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RISK ASSESSMENT TO IMPROVE FOOD SAFETY ALONG DIFFERENT FOOD CHAINS

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List of abbreviations

AC: Average Portion Size
ADI: Acceptable Daily Intake
Af: Accuracy Factor
AFB1: Aflatoxin B1
AFM1: Aflatoxin M1
AL: Attention Limit
ATCC: American Type Culture Collection
AU: Analytical Units
a_w: Water Activity
Bf: Bias Factor
$BMDL_{10}$: Benchmark Dose Level 10
CDC: Center for Disease Control
CFU: Colony Forming Units
CRA: Chemical Risk Assessment
DALY: Disability-adjusted Life Years
EA: Exposure Assessment
EC: European Commission
EDI: Estimated Daily Intake
EFSA: European Food Safety Agency
EOW: Electrolized Oxidizing Water
EU: European Union
EW: Electrolized Water
FAO: Food and Agriculture Organization
FBO: Food Business Operator
FDA: Food and Drug Administration
HALY: Health-Adjusted Life Years
HC: Hazard Characterization
HCC: fraction of hepatocarcinoma cases
HI: Hazard Identification
HI: Hazard Index
HPLC: High Pressure Liquid Chromatography
HPP: High Pressure Processing
IARC: International Agency for Research on Cancer
ID50: Infectious dose 50
IPCS: international program on chemical safety
ISO: International Organization for Standardization
LAB: Lactic Acid Bacteria
LCI: Liver Cancer Incidence
LOQ: Limit of Quantification
MAP: modified atmosphere packaging
MoE: margin of exposure
MPRM: Modular Process Risk Model
MRA: Microbial Risk Assessment
MS: member states
ORP: Oxidation-Reduction Potential
Pill: Probability of developing serious Illness
PM: Predictive Microbiology
PRM: Process Risk Model
QALY: Quality-Adjusted Life Years
QCRA: Quantitative Chemical Risk Assessment
QMRA: Quantitative Microbial Risk Assessment
QRA: Quantitative Risk Assessment
RA: Risk Assessment
RC: Risk Characterization
REW: Electrolized Reducing Water
RTE: Ready to Eat
SAEW: Slightly Acidic Electrolyzed Water

SD: Standard Deviation

UV: Ultraviolet

WHO: World Health Organization

WM: weighted mean

WTP: Willingness to Pay

Abstract

The exponential expansion of the world population and its demand for food, combined with climate changes and the transformation of the geopolitical situation, poses under great pressure the food chain. Intrinsic in this context is the issue of providing not only enough food to fulfill rising demand, but also safe and nutritious food that meets regulations in force as well as consumers' health standards. Every year, an estimated 600 million people get ill after eating contaminated food, and 420,000 die. In this context, Risk Analysis is used to produce an estimate of the risks to human health and to identify and implement effective risk-control measures. It can be used to support and improve standard development, as well as address food safety challenges caused by developing hazards or breakdowns in food control systems.

The main aims of this PhD work are 1) describe how quantitative risk assessment (QRA) is used to evaluate the risk for consumers health, due to microbiological and chemical hazards, connected to foods of animal origin; 2) address the methodology to obtain reliable models to apply in quantitative microbial risk assessment (QMRA), from data collection to model development; and 3) evaluate solutions to mitigate the risk for consumers.

The application of a quantitative chemical risk assessment (QCRA) to the Italian milk industry allowed the assessment firstly of the exposure of the consumers to Aflatoxin M1 through the analysis of consumption data and concentrations of the contaminant in milk, secondly of the impact of this exposure on different population categories, and finally to numerically evaluate the difference in efficacy of two risk-mitigation strategies applied by the industry to embrace consumer safety. More in detail, in Italy, an 'attention limit' of 40 ng kg^{-1} has been established in 2013 for aflatoxin M1, while a more stringent attention limit of 30 ng kg^{-1} was set voluntarily by different regions in the following years, both attention limits were associated with strategies to limit the presence of Aflatoxin M1 in milk.

The results highlighted how infants and toddlers are the most sensitive categories of the population due to the high milk consumption compared to their body weight, and how more stringent sampling plans, based on the actual risk, were able to reduce the risk for consumers to almost below the concern limits.

Along with this, the application of a QMRA to Spanish artisanal goat milk fresh cheeses evidenced how the contamination of this product with *Listeria monocytogenes* may generate a risk for the consumers, especially high-risk consumers, given the ability to grow even at low temperatures. Moreover, since these products are usually made of pasteurized milk, and the contamination is mainly due to post-pasteurization stages, the good manufacturing practices and the control of the productive environment showed to be fundamental to decrease the risk by reducing the contamination with the pathogen. Furthermore, was evaluated the impact of two risk-mitigation actions, i.e., intervening with a reduction of the shelf life and of domestic refrigerators temperature, with both solutions that proved to be effective in reducing the risk of listeriosis for consumers.

Then, a description of the most applied protocols for data generation, and specifically for predictive model development, was provided with the aim to expand the availability of information helpful to the process just mentioned. The standardization of methodologies for generating data would benefit the predictive microbiology community, by increasing transparency and reproducibility and, in return, the whole food chain community by providing the means to better QMRA and risk management. In addition, the development of a model describing the fate of *Salmonella* spp. in Italian *salami* during the production process and high-pressure processing (HPP) was described. A linear regression model was used to describe the decay of the microorganism during the production process. Furthermore, compliance with the 5-log reduction policy necessary to export to the U.S. was assessed for the process combined with HPP.

The contamination of the producing environment may generate risks for the consumers, and the control of this environment, through cleaning and good manufacturing practices, has proven to be fundamental to reduce the risks. Alkaline electrolyzed water, as low environmental impact and safe

for the user solution, was evaluated for its use in the food industry to reduce microbial loads on working surfaces. The results evidenced its effectiveness in reducing the contamination of food-contact surfaces, with different foodborne pathogens, prospecting a potential use, after more advanced trials, in the food industry as solution to reduce microbiological risks.

To conclude, this PhD thesis showed the relevance of QRA and, in its framework, but not exclusively, of predictive microbiology and of recently developed technologies to ensure food safety on a more integrated way. Further developments of this work may lead to improvements in the presented QRAs through the filling of data gaps, the development of better models and the inclusion of new risk-mitigation strategies.

Introduction

The exponential expansion of the world population and its demand for food, combined with climate changes and the transformation of the geopolitical situation, poses under great pressure the food industry, its stakeholders, and policymakers. Intrinsic in this context is the issue of providing not only enough food to fulfill rising demand but also safe and nutritious food that meets regulations in force as well as consumers' health standards. Every year, an estimated 600 million people, about one in every ten people in the world, get ill after eating contaminated food, and 420,000 die, resulting in the loss of 33 million healthy life years (disability-adjusted life year, DALYs) and hundreds of billions of dollars in medical expenses (1). Foodborne illnesses are usually infectious or toxic and caused by bacteria, viruses, parasites, or chemical substances entering the body through contaminated food. In 2021, 4,005 foodborne outbreaks, 32,543 cases of illness, 2,495 hospitalizations and 31 deaths were reported in the EU. Overall bacteria accounted for 28.5% of strong-evidence foodborne outbreaks, with *Salmonella* spp. being the most frequently identified (19.3 %) followed by *Campylobacter* spp. (6.2 %), even if *Listeria monocytogenes* had a relatively low identification rate (0.6 %), this was the highest number of outbreaks reported since EFSA started collecting data, and furthermore, was the causative agent with the highest number of deaths (12) (2). Bacterial toxins (17.0 %), viruses (6.8 %) parasites (0.2 %), were less frequently connected to foodborne outbreaks, with lower hospitalization and fatality rates. With 31 deaths in member states (MS) and 2 deaths in non-MS, foodborne outbreaks in Europe resulted in a significant mortality toll, *L. monocytogenes* was linked to 12 (36.4%) fatal cases, *Campylobacter* spp. to 6 (18.2%), *Salmonella* spp. and *Cronobacter sakazakii* to 1 each, bacterial toxins to 7, viruses to 1 and unknown agents to 3 (data for MS). Composite foods were associated with the highest number of illnesses (39.3%) in food outbreaks and were carrier of a wide range of pathogens, moreover, domestic consumption was the main source of outbreaks followed by public catering and restaurants (2). Food of animal origin (meat, fish, diary, and derived products, etc.) were involved in most of the strong-evidence outbreaks (accounting for 56.9%), more in detail fish and fish product accounted for 55/355 strong-evidence outbreaks, meat and meat product for

77/355, eggs and derived products for 42/355 and diary for 28/355 (data for MS) (3). As found by WHO (4), the highest impact on global public health is related to diarrhea agents (*Campylobacter* spp. and Norovirus, etc.), anyway a high burden on public health is associated with *Salmonella* spp., responsible for the highest number of hospitalizations and outbreaks, and with *L. monocytogenes*, associated with the most severe illnesses (5). In the context of this work, given the recent outbreaks and the relevance of *L. monocytogenes* in RTE cheeses was chosen to investigate the risk associated with this type of products, specifically artisanal fresh goat cheeses. Furthermore, the high number of cases and hospitalizations due to *Salmonella* spp. combined with its relative high prevalence in pork meat and its ability to survive in fermented products lead to the choice of investigating its fate in typical Italian *Salami*.

Chemical hazards in foods occasionally cause acute illnesses, and some food additives, residues of pesticides and veterinary drugs, and environmental contaminants may pose risks of long-term adverse effects on public health (6), an interesting example is given by mycotoxins. Mycotoxins are toxic compounds that are naturally produced by certain types of moulds which grow on numerous foodstuffs such as cereals, dried fruits, nuts, and spices. Of the several hundred mycotoxins identified so far, about a dozen have gained the most attention due to their severe effects on human health and their occurrences in food. Aflatoxins are among the most toxic mycotoxins, generated by moulds (*Aspergillus flavus*, *Aspergillus parasiticus and Aspergillus nominus*) thriving in soil, rotting plants, hay, and cereals, and found especially in areas with hot and humid climates. These specific compounds are of increasing interest due to climate change, which is expected to have an impact on their presence in food in Europe. Aflatoxin B1 is the most prevalent aflatoxin found in food and is one of the most potent genotoxic and carcinogenic aflatoxins. In humans and animals, aflatoxin M1 is the main metabolite of aflatoxin B1, and it may be detected in milk from animals fed aflatoxin B1 contaminated diet (7,8). Given what has just been reported, in chapter 1 of this thesis the combination aflatoxin M1/milk was chosen to conduct a quantitative risk assessment.

In this context falls one of the general principles of food law established by Regulation (EC) 178/2002 (9) “In order to achieve the general objective of a high level of protection of human health and life, food law shall be based on risk analysis except where this is not appropriate to the circumstances or the nature of the measure”. Risk analysis is used to produce an estimate of the risks to human health, to identify and implement effective risk-control measures, and to communicate with stakeholders about the risks and measures implemented. It can be used to support and improve standard development, as well as to address food safety challenges caused by developing hazards or breakdowns in food control systems. It gives food safety authorities the knowledge and evidence they need to make informed decisions, leading to better food safety outcomes and public health improvements. Additionally, undertaking a risk analysis allows authorities to identify all the points of control throughout the food chain where measures could be implemented, to weigh the costs and advantages of multiple alternatives, and to decide the most effective ones (6). Risk Analysis is defined in the Codex Alimentarius as “a process consisting of three components: risk assessment, risk management, and risk communication” (10). The first component in risk analysis is to identify risks connected with food safety (risk assessment), this is done following a scientifically based process consisting of four steps: (i) hazard identification, (ii) hazard characterization, (iii) exposure assessment, and (iv) risk characterization. The second element in risk analysis is risk management, the process of weighing policy alternatives, in consultation with all interested parties, considering risk assessment and other factors relevant to the health protection of consumers and for the promotion of fair-trade practices, and, if needed, selecting appropriate prevention and control options. The last part of risk analysis is risk communication, the interactive exchange of information and opinions throughout the risk analysis process concerning risk, risk-related factors, and risk perceptions, among risk assessors, risk managers, consumers, industry, the academic community, and other interested parties, including the explanation of risk assessment findings and the basis of risk management decisions (10).

For the purpose of this thesis, just the concepts related to risk assessment will be deepened, being this the major scientific component of risk analysis.

Risk assessment

The key scientific component of the risk analysis process is risk assessment, which is the qualitative and/or quantitative evaluation of the adverse effects associated with chemical, physical, and biological factors that may be present in foods (10). The structure of risk assessment is based on four key elements (figure 1): (i) hazard identification (HI), (ii) hazard characterization (HC), (iii) exposure assessment (EA), and (iv) risk characterization (RC); even if the same structure is used there are some differences between microbial risk assessment (MRA) and chemical risk assessment (CRA), mainly a matter of definitions since the MRA is defined by the *Codex Alimentarius* (10) and the CRA by the international Program on Chemical Safety (IPCS) (11). Anyway, the scientific rationale for CRA is somewhat different from that for biological hazards, since adverse health effects are usually predicted for long-term exposure to chemicals, whereas biological hazards are generally assessed in terms of a single exposure and an acute health risk. Moreover, the level of hazard present in a food after the point of introduction, which is usually the raw food or ingredients, often does not significantly change in CRA, differently from MRA in which the hazard can enter at many points of the food chain and vary in concentration and prevalence after the contamination (6).

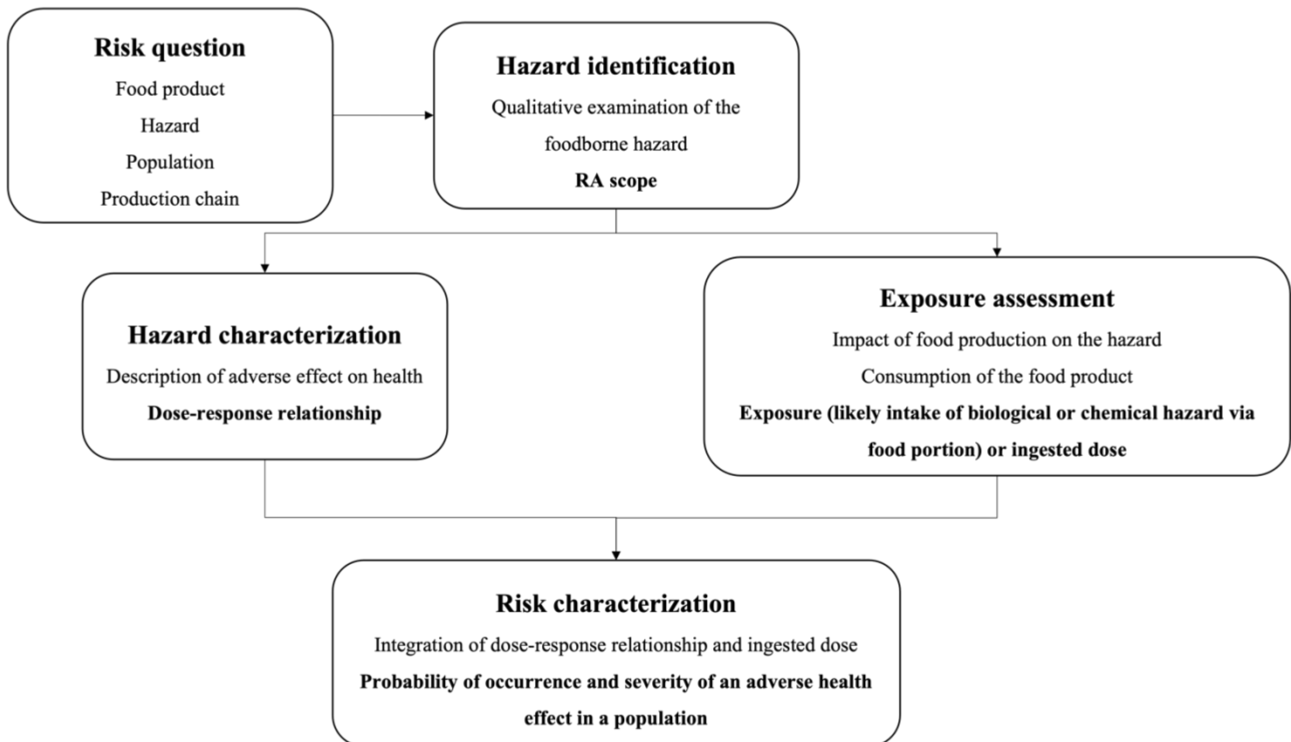

Figure 1: The main outputs (in bold) and the type of information described in each of the four components of a risk assessment (RA)

Hazard identification

HI is conventionally the first step of a risk assessment; its purpose is to identify hazards that are a cause of adverse health outcomes and whether this potential hazard is realistic for the food product of interest. More practically, HI is largely a qualitative examination of the foodborne hazard and associated potential adverse health outcomes due to the interaction between the hazard, the food, and the host. In the case of the MRA, HI includes information about invasiveness, virulence, pathogenicity, natural reservoir, transmissibility, and resistance to environmental factors (12), whereas in the case of CRA it describes the nature and the type of effects that could be caused by the hazard and identifies the affected target organs or target tissues as well as the circumstances under which the effects may be expressed (13). The microbiological hazard's survival and persistence in the food may be influenced by the physical and chemical characteristics of the food matrix. In addition to the hazard's growth, inactivation, and survival features, the exposure assessment may provide

observations on the food's attributes. For instance, high fat content in food can shield Salmonella from thermal inactivation (14, 15). Microbial hazards can be identified in numerous ways for risk assessment. Data on the presence and characteristics of microorganisms along the farm to fork supply chain can be found in published studies, clinical and epidemiological studies, surveillance and outbreak investigations, or reports of foodborne diseases (16). These data can be gathered via scientific literature, the food business databases, government agencies, relevant international organizations, and expert consultation (16)Decision support tools can be used in an organized way to identify microbiological hazards, and they also have the benefit of allowing the system to be automatically updated when new information becomes available. Currently available tools are Risk Ranger, sQMRA (semi-quantitative risk-ranking framework prototype), the FDA's fresh produce risk-ranking tool, and FDA-iRISK® (16). Other information usually included in the HI are impact on sensitive populations, acuteness of the illness (acute versus chronic disease), and other eventual complications.

Hazard characterization

The HC describes the adverse effects that may result from ingestion of a hazard, whether that is a microorganism or its toxin or a chemical compound, this element combined with the HI forms the hazard assessment of the RA. Where possible the hazard characterization should include an indication, for the population of interest, of the probability to cause an adverse health effect as a function of dose, in the case of MRA this usually is expressed as a dose-response relationship or using the Median Dose or Infectious Dose 50 (ID50), the dose at which 50 percent of consumers become infected, or ill (12). For CRA HC is important to identify the type of effect, which can be with a threshold under which there is no observed effect or without a threshold (mutagenic, genotoxic, and carcinogenic effects), in which case is considered just the probability or severity depending on the dose. In the first case, the most common metrics used to express the dose-response relationships are the Acceptable Daily Intake (ADI) for additives and pesticide residues or the Tolerable Daily Intake (TDI) for contaminants, exposures below these thresholds are usually considered safe (13).

In general, for well-established hazards (e.g., *Salmonella* spp., *L. monocytogenes*, Aflatoxins) this part of the RA may be well established and not needing deep revisions on the other hand for emerging hazards due to lack of information HC may be more uncertain and be more dependent on frequent update.

Exposure assessment

Defined as "the qualitative and/or quantitative evaluation of the likely intake of biological, chemical, and physical agents via food as well as exposures from other sources if relevant" (10), usually it is part of a RA but can also be a stand-alone process in the case, for example, the dose-response relationships are not well established or it is developed by food manufacturers aiming to assess the safety of their food products. Quantitative exposure assessments are mathematical evaluations of numerical data, whereas qualitative exposure assessments are descriptive or categorical treatments of information; even if qualitative assessments lack numerical precision, they are a valuable tool for decision-making, for example determining if the risk is significant enough to warrant a more detailed analysis (14).

The main goal of EA is to estimate the exposure of a certain population to a certain hazard, focusing on a certain age range or geographic area, for example, this leads to the prerequisite of developing a RA, answering the risk questions – which population? which food product? microbial or chemical hazard? Once clarified the objective of the EA, is to be identified every factor that has an impact on the consumers' exposure: frequency and levels of contamination with the hazard, the potential for microbial growth, inactivation during cooking (or other processes), meal size, etc. Furthermore, it is relevant to define the exposure pathways, these could be from production to consumption or maybe just from retail to consumers with a level of detail depending on the goal of the process (12).

Due to a lack of data on levels of hazard at consumption, usually EA rely on models, encompassing knowledge of the factors and their interactions that influence the number and distribution of hazards in foods, to estimate exposure at intake. In the EA context, the models synthesize data and knowledge derived from observations to infer what might happen in different conditions. Quantitative models can be categorized as deterministic or stochastic regarding how input variables are handled (14). Deterministic models use point estimates to describe variables and usually, just individual scenarios are analyzed, stochastic, or probabilistic, models on the other hand use probability distributions to

describe variables evaluating many different scenarios at the same time, and generally use Monte Carlo simulation for this purpose.

In both cases the quality of the data used as inputs is of uttermost importance, usually data to conduct EA, and more in general RA, can be retrieved from scientific literature or from expert advice but recently various software and database providing such information have been released to facilitate this task. An example is the EFSA Comprehensive European Food Consumption Database (18) which contains detailed information about food consumption for European countries, or two recently released web databases: Pathogens in Foods (19) reporting data on microbial responses in foods and on occurrence data of the most important biological hazards, and D-database (20) providing data on microbial inactivation in food.

Central elements in the development of EA of QMRA are predictive microbiology (PM) models, useful tools to assess the growth, survival or death (or time to toxin production) of microorganisms as a function of the food and environmental conditions encountered from raw materials to the food consumed, particularly important when making quantitative estimates. For their implementation into QMRA, PM models can be retrieved from various sources: from scientific literature, including previously developed QMRA, from experiments and surveys, and from databases or modelling tools. Examples of databases are the previously cited Pathogens in Foods (19) and D-database (20), but also others like open FSMR (21), a repository of PM models freely available online. PM models are also usually available in prediction software tools: e.g. MicroHibro (22), Sym'-Previus (23), Predicere Possum (24), ComBase Premium (25). Some tools like MicroHibro or FDA-iRisk (26), already offer a user-friendly interface to perform QMRA in which PM models, but also dose-response models, are already implemented and ready to use.

The term "conceptual model" refers to an understanding of the paths by which the population of interest is exposed to the hazard of concern, including all the components and their interactions that

determine the probability and level of exposure, it may be expressed in text or diagrams, or as a mathematical model (12). In MRA, an approach to the conceptual model is represented by the Process Risk Model (PRM), introduced by Cassin et al. in 1998 (15) and consists of breaking down a certain food chain or process into discrete events or steps that may be characterized by sequentially and linearly adding microbial predictive models to produce a final estimation. The Modular Process Risk Model (MPRM) (16) is an expansion of the PRM proposing a structured process to describe the fate of microorganisms along the food chain. MPRM is accomplished by applying different predictive modeling approaches to some basic microbial processes: growth, inactivation, mixing, partitioning, removal, and transfer (or cross-contamination), the level of complexity of the modeling applied is usually related to the existence of information on specific steps and the complexity of the process, an example is reported in figure 2. Conceptual modeling is applied also in CRA, but differently from MRA, dietary exposure assessment takes into consideration mainly the consumed quantities of foods that may contain the chemical and the levels and frequency of the chemical in those foods (13). The result of the EA is the quantification, expressed as likelihood and level of the pathogen in the food portion or as ingested dose, of the exposure of a population to a certain hazard, based on data input such as frequency and levels of contamination, potential microbial growth or inactivation, consumption data.

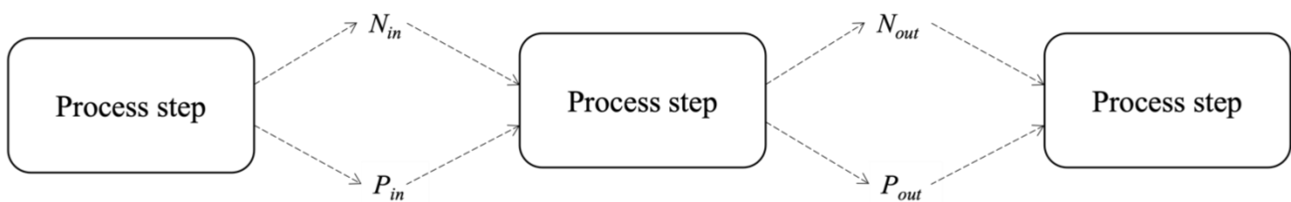

Figure 2: Example of a Modular Process Risk Model with several process steps and relative changes in number of microorganisms per unit (N) and prevalence (P).

Risk characterization

The last step of RA is RC which integrates the outputs of the three previous steps to estimate the level of risk, this estimate can later be used to address questions posed by risk managers. In comparison to

qualitative or semi-quantitative RC, quantitative RC addresses risk management issues at a finer level of detail and makes it easier to compare risks and risk management choices with higher accuracy. Quantitative evaluation might be stochastic or deterministic. Literature on CRA, contains more examples of deterministic QRA, while most of the research, guidelines, and most well-known instances of QMRA are stochastic.

The QRA combine the two quantitative components of risk: the measure of exposure and the severity of health effect. The measure of exposure is the result of the exposure assessment, usually expressed as the risk of an outcome (e.g. illness per portion), the measure of health effect, on the other hand, can be expressed in different ways: i) the number of adverse outcomes, ii) health adjusted life years, iii) monetary risk metrics (12).

The number of adverse outcomes (e.g. illnesses, hospitalizations, deaths) is the simplest metric that can be used in risk assessment and can be estimated “per serving” or “per annum” and standardized for population size (12).

Health-adjusted life years can characterize and compare the health effect of diverse risks and health outcomes. These are particularly useful when a risk assessment is considering different pathogens, in a case, for example, where is possible to take management options just for one pathogen and it is necessary to evaluate the differences in severity between those pathogens. The most common metrics are Health-Adjusted Life Years (HALYs), with DALYs and QALYs as the most representative. The DALY (Disability Adjusted Life Years) method presumes perfect health for the entire life span, therefore measures the loss due to ill health, and it is the summation of Years of Life Lost (YLL) and Years Lived with Disability (YLD). The QALY concept is analogous, but measures the increase in quality of life, and its duration, as a result of an intervention (17).

Foodborne and zoonotic diseases' effects on public health can also be expressed in monetary terms. There are three main methods: i) the human capital approach, which evaluates an individual's contribution to the market; ii) the cost of illness; iii) revealed and stated preferences of willingness to pay (WTP) (18).

At the end of this step, the result is the probability of occurrence and severity of known or potential adverse health effects in each population over a given period expressed using one of the above-described metrics (14).

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Objectives

The main aims of this PhD work are 1) describe how quantitative risk assessment (QRA) is used to evaluate the risk for consumers health, due to microbiological and chemical hazards, connected to different food products, with a focus on foods of animal origin; 2) address the process necessaire to obtain reliable models to apply in QMRA, from data collection to model development; and 3) evaluate new solutions to mitigate the risk for consumers along the food chains.

This PhD thesis will be divided in chapters as follows:

the first two chapters will deal with the first objective showing two application of quantitative risk assessment to evaluate and mitigate the risk, due to both chemical and microbiological hazards, connected with consumption of foods of animal origin.

- **Chapter 1**: "Evaluation of the influence of risk management solutions on exposure of a population to chemical hazards" describe an example of a QCRA, regarding aflatoxins M1 in cows' milk, collected in various Italian regions in a twelve-year period to evaluate the impact of the application of official and voluntary attention limits on the exposure of the consumers.

- **Chapter 2**: "Quantitative risk assessment of *Listeria monocytogenes* in Spanish fresh goat milk cheeses" present a QMRA, analyzing the risk connected with consumption of artisanal fresh cheese made with goat milk produced in the Spanish region of Andalusia and evaluating different scenarios and risk-mitigation actions.

The third and fourth chapters will focus on the second objective, describing the process to obtain reliable microbial predictive models to be applied in QMRA.

- **Chapter 3**: "Methodology for obtaining robust data of microbial responses in food" describe the methodology to collect data on microbial responses in food, fundamental for developing solid microbial predictive models to be used in QMRA.

- **Chapter 4**: “Developing and update of a predictive model” report the update of a model developed to describe the reduction of *Salmonella* spp. during the production process and high-pressure processing of Italian *salami*.

The last chapter will finally address the last objective, which will also partially be covered in chapter 1 and 2, the evaluation of solutions to mitigate the risk for consumers along the food chain.

- **Chapter 5**: “Novel solutions to control environmental contamination and to reduce microbiological risks” describe the evaluation of alkaline electrolyzed water as a tool for reducing the microbial load on surfaces intended to come into contact with food (for example food elaboration surfaces), considering the importance of sanitization methods as factors for environmental impact on exposure level to pathogens for the consumers.

Chapter 1

Evaluation of the influence of risk management solutions on exposure of a population to chemical hazards

This first chapter deals with the main aim of this PhD work describing the application of QRA to the evaluation of risk for consumers connected with consumption of milk in various Italian regions, specifically for a chemical hazard, aflatoxin M1 (AFM1). Furthermore, the outputs of this QCRA were used to evaluate risk management solution applied by the industry to mitigate the risk for consumers addressing also objective 3 described above.

Data relative to concentration of AFM1 in milk for a twelve-year period were obtained from six milk industries and statistical analysis was conducted to assess concentrations changes over time. Additionally, variation among processing plants which implemented different risk management solutions were assessed. In detail, the limit set for AFM1 by Regulation 1881/2006 (15) for milk is 50 ng kg^{-1}, nevertheless, in 2013 the Italian Ministry of Health issued a note defining an ‘attention limit’ (AL) of 40 ng kg^{-1} to increase the control over this hazard, while some producers, in the framework of their self-control plans, implemented an even more stringent AL of 30 ng kg^{-1}, both Als served as early detection of the problem to rapidly activate various mitigation strategies along the production chain to reduce the AFM1 contamination.

In this context the QCRA was used to evaluate if the implementation of a more stringent attention limit produced a significant reduction of AFM1 concentrations and thus in the exposure and in the risk for the consumers due to consumption of milk.

Overall, a total of 67,944 milk samples were taken into account, and the percentage of samples that were above the EU compliance limit ranged from 6.7% to 0% with a decreasing trend from 2004 to 2019. The same trend was also seen for the percentage of samples that were above the voluntary AL of 40 ng kg-1, which ranged from 35.3% to 0.3%, and the voluntary AL of 30 ng kg-1, which ranged from 35.3% to 0.3%. Concerning the QCRA, the estimated daily intake (EDI), the hazard index (HI),

and liver cancer incidence (LCI) values were calculated for the 2013-2019 period using AFM1 values from all the plants but dichotomized in two groups, one with a 40 ng kg^{-1} AL and one with a 30 ng kg^{-1} AL. Among the different population groups, infants and toddlers had the highest mean EDI value while adults the lowest. The same pattern was followed by the HI for which also infants and toddlers had the highest values and were the only groups to overtake the concern limit of 1. As the precedent indexes also the LCI showed the highest values in infant and toddlers indicating these two groups as the most at risk. The values of LCI, attributable to the intake of AFM1, ranged, respectively, from 0.0003 to 0.0038 and from 0.0004 to 0.0048 per 100,000 people in the 30 and 40 ng kg^{-1} ALs. Regardless of the attention limit used, infants and toddlers are confirmed to be more exposed than older consumers due to the relatively significant milk intake compared to their body weights. Nonetheless, the implementation of a more stringent attention limit, yield a decrease in EDI, HI, and LCI, which, even if not statistically significant, lowered the HI value for toddler to almost below (1.03) the concern limit and the LCI for both the more at risk groups by 0.001 per 100,000 people, showing its efficacy as strategy to reduce the risk related to AFM1.

This work shows how application of QRA is incredibly useful also when used to perform a retrospective analysis of the effectiveness of different risk reduction strategy applied, and how the information produced from such analysis can be extremely valuable for the risk manager, allowing him for example to remove high-cost but low-effect risk reducing measures or to implement new, more effective ones.

A detailed description of the methodology, results, discussion, and conclusion of this evaluation of the influence of risk management solutions on exposure of a population to chemical hazards is reported in the full-text paper[1] attached below.

[1] Notice: the following chapter represents the pre-print author's version of a work that has been submitted for publication, Federica Giacometti, Federica Savini, Valentina Indio, Andelka Bacak, Alessandra Canever, Paolo Bonilauri, Alessandra De Cesare and Andrea Serraino contributed in various forms to its development. The chapter

has been edited according to the formatting used for the remaining dissertation. Changes resulting from the publishing process, such as editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes could be made to this work before its acceptance for publication.

Introduction

Aflatoxins are bisfuranocoumarin compounds produced primarily by toxigenic strains of the fungi *Aspergillus flavus* and *Aspergillus parasiticus*, but also from *A. minisclerotigenes*, *A. korhogoensis*, *A. aflatoxiformans*, *A. texensis*, *A.novoparasiticus* and *A. arachidicola.* Their presence has been mainly reported in tropical and subtropical regions but is nowadays becoming an unavoidable problem due to climate change and the growing occurrence of hot and drought seasons in several regions of Europe. An increase is also evidenced in case the of bad agricultural practices (1) and in areas with a hot and humid climate (2). In addition, as an impact of climate change, the infected areas may further increase (3). Toxigenic strains of *Aspergillus* spp. are mainly responsible for the production of aflatoxins in many feed materials, causing the contamination of milk of lactating animals that are fed with the involved feedstuff (2, 4–6). In particular, among the different aflatoxins (B1, B2, G1, G2), the M1 hydroxylated metabolite (AFM1) of aflatoxin B1 (AFB1) is the most commonly occurring in milk, appearing after 2 or 3 days from the ingestion and clearing after 5-7 days depending on the amount and duration of the consumption (7). Procedures such as pasteurization or sterilization cannot eliminate or even vary the concentration of the AFM1 once the milk is contaminated, leading to withdrawal of consignments once the legal limit is exceeded.

Only safe food should be placed on the market (8) and, therefore, food safety could be considered one of the major risks for agribusiness firms, which have the social responsibility of ensuring food safety by following the necessary procedures established by the Food Safety Authorities as well as should incorporate food safety measures beyond baseline requirements demanded by regulation or governmental policy (9). Aflatoxins are genotoxic and carcinogenic compounds, specifically AFM1 is classified into Group 1 of carcinogenic substances for humans (IARC), with suggested exposure levels be kept as low as reasonably achievable. The exposure to AFM1 compromises both the health of animals and humans (10) imposing health risk for the consumers. Major concern is for children who are more susceptible to the toxic effects of aflatoxins, due to their underdeveloped metabolic and immune system.

In a previous study, the risk from exposure to AFM1 found in milk from April 2013 to December 2018 in the framework of a self-control plan of six milk processing plants in Italy as well as the risk characterization were calculated in terms of Estimated Daily Intake (EDI), the Hazard Index (HI), and the fraction of hepatocarcinoma cases (HCC) in different population groups.

Since the contamination of milk by mycotoxins poses issues not only regarding food safety and public health policies, but also for the economic sphere, encompassing agriculture and international trading, the most heavily regulated among natural toxins are mycotoxins throughout the globe (11).

Initially, in Italy the milk controls were only sporadic and only in the autumn of 2003, following an alarming amount of positivity in the self-control plan, special monitoring plans were coordinated for milk and feed (12). Probably the abnormal AFB1 contamination that occurred in maize grown in Italy, was the consequence of particularly unusual climatic conditions (high temperatures and drought lasting more than four months) that characterised the summer of 2003. Back in those days the Directive 2003/100/EC of 31 October 2003 on undesirable substances in animal feed (13) set limits for AFB1 in terms of mg/kg of feed with a humidity rate of 12%. In milk, a limit of 0.05 μg/kg for AFM1 was earlier set by European Legislation 466/2001/EC (14). The same maximum level is nowadays applied in Europe by Commission Regulation (EC) No 1881/2006 (15) for raw milk, heat-treated milk and milk for the manufacture of milk-based products, while for infant food it is limited to 0.025 μg/kg. Criteria for sampling and analysis of aflatoxins are specified in Commission Regulation (EC) No 401/2006 (16). In addition, specific import conditions have been put in place for certain feed and food commodities from selected third countries related to the presence of aflatoxins (i.e. Commission Regulation (EC) No 669/2009 (17) and Commission Implementing Regulation (EU) No 884/2014 (18)). In parallel to official controls, industries have been applying risk management strategies in order to detect unacceptable levels of contamination in the framework of self-control plans. However, as stated by Trevisani and colleagues (19), this regulation, similarly with other provisions worldwide, does not indicate the frequency of sampling or give an indication for seasonal or regional stratification. Therefore, the frequency of sampling must be evaluated on the

basis of acquired, previous knowledge on the risk for specific aspects. On the national territory, the Italian Ministry of Health issued a note in 2013 (20) defining an ‘attention limit’ (AL) of 40 ng kg^{-1}, to be applied every time that extreme weather conditions are registered. In addition, these guidelines, provide operators in the feed and food sectors with specific operational indications in order to allow the reduction of aflatoxin levels with a holistic approach for the dairy chain, focused not only on the food product but also on feed by means of cleaning techniques or other physical treatment. This because any milk sample with aflatoxin above this limit has to be regarded as suspect and preventive checks and measures at farm level must consequently be performed. Following the note of 2013, Regions are demanded to apply more stringent controls by means of regional plans when needed. In particular, in Calabria region, one dairy plant has applied the most stringent level of 30 ng kg^{-1}. In 2016 another note has been issued by the Italian Ministry of Health (Ministry of Health 2016) in order to declare the need to intensify aflatoxin official control and to underline the obligation of FBO (Food Business Operators). In 2017 also Emilia Romagna Region applied the more stringent AL of 30 ng kg^{-1}.

Based on these not ordinary events, the dairy industry performed several risk reduction strategies based on the specific scenarios observed in the different industries and well-programmed interventions have been defined from every dairy plant in its self-control plan, each year. Ten years have passed from the note of 2013, and what was previously an out of order strategy is now a routinely applied procedure. Thus, further data mining and analysis are needed to both define and update the actual real scenario in Italy for the hazard aflatoxin in milk as well as define appropriate sampling plans for milk and milk products.

This paper presents data on the concentration of AFM1 in milk sampled in 6 Italian dairy plants between 2004 and 2008 and between 2013 and 2019. In addition, a retrospective evaluation was performed to evaluate the effectiveness of the risk reduction strategy performed by these dairy industries and the evolution of AFM1 presence in milk during a long period of time. These data were used to update the information produced by a previous study (21) regarding human exposure and

potential risk of consumers in different age categories. Results allow both to identify potential different exposure and risk scenarios based on different AFM1 contamination data in milk in Italy, and to evaluate the effect of different AFM1 milk monitoring as a result of the implementation of more stringent AL in EDI, HI, and HCC cases reduction.

Materials and Methods

Aflatoxin M1 concentration data collection

The data on AFM1contamination in milk from the self-control plan records of six milk processing plants located in Northern, Central, and Southern Italy, were gathered in the years from 2004 to 2008 and from 2013 to 2019. The dairy plants involved in the study collected altogether almost 465 million liters of milk per year, comprising high quality milk, normal quality milk and organic milk, that were analysed within a self-control plan, following the same protocol. Data comprised a total of 67,944 samples that were tested for AFM1 concentration at arrival to the plant, using the ELISA kit Immunoscreen (Tecna srl, Trieste, Italy), in order to avoid the contamination of huge amount of milk at a later stage tank. The ELISA test was validated within the range of 5–100 ng kg^{-1} (22). Specifically, prior to unloading, milk samples were taken from the compartments of each truck, transporting milk provided from different farms. The procedures performed in the self-control plans and successive revisions by the six dairy plants to control the AFM1 in milk before and after 2013 and also into the several years considered in the study are different but a defined and rigorous framework is the same for all the dairy plants. Briefly, before milk is discharged on every milk truck entering the milk processing plant a bulk milk sample is analyzed with a commercial immunochromatographic rapid test (Charm), detecting AFM1 at or above 25 ng/kg in milk and suitable to indicate the compliance with EU limit of 50 ng/kg. In case the analytical record within the truck exceeds the AL, an ELISA test is further performed to better quantify the concentration of the mycotoxin. The milk can be processed only if the legal limit is not overcome, whereas in case levels result higher than 50 ng kg^{-1}, the milk truck awaits the AFM1 concentration measurement performed by HPLC method for the unloading. Liquid chromatography mass spectrometry and liquid

chromatography-fluorescence detection methods are also used for the determination AFM1 and the reported LOQs are typically between 0.0007 and 0.014 lg/kg. Whenever the analytical results show that AFM1 exceeds the legal limits the milk is discarded as Category 1 material as stated in Article 8 (d) of Regulation EC 1069/2009 (23). In parallel, the FBO must proceed with testing samples collected at charge of milk of every farm in order to identify the dairy farm or farms exceeding the limit, and the Veterinary Competent Authority is informed of the analytical record at the milk processing plant as well as at farms level in accordance with the Italian law (Ministry of Health, 2013). Immediately, FBO must adopt for the food product the procedures laid down in Regulation EC 178/2002 (8), article 9, whereas, at farm level, a supplementary in-depth analysis of the AFM1 level of contamination of milk is performed with programmed checks at fixed frequencies depending on the estimated AFM1 concentration until the full resolution of the non-conformity. Whenever the legal limit is exceeded at farm level, the milk consignment is not performed. The same procedure is applied when the milk exceeds the AL set by the Region.

In addition, following the visit of the veterinarian performed on the same day of the notification, the FBO has also to adopt corrective actions in all the implicated dairy farms. As mitigation measure, the feed provided to the animals is replaced in order to reduce the animal exposure to aflatoxins favoring the use of maize reserves from previous production seasons or changes in the components of the ration, with for example sorghum or other cereals such as barley and wheat. Finally, both cooperative and dairy plants perform further additional analysis by Charm and/or ELISA, with a minimum of twice a month, to test AFM1 concentration of milk of the different dairy farms.

Statistical analysis

Descriptive statistical parameters (mean, standard deviation, median, percentile) were calculated for all the years included in the study. Moreover, the percentages of sample above the EU compliance limit of 50 ng kg^{-1}, the AL levels of 40 ng kg^{-1} and 30 ng kg^{-1} were computed. Additionally a comparison between the AFM1 values of plants with different Als, for the 2013-2019 period, was made to investigate eventual differences. Data are illustrated in Tables 1-2-3-4.

The data were tested for normality using Kolmogorov-Simrnov test and for equality of variance using Levene's test, resulting not normally distributed and with non-equally distributed variances, hence were analysed using Chi-squared test, Mann–Whitney U test, Kruskal-Wallis test and Dunn's multiple comparison test considering significant a $p \leq 0.01$. All statistical analysis was made using *R Studio* (2022.2.3.492), *ggstatplot* (24) and *ggplot2* (25) packages.

Dietary exposure and risk characterization

For the risk assessment, the food consumption data used as well as the information for exposure assessment and hazard assessment were obtained as previously described (21). Briefly, food consumption data were obtained from the Comprehensive Food Consumption Database of EFSA (https://www.efsa.europa.eu/en/data-report/food-consumption-data), containing derived from the Italian National Food Consumption Survey (INRAN-SCAI) conducted in 2005-2006 (26). The exposure assessment is based on the mean "Cattle milk" consumption data of "consumers only" of six population groups: infants (0–0.9 years), toddlers (1–2.9 years), other children (3–9.9 years), adolescents (10–17.9 years), adults (18–64.9 years), elderly (65–74.9), and very elderly (>75).

The estimated daily intakes (EDI: ng kg^{-1} bw day^{-1}) of the population groups were calculated as:

$$EDI = \frac{\Sigma\left[WM_{AFM1}\, concentration\left(\frac{ng}{kg}\right) \times\ AC\left(\frac{kg}{day}\right)\right]}{[mean\ body\ weight\ (kg)]}$$

EDI values were calculated from the weighted mean (WM) AFM1 concentrations unloaded from the tankers in the given period and the average (AC) portion size (consumption data (kg/day), the calculations were carried on separately for plants using the more restrictive AL to assess whether this had an impact on the exposure to AFM1.

To calculate HI, the average EDIs were divided by 0.2 (27), in line with the approach of Serraino and Colleagues (21). $BMDL_{10}$ of AFB1 (870 ng kg−1 bw day−1) was used as a conservative value since no value for AFM1 is available. Margin of exposure (MoE) was calculated as reported by Serraino

(21) as well as risk potency (calculated assuming 2% prevalence of carriers of hepatitis B). The calculation was carried out for the same groups as for the EDI and HI.

Results

AFM1 results in milk

A total of 67,944 milk samples were considered in this study. All the statistics describing the distribution of AFM1 sorted by year and by season, are showed in Tables 1- 4. AFM1 mean values ranged between 25.9 and 7.9 ng kg^{-1} and the median between 24 and 7 ng kg^{-1} indicating a positive skewed distribution which implied a non-normal distribution of the data, as confirmed by the Kolmogorov-Simrnov test. We evidenced significantly ($p \leq 0.01$) different year-to-year variation in AFM1 prevalence and average contamination levels in the analysed milk samples. This result applies to the whole studied period, except for 2005 vs 2008, 2015 vs 2016 and 2018 vs 2019 which were not significantly different. The proportion of samples above the EU compliance limit (i.e., 50 ng kg^{-1}) varied between 6.7% and 0%, with a decreasing trend from year 2004 to year 2019. The same tendency was observed for the proportion of samples above the AL of 40 ng kg^{-1}, ranging from 13.1% to 0.04%, and 30 ng kg^{-1}, ranging from 35.3% to 0.3% (Table 1).

	2004	***2005***	***2006***	***2007***	***2008***	***2013***	***2014***	***2015***	***2016***	***2017***	***2018***	***2019***
Number of samples	5079	4930	5040	4619	4509	3826	9180	6114	6600	7062	6251	4734
Mean	25.94	15.18	13.98	11.41	15.08	17.63	11.21	11.72	12.86	8.84	8.31	7.95
SD	15.10	13.16	12.70	10.95	13.09	9.04	7.46	8.25	8.08	5.02	4.74	4.02
Median	24	12	11	9	13	16	9	9	11	8	7	7
P 0.95	54	35	36	28	32	35	26	29	28	17	17	16
P 0.99	75	62	53	45.82	60.92	46	37.21	40	40	26.39	25	22
Max ng kg $^{-1}$	175	181	228	197	280	50	50	48	50	47	58	48
CM1 ≥ 50 ng kg $^{-1}$ (%)	6.73	1.74	1.27	0.78	1.82	0.18	0.01	0.00	0.05	0.00	0.03	0.00
CM1 ≥ 40 ng kg $^{-1}$ (%)	13.15	3.20	2.88	1.56	3.30	2.59	0.84	1.82	1.08	0.14	0.10	0.04
CM1 ≥ 30 ng kg $^{-1}$ (%)	35.26	9.80	9.50	3.49	6.54	9.88	3.10	4.81	4.17	0.55	0.46	0.27

Note: P, percentile; C_{M1} ≥ 50-40-30 ng kg $^{-1}$(%) is the proportion of consignments above the limit in relation to number of samples.

Table 1: Descriptive statistics for the level of aflatoxin M1 (ng kg^{-1}) sorted by year.

Overall, in the studied period, 0.92% of the samples were above the 50 ng kg^{-1} EU limit, and 2.31% above the 40 ng kg^{-1}, while 6.66% above the 30 ng kg^{-1} ALs (Table 2).

	All data 2004-2018 / 2013-2019	***2004-2008***	***2013-2019***
Number of samples	67944	24177	43767
Mean	12.90	16.45	10.94
SD	10.6	14.05	7.38
Median	10	13	9
P 0.95	32	39	26
P 0.99	48	64	38
Max	280	280	58
CM1 ≥ 50 ng kg $^{-1}$ (%)	0.92	2.52	0.03
CM1 ≥ 40 ng kg $^{-1}$ (%)	2.31	4.93	0.86
CM1 ≥ 30 ng kg $^{-1}$ (%)	6.66	13.27	3

Note: P, percentile; C_{M1} ≥ 50-40-30 ng kg $^{-1}$(%) is the proportion of consignments above the limit in relation to number of samples.

Table 2: Descriptive statistics for the level of aflatoxin M1 (ng kg^{-1}) during 2004-2008/2013-2019.

A total of 36.4% of the samples above the EU compliance limit were detected during the critical season (September to November), with the highest levels of AFM1 detected in September 2006 and September 2008, respectively with a concentration of 228 and 280 ng kg^{-1}. Regarding seasonal variability, the observed AFM1 prevalence has shown an interesting periodic fluctuation over the surveyed period, as shown in Table 3.

	Jan	***Feb***	***Mar***	***Apr***	***May***	***Jun***	***Jul***	***Aug***	***Sep***	***Oct***	***Nov***	***Dec***
Number of samples	4764	4517	5415	5363	5829	5693	6197	6114	5948	6328	6233	5543
Mean	12.66	14.01	12.98	13.84	13.42	13.10	11.89	12.19	14.91	13.46	11.39	11.33
SD	9.57	11.56	10.31	10.47	10.10	9.87	8.64	9.93	14.63	11.63	9.70	8.66
Median	10	11	10	11	10	10	9	9	11	10	9	9
P 0.95	30	38	34	34	33	32	29	31	39	33	28	28
P 0.99	45.37	58	49.86	48.38	47	47	42	46.87	68	48	43	42
Max	185	95	91	105	111	95	105	112	280	247	175	125
CM1 ≥ 50 ng kg⁻¹ (%)	0.63	1.73	1.02	0.91	0.75	0.81	0.36	0.80	2.30	0.92	0.51	0.41
CM1 ≥ 40 ng kg⁻¹ (%)	1.64	3.74	2.53	2.18	1.99	1.88	1.37	2.11	4.69	2.78	1.67	1.28
CM1 ≥ 30 ng kg⁻¹ (%)	5.44	9.14	7.46	8.26	7.27	6.39	4.60	5.92	10.68	7.08	4.36	3.84

Note: P, percentile; C_{M1} ≥ 50-40-30 ng kg^{-1}(%) is the proportion of consignments above the limit in relation to number of samples.

Table 3: Descriptive statistics for the level of aflatoxin M1 (ng kg^{-1}) sorted by month of consignment in the 2013-2019 period.

Although significantly higher ($p \leq 0.01$) values were observed during the fall season (36.6%), the 23.8% and 21% of non-compliant samples (≥ 50 ng kg^{-1}) were observed in spring (March to May) and winter (December to February) respectively (Figure 1).

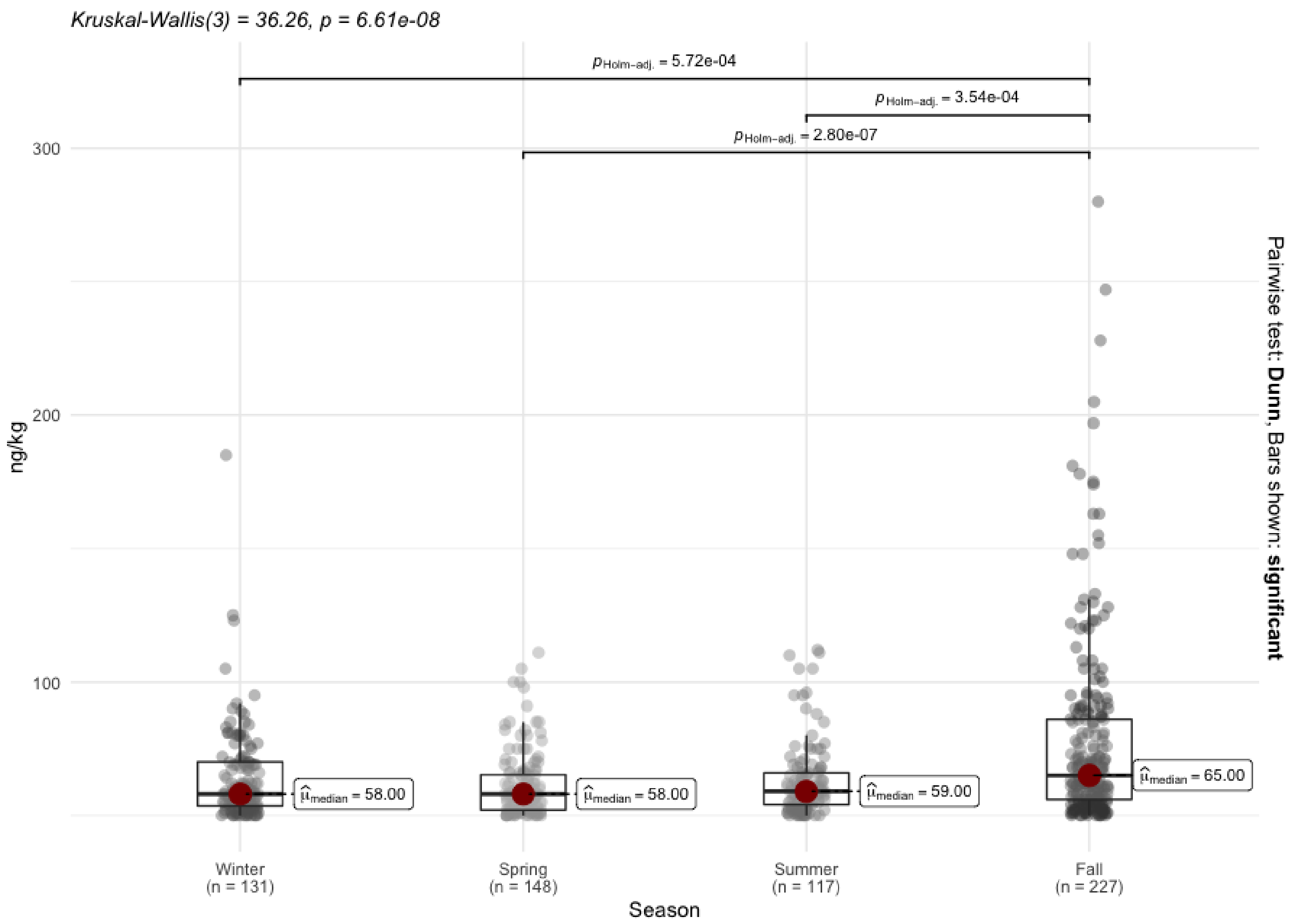

Figure:1 Information about statistics and distribution of samples above the EU 50 ng kg^{-1} limit, grouped by season. In the graph are reported median (μ̂median), number of samples (n), and statistically significant differences between groups (pHolm-adj.).

Almost all non-compliant samples (97.9%) were received between 2004 and 2008. Among them, > 50% of the samples was referred to 2004 (Figure 2) while 2.1% to the period 2013-2019. During the latter, only 13 of the 43,767 samples (i.e., 0.03%) were contaminated with levels above the 50 ng kg^{-1} limit and 376 (0.85%) above the AL of 40 ng kg^{-1}.

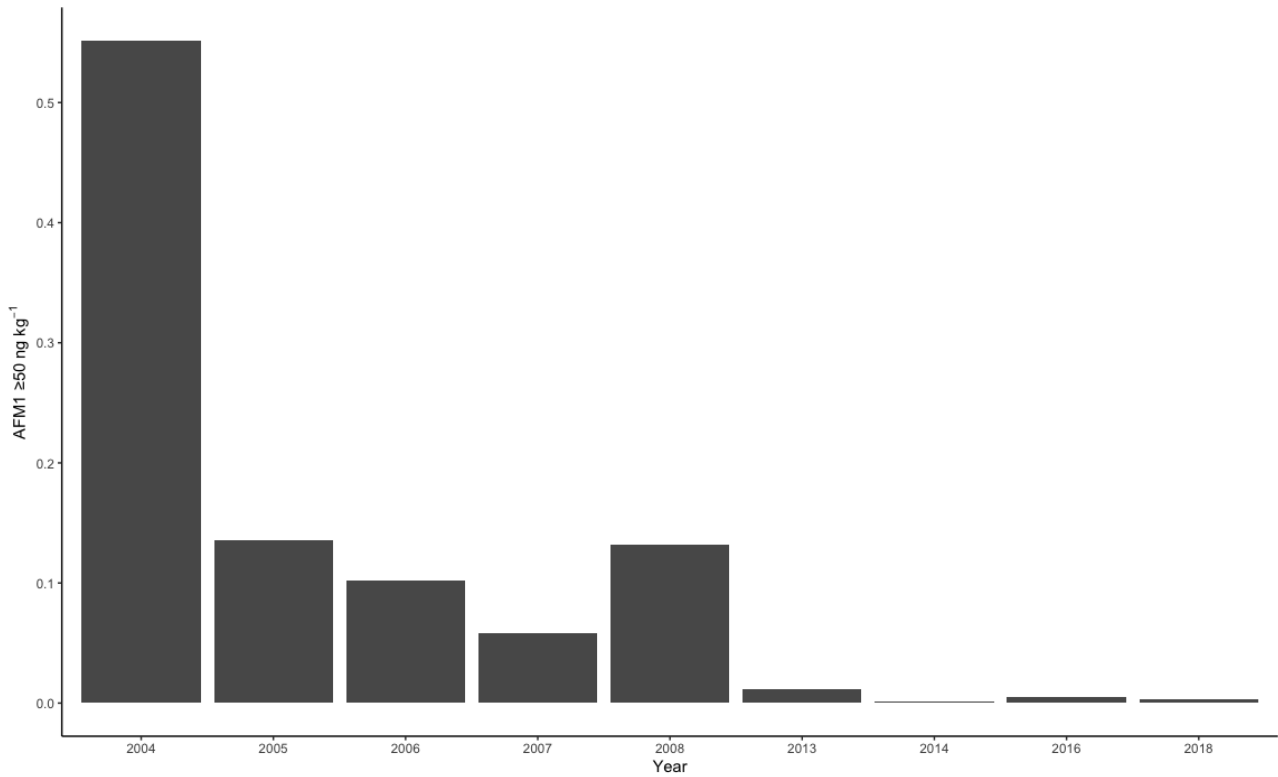

Figure 2: Frequency of samples above the EU 50 ng kg^{-1} limit, grouped by year.

For the 2013-2019 period an overall reduction of AFM1 levels was observed (Table 4). In particular, a statistically significant ($p \leq 0.01$) reduction in the proportion of samples above the 30 and 40 ng kg^{-1} AL limits was observed in plants with a 30 ng kg^{-1} AL compared to plants with 40 ng kg^{-1} AL.

	30 ng kg^{-1}	***40 ng kg^{-1}***
Number of samples	10572	34330
Mean	9.21	11.64
SD	5.44	7.90
Median	8	9
P 0.95	19	28
P 0.99	29	40
Max	58	50
CM1 ≥ 50 ng kg^{-1} (%)	0.019	0.032
CM1 ≥ 40 ng kg^{-1} (%)	0.36	1.06
CM1 ≥ 30 ng kg^{-1} (%)	0.99	3.84

Note: P, percentile; C_{M1} ≥ 50-40-30 ng kg^{-1}(%) is the proportion of consignments above the limit in relation to number of samples.

Table 4: Descriptive statistics for the level of aflatoxin M1 (ng kg^{-1}) sorted by attention limit in the 2013-2019 period.

Exposure Assessment

Average EDI, HI, and liver cancer incidence (LCI) values were calculated for the 2013-2019 period using AFM1 values from all the plants but dichotomized in two groups, one with lower AL (namely considering 30 ng kg^{-1} AL from 2013 in Calabria Region and from 2017 in Emilia Romagna Region) and the other with AL laid down by regulation in force (namely 40 ng kg^{-1} AL in the 5 remaining plants from 2013 to 2017 and all the remaining 4 plants since 2017). The result of EDI calculation, based on the mean "cattle milk" consumption data of "consumers only", sorted by different

population age, and for both AL values are reported in Figure 3. Among the different population groups, EDI values varied between 0.02 and 0.24 ng kg^{-1} bw day^{-1} for the 30 ng kg^{-1} AL and between 0.03 and 0.30 ng kg^{-1} bw day^{-1} for the 40 ng kg^{-1} AL, within both groups infants and toddlers had the highest mean EDI values while adults the lowest (Figure 3).

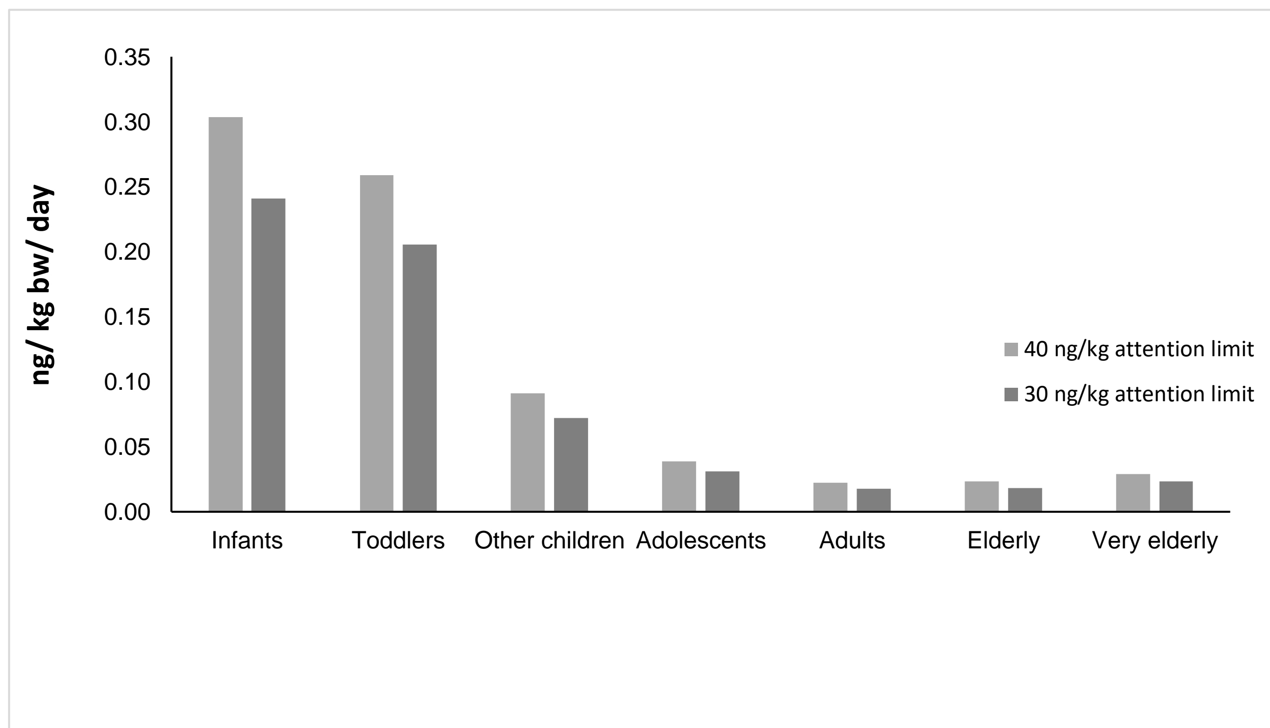

Figure 3: Estimated Daily Intake (EDI) values for different population groups and for different attention limits in the 2013-2019 period.

The results of mean HI for the different population groups are reported in Figure 4, as can be seen in the figure just infants and toddlers had values greater than 1 while all the other groups were well below the concern limit. Between the two AL groups there were no significant differences even though the group with lower limit had lower mean HI values, with toddlers, for example, being slightly above (1.03) the concern limit.

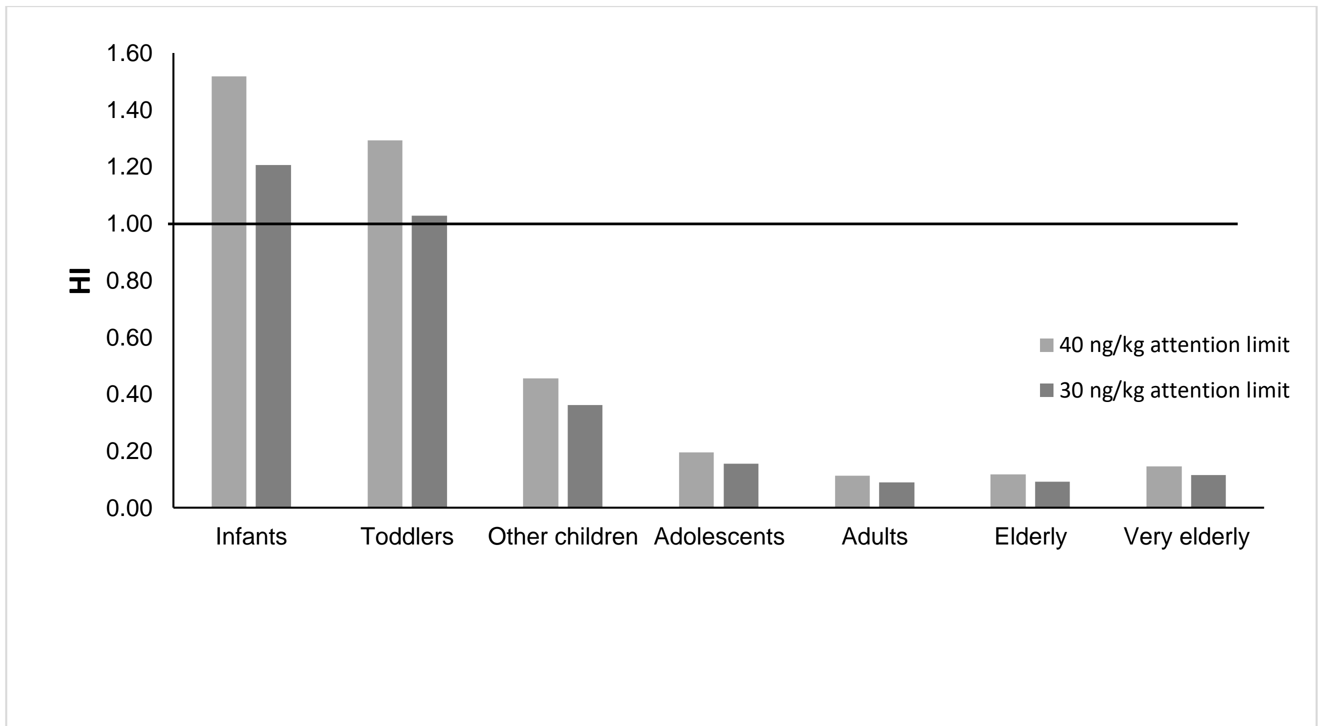

Figure 4: Mean Hazard Index (HI) values for different population groups and for different attention limits in the 2013-2019 period.

The fraction of incidence of HCC or liver cancer incidence (LCI) attributable to the intake of AFM1 was evaluated based on MoE considering the estimated mean exposure. The average LCI values calculated per 100,000 people for the studied period (Figure 5) showed, in alignment with EDI and HI results, the highest values in infant and toddlers. Among the two age groups, values for the 30 ng kg^{-1} ranged from 0.0003 to 0.0038, while for the 40 ng kg^{-1} from 0.0004 to 0.0048 per 100,000 people.

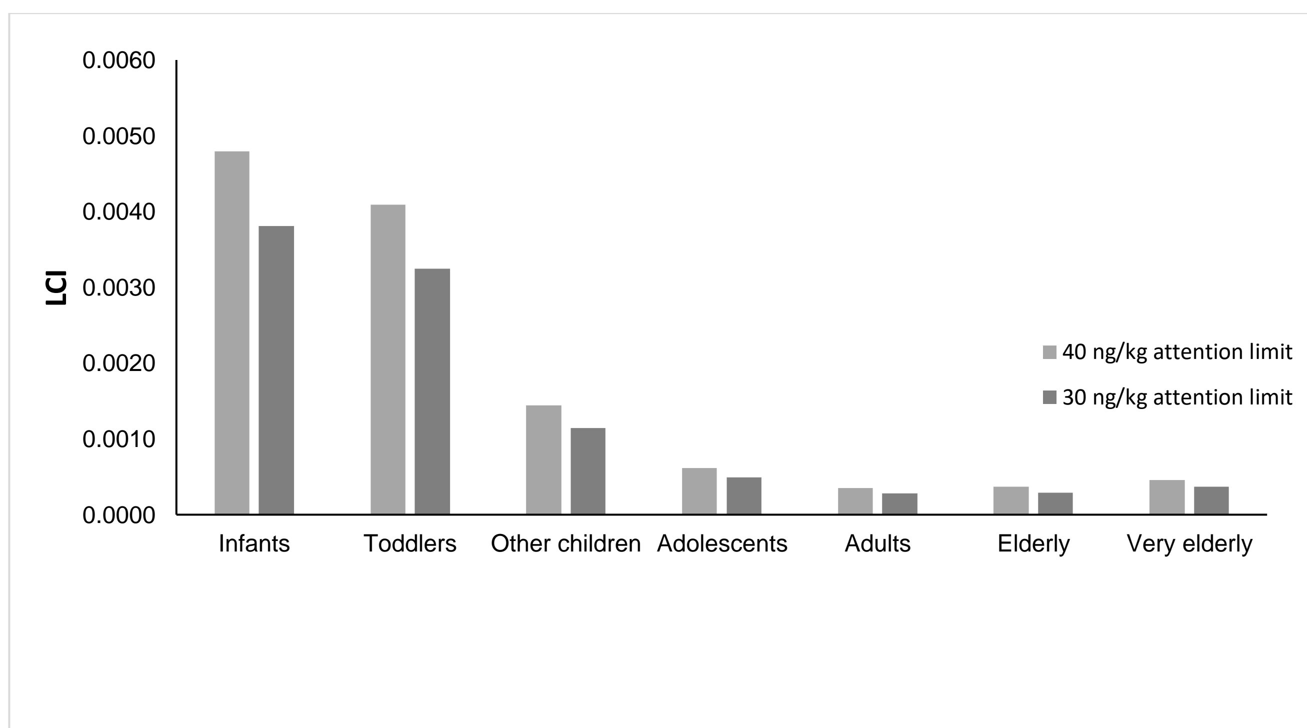

Figure 5: Estimated average liver cancer incidence (LCI) (cases per 100,000 people) in the Italian population by age groups and by attention limit during 2013-2019.

Discussion

The strict control of AFM1 in the commercialized milk is extremely relevant for protecting public health because this aflatoxin is a carcinogenic compound classified in the Group 1 (IARC). To this aim the implementation of a risk assessment approach can help to identify risk management strategies reducing the consumer exposure to AFM1.

The current EU Regulation sets the maximum levels of AFM1 in milk at 5 ng/kg but does not indicate the frequency of sampling nor give an indication for seasonal or regional sampling stratification. Therefore, the frequency of sampling must be evaluated on the basis of acquired previous knowledge on the distribution of the hazard. Figure 2 clearly shows three time-frames relevant in our dataset, during which the AFM1 concentrations in milk were significantly different: the year 2004 and the period 2005-2008 before the introduction of the AL and the period 2013-2019 after the introduction of the AL. The median of the AFM1 concentration from 2004 to 2019 was 10 ng kg^{-1} but is it

important to highlight that after the introduction of the AL it decreased ($p \leq 0.01$) from 13 ng kg^{-1} to 9 ng kg^{-1}.

Our results are in line (a bit lower considering values after 2013) with the ones reported by other authors in European countries such as France 14.3 ng kg^{-1} in raw milk (28) and Spain 9.69 ng kg^{-1} in UHT milk (29). Besides, our values are lower than Portugal 23.4 ng kg^{-1} in pasteurized milk (30), Croatia 46.6 ng kg^{-1} (31) and Serbia 71 ng kg^{-1} in raw milk (32) as well as other extra-EU countries such as Brazil 66.9 ng kg^{-1} (33) and China 51.9 ng kg^{-1} (34). However, it is important to consider that a higher maximal residual limit may be in force, for instance China and Brazil have a 500 ng kg^{-1} compliance limit.

The percentage of cow's raw milk sample noncompliant with the 50 ng kg^{-1} limit reported for the whole studied period was 0.92%, with a reduction from 2.52% to 0.03% before and after the introduction of the AL. Nations such as Greece (3.6%) (35), Croatia (9.36%) (31) extra-EU Serbia (30%) (32), as well as China (1.1%) (34) and Brazil (14%) (33) report higher values, while Spain (0%) and France (0%) (28, 29) lower ones. The wide variations in mycotoxin levels among studies could be related to the sample size, but also to the analysed geographic, temporal and climatic differences, as well as to the identification methods. Nevertheless, it is to notice that if we consider the studies conducted In Italy after 2013, our results (0.03%) are lower than those reported by Serraino (21) (0.20%) and Roila (36) (0.89%).

Our data, in accordance with (37) demonstrate how the application of a stringent self-control strategy, where the application of an AL and the subsequent accomplishment of corrective measures is performed, can significantly diminish the risk for public health due to AFM1in milk. These actions synergically intercept possible ascending trends enabling the application of early countermeasures, preventing health problems. This impact is clearly showed in Table 2 displaying that the AFM1 concentration in milk quantified after the application of the AL was significantly ($p \leq 0.01$) lower in comparison to the previous period. Moreover, the proportion of samples exceeding the EC limit in

2008 was 10 times higher in comparison to 2013 (Table 1) and a decreasing trend was kept up to 2019.

Temperature, humidity, rainfall patterns and the frequency of extreme weather events are already affecting farming practices, crop production and the nutritional quality of food crops, and therefore have an effect on aflatoxin presence. The impact of seasonality on AFB1 contamination in feed has been observed both in Italy (19) and in other countries (31, 34). Before 2013, our data report higher AFM1 levels in September 2006 and 2008. On the contrary, after the introduction of the AL of 40 ng kg^{-1} and the subsequent decrease of non-conformities, not only the seasonal trend was absent, but in addition the highest number of samples (n= 13) exceeding EC limit was detected during spring (n= 6), followed by summer (n= 5), autumn (n= 1) and winter (n= 1) (Data not shown). It is also important to highlight that we observed a lowering trend of AFM1 levels in the years 2015 and 2018 when the highest temperatures in comparison to the previous last 10 years where registered (38) in the area from which part of samples where tested. The decreasing trend in the presence of AFM1 is also certainly due to a new consolidated way of thinking of the farmers who are now used to manage the aflatoxin hazard as an ordinary and intrinsic problem for milk production and are aware of the impact climate changes on its occurrence.

In Italy, the regulation that introduced the AL dates back to 2013 and was specifically emitted in order to prevent and manage aflatoxin risk contamination "in case of extreme climatic conditions". After the note of Italian Ministry of Health, aflatoxins control was implemented by feed manufacturer, supplier and feed business operators in their respective fields. Moreover, it was implemented by farmers in relation to the drying procedures able to prevent aflatoxins contamination of feed leading to the consequent AFM1 reduction in milk. The effectiveness of the effort is showed by Ferrari et al 2022, who demonstrated that almost the totality of feed matrices analysed between 2013 and 2021 were compliant with the EU legal limit.

Considering that self-control strategies for limitation of the presence of mycotoxins in milk are expensive, our results provide the basis for redefining a risk-based sampling plan and assure an

appropriate level of compliance of milk and milk products with the legal limits. Based on the very low AFM1 concentrations in milk observed from 2013 to 2019, the probability of non-compliant milk could be considered negligible in this specific scenario. Therefore, an AFM1 monitoring plan based on a reduced sampling frequency but incorporating a precise early warning system able to intercept increasing trends in AFM contamination, allows to quicky identify the most critical dairy farms. Regretfully, the impact of rising feed costs as well as the farmers uncertainty in the relative tight feed supply, for which feed quality is not always ensured, might nullify the actually applied AFM1 risk reduction strategy.

Infant and toddlers, due to the relatively large milk intake compared to their body weights, confirmed to be more exposed than older consumers in line with literature (36, 39) and independently from the AL applied. Our results are in line with previously reported mean EDIs of 0.08 ng kg^{-1} bw day^{-1} (n = 40) in Portugal (30), 0.09 ng kg^{-1} bw day^{-1} (n = 16) in France (40), and 0.18–0.20 ng kg^{-1} bw day^{-1} (n = 1,233) in Serbia (32). The implementation of a more stringent AL, yield a decrease of EDI, HI, and LCI. Specifically the HI was lowered, even if not statistically significant, to a value (1.03) almost below the concern limit for toddlers, while the LCI for both infants and toddlers (the most at risk groups), by 0.001 per 100,000 people, showing its efficacy as strategy to reduce the risk related to AFM1.

Given that milk containing AFM1 $\leq$ 10 ng kg^{-1} should be used for producing milk and milk-based products specifically for young children because HI is estimated below 1 (21), the AL of 30 ng kg^{-1} would allow a mean and median AFM1 concentration respectively of 9.21 and 8 ng kg^{-1}, meaning that almost all these commingled milk batches might be used, stored and processed for the youngest population, with a remarkable advantage for milk industry to assure safety also of this population groups.

Conclusions

The results obtained in this study demonstrate the efficacy of the management strategies to limit the presence of AFM1 in milk implemented after the aflatoxin crisis in 2003 and 2013. Moreover, they represent baseline data to define risk-based sampling plans to detect AFM1 contamination in milk thus lowering the human exposure to AFM1. The application of tailored sampling strategies when FBO must face either expected situation, as global climate changes, or unexpected crises, as the disruption in the supply chain, due for instance to geopolitical reasons, can certainly help to limit the presence of aflatoxins in the food and feed systems.

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Chapter 2

Quantitative risk assessment of *Listeria monocytogenes* in Spanish fresh goat milk cheeses

This second chapter, as the first one, deals with the first aim of this thesis as well as the third, namely describing the application of QRA to evaluate the risk for consumers connected to consumption of foods of animal origin and to evaluate the risk-mitigation strategies applied.

Differently from the previous chapter it deals with those aims from the perspective of microbiological hazards, in fact, in this chapter a QMRA is conducted, and specifically the risk connected with *L. monocytogenes* in artisanal fresh cheeses, made of pasteurized goat milk, produced in the Spanish region of Andalusia. In the first part the hazard will be assessed as well as the consumers exposure, focusing mainly on the impact of refrigerated shelf-life. Successively, will be assessed the risk connected with the consumption of this product in two population groups, general and high-risk, evaluating two possible scenarios as well as risk-mitigation solutions based on the evaluation of the impact of the different steps of the shelf-life on the risk for consumers.

This QMRA was created under the assumption that *L. monocytogenes* would contaminate the cheese in the post-production stages and that changes in its concentration from cross-contamination would be virtually zero. Exposure assessment and risk characterization were the key areas of the QMRA's attention following the product's production, distribution, and up until the point of consumption (i.e. during retail, consumer handling, and storage prior to consumption). The model was built using the statistical software *R Studio* (4.2.1) running 1,000,000 iterations and using data retrieved from bibliographic research and form consultation with experts from the industry.

The primary outcome of this QMRA's exposure assessment is the ingested dose (the number of *L. monocytogenes* per serving). Data on the frequency of fresh cheese contamination, the predicted contamination level at consumption, and serving size were used to reach this result. To predict the contamination level at consumption a generalized linear regression model (15) was used to simulate the growth of the pathogen in the various steps of the refrigerated shelf life. The ingested dose was

then combined with the FAO/WHO (3) dose-response model for *L. monocytogenes* to assess the risk of developing a serious listeriosis in the two population groups (general and high-risk). This analysis was conducted for two scenarios, one simulating a "low" contamination and one simulating a higher initial concentration in the product.

The results showed the capacity of the pathogen to thrive under the condition of the distribution chain used to simulate this model, with both scenarios reaching high mean concentrations of *L. monocytogenes* at the time of consumption, 4.93 ± 2.97 log CFU/g and 6.43 ± 2.37 log CFU/g respectively for the "low" and "high" contamination scenario. Furthermore, the results support previous risk assessments in showing that fresh cheeses, in this case artisanal goat milk cheeses, pose a risk for listeriosis, in fact, both scenarios, despite presenting a very low median of risk, showed in various simulation a higher proportion of risk especially when considering the high-risk population and the high contamination scenario. Subsequently, through a correlation analysis, the influence of the different model units on the risk of contracting a serious case of listeriosis was assessed. The analysis showed how the domestic storage was the main step affecting the risk genesis followed by the initial concentration of the pathogen. Based on this evidence two risk-mitigation actions were evaluated 1) decreasing the fridge temperature at domestic storage and 2) shortening the shelf life of the product, after simulation both these solutions produced a reduction of the risk for both populations compared to the baseline scenario, with the action 1 reducing the risk of 93.8% and action 2 of about 98% and proving to be the most effective.

The overall results of this chapter evidence how the application of a QMRA can help the producer as well as the risk manager to improve the safety of the food chain, giving evidence, for example, on how good manufacturing practices and the control of the productive environment are fundamental to reduce the risk of listeriosis as well as interventions on the shelf-life duration or on domestic refrigerators temperature.

A detailed description of the methodology, results, discussion, and conclusion of this QMRA is reported below in the format of a scientific paper, which though as not yet been submitted for publication it might be in the future.

Introduction

Listeria monocytogenes is a common environmental pathogen that can thrive in a variety of challenging conditions, including refrigeration temperatures, a wide pH range, and high salt concentrations and that contaminate a wide range of foods of animal and non-animal origin (19).

Listeriosis is a relatively uncommon but one of the most serious foodborne illnesses in the EU, presenting 0.49 cases per 100,000 population (2). The infection typically manifests as a mild, febrile illness, but it can also appear as systemic (invasive) listeriosis, which has more severe symptoms, a greater incidence of hospitalization, and a high mortality rate (20-30%). In sensitive populations, such as pregnant women, the elderly, and people with weakened immune systems, systemic listeriosis is far more common (19).

Although a wide range of foods may be contaminated with *L. monocytogenes*, RTE foods are mostly linked to outbreaks and sporadic cases of listeriosis. In fact, RTE foods, such as "meat and meat products," "fish and fish products," and "milk and milk products," as well as "food of plant origin" and frozen meals, are frequently linked to human listeriosis(2,19,20). Among these RTE foods different types of cheese have been involved in several outbreaks worldwide, especially fresh unripened cheeses, made of both raw and pasteurized milk (21–28).

In 2021 in the EU, the overall occurrence of *L. monocytogenes* in RTE cheeses was 0.69%, in detail for soft and semi-soft cheeses, including fresh cheeses, made of pasteurized goat milk the occurrence was 0.95% (over 316 tested samples)(2).

A growing segment of the consumer market is particularly drawn to the gourmet experiences and sensory appeal of artisanal foods. Sensory qualities, along with others like food sustainability, can be found in artisanal food products. Among these, in terms of sensorial, nutritional, and functional qualities, artisanal cheeses are given a special place within the large market for traditional food products (29). Particularly, goat milk cheeses make up a sizeable portion of the traditional cheese production in Mediterranean regions like Spain, Greece, Italy, and Turkey, and are important elements for their economies (30). With 42.5% of the production in the Andalusian region and some

traditional products like goat milk cheeses, Spain is the second-largest goat milk producer in the European Union after Greece (31).

As a public health hazard and a threat to the reputation of artisanal producers, the occurrence of foodborne outbreaks connected to the consumption of artisanal cheeses raises concerns for both sanitary authorities and artisanal producers. Since fresh cheeses are highly susceptible to *L. monocytogenes* post-process contamination and proliferation, due to their high pH (5.0-6.3), high water activity (> 0.97), low salt content (1.4-1.6%), and refrigerated storage throughout shelf-life, they require special consideration(32). Given the recent notifications of *L. monocytogenes* in fresh goat milk cheeses in EU (25–28,33) and the lack of specific literature, it is essential to assess how these products affect public health. In this context lays the aim of this work, namely, to employ a QMRA model to predict the public health risk from *L. monocytogenes* following consumption of artisanal fresh goat milk cheeses. Moreover, a QMRA will frequently help to identify areas where there is insufficient information (knowledge gaps) to make decisions about a certain foodborne pathogen and food combination and may be helpful in evaluating the effectiveness of intervention initiatives intended to reduce threats to public health, providing helpful data for risk managers.

In this QMRA the impact of the refrigerated shelf-life of artisanal made fresh goat milk cheeses, produced in the Andalusia region of Spain, on the risk of listeriosis for the consumers was evaluated. A baseline scenario and an alternative scenario were used to suggest some data driven risk-mitigation strategies.

Materials and methods

Artisanal fresh goat cheese production process and pathway

The production of fresh goat milk cheese described is adapted to the artisanal practices and procedures of a traditional cheesemaker from Málaga (Andalusia, Spain). The ingredients used for the production mainly consist of pasteurized goat milk, salt (NaCl, 2% v/v), calcium chloride ($CaCl_2$, 0.28% v/v) and rennet (0.28% v/v). For this product, starter cultures are not used for cheese elaboration. The

cheese is made from pasteurized milk, which is usually pasteurized at the moment but can also be pasteurized the day before and refrigerated until use. The milk is then preheated at 30°C and $CaCl_2$ is added leaving the milk to ferment for an hour, then commercial liquid rennet is added, and coagulation takes place in approximately 40 minutes. Next the curd is cut and agitated for 10-15 min, then is transferred into molds, previously cleaned and disinfected, and pressed for 50-60 minutes to partially remove the whey. Once unmolded, the cheeses are placed in a salt-water solution (250g/L, 18º Beaumé) for brining, at a temperature of 7-9°C for approximately 2 hours. Then, cheeses are aired in a chamber conditioned at 0-9°C depending on the production season. Before of the distribution, the cut, vacuum-packaged and labelled cheeses, are stored under refrigeration at 0-6°C. The final product has a pH of 6.7 and a water activity of 0.998 circa.

Model overview

L. monocytogenes may be present in raw milk because of contact with environmental factors like animal feed, sewage, water, plants, and soil. Furthermore, the same bacterium that causes cow mastitis might be another important source of contamination in raw milk. Furthermore, *L. monocytogenes* can form environmentally stable biofilms that are resistant to sanitation and is cold tolerant (i.e., it can grow at refrigeration temperatures as low as –1.5 °C) (34). As a result, sources of contamination for pasteurized milk include the environment and the machinery used for milk storage or cheese production. For example, contamination may occur as a result of insufficient pasteurization, or as post pasteurization contamination during one or more of the following steps: culture addition, curd formation, cutting, stirring, washing, molding, draining, pressing, brining, salting, ripening, and packaging (34)

The intrinsic factors of fresh cheese do not prevent the growth of *L. monocytogenes*, and so, due to its capacity to thrive at standard refrigerator temperatures, post-process contamination may result in high numbers of the microbe at the time of consumption, even if the initial cell numbers are low.

This QMRA was developed assuming *L. monocytogenes* contamination of the cheese in post-production phases and that changes in its concentration due to cross-contamination after this step was

negligible. The main focus of the QMRA were exposure assessment and risk characterization after the product was manufactured and distributed and up until the point of consumption (i.e. during retail, consumer handling, and storage prior to consumption).

The risk assessment model is composed by two modules: a) refrigerated shelf-life, from manufacturing to consumption; b) consumption and risk characterization, using the output of the exposure assessment (*L. monocytogenes* per serving) and the dose-response function to predict risk of listeriosis. The model was built using the statistical software *R Studio* (4.2.1) running 1,000,000 iterations, the model flow chart is reported in figure 1.

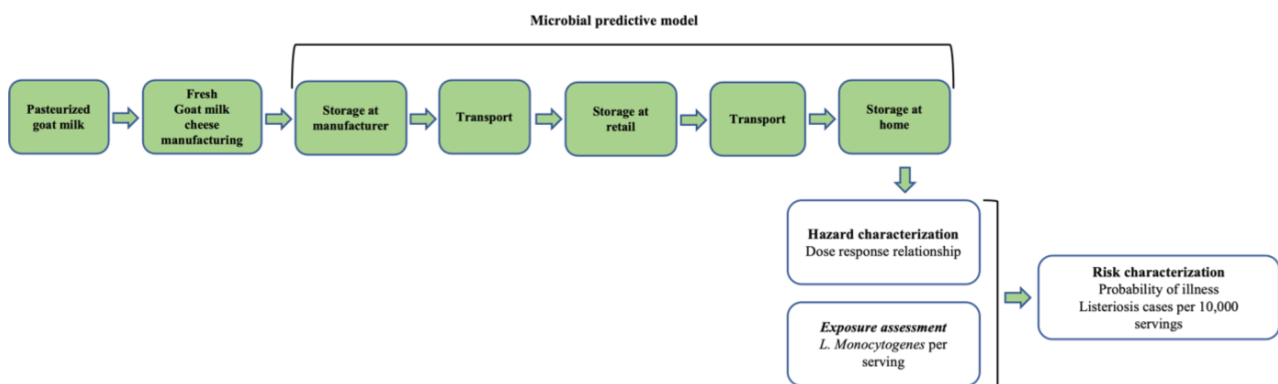

Figure 6 The product pathway and QMRA model flow chart.

Hazard identification

Listeria spp. includes 17 species identified so far, among these, *L. monocytogenes* is the most critical from human health perspective for the severity of the symptoms (meningitis, septicemia, abortion) and for the high fatality rate (20-30%) (35). Majority of the population's affected subgroups are those who are more vulnerable to infections. *L. monocytogenes* is basically an opportunistic pathogen that primarily affects people with a severe underlying disease or condition (for example, immunosuppression, HIV/AIDS, chronic conditions like cirrhosis that impair the immune system); pregnant women; unborn or recently delivered infants; and the elderly (20). An in-depth hazard identification for *L. monocytogenes* is given by EFSA and FAO/WHO (20,35). Despite the possibility of *L. monocytogenes* contamination in a wide range of foods, outbreaks and occasional cases of

listeriosis are primarily linked to RTE foods, with dairy products, and in particular cheeses among the most involved after meat and meat product and fish and seafood. Various listeriosis notification connected to goat milk cheeses were reported in the recent past in Europe, with two linked to artisanal made goat cheese from Andalusia (Spain) (25,26) and many others from other European countries (27,28,33).

Hazard characterization

Several QMRAs(32,36–39) have used the FAO/WHO(20) dose response model for *L. monocytogenes*:

$$P_{ill} = \left(1 - e^{(-r \cdot D)}\right)$$

Where P_{ill} is the probability of severe illness; D is the number of L. monocytogenes cells consumed per serving (the dose) calculated as $D = C \cdot S$ where C is the concentration of the pathogen (number of cells/g) and S is the serving size (g);

r is the parameter that expresses the probability of illness after the consumption of a single *L. monocytogenes* cell for a specific population group. Median r-values generated by FAO/WHO (20) for “general” and ‘high-risk’ populations (Table 1) were included in this QMRA. Based on the distribution of exposure levels, the predicted incidence of listeriosis per ingested serving was computed for the two risk sub-populations.

Because the exponential model assumes there is no minimum infectious dosage, it is a non-threshold model. Instead, the model assumes that there is a very small but limited likelihood that a single *L. monocytogenes* cell will cause disease. The model's linearity, or proportionality, between dose and likelihood of disease at low doses, is one of its important characteristics. This suggests that if the dose is decreased tenfold, the likelihood of getting sick is also decreased tenfold. Furthermore, it means that, excepting very high dosages, 1,000 meals of a given level of contamination have the same effect on public health as 10,000 servings of a lower level of contamination (20).

Dose-response model parameters					
Description	Type	Abbreviation	Value	Unit	Source
Servings	Stochastic	*S*	tri(10, 215.40, 56.73)	g	EFSA Comprehensive Food Consumption Database
Prevalence of *L. monocytogenes* in fresh cheese	Stochastic	*Pr*	Unif(0, 0.8)	%	Martinez-Rios et al., 2018
Dose-response parameter for general population	Constant	$r_{general}$	5.34E-14	/	FAO/WHO, 2004
Dose-response parameter for high-risk population	Constant	$r_{high\text{-}risk}$	5.85E-12	/	FAO/WHO, 2004
Dose	Stochastic	*D*	$10^{[LMC_5]}$ ・ servings	CFU/g	Calculated
Risk of listeriosis per serving	Equation	P_{ill}	(1-exp(-r ・ D)) ・ Pr	Probability	FAO/WHO, 2004

Table 5 Dose-response model parameters used in the model.

Exposure assessment

The ingested dose (number of *L. monocytogenes* per serving) is the main output of the exposure assessment of this QMRA. This result is obtained using data about prevalence of contamination of fresh cheese, estimated contamination level at consumption and serving size.

Data about prevalence (table 1) were obtained from Martinez-Rios and Dalgaard metanalysis (40) which reported the prevalence of *L. monocytogenes* in fresh cheeses, and no difference was assumed for different types of fresh cheeses.

The estimated contamination at consumption was calculated from initial contamination at the end of processing, using predictive microbiology, based on times and temperatures of refrigerated shelf-life. Two different initial contaminations were included as probability distributions, one as baseline scenario (-1 ± 1 log CFU/g) and one as alternative scenario (1 ± 1 log CFU/g).

The serving size was retrieved from EFSA Comprehensive European Food Consumption Database (41), which contains detailed information about food consumption for European countries. More specifically, data about consumption of "fresh uncured cheese", including goat cheese but non exclusively, for different population sub-groups obtained from "Spanish National dietary survey on children and adolescents" and "Spanish National dietary survey in adults, elderly and pregnant women" conducted respectively in 2012 and 2013 by Spanish Agency for Food Safety and Nutrition (AECOSAN). The data were included as a triangular distribution using the mean consumption, as maximum the highest 99th percentile value and as minimum the lowest 5th percentile across the different sub-groups, and assuming no difference for consumption between "high-risk" and "general" sub-populations.

All the variables included in the model (derived from literature or after discussion with experts of Spanish cheese industry) were included as distributions to account for variability and are reported in table 2.

Distribution of time and temperature of storage at warehouse were assumed based on expert opinion and industry confidential data; temperature of transport to retail was derived from Derens et al. (42) and truncated based on industry confidential data as well as time profile which was entirely based on industry data and expert opinions; distribution of temperature of storage at retail were obtained from Zubeldia et al.(43) which conducted a survey on the effectiveness of cold chain at retail level in southern Spain; while time profiles were obtained from Derens et al. (42) and truncated based on industry confidential data; for transport by consumers the same approach of transport to retail was used; and finally for storage at households temperature profiles where obtained from Carrasco et al. (44) which conducted a survey on temperature of domestic refrigerators in southern Spain, storage time was assumed based on expert opinion and product shelf life, which we assumed wasn't exceeded.

The growth of *L. monocytogenes* is described by the relationship between growth rate and temperature represented by a generalized linear regression model shown in Equation 2 (45):

$$\sqrt{\mu} = b(t - t_{min})$$

Where $\sqrt{\mu}$ is the square root of maximum growth rate, *b* is the slope of the regression line, *t* is the temperature (°C) and t_{min} is the theoretical minimum temperature for microbial growth. Kinetic parameters for the growth of *L. monocytogenes* in fresh cheeses were determined, as shown in Equation 3 (32), which calculate the growth rate per day (i.e. log CFU/day):

$$\sqrt{\mu} = 0.068(t + 2.765)$$

Equation 3 was used to calculate *L. monocytogenes* growth during storage and transport at the different stages described, lag time was assumed to be equal to zero, and the maximum population density was set to 9 log CFU/g.

Description	Type	Abbreviation	Value	Unit	Source	Description	Abbreviation	Value	Unit	Source	Description	Abbreviation	Value	Unit
Storage at werehouse														
Inputs						***Intermediate variables***					***Outputs***			
L. monocytogenes concentration in fresh cheese	Stochastic	*[LMC₀]* → $[LMC_0]$	U(-2, 0)	log CFU/g	Assumed	*L. monocytogenes* growth rate	μ_1	$\mu_1 = (b(t_1 - t_{min}))^2$	CFU/g*day	(15)	*L.monocytogenes* concentration	$[LMC_1]$	$[LMC_0] + \mu_1 \cdot t_1$	log CFU/g
Storage temperature	Stochastic	T_1	N(6, 2)	°C	Industry									
Storage time	Stochastic	t_1	U(0.5, 5)	days	Industry									
Transport to retail														
Inputs						***Intermediate variables***					***Outputs***			
Transport temperature	Stochastic	T_2	trN(2.9, 1.4, 0.1, 5.7)	°C	(25); Industry	*L. monocytogenes* growth rate	μ_2	$\mu_2 = (b(t_2 - t_{min}))^2$	CFU/g*day	(15)	*L. monocytogenes* concentration	$[LMC_2]$	$[LMC_1] + \mu_2 \cdot t_2$	log CFU/g
Transport time	Stochastic	t_2	U(0.08, 0.25)	days	Industry									
Storage at retail														
Inputs						***Intermediate variables***					***Outputs***			
Storage temperature	Stochastic	T_3	T(-3, 15, 4)	°C	(26)	*L. monocytogenes* growth rate	μ_3	$\mu_3 = (b(t_3 - t_{min}))^2$	CFU/g*day	(15)	*L. monocytogenes* concentration	$[LMC_3]$	$[LMC_2] + \mu_3 \cdot t_3$	log CFU/g
Storage time	Stochastic	t_3	trE(4.3, 0.5, 7)	days	(25); Industry									
Transport by consumers														
Inputs						***Intermediate variables***					***Outputs***			
Transport temperature	Stochastic	T_4	trN(7.8, 3.1, 1.6, 14)	°C	(25); Industry	*L. monocytogenes* growth rate	μ_4	$\mu_4 = (b(t_4 - t_{min}))^2$	CFU/g*day	(15)	*L. monocytogenes* concentration	$[LMC_4]$	$[LMC_3] + \mu_4 \cdot t_4$	log CFU/g
Transport time	Stochastic	t_4	U(0.01, 0.08)	days	Industry									
Storage by consumers														
Inputs						***Intermediate variables***					***Outputs***			
Storage temperature	Stochastic	T_5	trN(6.62, 2.56, 1.5, 11.74)	°C	(27); Industry	*L. monocytogenes* growth rate	μ_5	$\mu_5 = (b(t_5 - t_{min}))^2$	CFU/g*day	(15)	*L. monocytogenes* concentration	$[LMC_5]$	$[LMC_4] + \mu_5 \cdot t_5$	log CFU/g
Storage time	Stochastic	t_5	U(0.01, 28-(t1+t2+t3+t4))	days	Industry									

Note: Probability distributions used are Uniform (U), Normal (N), truncated Normal (trN), Triangular (T),and truncated Exponential (trE).

Table 6: Summary of variables for refrigerated shelf-life module of risk assessment model.

Risk characterization

This step combined the output of the exposure assessment with the dose response model and the contamination prevalence to estimate the probability of developing a serious illness (P_{ill}) per serving:

$$P_{ill} = \left(1 - e^{(-r \cdot D)}\right) \cdot Pr$$

Furthermore, was estimated the number of listeriosis cases in a population of 10,000, both these estimates were obtained for the two population sub-groups ("general" and "high-risk") and for the various scenarios.

Results

In the baseline scenario the simulated concentration of *L. monocytogenes* at the beginning of shelf-life of the fresh goat milk cheese showed a mean of -1 ± 0.58 log CFU/g and reached 4.93 ± 2.97 log CFU/g, ranging from -1.74 to 9 log CFU/g, just before consumption, after simulating the effects of the various stages. Descriptive statistics of the concentrations at various steps are reported in table 3a.

	[LMC₀]	*[LMC₁]*	*[LMC₂]*	*[LMC₃]*	*[LMC₄]*	*[LMC₅]*
Mean	-1.00	0.03	0.05	0.32	0.35	4.93
SD	0.58	0.91	0.91	0.94	0.94	2.97
Minimum	-2.00	-1.97	-1.97	-1.90	-1.89	-1.74
5th percentile	-1.50	-0.63	-0.61	-0.35	-0.33	2.39
Median	-1.00	-0.02	0.00	0.26	0.29	4.69
95th percentile	-0.10	1.64	1.66	1.98	2.01	9.00
Maximum	0.00	5.71	5.73	6.20	6.23	9.00

Table 7a: Results of simulation for L. monocytogenes on the various steps of the shelf-life for the baseline scenario.

Regarding the alternative scenario, the initial simulated concentration was on average 1 ± 0.58 log CFU/g and ranged from 0.22 to 9 log CFU/g with a mean of 6.43 ± 2.37 log CFU/g at the end of the refrigerated shelf-life, concentrations for all the steps are reported in table 3b.

	[LMC_0]	*[LMC_1]*	*[LMC_2]*	*[LMC_3]*	*[LMC_4]*	*[LMC_5]*
Mean	1.00	2.03	2.05	2.32	2.35	6.43
SD	0.58	0.91	0.91	0.94	0.94	2.37
Minimum	0.00	0.02	0.05	0.07	0.10	0.22
5th percentile	0.50	1.37	1.39	1.64	1.67	4.39
Median	1.00	1.97	2.00	2.26	2.29	6.68
95th percentile	1.90	3.63	3.66	3.98	4.01	9.00
Maximum	2.00	8.46	8.52	8.91	8.93	9.00

Table 3b: Results of simulation for L. monocytogenes on the various steps of the shelf-life for the alternative scenario.

The concentration at consumption was used to calculate the ingested dose, see table 1, which ranged from 0.64 CFU to $2.15 \cdot 10^{11}$ CFU with a mean of $2.04 \cdot 10^{10} \pm 4.23 \cdot 10^{10}$ CFU in the baseline scenario, and from 39.6 CFU to $2.15 \cdot 10^{11}$ CFU with a mean of $3.21 \cdot 10^{10} \pm 4.97 \cdot 10^{10}$ CFU in the alternative scenario (table 4).

	Baseline	**Alternative**
Mean	2.04E+10	3.21E+10
SD	4.23E+10	4.97E+10
Minimum	6.45E-01	3.96E+01
5th percentile	2.05E+04	2.04E+06
Median	4.08E+06	4.02E+08
95th percentile	1.25E+11	1.44E+11
Maximum	2.15E+11	2.15E+11

Table 8: Results of predicted ingested dose for baseline and alternative scenarios.

From the ingested dose, using the dose response model from FAO/WHO (table 1), was calculated the risk of developing serious listeriosis per serving, for both high-risk and general populations in both the baseline scenario and the alternative scenario. Results are reported in table 5. For the high-risk population the median risk was $2.23 \cdot 10^{-5}$ in the baseline scenario while $2.24 \cdot 10^{-3}$ in the alternative scenario, for the general population was $2.04 \cdot 10^{-7}$ and $2.05 \cdot 10^{-5}$ for the baseline and alternative scenario respectively.

	Baseline		Alternative	
	High-risk	**General**	**High-risk**	**General**
Risk of listeriosis per serving				
Mean	0.09	0.001	0.14	0.002
SD	0.19	0.002	0.22	0.003
5th percentile	1.13E-07	1.03E-09	1.12E-05	1.02E-07
Median	2.23E-05	2.04E-07	2.24E-03	2.05E-05
95th percentile	0.53	0.007	0.61	0.008
Maximum	1.00	0.021	1.00	0.021
Number of listeriosis cases per 10,000 servings				
Mean	890.47	10.84	1401.36	17.10
SD	1855.07	24.23	2179.52	28.76
5th percentile	0.00	0.00	0.00	0.00
Median	0.00	0.00	22	0.00
95th percentile	5254	67	6138	80
Maximum	10000	232	10000	221

Table 9: Output of QMRA model regarding the baseline and alternative scenarios

To assess the impact of the different variables on the risk of developing serious listeriosis, the Pearson correlation coefficient has been estimated, time and temperature of domestic storage showed the highest coefficients, 0.44 and 0.41 respectively, followed by initial *L. monocytogenes* concentration, storage temperature at warehouse and storage temperature and time at retail figure 2.

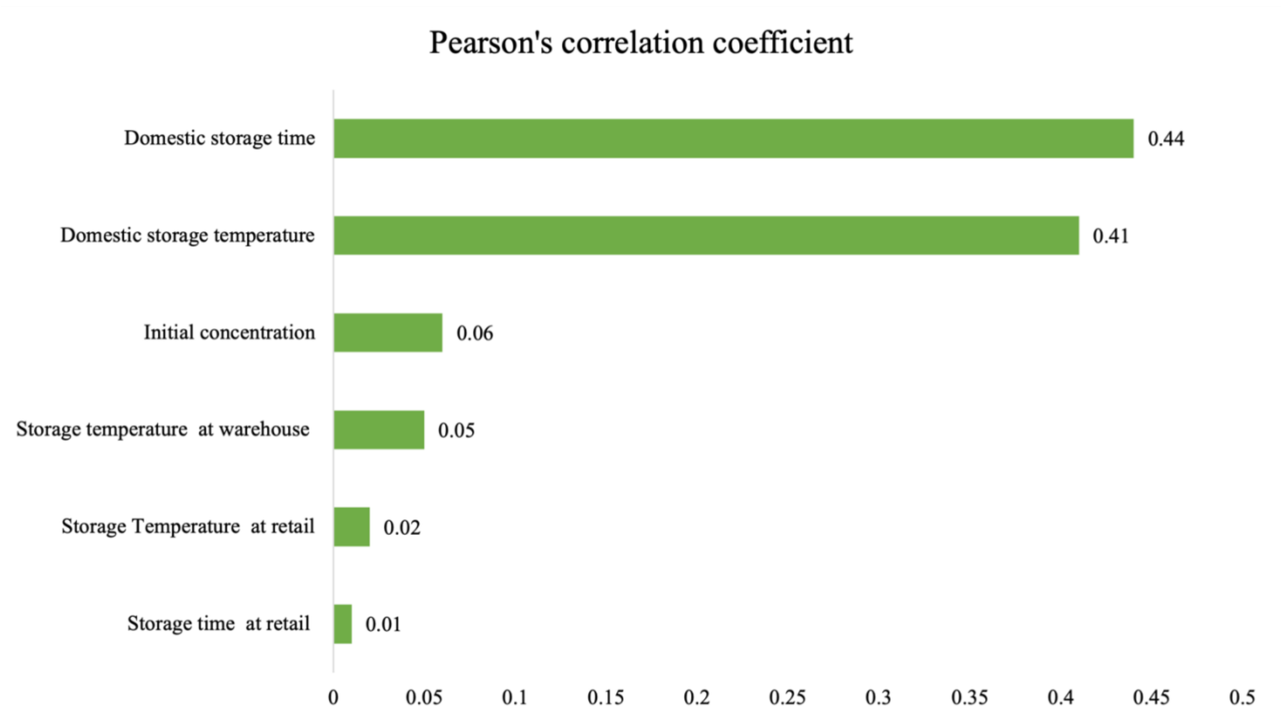

Figure 7: Correlation of different input variables with risk of listeriosis.

In the baseline scenario, consumption of fresh goat milk cheese resulted in 890.47 ± 10.84 and 1855.07 ± 24.23 cases of listeriosis in general and high-risk population respectively for 10,000 servings based on 1,000,000 iterations. In the alternative scenario the number of cases was higher with a mean of 17.10 ± 28.76 and 1401.36 ± 2179.52 for general and high-risk respectively, in both alternative and baseline scenario in some iterations all the high-risk population was predicted to fall ill (table 5). Anyway, the most informative statistics in this case is the median of the listeriosis cases, that in this case is 0.00, except for high-risk population in the alternative scenario for which is 22, indicating a right skewed distribution and a majority of the simulation yielding a very low number of cases.

Since a positive correlation, with a relatively high correlation coefficient, between domestic storage and the risk of listeriosis was found, some risk-mitigation actions to apply to this step were weighed. Two more scenarios were simulated: a) decreasing the temperature of domestic refrigerators and b) shortening the shelf-life of the product (shorter domestic storage). The inputs of the model were modified, considering the two scenarios separately, and the model re-run.

The fresh goat cheeses considered in this work have a use-by date of 28 days. According to literature statistics (46), chilled food is typically consumed before it exceeds its maximum shelf life, and very few consumers disregard the use-by date, so, in this work, was assumed that domestic storage never exceeded it and considered the domestic storage time as the use-by date minus the time taken by the previous steps. In this risk-mitigation scenario the maximum shelf-life was reduced to 24 days, this intervention reduced the median risk of listeriosis per serving for both general and high-risk populations by 98% (table 6 and figure 3).

For the second scenario, improving domestic refrigeration, the distribution of temperature of domestic refrigerators (originally a truncated normal distribution (6.62, 2.56, 1.5, 11.74)) was modified to a normal distribution with mean 5°C and SD 1.5°C reducing the mean temperature respect to the baseline scenario. Also in this second scenario the risk of developing serious listeriosis and thus the

number of cases was reduced by 93.8% for both general and high-risk populations (table 6 and figure 3).

	Baseline scenario		Improved domestic refrigeration		Reduced use-by date	
	High-risk	General	High-risk	General	High-risk	General
Risk of listeriosis per serving						
Mean	0.09	0.001	0.03	0.0003	0.01	0.0001
SD	0.19	0.002	0.10	0.001	0.06	0.0008
5th percentile	1.13E-07	1.03E-09	3.12E-08	2.85E-10	1.70E-08	1.55E-10
Median	2.23E-05	2.04E-07	1.38E-06	1.26E-08	4.42E-07	4.03E-09
95th percentile	0.53	0.007	0.19	0.002	0.01	0.0001
Maximum	1.00	0.021	1.00	0.02	1.00	0.02
Number of listeriosis cases per 10,000 servings						
Mean	890.47	10.84	251.37	2.99	104.68	1.22
SD	1855.07	24.23	1014.37	13.04	643.40	8.19
5th percentile	0.00	0.00	0.00	0.00	0.00	0.00
Median	0.00	0.00	0.00	0.00	0.00	0.00
95th percentile	5254	67	1895	20	144	1
Maximum	10000	232	10000	217	10000	222

Table 10: Output of QMRA model regarding the baseline and risk-mitigation scenarios.

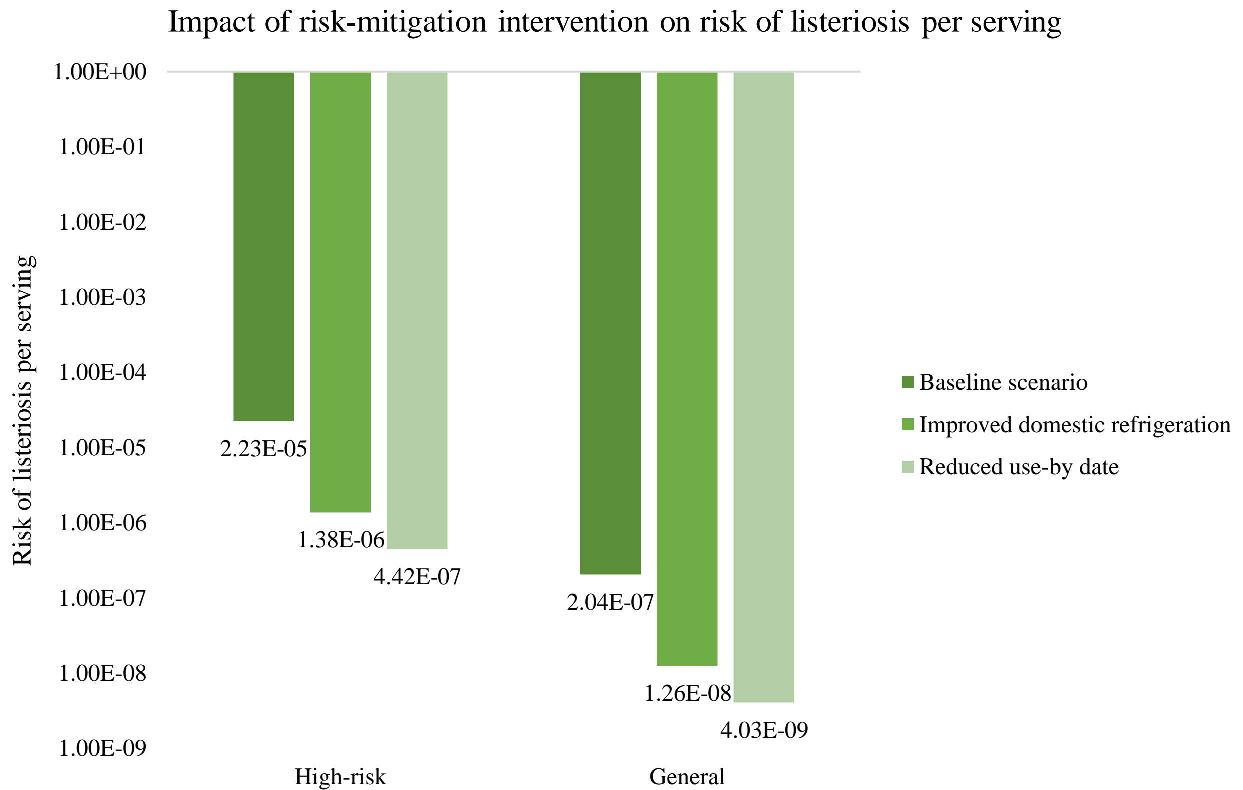

Figure 8: Impact on the risk of listeriosis of different mitigation strategies.

Discussion

Various authors reported how the use of raw milk in the production of fresh cheeses is a major risk factor for *L. monocytogenes* infections (47,48), nonetheless, it is reported how pasteurization can be insufficient in preventing the contamination of fresh cheeses. In fact, post-processing contamination represent a major cause of cheese contamination, occurring at different stages of processing (47,49). In this work the route of contamination of the product with *L. monocytogenes* was not evaluated, but rather was assumed a postprocessing contamination and the focus was set mainly on the refrigerated shelf-life of the product.

For the baseline scenario a “low” initial concentration ranging from -2 to 0 log CFU/g was assumed, based on the idea that sporadic postprocessing contamination may not result in very high concentrations. After simulation, the predicted *L. monocytogenes* concentration at the end of retail was below the 2 log CFU/g safety criterion set by Reg. 2073/05 for products placed on the market during their shelf-life in 95% of the cases, but after domestic storage reached concentrations much

higher in 95% of the cases with a mean concentration of around 5 log CFU/g. This result is not surprising given the ability of *L. monocytogenes* to grow in such RTE products (32,50,51) and since the domestic storage is the step were time and temperature are less under control this result goes along with those of other authors and other RTE products (35,37). In the alternative scenario was set a higher initial concentration of the pathogen in the product, from 0 to 2 log CFU/g, to account for a scenario in which the postprocessing contamination was less contained (52). In this case, there was 95% of simulations with little less than 2 log CFU/g at the beginning of the shelf-life but already after transport to retail this concentration was exceeded by at least 50% of the simulations, showing how the initial concentration has a great impact on the final contamination, even under more controlled environmental condition, since *L. monocytogenes* is very well adapted to grow at refrigeration temperatures, as also seen by Campagnollo et al.(32) on fresh Minas cheeses. Reading these results, it is important to keep in mind that was set an initial concentration which presume, even at its lowest concentration, the presence of the pathogen in the product, and therefore this is not a limiting factor to the growth during shelf life. An important consideration to make is regarding the predictive model used in the exposure assessment, given the absence of a specific model for pasteurized goat milk cheeses, was selected a model developed for a product with characteristics as similar as possible to reduce the inaccuracy of the predictions, of course the development of a specific predictive model for this product would greatly improve the reliability of this QMRA.

Regarding servings, the assumption that no difference is present in consumption of fresh cheeses between general and high-risk populations was made, same assumptions were made also on previous QMRA (32,37,38) This assumption may yield an increase of the predicted risk for the high-risk population in case the levels of consumption are lower than those of the general population. Furthermore, the variability on the serving size was accounted using a triangular distribution based on consumption data across the different sub-groups of the population, so the risk wasn't evaluated for those groups individually generating a higher risk for groups which usually have smaller serving sizes. In the same way were not included differences in the sensibility across the two populations,

among which there are individuals with different susceptibility which may result in differences in the illness severity.

The results of this QMRA confirm, as seen in other risk assessments, how fresh cheeses, in this case artisanal made goat milk cheeses, represent a risk for listeriosis. The median risk for the high-risk population was $2.23 \cdot 10^{-5}$ in the baseline scenario while $2.24 \cdot 10^{-3}$ in the alternative scenario, while for the general population was $2.04 \cdot 10^{-7}$ and $2.05 \cdot 10^{-5}$ for the baseline and alternative scenario respectively. A mean of 890.47 (± 1855.07) cases of listeriosis per 10,000 servings were predicted for the high-risk population and a mean of 10.84 (± 24.23) for the general population, with some cases in which the entire high-risk population was predicted to fall ill. The alternative scenario yields a higher risk of listeriosis showing a positive correlation between the initial concentration and the risk of listeriosis. Anyway, the median value of risk being extremely small in all populations and scenarios, shows how the distribution of risk is not normally distributed but more skewed to the right, indicating that even if the mean risk is high this is due to some more extreme simulations, while most simulations predict a lower risk for the consumers, data which well supports the numbers we see in real outbreaks. Nevertheless, the mean value of risk is of good use in giving us the idea of risk proportion between the two scenarios and populations.

Various QMRAs evaluated the risk of listeriosis connected to consumption of cheeses(32,38,39,53–55), including fresh cheeses. Campagnollo et al. (32) performing a QMRA for fresh Minas cheeses observed a mean risk of listeriosis of 0.34 for the general population and 0.49 for the high-risk population, values more than 300% higher than those reported in this work on the other hand Soto-Beltran et al. (56) reported lower risk for consumption on queso fresco in the Mexican region of Culiacan, $9.03 \cdot 10^{-9}$ for the general population and $1.72 \cdot 10^{-4}$ for the sensible population, with similar results reported by Condoleo et al. (39) in raw sheep's milk cheeses in Italy, $3.53 \cdot 10^{-10}$ for healthy and $1.58 \cdot 10^{-8}$ for the high-risk populations. Anyway, such comparisons should be considered with care, since those QMRA are developed under different assumptions which can greatly influence the

outcomes, furthermore, these authors evaluated products and consumption habits that may differ from one geographical area to another, generating even more differences in the risk outcomes.

In this work were evaluated some risk-mitigation strategies which were mainly aimed to the consumers handling of the product, since as also reported by other authors (37,55) those are the most impacting phases for risk generation. Reducing the shelf-life of the product was the most effective way of reducing the risk of listeriosis between the scenarios simulated and is the easiest way of intervention given the complexity of acting on consumers refrigerators temperatures, anyway care must be taken when reducing a product shelf-life in order to avoid unnecessary food waste. Campagnollo et al. (32) evaluated as mitigation strategy the addition of LAB with anti-listerial properties to Minas fresh cheeses during production with results ranging from a risk reduction of 1.5 to 4.6 fold, this option was not evaluated in this QMRA but it may be a good option to implement in this type of product to reduce the risk.

The goal of this QMRA was to evaluate the risk of listeriosis connected to consumption of artisanal goat milk fresh cheeses in Andalusia, some of the data used in the model were derived from other regions or assumed, the filling of these data gaps would be a great benefit for the models' predictions, as well as the development of a predictive model for *L. monocytogenes* in this specific product.

Conclusions

This work evidence how the contamination with *L. monocytogenes* may generate a risk for the consumers, especially high-risk consumers. Since goat milk fresh cheeses are usually made of pasteurized milk, the contamination is mainly due to post-pasteurization stages, and due to the ability of the pathogen to grow well in this type of product the good manufacturing practices and the control of the productive environment are fundamental to reduce the risk, since these practices reduce the initial concentration of the pathogen. Furthermore, intervening with a reduction of the shelf life or with an implementation of domestic refrigerators temperature is effective on reducing the risk of listeriosis. Even if this QMRA would benefit of more specific data and predictive models, it represents

a first approach of estimating the risk connected to this product and gives an insight on the most relevant stages for risk-mitigation, helping to improve the risk management.

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Chapter 3

Methodology for obtaining robust data of microbial responses in food

The aim of this chapter is to present a standardized data production methodology for obtaining robust data of microbial responses in food, to be used in the development of microbial predictive models. The reliability of predictive models is of utmost importance, particularly when used in the context of risk assessments. Since the development of a predictive model is mainly based on data, these two elements are closely linked, especially when it comes to their quality, so good quality data is fundamental to provide a reliable predictive model whose prediction can therefore be trusted.

Overall, using standardized data production methodologies to create predictive models improves repeatability and transparency in the predictive microbiology community. Therefore, procedures that encourage data production via standardized techniques are important tools for the modeling process. This chapter[2] provides a description of the data generating processes used for the creation of predictive microbiology models, including the methods for gathering information on both growth and inactivation/survival.

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[2] Notice: the following chapter represents the pre-print author's version of a work that has been submitted for publication in a book on Basic Protocols in Predictive Food Microbiology and written in collaboration with Antonio Valero, Andrea Serraino, and Aricia Possas. The chapter has been edited according to the formatting used for the remaining dissertation. Changes resulting from the publishing process, such as editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes could be made to this work before its acceptance for publication.

Introduction

The development of predictive models is based on observations of microbial responses in foods or culture media systems *(1)*. When evaluating the effect of intrinsic factors (e.g., pH, a_w, antimicrobial compounds) on the microbial responses in a food product or culture media, different formulations are obtained by adjusting these factors to values set at the experimental designs, by adding salt, organic acids, preservatives and so on. These foods or modified media are then artificially contaminated with the target microorganism and exposed to a series of environmental conditions of interest (e.g., temperature, modified atmosphere) for a given time. These environmental conditions are usually representative of the food production processes, including killing steps such as heat and high-pressure treatments *(2)*, and/or logistic distribution chain steps like storage at retail and household environments.

Collection of high-quality data, which rely mainly on experimental designs and microbiological methods, is essential to build efficient and accurate predictive models *(1)*. Experimental designs will depend on factors such as the purpose of the model to be developed and the economic resources available to generate data. Microbiological aspects, which englobe strain selection, inoculum preparation, inoculation, and quantification methods, will depend on a myriad of factors which include the objective of the study, limit of detection/quantification of the microbial analysis technique and the target microorganism and food product evaluated.

The number of strains, source and the physiological state of the microorganism are important factors to be considered when preparing inocula for challenge tests to obtain data for model development. Once the strains are chosen, different inoculation methods can be applied for samples contamination, depending on the food type, structure, and physical state. For instance, for food powders such as powder milk, dry inoculation methods have been tested *(3)*, while for solid and liquid foods such as liquid milk, wet inoculation methods have been employed *(4)*. Moreover, depending on the objective of the study, inoculation of solid foods can be performed on food surfaces by spreading or spraying the inoculums, whereas in other cases the inoculum can be introduced into the internal part of the

product with the aid of syringes or pipettes. Regarding liquid and paste foods, inoculation is usually carried out by homogenizing the product with the inoculums.

The classical plate count techniques have been the most widely applied methods for data collection in predictive microbiology and to date there are many ISO methods specific for detection and quantification of different microbial groups in foods *(1)*. Overall, the use of standardized methods for data generation to develop predictive models increase reproducibility and transparency within the predictive microbiology community. Hence, the development of protocols which foster data generation using standardized methods are valuable resources for the modelling process.

This chapters presents a description of the protocols applied for data generation for predictive microbiology models development, including procedures for obtaining both growth and inactivation/survival data.

1. Inoculation methods

In this section, relevant aspects related to the inoculum used for obtaining microbial data for model development are described, including strains selection, maintenance, inoculum preparation and levels inoculated.

1.1. Strains selection

The strains used in challenge tests to generate data for model development should be clearly identified and characterized through previous biochemical, and/or serological, and/or genetical methods *(5)*. The strains should be selected from the ones isolated from the evaluated food matrices (raw materials, ingredients, and final product), production environment or clinical, food, and environmental samples involved in outbreaks. In all cases the following requirements must be met:

- Original sources of the strains must be known.
- Strains must be properly identified and characterized.
- Strains must be available in national or international culture collections for future use.

To allow applying the gamma concept for growth modelling *(6)*, strains for which the cardinal parameters are known (e.g., minimal, optimum and maximum temperature, pH and a_w for growth) should be preferred whenever possible *(4, 7)*. Besides, previous knowledge on the cardinal parameters allows selecting strains which can grow within the ranges of environmental conditions considered in the study.

To account for the variability between strains which can be found in processing environments, a mixture of 3 to 5 strains of the same microbial specie is usually used in growth potential studies. However, in growth kinetics studies to estimate growth parameters in foods (e.g., growth rates) only one strain shall be used per challenge test *(5)*.

Surrogate microorganisms may be used in place of specific pathogens, for example in case the tests are carried out into a processing facility where for safety reason it is not possible to use pathogenic microorganisms. An ideal surrogate is a strain that retain all the characteristics of the pathogen except for the virulence and usually is closely related but not necessarily the same species of the target pathogen. If a surrogate microorganism is to be used, preliminary work should be done to characterize the strain before use. Some of the characteristics requested to a surrogate are *(8)*:

- Nonpathogenic.
- Similar behavior to the target pathogen when exposed to parameters such as pH, a_w, temperature.
- Stable and consistent growth characteristics.
- Easily prepared to yield high density populations and easily enumerated.
- Easily differentiated from background microbiota.
- Inactivation characteristics and kinetics that can be used to predict those of the target microorganism.
- Genetically stable so that results can be replicated independently of laboratory or time of experiment.

Examples of surrogate microorganisms used in predictive microbiology studies are listed in Table 1.

Pathogenic strain	Surrogate	Food/Media	Process	Reference
Escherichia coli O157:H7	*Escherichia coli* ATCC 25922	Apple cider	UV treatment	Quintero-Ramos et al. (16)
Escherichia coli O157:H7	*Escherichia coli* ATCC 25922	Apple cider	UV treatment	Duffy et al. (17)
Salmonella enterica	*Enterobacter aerogenes* B199A	Alfalfa seeds	Growth	Liu et al. (18)
Salmonella	*Enterobacter faecium* NRRL B-2354	Peanuts	Heat treatment	Casulli et al. (19)
Salmonella Typhimurium	*Salmonella Typhimurium* LT2 ATCC 700720	Cantaloupe	Growth	Chimbombi et al. (20)
Listeria monocytogenes	*Listeria innocua*	Poultry	Heat treatment	Li et al. (21)
Listeria monocytogenes	*Listeria innocua*	Parsley	Heat treatment	Miller et al., (22)
Listeria monocytogenes	*Listeria innocua*	Fermented sausages	High pressure	Bonilauri et al. (23)
Clostridium botulinum spores	*Clostridium sporogenes* PA 3679 spores	Ground beef	High pressure	Zhu et al. (24)
Clostridium botulinum spores	*Clostridium sporogenes* spores	Nutrient broth	Growth	Khanipour et al. (25)
Clostridium botulinum	*Clostridium sporogenes*	Ground beef	Heat treatment	Hong et al. (26)

Table 1: Examples of modelling studies in which surrogate microorganisms were used.

1.2. Strains maintenance

National and international reference laboratory (e.g., European Union Reference Laboratories), or culture collections (e.g., Spanish Type Culture Collection, American Type Culture Collection, see Table 2) if necessary, may provide appropriate strains to the laboratory that runs the test. These strains are usually obtained in the lyophilized form and may be activated following provider instructions.

The selected strains should be held in local to enable future testing if required, keeping them in culture broth supplemented with glycerol (20-25 %) or using cryopreservation beads immersed in cryo-solutions to prevent damage whilst freezing and to improve the survival during long term storage of microorganisms. In all cases the selected strains must be stored at temperatures ≤ -20 °C.

Culture collection	***Website***
American Type Culture Collection(ATCC)	https://www.atcc.org
Spanish Type Culture Collection (CECT)	https://www.uv.es/uvweb/spanish-type-culture-collection/en/spanish-type-culture-collection-1285872233521.html
Culture Collection University of Goteborg (CCUG)	https://www.ccug.se
Collection de L'Institut Pasteur (CIP)	https://www.pasteur.fr/en/public-health/biobanks-and-collections/collection-institut-pasteur-cip
National Collections of Industrial Food and Marine Bacteria (NCIMB)	https://www.ncimb.com
China General Microbiological Culture Collection Center (CGMCC)	http://www.cgmcc.net

Table 2: Example of culture collections

1.3. Inoculum preparation

Standardization of the preparation of the inoculum is particularly important to be able to inoculate the food matrix at the expected/desired concentration.

For the preparation of the vegetative cell suspensions, two successive cultures of the chosen strains shall be conducted. The first culture may be carried out in a medium under conditions to enable optimal growth of the strains, reaching the end of the exponential phase or the early stationary phase. The second culture is meant to adapt the strains to the natural conditions of the food, so should be

conducted in a medium mimicking those conditions (at least temperature, pH, a_w) again until the end of the exponential phase or the early stationary phase.

In case of fate studies (growth potential studies), if inoculation is performed in end products to evaluate microbial behavior during their storage at different conditions, it is convenient to consider the adaptation of bacterial cells to stress conditions that may take place during the production processes of the selected products (e.g., HPP treatment as part of inoculum preparation). To this end, strains shall be subjected to treatments that mimic food production processes, e.g., injury and/or stress to induce adaptation. Examples are treatment with hydrochloric acid to injury bacterial cells, and heat, cold or lactic acid stress *(5)*. If growth kinetics are being studied, there is no need to induce any adaptation on the selected strain *(5)*.

After this adaptation step, centrifugation and resuspension in a non-growth promoting diluent (e.g., phosphate buffer solution) is needed to avoid adding nutrients that could interfere with the microbial behavior *(5)*.

In case of using a multi-strain cocktail, aliquots of grown cultures of each individual strain selected stress-adapted or not should be transferred to a sterile tube and each strain must be present at the same concentration on the final cocktail.

The subcultures of the strains selected for the test should be carried out in advance, and the total volume of the inoculum suspension should be large enough to inoculate all the units with a sample – inoculum ratio not exceeding 1:100 *(5)*.

1.4. Inoculum level

The inoculum concentration used depends mainly on the objective of the study, namely, to determine growth or inactivation/survival of the microorganisms. Moreover, the inoculum concentration shall be justified and adjusted in accordance with the expected microbial behavior in the food matrix. Usually, for challenge tests in which microbial growth in the food matrix is expected, an inoculum level ranging from 10^2 to 10^3 colony-forming units (CFU)/g is used. It is important to consider that the product formulation could lead to a death of the strains before their adaptation and that also an

excessive concentration could overwhelm the stability of the product leading to an incorrect conclusion about the growth potential. Thus, these aspects should be considered when selecting the inoculum concentration.

Conversely, in fate studies where microbial survival or inactivation patterns are expected (e.g., heat treatments), the inoculum level shall be increased up to 10^5–10^7 CFU/g to enable the proper evaluation and characterization of the microbial reduction trend during the test, avoiding reaching, in samples, microbial concentrations below the detection limit over the period considered. In any case the inoculum concentration should be within the quantification limit of the enumeration method used. Determination of microbial suspensions counts shall be carried out, using for example the plate count method, the optical density at 420-660nm, or McFarland Turbidity Standards *(9)*, to aid in calculating the dilutions necessary to achieve the target inoculum in the product *(5, 8)*.

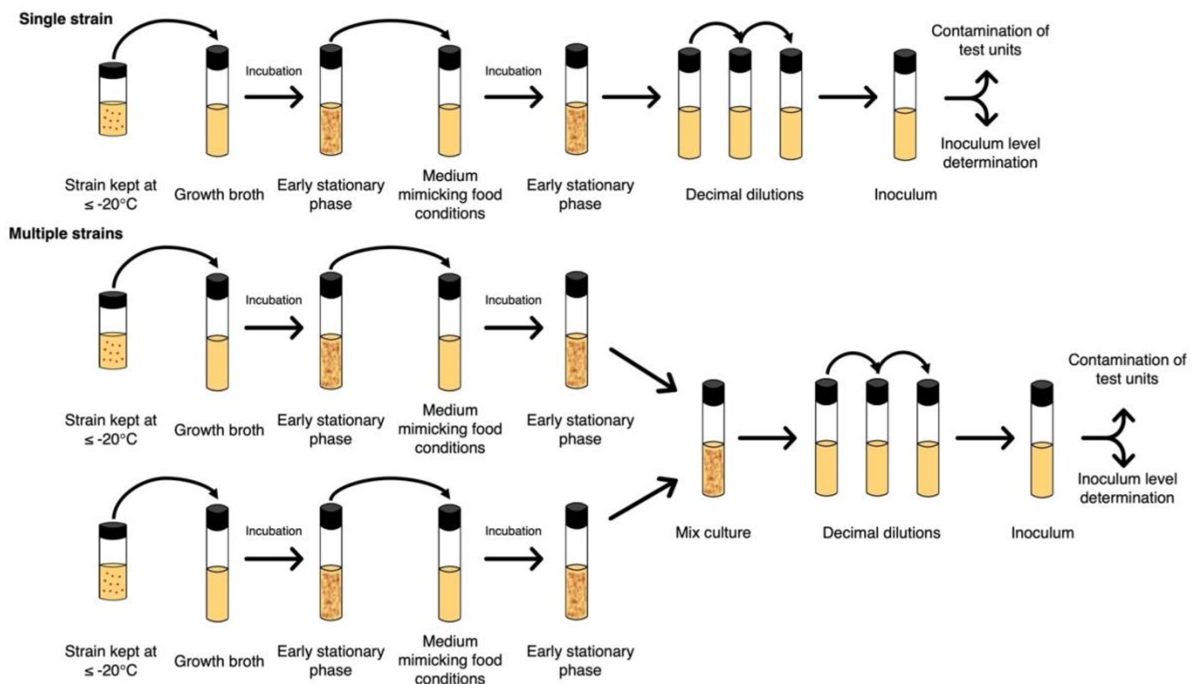

Figure 1: Inoculum preparation.

1.5. Inoculation techniques

The aim of this step is to achieve the required level of microorganism in each test unit without modifying the physico-chemical properties of the matrix (e.g., pH, a_w). Before inoculation, food

samples shall be adapted to the initial temperature of the test. The matrix can be inoculated on the surface, mimicking contamination during production process (e.g., slicing of smoked salmon), or in depth for homogeneous foods like ground food or creams, or for food prepared mixing different ingredients (e.g., mixed salad).

There are various inoculation methods that can be applied depending on the type of product being tested. For example, in liquid matrices with high a_w (> 0.96), the inoculum could be directly inoculated with mixing, using a negligible amount of carrier, and adjusting its a_w to that of the matrix. In solid matrices with high a_w (> 0.96), such as fresh meat or fish, the use of an atomizer represent a good option. In this technique, the inoculum is suspended in water or buffer and sprayed into the ground product or on the surface. This procedure should be done using devices to ensure personal safety (e.g., cabinet) and using the minimum amount of liquid possible. It is also possible to inoculate the sample using sterile pipettes, distributing the inoculum on the surface or into the ground product (e.g., in cases where it is not possible to ensure the personal safety spraying the inoculum) *(8)*.

For products with lower a_w (< 0.92), there are two options for inoculation. The first uses an inoculum in aqueous form, an example is the atomizer technique, above described, used paying special attention to using a minimal volume of the carrier solution, also applying a drying period after inoculation, or mixing the wet inoculum with a small amount of food sample, called a "seed" inoculum, which is then mixed with a larger food sample. The second option is the dry inoculation, suggested for products with even lower a_w (≤ 0.65), in which the inoculum can be prepared by suspension of the challenge microorganisms in water or buffer and adding it to sterile flour, or powdered product and allowed to dry until it reaches extremely low a_w (≤ 0.5). Alternatively, drying techniques such as the use of vacuum desiccator or freeze-dryer, spray dryer, or fluidized bed dryer can be applied to the microbial culture, adding protective agents (e.g., sucrose, skim milk, etc.) to increase the microbial survival. The dried inoculum then can be added to the product, under agitation for an even distribution. Despite the longer and more labor intensive procedure, dry inoculums allow a more uniform distribution of microorganism, with minimal impact on the properties of the food matrices*(8, 10)*.

Moreover, product can be inoculated through the packaging using a needle and some type of self-sealing rubber or silicon septum *(11)*. The package atmosphere is an important extrinsic factor to consider when inoculating food products. Ideally, the product should be first inoculated and then packaged under a condition that best mimic the ones used during commercial production (e.g., vacuum packaging, modified atmosphere packaging (MAP)), including the use of the exact gas mix used for MAP, same packaging material, and similar vacuum levels. However, in some cases the inoculation previously to packaging is not possible, and the use of syringe for inoculation have been reported in different studies.

2. Controls

Controls are essentials to assist in the proper microbial data interpretation and to understand the influence of the evaluated factors on microbial evolution. They can be used to evaluate relevant physico-chemical properties like pH, a_w, organic acid concentration, concentrations of other preservatives, background microbiota or/and added starter cultures.

Two kinds of controls are usually used: control units and food controls. For the preparation of control units, the same inoculation procedure applied for the test units may be performed using a non-growth promoting diluent, free of the pathogenic cells. It is recommended to analyze control units at least at the first (t_0) and the last (t_{end}) sampling point of the fate study, although it is better to analyze at least one control unit per sampling point. Food controls on the other hand are non-inoculated food units and are used to verify if the inoculation process modifies the physio-chemical properties of the food and/or to evaluate the natural contamination of the target microbial population or of the background microflora in the food matrices.

3. Replicates and number of units

Depending on the variability of the production process and food characteristics, i.e., intrinsic (e.g., pH, a_w, preservatives), extrinsic (e.g., gas composition) and microbiological properties, a different

number of replicates, using different batches, shall be used in the challenge study. The batches variability should be representative of the production process, based on historical data.

If the variability between different batches in the process is sufficient ($\Delta\varphi_{pHaw}$ or $\Delta\psi > 0.2$) to produce differences in microbial behavior, more than one batch is necessary to account for the variability in microbial responses *(12)*. In this case, at least three different batches should be used.

For growth rate studies, the "Inter-Batch Physico-Chemical Variability calculator" (URL: http://standards.iso.org/iso/20976/-1/ed-1/en) provided with ISO 20976-1:2019, can be used to assess the impact of inter-batch variability. Moreover, a detailed description of inter-batch variability assessment, based on pH and a_w, is described within the ISO 20976-1:2019. The use of one batch shall be justified (e.g., using the Calculator, using a "worst case" batch, etc.). Replicates should be independent trials using different batches of product and inoculum to account for variations in product, inoculum, and other factors *(4, 5, 11)*.

The number of analytical units (AU) greatly depends on the experimental design, mainly the duration, since a representative sampling regime need to be covered. The sampling interval, and so the number of AU, should be defined based on prior experience with similar products and in consideration of the likely duration of survival or rate of growth or inactivation and on the shelf life of the product. Depending on the product characteristics and expected outcomes for products with a long shelf life, it may be appropriate to test on a more frequent basis early in the study (e.g., daily) and at longer intervals later in the study. Anyway, a minimum of five to seven points are required to obtain an accurate description of the studied microbial behavior.

A minimum of two AU shall be analyzed at each time interval, during processing or storage, if possible, analysis of three or more AU shall be preferred.

Sources of intra-batch variability need to be considered when selecting the number of AU. Generally, the number of samples and replicates should be increased in situations of higher variability or uncertainty, but in cases where data from other studies exist the need for replication may be reduced.

Moreover, statistical experimental design (e.g., power analysis) can improve the validity of the study *(4, 5, 11)*.

4. Microbial enumeration

Microorganisms ‘quantification may be achieved in various ways: through inoculation of solid or liquid media, by real-time polymerase chain reaction, etc. In this chapter we’ll cover just the inoculation of solid media, being this the one suggested for general quantification test by the ISO 7218:2007/Amd.1:2013*(13)*.

For microbial detection and quantification, internationally accepted and validated protocols for the specific microorganism shall be applied (see examples in Table 3).

Microorganism	Enumeration method
Enterobacteriaceae	ISO 21528-2:2017(27)
Aerobic Mesophilic Bacteria	ISO 4833-1:2013(28) ISO 4833-2:2013/Corr.1:2014(29)
Coagulase-Positive Staphylococci	ISO 6888-1:2021(30)
Clostridium perfringens	ISO 7937:2004(31)
Lactic Acid Bacteria	ISO 15214:1998(32)
Thermotolerant *Campylobacter*	ISO 10272-2:2017(33)
Listeria spp.	ISO 11290-2:2017(34)
Salmonella spp.	ISO 6579-1:2017(35) ISO 6579-1:2017/AMD 1:2020(36)
Escherichia coli	ISO 16649-1:2018(37)
Yeast & molds	ISO 21527-1:2008(38) ISO 21527-2:2008(39)

Tables 3: Examples of enumeration methods by the International Organization for Standardization (ISO).

4.1. Sample preparation

Quantification of microorganisms is usually performed using Analytical Units (AU) of 25 g (25 mL) of the sample, a minimum of 10 g (10 mL) is suggested according to ISO 6887-1:2017*(14)*. Before withdrawing the AU, the sample should be well homogenized to ensure the representativity of the portion withdrawn.

The subsequent step is to dilute the AU with the appropriate diluent before plating into culture media. The recommended initial dilution is 1:10, obtained by adding *n* grams or milliliters of AU to 9 x *n* mL of diluent (e.g., 25 g/mL into 225 mL).

In the case of solid foods, a homogenization step is necessary to obtain a suspension that can be used for the subsequent analysis. Usually, the AU is transferred into a sterile bag, an amount of diluent necessary to obtain a 1:10 dilution is added and homogenized by agitation in a peristaltic homogenizer (stomacher) for 1 to 2 minutes (soft or pasty foods, ground or minced foods, poorly soluble powders). Alternatively, for hard foods homogenization can be done using sterile jars in a blender avoiding excessive heating of the AU. For liquid foods this homogenization process is not necessary since it is possible to use the food matrix as it is for the analysis, anyway, in case of viscous fluids it can be achieved by agitation in a flask containing the amount of diluent necessary for a 1:10 dilution.

In cases the number of microorganisms in the AU is expected to be high, a series of 10-fold dilutions to reduce the number of cells per unit of volume are necessary to allow quantification. The number of necessary dilutions depends on the expected microbial load and should allow counting between 25-30 and 250-300 CFU per plate *(14)*. Dilutions are carried out using saline peptone water or buffered peptone water. An example of this procedure is shown in Figure 2.

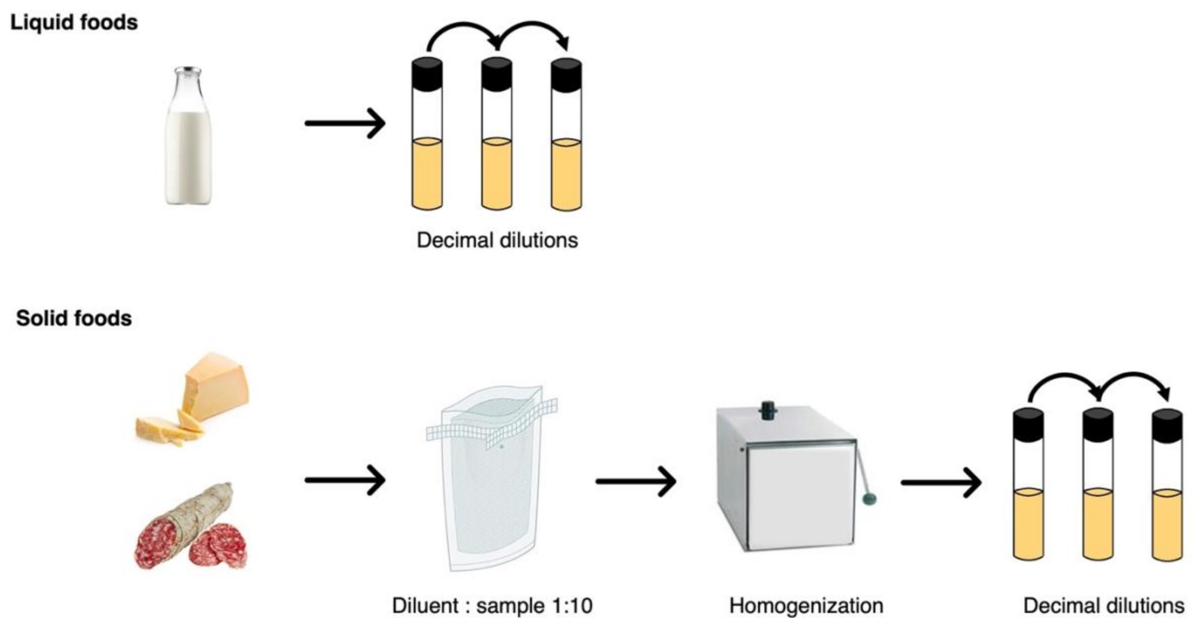

Figure 2: Sample preparation.

4.2. Enumeration techniques

Depending on the physiological characteristics of the microorganisms, expected levels to be encountered in the AU and the limit of quantification, different microbial enumeration techniques can be employed. The most applied ones are the pour and the spread plate techniques (Figure 3).

4.2.1. Pour plate technique

This technique has a detection limit of 10 CFU/g for solid and 1 CFU/mL for liquid food, when 1-mL aliquots of the homogenized AU are used for plating. Inoculating grater volumes distributed over several Petri dish (up to 2 mL per 90 mm Petri dish) allows to achieve a detection limit of 1 CFU/g for solid products.

The molten culture media should be rapidly cool down and kept in a water bath at a temperature of 44 °C to 46 °C until use. Attention should be paid when removing the tempered agar medium from the water bath, drying the bottle with a clean towel to prevent water from contaminating the plates. The molten agar medium at 44°C to 46°C must be poured into each Petri dish containing the AU homogenate (generally, 18 mL to 20 mL of agar in 90 mm Petri dishes to obtain at least 3 mm thickness) avoiding excessive agitation of the medium to prevent bubble formation. The molten

medium may not be poured directly onto the aliquot of diluted AU. The time elapsed between pipetting the aliquots of diluted AU and pouring the agar media shall not exceed 15 min, to avoid aggregation of colonies. Immediately after pouring, the molten medium and the aliquot shall be carefully mixed to obtain a homogeneous distribution of the microorganisms within the medium, e.g., by gently moving the dish backward and forwards, from side to side, and in a circular direction, and allowed to cool and solidify placed on a cool horizontal surface. Then incubation times, temperature, and atmosphere required by each microorganism can be found in the specific ISO methods (Table 3) *(13)*.

4.2.2. Surface plate technique

This standard procedure has a detection limit of 100 CFU/g for solids and 10 CFU/mL for liquids, but can be adapted, if necessary, to a detection limit of 10 CFU/g for solid products or 1 CFU/mL for liquid products, by inoculating 1 mL of the initial suspension onto the surface of three different 90 mm Petri dishes. For this method, the use of pre-poured plates with agar medium of at least 3 mm of thickness, level and free from air bubbles and surface moisture, is required.

The standard procedure uses 0.1 mL of the appropriate dilutions of the AU, which should be inoculated into the agar plates using a sterile pipette. Glass or plastic spreader (Drigalski) are usually used to spread the inoculum onto the entire surface of the medium as fast as possible, without touching the sidewalls of the Petri dish and ensuring a uniform distribution over the surface until all aliquot is

absorbed by the medium. Finally, plates must be dried before incubation, with the lid on for 15 minutes at room temperature *(13)*.

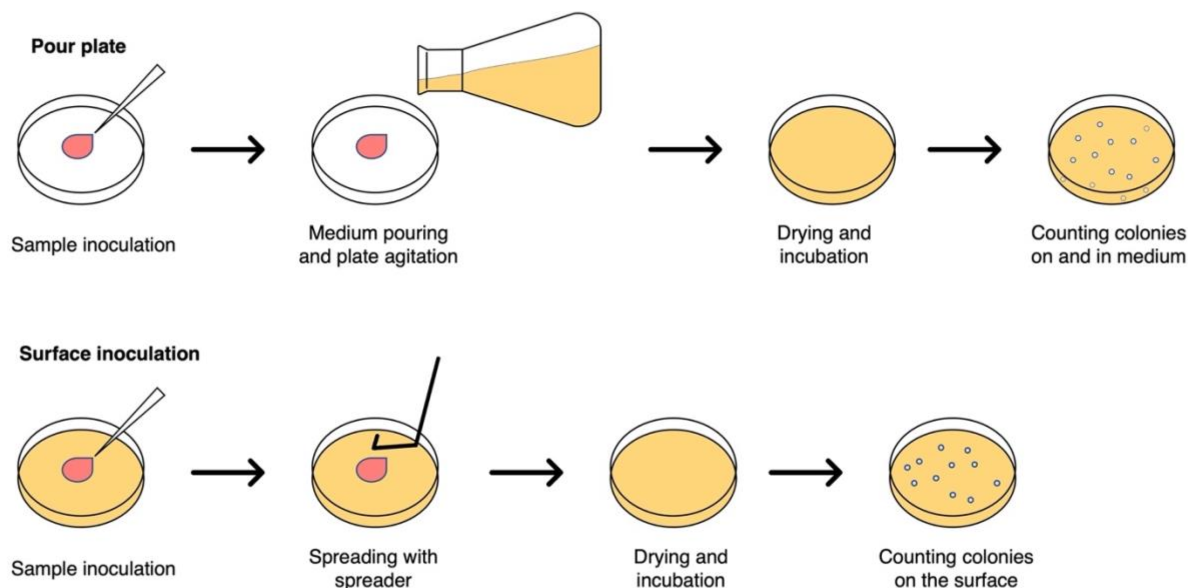

Figure 3: Enumeration techniques.

4.3. Calculation and expression of results

ISO 7218:2007/Amd.1:2013 requires the inoculation of one plate per dilution, of at least two successive dilutions, or two plates per dilution if only one dilution is used. The use of two plates per dilution may also be applied to improve reliability and it is mandatory for laboratories that do not operate under quality assurance principles. If two or more dilutions are used, the count at a given dilution should be approximately 10 % of the previous/following, with an upper limit of 15.6 % and a lower limit of 5.2% *(15)*.

The maximum number of colonies acceptable to count does not exceed 300 in 90 mm Petri dishes, while the minimum number of colonies shall not be less than 25. When dishes with a diameter different from 90 mm are used, the maximum number of colonies shall be increased or decreased in proportion to the surface area of the dishes.

The general rule for counting total or typical colonies reported in ISO 7218:2007/Amd.1:2013 is described by Equation 1.

$$CFU/g \ or \ CFU/mL \ = \frac{\Sigma C}{v \times [n_1 + (0.1 \times n_2)] \times d} \quad \text{Equation 1}$$

where:

S C = sum of colonies (or typical colonies) counted on the plates selected for counting from two successive dilutions.

v = volume inoculated on each plate (usually 0.1 mL for spread plating or 1 mL for pour plating).

n_1 = number of plates counted from the first dilution selected (usually 1 if no replicate was made or 2 when duplicate was made).

n_2 = number of plates counted from the second dilution selected.

d = first dilution retained for counting ($10^0 = 1$, $10^{-1} = 0.1$, $10^{-2} = 0.01$).

The ISO 7218:2007/Amd.1:2013 also reports other ways of counting CFU in unusual situations, such as plates with less than 10 or more than 300 colonies and establishes exponential notations for the presentation of the results.

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Chapter 4

Developing and update of a predictive model

This chapter deals mainly with objective 2 of this dissertation, addressing the process necessary to obtain reliable models to apply in QMRA, and partially with the evaluation of risk-mitigation solution to ensure food safety, in detail, reports an example of the development of a predictive model, more precisely the update of a previously developed model and its validation.

This work published on Meat Science in 2021, describes the fate of *Salmonella* spp. in Italian *salami* during the production process and high-pressure processing (HPP), a linear regression model was used to describe the decay of the microorganism. Furthermore, compliance with the 5-log reduction policy necessary to export to the U.S. was assessed for the process combined with HPP.

Salmonella is one of the most common causes of foodborne illnesses in the world. According to the Centers for Disease Control and Prevention (CDC), human salmonellosis cases result in about 1.2 million illnesses, 23,000 hospitalizations, and 450 fatalities in the United States (U.S.) each year and about one million of these illnesses are caused by food (57). In the European Union (EU) a total of 60.050 confirmed human salmonellosis cases were reported by member states in 2021, resulting in a notification rate of 15.7 cases per 100,000 population (2).

In swine abattoirs around the world, *Salmonella* is regularly found in fecal samples, feces, lymph nodes, the surroundings, and carcasses. *Salmonella* can contaminate raw materials used to make fermented meat products at the slaughterhouse, during the manufacturing process, and in the post-processing phases (58–65). Today, pig meat and its products continue to be a substantial source of human infection from *Salmonella* and are responsible for 9.7% of outbreaks with strong evidence, placing them sixth among the top 10 pathogen/food vehicle pairs that cause outbreaks with strong evidence (2). *Salmonella Typhimurium* is the most frequent serovar concerning animal-based food contamination (Ferrari et al., 2019) and the second serovar involved in outbreaks (Gossner et al., 2012; Scavia et al., 2013; Andreoli et al., 2017).

Several *Salmonella* outbreaks have been connected to ready-to-eat (RTE) pork meat products, including salami, sausages, and chorizo (66–72).

Salmonella Compliance Guidelines for Small and Very Small Meat and Poultry Establishments Manufacturing RTE Products (73) said that companies making RTE meat must validate their method to achieve at least a 5-log decrease in *Salmonella* spp (73). Cooking is the most common method for achieving the target, but other non-thermal inactivation procedures such as fermentation, drying, salt curing, alternative processing technologies, or a combination of these are permitted(73); the same requirement is considered necessary for exporting meat products to the United States (74). Scientific literature aids manufacturers in determining the efficacy of methods in minimizing foodborne infections; nonetheless, a large variety of fermented sausages with highly variable processing conditions exist. Furthermore, within the same type of fermented sausages, variances in the procedure may occur between distinct establishments, making the applicability of data collected from research studies challenging. Moreover, challenge studies, one of the most reliable tools available to assess the efficacy of the applied methods in reducing pathogens, can be very challenging in terms of time, money, and human resources.

Since scientific literature showed that the required 5-log reduction of *Salmonella* is not achievable by the fermented sausages production process alone (75), other methods need to be applied to conform to this limit. High-Pressure Processing (HPP) is a non-thermal food preservation technique that inactivates pathogens and vegetative spoiling organisms. At refrigeration or mild process temperatures (45°C), HPP treats liquid and solid meals to pressures ranging from 400 to 600 with little impact on flavor, texture, appearance, or nutritional value (76). HPP has been shown to lower *Salmonella* load in a variety of foods, including RTE meat (77).

In 2019, a model to predict the fate of *Salmonella* in fermented sausages during production and HPP treatment was developed by Bonilauri and colleagues (78). In their study, twenty challenge tests on nine different fermented sausages from six producers were conducted to analyze and record the following parameters: time, temperature, pH, a_w, and *Salmonella* counts. A linear regression model

was used to describe the *Salmonella* spp. decay: at the end of the process, the total *Salmonella* reduction was 0.97-5.84 log CFU/g, and it was significantly associated with pH at the end of the acidification/drying process, a_w at the end of the seasoning period, seasoning duration, and salami caliber. High-Pressure Processing (HPP) further reduced the *Salmonella* level by 2.41-5.84 log CFU/g, with an efficacy that was inversely related to the a_w reduction of salami after seasoning; in all cases tested the goal of 5-log reduction was always met by the manufacturing process plus HPP.

The goal of the work presented in this PhD thesis is to update the model described above, the linear regression model, used to describe the *Salmonella* spp. decay during the production process and HPP treatment, was further studied. To accomplish this, 19 additional fermented sausage challenge tests were conducted, involving three extra companies in addition to the ten previously involved, and six additional salami varieties in addition to the nine already examined (Pepperoni small caliber, Flattened pepperoni, Milano small caliber, Hungarian, Hungarian small caliber, and Garlic). In the univariate analysis, the findings of the new tests revealed that *Salmonella* spp. log CFU/g reduction was associated with pH after the acidification/drying process, a_w at the end of the seasoning period, the duration of seasoning, and the caliber of salami. The pH at the end of the acidification/drying process, a_w at the end of the seasoning period, and the duration of seasoning remained significant ($p < 0.001$) in the multivariate analysis, with a coefficient of determination $R^2 = 0.485$. This new model better describes the reduction of *Salmonella* spp. compared to the previous one within previous and recent challenge tests. The model was then validated using 8 supplementary challenge tests and calculating the Accuracy factor (Af) and the Bias factor (Bf). The Bf determines whether the predicted values are, on average, above or below the line of equivalence and, if so, by how much. As a result, it determines if the model is “fail-safe” or rather “fail-dangerous”; the Af averages the “distance” between each point and the line of equivalence to determine how close predictions are to observations on average. Thus, the accuracy factor is a measure of average deviation and can be used as a basic indicator of one's level of confidence in the model's predictions. A Bf of 1 indicates perfect agreement between predictions and observations; on the other hand, a bias factor of 1.1 indicates not only that

the model is "fail-dangerous", because, for example, it predicts longer generation times than are observed, but also that the predictions exceed the observations by 10% on average. A Bf less than one, on the other hand, implies that a model is generally "fail-safe", but a bias factor of 0.5 indicates a poor model that is unduly conservative because it predicts generation times that are half of what is observed. The Af, like the Bf, is a simple multiplicative factor that indicates the spread of data around the prediction. Thus, an Af of two shows that the predictions are, on average, a factor of two different from the actual value, i.e. half as large or twice as large, but a value of one indicates that all predicted and observed values agree perfectly (79)

The results of this validation showed the model to be safe, with a Bf < 0.95, and with good accuracy, with the predictions on average within 11% of observations (Af 1.11), overall, the production process reduced *Salmonella* spp. by 2.477 log CFU/g (± 0.898 SD), while the updated model estimated 2.476 log CFU/g (± 0.622 SD), with a mean residual of 0.002.

As in the work of Bonilauri et al (78), HPP was able to increase *Salmonella* spp. decay, with a mean reduction of 3.561 log CFU/g (± 0.637 SD); also, in this updated model, HPP efficacy was inversely associated with a_w decay at the end of seasoning.

The model presented here to describe the fate of *Salmonella* during the manufacturing of Italian salami was built considering the pH at the end of fermentation drying, the length of seasoning, and the a_w value before HPP. The model predicts the log reduction in *Salmonella* count throughout processing and is a useful tool for businesses and risk managers to assess the efficacy of processes to reduce *Salmonella* load. This model better predict the fate of *Salmonella*, being more precise than the previous with a mean of residuals a hundred times smaller (0.251 in the old model vs 0.002 in the updated one).

These results underline the dependency of predictive models from the data used to generate them, and, in this case, that increasing the number of data used to develop the model is of great use in improving its quality, especially when a great variability is present in the process and needs to be described by the model.

Reported below is the scientific publication resulting from this update on the model to describe the fate of *Salmonella* in Italian *salami* during production and HPP, in the form of a letter to the editor, in which more detailed results can be seen.

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Update on a model to describe *Salmonella* spp. population reduction in Italian salami during production and high-pressure processing

Dear Dr. Hopkins,

I am submitting this letter in order to update our recently published article entitled "Reduction of *Salmonella* spp. populations in Italian salami during production process and high-pressure processing treatment: validation of processes to export to the U.S." in Meat Science Journal (Bonilauri et al., 2019).

In our previous work ten enterprises producing Italian salami were involved, and 20 challenge tests on different fermented sausages were performed in order to assess the reduction of *Salmonella* spp. during manufacturing and High-Pressure Processing (HPP). A linear regression model was used to describe the *Salmonella* spp. decay: the total *Salmonella* spp. reduction during manufacturing was significantly associated with pH at the end of acidification/drying process, a_w at the end of the seasoning period, the duration of seasoning and the caliber of salami. HPP showed to further reduce the *Salmonella* spp. count with an efficacy that resulted inversely associated with a_w of salami at the end of seasoning. The objective of 5-Log reduction was always reached after manufacturing and HPP in all the tested salami types.

We further studied the linear regression model used to describe the *Salmonella* spp. decay during the production process and high-pressure processing treatment. To do so, 19 additional challenge tests on fermented sausages were performed, adding three enterprises to the ten previously involved, and six supplementary salami types to the nine already tested (Pepperoni small caliber, Flattened pepperoni, Milano small caliber, Hungarian, Hungarian small caliber and Garlic). The results of the new tests confirmed that *Salmonella* spp. Log Colony Forming Units (CFU)/g reduction was associated with pH at the end of the acidification/drying process, a_w at the end of the seasoning period, the duration of seasoning, and the caliber of salami in the univariate analysis. In the multivariate analysis pH at the end of the acidification/drying process, a_w at the end of the seasoning period and the duration of seasoning remained significant ($p < 0.001$) with a coefficient of determination ($R2 = 0.485$).

An updated model that better encompasses all variables was obtained:

$$\Delta s = 33.06725 + 0.0193796\ T2 - 2.4978\ pH\ 2 - 20.211\ a_w3$$

where: pH 2 = pH at the end of the acidification/drying process; a_w3 = a_w at the end of the seasoning period and T2 = the duration of seasoning.

Compared to the previous model, the new one describes more accurately the decay of *Salmonella* spp. Log reduction within the previous and the latest challenge tests. Overall, the production process showed a mean *Salmonella* spp. reduction of 2.477 Log CFU/g (SD 0.898), the mean decay predicted by the updated model was 2.476 Log CFU/g (SD 0.622), with a mean of residuals of 0.002.

A validation of the updated model was performed using eight challenge tests further enrolled in 2020 (Table 1) following the methods proposed by Ross (1996), calculating the Accuracy factor (Af) and the Bias factor (Bf) of the model. The Af indicates to what extent the prediction of the model differs from the observed data, thus providing an indication of the goodness of fit. The closer the value of Af is to 1, the better the model makes the description of the experimental data. The Bf indicates whether the predicted data are numerically above or below the experimental data, thus marking a structural deviation of the model. The model showed to be safe (Bf <0.95) with an Af of 1.11 which means that

Table 1
validation of the predictive model updated from Bonilauri et al., 2019 with the calculation of bias and accuracy factors.

	Variables			Observed Δs (log)	Predicted Δs (log)	Predicted/ Observed	Log (pred/obs)	Absolute value
Salami type	pH 2	Aw3	Ts (days)					
Felino low salt	4.89	0.9	30	3.31	3.24	0.980	−0.009	0.009
Pepperoni	4.88	0.923	35	2.78	2.90	1.043	0.018	0.018
Pepperoni	4.93	0.904	30	3.19	3.06	0.960	−0.018	0.018
Pepperoni large caliber	4.86	0.912	80	5.21	4.04	0.776	−0.110	0.110
Pepperoni	4.91	0.92	35	2.59	2.89	1.114	0.047	0.047
Milano	4.44	0.924	45	4.6	4.17	0.907	−0.042	0.042
Smoked Hungarian	4.79	0.929	62	4.27	3.53	0.826	−0.083	0.083
Pepperoni small caliber	4.98	0.918	19	2.27	2.44	1.075	0.032	0.032
						Bf*	−0.021	0.045
							0.95	
						Af*	1.11	

*Af = Accuracy factor; *Bf = Bias factor.

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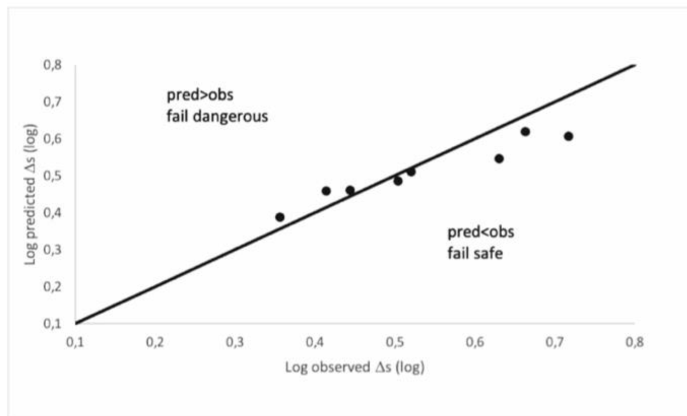

Fig. 1. Predictions of the updated model of Bonilauri et al. (2019) for *Salmonella* spp. describing the *Salmonella* spp. decay (Δs) observed at the end of the seasoning period in Italian salami compared to independent *Salmonella* spp. decay data obtained in late 2020. The diagonal line is the line of identity. Points above this line represent decay predictions smaller than the observed and are hence considered 'fail-safe' predictions. Conversely, points below the line of identity are 'fail-dangerous' predictions. The data are detailed in Table 1.

the predictions are, on average, within 11% of the observation. (See Fig. 1.)

As previously observed HPP was able to further reduce *Salmonella* spp. count, mean 3.561 Log CFU/g (SD 0.637); in the new model, HPP efficacy was inversely associated with a_w at the end of seasoning, confirming the previous observations, but without giving any better description.

Processing, in combination with HPP, led to a 5 Log *Salmonella* spp. decay.

This new model, being more precise than the previous (mean of residuals of the first model applied to all the dataset 0.251> > 0.002 mean of residuals with updated model), shows to be safe with a good accuracy in validation step, representing a better tool for enterprises and Authorities for evaluating the efficacy of the processes and HPP to reduce *Salmonella* spp. load.

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Chapter 5

Novel solutions to control environmental contamination and to reduce microbiological risks

This last chapter face the objective of evaluating risk-mitigation solution to ensure food safety along the food chain. As discussed in previous chapters, the contamination of the producing environment may generate risks for the consumers and the control of this environment is fundamental to reduce the risks, reason why applying cleaning and disinfection practices to reduce the initial concentration or to assure absence of the pathogens is crucial in the risk management process. In this work published in 2021 on the Italian Journal of Food Safety, alkaline electrolyzed water, as low environmental impact and safe for the user solution was evaluated for its use in the food industry to reduce microbial loads on working surfaces.

Electrolyzed water (EW) has recently gained popularity as a novel disinfection and cleaning solution. EW is produced by electrolysis of a salt solution, typically NaCl n (2 g/L), when electricity is applied to the solution, two types of water are produced: the cathode produces alkaline electrolyzed water (REW) containing sodium hydroxide (pH 11,6; ORP -795 mV), and the anode produces acidic electrolyzed water (EOW) containing hypochlorous acid (pH 2.4-2.7; ORP 1150 mV); the concentration of residual chlorine depends on the EW machine setting (80,81).

The EOW has been widely employed in different industries as an effective yet safe-to-handle sanitizer, as a disinfectant for food processing equipment on various materials (stainless steel, glass) (80,82), or directly on food (vegetables, poultry, eggs, fish) (83–85). The REW has not seen as widespread use as EOW and has been primarily regarded as a waste, but some studies have shown that it can be used in a variety of ways within the food industry, particularly as a cleaning solution; additionally, it has been demonstrated that the combined use of REW and EOW improves microbial load reduction (81,83,85). In this work was tested an alkaline electrolyzed water (REW) obtained from Aquasol S.r.l (Bologna, Italy), which was generated through the electrochemical process described above using reverse osmosis water and potassium carbonate (K_2CO_3) as electrolyte. The

final product composition is 99.83% pure water and 0.17% potassium hydroxide (KOH) with a pH of 12.2-12.5 and an oxidation – reduction potential of -40/-90 mV. The experimental design was aimed at evaluating its efficacy in reducing, in experimentally contaminated stainless-steel plates, the load of four different pathogens commonly connected with food infections, two Gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*) and two Gram-negative (*Escherichia coli* and *Salmonella* spp.). The ultimate aim was to evaluate its possible application under operating conditions as a low-risk solution for the user but still effective in reducing the risk of food contamination through the environment.

The results of this work evidenced an overall ability of REW to reduce the contamination in the experimental conditions, thus with different degrees of microbial load reduction in the different tests with the various pathogens. More in detail Gram-positive bacteria showed a significantly ($p < 0.01$) higher resistance to the treatment with REW, being reduced on average by 1.78 (± 1.21 SD) log UFC/cm^2, compared to Gram-negative, which were reduced on average by 3.49 (± 1.31 SD) log UFC/cm^2 with a high frequency of no bacterial recovery from plates after treatment. These results were in accord with those of other authors, which evidenced a higher resistance of Gram-positive bacteria to treatment with REW (86) and slightly acidic electrolyzed water (SAEW) (87,88), and with the widespread knowledge of the higher resistance of Gram-positive to sanitizing treatment, probably due to the increased thickness of the cell wall. While these are just preliminary results, in literature no other work evaluated the effectiveness of REW in reducing pathogens loads from stainless-steel surfaces, at least as the only treatment applied, since there is evidence of its effectiveness in combination with slightly acidic electrolyzed water (89)

Although its application in a more operational perspective is still to be tested, its efficiency as a detergent, the absence of risks for users, the lack of environmental pollutants, combined with its effectiveness in reducing the microbial load in experimental conditions could adapt to its use in the food industry to reduce the risk of contamination in particular production processes, despite the not so competitive price compared to other products. Moreover, in a larger perspective, even if the use of

a product reducing the contamination of the food processing environment by pathogens is of great interest when evaluating risk-mitigations solutions, often these solutions may pose collateral risks for consumers, as the presence of contaminants in the final products, regarding this, REW, being composed by salt and water and to which returns rapidly after use, reduces this additional risk.

Further studies on REW may be directed in evaluating it in operative condition, as well as modeling the fate of specific microorganisms when treated with it in order to, for example, include this step in a QMRA, and also, evaluating its effectiveness in reducing the formation of biofilms in the food chains.

A detailed report of the methodologies, results, and discussions is attached below in the form of a published scientific paper.

Effectiveness of alkaline electrolyzed water in reducing bacterial load on surfaces intended to come into contact with food

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Abstract

Alkaline electrolyzed water (REW) is known for its cleaning action. The aim of this work was to assess REW effectiveness in reducing microbial load on surfaces intended for contact with food. Stainless-steel surfaces were experimentally contaminated, bacterial inactivation was tested before and after treatment with REW. Treatment with REW was operated spraying it on the contaminated plates until drying. Tests were conducted for *Salmonella* spp., *Listeria* spp., *Staphylococcus aureus* and *Escherichia coli*. The treatment revealed different degrees of sanitizing activity of REW on different bacterial species, with higher efficacy on *E. coli* and *Salmonella* spp. than *S. aureus*, *Listeria* spp.. Statistical analysis revealed a significant microbial load reduction ($p<0.01$) after treatment with REW, suggesting that it has a good disinfectant activity which, along with its easy and safe use, makes it a good alternative to many other more widely used disinfectants.

Introduction

Foodborne pathogens are currently estimated to be responsible for one third of human diseases in the developed world. In Europe during 2019, 27 Member States reported 5,175 food-borne outbreaks involving 49,463 cases of illness, 3,859 hospitalizations and 60 deaths: 45.7% were caused by bacteria and bacterial toxins and mainly linked to consumption of food of animal origin (EFSA and ECDC, 2021).

Although animals and humans are a major source of bacterial food contamination, in the food industry, products are often biologically contaminated through contact with the surfaces of equipment, shredders, slicers, and cutting boards (Fukuzaki *et al.*, 2004; Serraino *et al.*, 2010). Procedures aiming at reducing or even eliminating pathogens from surfaces are one of the key points of an effective HACCP program in the food industry and also in controlling food contamination in homes, food markets, restaurants, health facilities and public areas (Venkitanarayanan *et al.*, 1999).

Recently, electrolyzed water (EW) has been receiving attention as a novel disinfectant and cleaning solution. Electrolyzed water is obtained from the electrolysis of a salt solution, generally NaCl (≈2 g/L). When electricity flows through the solution two types of water are generated: the cathode produces alkaline electrolyzed water (REW) containing sodium hydroxide (pH 11.6; ORP ≈ -795 mV), while the anode produces acidic electrolyzed water (EOW) containing hypochlorous acid (pH 2.4-2.7; ORP≈1150 mV); the concentration of the residual chlorine depends on the EW machine setting (Fukuzaki *et al.*, 2004).

As a potent yet safe-to-handle sanitizer, the EOW has been easily applied into various industries, as disinfectant for food processing equipment either on various materials (stainless steel, glass) (Park *et al.*, 2002; Serraino *et al.*, 2010), or directly on food (vegetables, poultry, eggs, fish) (Athayde *et al.*, 2018; Fabrizio *et al.*, 2002; Huang *et al.*, 2008). The REW has not seen as widespread use as EOW and has been mainly considered a waste, but some research have demonstrated that it can be employed in different manners within the food industry, in particular as a cleaning solution; moreover it has been demonstrated that the combined use of REW and EOW enhances the microbial load reduction (Athayde *et al.*, 2018; Fukuzaki *et al.*, 2004; Huang *et al.*, 2008). The aim of this work was to evaluate the efficacy of the REW in reducing the microbial load on experimentally contaminated stainless-steel surfaces intended for contact with food, to define its possible use in operating conditions as a sanitizer as well as a detergent of surfaces.

Materials and methods

Alkaline electrolyzed water (REW) was obtained from Aquasol S.r.l (Bologna, Italy), generated through an electrochemical process that uses reverse osmosis water and potassium carbonate (K_2CO_3) as electrolyte. REW is made up of 99.83% pure water and 0.17% of potassium hydroxide (KOH) with a pH of 12.2-12.5 and an oxidation – reduction potential of -40/-90 mV. Chemical characteristics, like pH and oxidation-reduction potential (ORP), were measured with a pH-meter (FiveGo F2, Mettler – Toledo, Greifensee, Switzerland), using LE427 and LE510 electrodes to measure pH and ORP, respectively.

Four tests were performed to evaluate the disinfectant activity on *Salmonella* spp., *Listeria* spp., *Staphylococcus aureus* and *Escherichia coli*.

A mix of three different strains was used for each test (Table 1), strains were selected including one reference strain and two strains isolated from animal production chains.

For each test eight stainless steel plates were used, representing eight repetitions for each bacterial species: an area of 100 cm^2 was defined on the plates previously sterilized. Each plate was contaminated with a 10^{11} UFC/ml suspension of the above-mentioned microorganisms, using a sterile swab, to a total of about 10^4 UFC/cm^2 in the plate. The 100 cm^2 area was divided by two and, after drying, half of the plate was sampled with a sterile sponge, to evaluate the pre-treatment microbial load. REW was then applied with a low-pressure pump, left to dry for about 15 minutes and the second half of the plate was sampled through sterile sponge, to evaluate the microbial load after

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treatment. In detail, sterile sponges were rubbed over the previously described areas 10 times and then put in a sterile plastic bag containing 100 mL of sterile saline solution. After mixing in a stomacher (BagMixer®, Interscience, St Nom, France) 0.1 mL of the sample obtained and 0.1 mL of four serial 10-fold dilutions were seeded in plastic Petri dishes containing PCA and incubated at 37°C for 24 h. After incubation, colonies were identified with matrix-assisted laser desorption/ionizationtime-of-flight (MALDI-TOF, Bruker, Massachusetts, USA) and then counted.

Results were expressed as log UFC/cm², and statistical analysis was made by paired T-test, one-way ANOVA and Tukey post-hoc test ($p \leq 0.01$).

Results

Treatment of artificially contaminated surfaces with REW revealed different degrees of microbial load reduction in different tests.

Overall, a 2.64 (±1.52 SD) log UFC/cm² reduction was observed, resulting in a significant ($p<0.01$) microbial reduction. A minimum of 0.43 log UFC/cm² in one out of the eight tests for *S. aureus* and a maximum of 5.63 log UFC/cm² in one of the eight tests for *Salmonella* spp.. In detail *Salmonella* spp. was reduced by 3.70 (±1.35 SD) log UFC/cm², *E. coli* by 3.29 (±1.35 SD) log UFC/cm², *Listeria* spp. by 2.41 (±1.22 SD) log UFC/cm², *S. aureus* by 1.16 (± 0.90 SD) log UFC/cm² on average. Details of the results are reported in Table 2 and Figure 1.

Discussion

REW is well known as detergent, dissolving fats and proteins. Some authors have suggested that using REW as pre-treatment before using disinfectants might enhance the action of the latter (Ayebah *et al.*, 2005; Fabrizio *et al.*, 2002; Jiménez-Pichardo *et al.*, 2016), therefore the cleaning action of REW could improve its disinfectant efficacy by removing organic material that could shelter microorganisms.

In this work, treatment with REW led to a significant ($p<0.01$) overall reduction of microbial load compared to the pre-treatment samples. Results showed different degrees of microbial load reduction on different bacterial species. Specifically, REW showed to be significantly ($p<0.01$) effective on *Salmonella* spp., *E. coli, S. aureus* and *Listeria* spp.. Moreover, it appeared to be significantly ($p<0.01$) more effective on Gram negative bacteria (*Salmonella* spp., and *E. coli),* with an average microbial reduction of 3.49 (±1.31 SD) log UFC/cm² and a high occurrence (75%) of no microbial growth after treatment, being instead less effective on gram positive microorgan-

Table 1. Bacterial strains used and their biological matrices of origin.

Microorganism species	Strain	Biological matrices of origin
Escherichia coli	ATCC 25922	
Escherichia coli	VeLaBac 444	Bovine faeces
Escherichia coli	VeLaBac 445	Meat
Salmonella Thyphimurium	ATCC 14020	
Salmonella Thyphimurium monophasic	118174/1	Pork sausage
Salmonella Derby	106463/1	Pork meat
Listeria monocytogenes	ATCC 15313	
Listeria innocua	257529/1	Pork sausage
Listeria innocua	257529/2	Pork meat
Staphylococcus aureus	ATCC 25923	
Staphylococcus aureus	B/122/2	Raw milk
Staphylococcus aureus	22-7-16/2	Goat skin

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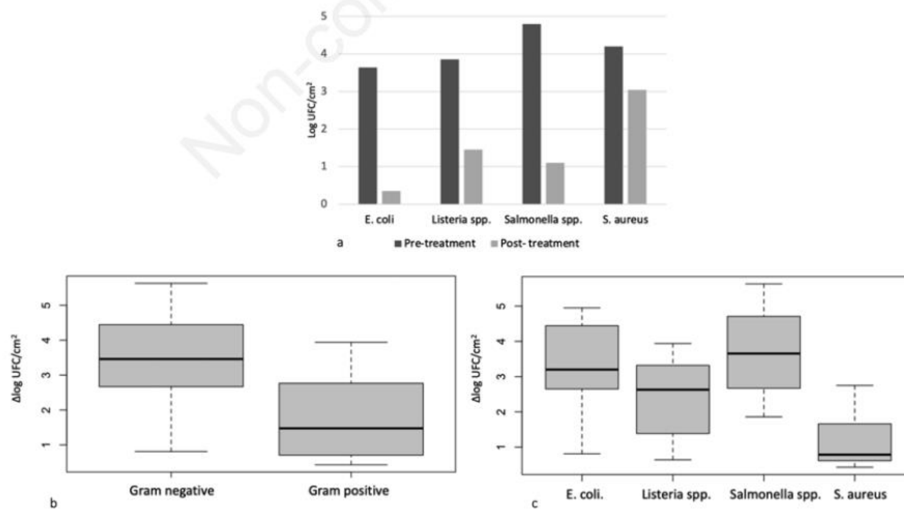

Figure 1. a) Mean Bacterial counts before and after treatment with REW for each microorganism and mean microbial load reduction values (Δlog UFC/cm²) for b) gram-negative and gram-positive bacteria, and c) each microorganism.

Table 2. Bacterial counts before and after treatment with REW, and microbial reduction expressed as mean (±SD), minimum and maximum (Δlog UFC/cm²).

Microorganism	Pre – treatment Log UFC/ cm² mean (±SD)	Post- treatment Log UFC/ cm² mean (±SD)	Microbial reduction ∅Log UFC/ cm² mean (±SD)	Minimum microbial reduction ΔLog UFC/ cm²	Maximum microbial reduction ΔLog UFC/ cm²
Escherichia coli	3.64 (± 0.79)	0.35 (± 0.98)	3.29 (± 1.35)	0.81	4.95
Salmonella spp.	4.80 (± 1.29)	1.10 (± 1.58)	3.70 (± 1.35)	1.86	5.63
Listeria spp.	3.85 (± 0.49)	1.45 (± 1.61)	2.41 (± 1.22)	0.64	3.94
Staphylococcus aureus	4.20 (± 0.20)	3.04 (± 0.90)	1.16 (± 0.90)	0.43	2.75

isms (*S. aureus* and *Listeria* spp.) with an average microbial reduction of 1.78 (±1.21 SD) log UFC/cm². There's large evidence in literature of the major resistance of Gram positive bacteria to sanitizing treatments than Gram negative bacteria, and being the major difference between the two groups the thickness of the cell wall, responsible for preserving the integrity of the cell, also the results of this work suggest that the wall thickness may play a role in the REW bacterial inactivation efficacy (Koike *et al*., 2009; Mai-Prochnow *et al*., 2016; Vollmer *et al*., 2008).

Other authors found similar results when testing slightly acidic electrolyzed water (SAEW) and strongly alkaline electrolyzed water, with Gram positive bacteria showing relatively more resistance to the treatment than Gram negative bacteria (Issa-Zacharia *et al*., 2010; Koike *et al*., 2009; Tango *et al*., 2015). In addition, different microorganisms may have different sensibility to sanitizers, for example *Listeria* spp. is generally more resistant to chlorine than *Salmonella* spp. and *E. coli* (Burnett & Beuchat, 2000).

In literature no other work evaluated the efficacy of REW alone in reducing the microbial load on stainless steel surfaces; other authors evaluated its efficacy in association with SAEW and ultrasound to improve the sanitation of knives in the meat industry, but due to the different treatments applied we could not compare the data (Brasil *et al*., 2020).

It is important to notice that the tests underwent microbial load conditions that are not the ones typical of working conditions. Indeed, contamination levels in the food industry are usually much lower than those considered in this work, especially considering pathogenic microorganisms. In addition, in this work it was considered just the REW action, while in operating conditions it could be associated to a mechanical bacterial removal action, that could make the treatment even more effective.

Economically REW is not competitive with the most common commercial sanitizers, yet, besides its efficacy as detergent and disinfectant, REW is safe to handle, bearing no risks for the users and has low environmental impact, being composed by 99.83% pure water and 0.17% of potassium hydroxide (KOH) and not containing environmental pollutants, moreover, its instability makes unnecessary to rinse surfaces with water after its use and hence saving time during working activities (Athayde *et al*., 2018; Fabrizio *et al*., 2002; Huang *et al*., 2008). All this makes REW a suitable alternative to the most common commercial formulation for use in some particular productions process.

Conclusions

The results of this study showed its efficacy in reducing the microbial load on stainless-steel surfaces under experimental conditions, giving a good perspective on its use as sanitizing under operative conditions on surfaces intended for contact with food.

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Conclusions

Each chapter in this thesis is "self-conclusive," or designed to be read independently as a thorough exposition of the topic, of the techniques employed and outcomes produced, as well as their analysis and conclusions. Therefore, the goal of this general conclusion is to integrate all the studies presented in the body of this thesis and draw general conclusions about their significance in relation to its goals. In this thesis the methodology of QRA applied to food safety under different aspects was addressed: firstly describing the process of application of QRA for chemical and microbiological hazards to target specific questions and to propose effective solutions; secondly deepening the composition of QMRA, through the inspection of one of its structural elements, the predictive models, aiming to make knowledge about this tool more accessible and facilitating its use in QMRA; and lastly producing evidences, both using the outputs of QRA or of specific researches, on risk reduction strategies to be applied to ensure safety of consumers.

The first objective of this thesis was to apply QRA along different food chains, with a focus on foods of animal origin. The analysis of data on AFM1 concentration in milk was a retrospective approach to the application of QRA to the Italian milk supply chain. This approach allowed the comparison of the effectiveness of different risk-based sampling plans, already in force, in reducing the risk for consumers. The application of a QCRA permitted the assessment: first of the exposure of the consumers to AFM1 through the analysis of consumption data and concentrations of the contaminant in milk, secondly of the impact of this exposure on different population categories, finding the most sensitive to this hazard, and finally this quantitative approach gave the opportunity to numerically evaluate the difference in efficacy of two risk-mitigation strategies applied by the industry to embrace consumer safety. This permitted to assess how more stringent sampling plans, based on the actual risk, were able to reduce the risk for consumers, even the most sensitive, to below or almost below the concern level, giving evidence that can be used by the industry or by the Authorities to take risk-management decisions. In the same context, the application of a QMRA to the Spanish supply chain

to assess the risk connected with consumption of artisanal fresh cheeses was a way of tackling the microbiological risk posed by *L. monocytogenes* using the knowledge about the food chain. This allowed to first assess whether this pathogen/food pair pose an actual risk and secondly predict which step of the food chain would impact more the risk for the consumers.

The application of predictive microbiology in this path was fundamental, giving the ability to predict the behavior of the pathogen in the food and allowing the estimation of exposure of consumers. This highlights the necessity of having always highly reliable predictive models, and thus the need of generating new and accurate data on pathogens behavior in foods to allow the developing of solid QMRAs. Specific results of this QMRA show how the implementation of good manufacturing practices, aimed at lowering the environmental contamination and consequentially the contamination of the products, have an important impact on the risk for consumers, especially when the product allows an easy growth of pathogens. This is also shown by the evidences provided by the epidemiological investigations conducted in recent outbreaks of *L. monocytogenes* in RTE foods, showing that often the episodes were due to incorrect sanitization of the equipment of the manufacturing companies (delicatessens, dairies, etc.) or at the retail vending stage, when manipulating or slicing the products (slicers, cutting boards, tables, etc.) (1). Furthermore, the QMRA gave evidence on how the consumers behavior is the most impacting factor on the risk, highlighting how consumers education is one of the first risk-mitigation strategies to be applied, but also how the knowledge of this can be used by the FBOs and the risk managers to take effective countermeasures. In fact, the use of QRA, allowing the simulation of various scenarios through the use of real data from the supply chain, offers many practical advantages to all the stakeholders: first supporting the FBOs by giving evidence of the impact of specific measures on the contamination of their products and thus helping them to set specific Performance Objectives (POs), Performance Criteria (PC), and Process and Product Criteria (PrC) to reach the Food Safety Objectives (FSOs); second helping the risk managers to set those FSOs in order to aim to the Appropriate Level of Protection (ALOP) through, for example, identification of potential risk factors contributing to food illnesses, determination of

risk factor safety threshold values and application of risk-mitigation solutions (2). Moreover, in the case of RTE foods, the FBO is required by EC Regulation 2073/2005 (3), to evaluate the physico-chemical characteristics of its product (i.e. pH and a_w values) to classify its ready-to-eat food among those a) able to support the growth of *L. monocytogenes*, or alternatively b) unable to support the growth of *L. monocytogenes* (1). Apart from this, in the regulation is also stated that the FBO can classify a RTE food as unable to support the growth of *L. monocytogenes* regardless of the physico-chemical characteristics providing solid scientific justification, using for example challenge tests but also predictive modeling as additional studies (1). Furthermore, to comply with the criteria established by the EC Regulation 2073/2005 (3), each FBO is bound to evaluate the effectiveness of its systems in all stages of production, processing, and distribution of the foods subject to their control and to implement in its HACCP system preventive measures and/or corrective actions, to avoid non-compliance such as failure to comply with a microbiological criterion (1). In this sense, the use of QMRA, and more specifically microbiological exposure assessments along with predictive modeling, is very useful since allows to evaluate different scenarios and the efficacy of preventive/corrective measures without having to conduct several challenge tests but still providing authorities with scientific evidence. QRA, moreover, provides a solid foundation for increasing trade access to new markets by helping to objectively establish the absence of hazards or their effective control to create safe food. For instance, QRA identifies gaps and uncertainties in scientific knowledge on risks, which can assist determine research priorities and lead to a better understanding of food-related implications on public health in the long term. Moreover, the identification on data gaps on the food chain and on consumers behavior is essential to promote targeted surveys to fill those gaps. For all these reasons, QRA is incredibly useful as method for developing food safety control measures.

As previously stated, QRA are highly dependent on data and especially the modelling of microbial behavior on a QMRA through the farm-to-fork chain, which is an extremely complex process, relies on data availability and on validated predictive model present in scientific literature. The processes of development and validation of new and more robust predictive models able to describe more

accurately the microbial behavior in food, including an increasing number of variables (e.g. inputs from omics technologies), are of great importance, since the final output of the model is significantly influenced by the development process itself (4). The description of the most applied protocols for data generation was provided in the body of this thesis to try to improve the availability of information helpful to the process just mentioned, since the standardization of methodologies for generating data would benefit the predictive microbiology community, by increasing transparency and reproducibility and in return the whole food chain community by providing the means to better QMRA and risk management. In addition, these predictive models can be used in food processing industries as alternative to challenge tests, highly consuming in terms of time, human resources and money, supporting existing production processes and developing new ones, helping the increase of trades and the development of food manufacturers.

Finally, the development of new solutions to mitigate the risk along the food chain is an essential part of the risk management process, allowing to increase the efficiency of the actions implemented to counter specific hazards. The research of new technologies more sustainable for both the environment, the manufacturers, and the consumers is today of outmost importance given the competitiveness of the markets, the rapid changes in the climate, in the geopolitical situation, and in the consumers habits, to allow the FBOs to keep ensuring high quality and safety products. In this thesis several risk-mitigation solutions were presented, but more specifically in the last chapter, a recently developed solution with interesting characteristics in terms of sustainability for all the parts above mentioned, and effectiveness in terms of reducing the contamination of food-contact surfaces was described. Its potential use, after more advanced trials, seemed realistic and with concrete advantages in terms of risk reduction.

To conclude, this PhD thesis showed the relevance of QRA, and in its framework, but not exclusively, of predictive microbiology and of recently developed technologies to ensure food safety on a more integrated way. Nonetheless, the results of these QRAs are highly dependent on the availability of specific data, so further developments of this work may lead to improvements through the filling of

data gaps, the development of better models and the inclusion of new and more adequate risk-mitigation strategies.

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