public health priority: at least one-third of the populations in developed countries are affected by foodborne illnesses every year, and the proportion is likely to be even more prominent in developing countries. The availability of safe food should be a basilar human right and the reduction of social and economic burdens of foodborne disease, a primary objective in all the countries.

In theory, foodborne diseases are preventable, a deep knowledge about foodborne hazards and the nature of the risks that these hazards pose to human health, combined with the capacity to take appropriate interventions, should result in a significant reduction of the food-borne disease. Whereas in the past, the hazards associated with certain foods were not formally linked to specific disease because of lack of evidences and/or epidemiological data, nowadays, new science-based approaches provide an effective way for government and food safety authorities to protect the consumers and to plan appropriate preventive measures or mitigation strategies when necessary. In this context, the risk analysis represents the systematic procedure allowing the data on hazards in food to be linked to epidemiological evidences related to foodborne disease, making possible a reliable evaluation of the risk for human health. In recent years, several practical examples (3-5) demonstrated the value of the risk analysis as a structured and systematic approach for the improvement of the decision-making processes and such system became the standard practice for food safety management systems to ensure that regulatory decisions about foods are science-based and transparent.

Besides improving the public health, the adoption of standardized frameworks to systematically assess, manage and communicate the food safety risks is also important to maintain the consumer confidence and provides a sound and scientific-based regulatory foundation for domestic and international trade in such a globalized food system. At this respect, it is important to recognize the role of the risk analysis as an 'instrument of guarantee' against protectionism or unjustified barriers to the international trade of food; in fact, the 'Agreement on the Application of Sanitary and Phytosanitary Measures' (8) entered into force with the establishment of the World Tread Organization (WTO) in 1995, in Article 5 specifies: *"Members shall ensure that their sanitary or phytosanitary measures are based on an assessment, as appropriate to the circumstances, of the risks to human, animal or plant life or health, taking into account risk assessment techniques developed by the relevant international organizations."*. Thus, the Agreement clearly states that restrictive measures adopted because of sanitary reasons by the members of the WTO must be not only *appropriate* but also undertaken only if *based on results* obtained by recognized methodologies for the assessment of the risk.

Following the publication of the Agreement and the recognition of the need for standard references for the assessment of the risks, different documented methodologies have been developed and transparent processes emerged.

The World Organisation for Animal Health (OIE), together with other standard-setting organisations recognised by the SPS Agreement such as

the Codex Alimentarius Commission (CAC) and the International Plant Protection Convention (IPPC), have all developed guidelines on the risk analysis methodology to assist decision-makers.

Approaches to Food Safety Risk Analysis.

The Risk Analysis, as systematic process, finds its application in a wide range of very different contexts (e.g. financial, engineering, insurance, military...) and several definitions have been proposed; regardless the context of application, the glossary of the *Society for Risk Analysis* (Accessed 17/10/2015) defines the Risk Analysis as: *"A detailed examination including risk assessment, risk evaluation, and risk management alternatives, performed to understand the nature of unwanted, negative consequences to human life, health, property, or the environment; an analytical process to provide information regarding undesirable events; the process of quantification of the probabilities and expected consequences for identified risks."*. With particular reference to the field of food safety, different systematic procedures to evaluate and manage potentially harmful effects are used and the choice of one system from another is a function on the type or the risk question under consideration; three frameworks are distinguished:

1. *World Organization for Animal Health.*

The World Organization for Animal Health (or Office International des Epizooties - OIE) provided a versatile standard framework for the assessment of the risk posed by the importation of animals and animal products (6). This framework is based on the standards described by *the*

Codes[1] and it is mainly designed to assess the magnitude of the risk for specified consequences in a given situation. In this system, the risk assessment follows hazard identification, which is considered a separate step and is completed first. In the risk assessment process, four steps are formally recognized: (i) Entry assessment, (ii) Exposure assessment, (iii) Consequence assessment and Risk estimation.

2. *Codex Alimentarius commission.*

The framework developed by the Codex Alimentarius Commission (CAC) is mainly designed to answer the question related to the maximum amount of a substance (or pathogen) to which a person can be exposed from a particular source; therefore, this system is usually adopted for setting allowed, acceptable or tolerable levels of contaminants and pathogens in food (2). This framework is mainly used in quantitative microbiological food safety risk assessment models (QMRA) and adopts the terminology of the National Academy of Sciences-National Research Council (NAS-NRC) in which the risk assessment is divided into the four steps: (i) Hazard identification, (ii) Hazard characterisation, (iii) Exposure assessment, and (iv) Risk characterisation.

3. *International Plant Protection Convention (IPPC).*

The International Plant Protection Convention (IPPC) is part of the Food and Agriculture Organization of the United Nations (FAO), and it is

[1] *Terrestrial Animal Health Code* (*Terrestrial Code*) and the *Aquatic Animal Health Code* (*Aquatic Code) are* known together as '*the Codes*'.

responsible for the development of the International Standards for Phytosanitary Measures (ISPMs) to guide governments in protecting their plant resources from harmful pests as a result of international trade in plants and plant products (7). At its simplest, the pest risk analyses (PRAs) process is aimed to determine whether a pest designated as 'quarantine pest' is of potential economic/health importance to an area in which it is not present. In this system, the risk assessment includes: (i) Pets categorisation, (ii) Assessment of probability of introduction and spread, (iii) Consequence assessment. The steps in this framework are conceptually similar to those reported in the OIE's one, with the main exception that in the IPPC framework, the 'pest categorisation' (the equivalent to Hazard identification) is not a separate procedure.

Under the premise that in the process of the risk analysis, the evaluation (risk assessment) and the management (risk management) of the risk are aspects of equally importance, the risk assessment is the module leading to the practical estimation of the risk, hence, it is strictly and solely related to scientific aspects of the whole process. Several definitions have been proposed for the word 'risk', but the most relevant in food safety is the one proposed by the CAC who defines the risk as: '*A function of the probability of an adverse health effect and the severity of that effect, consequential to a hazard(s) in food*'(1).

At its simplest, the risk can be considered as a function of: (i) the probability that an unwanted event occurs, and (ii) the consequences of the event if it occurs.

Irrespectively to the framework adopted (OIE, CAC or PRAs) for the estimation of the risk, qualitative and quantitative approaches are distinguished: in the first, qualitative terms such as 'high', 'medium', 'low' or 'negligible' are used for the expression of the outcome whereas in the second, the risk estimates are expressed by numbers; in the middle, semi-quantitative (or semi-qualitative) approaches can be used.

The qualitative risk assessment can be considered as a reasoned and logical discussion of the available scientific evidences, epidemiological and biological information associated with the hazard of interest, and all the factors involved. This approach is typically adopted in risk analysis models aimed to guide the food safety authorities in the decisions related to risks associated with the importation of live animals or animal products.

Qualitative models are usually less expensive, quicker and easier to present than the quantitative ones, this make those model the first option in routine decision-making processes and the favoured approach to be undertaken in situations in which food safety or health-related decisions are required but data are insufficient/absent (new emerging risks) or time is few (health emergency). Qualitative models are also chosen as a transparent option to define/rank risks priorities and thus, evaluate where the resources should be allocated and whether a more detailed quantitative approach is necessary.

The quantitative approach for the assessment of the risk foresee the implementation of mathematical models to link the steps along the risks

pathway(s), those models are usually complex and time-consuming, require exhaustive data, and quite advanced mathematical competences. Quantitative models can be either deterministic or stochastic, but normally, the complexity of the real world and biological dynamics requires the adoption of probability distributions to describe the variability and the uncertainty surrounding the factors (inputs) involved in the model. In fact, when a probabilistic approach is adopted, the probability of an unwanted event occurring is quantified by using simulation techniques (e.g. Monte Carlo) and consequently, the model output is a probability distribution of the possible outcomes. Conversely, in deterministic models, the output is always a single value rather than a distribution; as in this case, the effect of the variability and uncertainty is completely ignored, the use of those models is limited to the evaluation of specific (or 'what if?') scenarios. With respect to quantitative approaches, it is important to emphasize that although both the inputs and outputs are expressed numerically, quantitative models are not necessarily more objective or precise than a clear and transparent qualitative one, and there are inevitably significant challenges in describing the model itself, as well as interpreting and communicating the results.

In the field of food safety, any risk assessment model should ultimately give access to information on the level of the risk(s) related to a certain contaminant in the food supply and enable the decision makers to understand the current situation and take appropriate decision (e.g. setting or revising a maximum limit for that contaminant, improve the

surveillance system, review/establish label requirements, provide targeted advice to a specific population subgroup etc.).

The availability of a model reproducing the real system, enables authorities to identify the various points of control along the food chain at which the measures could be applied, weigh up the costs and benefits of these different options, and choose the most effective one(s). As such, it offers a systematic approach to consider the likely impact of the possible mitigation strategies or control measures, contributing towards enhanced utilization of public resources by focusing on the highest food safety risks. Not less importantly, qualitative and quantitative models can be efficiently used to identify the critical points in the system and the areas where more research and data collection is suggested (or necessary) to reduce the uncertainty surrounding the risk estimates.

The Risk analysis offers a systematic approach that all food safety authorities can use to make significant achievements in food safety issues, however, regardless of which system is chosen, it is essential for the analysis to be transparently documented.

AIM OF THE WORK

This thesis explores the use of the probabilistic modelling in the field of food safety with the major objective of using the systematic risk assessment methodologies to: (i) improve the current level of understanding of the dynamics of the biological system and (ii) provide new and science-based information for the animal derived food safety management.

OUTLINE OF THE THESIS

The thesis is divided into two main parts, the first (chapters 2, 3 and 4) deals with the *quantitative* probabilistic modelling while the second (Chapter 5) with the *qualitative* probabilistic modelling. For each chapter, a general introduction explaining the rational of the work is reported.

Chapter 2 – Overview

In chapter 2, a typical 'from farm-to-fork' probabilistic model is presented and a new approach aimed to model (stochastically) the variability and the uncertainty in the pathogenic potential at bacteria level is proposed. Moreover, the model includes Bayesian methodologies used to fit probability distributions to questionnaire-based dataset, this allowed the model to capture and include the variability and the uncertainty in the consumers' behaviours at household level.

Chapter 3 – Overview

The study presented in chapter 3 deals with the consequences that the acritical use of probability distributions and/or the overlook of relationship/dependency between distributions may have on the output(s) of quantitative models. This work is of particular relevance besides the strictly scientific aspect, in fact, considering that in the risk analysis process, the risk manager takes decisions trusting the results of a model provided by the risk assessor, this study highlights that not modelling the process correctly may lead to alarmistic but unrealistic scenarios.

Chapter 4 - Overview

The work presented in chapter 4 is a spin-off of an extended project promoted by the Food and Standard Agency (FSA) in collaboration with the Joint Working Group on *Campylobacter* (JWG) aimed at reducing levels of *Campylobacter* spp. colonisation in poultry at farm level in the UK. The quantitative model implemented in chapter 4 reproduces the dynamics underlying the spread of infection in chicken broiler flocks and integrates the result of the epidemiological study to assess the effects of interventions to control campylobacter and to reduce the incidence of highly contaminated flocks at slaughter. This work represents a practical example of an applied use of probabilistic modelling to show decision-maker the potential effect of different options and evaluate different scenarios.

Chapter 5 – Overview

The qualitative study presented in chapter 5 is a generalization of an in-field model commissioned to the author by a private company. The model is implemented to assess the likelihood of introduction of anisakids in Atlantic salmon farms and the consequent commercialization of infested products, but the general approach proposed lead itself to be adapted to other parasites as well as other farmed species.

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Part 1

QUANTITATIVE MODELLING

Chapter 2

Multiple Strain Approach and Probabilistic Modelling of Consumer Habits in Quantitative Microbial Risk Assessment: A Quantitative Assessment of Exposure to Staphylococcal enterotoxin 'A' in raw milk

General introduction

The increasing ability of bacterial characterization highlights differences in the pathogenic potential at bacteria level, indicating that not all the strains of a given pathogen are equally capable of causing disease in humans. This heterogeneity is often overlooked in quantitative microbiological risk assessments (QMRA).

Explicit inclusion of differences in pathogenicity across strains into QMRA Models in food safety, allowing the models to be updated as new information becomes available, would help to make models more realistic and to increase validity of their outputs. In this work, a probabilistic assessment of exposure to staphylococcal enterotoxin 'A' in raw milk was implemented to illustrate –methodologically - how the biological variability at bacteria level can be stochastically modelled and included in practice.

The second objective of the work was to explore the importance of the so-called 'Consumer Phase Module'. Despite a number of key steps determining the exposure take place at household level, this is another aspect often overlooked in quantitative microbial risk assessment. At this

respect, different stochastic processes were used to describe the variability and the uncertainty in the consumer behaviour and/or informative opinions where data were not available. This allowed the extension beyond the evaluation of the worst or "what if" scenarios only.

For a better appreciation of the contribution of the uncertainty in the factors under investigation, the uncertainty components were clearly shown and separated from the variability by using second order plots.

Multiple Strain Approach and Probabilistic Modelling of Consumer Habits in Quantitative Microbial Risk Assessment: A Quantitative Assessment of Exposure to Staphylococcal enterotoxin 'A' in raw milk

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ABSTRACT

Quantitative microbial risk assessment (QMRA) models are extensively applied to inform management of a broad range of food-safety risks. Inevitably, QMRA modelling involves an element of simplification of the biological process of interest. Two features that are frequently simplified or disregarded are the pathogenicity of multiple strains of a single pathogen and consumer behaviour at household level. In this study, we developed a QMRA model with a 'multiple strain' approach and a consumer phase module (CPM) based on uncertainty distributions fitted from field data. We modelled exposure to staphylococcal enterotoxin 'A' in raw milk in Lombardy; a specific enterotoxin production module was thus included. The model is adaptable and could be used to assess the risk related to other pathogens in raw milk as well as other staphylococcal enterotoxins. The multiple-strain approach, implemented as a multinomial process, allowed the inclusion of variability and uncertainty with regard to pathogenicity at bacterial level. Data from 301 questionnaires submitted to raw milk consumers were used to obtain uncertainty distributions for the CPM. The distributions were modelled to be easily updatable with further data/evidence. The sources of uncertainty due to the multiple strain approach and the CPM were identified and their impact on the output was assessed by comparing specific scenarios to the baseline. When the distributions reflecting the uncertainty in consumer behaviour where fixed to 95th percentile, the risk of exposure increased up to 160 times. This reflects the importance of taking into consideration the diversity of consumers' habits at household level and the impact that the

lack of knowledge on the variables in the CPM can have on the final QMRA estimates. The multiple-strain approach lends itself to use in other food matrices besides raw milk and allows the model to better capture the complexity of the real world and to be capable of geographical specificity.

1. INTRODUCTION

Probabilistic modelling is being used with increasing frequency to address food safety issues. In recent years, quantitative microbial risk assessment (QMRA) models have been applied extensively in this area and risk analysis has become standard practice for food safety management systems to ensure that regulatory decisions about foods are science-based and transparent (41, 42).

Depending on the scope of the analysis, the probabilistic model is not necessarily "from farm to fork" (16, 18, 23), but irrespective of the starting and the end point on the food chain, the common thread from a modelling perspective is the representation of the pathways that bacteria may take and the ascertainment of the fate of the microbial cells along the way. Thus, the complexity of a QMRA model is related to the question that the model aims to answer, and the main challenge for risk assessors is to capture the complexity of reality with the available scientific evidence and data. In this work, we focused on two aspects that, if included in QMRA models, can enhance their ability to capture the complexity of real food safety scenarios: differences in pathogenicity between strains and consumer behaviour at household level. Several studies have revealed a remarkable degree of diversity on the pathogenic

potential across different strains of food-related pathogens such as *Campylobacter jejuni* (19, 43) *Listeria monocytogenes (7, 10, 22) Escherichia coli (6, 27)* or *Staphylococcus aureus (12, 15, 24)*

The first aspect we considered was the uncertainty and variability in pathogenic potential at bacteria level.

The second aspect relates to consumer behavior at household level. The estimation of the changes in bacteria concentration along the steps of the food chain is a cardinal point in any QMRA model; usually, because of environmental conditions regulating bacterial growth and death, it is an intricate problem.

The inclusion in a QMRA model of consumer behavior at household level is usually dealt with by including a 'consumer phase module' (CPM) which is often characterized by a large variation in consumer habits and limited data availability (32).

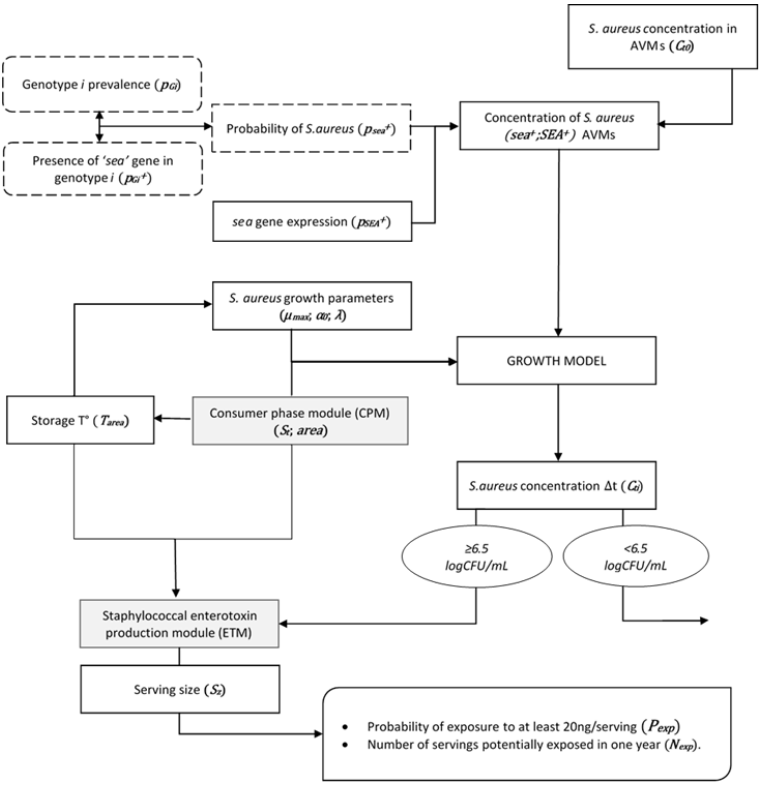
Following these considerations, the objectives of this study were (i) to develop a QMRA model with a multiple strain approach and (ii) to develop a CPM using uncertainty distributions fitted from field data.

A probabilistic assessment of exposure to staphylococcal enterotoxin 'A' in raw milk from automatic vending machines (AVMs) in Lombardy (Italy) was performed. The toxin-mediated virulence of the bacteria required a specific module describing the production of the staphylococcal enterotoxin.

The model, outlined as a flowchart in Figure 1, is described in detail to

illustrate the inclusion of the multiple strain approach and a CPM in a QMRA.

Figure 1 Flowchart of the exposure assessment model showing the steps involved in the multiple strain approach (dotted line), the consumer phase module and the enterotoxin production module (both shown with grey background)



2. MATERIAL AND METHODS

2.1. Staphylococcus aureus in AVMs.

In Italy, raw milk sold in an AVM comes directly from the bulk tank of a single dairy farm, as farms are not allowed to mix their milk with that of any other farm into the AVMs (37). In Lombardy, similarly to all other regions, the veterinary services regularly test milk samples from all the AVMs. The legal requirement for pathogens such as *Listeria monocytogenes*, *Campylobacter jejuni* or verocytotoxigenic *Escherichia coli* in drinking raw milk is 'absence in 25ml' established by means of highly sensitive tests such as PCR. In contrast, the legal requirement for *S.aureus* is quantitative: '<100 CFU/ml' (37). Therefore, the first step in this study was to estimate the level of contamination in purchased raw milk. To this end, assuming independency between herd size, volume of milk sold through AMVs and level of contamination, data from the regional monitoring program for raw drinking milk were used. The final dataset consisted of 3382 official samples collected from 420 different AVMs in the region between 2011 and 2014. Samples were analysed by the twelve agencies of the Experimental Zooprophylactic Institute of Lombardy and Emilia Romagna (IZSLER) on the territory, each agency analysed the samples of AVMs located in its area of competence.

Quantitative data of *S.aureus* in the dataset were reported as '*x* CFU/ml' with *x* being the number of counted colonies (based on the dilution that was applied) or '<*n* CFU/ml' with *n* being the threshold of the detection limit. Results were not homogeneous with respect to the sensitivity (different thresholds were reported) reflecting differences in the method

or dilution applied by the labs during the years. To parameterize the distribution describing the uncertainty in mean log CFU/ml, taking into consideration the proportions of samples analysed with different detection limits, the maximum likelihood estimation (MLE) method for a Gamma distribution with left censored data was used (40). Assuming that a given set of data can be described by a certain distribution (e.g. Gamma), the method of maximum likelihood is aimed to provide an estimation of the distribution's parameter(s) so that the joint probability of the observed data under the resulting distribution is maximized:

$$\log L(X|\alpha)=\sum \log(f(x_i,\alpha)) +\sum(n_{ti} * \log(F(T_i,\alpha))) \quad \text{(Eq.1)}$$

Where α represents the parameter(s) of the distribution of the likelihood function (α and β of the Gamma distribution), $logL(X|\alpha)=\sum log(f(x_i, \alpha))$ is the likelihood of observing the *n* observations recorded given α, and $logL(X<T|\alpha) =n_{ti}*log(F(T_i, \alpha))$ is the likelihood that n_{ti} observations fall below each minimum threshold T_i given α. The gamma distribution was chosen because data are continuous and its parameters α (shape) and β (scale) allow great flexibility making possible for the distribution to assume a range of different shapes.

2.2. Prevalence of S. aureus with 'enterotoxin A' gene (sea) in AVM, the multiple strain approach.

The multiple strain approach was aimed to take into account the variability and the uncertainty at bacteria level, therefore, the prevalence of *S. aureus* harbouring the staphylococcal enterotoxin 'A' gene ($S.aureus_{sea+}$) was estimated in the model by combining multiplicatively

the frequency of the different genotypes, with the probability of detecting the gene in each genotype.

Geographical differences in the prevalence of some *S.aureus* strains have been observed in previous studies (21, 38), therefore, results from an extensive regional survey funded by the Italian Ministry of Health, and published by IZSLER in 2014 were used to estimate the frequency of *S. aureus* genetic clusters in Lombardy (8). This made the model specific for the geographical area of interest. In that study, 1099 *S.aureus* isolates were analysed and 471 different strains distributed in 44 genotypes were recognised. The uncertainty about the true proportion of each genetic cluster p_{Gi} was modelled by the Dirichlet distribution:

$$(p_{G1}, p_{G2}, p_{Gk}) = \text{Dirichlet}\ (s_{G1}+1; s_{G2}+1; \ldots ; s_{GK}+1) \qquad \text{(Eq.2)}$$

The Dirichlet distribution was selected because it is the conjugate to the multinomial process. s_{Gi} were the number of strains recorded in each of the genetic clusters.

In a recent study, *S. aureus* strains isolated from cow milk samples in 10 European countries were genotyped and the virulence genes (enterotoxin genes, polymorphisms of *coa*, *lukE*) analysed(9), the results from this study were used to estimate the probability of *sea* gene being present in each genotype.

A total of 393 strains were included in this study; of them there were 51 strains positive for *sea* whereby 47 observations resulted from strains positive for GTB, 2 from GTAM-, 1 from GTE-, and 1 from GTAH-.

For each combination genotype - *sea*$^{+}$, a Beta distribution was used to include the uncertainty:

$$p_{Gi}^{sea+}=\text{Beta}\ (s_{sea+} + 1;\ n_{Gi} - s_{sea+} + 1) \qquad \text{(Eq.3)}$$

Where s_{sea+} was the number of genotype *i* isolates positives to *sea* and n_{Gi} was the total number of genotype *i* isolates.

Both, the study conducted in Italy (8), and the European study (9) genotyped the samples using the same RS-PCR protocol (12), therefore, results were comparable and for each cluster, the probability for each i^{th} genotype to be *sea*$^{+}$ was estimated by the joint probability:

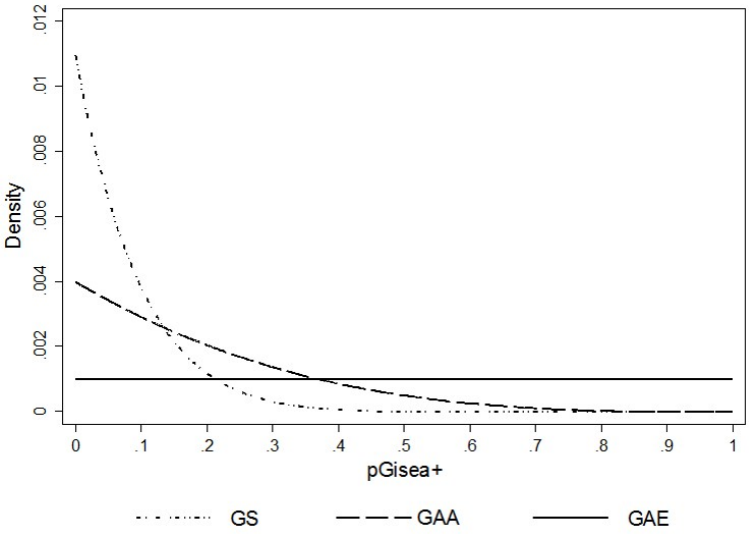
$$p_{Gi}^{+} = p_{Gi} \cap p_{Gi}^{sea+} \qquad \text{(Eq.4)}$$

Consequently, the overall probability to find *S.aureus* isolates with *sea* gene (*S.aureus*$_{sea+}$) in Lombardy was estimated as:

$$p_{sea}^{+} = p^{+}_{G1} \cup P^{+}_{G2} \cup \ldots \cup p^{+}_{Gk} \qquad \text{(Eq.5)}$$

Since p_{sea}^{+} depends on several uncertainty distributions (Fig.2), a second order plot was used to separate the uncertainty from the randomness of the system and evaluate the impact on the model output (see next).

Figure 2 Uncertainty distributions describing the probability of harbouring the sea gene in genotypes S, AA and AE. The shape of the distributions reflect the lack of knowledge about the presence of the gene on certain genotypes. Where no information was available, the uninformative prior Beta(1; 1) was assumed, that was the case, for example, in genotype AE.



2.3. sea gene expression.

Previous studies support the existence of a good correspondence between the presence of *sea* and production of enterotoxin 'A'(21, 26).

The uncertainty in this correlation was assessed by including the results of an Italian study (30). In that study, a non-correspondence between the presence of *sea* and the enterotoxin 'A' was observed in 4 out 32 raw milk samples with *S.aureus*$_{sea+}$ isolates.

A Beta distribution was used to take into account the probability of there being no correspondence between the presence of the gene and

enterotoxin production:

$$p_{SEA}^{+} = Beta(s_{SEA+} +1; n - s_{SEA+} +1) \quad \text{(Eq.6)}$$

Where s_{SEA+} was the number of samples showing enterotoxin production (s_{SEA+}=28) and *n* was the total number of samples harbouring the *sea* gene (n=32).

Because of previous considerations, consumers were expected to purchase raw milk from a random AVM in Lombardy with at least one *S. aureus*$_{sea+;\ SEA+}$ according to:

$$Bernoulli(p_{sea}^{+} * p_{SEA}^{+}) \quad \text{(Eq.7)}$$

2.4. Consumer Phase Module.

From October 2013 to November 2014, a questionnaire aimed to assess the habits of raw milk consumers was used to gather information from 301 raw milk consumers interviewed while they were purchasing raw milk at vending machines.

Respondents were asked about their habits related to the raw milk they purchase from AVMs. Results for selected key variables of relevance for QMRA modelling (position of the milk in the refrigerator, storage time, litres purchased weekly, whether the milk was boiled before consumption or not, estimated transport time and utilization of thermal bags) were summarized in the form of probability distributions and associations between pairs of variables assessed by means of chi-squared tests of association.

One important information in the CPM is the position of the milk in domestic refrigerators; in fact, the usual area where milk is kept (*area*) is important because the mean temperature may vary considerably across different areas. Interviewees were asked to choose between: Upper shelf (u), Middle shelf (m), Lower shelf (l), Door shelf (d) or Do not know/indifferent (x). The Dirichlet distribution was selected to include the uncertainty surrounding the chances that a milk bottle is kept in a given refrigerator area:

$$(p_u, p_m, p_l, p_d, p_x) = \text{Dirichlet}\ (s_u+1; s_m+1; s_l+1\ s_d+1; sx+1) \qquad \text{(Eq.8)}$$

Where s_u, s_m, s_l, s_d, and s_x were the number of observations recorded in each area, therefore, In each iteration, the position of the milk in the refrigerator was modelled with a Multinomial process:

$$\text{area}=\text{Multinomial}\ (1\ ;\{p_u, p_m, p_l, p_d, p_x\}) \qquad \text{(Eq.9)}$$

When position *x* was sampled, it was redistributed in one of the other categories according to the Discrete distribution: Discrete(u,m,l,d; p_u,p_m,p_l,p_d,) with p_u, p_m, p_l and p_d being the point estimated prevalences observed in the survey.

Respondents were asked to say how many days the milk is usually kept in the refrigerator. Answers were converted to hours and MLE for a Gamma distribution with interval-censored data was used to estimate the uncertainty in the mean of storage time per bottle (S_t).

$$\log L(X|\alpha)=\sum n_i * \log(F(h, \alpha)-F(l, \alpha)) \qquad \text{(Eq.10)}$$

Where *logL(X|α)* is the likelihood of randomly observing the *n*

observations recorded between the *I* intervals, given the parameter ⍰⍰ and *log(F(h, α)-F(l, α))* is the difference between the cumulative distributions of the high (*h*) and low (l) intervals. A truncation limit of 120h was assumed considering that the shelf life for raw milk in Italy is legally three days (72h) and none of the interviewees reported keeping the milk more than five days (120h). A Poisson(λ*t) with λ = S_t and t = 1, was then used to take inter-variability into account.

The '*usage of thermal bag'* was modelled by a Dirichlet and Multinomial process (eq. 8-9) with possible outcome: 'Always' (s_a), 'Only in summer-hot days' (s_s) and 'Never' (s_n), and, similarly to S_t, MLE for a Gamma distribution with interval-censored data (Eq.10) was used to model the transport time (T_t). A truncation limit to 60 minutes was assumed in this case.

Boiling the milk before consumption to prevent intoxication by heat-sensible pathogens like *Listeria monocytogenes, Salmonella, Campylobacter jejuni* is strongly suggested and visible specific warning labels are a legal requirement on every AVM (28). However, out of 301 interviewed consumers, 203 declared to boil the milk before consumption, the remaining 98 stated to drink the milk raw or heated but without reaching the boiling point. A Beta distribution was assumed to describe the true prevalence of consumers who boil milk before consumption:

$$P_{boil}=Beta(s_b+1;\ n-s_b+1) \qquad \text{(Eq.11)}$$

Where s_b is the number consumers who declared to boil the milk (s_b=203)

and *n* is the total number of consumers interviewed (n=301).

Like the storage time, the temperature at which the milk is kept at household level is an important parameter to estimate the microbial growth. To date, no official estimation of domestic refrigerator temperatures in Italy has been published to our knowledge.

Distributions describing the mean temperature in different refrigerator areas (T_l, T_m, T_d, T_u) were obtained from the opinion of seventeen professional refrigerator repair services operating in Lombardy. Specialists were asked to provide their best estimations about the minimum (MIN), maximum (MAX), and most probable (MLIKE) mean temperatures that people had in each area of their refrigerators. For each estimation, interviewees were also asked to give a score ranging from 1 (not sure) to 4 (sure) to describe how confident they were with their own estimates. Results were then included into a discrete distribution: Discrete($\{x_i\}$;$\{p_i\}$) where the $\{x_i\}$ are the Pert distributions and $\{p_i\}$ are the weights given to each opinion according to their own confidence level. In this way, each Pert distribution has a chance to be sampled proportional to its score.

$$T_{area} = \text{Discrete}(Pert_1, Pert_2 \ldots Pert_{17;}\ Score_1, Score_{2\ldots}\ Score_{17}) \quad \text{(Eq.12)}$$

Where $Pert_i$ represents the estimation of the i^{th} interviewee, and $Score_i$ is the level of confidence of the i^{th} interviewee with their own estimation.

2.5. Growth parameters: Specific growth rate, Lag phase.

In the model, the growth of *Staphylococcus aureus* in milk was estimated as follows:

$C_{t1}=C_{to}$ for $S_t \leq \lambda$; (Eq.13)

$C_{t1}=C_{to}+[(\mu^*(S_t - \lambda)]$ for $\lambda < S_t < T_{max}$ and (Eq.14)

$C_{t1}=C_{max}$ for $S_t \geq T_{max}$ (Eq.15)

where C_{t1} (log CFU/ml) is the population density at time S_t; C_{to} (log CFU/ml) is the initial population density; C_{max} (log CFU/ml) is the maximum population density; T_{max} is the time at which the maximum population density is reached (h); $\mu=\mu_{max}/ln\ (10)$ *with* μ_{max} (log CFU/ml*h^{-1}) being the maximum specific growth rate and λ is the lag phase (h).

Growth rates at different temperatures (T_{area}) were estimated by the square root model described by Ratkowsky (36):

$\mu_{max}^{0.5}=b(T- T_{min})$ (Eq.16)

where *b* and T_{min} were regression parameters.

Those parameters were obtained by plotting experimental μ_{max} values against temperature. Briefly: Eight *S.aureus* strains were inoculated in eight whole fresh milk cartons after purification at a concentration of 2 log CFU/ml. Cartons were kept in isothermal conditions at 10°, 12° and 16°C for seven days. Samples were taken from each carton at each temperature three times a day and *S.aureus* enumerations according to ISO 6888:1983 were recorded. Experimental μ_{max} values were obtained

analysing the resulting growth curves with DMfit software.

The parametric bootstrap was used to include uncertainty in the regression parameters and consider the growth variability among different *S.aureus* strains. Lag phase values at different temperatures were estimated by using the parameter expressing the physiological state of the cells (α_0). This parameter is a dimensionless number ranging from 0 to 1 expressing the idea that λ is inversely proportional to μ_{rate} and depends on the physiological state of the inoculum as well as on the actual environment (3, 5):

$$\lambda = [-\ln(\alpha_0)/\mu_{max}] \qquad \text{(Eq.17)}$$

In order to take into account the variability in α_0, a survey on forty *S. aureus* strains was carried out in 2014 by IZSLER (Bergamo section) and experimental data were included into a Cumulative(0;1{x_i},{p_i}) distribution where {x_i} and {p_i} represented the vectors of the forty α_0 values and the respective probabilities on cumulative scale respectively.

Briefly: forty *S. aureus*$_{sea+}$ strains, isolated from raw milk, were selected and supplied by IZSLER (Lodi section). For each strain, a well-isolated colony was transferred in 10ml of BHI and incubated at 37°C for 24h and pure cultures were obtained. Appropriate dilutions were calculated and fresh pasteurized whole milk cartons (commercial product) were inoculated to obtain a target level of 2 log CFU/ml. Assuming independency of α_0 from the temperature if pre-inoculation history of cultures is identical (4, 35), cartons were stored under controlled isothermal conditions at 12°C for practical reasons. Duplicate samples

were taken at appropriate time intervals to allow an efficient kinetic analysis of microbial growth parameters. Growth curves and kinetic parameters were estimated using the curve-fitting program DMfit based on (4) and α_0 values were calculated by the inverse formula of Equation 17.

A maximum population density of C_{max}=8.7 log CFU/ml was assumed because it was the higher density observed amongst the forty *S.aureus* in milk in the trial (result not shown).

2.6. SEA production model.

Several conditions were required and assumed in the model to enable SEA production:

1. *S. aureus* density must be >6.5 log CFU/ml (13).

2. S_t must be greater than λ to let *S. aureus* growth if C_{t0}<6.5 log CFU/ml;

3. The difference: S_t - λ has to be large enough to allow the quorum density achievement if C_{t0}<6.5 log CFU/ml, and S_t >λ.

4. Once the quorum density is achieved, the remaining time during which SEA may be produced (t_{sea}) is equal to:

   $$t_{sea} = (S_t - \lambda) - [(6.5 - C_{t0})/ \mu_{rate}] \quad \text{(Eq.18)}$$

   where the second term represents the required time to reach the quorum density.

Even if the conditions above are met, the temperature must be greater than 14.95° C (13).

2.7. Enterotoxin A production model.

According to (13), the SEA production rate in the model (ng/ml*h^{-1}) increased linearly at temperatures between 14.95° and 32°C. The regression line in this temperature range was described as:

$$p = 0.0376*T° - 0.599 \quad \text{(Eq.19)}$$

Therefore, in our model, the amount of enterotoxin produced (ng/ml) in the available time (t_{SEA}) was calculated as:

$$p = (0.0376*T_{area} - 0.599)*t_{SEA} \quad \text{(Eq.20)}$$

In that study, highly virulent strains were used; this makes our model conservative.

2.8. Number of raw milk servings.

To our knowledge, no official estimates exist for the total number of raw milk consumers and/or the total amount of raw milk sold in Lombardy from AVMs. Considering that from the survey, 96% of consumers reported buying at least 1L per week, and assuming a mean of 30 L of raw milk sold daily (Personal communication from Local Health Authority, Lombardy Region) from each of the 338 AVMs registered in Lombardy in 2015, a total conservative number (N_{pop}) of $2.55x10^7$ servings/year in Lombardy was estimated.

Since herd size is not necessarily correlated to volume of milk sold

through AVMs, in the exposure assessment, it was assumed that the relative contribution of the farm to the exposure is independent of the herd size.

2.9. Serving size.

No specific information about raw milk consumption (ml/person per day) in Italy has been published to our knowledge. Assuming a daily consumption comparable to that of pasteurized milk, data from the National Institute for Food and Nutrition Research were used (34) to estimate the serving size (S_z).

It was assumed that data from the age category 0-2 years came from breast milk consumption or reconstituted milk for babies and the contribution of this category was excluded. S_z was thus modelled as:

Normal[145; 104(truncate (0)] ml (Eq.21)

2.10. Risk Output.

The limited dose-response information available for humans did not allow the development of a complete dose-response model. Therefore, the output of the model was an estimate of the probability (p_{exp}) of a serving carrying the minimal dose of SEA deemed sufficient to be harmful to humans.

The conservative threshold of 20ng/serving was chosen (1) and the total amount of enterotoxin per serving was obtained by multiplying the amount of enterotoxins produced in one ml (Eq.20) by the serving size (Eq.21).

2.11. Simulation.

Because of the large number of inputs and distributions included, the output risk was estimated as a mean of 1,000,000 Monte Carlo iterations. The software @Risk (Palisade Corporation, Version 6.3 for Excel) and STATA/SE 14 were used. The flowchart of the baseline model was shown in Figure 1. Input distributions and functions are presented in Table 1.

To better assess the impact of the exposure in the target population, the estimated probability was used to obtain the distribution of the number of servings carrying a dose ≥ 20ng/ml yearly in Lombardy p(N_{exp}):

$$p(N_{exp})=Poisson(N_{pop}*p_{exp}) \quad \text{(Eq.22)}$$

In order to assess the impact of the uncertainty distributions included in the model with the multiple strain approach, and the CPM, two stressed scenarios were compared to the baseline model. In the first (Scenario1) all the uncertainty distributions p_{Gi}^{sea+} were fixed to the 95th percentile. In the second (Scenario2) S_t and T_{area} were both fixed to 95th percentile.

To better evaluate the effect of the uncertainty in the multinomial process, Scenario2 was used as a baseline and two additional scenarios with all the uncertainty distributions p_{Gi}^{sea+} fixed to the 5th (Scenario2a) and 95th (Scenario2b) percentile respectively were compared.

Table 1 Baseline model input description, unit and data source

Parameter	*Model id*	Unit	Assumed distribution / Function	Data source
Genotypes prevalences	p_{Gi}	%	Dirichlet (s_{G1}+1; s_{G2}+1; … ;s_{Gk}+1)	(8)
Presence of '*sea*' gene	p_{Gi}^{sea+}	%	Beta(α;β)	(9)
Probability of a given genotype being *sea*$^+$	p_{Gi}^{+}	%	$p_{Gi} \cap p_{Gi}^{sea+}$	/ Function
Overall Probability of *S.aureus* being *sea*$^+$	p_{sea}^{+}	%	$p^{+}_{G1} \cup p^{+}_{G2} \cup \ldots \cup p^{+}_{Gk}$	/ Function
Probability of gene expression	p_{SEA}^{+}	%	Beta(α;β)	(30)
Probability of *S.aureus*$_{sea+;SEA+}$	$p_{sea}^{+};_{SEA}^{+}$	%	Bernoulli($p_{sea+} * p_{SEA+}$)	/ Function
S.aureus concentration	C_{t0}	log CFU/ml	Gamma(α;β)	Surveillance data
Storage time	S_t	h	Gamma(α;β)+truncation	Survey
Position in fridge	*Area*	(u,m,l,d)	Dirichlet(s_u+1; s_m+1; s_l+1 s_d+1;s_x+1) Multinomial(1;{p_u, p_m, p_l, p_d, p_x})	Survey
Storage temperature	T_{area}	°C	Discrete(data;{p})	Personal comm.
Growth parameters	a_0 μ_{max};$^{0.5}$ λ	/ log CFU/ml*h^{-1} h	Cumulative(data; (P_{data})) b(T_{area}; T_{min}) [-ln(α_0)/μ_{max}]	Experimental data / Function / Function
Available time to produce SEA	t_{SEA}	H	(St – λ) - [(6.5- Ct_0)/ μrate]	/ Function
SEA production	p	ng/ml*h^{-1}	(0.0376*T_{area}- 0.599)*t_{SEA}	/ Function
Serving size	S_z	ml	Normal(μ; σ)+truncation	(25)

3. RESULTS

3.1. S.aureus concentration in purchased raw milk.

Following the estimation of the parameters obtained by the MLE (Eq.1), the Gamma distribution describing C_{t0} resulted:

C_{t0}=Gamma(0.10; 4.52) log CFU/ml (Eq.23)

Thus, estimated levels of *S.aureus* in purchased raw milk showed a mean of Ct_0=0.39 log CFU/ml and 93.8% of simulated results below 2 log CFU/ml.

3.2. Prevalences.

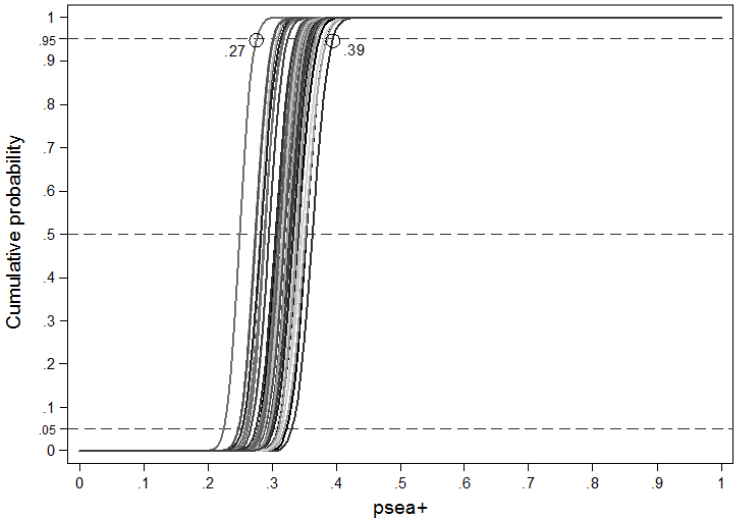
After simulation p_{sea}^{+} ranged from a minimum sampled value of 14.28% to a maximum of 38.73% with μ=25.17% 5th and 95th percentile of 26% and 36% respectively. The second order plot for p_{sea}^{+} (Fig.3), showed the contribution of the uncertainty component.

This uncertainty could be explained by the lack of knowledge surrounding both the occurrence of different genotypes (Eq.2) and the uncertainty surrounding the incidence of *sea* in each of them (Eq.3). In fact, identified genetic clusters showed high diversity with respect to the presence of the gene; three representative uncertainty distribution for p_{Gi}^{sea+} were reported (Fig.2). The uninformative distribution Beta(1;1) was used to describe p_{Gi}^{sea+} in genotypes where the presence of *sea* was never tested.

p_{SEA}^{+} ranged from a minimum sampled value of 45.24% to a maximum of 99.42%. Because of the few samples showing a non-correspondence between the presence of *S.aureus*$_{sea+}$ and the detection of SEA, the

uncertainty distribution for p_{SEA}^{+} resulted skewed to the left with mean and mode 85.29% and 87.23% respectively.

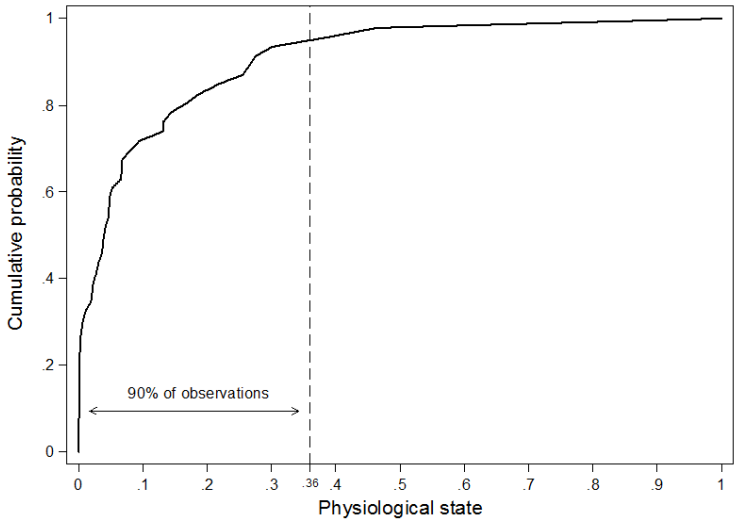
Figure 3 Second order plot for psea+. The graph shows how the uncertainty in the presence of sea in considered genetic clusters affects the cumulative distribution expressing the overall probability for S.aureus isolates to be S.aureussea+. Over 50 simulated scenarios in which each of the beta distributions describing PGisea+ were fixed to randomly sampled percentiles; a difference of more than 10 percent points was recorded at its widest. On the 95th percentile psea+ ranged from 27.52% to 39.46%.



3.3. Growth model.

The cumulative distribution describing the variability in α_0 is reported in Figure 4, the inclusion of the parametric bootstrap in the square root model (Eq.16) resulted in a variation of predicted μ_{max} and λ at each sampled temperature at each iteration; in fact, λ depends directly on μ_{max} and α_0 (Eq.17).

Figure 4 Cumulative distribution for the dimensionless physiological state parameter α_0 estimated from experimental data. 90 % of the observations ranged from 0 to 0.36



3.4. Consumer Phase Module results.

Answers concerning the area where the milk is usually kept (Eq.8-9), storage and transport time (Eq.10), proportion of consumer who boil the milk before consumption (Eq.11) and the usage of thermal bags, were reported although not all these information were used in the model. Results recovered for s_u, s_m, s_l, s_d, s_x were 15, 16, 60, 182 and 28 respectively; point estimate for P_{boil} was 67,4% and answers recovered for s_a, s_s, s_n were 177, 82 and 42.

The result of Chi-squared tests did not shown evidence of associations between the frequencies of pairs of risky behaviours (results not shown).

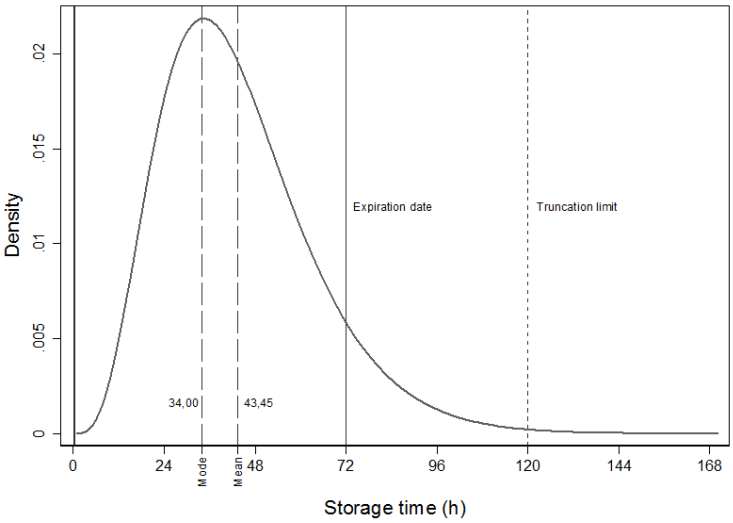
The Gamma distributions describing the uncertainty in S_t (Fig.5) and T_t

resulted respectively in:

S_t = [Gamma(4.85; 8.89); truncate(120)] h (Eq.25)

T_t = [Gamma(1.82; 8.19);truncate(60)] min (Eq.26)

Figure 5. Distribution describing the uncertainty in St (h). Assumed truncation limit (120h), expiration date (72h) and location parameter mean and mode are shown.



3.5. Temperature distributions.

After the simulation, as expected, the highest temperature was assigned to the door (μ=9.8, 95th =13.28) followed by the upper shelf (μ=7.3, 95th =10.16), the middle (μ=6.3, 95th=9.2) and the lower shelf (μ=5.5, 95th=8.3).

3.6. Risk output.

After simulation, in the baseline model, the estimated risk of P_{exp}≥20ng/serving resulted in $1.9x10^{-5}$ indicating that the 99.99^{th} percentile of servings are not likely to contain 20ng or more of staphylococcal enterotoxin 'A'.

After 1,000,000 simulations, Over $2.55x10^{7}$ servings sold in Lombardy per year, the median of p(N_{exp}) was 485 servings/year; the maximum estimated value for N_{exp} resulted 589.

In Scenario1, P_{exp}≥20ng/serving resulted $2.9x10^{-5}$ indicating that the 99.99^{th} percentile of servings are not likely to contain 20ng or more of staphylococcal enterotoxin 'A'. The median of p(N_{exp}) resulted in 740 servings/year; the maximum simulated value for N_{exp} was 890.

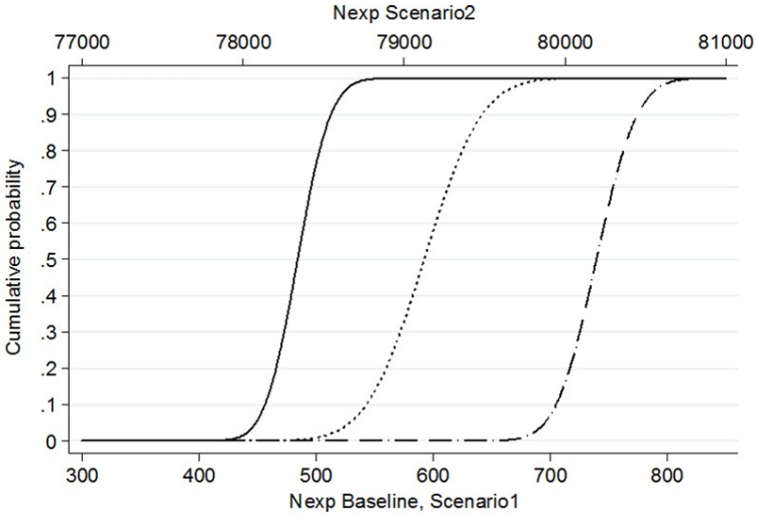
In Scenario2, P_{exp}≥20ng/serving was $3.1x10^{-3}$ indicating that the 99.69^{th} percentile of servings would not likely contain 20ng or more of staphylococcal enterotoxin 'A', while the median value recovered for p(N_{exp}) was 79,127 servings/year; the maximum estimated value for N_{exp} was 80,660.

Results of estimated P_{exp} for the computed scenarios together with corresponding values of p(N_{exp}) at 50^{th} , 95^{th} and 99^{th} percentile are summarized in Table 2 and Figure 6.

Table II Baseline, Scenario1 and Scenario2 model outputs: P_{exp}≥20ng/serving and values at 50th, 95th and 99th percentile of $p(N_{exp})$ are reported.

SCENARIO	P_{exp}	$p(N_{exp})$		
		Median	95th	99th
BASELINE MODEL	1.9 x10^{-5}	485	522	537
SCENARIO 1	2.9 x10^{-5}	740	785	804
SCENARIO 2	3.1 x10^{-3}	79,127	79,590	79,782

Figure 6 Cumulative distributions describing N_{exp} after simulation. Results for the baseline (solid black), Scenario1 (long-dash line) and Scenario2 (dotted line) are reported. Because of the different scales of the scenario outputs, an additional x axis was used at the top to represent the cumulative distribution of N_{exp} for scenario2.



When all the uncertainty distributions p_{Gi}^{sea+} were fixed at the 95th percentile (Scenario1), the risk increased by approximately 1.5 times,

while when the second stressed scenario was simulated, the risk increased by approximately 160 times compared to the baseline. Results from scenario2a-2b represented the potential contribution of the uncertainty at bacteria level. The median of p(N_{exp}) resulted 35,709 and 128,186 servings/year in Scenario2a and 2b respectively; that is, about -55% and +62% of the median of p(N_{exp}) in Scenario2 (79,127).

4. DISCUSSION

The multiple strain approach, implemented as a multinomial process, allowed us to include in the model the variability and the uncertainty in pathogenic potential at bacteria level. The same approach can be easily applied to other pathogens or food matrices without substantial modifications.

In fact, the occurrence of the pathogenic factor of interest in the food matrix under consideration is fully determined in the simulation by the uncertainty distribution describing the occurrence of each genetic cluster in the population (Eq.2) and the uncertainty distributions describing the occurrence of the pathogenic factor in each of them (Eq.3).

Consequently, considering the increasing understanding of virulence factors at genetic level, our approach may be used to: (i) account for the fact that different genotypes may represent different magnitude of public health risk and (ii) assess specific scenarios in which the frequencies of particular genotype increase (or decrease) across the geographic area of interest.

In the CPM, the inclusion of consumers' habits as uncertainty

distributions fitted to data allowed extension beyond the evaluation of the worst or "what if" scenarios, including every possible scenario in the output (baseline).

As expected, the average value for S_t was around 2 days (43.4h). Despite the requirement that the expiry date of 72h must be clearly shown on all the AVMs (11), it appears that 9.5% of respondent still kept the milk up to 120h.

To our knowledge, the first QMRA related to raw milk consumption in Italy involving information on consumers' habits (obtained from 100 interviewee) is the one published by Giacometti et al. in 2012 (8). Some of their results differ substantially from our findings and data were elaborated differently. (i) the proportion of consumers who did not boil the milk before consumption resulted 33% in our study, Giacometti et al. estimated 43%; (ii) variability in refrigerators' temperature and storage area were not considered in that study; (iii) Giacometti et al. described the 'storage time' by a triangular distribution, we used the MLE to fit a distribution to data. The difference is not in the distribution's means, which differ by less than 2 hours, but in distribution's shapes. In fact, 31.4% of simulated values are included between 60 and 120h in the triangular distribution while, for the same range, the gamma distribution included 17.8% of observation (results not shown). The results differ substantially and the discrepancy in the distributions' shapes is likely to have a significant effect on an output obtained by means of Monte Carlo simulation. Moreover, considering that: (i) the variables included in a generic CPM of a raw milk-related QMRA (storage time and temperature,

heat treatment) are usually critic for microbial growth and/or survival and (ii) health authorities have no practical options to control these factors at household level; the distributions included in the CPM has a crucial effect on the final output and should be described accurately.

By using uncertainty distributions, the lack of knowledge that characterizes these parameters can easily be shown to decision makers by means of second order plots or, alternatively, by comparing the results of the best and worst scenarios with the baseline. As an example, results of Scenario2, clearly showed the extreme impact that the variability and uncertainty surrounding the data that generated the distributions in the CPM may have on the outcome.

Modelling the risk related to staphylococcal enterotoxin 'A' in raw milk gave us the opportunity to develop a QMRA model able to operate with a considerable number of biological variables and to model them from a probabilistic point of view. Moreover, in contrast to other pathogens, the risk related to *S.aureus* in raw milk was linked to its ability to produce enterotoxins; therefore, the model required additional steps beyond the microbial growth. This made our model a manageable, adaptable and useful tool that can be used to assess the risk related to other pathogens in raw milk as well as other staphylococcal enterotoxins.

Predicted N_{exp} in the baseline model was in agreement with the fact that no strong evidence of, or suspected *S.aureus* intoxication related to raw milk consumption have been reported in Lombardy since the sale of raw milk was allowed in 2004.

In addition to the previous considerations, some others should be recognised. First, the scope of the model was limited to assessment of exposure. Second, the requirements for enterotoxin 'A' production in raw milk were very restrictive. Third, the result found for p_{exp} was low, despite the conservative assumption underlying the model. Fourth, information about *S.aureus* intoxications reveal that the disease is usually self-limiting and typically resolves within 24–48 h after onset (1, 12).

As for the model output, we concluded that estimated p_{exp} can be considered negligible. In fact, even though raw milk is known to be an excellent medium for *S.aureus* growth, an enterotoxin production sufficient to warrant a threshold of concern was linked to very unlikely S_t-$T°$ combinations only. Even when the worst storage conditions were simulated in scenario2 (with the distributions involved in the CPM fixed to 95th percentile); P_{exp} ≥20ng/serving was found only above the 99th percentile.

However, great care should be taken to extend our findings to other dairy products or fluid milk. Staphylococcal enterotoxins are thermostable, the estimation of the public health risk due to SEA in industrial products with extended shelf life or intended to be used as ingredient for other products would require consideration of other pathways. That was the case of the skim milk powder, which was the raw material for the reconstituted milk that caused the outbreak in Osaka (20) or the chocolate milk that caused an outbreak in United States (11).

The apparent rarity of the scenarios that generate at least 20ng/serving, together with the predominant effect that the variables S_t and $T°$ have on

these as previously discussed; could be the reasons why the contribution of the uncertainty on p(N_{exp}) due to the multiple strain approach becomes appreciable only when Scenario2a-2b were compared to Scenario2 used as baseline.

A similar QMRA for staphylococcus aureus and staphylococcal enterotoxin 'A' in milk was developed in the United States (17); although our results did not differ substantially, there are some important structural differences between the two models.

(i) In our model we estimated the probability of enterotoxigenic isolates from field data obtained within a region (Lombardy); Heidinger et al. used a Pert distribution estimating the distribution's parameters by using four different studies from several countries (Brazil, Italy, France and United States).

(ii) Our model admits the possibility that the gene is not expressed, Heidinger et al. implicitly assumed a probability of enterotoxin expression equal to 100%.

(iii) The storage practices of consumers of raw milk were inferred from storage practices of consumers of pasteurized milk in the United States with a storage time up to one week.

4.1. Main Assumptions and limitations.

Results of the regional survey gave exhaustive information about the genotypes established in the area, with 7 out of 44 genotypes accounting for most identified isolates (66.6%); unobserved genetic clusters were not included in equation 2. In fact, the proposed approach finds its

application only if representative data about the genetic clusters established in the area of interest are available. However, in the Dirichlet distribution, the probabilities assigned to each outcome (genotype) are inter-related and must add to 1, therefore, if variations in genotype proportions or the establishment/eradication of a particular genetic cluster is of interest, this can be assessed by including the frequency of the genotype of interest in the equation evaluating specific scenarios. The inclusion of an unobserved genotype (s_{G1}=0) can also be assessed.

The model estimates the probability of an AVM being contaminated with a generic *S. aureus*$_{sea+;SEA+}$ regardless of the genotype (Eq.5); thus, it is assumed that *S. aureus*$_{sea+}$ from different genotypes are equally virulent with respect to SEA and independent with respect to p_{SEA+}.

Another assumption underlying the model's structure is that contamination is due to *S.aureus* isolates attributable to a single genotype. However, contamination of individual AVMs by different genetic clusters could be addressed by attributing quotes of C_{t0} to the genotypes according to their proportions and run *n* parallel models (with *n* being the number of considered genotypes) summing up the outputs in the final step.

We have modelled p_{SEA+} by using data from a study in which *S.aureus*$_{sea+}$ strains were incubated in optimal conditions; enterotoxin production ratio under sub-optimal and in-field conditions are unknown but likely to be lower, this made our estimated p_{SEA+} conservative.

The lack of official estimation of the number of raw milk consumers or

any other data useful to estimate this parameter forced the estimation of the number of servings from informed opinion and data on consumption of pasteurized commercial milk, moreover, p(N_{exp}) is highly influenced by the number of AVMs in Lombardy which has steadily decreased in recent years (9). Furthermore, with respect to N_{exp} it should be noted that because of the independency assumption between the size of a herd and the amount of milk originating in this herd and purchased by consumers through AVMs, the model does not allow for large herd to result in a cluster of events as a result of a larger volume from this herd being sold through AVMs.

The temperature distributions at household level were based on informed specialists' opinion. Despite the attempt to take into account the uncertainty in their estimations with the score methods, that parameter remained an important data gap, and further specific research on this is strongly advised.

Fujikawa et al. using the strain n°12057 (isolated from a staphylococcal food poisoning outbreak in Tokyo) experimentally observed the threshold used in this study. The process for toxin 'A' production is known to not be regulated by the quorum sensor '*agr* system' like some other staphylococcal enterotoxins (2, 33, 39), consequently, the model assumes that once it is established that the milk is contaminated by a *sea*$^{+}$ strain (Eq.7) the threshold refers to that strain only. No other experiments have been carried out with respect to enterotoxin 'A' production in milk, different strains may show different thresholds and results are highly sensitive to this value, decreasing the threshold by 1 log would increase

p_{exp} 1.5 times in the baseline model.

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Chapter 3.

Consumers' behaviour in quantitative microbial risk assessment for pathogens in raw milk: incorporation of the likelihood of consumption as a function of storage time and temperature

General introduction

The sale of raw milk for human consumption is currently a hotly debated issue worldwide. The demand for raw milk has increased in recent year as groups of consumers in Europe and North America claim a variety of health benefits attributable to untreated dairy products. However, there are food-safety concerns and on May 2015, the EFSA panel on biological hazards (BIOHAZ) released the last scientific opinion on the public health risks related to the consumption of raw drinking milk. Public health concerns have been the motivation for a number of probabilistic models aimed to assess the risk of human illness from different pathogens related to raw milk consumption but none of these models has considered that under certain extreme storage conditions at household level, the product is likely to deteriorate becoming clearly unfit for consumption. Failure to identify as unrealistic these extreme scenarios may have result in an overestimation of the risk. In this work, this issue has been assessed.

A sensorial analysis to evaluate the organoleptic characteristics of raw milk conserved at different storage conditions at household level was carried out and an equation describing the changes in the probability of

milk of being perceived as spoiled as function of the time-temperature of storage was obtained.

In order to test the impact of this relationship, two recently published models aimed to assess food safety risk related to raw milk consumption were reproduced: (i) as they were published and (ii) with our equation included. Model outputs changed significantly, suggesting that results published so far are likely to have overestimated risk due to the inclusion of scenarios that, in practice, would not occur.

This study provides, for the first time, a concrete and objective tool to model the time-temperature relationship in quantitative risk assessment models related to raw milk and shows that this relationship should be taken into account in the future when assessing the risk related to raw milk.

The results are also relevant for other studies of the public health risk associated with the consumption of other food products subjected to fast deterioration if not stored properly.

Consumers' behaviour in quantitative microbial risk assessment for pathogens in raw milk: incorporation of the likelihood of consumption as a function of storage time and temperature

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ABSTRACT

Foodborne disease as a result of raw milk consumption is an increasing concern in Western countries. Quantitative Microbial Risk Assessment (QMRA) models have been used to estimate the risk of illness due to different pathogens in raw milk. In these models, the duration and temperature of storage before consumption have a critical influence in the final outcome of the simulations and are usually described and modelled as independent distributions in the Consumer Phase Module (CPM). We hypothesize that this assumption can result in the computation, during simulations, of extreme scenarios that ultimately lead to an overestimation of the risk. In this study, a sensorial analysis was conducted to replicate consumers' behaviour. The results of the analysis were used to establish, by means of a logistic model, the relationship between time-temperature combinations and the probability that a serving of raw milk is actually consumed. To assess our hypothesis, two recently published QMRA models quantifying the risks of listeriosis and salmonellosis related to the consumption of raw milk were implemented. Firstly, the default settings described in the publications were kept, secondly, the likelihood of consumption as a function of the length and temperature of storage was included. When results were compared, the density of computed extreme scenarios decreased significantly in the modified model, consequently, the probability of illness and the expected number of cases per year also decreased. Reductions of 11.6% and 12.7% in the proportion of computed scenarios in which a contaminated milk serving was consumed were observed for the first and the second study

respectively. Our results confirm that overlooking the time-temperature dependency may yield to an important overestimation of the risk. Furthermore, we provide estimates of this dependency that could easily be implemented in future QMRA models of raw milk pathogens.

1. INTRODUCTION

Probabilistic modelling is becoming established as one of the main tools to inform risk management decisions with regard to foodborne hazards. Quantitative Microbial Risk Assessment models (QMRAs) are increasingly applied to scenarios involving established and emerging food safety hazards as risk analysis becomes standard practice to manage food safety and ensure that regulatory decisions about foods are science-based and transparent (5, 19).

One of the most significant examples from the public health perspective in recent years has been the use of QMRAs to estimate risks associated with the consumption of unpasteurized milk. Growing interest on raw milk consumption by some groups of consumers and an increasing number of foodborne incidents in which raw milk has been identified as the source, have lead agencies such as the UK Food Standards Agency (FSA), the European Food Safety Authority (EFSA) or the US Centres for Disease Control (CDC) to conduct consultations and issue scientific opinions on the risk posed by milk-borne hazards (1, 2, 6).

The public health risk related to consumption of raw milk is a particularly relevant (and debated) topic. Raw milk can contain human pathogens

which can be inactivated by appropriate heat treatment (pasteurization or sterilization). However, the perception of raw milk as a "more natural" product has led to a number of consumers opting for raw as opposed to heat-treated milk. In light of this trend, models have been developed in recent years to assess probability of exposure or infection by pathogens such as *Salmonella*, *Listeria monocytogenes*, *Campylobacter jejuni*, *E. coli* O157 or *Staphylococcus aureus* as a result of raw milk consumption (8-10, 13).

QMRA models aimed at assessing the risk from farm-to-table include a consumer phase module (CPM), a stage of the model that occurs at household level, where the food is no longer controlled by professionals and where control of storage conditions or application of sufficient heat treatments cannot be enforced by legislation (15). In QMRAs related both to unpasteurized or pasteurized (12) milk, the time and temperature of storage in the CPMs are usually described and modelled as independent distributions. Time and temperature are the most important parameters that regulate microbial growth in milk and are regularly identified in sensitivity analysis as the factors with greatest effect on the model output (12, 13).

When both, storage time and temperature, are modelled as independent probability distributions (most often Triangular or Pert) there will be instances during simulations in which values from the tails of the distributions are sampled together yielding scenarios with high bacteria concentration at the time of consumption. An implicit assumption underlying the cited models is that 100% of the computed scenarios will

result in milk being consumed, whatever the time-temperature combination is. However, in reality some time-temperature combinations are unlikely to result in milk being consumed as it would be perceived by the consumer as unsuitable (raw milk stored at high temperature for extended periods might be spoiled and thus not actually consumed). Therefore, given that in microbial Dose-Response models the probability of illness is directly dependent to the number of bacteria ingested per serving (i.e. each bacteria has the same probability to generate infection), the amount of simulated scenarios under extreme conditions may have a significant impact on the final output.

This limitation was already highlighted by Latorre *et al.* (13) who noted that some correlation between these variables may exist and that without any restriction, the model cannot take into account that some extreme scenarios may not occur or end with milk not being consumed. However, to our knowledge, this limitation and the effect that this assumption may have on model output have never been formally assessed.

Following these considerations, the objectives of this work were to (i) model the dependencies between time and temperature in order to express the likelihood for a raw milk serving to be actually consumed for any computed storage time-temperature combination and (ii) assess the extent to which this dependency would affect the output of a QMRA model.

To this end, results of a simplified sensorial analysis on raw milk stored

for five days at different temperatures were used to estimate the probability that at given time-temperature combinations, the milk is spoiled, recognized as such, and thus not consumed. The potential effect of the estimated time-temperature relationship on model output was than evaluated by its inclusion in two recently published QMRAs of raw milk consumption and comparing published results with those of the modified model.

2. MATERIAL AND METHODS

2.1 Raw milk sample collection for sensorial analysis

One litre and an half of raw milk was collected from thirty automatic vending machines (AVMs) in Lombardy by the public veterinary services, univocally coded, placed in cold boxes at 5°C±3 and taken to the laboratory within 30 min. Upon arrival, five aliquots of 200 mL were obtained from each sample and kept in different isothermal conditions at 3°C, 5°C, 8°C, 12°C, and 16°C for five days (temperatures were chosen in order to reflect the range of temperatures at which the domestic refrigerators can be expected to operate).

500 mL from each sample were used to test the samples for: pH, somatic cell count (SCC), Lactic Acid Bacteria (LAB) Total Mesophilic Flora (TBC), enterobacteriacee (EB) and the major pathogens to ensure operator's safety. An instrument with automatic temperature compensation (HANNA instrument HI9321) was used for pH measurement; SCC was determined by an Optofluorimetric accredited internal method MP02/063 (Fossomatic, Foss Electric, Hilleroed, DK); the ISO standards ISO4833-2, ISO21528-2 and ISO16649-2; were used for surface plate

enumeration of TBC, EB and *E. coli*, while the standards AFNOR BRD 07/10 and AFNOR BRD 07/06 were used for PCR REAL-TIME detection of *L. monocytogenes* and *Salmonella.* Enumeration of LAB was performed by the accredited internal method MP01/048 (decimal dilution and plating in MRSA agar plate incubated under microaerophilic condition at 37±2°C for 72±2h and decimal dilution and plating on M17 agar plate at 37±2°C for 48±2h for enumeration of *Mesophilic Lactic Flora and Lactococci* respectively. The accredited internal method (MP 09/135) was used to test the samples for the presence of *Campylobacter jejuni by PCR REAL-TIME (Campylobacter Kit (Bio-Rad)).*

2.2 Sensorial analysis

In order to replicate consumers' behaviour, a simplified descriptive sensorial analysis of the milk samples stored at different temperatures was performed. The evaluation was carried out independently by two internal panellists experienced with sensory evaluation of milk[2]. Descriptors used in the evaluation sessions were selected following consultation with the panellists and based on their experience and the scope of the analysis (Table I).

Panellist were asked to evaluate all the milk samples every day at the same hour for five days.

Each raw milk sample required the judgment of five subsamples per session (one sample for each temperature), thus, for practical reason, no

[2] Experimental Zooprophylactic Institute of Lombardy and Emilia Romagna

more than five samples/week were processed and a total of six weeks were necessary to complete the experiment.

Table I Descriptors used in the sensorial analysis of raw milk samples stored at different time/temperature combinations.

	Description	Score
Aroma	None	1
	Acid aroma perceived when poured from the bottle	2
	Acid aroma perceived immediately at the opening of the bottle	3
Texture	Milk appears homogeneous when observed through the bottle. When poured from the bottle, milk appears smooth without any visible flake or residual on the bottle surface.	1
	Milk appears homogeneous when observed through the bottle. Small flakes are observed on the surface. Small flakes adhered to the bottle are clearly visible when milk is poured	2
	Milk in advanced coagulation phase, clear phase separation is observable through the bottle	3

All the milk samples were presented in transparent plastic bottles and panellist were asked to spill the milk into glasses in order to simulate consumers' behaviour. As reference, a 500mL of fresh raw milk was also taken to the lab every day from the nearest AVM and presented to the panellists prior to each evaluation.

Samples were presented in random order and panellists were asked to give their scores independently.

2.3 Data analysis

Following a conservative approach, the time at which a sample kept at a given temperature was considered 'spoiled' was the moment when at least one descriptor was scored as 3 or both the descriptors were scored as 2 or more.

Results from the panellists were analysed separately by means of binomial multiple logistic regression with time (h) and temperature (T°) as covariates:

$$logit(p_i) = ln\left(\frac{p_i}{1-p_i}\right) = \alpha + \beta_1 T° + \beta_2 h \quad \text{(Eq.1)}$$

$$logit^{-1}(p_i) = \frac{e^{\alpha+\beta_1 T°+\beta_2 h}}{1-e^{\alpha+\beta_1 T°+\beta_2 h}} \quad \text{(Eq.2)}$$

With $logit^{-1}(p_i)$ being the probabilities of the outcome events (i.e. the milk is considered spoiled and not to be drunk by consumers).

The potential interaction between time and temperature was tested by comparing models with interaction term with those without the interaction term by means of the Likelihood Ratio Test.

The Cohen's Kappa statistic for agreement was used to estimate the index of interrater agreement between the two panellists.

For inclusion in the QMRA model, the most conservative equation (i.e. the one that implies later detection of spoilage) was chosen; Statistical analysis was performed in R 3.1.2 (16) using packages 'lmtest' (11) and 'irr' (7).

2.4 Implementation of QMRAs

In order to evaluate the effect of including our estimates of association between time-temperature combinations and likelihood of milk being spoiled (and as a result not consumed), the two most recently published QMRAs related to raw milk and indexed in PubMed were identified and reproduced by using the Excel tool @Risk 6.3 (Palisade Corp.).

The query: 'Quantitative Risk Assessment Raw Milk', with the filter: 'published in the last 5 years' was used and 9 items were found (search date April 2015). The two more recently published studies (from different authors) including a formal QMRA were selected.

The more recently published studies were used without further consideration of their specific formulation. Use of the most recently published studies rather than purposively selected QMRAs was considered the more transparent and sound approach to illustrate the potential impact and highlight the relevance and timeliness of our proposal of incorporating time-temperature dependency in future QMRAs.

In the first work(13), the risk of listeriosis due to raw milk consumption in the United States was estimated for different scenarios and different susceptible population groups (Intermediate-age, Perinatal/Pregnant woman, Elderly), the scenario related to raw milk purchased at retail stores was chosen.

In the second (8), the risk of salmonellosis linked to consumption of raw

milk sold in vending machines in Italy was estimated for the best and worst storage conditions.

The 'worst conditions' scenario was selected (none heat treatment before consumption and worst storage conditions).

Both models were reproduced as described by the authors, and results (Baseline1, Baseline2) were compared with the ones obtained by the modified models (Model1, Model2) in which the probability that the milk is actually consumed given the sampled values for the time-temperature pair, was considered by including Eq. 2 (Figure1).

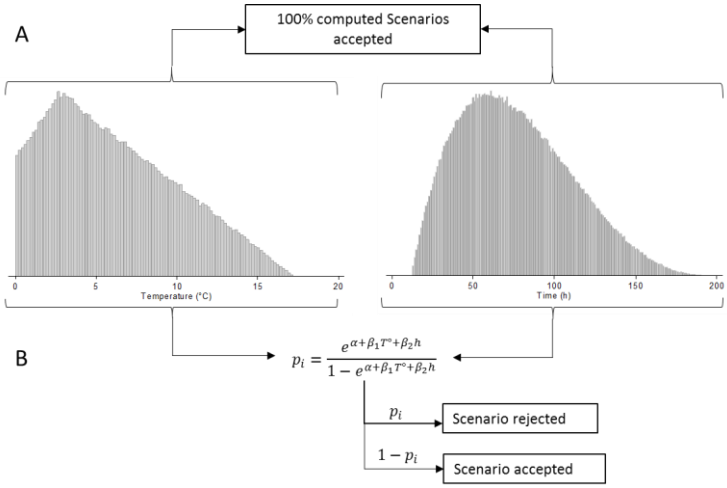
In the first study, the probability of infection per serving (p_{ill}) was calculated assuming an exponential dose response model (18) and combining multiplicatively the probability of illness given the dose with the assumed overall prevalence of *L.monocytogenes* in raw milk:

$$P = 1 - e^{(-rD)} \quad \text{(Eq.3)}$$

$$p_{ill} = P * prev \quad \text{(Eq.4)}$$

Where *P* is the probability of illness, *D* is the dose per serving (CFU per serving) and *r* is the parameter describing the probability that one *L.monocytogenes* cell causes illness (18). P_{ill} is the probability of illness per serving and *prev* is the assumed prevalence of *L.monocytogenes* in raw milk (proportion of raw milk positive servings).

Figure 1 Distributions describing the storage time and temperature assumed by Latorre et al. in QMRA related to risk of listeriosis due to raw milk in US. (A) in the original model all time-temperature combinations can yield a serving that could be consumed; (B) inclusion of eq. 2 implies that at any time-temperature combination the milk has a certain probability (p_i) to be recognised as spoiled by the consumer and thus not actually consumed.



Thus, in Model1, p_{ill} was estimated as:

$$p_{ill} = P * prev * (1 - p_i) \quad \text{(Eq.5)}$$

Where the *correction factor* ($1\text{-}p_i$) expresses the probability that the serving is actually consumed according to time and temperature.

In the second QMRA, the beta-Poisson relationship proposed by WHO/FAO (17) was used to calculate p_{ill} for the ingested dose:

$$p_{ill} = 1 - (1 + dose/b)^{-a} \quad \text{(Eq.6)}$$

Where *dose* is the ingested dose (CFU per serving), *a* and *b* are two

coefficients described by triangular distributions with parameters (minimum, most likely and maximum) 0.0763, 0.1324, 0.2274 and 38.49, 51.45, 57.96, respectively. In Model2, p_{ill} was estimated by shifting the sampled dose to 0 according to:

$$Bernouilli(p_i) \quad \text{(Eq.7)}$$

In this way, rejected scenarios are not considered 'at risk scenarios' by the model. For both models, as described by the authors, the number of expected cases per year (N_{exp}) were estimated by multiplying p_{ill} by the number of servings per year.

3 RESULTS

3.1 Analytical results

The initial (Time 0) values for: pH, SCC, TBC, LB, and EB are presented in Table II.

No pathogen were found in any sample and no inhibitory substances were detected. According to regional regulation (14), the microbiological and chemical quality of the samples was on average good.

Table II Analytical results (mean, standard deviation, minimum and maximum) of microbiological and chemical tests (pH, SCC, TBC, LAB and EB) of raw milk samples collected from automatic vending machines in Lombardy (n=30) for purpose of sensorial analysis; tests carried upon arrival to the laboratory.

Parameter	Unit	MIN	MAX	Mean	Std. Dev
pH	-log [H(+)]	6.69	7.7	6.9	0.28
SCC[1]	cells*ml^{-1}	2,000	371,000	176,367	100,438
TBC[2]	log CFU/ml	3.38	5.04	4.24	0.48
LAB[3]	log CFU/ml	1.3	4.2	2.88	0.62
EB[4]	log CFU/ml	1	4.3	2.61	0.92

[1]Somatic Cell Count [2]Total bacteria count [3]Lactic Acid Bacteria [4]Enterobacteriaceae

3.2 Sensorial analysis results

Results of the binomial multiple logistic regression analysis are reported in Table III. Only the results of the models without interaction are presented as the inclusion of an interaction term did not significantly

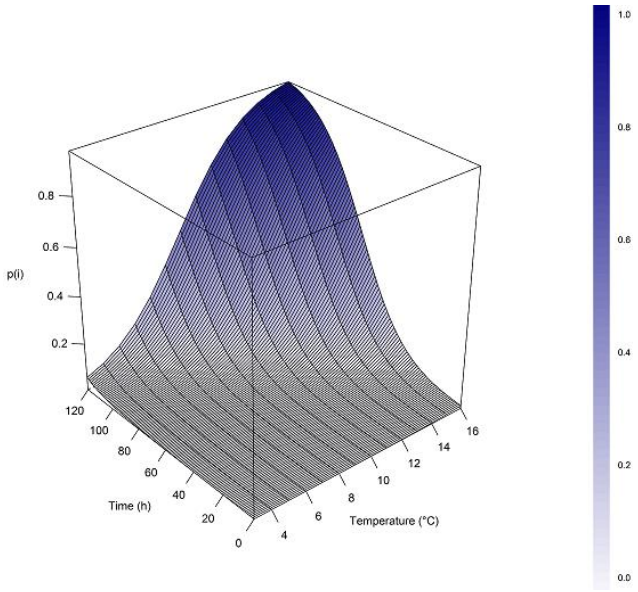
improved the models.

With an overall interrater agreement of 99.44%, the K coefficient for agreement resulted 0.98 confirming an excellent strength of agreement between the panellists. As expected, the model predicted that when the storage time and/or the storage temperature increases, the probability for the milk to spoil and being recognized by the consumer as expired also increases (Figure.2).

*Table III Coefficients of multiple logistic regression models for the association between the probability of raw milk being recognised as spoiled and the storage time-temperature combination. The regression curves were fitted to data from the evaluation of 30 samples of milk stored at different time-temperature combinations by two panellists. Results of each panellist (A and B) are reported independently. * indicates the equation coefficients selected to be included in QMRAs.*

Equation	Independent variable	Coefficient	2.5%	97.5%
A*	Constant	-12.273	14.150	10.395
	Time (h)	0.4883	0.403	0.573
	Temperature (°C)	0.0661	0.054	0.078
B	Constant	-13.004	15.025	10.983
	Time (h)	0.5161	0.426	0.606
	Temperature (°C)	0.0718	0.058	0.085

Figure 2 Graphical representation of the modelled relationship between storage time and temperature on probability of milk being perceived as spoiled (p_i)



3.3 Implementation of QMRAs

After 500,000 simulation of the first study (Baseline1) and according to an assumed prevalence of *L. monocytogenes* of 2.1%, 10,445 iterations (2.1%) yielded scenarios in which contaminated raw milk servings are ultimately drank by consumers, for the same study, 9,232 scenarios (1.8%) were predicted when the correction was applied (Model1). An overall reduction of about 11.6% of scenarios ending with consumption of a contaminated serving was observed.

The same approach applied to the second study (Baseline2 Vs Model2), generated a similar difference (12.7%).

The effect of this dependency is immediately evident when the densities of the sampled time-temperature pair combinations are compared between Baseline 1 and Model 1 (Figure 3) and between Baseline 2 and Model 2 (Figure 4).

Figure 3 Retrospective density plot representing the density of the time-temperature pair combinations behind the computed scenarios characterized by presence of L.monocytogenes in raw milk servings. In Baseline1 the time-temperature dependency is not modelled, thus, the occurrence of Time-Temperature combinations only depends on the individual Time and Temperature distributions; In Model1, each sampled combination generates a specific probability of milk being recognized as spoiled and, ultimately, not consumed. A decrease in the intensity of the extreme scenarios in the Model1 with respect to Baseline1 (upper right corner) is evident.

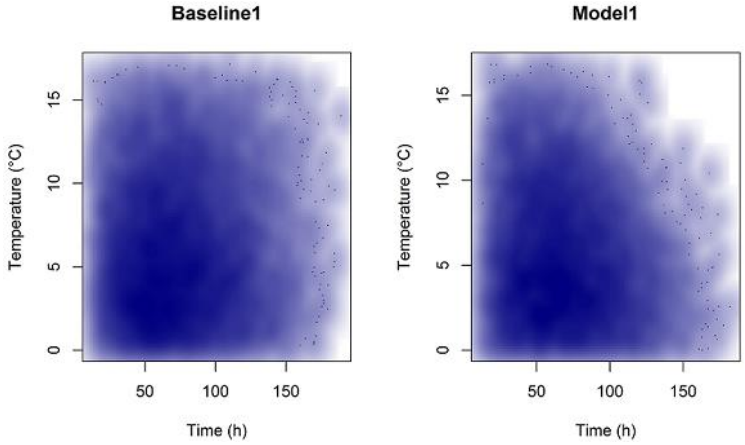
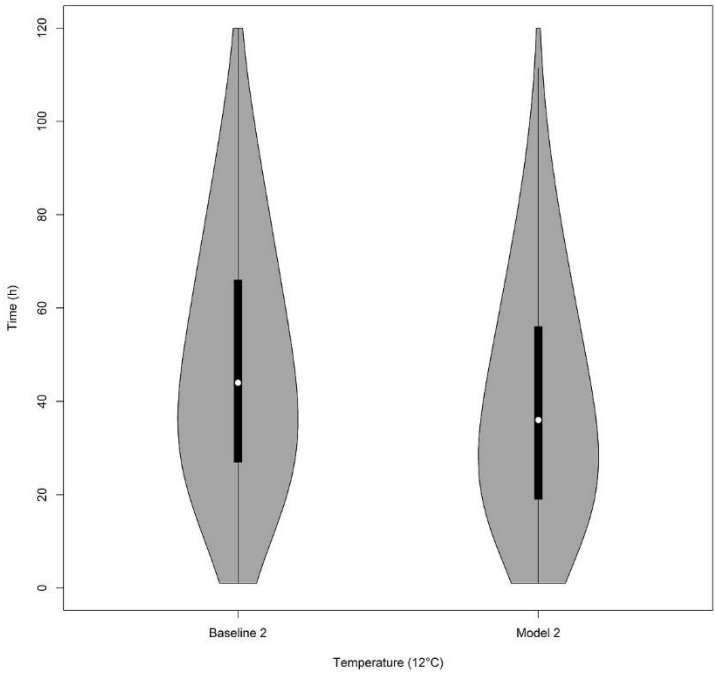


Figure 4 Retrospective violin density plot representing the density of the time–temperature (temperature was fixed to 12°C in this study) pair combinations behind the computed scenarios characterized by presence of Salmonella in a raw milk serving. In Baseline 2 the time–temperature dependency is not modelled. In Model 2, each sampled combination generates a specific probability of milk being recognized as spoiled and, ultimately, not consumed. A decrease in the intensity of extreme scenarios can be observed in Model 2 with respect to Baseline 2 approaching the apexes of the violins.



As expected, the most evident effects are noticed when the extreme time-temperature combinations are computed.

As a consequence, considering that: (i) the probability of illness per serving depends on the dose of the pathogen at the time of consumption (Eq.3, 6); (ii) the dose at the time of consumption depends on microbial

growth and (iii) microbial growth is regulated by time and temperature; if extreme time and/or temperature scenarios are unlikely to result in consumption, (Figure.2) there is a direct effect of including Time-Temperature dependency on the number of expected cases N_{exp} (Table IV).

Table IV Probability of illness per serving and number of cases per year associated with consumption of raw milk. Results from two published QMRAs with time and temperature as independent distributions (Baseline1, Baseline2) and with inclusion of time-temperature relationship (Model1, Model2). The effect on the shape of the output distributions is mainly shown from the values at 95th percentile.

Model	Probability of illness per serving Median (95th %ile)	Number of expected cases Median; (95th %ile)
Baseline1[1]		
Intermediate	1.4 x 10-13 (3.9 x 10-8)	4.1 x 10-5 (14)
Perinatal	8.0 x 10-12 (2.3 x 10-6)	2.0 x 10-5 (6)
Elderly	1.3 x 10-12 (8.8 x 10-7)	1.0 x 10-4 (29)
Model[1]		
Intermediate	1.3 x 10-13 (1.1 x 10-8)	4.5 x 10-5 (4)
Perinatal	7.4 x 10-12 (6.6 x 10-7)	1.9 x 10-5 (2)
Elderly	1.2 x 10-12 (1.1 x 10-7)	9.3 x 10-5 (8)
Baseline2[2]	2.6 x 10-4 (1.4 x 10-2)	28,558 (28,838)
Model[2]	1.5 x 10-4 (1.0 x 10-2)	16,243 (16,455)

The effect of explicitly including in the model the probability of consumption (1-p_i) as a function of the storage time and temperature on

p_{ill} and N_{exp} was evident in Model1 at 95th percentile where: p_{ill} was reduced by about 3.5 times for the categories 'intermediate' and 'perinatal' and up to 8 times for the category 'elderly'; N_{exp} resulted 3.5, 3 and 3.6 times smaller with respect to Baseline1 for the categories 'Intermediate', 'Perinatal' and 'Elderly' respectively.

In Model2 the effect of modelling the time-temperature relationship was evident even on the median values were a reduction of 1.7 times with respect to results from Baseline2 were observed for both p_{ill} and N_{exp}.

4 DISCUSSION

Raw milk spoilage is a natural phenomenon and the time at which it occurs depends on several factors like the type and initial load of microbial contaminant(s), pH, enzymes and time temperature conditions.

The processes leading to modification of organoleptics properties of milk are time temperature dependent, therefore, as for the majority of the fresh products, the spoilage occurs more rapidly if the products is not stored at low temperatures. Ignoring spoilage of raw milk in QMRA models and therefore assuming that milk will always be consumed regardless of its organoleptic modifications during storage is not realistic and can have a significant impact on model outputs.

In this study we have demonstrated that overlooking the time-temperature relationship may result in those scenarios in which contaminated raw milk servings are consumed being significantly

overestimated (by approximately 11.6 and 12.7% in the case studies we selected).

Coping with all the possible dynamics that might influence raw milk's spoilage, would require such level of complexity that analytical solutions might not be possible. An alternative would be the incorporation of a dependency such as the one described in our logistic model. Our equation simplifies the complex dynamics that ultimately determine the spoilage of milk considering only the relationship between storage time and temperature on likelihood of spoilage (and of consumption being adverted). It provides, for the first time, a concrete and objective basis to explicitly include the logical relationship between storage time-temperature combinations and likelihood of milk being consumed, that is: 'As the storage conditions became extreme the likelihood of raw milk being perceived as spoiled increases'.

For practical reasons, it will always be difficult to gather accurate information about storage conditions at household level or about consumers' behaviour; however, the proposed approach will mitigate the effect of too conservative assumed distributions. In fact, with the incorporation of the proposed equation, if very conservative storage time and/or temperature distributions are used (i.e. more extreme values are allowed), when high values are sampled, the predicted likelihood of milk being perceived as spoiled will be high (Figure 2) and the amount of rejected scenarios will increase consequently, mitigating the effect of conservative distributions. Conversely, if this dependency is ignored, the effect of too conservative distributions might lead to alarming but poorly

representative risk estimates. With the inclusion of this equation, QMRAs for hazards in raw milk would be more realistic and their outputs would not be inflated by ignoring the correlation between storage conditions that favour microbial growth and likelihood of milk being perceived as deteriorated and thus not consumed.

The probabilistic modelling of exposure to hazards present in raw milk should explicitly include this relationship and in the absence of more extensive empirical data on the relationship between storage conditions and perception of spoilage in milk from other sensorial evaluations, it is reasonable for future studies to make use of the estimates provided in this study.

Considering that the main objective of probabilistic risk modelling in food safety is to represent what happens in the real world in order to provide science-based information to decision makers, our equation improves the current level of understanding, making it closer to reality by excluding consumption scenarios that would not occur in practice. Inclusion of the logistic equation presented in this study would be a simple, transparent and sound approach and an improvement with respect to previously used QMRAs of raw milk.

In many European countries raw milk can be sold at the farm directly to the consumer (2) and according to the European legislation EU Regulation 852/2004, 853/2004 (3, 4), direct sale of milk is regulated by the national law of the member states and, in some cases, additional regulations at subnational level. Although some differences may exist in

national or sub-national regulations, farms allowed to sell raw milk for human consumption are asked to comply with strict criteria and operate with high quality standards. Consequently, a substantial homogeneity in the microbiological and biochemical quality of *raw milk for human consumption* from different regions with similar regulations might be assumed, making the results presented in this paper more directly applicable to future QMRA models aimed to assess the risk for human health related to consumption of raw milk in different European countries.

However, if the raw milk characteristics, hygienic practices or regulations are likely to be significantly different or subjected to high variability, the coefficients estimated in this study might not be appropriate (e.g. milk produced in systems and geographic regions where the initial bacterial count can be expected to be considerably higher). Furthermore, considering that the equation is aimed to predict consumers' behaviour through a sensorial evaluation, the social context of the country where the QMRA is to be implemented plays a critical role. In fact, the perception of 'suitability' might be different due to a number of traditional and social factors; therefore, even the parameters used to score the organoleptic characteristics should be revised accordingly. Besides raw milk, our approach can be applied to other food products for which the storage conditions at household level are critical: raw meat and fish, eggs, vegetables, soft cheese, and fresh products in general which are all subjected to a fast deterioration if not conserved properly.

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Chapther 4

Quantitative Risk Assessment of Campylobacter in broiler chicken - assessing interventions to reduce the level of contamination at the end of the rearing period

General introduction

Campylobacter is a long standing problem in the poultry industry worldwide and according to several publications and a number of dedicated reports published by the EFSA it is generally accepted that controlling the contamination at farm level would result in greater benefit (with respect to public health) than acting on the further steps of the food chain.

In this context, the Royal Veterinary College (UK) conducted an epidemiological study in supports of the activities of the Food and Standard Agency (FSA) and the Joint Working Group on *Campylobacter* (JWG) aimed at reducing levels of *Campylobacter* spp. colonisation in poultry at farm level in the UK. The study estimated the relative risks of contamination associated to a number of management activities with particular focus on the practice of thinning and the adoption of biosecurity measures.

In this work, the dynamics describing the campylobacter infection in broiler flocks were reproduced and a baseline model was used to: (i) show how epidemiological results can be integrated in quantitative models (ii)

explore the potential effects that different mitigation strategies or management options have on the level of contamination at slaughter (iii) provide information about the relative effects of the model inputs on the outcome.

Quantitative Risk Assessment of *Campylobacter* in broiler chicken - assessing interventions to reduce the level of contamination at the end of the rearing period

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ABSTRACT

The European Food Safety Authority (EFSA) has estimated that a proportion ranging from 20% to 30% of campylobacteriosis in humans may be attributed to the consumption of broiler meat and a reduction in the numbers of Campylobacter in the intestines of infected birds at slaughter by 3 log units would reduce the public health risk by at least 90%. In this study, a stochastic model aimed reproduce the dynamics of Campylobacter transmission in broiler flocks was developed and the effects of several management conditions and/or on-farm mitigation strategies on the level of contamination of infected flocks at slaughter were explored. Results were expressed as 'proportion of highly contaminated flocks' and quantified as a function of: (i) the proportion of infected birds in the flock the day of final depopulation and (ii) the individual level of contamination in infected birds. The potential effects of the mitigation strategies were modelled assuming that the effects are explicated on the distribution describing the bacterial load in infected birds whereas the impact of management conditions such as the adoption of enhanced biosecurity measures and/or partial depopulation during the production cycle were quantified by using results of an extensive epidemiological study conducted in UK. A standard broiler flock was reproduced in the baseline scenario but the model was developed to be flexible, easily reproducible and updatable so to be adapted to several baseline scenarios. The main assumptions underlying the transmission model were tested and shown with a sensitivity analysis and the major sources of uncertainty together with the impact that the baseline

information might have on the outcome were discussed.

1. INTRODUCTION

Campylobacter is one of the major agent of foodborne disease worldwide and it continues to be one of the most commonly reported gastrointestinal pathogen in humans In European Union (EU) (4). At European level, the pathogen is believed to be responsible for about nine million cases per year with an impact for the public health systems and to lost productivity estimated by EFSA to be around EUR 2.4 billion a year. In this context, the chicken meat is a well-known source of many cases of campylobacteriosis, in 2010 EFSA estimated that a proportion ranging from 20% to 30% of the total cases is to be attributed to chicken and chicken meat (1).

Considering the impact of the poultry industry on the risk posed by *Campylobacter* in human health, the European Commission required the Panel on Biological Hazards a scientific opinion about the control options and performance objectives and/or targets at different stages of the food chain with respect to *Campylobacter* in broiler meat production. The major conclusions reported by EFSA (2) were: (i) there is a linear relationship between prevalence of *Campylobacter* in broiler flocks and public health risk and (ii) reducing the numbers of *Campylobacter* in the intestines at slaughter by 3 log units would reduce the health risk by at least 90%. Hence, the opinion indicates that the public health benefits of controlling *Campylobacter* in primary broiler production are expected to be greater than control later in the food chain.

Following these considerations, the aim of this study was to quantify the

effect that different management conditions and/or 'on-farm' mitigation strategies have on the level of contamination of infected flocks at slaughter.

With respect to the options explored, two categories can be distinguished: (i) management conditions affecting the introduction of pathogen or the spread of the infection (enhanced biosecurity, partial depopulation) and (ii) interventions aimed to reduce the pathogen's load in the caecal contents of infected birds (vaccine, bacteriophage therapy and treatment with organic acids).

The assessment was made by developing a baseline probabilistic model aimed to reproduce the dynamics of the within flock transmission of *Campylobacter* into a broiler chicken flock at farm level and comparing the baseline output with the ones obtained when different scenarios were tested.

The baseline model was aimed to estimate the level of contamination in infected flock at slaughter with the outcame assumed to be directly related to two main factors: (i) the within flock prevalence (WFP) expressing the proportion of infected birds at the end of the rearing period and (ii) the individual level of contamination (logCFU/g) in infected birds.

The baseline model was implemented with the available information and/or data included in studies related to broiler chicken raised in intensive system.

The assessment of the management conditions affecting the introduction of the pathogen was made by using the results of a comprehensive epidemiological study conducted in supports of the activities of the Food and Standard Agency (FSA) and the Joint Working Group on *Campylobacter* (JWG) aimed at reducing the levels of *Campylobacter* spp. colonisation in poultry at farm level in the UK (15).

The assessment of the mitigation strategy affecting the pathogen's load in the caecal contents of infected birds was made by adopting the overall effects of the interventions already summarized by EFSA (3).

2. MATERIAL AND METHOD

The baseline model and subsequently the effects that different on-farm mitigation strategies and/or management conditions have on the probability for infected flock of being highly contaminated at slaughter were quantified through the model outlined as a flowchart in Figure 1.

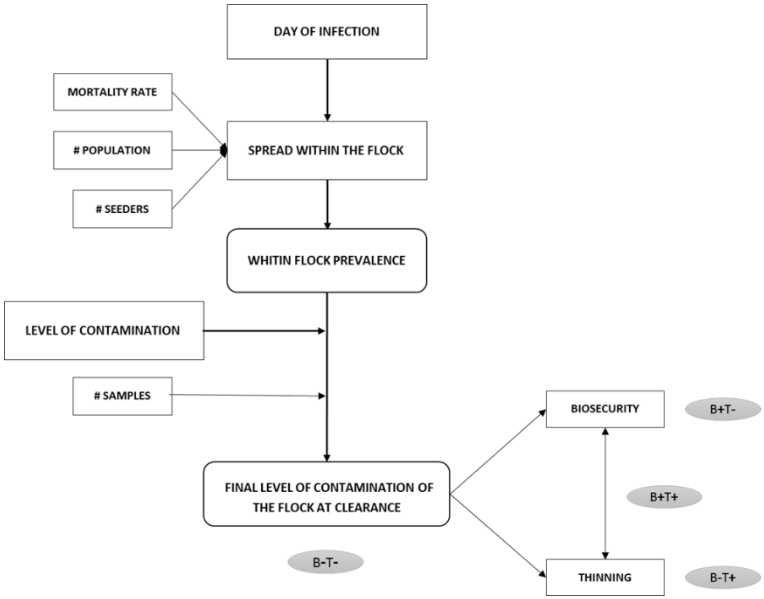
One of the main factors driving the model outcame is the *WFP* which can be expressed as the ratio between the number of birds colonized with *Campylobacter* over the total population in a positive flock.

This value is calculated at the day of the final depopulation (*dpday*) and it is assumed to be dependent on two main factors:

1. The age at which the flock became infected

2. The spread of the infection within the flock

Figure 1 Flowchart of the model implemented to assessment the probability for infected flock of being included in the category 'Highly contaminated' at slaughter. The steps describe the baseline scenario in which simulated flocks are raised under a standard biosecurity regime and not partially depopulated during the production cycle (B-T-). Additional scenarios involving the partial depopulation (T+) and/or the application of biosecurity measures (B+) were assessed operating on the baseline estimation.



Intuitively, the first day of infection defines the moment at which the spread starts and the spread of the infection, implemented with a logistic growth model, is in turn dependent on a number of biological variables such as: the mortality rate (*d_rate*), the total number of birds in the flock (*Nb*) and the number of infected birds at t_0 (It_0).

2.1. The age at which the flock became infected.

In our model, we assume the start of the growth model the first day at which infected feces are detected in the environment; in fact, broilers are coprophagic and when birds consume contaminated droppings, they become infected themselves.

The dynamics describing the broiler infection by Campylobacter and the time at which this occurs is generally unknown, however, In-field studies reported that *Campylobacter* is rarely detected in the flock form 10 to 14 days after the beginning of the production cycle (1, 5, 12). On the basis of this evidence, under a modelling prospective and in absence of further information, the first day at which the flock become colonized has been proposed to be modelled as a uniform random variable between fourteen days and the day of depopulation (6, 11).

Although the assumption related to the minimum age infection may be acceptable, the one that each day of the cycle has the same chance to be the day of infection conflict with in-field evidences.

In this work, results from three longitudinal (1, 5, 20) and two left-censored (16, 24) studies were combined to estimate the day at which a broiler flock become infected ($Iday^{+}$).

In the longitudinal studies, results were reported as 'range' of days in which a flock or *n* flocks were firstly detected infected; therefore, five ranges (0-28; 29-35; 36-42; 43-49; >50) were identified from the first study and the data were combined to estimate the overall probability of

the flock becoming infected in each i^{th} range (p^{+}_{rgi}). In order to incorporate the knowledge from the left-censored datasets, a Bayesian approach was adopted with the distributions of p^{+}_{rgi} used as informative priors and the left-censored datasets used for the likelihood function of a binomial process describing the likelihood of having observed s_i positive flocks on range i given p^{+}_{rgi} . The posteriors (i.e. revised) estimates of p^{+}_{rgi} were finally calculated by multiplying the prior and the likelihood function.

2.1.1. Longitudinal studies.

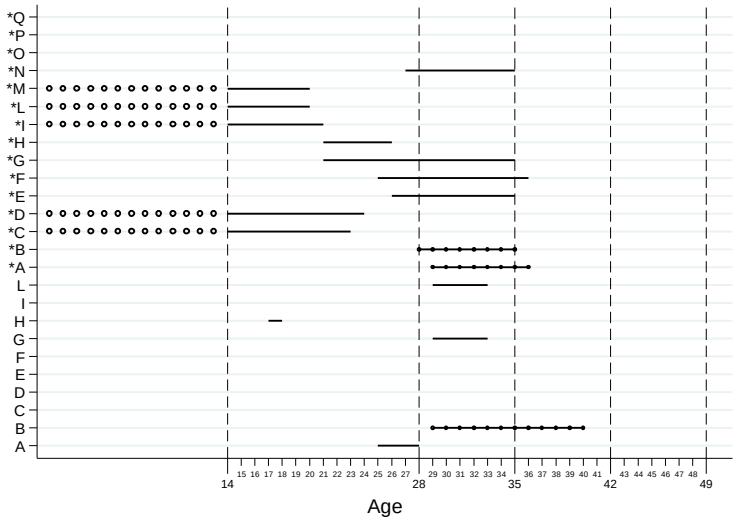
The first longitudinal study (5), consisted in a total of 100 broiler flocks ($N1_{t0}$) monitored for the presence of *Campylobacter* at weekly intervals (the weekly testing schedule meant that it was only known that infection occurred within a specific time interval). Sixteen birds were swabbed on each sampling time (four birds from each quarter of each broiler house) and a flock was defined infected if the presence of *Campylobacter* was detected in at least one bird.

The second work (1), consisted in a longitudinal study conducted on 10 ($N2_{t0}$) broiler flocks. From 10 to 14 individual fresh feces or cloacal droppings were collected at least weekly from the broiler houses. This study was characterized by a leak of two days between every sampling period; those days were conservatively included in our estimation as possible day of infection (i.e. if a flock was negative on day *n* but positive on day *n+3*, the days *n+1* and *n+2* were considered as possible days of infection).

In the third study (20), 15 broiler flocks ($N3_{t0}$) were followed in one farm

and visited three times during the production cycle. Fifty-eight environmental samples were collected in each visit and the last sampling time coincided with the flock clearance. In this work, the days at which the flocks were visited were univocally recorded, therefore, the range of days in which each flock has become infected was estimated from the day at which the flock was found positive back until the last day at which the flock was tested negative (A*-Q* in Figure 2).

*Figure 2 Black lines represent the estimated ranges of day in which the flocks could have become infected. Results from the second (A-L) and third (*A-*Q) longitudinal studies are shown. Solid lines with circle represent the flocks who could have become infected because of thinning. Vertical dotted lines delimiting the identified ranges are shown.*



Five flocks from the second study and two from the third were partially depopulated at some point of the growing cycle. In order to exclude from the baseline probabilities the flocks that could have become infected

because of thinning, those flocks were not included in the total number of exposed flocks ($N_i t_i$) from the i^{th} range in which the thinning was practiced.

None of the surveys reported evidences of positive samples in the first two weeks of the cycle, according to several studies indicating that *Campylobacter* is not usually detected in the flock environment form 10 to 14 days after the beginning of the cycle (3, 7, 8); it was assumed that the flocks do not become infected before the fourteenth day.

For each i^{th} range, p^{+}_{rgi} was estimated by using the conjugate formula for the beta distribution:

$$p^{+}_{rgi} = \text{Beta}(\alpha; \beta) \qquad \text{(Eq.1)}$$

Where:

$$\alpha = s1_{rgi} + s2_{rgi} + s3_{rgi} + 1$$

$$\beta = [(N1_{t0rgi} + N2_{t0rgi} + N3_{t0rgi}) - (s1_{rgi} + s2_{rgi} + s3_{rgi}) + 1]$$

With $s1_{rgi}$ $s2_{rgi}$ $s3_{rgi}$ being the total number of positive flocks that could have become infected in the i^{th} range in study 1, 2 and 3; and $N1_{t0rgi}$ $N2_{t0rgi}$ $N3_{t0rgi}$ the total numbers of considered flocks at t_0 for the i^{th} range.

2.1.2. Left-censored studies.

The Bayes' theorem is a method for revising belief about the parameter of interest after observing data. The posterior distributions of p^{+}_{rgi} for each i^{th} range were estimated by using two left censored datasets.

The first study (24) involved 291 broiler flocks from 134 broiler farms while the second (16), 389 broiler flocks from 88 farms. Authors kindly provided their original dataset for our estimations.

The posterior distributions of p^{+}_{rgi} for each range was estimated as follow:

$$f(\theta_{\mathrm{rg}i}|s_{\mathrm{rg}i}) \propto \pi(\theta_{\mathrm{rg}i}) * L(s_{\mathrm{rg}i}|\theta_{\mathrm{rg}i}) \quad \text{(Eq.2)}$$

Where $\pi(\theta_{rgi})$ is the density function of the prior belief about the parameter value θ_{rgi} and $L(s_{rgi}|\theta_{rgi})$ is the likelihood function for a binomial process expressing the calculated probability of observing s_{rgi} infected flocks given n_i and a given value of θ_{rgi}. In the formula, $f(\theta_i|s_i)$ is the posterior distribution of p^{+}_{rgi} describing the state of knowledge of p^{+}_{rgi} after having observed s_i positive flocks on range i and given our prior information about the value of the parameter before s_i was observed.

As the data from the left-censored datasets were informative about the probability of *being* infected in a given range, the probabilities of the binomial process of the likelihood function were modified as follow (the example for rg_2 is reported):

$$\theta_{\mathrm{rg2}} = \left[\theta_{\mathrm{rg1}} + \left(\left(1 - \theta_{\mathrm{rg1}}\right) * \theta_{\mathrm{rg2}}\right)\right] \quad \text{(Eq.3)}$$

So that $f(\theta_{rg2}|s_{rg2})$ in equation 2 defines the actual state of knowledge about the probability of the flock becoming infected in the range 29-35 after having observed s_{rg2} positive flocks and given the prior information about the probability of the flock *being* (θ_{rg1}) and *becoming* ($(1-\theta_{rg1})*\theta_{rg2}$) infected in the range 29-35 before s_{rg2} was observed.

Once the posterior probabilities were obtained, results were normalized for modelling purpose. Furthermore, it was assumed that within each i^{th} range, each i^{th} day has the same chance to be the day of infection:

$$P^{+}_{dayi\_rgi} \cup P^{+}_{dayi+1\_rgi} \cup \ldots \cup P^{+}_{dayn\_rgi} = p^{+}_{rgi} \quad \text{(Eq.4)}$$

Therefore, $Iday^{+}$ is modelled as:

$$Iday^{+} = Discrete\left(14, \ldots, dpday; p^{+}_{14}, \ldots, p^{+}_{dpday}\right) \quad \text{(Eq.5)}$$

Where *dpday* is the day of final depopulation and $p^{+}_{14} \ldots p^{+}_{dpday}$ are the estimated probabilities according to (Eq.4).

2.2. Spread of infection.

Once the flock became infected on $Iday^{+}$, the infection quickly spread through the flock and the horizontal spread describing the transmission of *Campylobacter* within the flock was parameterized by fitting the results of two experiments (26) to a logistic growth curve:

$$Ib_t = \frac{K\ Nb\ Ib_0}{Ib_0 + (kNb - Ib_0)e^{-rt}} \quad \text{(Eq.6)}$$

Where Ib_t is the number of infected birds at time *t*, *Nb* is the flock size, K the carrying capacity of the environment (assumed equal to 1) and *r* is the coefficient representing the growth rate (*rate*) of infected birds in the total population.

In both the experiments, 400 broiler chicks were housed on fresh litter in a density of 20 chicks/m^2 and 4 chicks per group were orally challenged

at the age of 2 days. The colonisation of chicks was determined at fixed time points by sampling 50 random birds. When all samples appeared to be *Campylobacter* positive, the sample size was reduced to 10 chicks per group in both the experiments.

The parameterization of logistic function was already used in a previous work (13) where *r* was estimated extrapolating from the original work the actual number of infected birds in the population at each data point. Using the original dataset, we used the hypergeometric process in order to include the uncertainty surrounding the number of infected birds detected in each sampling time given the sample size. Hence, given that at different sampling time, samples of size n_i were collected from a finite population *M,* we parameterized the total number of infected $D_i(\theta)$ in the population at each time point *i*, given that s_i positive samples were observed. Assuming the uninformative prior for the parameter ($\pi(\theta)=1$), the Likelihood of observing s_i infected for a given value of θ was estimated with the hypergeometric probability mass function:

$$L(s_i|n,\theta,M) = \frac{\binom{\theta}{s_i}\binom{M-\theta}{n-s_i}}{\binom{M}{n}} \quad \text{(Eq.7)}$$

Therefore, for each sampling time, the posterior distribution describing the actual state of knowledge about θ was estimated as:

$$f(\theta|x)_i \propto \pi(\theta) * L(s_i|n,\theta,M) \quad \text{(Eq.8)}$$

Indicating that the posterior distribution describing the expected number of infected birds in the population at each i^{th} sampling point (*x*) is

proportional to: (i) the prior believe about the parameter (π) and (ii) the likelihood function for a hypergeometric process expressing the calculated probability of observing s_i infected birds given n, M, and a given value of θ.

The distribution describing the number of infected birds allowed the simulation of alternative outcomes for each i^{th} sampling point: ten thousand simulated dataset were fitted to the logistic growth function (Eq.6) and as many values for *rate* were obtained. The values were used to parameterize the distribution describing the uncertainty in *rate,* to this end, the maximum likelihood estimation (MLE) method for a Gamma distribution was used (27). Assuming that a given set of data can be described by a certain distribution (e.g. Gamma), the method of maximum likelihood provides an estimation of the distribution's parameter(s) so that the joint probability of the observed data under the resulting distribution is maximized:

$$\log L(X|\alpha)=\sum \log(f(xi,\alpha)) \qquad \text{(Eq.9)}$$

Where α represents the parameter(s) of the distribution of the likelihood function (α and β of the Gamma distribution) and $\log L(X|\alpha)=\sum \log(f(xi,\alpha))$ is the likelihood of observing the n observations recorded given α. The gamma distribution was chosen because data are continuous and its parameters α (shape) and β (scale) allow great flexibility making possible for the distribution to assume a range of different shapes.

2.3. Within flock prevalence estimation.

In each simulated scenario, the *WFP* was defined as the predicted proportion of infected birds on *dpday*.

The probability distribution describing the *WFP* was obtained through the simulation of 100,000 production cycles in which $Iday^{+}$ was randomly sampled according to Equation 5, and the spread of the infection modelled by fitting a logistic growth model in which the coefficient *rate* was sampled from its uncertainty distribution.

2.4. Infected birds in infected flock at slaughter.

The actual number of infected birds in the flock *N(Ib)* was estimated after each iteration by Binomial distribution:

$$N(Ib)_i = Binomial\ (Nb;\ WFP_I) \qquad \text{(Eq.10)}$$

Were *Nb* is the number of birds in the flock and WFP_i is the estimated within flock prevalence in the flock after iteration i^{th}.

2.5. Level of contamination of the flock.

The level of contamination of the flock is generally estimated by bacteriological count of a number of pooled caeca (N_c) randomly sampled from the slaughter line, therefore, the final result is a function of: (i) the number of contaminated caeca sampled and (ii) the level of contamination in positive sample.

2.5.1. *Number of contaminated caeca samples.*

The Hypergeometric process was used to estimate number of contaminated caecal sampled (N_c^+) as a function of *Nb*, N_c and $N(Ib)_i$:

$$N_c^+ = Hypergeometric\ (N_b; N(Ib)_i; N_c) \quad \text{(Eq.11)}$$

2.5.2. *Level of contamination in caeca.*

The ability of *Campylobacter* in reaching high level in caecal contents after infection has been widely reported and according to several works (17, 23, 25). The Intestinal carriage of *Campylobacter* in contaminated chicken carcasses at slaughter (C_c) was estimated from a previous study (22) and described by the normal distribution:

$$C_c = Normal(\mu_c; \sigma_c) \quad \text{(Eq.12)}$$

With parameters μ_c and σ_c equal to 7.63 and 1.02 logCFU/g respectively (22). The final level of contamination of the flock (*Fl*) was inferred from the estimated level of contamination of a standard pooled sample of 10 caeca samples/batch:

$$Fl = \frac{Normal((\mu_c * N_c^+); \left(\sqrt{N_c^+} * \sigma_c\right))}{N_c} \quad \text{(Eq.13)}$$

Where the numerator represents the central limit theorem applied on the positive caeca samples taken (i.e. it is assumed that the level of contamination in each positive sample can be described by the same distribution), and the denominator the total number of caeca samples. A test sensitivity close to 100% is assumed.

2.6. The baseline model.

In the baseline model, 100,000 infected flocks coming from a standard broiler house with 20,000 birds (*Nb*), raised under a standard biosecurity management (B-), with a mortality rate (*d_rate*) of 5% assumed to be equally distributed along the cycle and not partially depopulated (T-) were simulated.

The simulation was initiated assuming that the infection was due to one initially colonized chicken –shedder- (Ib_0=1*)* and according to the industry dataset (15), the thirty-eighth day of the cycle was selected as the most likely day of clearance (*dpday*) in not partially depopulated flocks.

2.7. Risk outputs.

At the end of the simulation, the cumulative probability distribution obtained for *Fl* was used to estimate the expected proportion of highly colonized batches at slaughter. According to (15) the threshold level for the classification of the batches as 'highly colonised' was set to 5.09 logCFU/g ≈ 123,000 CFU/g. once the baseline output was obtained, different management conditions and/or mitigation strategies were tested and results compared to the baseline scenario. Moreover, in order to assess the relative effects on the output of the distributions included as model inputs ($Iday^+$; C_c; *r*), a sensitivity analysis was performed.

2.7.1. Enhanced biosecurity.

The hypothesis that enhanced farm biosecurity contributes to a decrease

in the risk of *Campylobacter* colonisation was tested in a dedicated epidemiological study (15) where the adjusted Relative Risk (RRa) expressing the ratio of the probability of an event occurring in an exposed group versus non-exposed was estimated. Results from that study indicate that batches raised under standard biosecurity are significantly more likely to be colonised at high level than batches raised under enhanced biosecurity. In fact, the estimated RRa for the effect of standard biosecurity at depopulation resulted 1.30 (CI 1.05 – 1.48).

Since the baseline model assumed a standard level of biosecurity (B^-), the effect of enhanced biosecurity on the proportion of highly contaminated flocks at slaughter was obtained using the RRa as multiplicative coefficient as follow:

$$(B+T-)= (B-T-)*1/RRa_{(B-)} \quad \text{(Eq.14)}$$

Where, (B-T-) is the proportion of highly contaminated flocks obtained from in the baseline model. In this case, the scenario (B+T-) estimates the proportion of highly contaminated flock at slaughter if all the infected flocks were grown under enhanced biosecurity management.

2.7.2. Thinning.

Similarly to the biosecurity, the estimated RRa for the factor of thinning (T+) resulted 1.55 (CI 1.18-1.87) for the flocks grown under enhanced biosecurity management. In the baseline model the partial depopulation was not practiced, therefore, the effect of thinning on the proportion of

highly contaminated flocks was estimated through the scenario (B-T+) in which 100% of the flocks are partially depopulated before the end of the production cycle:

$$(B\text{-}T+) = (B\text{-}T-) * RRa_{(T+)} \quad \text{(Eq.15)}$$

An additional scenario (B+T+) in which the flocks are all assumed to be partially depopulated and raised under enhanced biosecurity measures was also assessed.

$$(B+T+) = (B\text{-}T-) * RRa_{(T+)} * 1/RRa_{(B-)} \quad \text{(Eq.16)}$$

2.7.3. Vaccine, bacteriophage therapy and treatment with organic acids.

The Interventions aimed to reduce the bacterial load in infected birds have been recognized as important on-farm mitigation strategies to reduce the proportion of high-contaminated flocks at slaughter (3) and the available options such as Vaccination, Bacteriophage therapy, Bacteriocins or anti-*Campylobacter* additives in feed or drinking water have been recently reviewed (3, 21). As the efficacy of those interventions depends on a number of biological and technical factors their effect is difficult to estimate quantitatively, in fact, vaccines are still in the development phase and the other options are characterized by variable results and/or limited *in vivo* experiments. However, a generic modelling approach to evaluate the reduction of highly contaminated flock at slaughter due to a reduction in the number of *Campylobacter* in bird's intestines was performed to assess the potential benefit of interventions

with this general aim. The estimated effects on *Campylobacter* reduction for the interventions are summarized in table 1.

Table 1 Overall summary of the effects of the interventions aimed to reduce the bacteria load in Broiler chicken intestine.

Intervention	Effect	Reference
Vaccination	2 logCFU/g reduction in caecal contents	(3)
Bacteriocins	Uniform(5.1;5.9) logCFU/g reduction in caecal contents	
Bacteriophages	3 logCFU/g reduction in caecal contents	
organic acids	Uniform (0.5;2) logCFU/g reduction in caecal contents	

All the mitigation strategies affecting the level of contamination in infected birds are assumed to act on the μ_c (Eq.12).

2.8. Uncertainty in the baseline scenario.

The effects of the interventions under investigation on the proportion of highly contaminated flocks were estimated by comparing the outputs of the different scenarios obtained by means of Monte Carlo Simulations with that of the baseline.

The effects were estimated using a standard broiler flock as baseline; a number of initial information were assumed and despite the fact that the production process of broiler chickens is highly standardized, in reality, some inputs like *Nb, d_rate* or *dpday* might be different amongst the farms. The same goes for the initial number of infected, where the possible sources of *Campylobacter* infection and their effects on It_0 are

unknown and hard to quantify. Those inputs are expected to have an impact on the *WFP* and consequently on *Fl (*Figure 1*)*, therefore, the baseline values were replaced by distributions (Table 2) describing the variability and the uncertainty surrounding the parameters and a sensitivity analysis was performed in order to assess the effect that each input has on outcame.

The distributions describing *Nb* and *d_rate* were obtained assuming a conservative discrepancy of ±100% from the baseline information while the effect of the uncertainty surrounding the initial number of shedders was tested assuming that It_0 may ranges from 0.05% (It_0=1) to 5% (It_0=1000) of the total population.

The day of final depopulation depends on several biological, economical and practical factors; industry data were used to estimate the parameters (Minimum; Most Likely; Maximum) of the Pert distribution describing the uncertainty in *dpday*.

Table 2 distributions used to evaluate the impact of the input on the model output.

Input	Unit	Distribution	Assumption
Nb	Unit	Uniform(5000;40000)[1]	±100% discrepancy from the baseline
d_rate	%	Uniform(1;10)[1]	±100% discrepancy from the baseline
It_0	%	Uniform (0.5;5)	+100% discrepancy from the baseline
dpday	Unit	Pert (36;38;50)	Industry data

[1]The minimum values of 5000 and 1% were maintained for the uncertainty distribution representing *Nb* and *d_rate respectively.*

The output of the model obtained with those inputs was used to perform a sensitivity analisys and tornado charts were used to represent the inputs ranked by effect on the output mean.

3. RESULTS

Following the flowchart reported in Figure 1, the results of the steps driving to the proportion of highly contaminated flocks in the baseline model are reported.

3.1. Baseline model - the age at which the flock became infected.

The risk analysis software @Risk 6.3 (Palisade Corporation) was used to simulate 10,000 values from each prior distribution (Eq.1) and to store results of computed posteriors (Fig.3).

Figure 3 Normalized posterior distributions for p^{+}_{rgi}. The median values together with the 5th and 95th percentiles are shown.

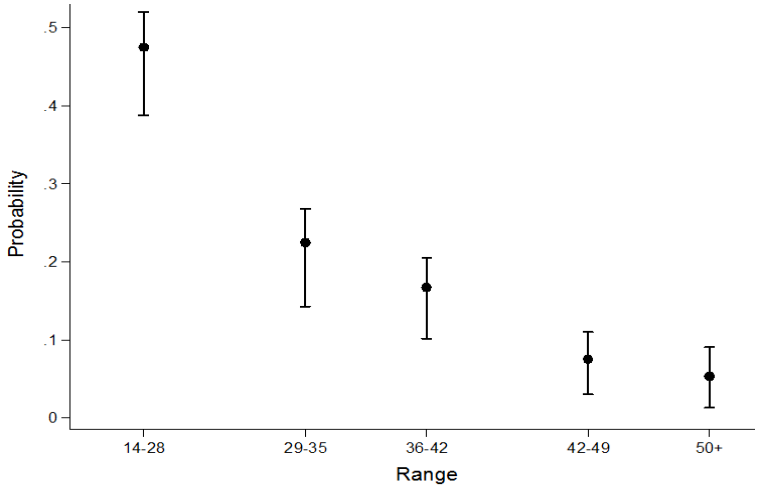
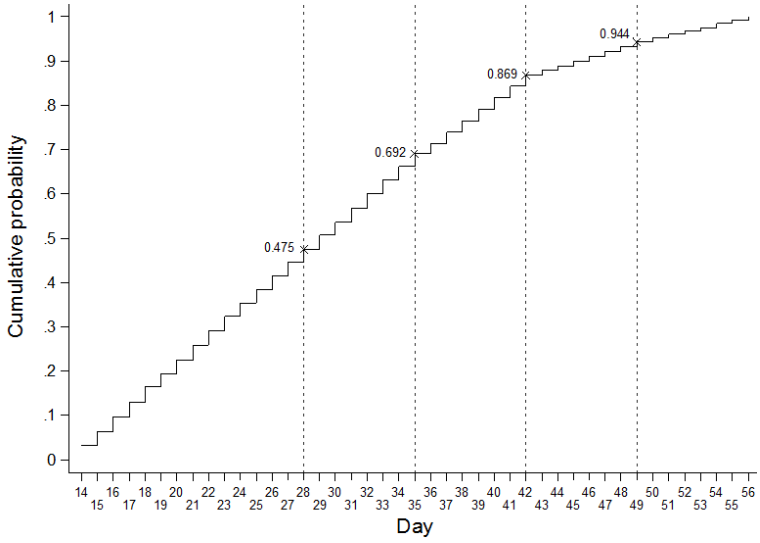


Figure 4 Cumulative probability describing the day of infection in positive flocks at slaughter. For the infected flocks, the cumulative probability of being infected before day 28 was 47.5%. This rose to 69.2%, 86.9% and 94.% for times of infection before days 35, 42 and 49, respectively

The cumulative probability distribution representing the chances that each day has to be the day of infection is reported in Figure 4.



3.2. Spread of infection.

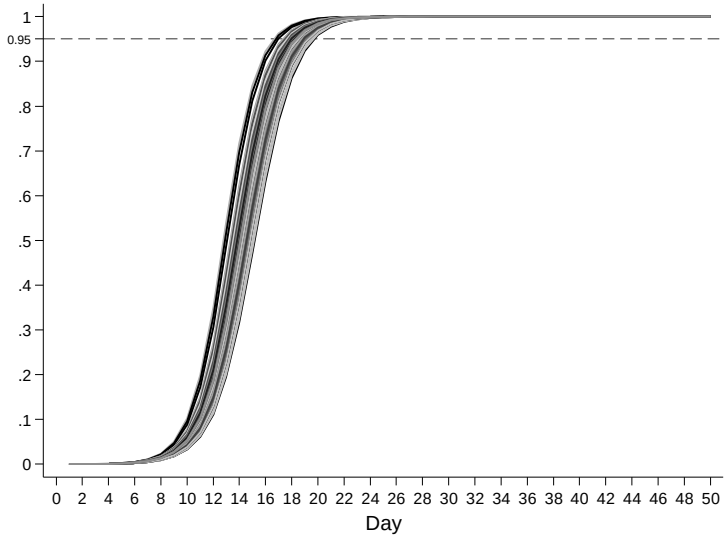
Following the estimation of the parameters obtained by the MLE (Eq.9), the Gamma distribution describing the *r* resulted:

$$r = Gamma(652.2; 0.0010) \qquad \text{(Eq.17)}$$

The distribution shown a mean of 0.698 with a standard deviation of 0.027. The effect of the uncertainty surrounding the parameter when the

logistic growth model was adapted to the baseline scenario, (Nb=20,000 chicken broilers with one initial infected at t_0) is reported in Figure 5.

Figure 5 The effect of the uncertainty in the coefficient 'r' on the horizontal spread. If the infection starts at day 0, the day at which the flock reaches a WFP of 95% ranges from day 15 to day 20 because of the uncertainty surrounding the parameter.

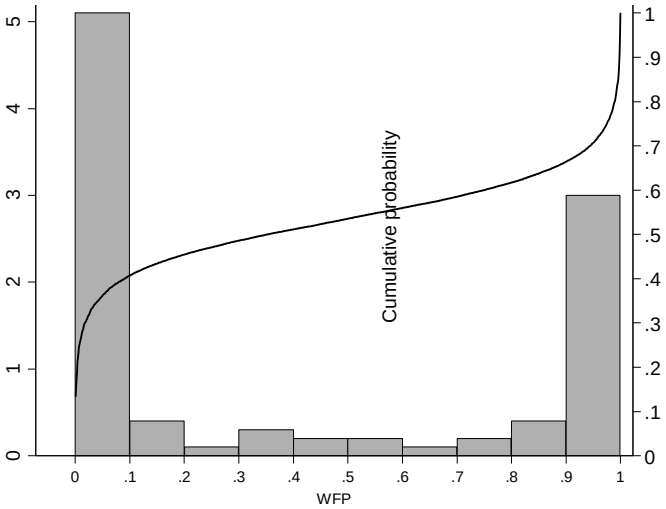


For the effect of the variability and the uncertainty, it takes from two to three weeks from the day of infection before the WFP reaches the 100%.

3.3. Baseline model - within flock prevalence.

Over 100,000 simulated flocks, the *WFP* at slaughter in contaminated flocks resulted equal to 46.35% on average. The cumulative distribution together with the probability density was reported in Figure 6.

Figure 6 Cumulative distribution and overlapped frequency of the WFP at slaughter in contaminated flocks. The probability density is reported on the y-axis on the left and the cumulative distribution on the y axis on the right.

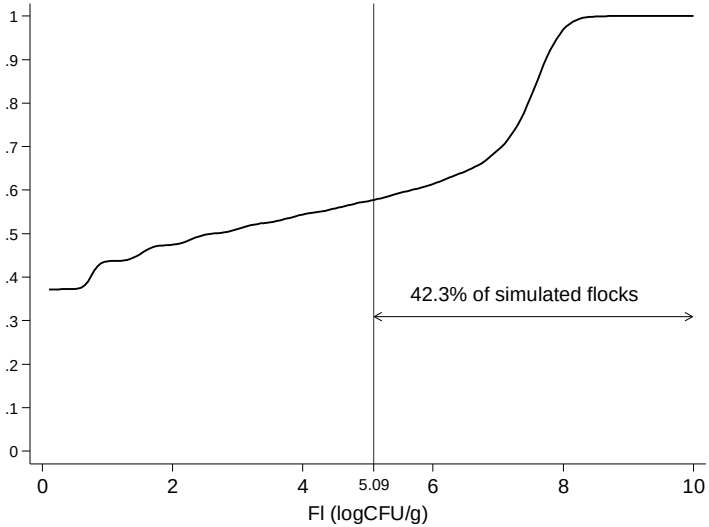


The WFP resulted below 50% in 53.6% of simulated scenarios but close to 90% at 65th percentile.

3.3.1. *Baseline model - level of contamination.*

As for the WFP, the cumulative distribution describing *Fl* (Eq.14) is reported (Figure 7). In the baseline model, the average value recovered for *Fl* in infected flocks was 3.51 logCFU/g, with a standard deviation of 3.43logCFU/g. The value at 95th percentile was 8.66 log CFU/g with 42.3% of infected flocks showing a contamination greater to 5.09log CFU/g.

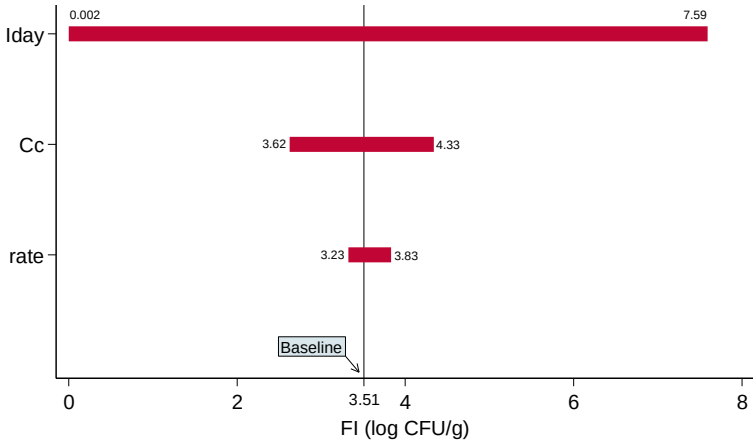
Figure 7 cumulative distribution of Fl, the reference line indicating the threshold for the 'highly contaminated flocks' is reported



The result of the sensitivity analysis outlined as tornado chart with the inputs ranked by effect on the output mean is reported in Figure 8.

Considering that *Fl* is calculated from the estimated level of contamination of a pooled sample (Eq.13), this value is directly dependent on the number of infected birds in the flock (Eq.10-11). In fact, the tornado chart clearly shown that the $Iday^{+}$ (and thus the WFP) is the input with the greater influence on the output mean when all the other inputs are fixed to the baseline values. The effect of C_c is also important, being able to move the average from 2.62 logCFU/g to 4.33logCFU/ml while the distribution describing *r* has a minimal impact on the outcome, being able of moving the average by 0.7 logCFU/g only.

Figure 8 Tornado chart representing the model inputs ranked by effect on the output (Fl) mean. Each bar represents how much the respective input is able to move the mean of Fl when all the other ones are fixed to the baseline value.



3.4 Alternative scenarios

For each on-farm mitigation strategy explored (Table 1), the distributions describing *Fl* (mean, 5^{th} and 95^{th} percentile) and the proportion of highly contaminated flocks at slaughter were reported in Table 3.

The estimated proportion of highly contaminated flocks for the scenarios in which the enhanced biosecurity (B+T-), the partial depopulation (B-T+) or both the management option were enabled (B+T+), are reported in Table 4. The confidence limits associated to the RRa of the factors under investigation were used in Eq.15-17 so that the 'best' and the 'worst' scenarios reflecting the uncertainty surrounding the estimates were reported.

Table 3 results obtained for Fl and proportion of flocks included in the category '>5.09 logCFU/g' at slaughter when the effect of interventions aimed to reduce the bacteria load in infected birds were simulated. Numbers in brackets represent the ±deviation from the baseline output in percentage.

	Fl (logCFU/g)			Flocks >5.09 logCFU/g* (%)
	Output (mean)	5th p.ile	95th p.ile	Output
Baseline (B-T-)	3.52	0.00	8.65	42.3%
VACCINE	2.59	0.00	6.67	31.17% (-26.31%)
BACTERIOCINES	0.97	0.00	3.26	0% (-100%)
ORGANIC ACIDS	2.92	0.00	7.51	37.1% (-12.29%)
BACTERIOPHAGE	2.13	0.00	5.69	1.92% (-95.46%)

*proportion over 100,000 simulated flocks

Table 4 resulting proportion of flocks included in the category '>5.09 logCFU/ml' at slaughter when the effect of management conditions affecting the introduction of pathogen and/or the spread of the infection (enhanced biosecurity, thinning) were simulated. Numbers in brackets represent the ±deviation from the baseline output in percentage.

	Flocks >5.09 logCFU/g* (%)		
Scenario	Output	BEST SCENARIO	WORST SCENARIO
Baseline (B-T-)	42.30%	//	//
B+T-	32.54% (-22.96%)	28.58% (-32.39%)	40.29% (-4.49%)
B-T+	65.6% (+55.08%)	49.91% (+17.99%)	79.10% (+87.00%)
B+T+	50.43% (+19.22%)	33.73% (-20.26%)	75.33% (+78.09%)

*proportion over 100,000 simulated flocks

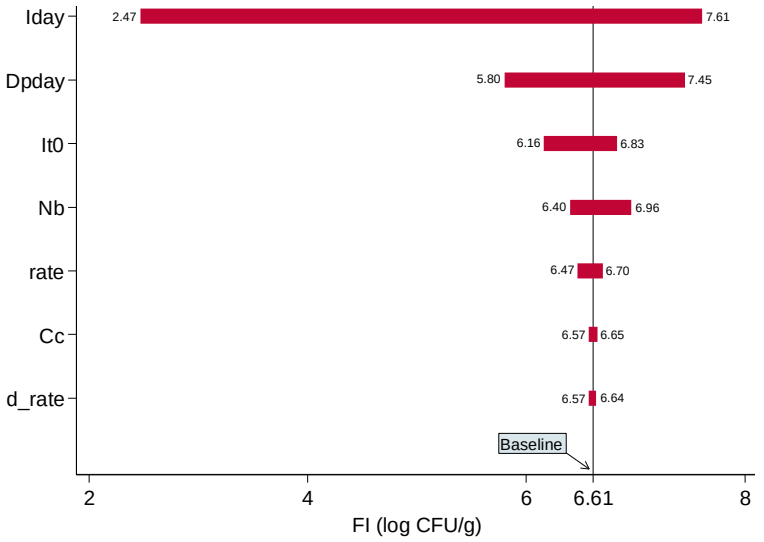
As expected, the application of biosecurity measures reduced the predicted proportion of flocks included into the category '>5.09 logCFU/g'. Conversely, the thinning practice had a negative impact. Interestingly, when both, the biosecurity measures and the thinning practice were

adopted, the combined effect of the factors was not conclusive, in fact, the uncertainty surrounding the effects led to a reduced and increased proportion of highly contaminated flocks when the best and the worst scenarios respectively were assessed.

3.5 Uncertainty in the baseline scenario.

In order to evaluate the effect that the fixed inputs have on the model output, the baseline values were replaced by the distributions reported in Table 2 and a sensitivity analysis was performed (Fig. 9).

Figure 9 Tornado chart representing the model inputs ranked by effect on the output (Fl) mean.



In this case, *Iday*$^{+}$ remained the input with the greater effect on the output mean, while all the newly introduced distribution (with the exception of

d_rate), replaced C_c and *r* in the upper positions suggesting that the uncertainty and variability underling those inputs, are likely to have a significant effect on *Fl*.

4. CONCLUSIONS AND DISCUSSION

The development of a probabilistic model for the transmission of *Campylobacter* infection in broiler flocks and the consequent estimation of the chances for a flock of being contaminated at high level as a function of the *WFP* and C_c (and related baseline inputs) provided a useful tool of practical use.

In fact, the model can be used to quantify: (i) the effect of mitigation strategies of which the effect and the specific point of action in the model is known and (ii) the effects of factors of which the specific point(s) of actions are not directly identifiable but the overall effect on the output is known.

When different mitigation strategies were tested, results clearly indicated that the potential effects of treatment with Bacteriocins and bacteriophages were significantly higher than vaccination or integration with organic acids. However, great care should be taken in considering these estimations; as previously stated, the effects of those mitigation strategies were estimated by using not definitive results. Nevertheless, researches on measures to combat the survival of *Campylobacter* in broilers seem to be promising (21), and the simple approach proposed to quantify those effects might be easily utilized as soon as new evidences will be available.

Conversely, the coefficients used to correct the baseline estimation as a function of the adoption of enhanced biosecurity measures or/and the partial depopulation practice, were obtained from an exhaustive epidemiological study conducted in UK in 2014; therefore, these effects can be considered a sound representation of the reality. At this respect, it should be noted that the results recovered for the scenarios under investigation (B-T-; B+T-; B-T+ and B+T+) were obtained assuming that all the simulated flocks operated at the same conditions. However, if the actual proportions of the flocks operating under each management condition in the population are known, those fractions might be used to weight the results and obtain an estimation of the overall prevalence of highly contaminated flocks in the whole population.

The on-farm model, although relatively simple, provided an exhaustive understanding of the dynamics leading to the *WFP* and the *Fl* in infected flocks and the related biological factors involved (i.e. *Nb*, It_0, *rate*, and C_c). The data used to parameterize the model inputs were collected from epidemiological studies related to commercial broiler chicken grown in intensive system and experiments in which the intensive conditions were reproduced; therefore, we believe the model is not likely to be inflated by sources of information that could have biased the estimation of the parameters.

With respect to the cumulative distribution describing $Iday^{+}$, our estimation differed slightly from those reported in a previous study (9). This could be explained by the fact that in that study, different longitudinal studies were collated and the final dataset included information from

broiler chicken grown under different management system (free range or organic). Moreover, left censored data (i.e. flocks that were positive at the first sampling point) were excluded from the analysis.

For the transmission of *Campylobacter* within the flock, the logistic growth model proposed by Katsma et al. (13) was adopted, with the only difference that with the Bayesian approach applied on the original datasets we included in the model a distribution for *r* instead of a fixed value. This gave us the opportunity to formerly consider the uncertainty and the variability underlying this input and assess its influence on the outcome with the sensitivity analysis.

The on-farm model was developed not only with the intent of being a flexible and easily reproducible tool for the assessment of the mitigation strategies at farm level, but also for the quantification of the impact that variations in the baseline characteristics of a broiler flock might have on the output. In fact, a number of baseline information (*Nb*, *d_rate*, It_0 and *dpday*) were included in the model as initiative inputs (Eq.6, *WFP*) and the potential impact on the outcome as a function of a variation in those values should be taken into account. In fact, the sensitivity analysis reported in Figure 9, clearly shown how variations in those information might lead to significant consequences. As a practical example, if *dpday* is anticipated by two days or *Nb* decrease of 5000 units, the baseline proportion of highly contaminated flocks at slaughter decreased by 13.7% and increased by 3.8% respectively from the baseline (result not shown). Consequently, one of the practical value of this model is that its flexibility lends itself to be adapted to very specific baseline scenarios besides the

standard condition assumed in this study.

4.1. Main assumptions and limitations.

As in any model aimed to describe the complexity of the real world, some assumptions and limitation are recognized.

The first assumption is related to $Iday^{+}$ where the baseline model assumes that the transmission never starts before the fourteenth day of the cycle. The sensitivity analysis (Figure 8-9) highlighted the importance of this inputs, but the threshold assumed by the model finds its justification from in-field studies and multiple biological factors (14, 18). However if new evidences and data become available, the model can be easily updated operating on Equation 4 and 5.

Another assumption is that the simplified transmission model do not admits that infected birds can recover. Even tough cases of self-limitation of the infection have been occasionally reported (8), considering the chicken broiler reared in intensive system and the length of the production cycle (usually less than 40 days), It is generally accepted that once a bird is infected the infection persists until clearance.

An important limitation highlighted by the sensitivity analysis in figure 9, concerned the effect of the uncertainty related to It_0. Our transmission model, was initiated assuming one initial infected bird but in reality, the initial number of shedders is likely to be strictly related to the source of contamination (i.e. if the source of contamination is the drinking water rather than feces of wild animals, the number of infected birds at t_0 is

likely to be very different). The identification of the on-farm risk factors for the introduction of *Campylobacter* has been assessed in several studies by risk factor-based surveys using structured questionnaires (5, 7, 10, 19) but the relationship: source of contamination-number of infected birds at t_0 has never formerly investigated, however, even this information can be easily included once available. Given the potential impact of this factor, further researches focused on this relationship are strongly needed.

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Part 2

QUALITATIVE MODELLING

Chapter 5

Qualitative risk assessment of introduction of Anisakidae nematodes in Atlantic salmon (salmo salar) farms and commercialization of fishery products infested by vital larvae

General introduction

The presence of alive parasites in fish with special reference to nematodes of genus *Anisakis spp.* is a worldwide food-safety concern and on April 2010, the EFSA Panel on Biological Hazards released a scientific opinion concerning the assessment of parasite in fishery products in response of a specific request of the European Commission. On the basis of that opinion, the Regulation (EC) No 1276/2011, allows food business operator to not apply freezing treatment on fishery products if procedures approved by the competent authority are used to verify that the product do not represent a health hazard with respect to viable parasites.

In this study, we adapted the general approach recommended by the OIE for the assessment of the risk posed by the importation of live animal to formally assess the risk of introduction and establishment of Anisakids nematode in Atlantic salmon farms and consequently, the commercialization of infested fishery products. We explored several plausible biological pathways beside the 'feed' (the only route considered by EFSA) and all the key steps along each route of introduction were identified and assessed by reviewing the most recent

evidences/information concerning the parasite, its primary and paratenic hosts and the farming practices. The overall probabilities of introduction along each route were qualitatively assessed taking into account the uncertainty surrounding all the available information; therefore, we believe our approach is of critical interest for both food business operator and researchers.

Our study provides, for the first time, a formal and transparent qualitative assessment of the risk of introduction and establishment of live nematodes of genus Anisakis in farmed Atlantic salmon and commercialization of infested animals, therefore, the general model is easily adaptable to different production companies making the model of immediate practical use. Furthermore, the framework can be adapted to assess the risk related to commercialization of infested aquaculture fishery products beside the Atlantic salmon.

Qualitative risk assessment of introduction of Anisakidae nematodes in Atlantic salmon (*salmo salar*) farms and commercialization of fishery products infested by vital larvae

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ABSTRACT

A qualitative risk assessment was adopted to formally assess the likelihood of introduction of anisakids larvae in farmed Atlantic salmons (Salmo salar) and the commercialization animals infested by at least one vital parasite. Several plausible pathways were identified and the most recent information concerning the parasite, its primary and paratenic hosts and the farming practices were reviewed and used to assess the likelihoods of each key step. A matrix for the conditional probabilities was adopted to combine the qualitative estimations and obtain an objective and transparent overall risk of introduction along each route. In order to avoid misinterpretation and the overconfidence of the outcome, the uncertainties surrounding the estimations were qualitatively assessed and associated to each estimation. The likelihood of parasite being introduced into a generic Atlantic salmon farm resulted higher than 'negligible' only when the pathway outlining the introduction of the parasite through the ingestion of infested hosts who have penetrated the harvesting cages was assessed. In that pathway, the overall risk resulted 'very low' with a high degree of uncertainty; the uncertainty resulted 'high' because of the scarcity of information in some of the key steps of the pathway; however, the scientific evidences in support of the overall estimation suggest that the availability of additional data would be unlikely to change the final estimation upward. The proposed qualitative approach is an objective and transparent method to assess the risk when data and information are scarce, it can be easily adapted to other species besides farmed Atlantic salmons and other parasite besides Anisakids.

1. INTRODUCTION

Fish-borne parasite zoonosis represent a global emergent threat, among these, anisakidosis is showing a generalised increase in the last two decades (7). The family Anisakidae includes zoonotic parasitic nematodes among which, the species belonging to the genera *Anisakis* and *Pseudoterranova* are the most commonly associated with infestations in human due to consumption of raw or undercooked fishery products.

The effects of anisakids in terms of decreasing the commercial value of fishery products and the impact on human health have resulted in these parasites becoming both an economic and a public health concern worldwide (4, 5, 14, 56).

The life cycle of Anisakidae of zoonotic interest, is developed in seawaters and involves marine mammals (cetaceans and pinnipeds) and piscivorous birds as definitive hosts. In natural conditions, the predation of infested fishes lead to bioaccumulation along the predation chain resulting in certain fish species being characterized by higher chance to be infested (64) and thus, represent a risk for human health.

In order to prevent and control transmission of fishery product-borne parasites, the Section VIII of Annex III to Regulation (EC) No 853/2004 lays down provisions for fishery products to be consumed raw or almost raw. The Regulation indicates that fishery products intended to be eaten after a process that is not sufficient to destroy nematode larvae must be frozen at a temperature of not more than -20°C in all parts of the product for not less than 24 hours.

In April 2010, the European Food Safety Authority published a scientific opinion on risk assessment of parasite in fishery products (14) providing criteria for determining the conditions under which fishery products from aquaculture can be recognized as being free of viable parasite and that may represent an hazard for human health. With particular reference to farmed Atlantic salmon (*salmo salar*), the Opinion concluded that farmed Atlantic salmons reared in floating cages or onshore tanks and fed compound feedstuffs are unlikely to contain live parasite, however, the Panel on Biological Hazards did not considered routes of infection other than the feed and the risk was never assessed formally.

Following that Opinion, in 2011, the Regulation (EC) No 1276/2011 modified the requirements set out in Annex III, Section VIII, Chapter III, Part D of Regulation (EC) No 853/2004 allowing food business operators to not apply freezing treatment if procedures approved by the competent authority are used to verify that the product do not represent a health hazard with respect to viable parasites.

In the present study, the general approach recommended by the World Organization for Animal Health (OIE) for the assessment of the risk posed by the importation of live animal and animal products (43) was adapted to formally investigate the potential for live zoonotic nematodes to represent a risk for human health in farmed Atlantic salmon (35).

2. MATERIAL AND METHOD

The likelihood of the commercialization of a farmed Atlantic salmon (*salmo salar*) infested by at least one vital anisakids larva was qualitatively

assessed. In the approach proposed by the OIE, the hazard identification precedes the risk assessment, which is composed of three components: (i) Release assessment, (ii) Exposure assessment and (iii) Consequence assessment. Generally, the final risk estimate is the result of the integration the steps but because of the purpose of this study, the consequences of the parasite's establishment are not considered.

Several pathways outlining the sequence of sufficient and necessary events leading to the introduction of the parasite into a general fish farm were identified and the likelihoods of introduction assessed for each pathway considering the farming practices of the Atlantic salmon. The qualitative risk assessment models foresee the use of subjective risk levels to describe the likelihood of unwanted events; in this work, the qualitative terms proposed by Kahn et al. (25, 26) were adopted (Table1). The biological and epidemiological characteristics of the parasite, its primary and accidental hosts together with the biosecurity technologies and measures applied in the Atlantic salmon's farms were reviewed and discussed to assign the likelihood at each step of each identified pathway.

Table 1 Definition of the likelihood terms

Likelihood	Decription
High (H)	Expected to occur
Moderate (M)	Occurrence less than 50% probability
Low (L)	Unlikely to occur
Very low (VL)	Rarely occur
Extremely low (EL)	Very rarely occur
Negligible (N)	Chance of occurrence so small that can be ignored

In each pathway, the likelihoods assigned at each event were combined to derive the overall risk estimate the introduction and establishment of the parasite in farmed salmon.

The risk estimates were expressed as cumulative likelihoods obtained combining the qualitative estimates of the inputs according to the matrix for the conditional probabilities (Table II) previously applied by EFSA and other qualitative risk assessment (13, 47).

This matrix defines a likelihood estimate for any binary combination of conditional events and in order to avoid an overconfidence of the outcomes and prevent misinterpretation, an assessment of the uncertainty surrounding each estimation was also reported (Table 3), and expressed as: High, (H) Moderate (M) or Low (L).

Table 2 Combination matrix used for the estimation of the conditional likelihoods. The product of two probabilities is always less than the lowest probability and is sometimes given as a range (e.g. N-EL). However, as explained in the EFSA report, since qualitative term covers a wide range of likelihoods the combined estimate is in some case equal to the lower estimate (e.g. a step 'n' with an estimate of VL with a step 'n+1' with an estimate of EL produces and an overall estimate of N-EL).

Likelihood step '*n+1*'	Conditional Likelihood step *'n'*					
	N	**EL**	**VL**	**L**	**M**	**H**
H	N	EL	VL	L	M	M
M	N	EL	VL	VL	L	M
L	N	EL	EL	VL	VL	L
VL	N	N-VL	EL	EL	VL	VL
EL	N	N	N-EL	EL	EL	EL
N	N	N	N	N	N	N

Table 3 Definition of uncertainties

Uncertainty	Interpretation
Low (L)	The estimation is strongly supported by data-evidences, Agreement by different authors
Medium (M)	The estimation is supported by few or Incomplete data. Some authors report conclusions slightly different from some other
High (H)	The estimation is supported only by scarce data or it is based on Hypothesis. Strong disagreement from different authors

If two or more independent risk factors contributed to the likelihood estimation for a single step, the likelihoods for each factors were estimated and the same matrix for the conditional probabilities was used to outline the overall likelihood for the step. With respect to the uncertainties, the worst estimate was conservatively considered among the risk factors and along the steps of the pathways; in this way, a high uncertainty in one level is enough to lead to a high uncertainty in the overall outcame. An exception was made if the occurrence of the event in step n+1 is *Negligible* with *Low* uncertainty.

2.1 Hazard identification and Characterization.

The different species belonging to genus *Anisakis spp.* and *Pseudoterranova spp.* are not reliably distinguishable morphologically but several species were identified at molecular level (38, 39, 44, 45). The morphospecies most commonly associated to human infection are:

(i) *Anisakis simplex,* worm-like parasite, usually 1 to 3cm length,

thin, characterized by a pinkish-white colour and that usually appear rolled up on itself. Larvae are usually localized in the viscera where are generally easily visible but can migrate into the muscle or the abdominal wall where the parasite is more difficult to identify, especially in the white fish (13);

(ii) *Pseudoterranova decipiens*, worm-like parasite, usually of 1 to 4cm length and characterized by a reddish-brown colour it tends to present a large-rolled coil. In infested specimens it is usually located at muscular level (40).

The life cycle of the species belonging to genus *Anisakis* takes place in seawater and proceeds in several steps. In the first step, larvae at the first stage (L1), are released in seawater with the feces of the definitive hosts (mainly cetaceans such as whales, dolphins and porpoises); in marine environment they develop to L1-L3 stage in the eggs after which, larvae are released in seawater (29, 40, 60).

Newly hatched larvae can survive in marine environment for weeks and be eaten by a wide range of different primary hosts (crustaceans and molluscs). When fish or cephalopods eat primary infested hosts, the parasite migrates to the coelomic cavity of the predator, which act as paratenic (i.e. intermediate) host. In paratenic hosts, the number of parasites is regulated by bioaccumulation along the predation chain; consequently, big and/or old fishes may host even thousands of nematodes (60). Humans are act as accidental hosts when they eat undercooked infected fish or squid.

The larval stages and the biological cycle of *Pseudoterranova spp.* do not

differ from the ones described for *Anisakis spp.* even though the definitive hosts are usually pinnipeds like sea lions or wales instead of cetaceans. Moreover, larvae of *Pseudoterranova.spp,* lack of cuticular sheaths with lateral extremities that increase the buoyancy, thus, conversely to larvae of *Anisakis spp.*, are not able to swim (46).

Although the dynamics underlying the geographical distributions of the most important primary and intermediate hosts of *Anisakis spp.* and *Pseudoterranova spp.* are complex and still largely unknown (14), considering the differences in the habitat of the hosts involved; it is generally recognized that *Anisakis spp.* has an essentially pelagic life-cycle, whereas *Pseudoterranova spp.* has a more benthic habit. Consequently, with particular reference to the Atlantic salmon, parasites belonging to the genus *Anisakis spp.* represent a greater concern than *Pseudoterranova spp.(69).*

Following these considerations, nematodes of genus *Anisakis spp.* were formally identified as the hazard of interest while *Pseudoterranova spp.* is not considered further.

2.1.2. Hazard characterization *Anisakis spp.* – prevalences.

The prevalences of the parasite in different wild fishes and data related to the occurrence of the different species of *Anisakis* in infected fish shown high variability according to both geographical region and the hosts species (14, 41, 48, 50). The complexity of the dynamics leading to different proportions of the parasite's species in different hosts and in different areas led to the cautionary conclusion that none of the fishing area worldwide should be considered as *Anisakis*-free, and thus, all the

wild saltwater fishery products must be considered potentially infested (14).

2.1.3. Hazard characterization Anisakis spp. – Pathogenesis.

In humans, the accidental ingestion of live nematodes belonging to the family of Anisakidae, causes parasitic zoonosis known as anisakidiosi or anisakisiasi, described for the first time in 1960 by Van Thiel (4). The minimum infectious dose is a single nematode (11, 16) and after ingestion, vital larvae may be excreted up to 48 hours with feces, or turns to the acute form of anisakidiosi, the most frequently observed form and characterized by violent abdominal pain, nausea and vomiting, sometimes with the presence of the larvae (2, 52, 65). The acute form might degenerate into chronic if misdiagnosed or untreated. In the chronic form, the larvae penetrate the gastrointestinal mucosa, causing the formation of abscesses and granulomas with eosinophilic infiltrate. Granulomas and the inflammation process remain even after the death of the worm that in human body usually happen after 3 weeks after ingestion. Complications are rarely reported in literature, few episodes involved intestinal obstruction (53), colic intussusception (21, 70) and pneumoperitoneum (24). Moreover, the consumption of fishes harbouring dead Anisakis spp. larvae has been reported to be potentially dangerous because of potential allergenic reactions (3).

2.2. Release and Exposure assessment for the Introduction of Anisakis spp into a generic Atlantic salmon farms

The risk of the introduction of *Anisakis spp.* into a generic Atlantic salmon

farm was assessed considering five pathways.

1. *Capture and harvest of wild Atlantic salmon*
2. *Presence of Anisakis spp. in feed*
3. *Accidental introduction of wild salmon in the floating cages*
4. *Re-introduction of escaped salmon that have been infested offshore*
5. *Ingestion of infected intermediate/paratenic hosts*

For each pathway the sequence of sufficient and necessary events leading to the release of the parasite into a general farm were identified and reported (Figure 2-5).

2.2.1 Pathway 1: Capture and Harvest of wild Atlantic salmon

The occurrence of Anisakis in wild salmon is known to be high and above 70% (14, 17), therefore, the harvest of wild animals would represent an important pathway for the commercialization of risky products. However, unlike the farming methods applied for other species (i.e. Cod or Eels), the production cycle of Atlantic salmon is totally closed and neither the capture of juvenile form is required. This pathway was not further explored.

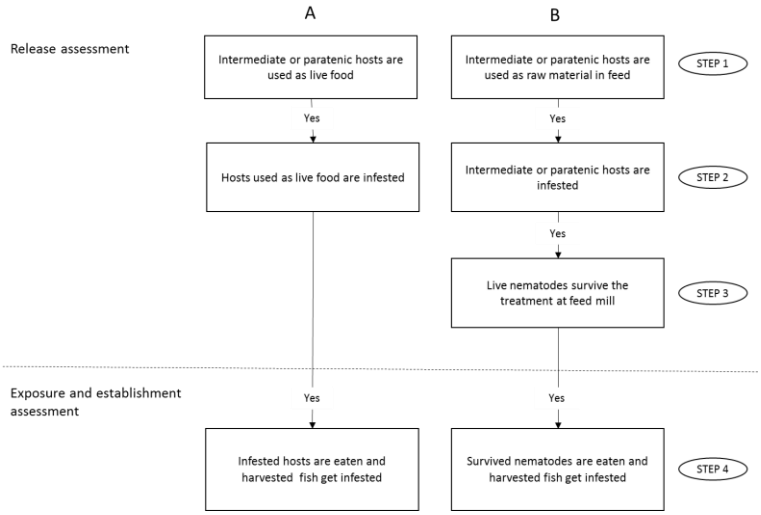
2.2.2 Presence of Anisakis spp. in feed

The likelihood of the introduction and establishment of the parasite into a generic farm trough the feed depends on: (i) the source and the nature of the raw material and (ii) the thermal/physical treatments to which raw material has been subjected.

The scenario trees outlined in Figure 1 represent the pathways leading to

the introduction of the parasite into a generic farm by feed. Both the use of live food (A) and treated feed (B) were considered.

Figure 1 Pathways outlining the required steps for the introduction of Anisakis spp. into a generic farm through feed. Both live food (pathway A) and feed were considered (pathway B).



The use of live food would lead to an evident risk of introduction of the parasite (68), however, the farming of the Atlantic salmon foresees the use of treated feed only; therefore, the pathway A was not considered. Moreover, since farmed salmon are fed with composite feed which includes wild species like herring (*Clupea harengus*), capelin *(Mallotus villosus)*, Chilean anchovies *(Engraulis ringens)* etc., the first step of the pathways B was considered as an event that always occur, and its likelihood was not included in the assessment.

Step 2

Considering the wide range of intermediate/paratenic hosts species at which *Anisakis* have adapted (14, 28), together with the variability and the uncertainty underlying the presence of the parasite in wild species (Section 2.2.1); the likelihood of *Anisakis spp.* being present in wild species used as raw material for the farmed salmon feed is considered ***High*** with ***Low*** uncertainty.

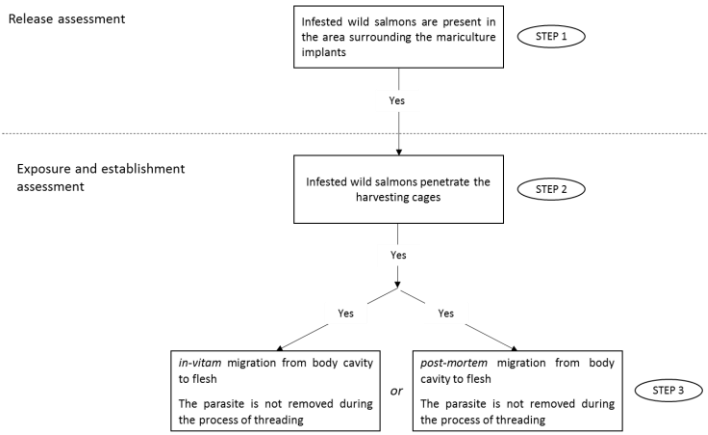
Step 3

Farmed salmon are fed with dry pellet produced by extrusion and temperature above 150°C. The likelihood of parasite surviving the treatment is ***Negligible*** with ***Low*** uncertainty.

2.2.3 Accidental introduction of wild salmon in the farm

The pathway leading to the introduction of *Anisakis spp.* by the accidental introduction of wild salmons in floating cages is outlined in Figure 2.

Figure 2 Pathway outlining the required steps for the introduction of Anisakis spp. into a generic farm by accidental introduction of wild salmon in floating cages.



Step 1

As mentioned in section 2.2.1. The occurrence of *Anisakis* in wild salmon is known to be high; however, although the presence of wild salmons in the areas surrounding the salmon farms cannot be excluded, a low density of wild salmon is usually recorded in the areas bordering the mariculture implants (18, 20). From these evidences, the estimated likelihood of wild salmons being present in the area surrounding the farm is ***Low*** whilst the likelihood of wild salmon being infected is ***Medium;*** consequently, the combined likelihood for the presence of infected wild salmons in the area surrounding the farms is ***Very Low***.

The level of uncertainty was considered ***Medium*** for the first condition and ***Low*** for the second one leading to an overall conservative ***Medium*** level of uncertainty for this step.

Step 2

Atlantic salmons are grown to marketable size in floating nets offshore; the possibility for a wild salmon to penetrate the harvesting nets and to mingle with the reared salmons is linked to the presence of a hole in the floating cages. However, the firsts consequences of a hole in a floating net would be the escape of the raised fish, with sensible economical loss and huge environmental consequences (10, 19, 22, 66); consequently, it is of industry interest to apply all the biosecurity measures aimed to prevent/avoid the escape of the reared fishes, and indirectly, the introduction of wild animals. At this respect, the major food business operators in salmon harvesting invest many resources to pursue the so-called 'zero escape' objective and public reports shown how the efforts resulted in a steadily decreasing occurrence of incidents leading to 'escape' events (30-32, 34-36).

Moreover, incidents resulted in an escapes should not be interpreted as events favouring at the same time the introduction of wild individuals. In fact, all the reported incidents were one-way oriented in determining the 'escape' without favouring the 'introduction' in any way.

It can be surmised that only a so limited breakage in nets as to prevent a massive escape of reared might not be immediately noticed by the operators through the underwater cameras surveillance can be the opportunity for wild animals to penetrate the floating cages; but even then, the wild salmon should penetrate exactly from that specific point.

Following these considerations, the estimated likelihood of the wild

salmon accidentally penetrating the floating cage is ***Negligible*** with ***Low*** uncertainty.

Step 3

At this stage, the combined likelihood of the parasite migrating (*intra-vitam* and/or *post-mortem*) from the coelomic cavity to edible muscles, and not being removed during the process of threading is assessed.

The *intra-vitam* migration of the parasite is not a certain event and the frequency distributions of *Anisakis* third stage larvae in hosts' tissues are believed to be affected by a number of conditions encountered within the hosts themselves (62) among which, the lipid contents is believed to play an important role (61, 63).

Several recent studies reported the presence of *Anisakis* nematodes in muscles surrounding the body cavity of freshly caught salmonids (27, 54, 55, 67) or sibling species (51) indicating that the *intra-vitam* migration of the parasite is an event that it is likely to occur in salmonids. Following these considerations, the estimated likelihood of parasite *intra-vitam* migration from the coelomic cavity is ***High,*** with ***Low*** uncertainty.

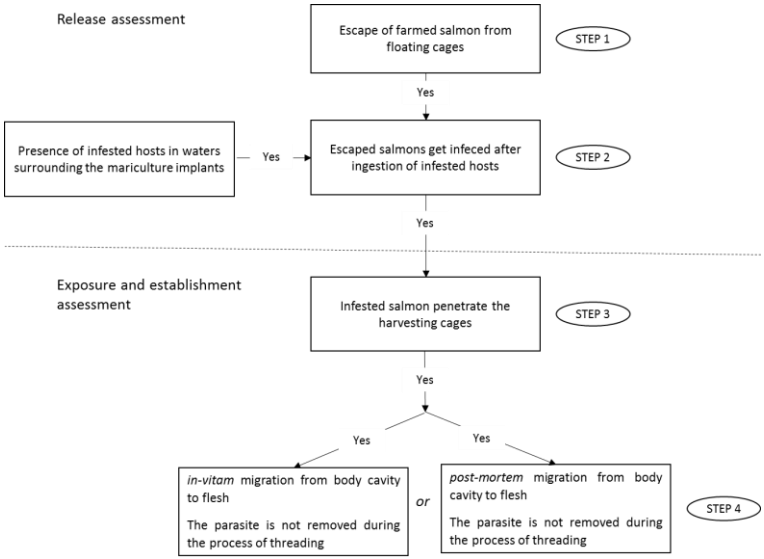
The *post-mortem* migration of the parasite from the viscera to flesh is still a debated topic, the scientific opinion from the Panel Biological Hazards (14) reported: "… based on scientific evidence it is not clear when, under what conditions and in which fish species, *post-mortem* migration of *Anisakis simplex* larvae occurs …". However, factors stimulating the migration of the parasite after host death are presumably related to physio-chemical changes in viscera (59) and time-temperature storage

conditions (8,9). At this respect, it should be considered that opposite to wild salmons, reared fishes are processed immediately after collection from the floating cages; consequently, the likelihood of *post-mortem* migration in farmed Atlantic salmon is ***Negligible*** with ***Low*** uncertainty. The worst scenario (intra-vitam migration) was conservatively considered in this step

2.2.4 Re-introduction of escaped salmon that have been infested offshore

Although the 'escape' events are rare (Section 2.2.3), the likelihood of the re-introduction of escaped salmon that have been infested offshore is assessed (Figure 3).

Figure 3 Pathway outlining the required steps for the introduction of Anisakis spp. into a generic farm by Re-introduction of escaped salmon that have been infested offshore.



Step 1

From consideration in Section 2.2.3. (Step 2), the estimated likelihood of farmed Atlantic salmons escaping in seawaters is ***Low*** with ***Low*** uncertainty.

Step 2

Anisakis spp. larvae can survive in seawaters for extended period and be eaten by a wide variety of different hosts. Although the parasite mainly uses *euphausiids* (krill) living in deeper water offshore as first intermediate host (58), the parasite is able to select host species depending on the locality (28). Therefore, the estimated likelihood for the presence of infested hosts in the areas bordering the implants is ***High***, with a ***Low*** .

Since escaped salmons are forced to prey to survive, the estimated likelihood of escaped salmons preying infested hosts is ***High*** with ***Low*** uncertainty.

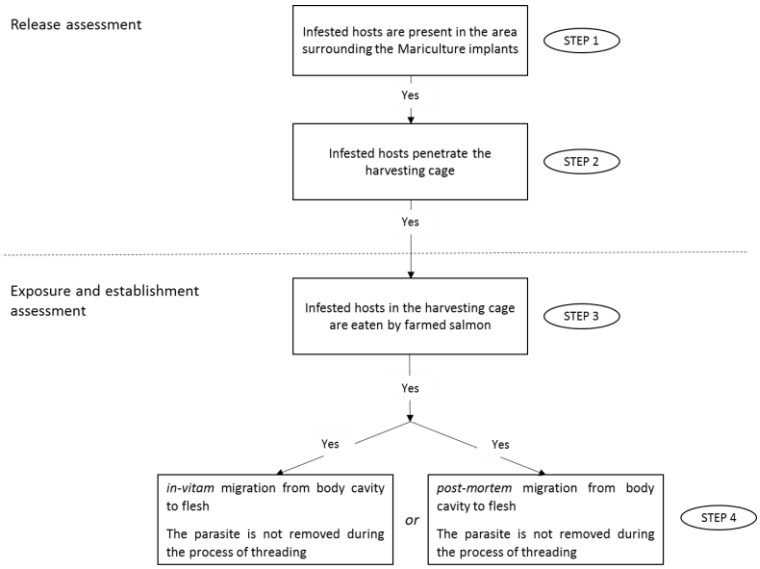
Step 3 - 4

Estimated likelihoods and uncertainties for these steps are identical to the ones reported in pathway 3.

2.2.5 Ingestion of infected intermediate/paratenic hosts

The pathway leading to the introduction of *Anisakis spp.* by ingestion of infected intermediate/paratenic hosts is outlined in Figure 4.

Figure 4 Pathway outlining the required steps for the introduction of Anisakis spp. into a generic farm by ingestion of infected hosts.



Step 1

Following considerations in Section 2.2.4. (Step 2), the estimated likelihood of infested hosts being present in areas bordering the implants is ***High*** with ***Low*** uncertainty.

Step 2

The access for infested hosts into the floating cages is strictly dependent by the size of the hosts themselves. In fact, while the introduction of large hosts like is physically prevented by the meshes' size, none barriers are applicable to hosts smaller than the meshes. Thus, the estimated likelihood for this step is ***Medium***, with a ***High*** level of uncertainty due to the lack of information about the occurrence of the different host species

(with particular interest in host size) in the area bordering the implants.

Step 3

In this step, the ingestion of *Anisakis spp.* is linked to the predation of the hosts.

Even though farmed salmon are fed with dry pellet, results of two recent studies (37, 42) reported the presence of *Anisakis* larvae in runts of farmed Atlantic salmon (Fishes with clear signs of poor performance and/or abnormal appearance, emaciated and not suitable to be marketed for human consumption); suggesting that farmed salmonids in open cages may feed even on live food.

However, it is important to emphasize that the nematodes were found only in discarded animals and not in harvested quality salmons. The authors explained their findings hypnotizing that in floating cages weak animals undergo competition phenomena that limit their access to feed and thus, runts must feed with 'anything' that can be eaten in order to survive. It is assumed that the likelihood of high quality salmons preying live food to supply their feed intake is ***Low*** with ***High*** uncertainty

Step 4

As discussed in Section 2.2.3 (Step 4), the presence of the parasite in infested salmons represents a risk for human health only if it is not physically removed during the process of threading. According to previous estimations, the likelihood for the event is ***Low*** with ***Low*** uncertainty.

3. RESULTS

Results of estimated likelihoods and uncertainties, together with the cumulative likelihoods (in parenthesis) were reported for each considered pathways (Table 4-7).

Table 4 summary of risk estimates for the Introduction of Anisakis by feed (H=High, M=Medium, L=low, VL=Very Low, EL= Extremely Low N=Negligible).

Step	Description	Likelihood (conditional)	Uncertainty
Release assessment			
2	Infested hosts were used as raw material for the feed	H	L
3	The parasite survive the treatment in feed mill	N (N)	L (L)
Exposure and establishment assessment			
4	Survived larvae are ingested	//	//

Table 5 summary of risk estimates for the Introduction of Anisakis by accidental introduction of infested wild salmons in floating cages.

Step	Description	Likelihood (conditional)	Uncertainty
Release assessment			
1	Wild infested salmons are present in the area surrounding the farm	VL	M
Exposure and establishment assessment			
2	The wild salmons penetrate the floating cage	N (N)	L (M)
3	The parasite is not removed during the process of threading	//	//

Table 6 summary of risk estimates for the Introduction of Anisakis by the Re-introduction of escaped salmons who have been infested in open waters.

Step	Description	Likelihood (conditional)	Uncertainty
Release assessment			
1	Escape of farmed salmons	L	L
2	Escaped salmons prey infested hosts	H (L)	L (L)
Exposure and establishment assessment			
3	Escaped salmons get infested and re-enter the floating cages	N (N)	L (L)
4	The parasite is not removed during the process of threading	//	//

Table 7 summary of risk estimates for the Introduction of Anisakis by ingestion of infested hosts.

Step	Description	Likelihood (conditional)	Uncertainty
Release assessment			
1	Infested hosts are present in the area surrounding the farm	H	L
2	The infected hosts penetrate the floating cages.	M (L)	H (H)
Exposure and establishment assessment			
3	Infected hosts in floating cages are eaten by high quality harvested salmons	L (VL)	H (H)
4	The parasite is not removed during the process of threading	H (VL)	L (H)

4. DISCUSSION

In our study, the estimated cumulative likelihoods defined the risk of introduction of *Anisakis spp*. into Atlantic salmon farms (and

commercialization of infested products) as *Negligible* or *Very Low* depending on the considered pathway. Our formal qualitative estimations agreed with the available scientific evidences (1,14,33,57,69) who generally considered the presence of vital Anisakids in farmed salmon as a very unlikely event.

With respect to the second pathway, our estimation coincides with the conclusions reported by EFSA, (14) and the outcome was characterized by a Low level of uncertainty, indicating strong evidences in support of the result. In fact, to date, there are no evidences or reported cases indicating that nematodes of genus *Anisakis spp.* are able to survive the processes at which the raw materials are subjected (1, 6, 23, 33). Recently, some proteins attributable to *Anisakis simplex* have been found in processed fish products (15), but the risk related to allergic reactions due to the presence of heat-resistant proteins (12, 49), was beyond the scope of this study.

The cumulative likelihood obtained for the pathway 3 and 4 led to a N*egligible* risk of introduction and the estimation would not changes neither in presence of further evidences moving the likelihood for the first step of pathway 3, (characterized by *Medium* uncertainty), to *High*.

The cumulative likelihood of the pathway 4 resulted *Very Low* but this outcame was characterized by *High* uncertainty.

The route of introduction by ingestion of infested hosts, although characterized by high uncertainty, was the only pathway leading to an overall estimation of the risk greater than 'Negligible'. Our formal findings

seem to support the hypothesis of the authors who recovered larvae of *Anisakis simplex* from farmed salmons (37, 42). Although characterized by a high level of uncertainty (because of the uncertainty in step 2 and 3), it is unlikely that the overall estimate is not representing the real risk, otherwise very different evidences would be reported in literature. Consequently, it can be hypothesized the high uncertainties in step 2 and 3 are the result of a lack of data who is likely to do not have the potential to move the overall estimation upward (i.e. greater than *'Very Low'*).

4.1. Main assumptions and limitations.

As outlined by the hazard identification, the assessment was made considering nematodes belonging to *Anisakis spp.* without distinguishing between the different species, thus, similar properties amongst the species of genus *Anisakis* were assumed.

Moreover, it should be noted that because of the differences in the typical habitat between *Anisakis spp.* and *Pseudoterranova spp.* results obtained for *Anisakis spp.* could be extended to *Pseudoterranova spp.*

In the study, only plausible routes of introduction were considered in the release and exposure assessment; nevertheless, since science cannot prove that a particular pathway does not exist there will always be a degree of uncertainty.

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Chapter 6.

General Discussion

General conclusions

Risk analysis is an internationally recognised process adopted by the food regulatory bodies worldwide, under the premises that the process is conducted according to the principles of 'good policy', the purpose of the risk analysis in food safety is to provide a systematic procedure to examine and assess public health and safety risks associated with food.

Following the definition that the CAC provides for the 'risk management': *"The process, distinct from risk assessment, of weighing policy alternatives in consultation with all interested parties, considering risk assessment and other factors relevant for the health protection of consumers and for the promotion of fair trade practices, and, if needed, selecting appropriate prevention and control measure"* it is clear that the *appropriate* decisions are ultimately the result of a risk-benefit assessment in which the impact of managerial factors outside the scientific context might be determinant.

In this system, the role of the probabilistic modelling (and the figure of the 'risk assessor' or 'risk modeler'), is crucial and explicated in the risk assessment module where a transparent representation of the biological dynamics of the real world are reproduced and used to estimate the risks, and eventually, the effects of mitigation strategies or control measures.

The studies reported in chapter 4 and 5, although very different in the approach, are both clear and complete examples of how those models find their practical application in the field of the food safety management

systems.

In fact, those models, not only provided scientific-based information to take practical decisions, but also gave to decision-maker the possibility to consider both the relative weight of the factors involved and the impact that the variability and the uncertainty surrounding each of them has on the final estimate. This second opportunity is particularly relevant even for the scientific community; in fact, the identification of the inputs characterized by the major 'lack of knowledge' might be helpful to drive the efforts on targeted objectives.

With particular reference to the model's structure, qualitative and quantitative models are at their basis, a simplified description of the complexity of the biological dynamics and the role of the modeller is to integrate the information from different field into a mathematical model with the main objective to represent what happens in reality as correctly as possible.

Intricate dynamics are a characteristic of the biological systems and the attempt to translate them into equations might easily lead to very complicate models; it should be considered that the level of complexity of a model is not necessarily proportional to the quality of the results. Analysis that are so complex that transparency is lost, or make wide use of vague or implicit assumptions are clearly not any better than simple models. As demonstrated by the study in chapter 3, an implicit assumption in a single step is enough to lead to misleading results or alarmistic scenarios. Therefore, is important for a model to be simple (but

not simplistic).

Usually, the extent and the depth of the risk assessment model depends on a number of factors such as the time constraints on responding to the issue and the availability of resources and data. At this respect, beside the practical value of the methodological approaches proposed, the study reported in chapter 2 is an example of how, the availability of data, time and resources can lead to extremely deep and detailed models. Moreover, as a quantitative model covering the whole food chain, this study also points out how important is the collaboration among experts from different field of research. That work required the inclusion of so many biological aspects that the model could not have been implemented without the contribution of co-authors with different expertise (i.e. microbiologist, epidemiologist, and geneticists).

Personal Remarks

The projects presented in this thesis have all requested since the very beginning a constant dedication to the topic and the understanding of -sometimes- not very friendly concepts.

As any other researcher, even the risk modeler must be a lifelong learner, but the good news for me, is that in these years, more importantly than the gratification of having provided works of scientific interest, is the consciousness of having found something that I really love to do.