CHILDREN EXPOSURE TO MULTIPLE MYCOTOXINS THROUGH FOOD CONSUMPTION:
A HOLISTIC APPROACH FOR RISK ASSESSMENT

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To my beloved grandparents, Alice and Zé...

May we meet again...

"Here is my secret. It is very simple: It is only with the heart that one can see rightly;

what is essential is invisible to the eye." – *Antoine de Saint-Exupéry*

FOREWORD

The present study was developed under the Doctoral Programme in Veterinary Sciences of University of Évora. The research work was mainly developed at the Food and Nutrition Department of the National Institute of Health Dr. Ricardo Jorge, Lisbon, Portugal (INSA) and also at the Department of Chemistry, Biotechnology and Food Science of Faculty of Veterinary Medicine and Biosciences, Norwegian University of Life Sciences, from September 2013 to December 2016. Additional training was obtained at National Food Institute of the Technical University of Denmark, Utrecht University, Wageningen University and Leiden University.

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The present thesis is organized into five main chapters. Chapter I pertains to the introduction and background of the thesis. Chapter II presents the justification, aims and objectives of the thesis. Chapter III presents the experimental work, gathering results and related objectives, organized in seven different studies corresponding to seven scientific papers, already published or submitted in international journals. Chapter IV presents the general discussion and Chapter V summarizes the main conclusions and future perspectives.

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"O valor das coisas não está no tempo que elas duram, mas na intensidade com que acontecem. Por isso, existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis." – *Fernando Pessoa*

Refletindo as palavras de Fernando Pessoa, sinto que o período do meu Doutoramento se revestiu de grande intensidade e de "momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis". Este período caracterizou-se pela aprendizagem, busca pelo conhecimento e aplicação do que aprendi. Tive a oportunidade de contactar com pessoas únicas pela forma como me acolheram e também pelo que me transmitiram. Sei, que apesar de sentidas, estas palavras serão insuficientes para reconhecer o quão importantes e significantes foram para mim e para o meu crescimento. Mas, como referido por São Francisco de Assis,

"Começa por fazer o que é necessário, depois o que é possível e de repente estarás a fazer o impossível" – *São Francisco de Assis*

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Para concluir, cito um pensamento que expressa o que sinto:

"Cada um que passa na nossa vida passa sozinho... Porque cada pessoa é única para nós, e nenhuma substitui a outra. Cada um que passa na nossa vida passa sozinho, mas não vai só... Levam um pouco

de nós mesmos e deixam-nos um pouco de si mesmos. Há os que levam muito, mas não há os que não levam nada. Há os que deixam muito, mas não há os que não deixam nada. Esta é a mais bela realidade da vida... A prova tremenda de que cada um é importante e que ninguém se aproxima do outro por acaso..." – *Antoine de Saint-Exupéry*

A todos os que passaram na minha vida, e me deixaram algo, que construiu a pessoa que sou hoje, a minha sincera e profunda gratidão!

ABSTRACT

CHILDREN EXPOSURE TO MULTIPLE MYCOTOXINS THROUGH FOOD CONSUMPTION: A HOLISTIC APPROACH FOR RISK ASSESSMENT

Food ingestion is considered a major route of human exposure to chemical contaminants, namely mycotoxins. Considering their toxic and carcinogenic effects, mycotoxins exposure assessment assumes particular importance, especially when vulnerable populations as children, are involved. Although there are increasing evidences of mycotoxins co-contamination in food, scarce data are available concerning children exposure to multiple mycotoxins, their bioaccessibility and the potential toxic effects resulting from intestinal exposure. Addressing these considerations, this thesis main objectives were: to characterize Portuguese children (under 3 years old) exposure to multiple mycotoxins through food consumption; to determine mycotoxins bioaccessibility in foods usually consumed by children; to evaluate intestinal toxic effects associated to mycotoxins exposure; and, to characterize the risk associated to the consumption of foods, considering data from exposure assessment, bioaccessibility and intestinal toxicity assays. The present thesis applied for the first time a holistic approach, gathering results obtained through different first-line methodologies, comprising probabilistic tools to estimate mycotoxins daily intake and *in vitro* assays to determine bioaccessibility and toxic effects. Results revealed a potential health concern for aflatoxins exposure with regard to the high percentiles of children intake. For the first time, a potential synergism was described relatively to the combined intestinal toxic effects of patulin and ochratoxin A, two known enterotoxins. The obtained results reinforce the importance to use a holistic approach to multiple mycotoxins risk assessment, especially for vulnerable populations as children. Particular attention should be dedicated to evaluate the consequences of intestinal exposure to mycotoxins, in particular to their impact on the development of intestinal diseases.

Keywords: Multiple mycotoxins, Risk assessment, Children, Food safety, Food toxicology

RESUMO

EXPOSIÇÃO DE CRIANÇAS A MÚLTIPLAS MICOTOXINAS ATRAVÉS DO CONSUMO DE ALIMENTOS: UMA ABORDAGEM HOLÍSTICA À AVALIAÇÃO DE RISCO

A ingestão de alimentos é considerada a principal via de exposição humana a contaminantes químicos, dos quais se destacam as micotoxinas. Em virtude dos seus efeitos tóxicos e carcinogénicos, é fundamental avaliar a exposição a estes compostos, particularmente em populações vulneráveis, como é o caso das crianças. Nos últimos anos, alguns estudos têm dedicado especial atenção à co-contaminação de alimentos por micotoxinas. No entanto, são ainda escassos os dados disponíveis relativos à exposição das crianças a múltiplas micotoxinas assim como a sua bioacessibilidade e potenciais efeitos tóxicos a nível intestinal. Neste contexto, a presente Tese teve como principais objetivos: caracterizar a exposição das crianças portuguesas (idade inferior a 3 anos) a múltiplas micotoxinas através da alimentação; determinar a bioacessibilidade das micotoxinas nos alimentos habitualmente consumidos pelas crianças; avaliar os efeitos tóxicos intestinais associados à exposição simultânea a micotoxinas; e, caracterizar o risco associado ao consumo de alimentos, coligindo resultados da avaliação da exposição, bioacessibilidade e toxicidade intestinal. A presente Tese desenvolveu pela primeira vez uma abordagem holística, reunindo resultados obtidos através de diferentes metodologias, nomeadamente ferramentas probabilísticas para estimar a ingestão diária de micotoxinas e ensaios *in vitro* para determinar a bioacessibilidade e efeitos tóxicos. Os principais resultados revelaram, pela primeira vez, que a exposição a aflatoxinas pode representar um risco potencial para as crianças que apresentaram os percentis mais elevados de ingestão. Foram também descritos efeitos sinérgicos inéditos decorrentes da exposição intestinal simultânea a duas enterotoxinas: patulina e ocratoxina A. Os resultados obtidos reforçam a importância da implementação de uma abordagem holística para a avaliação de risco de misturas de micotoxinas, em especial quando envolvidas populações vulneráveis como as crianças. As potenciais consequências da exposição intestinal a micotoxinas deverão ainda ser ponderadas, principalmente no que respeita às implicações no desenvolvimento de doenças intestinais.

Palavras-chave: Múltiplas micotoxinas, Avaliação de risco, Crianças, Segurança Alimentar, Toxicologia Alimentar

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Food Chemistry (under revision)

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Single-compound and cumulative risk assessment of mycotoxins present in breakfast cereals consumed by children from Lisbon region, Portugal

Ricardo Assunção, Elsa Vasco, Baltazar Nunes, Susana Loureiro, Carla Martins, Paula Alvito

Food and Chemical Toxicology, 2015, 86, 274–281. http://doi.org/10.1016/j.fct.2015.10.017

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15-Ac-DON	15-acetyl deoxynivalenol
3-Ac-DON	3-acetyl deoxynivalenol
ADME	Absorption, distribution, metabolism (biotransformation) and excretion
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFM_2	Aflatoxin M_2
AFT	Aflatoxins
AJs	Adherence junctions
ALARA	As low as reasonably achievable
AMP	Antimicrobial proteins
BEN	Balkan Endemic Nephropathy
$BMDL_{10}$	Benchmark dose lower confidence limit 10
bw	Body weight
CA	Concentration addition
CAC	Codex Alimentarius Commission
CI	Combination index
CLDNs	Claudins
CYP	Cytochrome P450
DAD	Diode array
DAS	Diacetoxyscirpenol
DC	Dendritic cells
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
E. coli	*Escherichia coli*
EC	European Commission
ECD	Electron capture
EFSA	European Food Safety Authority

ELISA	Enzyme linked immunosorbent assays
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
FB_4	Fumonisin B_4
FD	Fluorescence
FID	Flame ionisation
FITC	Fluorescein isothiocyanate
GALT	Gut-associated lymphoid tissues
GC	Gas chromatography
GFR	Glomerular filtration rate
GIT	Gastrointestinal tract
GSH	Glutathione
HBGV	Health-based guidance values
HI	Hazard Index
HPLC	High-performance liquid chromatography
HQ	Hazard Quotient
HRP	Horseradish peroxidase
HT-2	HT-2 toxin
IA	Independent action
IARC	International Agency for Research on Cancer
IEC	Intestinal epithelial cells
IESC	Intestinal epithelial stem cell
JAM	Junctional adhesion molecule
LOD	Limit of detection
LOQ	Limit of quantification
LP	*Lamina propria*
LY	Lucifer yellow
M cells	Microfold cells
M. tuberculosis	*Mycobacterium tuberculosis*

MLC	Myosin light chain
MLCK	Myosin light chain kinase
MoA	Mode of Action
MOE	Margin of exposure
MOET	Combined Margin of Exposure Index
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
N/A	Not applicable
ND	Not determined
NIV	Nivalenol
NK cells	Natural killer cells
OCLN	Occludin
OTA	Ochratoxin A
PAT	Patulin
Phe	Phenylalanine
PMTDI	Provisional maximum tolerable daily intake
PODI	Point of Departure Index
PP	Peyer's patches
PTWI	Provisional tolerable weekly intake
RASFF	Rapid Alert System for Food and Feed
RNA	Ribonucleic acid
ROS	Reactive oxygen stress
RPF/TEF	Relative Potency Factors/Toxic Equivalency Factors
***S.* Typhimurium**	*Salmonella enterica* subsp. *enterica* serovar Typhimurium
T	Food-borne toxins
T-2	T-2 toxin
TDI	Tolerable daily intake
TDS	Total Diet Studies
TEER	Transepithelial electrical resistance
TJ	Tight junction
TLC	Thin layer chromatography
TUS	Toxic Unit Summation

UV	Ultraviolet
WHO	World Health Organization
ZEA	Zearalenone
ZO	Zonula occludens
α-ZOL	Alpha-zearalenol
β-ZOL	Beta-zearalenol

I. GENERAL INTRODUCTION

1. FOOD SAFETY AND FOOD CONTAMINANTS

There can never be an absolute guarantee that our food is safe. It is simply impossible to determine each single item for every imaginable toxin, contaminant, adulterant, or foodborne pathogen, not to mention that this would make our food prohibitively expensive (Borchers et al., 2010). Over the last years a significant number of food safety crises that included the bovine spongiform encephalopathy, dioxins, high pesticide and antibiotic content in several foods, high nitrate content, presence of coliforms in drinking water and formation of acrylamide, among others, occurred within the European Union (EU), resulting in losses of human lives and resources. These crises made EU citizens more alert but also considerably increased the EU legislative task in an attempt to undertake preventive instead of corrective measures (Arvanitoyannis et al., 2005). At European level, the EU integrated strategy aims to ensure a high level of food safety, animal health and welfare, and plant health, through coherent measures and adequate monitoring, while guaranteeing the effective functioning of the internal market. The EU food safety strategy considers a farm-to-fork approach, including all aspects of the food production chain, from and including primary production, animal feed production and sale or supply of food to the consumer. This approach involves the development of legislative and other actions in order to assure effective control systems, evaluate compliance with EU standards in the food chain within the EU and in third countries in relation to their exports to the EU, manage international relations with third countries and international organisations, manage relations with the European Food Safety Authority (EFSA), and ensure science-based risk management (Arvanitoyannis et al., 2005; Cheli et al., 2014; European Commission, 2016a). EFSA is a European agency funded by the EU that operates independently of the European legislative and executive institutions (Commission, Council and Parliament) and EU Member States. EFSA was set up in 2002 following a series of food safety crises in the late 1990s to be a source of scientific advice and communication on risks associated with the food chain. The agency was legally established by the EU under the General Food Law – Regulation 178/2002 (EC, 2002). The aim of this Regulation is to provide a framework to warrant a coherent approach in the development of food legislation. At the same time, this Regulation acts as the milestone of the legislative structure in the field of feed and food legislation, providing a general framework for those areas not covered by specific harmonised rules, but where internal market functioning is ensured by mutual recognition (Cheli et al., 2014; EC, 2002). The General Food Law created an European food safety system in which responsibility for risk assessment (science) and for risk management (policy) are kept separate. EFSA is responsible for the former area and plays an important role in collecting and analysing data to ensure that European risk assessment is supported

by the most comprehensive scientific information available. Moreover, as the risk assessor, EFSA produces scientific opinions and advice that form the basis for European policies and legislation and has the duty to communicate its scientific findings to the public.

One of the main priorities of the EU is the control of possible associated risks regarding chemical contaminants in food and undesirable substances in feed. Although the term "chemical contaminant" is preferably used for food, whereas, in the case of feed the terminology more widely used is "undesirable substance", both refer to unintentionally present chemical substances that constitute a potential hazard for human/animal health or the environment (Silano and Silano, 2015). A plethora of chemicals from anthropogenic and natural origins enter animal feed, human food and water either as undesirable contaminants or as diet components. Anthropogenic contaminants of public and animal health importance include, amongst others, persistent organic pollutants (*i.e.*, dioxins, polychlorinated biphenyls, brominated flame retardants, perfluoroalkyl acids), Maillard reaction products (acrylamide, furans), phthalates, and pharmaceuticals. Also residues from production aids and chemicals authorised for use following a pre-marketing approval in food and feed productions such as pesticides/biocides, and food and feed additives are included (Dorne et al., 2009). Important classes of natural contaminants include heavy metals such as lead, cadmium, uranium, mercury; and metalloids such as arsenic and natural toxins produced by bacteria, protozoa, algae, plants, and fungi as mycotoxins (Dorne et al., 2009; Dorne and Fink-Gremmels, 2013). A key tool to ensure the cross-border follow of information to swiftly react when risks to public health are detected in the food chain is the Rapid Alert System for Food and Feed (RASFF). Created in 1979, RASFF enables information to be shared efficiently between its members (EU-28 national food safety authorities, Commission, EFSA, ESA, Norway, Liechtenstein, Iceland and Switzerland) and provides a round-the-clock service to ensure that urgent notifications are sent, received and responded to collectively and efficiently (European Commission, 2016b). According to the preliminary annual report by RASFF, the 2015-notifications by hazard category of chemical contaminants were mainly for mycotoxins, pesticide residues, heavy metals, food additives and flavourings and allergens (Table 1)(RASFF, 2015). Mycotoxins, within the chemical compounds, represented the main reason to notify RASFF.

Table 1. Top 10 of total 2015-notifications by hazard category and by classification in the EU (RASFF, 2015).

Hazard category	Alert[1]	Border rejection[2]	Information for attention[3]	Information for follow-up[3]	Total
Pathogenic micro-organisms	261	265	136	83	745
Mycotoxins	74	388	29	4	495
Pesticide residues	24	292	71	18	405
Heavy metals	73	73	57	16	219
Food additives and flavourings	17	55	32	36	140
Allergens	114	3	18	2	137
Composition	51	19	22	26	118
Foreign bodies	43	23	14	30	110
Adulteration/fraud	1	89	3	6	99
Poor or insufficient controls	2	70	7	9	88

[1]Alert: when a food, feed or food contact material presenting a serious risk is on the market and when rapid action is or might be required in another country than the notifying country; [2]Border rejection: a consignment of food, feed or food contact material that was refused entry into the Community for reason of a risk to human/animal health or to the environment; [3]Information notification: food, feed or food contact material for which a risk has been identified that does not require rapid action either because the risk is not considered serious or the product is not on the market at the time of notification.

2. RISK ANALYSIS

Ensuring food safety to protect public health and promote economic development remains a significant challenge in both developing and developed countries. Considerable progress to strengthen food safety systems has been achieved in many countries, highlighting the opportunities to reduce and prevent foodborne disease. However, foodborne disease remains a real and formidable problem in both developed and developing countries, causing great human suffering and significant economic losses. Up to one third of the population of developed countries may be affected by foodborne diseases each year, and the problem is likely to be even more widespread in developing countries, where food and water-borne diarrhoeal diseases kill an estimated 2.2 million people each year, most of them children (FAO/WHO, 2006). During the past decades, most systems for regulating food safety were based on legal definitions of unsafe food, enforcement programmes for the removal of unsafe food from the market and sanctions for the responsible parties after the fact. These traditional systems cannot respond to existing and emerging food safety challenges because they do not provide or stimulate a preventive approach. In the last years, there was a transition to risk analysis based on better scientific knowledge of foodborne illness and its causes (WHO, 2002). Nowadays, risk analysis is considered a key discipline for further reducing foodborne illness and strengthening food safety systems (FAO/WHO, 2006). In the context of food safety, risk is defined as an estimate of the likelihood/probability of the occurrence of an adverse health effect

in humans, weighted for its severity that may result from exposure to a hazard(s) in food. A hazard is defined as a biological, chemical or physical agent in, or condition of, food with the potential to cause an adverse health effect (FAO/WHO, 2009). Risk analysis has been defined by Codex Alimentarius Commission (CAC) as a process consisting of three components: risk assessment, risk management and risk communication, which are themselves defined as follows (FAO/WHO, 2015, 2009): i) risk assessment: a scientifically based process consisting in a detailed analysis of the occurrence of known or potential adverse health effects from human exposure to food hazards; ii) risk management: a process of weighing policy alternatives in light of risk assessment and other factors relevant for the health protection of consumers, promotion of fair trade practices and selecting strategies of control and regulatory actions; and iii) risk communication: an interactive exchange of information and opinions concerning risk analysis process regarding management options and actions among risk managers, consumers and other interested subjects, including the explanation of risk assessment findings and the basis of risk management decisions. A formal description of the risk analysis process, that emphasizes the functional separation of its three components, while at the same time demanding the need for communication and interaction between those with responsibility for each of the three components, was represented in the risk analysis paradigm (Figure 1).

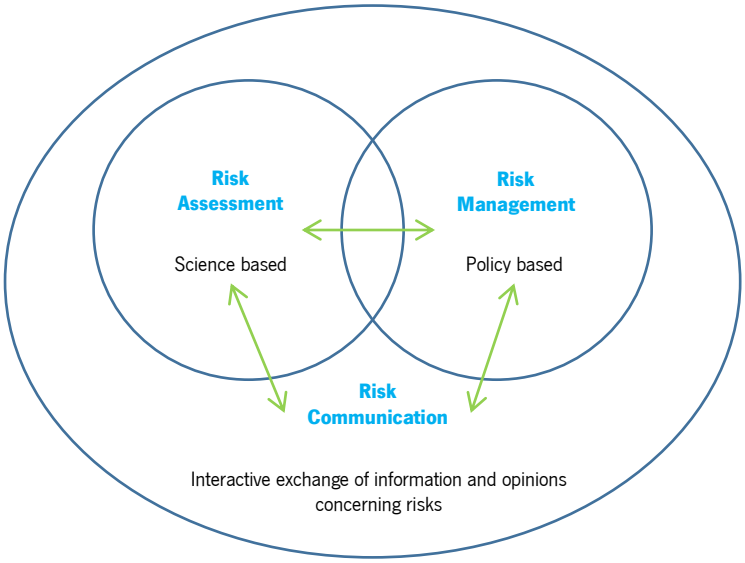


Figure 1. Generic components of risk analysis paradigm (adapted from FAO/WHO, 2009).

Within risk analysis, the functional separation between risk assessors and risk managers is essential to ensure scientific objectivity of the risk assessment process. All these components are internationally well spread by CAC as the basis for setting science based standards, criteria on food safety hazards, e.g.

setting maximum limits for contaminants in foodstuffs. Governments have moved quickly to incorporate much of this international work in national legislation and further developments in food safety risk analysis at the national level are ongoing (FAO/WHO, 2006). At the European level, risk analysis is the process chosen by the European Commission to act as the frame for Food Law, becoming an important tool for the management of food safety (EC, 2002). The General Food Law Regulation established the principle of risk analysis in relation to food and feed and established also the structures and mechanisms for the scientific and technical evaluations undertaken by EFSA. Depending on the nature of the measure to be taken, food law, and in particular measures relating to food safety must be underpinned by strong science. According to Regulation (EC) 178/2002, measures adopted by the Member States and the Community governing food and feed should generally be based on risk analysis, except when unappropriate to the circumstances or the nature of the measure. Additionally, when food law aims the reduction, elimination or avoidance of health risks, the three interconnected components of risk analysis provide a systematic methodology for the determination of effective, proportionate and targeted measures or other actions to protect health (EC, 2002).

For instance, risk analysis can be used to obtain information and evidence on the risk level of a certain contaminant in the food supply, helping governments to decide which actions should be taken in response (e.g. setting or revising a maximum limit for that contaminant, increasing testing frequency, labelling requirements review, advice provision to a specific population subgroup, issuing a product recall and/or a ban on imports of the product in question). Furthermore, the process of conducting a risk analysis enables authorities to identify the various control points along the food chain, where measures could be applied, and to weigh up the costs and benefits of these different options, and determine the most effective one(s). As such, it offers a framework to consider the likely impact of the possible measures (including on particular groups such as a food industry subsector) and contributes towards a consciousness use of public resources by focusing on the highest food safety risks. This approach has now gained a wide acceptance as the preferred way to assess possible links between food chain hazards and actual risks to human health, and takes into account a wide range of inputs for decision-making on appropriate control measures (FAO/WHO, 2006).

2.1. RISK ASSESSMENT

Risk assessment is the central scientific component of risk analysis and was developed primarily because of the need to take decisions in order to protect health in face of scientific uncertainty. Risk assessment of food chemicals can be generally described as characterizing the potential hazards and the associated risks to life and health resulting from human exposure to chemicals present in food over a specified period (FAO/WHO, 2009). In the context of food chemical safety, risk assessment is a conceptual framework that provides mechanisms for a structured review of relevant information to estimate health outcomes in relation to chemicals exposure through food. Risk assessment process comprises four steps: i) hazard identification; ii) hazard characterization (including dose–response assessment); iii) exposure assessment; and iv) risk characterization, as summarized in Figure 2. Usually, both hazard identification and characterization are considered as hazard assessment. In practice, once a chemical has been identified, its content in food measured through validated analytical techniques, its biological (toxicological) effects characterized and a safe level derived, one can relate exposure to biological effects for human risk assessment (Dorne et al., 2009).

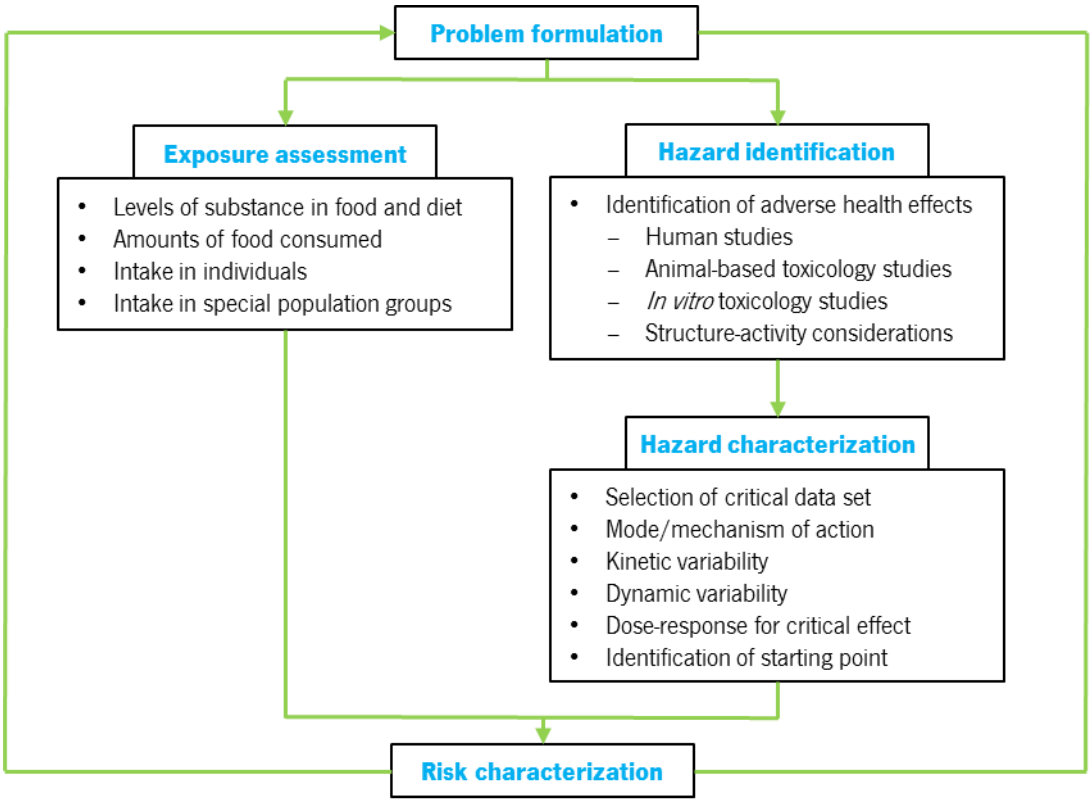


Figure 2. Risk assessment framework (adapted from Renwick et al., 2003)

2.1.1. Hazard identification

The first step in a risk assessment process is hazard identification and aims to address two questions: i) the nature of any health hazard to humans that an agent may pose; and ii) the circumstances under which an identified hazard may be expressed. Hazard identification is based in the process of describing the adverse effects of the substance, the possibility of causing an adverse effect as an inherent property of the chemical, and the type (e.g. age group, gender) and extent of the population that may be at risk. Because sufficient human data from epidemiological studies are not often available, risk assessors frequently rely on results from toxicological studies in experimental animals and *in vitro* studies (FAO/WHO, 2009, 2006).

2.1.2. Hazard characterization

Hazard characterization describes the relationship between the administered dose of, or exposure to, a chemical and the incidence of an adverse health effect. The critical effect – that is, the first adverse effect observed as the dose or exposure is increased – is determined, and if possible, a dose-response relationship is established (FAO/WHO, 2009). Types of data that can be used to establish dose-response relationships include animal toxicity studies, clinical human exposure studies and epidemiological data from disease investigations. Within this step, mechanistic aspects could be included (e.g. whether the chemical mechanism of action observed in often high-dose experimental studies is also relevant to human exposure at lower levels) (FAO/WHO, 2006).

In cases where the toxic effect is assumed to have a threshold, hazard characterization usually results in the establishment of health-based guidance values (HBGV) – a tolerable intake for contaminants as tolerable daily intake (TDI). The estimation of TDI includes the application of default "uncertainty factors" to a no-effect-level or low-effect level, observed in experimental or epidemiological studies, to account for uncertainties inherent to the extrapolation from an animal model to humans and to account for inter-individual variability. The conservatism considered to be inherent in such a safety evaluation is generally thought to ensure the sufficient protection of human health. Toxicological reference values used by different authorities for genotoxic and carcinogenic chemicals vary. Some are based on a combination of epidemiological and animal data, some may be based on animal data alone, and different mathematical models may be used to extrapolate risk estimates to low doses. These differences can lead to significant

variability in cancer risk estimates for the same chemical (Dorne and Fink-Gremmels, 2013; FAO/WHO, 2009, 2006). Some additional aspects within genotoxic/carcinogenic domains will be presented later.

Within hazard identification and characterization, it is important to refer the relationship between the external, or administered, dose of a substance and its biological responses. These can be divided into two aspects: i) toxicokinetics, which relates to the delivery of the chemical to the site of action and its removal as the parent substance and/or any active metabolites; and ii) toxicodynamics, which relates to the interaction between the chemical and/or any active metabolites at the site of action and the final outcome or toxicological response (FAO/WHO, 2009). The term toxicokinetics describes the movement of a substance within the body and therefore relates to its absorption from the administration site, its distribution from the general circulation into, and out of body tissues and its elimination, usually by metabolism and excretion [ADME, absorption, distribution, metabolism (biotransformation) and excretion].

2.1.3. Exposure assessment

Exposure assessment characterizes, qualitatively or quantitatively, the likely intake of chemical agents via food as well as exposure from other sources if relevant (FAO/WHO, 2015, 2009). As represented in Figure 3, in the case of food chemicals, dietary exposure assessment takes into consideration the occurrence and concentrations of the chemical in the diet, the consumption patterns of foods containing the chemical and the likelihood of a large intake by consumers (high consumers) and the presence of high levels of the chemical. Usually a range of intake or exposure estimates will be provided (e.g. for average consumers and for high consumers), and estimates may be broken down in subgroups of the population (e.g. infants, children, adults) (FAO/WHO, 2009, 2006). The resulting dietary exposure estimate may then be compared with the relevant HBGV for the concerned food chemical, if available, as part of the risk characterization step. The general equation (1) used to estimate the dietary exposure combines occurrence and consumption data.

(1)

$$Dietary\ exposure\ = \frac{\sum(Concentration\ of\ chemical\ in\ food\ \times Food\ consumption)}{Body\ weight}$$

Depending on the nature of the available data we can perform these calculations in a deterministic (point estimate) way (e.g. minimum, mean or maximum), or in a probabilistic (stochastic) manner (attribution

of distributions on concentration and consumption data), resulting in a distribution of the population exposure (Jacxsens et al., 2016) (Figure 3). The term "deterministic" describes an approach in which numerical point values are used at each step of the risk assessment. For example, the mean or the 95th percentile value of measured data (such as food consumption or chemical contents) may be used to generate a single risk estimate. In probabilistic approaches, scientific evidence is used to generate statements of probabilities of individual events, which are combined to determine the probability of an adverse health outcome. This requires mathematical modelling on the variability of the phenomena involved, and the final estimate is a probability distribution. Probabilistic models are used to create and analyse different exposure scenarios. The structure of a probabilistic model is similar to that of the deterministic models and it is based on the same basic equations, whereby food consumption data are combined with concentration data to estimate dietary exposure. The fundamental difference is that at least one variable is represented by a distribution function instead of a single value and the model sample from each distribution is a distribution of potential dietary exposures generated using several thousand iterations. This approach is generally viewed as being most reflective of the real world, but probabilistic models are often complex, difficult to generate and require specific software (FAO/WHO, 2009).

A widely applied software is @Risk® (Palisade Corporation, USA), where Monte Carlo simulations are conducted, by multiplying randomly a point on the contamination data distribution and a consumption pattern distribution shown by the studied population (FAO/WHO, 2009; Jacxsens et al., 2016). In fact, Monte Carlo simulation is one of the most popular approaches to develop probabilistic models of dietary exposure assessments and involves the use of random numbers to select values from the input distributions.

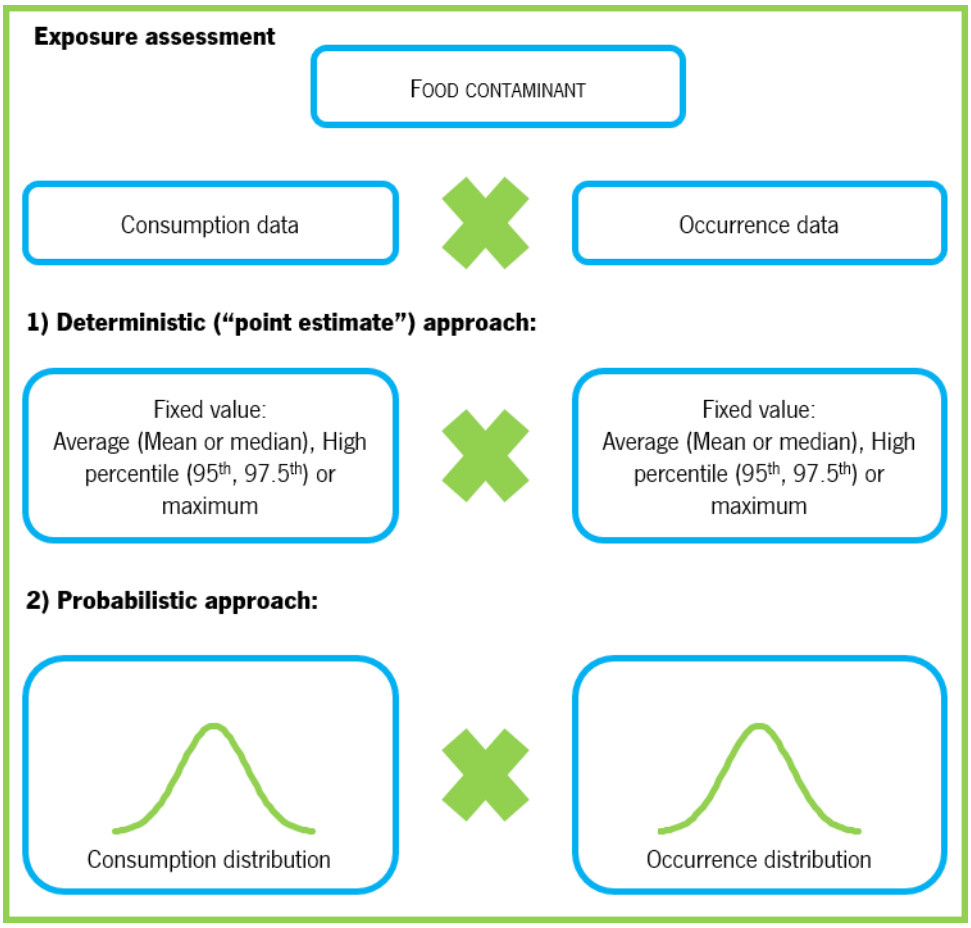


Figure 3. Human exposure assessment to chemical contaminants present in food (adapted from Dorne et al., 2009).

2.1.4. Risk characterization

Risk characterization, the fourth step of the risk assessment process, has been defined as the qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterization and exposure assessment. During risk characterization, outputs from the previous three steps are integrated to generate an estimate of risk (FAO/WHO, 2015, 2009, 2006). Different approaches have been used for risk characterization of toxic compounds, according to their genotoxic and carcinogenic potential. In the risk characterization for non-genotoxic and carcinogenic substances, a HBGV is compared with estimates of dietary exposure. Generally, if the exposure does not exceed the HBGV, it is generally assumed that the chemical does not pose a health concern (Moy, 2014). The calculation of the hazard quotient (HQ) is commonly done and is derived by comparing the respective reference dose (e.g. TDI) with the exposure to evaluate whether the exposure level is tolerable or not. A ratio of HQ <1 indicates a tolerable exposure level and a ratio of HQ >1 indicates a non-tolerable exposure level (EFSA, 2013). For those substances that are genotoxic and carcinogenic, the traditional assumption

is that some degree of risk may exist at any level of exposure and it is recommended that the exposure should be as low as reasonably achievable (ALARA). However, this approach presents limited value, because it does not allow risk managers to prioritize different contaminants or to target risk management actions. The margin of exposure (MOE) approach, which is the ratio between an amount of a substance producing a small but measurable effect in laboratory animals or humans and the estimated human exposure, has been proposed by WHO and EFSA as the methodology for risk characterization of genotoxic and carcinogenic compounds (EFSA, 2013; FAO/WHO, 2009). The Scientific Committee of EFSA considers that MOE values of 10,000 or more, when based on a benchmark dose lower confidence limit 10 ($BMDL_{10}$) from an animal study and taking into account overall uncertainties in the interpretation, are considered "of low concern from a public health point of view". Benchmark dose lower confidence limit corresponds to the lower boundary of the confidence interval on the benchmark dose. EFSA's Scientific Committee notes that the magnitude of a MOE only indicates a level of concern and does not quantify risk and, consequently, the larger the MOE, the smaller the potential risk posed by exposure to the compound under consideration (Benford et al., 2010a; EFSA, 2012; EFSA, 2013).

The information generated within risk characterization supports the decision making regarding an eventual necessary management action. Sometimes, the risk manager may request additional information related to the risk assessment. For example, the likely health outcomes of various maximum permitted levels of a contaminant in food may be requested by the risk manager to allow balancing the risk of a chemical with the health benefits of food availability. Finally, it should be noticed that any risk assessment is based on the scientific evidence available at the time of the assessment and that whenever significant new information warrants, the chemical should be re-evaluated regarding its risk (Moy, 2014).

2.2. RISK ASSESSMENT AND RISK MANAGEMENT INTERFACE

Risk assessment and risk management are closely related although different processes describe them. Risk assessment is usually seen as the objective/scientific component and risk management as the subjective/political part of risk analysis (van Leeuwen, 2007). The relations between risk assessment and risk management are depicted in Figure 4. Risk management should follow a structured approach including preliminary risk management activities, evaluation of risk management options, monitoring and review of the decision taken. The decisions should be based on risk assessment and take into account, when appropriate, other legitimate factors relevant for the health protection of consumers and for the

promotion of fair practices in food trade (FAO/WHO, 2015). Actions and decisions within risk management should also consider legal, political, social and economic issues (van Leeuwen, 2007). Risk management sets priorities for risk reduction in line with a variety of available options. Priorities depend on the extent and frequency that the TDI is exceeded, or on the extent of the margin of exposure. In these deliberations, one considers the severity of the health effect, the impact of an elevated exposure on eventual health consequences, as well as the size and vulnerability of the population affected (Kuiper-Goodman, 2004).

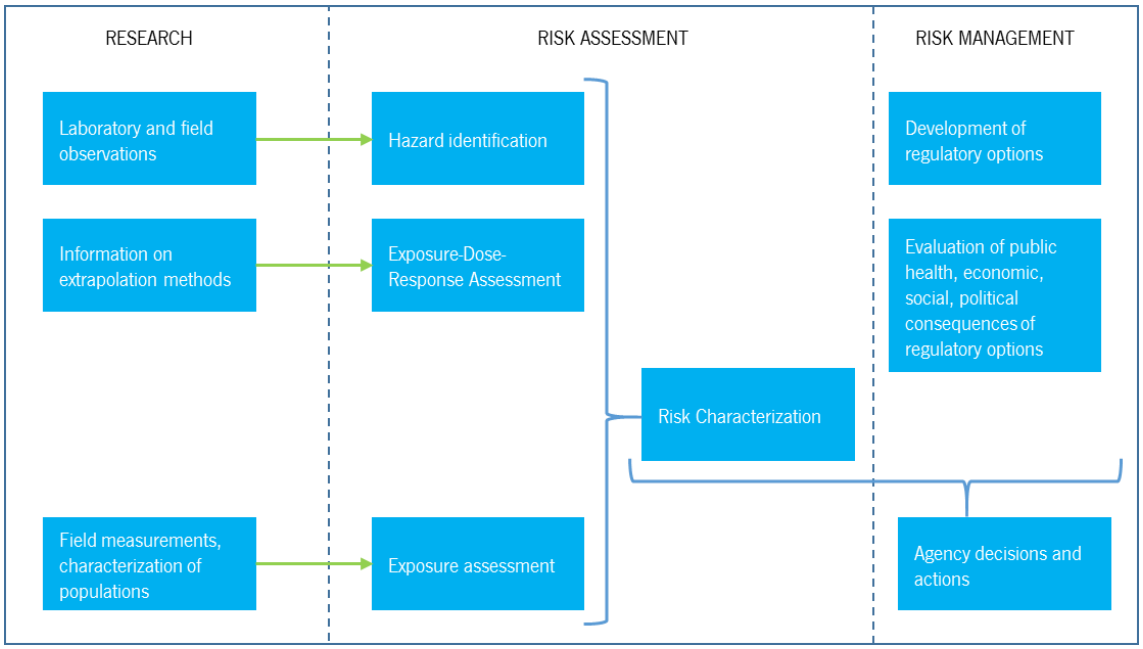


Figure 4. Interplay of science and policy in risk analysis: the elements of risk assessment and risk management (Sherif et al., 2009)

In the European food safety system, risk assessment is performed independently from risk management. As the risk assessor, EFSA produces scientific opinions and advice to provide a sound foundation for European policies and legislation and to support the European Commission, European Parliament and EU Member States in taking effective and timely risk management decisions (Silano and Silano, 2015).

2.3. RISK COMMUNICATION

Risk analysis include a clear, interactive and documented communication amongst risk assessors, risk managers and all the stakeholders involved in this domain, as represented in Figure 1. Risk communication is a powerful but often underused element of risk analysis (FAO/WHO, 2006; van Leeuwen, 2007). This communication step of risk analysis is defined as an 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 (FAO/WHO, 2006). Risk communication helps to provide timely, relevant and accurate information to, and to obtain information from, members of the risk analysis team and external stakeholders, improving knowledge about the nature and effects of a specific food safety risk. Successful risk communication is a prerequisite for effective risk management and risk assessment. It contributes to the transparency of the risk analysis process and promotes a broader understanding and acceptance of risk management decisions (FAO/WHO, 2006).

3. RISK ASSESSMENT OF MYCOTOXINS IN FOOD

Food is an important source of exposure to different intentionally added food ingredients or additives, and also to unintentionally added substances such as abiotic and biotic contaminants. As refereed previously, among substances that can be found in different foodstuffs and processed foods, chemicals are a source of health concern, including those that appear in food naturally, such as mycotoxins (Vettorazzi and López de Cerain, 2016). Ingestion of food is considered a major route of exposure to mycotoxins and, consequently, a deep knowledge within this domain is crucial for an accurate risk assessment.

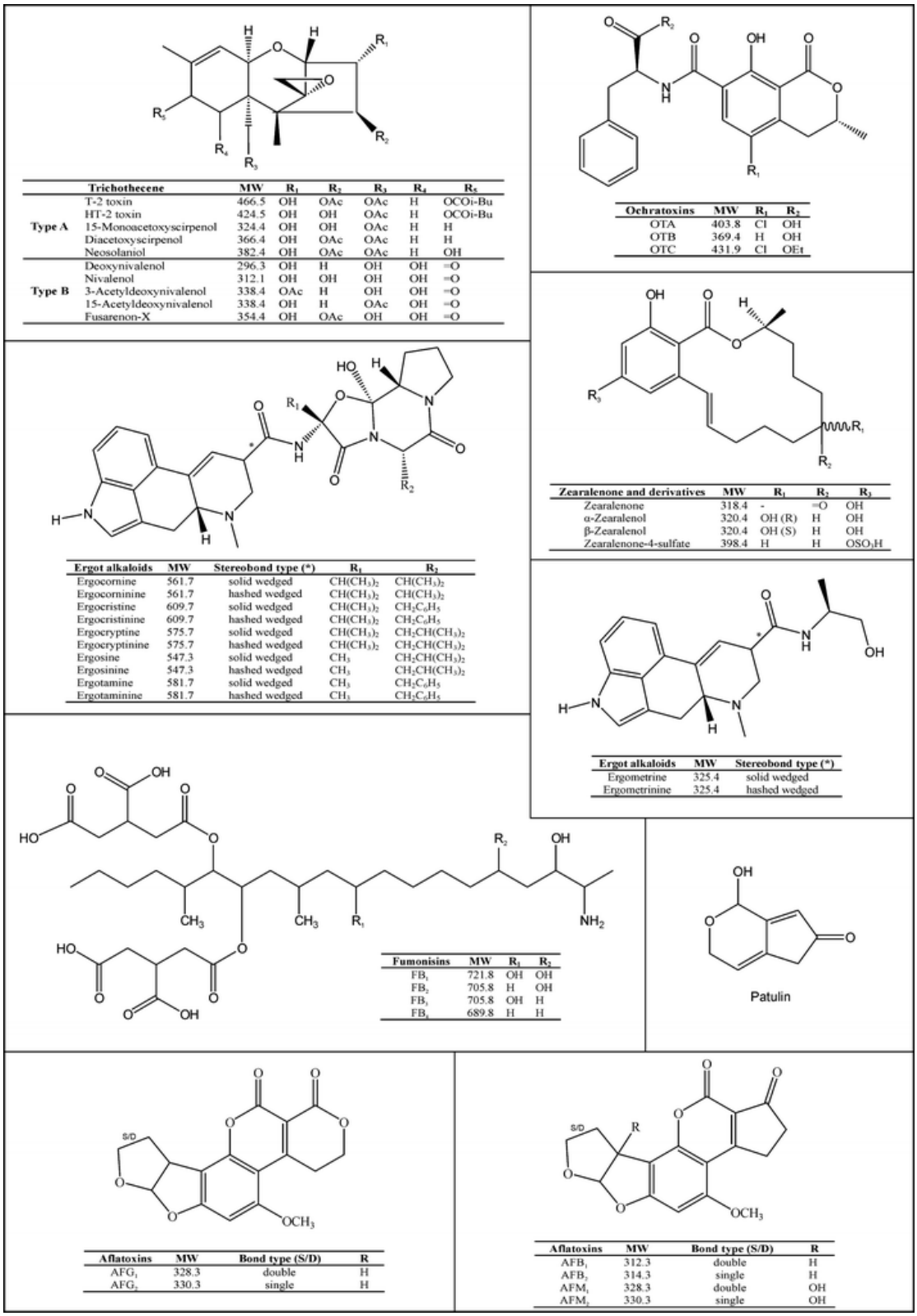
3.1. MYCOTOXINS – A GENERAL OVERVIEW

The name mycotoxin is a combination of the Greek word for fungus “mykes” and the Latin word “toxicum” meaning poison. The term mycotoxin is usually reserved for the relatively small, low-molecular-weight (mostly below 700 Da) secondary metabolites produced by filamentous fungi (Bennett and Klich, 2003;

Turner et al., 2009). These metabolites, with no apparent function in the normal metabolism of fungi, are produced mainly, although not exclusively, when the fungus reaches maturity. More than 500 different mycotoxins are known. They vary considerably in their chemical structure, from simple heterocyclic rings with molecular weights of up to 50 Da, to groups with 6 – 8 irregularly arranged heterocyclic rings with a molecular weight of over 500 Da (Rocha et al., 2014). This structural diversity results in different chemical and physicochemical properties. Generally, mycotoxins are chemically and thermally stable compounds, surviving storage and most production processes (Köppen et al., 2010). From all known mycotoxins, aflatoxins (AFT), ochratoxins, fumonisins, trichothecenes, zearalenone (ZEA), patulin (PAT), and ergot alkaloids receive a particular attention due to their frequent occurrence and their severe effects on animal and human health (Bennett and Klich, 2003; Krska et al., 2008). Figure 5 presents the molecular basic structures of these most prominent mycotoxins.

Mycotoxins and their clinical manifestations were relatively unclear in the scientific literature, until the discovery of aflatoxin in the early 1960s, in the aftermath of an unusual veterinary crisis near London, United Kingdom, during which approximately 100,000 turkey poults died (Asao et al., 1963; Blout, 1961; Forgacs, 1962). When this mysterious "turkey X disease" was linked to a peanut meal contaminated with secondary metabolites from *Aspergillus flavus*, it sensitized scientists to the possibility that other occult mould metabolites could be deadly. Soon, the mycotoxin subject was extended to include a number of previously known fungal toxins (e.g., the ergot alkaloids), some compounds that had originally been isolated as antibiotics (e.g., PAT), and a number of new secondary metabolites revealed in screens targeted at mycotoxin discovery (e.g., ochratoxin A) (Bennett and Klich, 2003; Vettorazzi and López de Cerain, 2016). Since then, there has been growing interest in research related to them, which has resulted in an increase in the number of publications regarding mycological, chemical, toxicological, and epidemiological aspects of mycotoxins. Thus, the possibility of foods being contaminated by mycotoxins was known early on. Consequently, governments across the world have implemented control measures directed to specific mycotoxins in foodstuffs (Vettorazzi and López de Cerain, 2016).

All humans and animals are at risk of mycotoxin exposure, mainly via the ingestion of contaminated foods. However, alternate routes include dermal absorption and inhalation of toxicogenic moulds containing mycotoxins. The latter has been still difficult to link to diseases other than upper and low respiratory tract infections, but it represents a feasible risk since growth of some trichothecene-producing fungi like *Stachybotrys* sp. has been documented in water damaged constructions and deleterious health effects in humans in contact with damp damaged buildings have been described (CAST, 2003; Pestka et al., 2008).



	Trichothecene	MW	R_1	R_2	R_3	R_4	R_5
Type A	T-2 toxin	466.5	OH	OAc	OAc	H	OCOi-Bu
	HT-2 toxin	424.5	OH	OH	OAc	H	OCOi-Bu
	15-Monoacetoxyscirpenol	324.4	OH	OH	OAc	H	H
	Diacetoxyscirpenol	366.4	OH	OAc	OAc	H	H
	Neosolaniol	382.4	OH	OAc	OAc	H	OH
Type B	Deoxynivalenol	296.3	OH	H	OH	OH	=O
	Nivalenol	312.1	OH	OH	OH	OH	=O
	3-Acetyldeoxynivalenol	338.4	OAc	H	OH	OH	=O
	15-Acetyldeoxynivalenol	338.4	OH	H	OAc	OH	=O
	Fusarenon-X	354.4	OH	OAc	OH	OH	=O

Ochratoxins	MW	R_1	R_2
OTA	403.8	Cl	OH
OTB	369.4	H	OH
OTC	431.9	Cl	OEt

Zearalenone and derivatives	MW	R_1	R_2	R_3
Zearalenone	318.4	-	=O	OH
α-Zearalenol	320.4	OH (R)	H	OH
β-Zearalenol	320.4	OH (S)	H	OH
Zearalenone-4-sulfate	398.4	H	H	OSO_3H

Ergot alkaloids	MW	Stereobond type (*)	R_1	R_2
Ergocornine	561.7	solid wedged	$CH(CH_3)_2$	$CH(CH_3)_2$
Ergocorninine	561.7	hashed wedged	$CH(CH_3)_2$	$CH(CH_3)_2$
Ergocristine	609.7	solid wedged	$CH(CH_3)_2$	$CH_2C_6H_5$
Ergocristinine	609.7	hashed wedged	$CH(CH_3)_2$	$CH_2C_6H_5$
Ergocryptine	575.7	solid wedged	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$
Ergocryptinine	575.7	hashed wedged	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$
Ergosine	547.3	solid wedged	CH_3	$CH_2CH(CH_3)_2$
Ergosinine	547.3	hashed wedged	CH_3	$CH_2CH(CH_3)_2$
Ergotamine	581.7	solid wedged	CH_3	$CH_2C_6H_5$
Ergotaminine	581.7	hashed wedged	CH_3	$CH_2C_6H_5$

Ergot alkaloids	MW	Stereobond type (*)
Ergometrine	325.4	solid wedged
Ergometrinine	325.4	hashed wedged

Fumonisins	MW	R_1	R_2
FB_1	721.8	OH	OH
FB_2	705.8	H	OH
FB_3	705.8	OH	H
FB_4	689.8	H	H

Patulin

Aflatoxins	MW	Bond type (S/D)	R
AFG_1	328.3	double	H
AFG_2	330.3	single	H

Aflatoxins	MW	Bond type (S/D)	R
AFB_1	312.3	double	H
AFB_2	314.3	single	H
AFM_1	328.3	double	OH
AFM_2	330.3	single	OH

Figure 5. Molecular basic chemical structures of the most important mycotoxins (Köppen et al., 2010).

Mycotoxins can appear in the field before harvest, post-harvest or during processing, storage and feeding, adversely affecting the quality of the food (Rocha et al., 2014). FAO estimated that approximately 25% of

cereals produced in the world are contaminated with, at least, one mycotoxin (Rice and Ross, 1994). These toxins can enter the human and animal food chains through direct or indirect contamination. Direct contamination occurs when the product, food or feed, becomes infected by a toxigenic fungus, with the subsequent production of mycotoxins. On the other hand, the indirect contamination of foodstuffs and animal feed occurs when any ingredient has been previously contaminated by a toxigenic fungus, and even though the fungus has been eliminated during the processing, the mycotoxins remain in the final product (Rocha et al., 2014). Consequently, humans could be exposed to mycotoxins directly through the intake of contaminated agricultural products (e.g. cereal-based products, fruits) or indirectly through the consumption of products of animal origin (e.g. meat, milk, eggs) prepared or obtained from animals that were fed with contaminated material (Capriotti et al., 2012; Flores-Flores et al., 2015).

Mycotoxins are produced by a number of fungal genera and *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* and *Claviceps* genus are the predominant mycotoxin producers. The major food commodities affected are cereals, nuts, dried fruit, coffee, cocoa, spices, oil seeds, dried peas, beans and fresh fruit, particularly apples. Mycotoxins may also be found in beer and wine resulting from the use of contaminated barley, other cereals and grapes in their production. They can also enter the human food chain via meat or other animal products such as eggs, milk and cheese as the result of livestock contaminated feed intake (Turner et al., 2009). Table 2 summarizes the major mycotoxins that affect human health worldwide, the fungal species that produce them, the food commodities in which they are common contaminants, the respective EU legislative limits and the main health effects.

The clinical manifestations caused by mycotoxins, known as mycotoxicoses, do not necessarily involve the toxin-producing fungus. Thus, they are abiotic hazards but with biotic origin. Diagnostic features characterizing mycotoxicoses are i) no transmissibility; ii) drug and antibiotic treatments have little or no effect; iii) outbreaks are often seasonal; iv) outbreaks are usually associated with a specific foodstuff; and examination of the suspected food or foodstuff often reveals signs of fungal activity (Marin et al., 2013). Like all toxicological syndromes, mycotoxicoses can be categorized as acute or chronic. Acute toxicity generally has a rapid onset and is more likely following exposure to high levels of a mycotoxin. Chronic toxicity is characterized by low-dose exposure over a long time-period, resulting in cancer and other general irreversible effects. Currently, the main human and veterinary health burdens of mycotoxin exposure are related to chronic exposure (e.g., cancer, kidney damage, immune suppression) (Bennett and Klich, 2003; CAST, 2003; De Ruyck et al., 2015). Mycotoxins can affect single or multiple target organs, with a varying degree of cytotoxic, mutagenic, teratogenic, carcinogenic, and/or immunosuppressive potency (Lee and Ryu, 2015). Several food-contaminating mycotoxins have been

evaluated by the International Agency for Research on Cancer (IARC) relatively to their potential human carcinogenicity. The IARC classifies five different groups, depending on the strength of evidence of carcinogenicity in humans and experimental animals, using standard terms. Mechanistic and other relevant data are also taken into account. Group 1 classification is used when there is sufficient evidence of carcinogenicity in humans. Group 2 includes agents for which, at one extreme, the degree of evidence of carcinogenicity in humans is almost sufficient, as well as those for which, at the other extreme, there are no human data but for which there is evidence of carcinogenicity in experimental animals. Agents are assigned to either Group 2A (probably carcinogenic to humans) or Group 2B (possibly carcinogenic to humans) on the basis of epidemiological and experimental evidence of carcinogenicity and mechanistic and other relevant data. Group 3 is commonly used for agents for which carcinogenicity evidence is inadequate in humans and inadequate or limited in experimental animals. Group 4 is used for agents for which there is evidence suggesting a lack of carcinogenicity in humans and in experimental animals (Lerda, 2011; Vettorazzi and López de Cerain, 2016). Table 3 presents IARC classification of food mycotoxins as human carcinogens or potential human carcinogens. Aflatoxins were classified as carcinogenic to humans (Group 1), whereas OTA, aflatoxin M_1 (AFM_1), and fumonisin B_1 were classified as possible carcinogens (Group 2B). Trichothecenes, ZEA and PAT were not classified as human carcinogens (Group 3). However, new fungal metabolites are still being discovered, and their potential and synergistic contributions to toxicity for humans and animals are yet to be assessed.

Table 2. Major food borne mycotoxins or mycotoxin groups, their main producing fungal species, the commodities most frequently contaminated, the range of legal limits (EU) and their major health effects (adapted from Köppen et al., 2010).

Mycotoxin	Fungal species	Food commodity	Legal limit range (EU), µg/kg[a]	Major health effects[b]
Aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFM_2)	*Aspergillus flavus, A. nomius, A. parasiticus, A. arachidicola, A. bombycis, A. pseudotamarii, A. minisclerotigenes, A. rambellii, A. ochraceoroseus, Emericella astellata, E. venezuelensis, E. olivicola*	Maize, wheat, rice, spices, sorghum, ground nuts, tree nuts, almonds, milk, oilseeds, dried fruits, cheese, spices, eggs, meat	Sum of AFB_1, AFB_2, AFG_1 and AFG_2: 4 (dried fruits, cereals, nuts) – 15 (groundnuts) AFM_1: 0.025 (infant and dietary foods) – 0.05 (milk) AFB_1: 0.1 (groundnuts) – 8.0 (dietary, processed cereal- based and infant foods)	Carcinogenic, mutagenic, teratogenic, hepatotoxic, nephrotoxic, immunosuppressive, haemorrhage of intestinal tract and kidneys, liver disease
Fumonisins (FB_1,FB_2,FB_3)	*Alternaria alternata, Fusarium anthophilum, F. moniliforme, F. dlamini, F. napiforme, F. proliferatum, F. nygamai, F. verticillioides*	Maize, maize based products, corn based products, sorghum, asparagus, rice, milk	Sum of FB_1 and FB_2: 200 (processed maize-based foods and infant foods) – 4000 (unprocessed maize)	Carcinogenic (liver tumors), hepatotoxic, cerebral oedema, cause necrosis, immunotoxic
Type A trichothecenes (T-2 and HT-2 toxin, diacetoxyscirpenol, neosolaniol)	*Fusarium sporotrichioides, F. poae, F. langsethiae, F. acuminatum, F. culmorum, F. equiseti, F. graminearum, F. moniliforme, F. myrothecium, Cephalosporium sp., Myrothecium sp., Trichoderma sp., Trichothecium sp., Phomopsis sp., Stachybotrys sp., Verticimonosporium sp.*	Cereals, cereal based products		Immuno-depressants, mutagenic, gastrointestinal haemorrhaging, neurotoxic
Type B trichothecenes (nivalenol, deoxynivalenol, 3-acetylDON, 15-acetylDON, fusarenone X)	*F. graminearum, F. culmorum, F. sporotrichioides, F. cerealis, F. lunulosporum*	Cereals, cereal based products	Deoxynivalenol: 200 (processed cereal-based foods and infant foods) – 1750 (unprocessed durum wheat and oats, unprocessed maize)	Immuno-depressants, mutagenic, gastrointestinal haemorrhaging, neurotoxic
Zearalenone (ZEA)	*F. graminearum, F. culmorum, F. crookwellense, F. equiseti, F. sporotrichioides*	Barley, oats, wheat rice, sorghum, sesame, soy beans, cereal based products	20 (processed cereal-based foods and processed maize-based infant food) – 400 (refined maize oil)	Estrogenic activity (infertility, vulval oedema, vaginal prolapse, mammary hypertrophy in females, feminisation of males)

Ochratoxins (OTA, OTB, OTC)	*A. alutaceus, A. alliaceus, A. auricomus, A. glaucus, A. niger, A. carbonarius, A. melleus, A. albertensis, A. citricus, A. flocculosus, A. fonsecaeus, A. lanosus, A. ochraceus, A. ostianus, A. petrakii, A. pseudoelegans, A. roseoglobulosus, A. sclerotiorum, A. steynii, A. sulphureus, A. westerdijkiae, Neopetromyces muricatus, Penicillium viridicatum, P. verrucosum, P. cyclopium, P. carbonarius*	Cereals, dried vine fruit, wine, coffee, oats, spices, rye, raisins, grape juice	OTA: 0.5 (processed cereal-based and infant foods) – 10 (dried vine fruits and instant coffee)	Carcinogenic, mutagenic, nephrotoxic, hepatotoxic, teratogenic, immunodepressants, carcinogenic (urinary tract tumors), inhibition of protein synthesis
Patulin (PAT)	*A. clavatus, A. longivesica, A. giganteus, P. expansum, P. griseofulvum, Byssochlamys* sp.*, Paecilomyces* sp.	Apples, apple juice, cherries, cereal grains, grapes, pears, bilberries	10 (apple juice, solid apple, infant foods)– 50 (spirit drinks derived from apples or containing apple juice, fruit juices)	Immuno-depressant, pulmonary and cerebral oedema, nausea, gastritis, paralysis, convulsions, capillary damage, carcinogenic
Ergot alkaloids (Ergocornine/inine, Ergocristine/inine, Ergocryptine/inine, Ergosine/inine, Ergotamine/inine)	*Claviceps africanana, Claviceps purpurea, Claviceps fusiformis, Claviceps paspali, Neotyphodium coenophialum*	Wheat, rye, hay, barley, millet, oats, sorghum, triticale		Gangrenous form: vasoconstrictive activity (oedema of the legs, paraesthesia, gangrene at the tendons) Convulsive form: gastrointestinal symptoms (nausea, vomiting), effects on the central nervous system (drowsiness, ataxia, convulsions, blindness, paralysis)
Alternaria mycotoxins (altenuene, alternariol, alternariol monomethyl ether, altertoxin I, altertoxin II, altertoxin III, tenuazonic acid)	*Alternaria alternata, Alternaria dauci, Alternaria cucumerina, Alternaria solani, Alternaria tenuissima, Alternaria citri*	Wheat, rice, rye, olives, sorghum, tobacco, apples, peppers, sunflower seeds, oilseed rape, pecan nuts, tomatoes, mandarins		Teratogenic, mutagenic, carcinogenic, cytotoxic

[a] According to Regulation (EC) No 1881/2006; [b] In animal experiments

Table 3. IARC classification of food mycotoxins as human carcinogens or potential human carcinogens (adapted from Lerda, 2011; Vettorazzi and López de Cerain, 2016).

IARC classification	Definition	Mycotoxins	IARC Monograph (Volume, Year)
1	Carcinogenic to humans	Aflatoxins (B_1, B_2, G_1, G_2)	100F, 2012[a]
2A	Probably carcinogenic to humans	-	-
2B	Possibly carcinogenic to humans	Aflatoxin M_1	56, 1993[b]
		Ochratoxin A	56, 1993[b]
		Fumonisin B_1	82, 2002[c]
		Sterigmatocystin	Suppl. 7, 1987[d]
3	Not classifiable as to its carcinogenicity to humans	Patulin	Suppl. 7, 1987[d]
		Deoxynivalenol	56, 1993[b]
		Nivalenol	56, 1993[b]
		Zearalenone	56, 1993[b]
		Fusarenone X	56, 1993[b]
		T-2 toxin	56, 1993[b]
		Citrinin	Suppl. 7, 1987[d]
4	Probably not carcinogenic to humans	-	-

[a] IARC, 2012; [b] IARC, 1993; [c] IARC, 2002; [d] IARC, 1987.

To protect consumers' health, many countries have also implemented legislation that imposes limits on major mycotoxins presence in food commodities. The limits can change in each country as a result of several factors, as the different perceptions about levels considered safe for health, or economic interests related to local cultures (Abrunhosa et al., 2016; van Egmond and Jonker, 2008). However, the limits guarantee that highly contaminated products are not traded and introduced into the human food chain. In EU, and regarding food, the maximum limits of mycotoxins permitted are presented in Regulation 1881/2006 (EC, 2006). Table 2 presents the range of legal limits for the major food borne mycotoxins or mycotoxin groups.

In the following sub-sections, the main characteristics of major food borne mycotoxins are summarised.

3.1.1. Aflatoxins

AFT are difuranocoumarin derivatives produced by a polyketide pathway by many strains of *Aspergillus flavus*, *Aspergillus parasiticus* and the rare *Aspergillus nomius*, which contaminate agricultural commodities. Other species of fungi also produce AFT as presented in Table 2. The aflatoxin-producing fungi are ubiquitous in nature, however, they are primarily found in hot and humid climates, when

temperatures are between 24 and 35 °C, and whenever the moisture content exceeds 7% (10% with ventilation) (Creppy, 2002; Marin et al., 2013; Sherif et al., 2009; Williams et al., 2004). Developing nations, including most of Africa, Latin and South Americas, and Asia are identified as high risk areas for aflatoxin exposure, leading to aflatoxicosis (the mycotoxicosis caused by aflatoxin consumption) (Williams et al., 2004). There are six predominant aflatoxins, named aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1), and aflatoxin M_2 (AFM_2) (De Ruyck et al., 2015). In this nomenclature, "B" and "G" are used to denote compounds' fluorescence under ultraviolet light, respectively blue and green. The "M_1" and "M_2" compounds are usually not found on cereal products themselves, but are the hydroxylated metabolites present in the milk of mammals whose diet was contaminated by aflatoxins B_1 and B_2, respectively. Finally, "1" and "2" numbers refers to structural isomers, presenting one double or single bound respectively (De Ruyck et al., 2015), as presented in Figure 5. Many substrates support the growth and aflatoxin production by aflatoxigenic moulds. Natural contamination of maize, wheat, rice, spices, sorghum, ground nuts, tree nuts, almonds, milk, oilseeds, dried fruits, cheese, spices, eggs, meat is of common occurrence (Bennett and Klich, 2003; Köppen et al., 2010). AFT are very stable and may resist to quite heavy processing like roasting, extrusion, baking, and cooking. For this reason, they are considered a problem in processed foods, such as roasted nuts and bakery products (Marin et al., 2013).

AFT have toxic, carcinogenic, mutagenic, teratogenic and immunosuppressive effects (IARC, 2012; Sherif et al., 2009). The liver is the main target organ for aflatoxin toxicity and carcinogenicity. AFB_1 is the most potent carcinogenic substance naturally produced by *Aspergillus* species and is classified by IARC as a Group 1 human carcinogen (Table 3). AFB_2, AFG_1 and AFG_2 have respectively 20, 50 and 10% of the toxicity potency of AFB_1, but are also classified within Group 1 of human carcinogens, as presented in Table 3 (Hussein and Brasel, 2001).

The metabolic pathway of AFB_1 is summarized in Figure 6. AFB_1 is metabolized mainly by the liver to AFB_1-8,9-exo-epoxide and 8,9-endo-epoxide. The exo-epoxide binds to DNA (deoxyribonucleic acid) to form the predominant 8,9-dihydro-8-(N_7-guanyl)-9-hydroxy AFB_1 (AFB_1-N_7-Gua) adduct. AFB_1-N_7-Gua may be converted to two secondary forms, an apurinic site and a more stable ring opened AFB_1-formamidopyrimidine (AFB_1-FAPY) adduct; the latter is far more persistent *in vivo* than AFB_1-N_7-Gua (IARC, 2012).

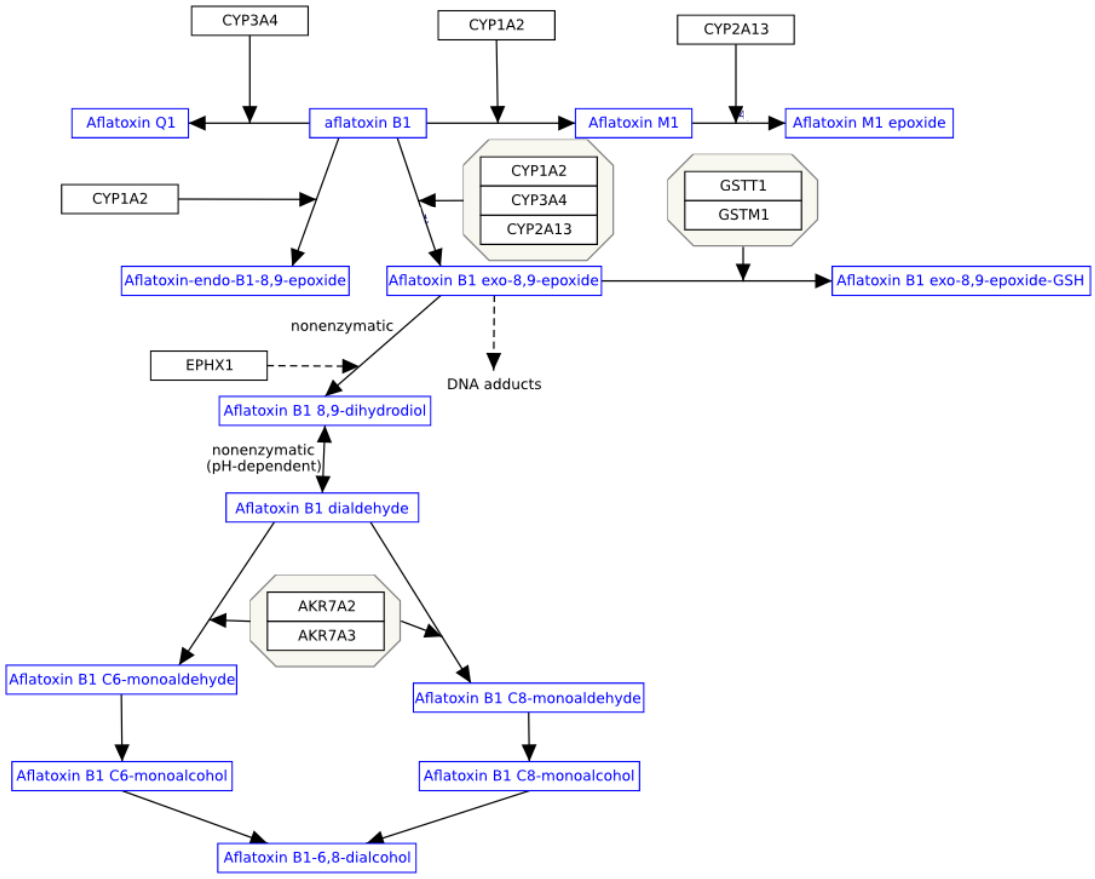


Figure 6. Metabolic scheme of aflatoxin B_1 (Wikipathways, n.d.).

The major human cytochrome P450 (CYP) enzymes involved in aflatoxin metabolism are CYP3A4, 3A5, 3A7, and 1A2. CYP3A4 is predominant in human liver and mediates the formation of AFB_1-exo-8,9-epoxide, the highly reactive metabolite that binds to DNA, and AFQ_1. CYP1A2 can also lead to the formation of exo-epoxide but mainly generates a high proportion of endo-epoxide, which does not bind to DNA, and AFM_1. However, CYP1A2 has been reported to be more efficient in producing AFB_1-exo-8,9-epoxide at low AFB_1 concentrations that may be found after dietary exposures. Finally, CYP3A5 has been described to metabolize AFB_1, mainly to the exo-8,9 epoxide, but it is much less efficient at forming the detoxification product, AFQ_1. Some studies in human liver samples have reported the importance of CYP3A5 in liver with low CYP3A4 expression. The DNA reactive metabolite, AFB_1-exo-8,9-epoxide, can hydrolyse to AFB_1-8,9-dihydrodiol, an unstable intermediate that undergoes base-catalysed rearrangement to a dialdehyde. This AFB_1 dialdehyde can react with proteins, such as albumin, but not with DNA.

The epoxide adduct is reported to catalyse a G → T mutation in the *p53* tumour suppressor protein gene, where it causes a missense mutation effectively inactivating the gene's protein product (De Ruyck et al., 2015). This mechanism, as well as data from previous studies in animal models, has lead the IARC to

classify AFB_1, AFB_2, AFG_1, and AFG_2 as Group 1 carcinogens, denoting their explicit carcinogenicity to humans.

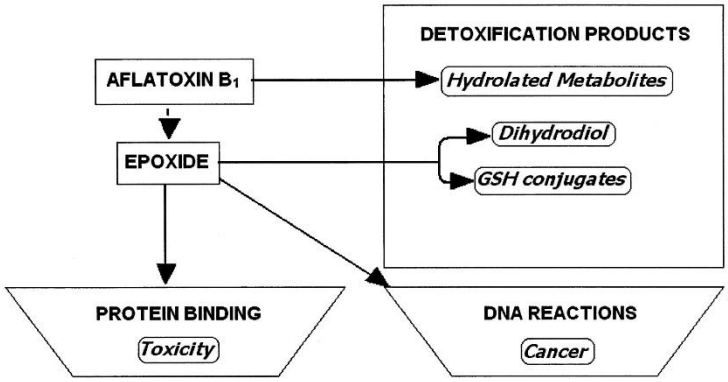


Figure 7. Pathways and consequences for aflatoxin B_1 in metabolism (GSH, glutathione) (Eaton and Groopman, 1994).

As shown schematically in Figure 7, the fate of AFB_1 is dependent on the relative activity of several biotransformation pathways, in addition to other factors such as DNA repair rates. The amount of the mycotoxin that is going to exert carcinogenic or toxic effects will depend on the amount converted to various metabolites as mentioned before, as well as the biological activity of these metabolites. With respect to carcinogenicity, AFB_1-8,9-epoxide is the key active metabolite. As indicated in Figure 7, hydroxylated metabolites of AFB_1 (AFM_1, AFP_1, AFQ_1) are assumed to represent detoxification products. Detoxification of the reactive epoxide also may occur through conjugation with glutathione (GSH). Hydrolysis of AFB_1-8,9-epoxide forms a dihydrodiol that probably still retains toxicity (via binding to protein) but is a less potent carcinogenic species than the epoxide (Eaton and Groopman, 1994; Vettorazzi and López de Cerain, 2016).

As refereed previously, aflatoxin M_1 is the main monohydroxylated derivative of AFB_1 formed in the liver via CYP-associated enzymes. Mammals that ingest AFB_1-contaminated diets excrete amounts of the main 4-hydroxylated metabolite AFM_1 into milk (Marin et al., 2013). It is estimated that about 1–2% of AFB_1 in animal feed is transformed to AFM_1 in milk with variations from animal to animal, from day to day and from one milking to the next. When the intake of AFB_1 is stopped, the AFM_1 concentration in the milk decreases to an undetectable level after 72 h (Egmond, 1989; Martins et al., 2005). This aflatoxin is heat stable, suffering no reduction after pasteurization, and was even detected in UHT milk, and milk derivatives like yogurt and cheese (Martins et al., 2005). This toxin has been categorized by IARC as a class 2B toxin, a possible human carcinogen (Table 3). AFM_1 is cytotoxic, as demonstrated in human

hepatocytes *in vitro* and its acute toxicity in several species is similar to that of AFB_1. In ducklings and rats, the acute and short-term toxicity of AFM_1 was similar to or slightly less than that of AFB_1. AFM_1 can also cause DNA damage, gene mutation, chromosomal anomalies and cell transformation in mammalians cells *in vitro*, in insects, lower eukaryotes and bacteria. However, AFM_1 is less mutagenic, and genotoxic than AFB_1 (Prandini et al., 2009).

3.1.2. Ochratoxin A

The ochratoxins were the first group of mycotoxins to be discover after AFT. This group consists of related pentaketide metabolites, comprised of a dihydroisocoumarin bonded to phenylalanine (Phe), which comes in three secondary metabolite forms (OTA, OTB, OTC). The structure of ochratoxins is depicted in Figure 5. Ochratoxin A (OTA) is the most common congener and is mainly produced by *Aspergillus ochraceus*. The related compound ochratoxin B differs only in having hydrogen in place of the chlorine atom, and ochratoxin C is an ethyl ester form of OTA produced in presence of rumen fluid (Figure 5) (Bennett and Klich, 2003; De Ruyck et al., 2015). OTA is one of the most commonly encountered mycotoxins and, in European countries, this mycotoxin is probably the most ubiquitous, particularly in Central Europe (De Ruyck et al., 2015; Marin et al., 2013; Sherif et al., 2009). Not all *A. ochraceus* isolates are capable of producing OTA. Besides this species, *Aspergillus alliaceus*, *A. auricomus*, *A. carbonarius*, *A. glaucus*, *A. meleus* and *A. niger*, as well as *Penicillium nordicum* and *P. verrucosum*, among others (Table 2), are also producers of OTA (Bui-Klimke and Wu, 2015; Köppen et al., 2010; Malir et al., 2016; Rocha et al., 2014). The fungi species vary in their optimal growing temperatures and water activity as summarized in Table 4.

Ochratoxins have been isolated from foods all over the world, in both warm and cool climates, and are common contaminants of grains, coffee, cocoa, wine, beer, and foods from animal origin, particularly pork (Bennett and Klich, 2003; Marroquín-Cardona et al., 2014; Rocha et al., 2014; Sherif et al., 2009). OTA is a chemically stable compound, hence, ordinary food processing measures fail to substantially reduce its presence in foods and beverages (Bui-Klimke and Wu, 2015; Marin et al., 2013).

Table 4. Ochratoxin-producing fungi and their optimal growth conditions (adapted from Bui-Klimke and Wu, 2015).

OTA-producing species	Optimal temperature range (Min-Max) (°C)	Water activity
Aspergillus ochraceus	24 - 31 (8 - 37)	0.95 - 0.99
A. carbonarius	32 - 25 (N/A - 40)	0.82
A. niger	35 – 37 (6 - 47)	0.77
Penicillium verrucosum	20 (0 - 30)	0.80

N/A: not applicable

OTA is recognized as nephrotoxic, hepatotoxic, embryotoxic, teratogenic, neurotoxic, immunotoxic, genotoxic, and carcinogenic in many species with species and gender related differences (IARC, 1993; Malir et al., 2016; Marroquín-Cardona et al., 2014; Sherif et al., 2009). The kidney is the primary target organ for OTA, which is considered a nephrotoxin to all animal species studied to date and is most likely toxic to humans, who have the longest half-life for its elimination among the species examined (Bennett and Klich, 2003). Other adverse effects of OTA include inhibition of macromolecular synthesis, increased lipid peroxidation, and inhibition of mitochondrial respiration. OTA has been suspected as a cause of various human nephropathies since the 1970s including Balkan Endemic Nephropathy (BEN) and chronic interstitial nephropathy (Bui-Klimke and Wu, 2015). However, in recent years, some authors have reported that BEN was rather due to aristolochic acid exposure (Grollman et al., 2007). OTA disturbs cellular physiology in multiple ways, but it seems that the primary effects are associated with the enzymes involved in phenylalanine metabolism, mostly by inhibiting the enzyme involved in the synthesis of the phenylalanine-tRNA complex. OTA has been hypothesized to cause oxidative damage to DNA, leading to mutagenesis and potential carcinogenesis. As reviewed by De Ruyck et al. (2015), recent papers also propose direct genotoxic mechanisms for OTA, describing a pathway that metabolizes OTA into an electrophilic species, capable of directly binding to some nucleotide bases. Consequently, OTA is classified as a possible human carcinogen by IARC (category 2B), citing sufficient evidence of carcinogenicity in animal models, but insufficient evidence from human studies. A recent *in vivo* study using mice reported that *p53* tumour suppressor protein was upregulated during OTA treatment, and

investigated the extent to which the *p53* protein inhibits progression of OTA-induced DNA damage (De Ruyck et al., 2015; Kuroda et al., 2015).

The mode or modes of action involved in OTA-mediated tumorigenicity and toxicity are still unknown and under continuous debate: a direct genotoxic mechanism (DNA binding of OTA), indirect oxidative DNA damage and several epigenetic mechanisms (such as disruption of mitosis, cell proliferation, activation of cell signalling pathways, and protein synthesis inhibition) have been proposed (Vettorazzi et al., 2013; Vettorazzi and López de Cerain, 2016). The controversy regarding OTA is that evidence supporting each of these mechanisms has been provided. The latest evaluation performed by the WHO (Joint FAO/WHO Expert Committee on Food Additives (JECFA), 2008) takes into account the different scientific evidence published to date and summarizes the main hypotheses that may contribute, totally or partially, to the possible mode of action of OTA, namely, i) genotoxicity from direct interaction of OTA or a reactive metabolite with DNA; ii) generation of tumours resulting from chronic renal toxicity and compensatory cell proliferation; iii) generation of tumours resulting from inhibition of phenylalanine-tRNAPhe synthetase and protein synthesis; iv) disruption of cell-cell signalling pathways and the cell division process; v) alteration of intracellular calcium homeostasis; and, vi) mitochondrial dysfunction leading to oxidative stress and indirect induction of DNA damage (reviewed by Vettorazzi and López de Cerain, 2016).

3.1.3. Patulin

Patulin (PAT) is a mycotoxin included in a group of compounds commonly known as toxic lactones (4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one). The structure of PAT is shown in Figure 5. This mycotoxin is produced by a wide range of fungal species of the *Penicillium*, *Aspergillus*, *Byssochlamys* and *Paecilomyces* genera from which *Penicillium expansum*, a common contaminant of damaged fruits and the principal cause of apple rot, is the most important (Azizi and Rouhi, 2013; Marin et al., 2013; Puel et al., 2010; Sant'Ana et al., 2008; Sherif et al., 2009). Table 2 presents the major fungi-producers species of PAT, however, other species were described as PAT-producers, as reviewed by Puel and colleagues (2010). Among the *Aspergillus* species, the number of PAT producing species is limited to three of the Clavati group: *Aspergillus clavatus*, *A. giganteus* and *A. longivesica*. For the *Penicillium* genus, 13 PAT producing species were identified: *P. carneum*, *P. clavigerum*, *P. concentricum*, *P. coprobium*, *P. dipodomyicola*, *P. expansum*, *P. glandicola*, *P. gladioli*, *P. griseofulvum*, *P. marinum*, *P. paneum*, *P. sclerotigenum* and *P. vulpinum*. In the case of the *Paecylomyces* sp., *Paecilomyces saturatus* was identified as a PAT producer. Relatively to *Byssochlamys* sp., two species were identified as PAT producers, *B. fulva* and *B. nivea* (Puel et al., 2010).

Historically, PAT was firstly isolated in 1943 from *Penicillium griseofulvum* and *Penicillium expansum* (Birkinshaw et al., 1943). This was part of the screening effort to find new fungal molecules with antibiotic properties, in the general enthusiasm following the discovery of penicillin by Fleming. This compound was tested in clinical trials by a British company, however, the interest in this potential antibiotic soon waned due its toxicity to humans and animals (Puel et al., 2010). For example, it was tested as both a nose and throat spray for treating the common cold and as an ointment for treating fungal skin infections. However, during the 1950s and 1960s, it became apparent that, in addition to its antibacterial, antiviral, and antiprotozoal activity, PAT was toxic to both plants and animals, precluding its clinical use as an antibiotic. During the 1960s, PAT was reclassified as a mycotoxin (Bennett and Klich, 2003; Reddy et al., 2010). As described by Azizi and Rouhi (2013), PAT is heat resistant, stable in acidic environment and unstable in alkaline environment and soluble in water. This toxin is a white crystalline substance with a melting point of 110.5 °C, molecular weight of 154 Dalton and maximum UV absorption at 276 nm (Azizi and Rouhi, 2013).

Juices of low-acid fruits (notably apple, grape, and pear) are the commodities most commonly contaminated by PAT, because these fruits are most often infected by the fungi that produce PAT. Patulin-producing strains have been isolated from a variety of fruits including apples, grapes, cherries, crab apples, pears, apricots, strawberries, nectarines, black mulberries, white mulberries, lingonberries, peaches, and plums (as reviewed by Reddy et al., 2010). Despite apples and their derived food products, such as juices and purees, are the main dietary source of PAT, other food commodities were contaminated by PAT, including vegetables, cereals and cheese (Abrunhosa et al., 2016; Azizi and Rouhi, 2013; Bennett and Klich, 2003; de Melo et al., 2012; De Ruyck et al., 2015; Raiola et al., 2015; Reddy et al., 2010; Sant'Ana et al., 2008). Despite PAT is regularly found in unfermented apple juice, it does not survive the fermentation into cider products (Reddy et al., 2010).

Forasmuch as PAT damage fruits and consumption of contaminated foods with this toxin is harmful for humans and animals health. PAT is recognized as mainly inducing gastrointestinal disorders with ulceration, distension and bleeding, and at higher doses, alterations in renal function. The ingestion of PAT can cause acute symptoms, such as seizures, shaking, bowel bleeding, oedema, and vomiting. Chronic symptoms include neurotoxic, immunotoxic, immunosuppressive, genotoxic, teratogenic, and carcinogenic effects. At the cellular level, some examples of these effects are plasmatic membrane rupture, protein synthesis inhibition, and DNA and RNA (ribonucleic acid) synthesis inhibition (Abrunhosa et al., 2016; de Melo et al., 2012; Mahfoud et al., 2002; Marin et al., 2013; Puel et al., 2010; Sant'Ana et al., 2008; WHO, 1998; Yang et al., 2014).

PAT has a strong affinity for sulfhydryl groups. This toxin exerts its toxic effect by covalently binding to reactive sulfhydryl groups in cellular proteins, as well as by glutathione depletion, resulting in oxidative damage and generation of reactive oxygen stress (ROS). Although several studies have shown PAT induced DNA strand breaks in mammalian cells, the mechanism of all these effects including apoptosis, however, is still poorly understood (Kwon et al., 2012). Although, the data on genotoxicity were variable, most assays carried out with mammalian cells were positive while assays with bacteria were mainly negative. In addition, some studies indicated that PAT impaired DNA synthesis. These genotoxic effects might be related to its ability to react with sulfhydryl groups and to induce oxidative damage (de Melo et al., 2012; Puel et al., 2010). Saxena and co-workers, using the comet assay, reported that topical application of PAT has the potential to induce DNA damage in mice skin cells, which may lead to apoptosis induction (Saxena et al., 2009). De Melo and colleagues studied for the first time the systemic genotoxicity of PAT and the results showed that this mycotoxin induces DNA damage in the brain, liver and kidneys in a dose-dependent manner and over a broad dose range (de Melo et al., 2012). These authors established a correlation between the pro-oxidant action and genotoxic effects of PAT and confirmed the positive relationship between PAT-induced GSH depletion and the effect of DNA damage derived by systemic administration of PAT. The mutagenicity of PAT was also demonstrated *in vitro* using V79 Chinese hamster cells, where exposure above threshold concentrations was observed to produce significant DNA strand breakage and interstrand cross-links (Schumacher et al., 2006). It is worth noting that cells exposed to genotoxic concentrations of PAT in that study produced other important cytotoxic effects, such as severe impairment of plating efficiency, whereby cell adhesion and colony formation was no longer observed. Interestingly, a previous *in vitro* study on PAT's genotoxicity using the same cell type reported abolition of mutagenic effects when simultaneously exposed to ascorbic acid (Alves et al., 2000). Additionally, PAT may increase permeability across intestinal Caco-2 monolayers, and it has been reported to have cytotoxic effects on colon cancer cell lines *in vitro* (Assunção et al., 2014; Katsuyama et al., 2014; Kawauchiya et al., 2011; Mahfoud et al., 2002; Maresca et al., 2008; McLaughlin et al., 2009; Mohan et al., 2012). All these findings may have important implications for risk analysis of real-world dietary exposure to this mycotoxin (De Ruyck et al., 2015). PAT is currently classified by the IARC as Group 3, that is, not classifiable with regard to its carcinogenicity to humans (Table 3), citing poor experimental designs providing inadequate evidence for carcinogenicity in animals, and preventing its evaluation with regard to carcinogenicity in humans.

3.1.4. Fusarium toxins

Fusarium toxins are produced by several field fungi and are commonly found in cereals, particularly in wheat, barley and maize and grown in temperate regions of America, Europe and Asia. Infection of the grain takes place in pre-harvest, whereby climatic conditions during growth, and in particular during flowering, shows major impact on the mycotoxin contents of final products. Most of the toxin-producing *Fusarium* fungi are capable of generating a variable range of two or even more toxins. The major *Fusarium* mycotoxins are fumonisins, trichothecenes, zearalenone and a group of mycotoxins known as the emerging mycotoxins that include beauvericin, enniatins, fusaproliferin, and moniliformin (Bennett and Klich, 2003; Biselli and Hummert, 2005; Creppy, 2002; Ferrigo et al., 2016).

3.1.4.1. Trichothecenes

The trichothecenes constitute a family of approximately 170 structurally related compounds. These metabolites are produced by a number of fungal genera, including *Fusarium*, *Myrothecium*, *Phomopsis*, *Stachybotrys*, *Trichoderma*, *Trichothecium*, among others. The term trichothecene is derived from trichothecin, which was first isolated from *Trichothecium roseum* and described by Freeman and Morrison in 1949. The discovery of trichothecin was followed by the isolation and description of other trichothecenes, such as diacetoxyscirpenol, T-2 toxin, nivalenol and deoxynivalenol (Bennett and Klich, 2003; Bosco and Mollea, 2012; Wu et al., 2014). All trichothecenes can be classified based on the substitution pattern of the tricyclic 12,13-epoxytri-chothec-9-ene, resulting in 4 groups, A, B, C and D. The structures of the key trichothecenes are shown in Figure 5. The 12,13-epoxytri-chothec-9-ene structure is shared by all trichothecenes and is considered essential for toxicity (Bennett and Klich, 2003; Ferrigo et al., 2016). Type A is represented by HT-2 toxin (HT-2) and T-2 toxin (T-2), and type B mainly includes deoxynivalenol (DON), nivalenol (NIV), 3-AcetylDON, 15-AcetylDON and fusarenone X. The trichothecenes are in general very stable compounds, both during storage/milling and the processing/cooking of food, and are not degraded by high temperatures. Types A and B are mainly characterized by the presence of different functional groups in the C-8 position of the trichothecene backbone. C and D types include some trichothecenes of lesser importance (Ferrigo et al., 2016; Marin et al., 2013). Trichothecenes of type C (e.g., crotocin and baccharin) have a second epoxy ring between C-7 and C-8 or between C-9 and C-10. Trichothecenes of type D, such as satratoxin and roridin, contain a macrocyclic ring between C-4 and C-15 (Li et al., 2011).

The trichothecenes are extremely potent inhibitors of eukaryotic protein synthesis by affecting the 60S subunit of the ribosome interfering with the peptidyl transferase activity and are known to cause

neurotoxicity, immunosuppression and renal toxicity. Different trichothecenes interfere with initiation, elongation, and termination stages (Bennett and Klich, 2003; Raiola et al., 2015; Sherif et al., 2009).

Type A trichothecenes are mostly represented by T-2 and HT-2 and do not contain a carbonyl group at the C-8 position. In type B trichothecenes, a carbonyl group is present at the C-8 position (Li et al., 2011). T-2 and HT-2 toxins are produced by several *Fusarium* spp., mainly *Fusarium langsethiae*, *F. sporotrichioides* and *F. poae* which may grow on a variety of cereal grains, especially in cold climate regions or during wet storage conditions (FAO/WHO, 2002; Li et al., 2011; Pettersson, 2011). The most frequently contaminated cereal samples are maize, wheat, and oats (SCOOP, 2003). T-2 has received much attention because it has the highest toxicity of all the trichothecenes, although it is less frequently detected compared to the other trichothecenes. T-2 causes a large range of toxic effects in animals, such as weight loss, decreases in blood cell and leukocyte count, reduction in plasma glucose, and pathological changes in the liver and stomach. Furthermore, T-2 is associated with an increased infection rate, alimentary toxic aleukia, DNA damage, and induction of apoptosis (Li et al., 2011). T-2 is rapidly metabolized *in vivo* to HT-2, which induces adverse effects similar to T-2, with non-remarkable differences in terms of potency. As a potent inducer of oxidative stress and an inhibitors of DNA, RNA, protein synthesis and mitochondrial function, T-2 and HT-2 toxins represent contaminants that are of considerable concern for human and animal health (Creppy, 2002; Ferrigo et al., 2016; Li et al., 2011; Pettersson, 2011).

Deoxynivalenol (DON) is one of the most commonly encountered mycotoxins and it is widely detected in feed and food products in both developed and developing countries. DON is produced by *Fusarium graminearum* and *Fusarium culmorum* on cereal crops, which are responsible for *Fusarium* head blight in wheat, and may be identified as a type-B trichothecene by the carbonyl group at C-8 (De Ruyck et al., 2015; Ferrigo et al., 2016; Hassan et al., 2015; Lee and Ryu, 2015). DON is mainly found in cereals like wheat, maize, barley, and less often in rice, oats, rye, triticale and sorghum (Bennett and Klich, 2003; Pestka, 2010; Raiola et al., 2015). Colloquially known as “vomitoxin” because of its emetic effects in pigs, DON has been associated with human gastroenteritis. IARC listed DON as a Group 3 (not classifiable) human carcinogen due to inadequate evidence of animal carcinogenicity, and lack of investigation in humans (Table 3). Acute dietary exposure to DON is characterized in animals by emesis and feed refusal. DON is rapidly metabolized for urinary excretion as glucuronide conjugates within 24 hours of dietary exposure (De Ruyck et al., 2015). DON is capable of disrupting macromolecular synthesis, cell signalling, differentiation, proliferation, gene upregulation, and programmed cell death contributing to acute poisoning and chronic pathological sequelae in experimental animals. These observations shed light on

DON's potential to adversely affect human health. At the immune system level, as demonstrated in animals and culture models, DON can act on the viability and proliferation of immune cells, inhibiting protein biosynthesis and altering the pro-inflammatory cytokine production. Additionally, DON binds to peptidyl transferase, inhibiting the synthesis of RNA and DNA and modifying cell membranes (Hassan et al., 2015; Pestka, 2010; Raiola et al., 2015). Moreover, a very recent study has proven that DON facilitates allergic sensitization to whey in mice and concluded that these obtained data illustrate the possible contribution of food contaminants as DON in allergic sensitization in humans (Bol-Schoenmakers et al., 2016). Relative to DON's chronic toxic effects, experimental animal studies indicate that growth is the most likely parameter to be affected (Hassan et al., 2015; Pestka, 2010).

3.1.4.2. Fumonisins

Fumonisins consist of similar analogues composed of a carbon-chain backbone with two tricarboxylic acid groups esterified at the C-14 and C-15 position. Although up to 13 *Fusarium* species are able to produce fumonisins, the major producers, *Fusarium verticilliodies* and *F. proliferatum* fungi, frequently contaminate maize and other cereal grains growing best at high temperatures in humid climates (Table 2) (Creppy, 2002; FAO/WHO, 2002; Ferrigo et al., 2016; Marin et al., 2013; Szabó-Fodor et al., 2015). Fumonisins can be separated into four main groups, identified as the fumonisin A, B, C, and P series. The B group includes the most active fumonisin B_1 (FB_1) and its isomers FB_2, FB_3 and FB_4 (depicted in Figure 5). In particular, FB_1, the most predominant and well-studied isoform, causes considerable toxicological concern, and is the most abundant fumonisin produced in maize. FB_1 accounts for 70% - 80% of total fumonisins compared with 15% - 25% FB_2, 3% - 8% FB_3 and 1% - 2% FB_4 (Creppy, 2002; FAO/WHO, 2002; Szabó-Fodor et al., 2015; Szécsi et al., 2010). FB_1 is nephrotoxic and hepatotoxic in several species and has been classified as a Group 2B, *i.e.* a possible human carcinogen (Table 3). FB_1 exposure has been associated with liver and oesophageal cancers in high-risk populations. Fumonisins have also been implicated as a risk factor for neural tube defects. The mechanism of action of FB_1 found to induce neural tube defects is the inhibition of folic acid uptake and metabolism, while its carcinogenic effects are related to the overall disruption of lipid metabolism, membrane structure and cellular signal pathways resulting from ceramide synthases inhibition. This group of mycotoxins, especially FB_1, is known to strongly inhibit ceramide synthase, the enzyme that catalyses sphinganine acylation and sphingosine recycling. This imbalance has been proposed as the main responsible for the toxicity, and possibly carcinogenicity, of fumonisins, based on mechanistic studies with cell cultures and borne out by animal studies (Gelderblom and Marasas, 2012; Norred et al., 1992; Riley et al., 1993). A recent etiological

study also found a relation between prevalence of FB_1 contamination in rice and incidence of oesophageal cancer, though this relationship was not seen with other contaminated staple foods, such as corn, in the same region of Iran (Alizadeh et al., 2012).

3.1.4.3. Zearalenone

Zearalenone (ZEA) is a phenolic resorcyclic acid lactone mycotoxin produced by several *Fusarium* species, particularly *F. graminearum* and also *F. culmorum*, *F. equiseti* and *F. verticillioides*. These species can produce small amounts of several related metabolites, α-zearalenol and β-zearalenol, which are the most important derivatives (Figure 5). ZEA is a common *Fusarium* mycotoxin in the temperate regions and high levels of ZEA in cereals are generally associated with wet temperate weather and improper storage in high moisture environments (Marroquín-Cardona et al., 2014). ZEA is commonly found in maize but can also be found in other crops such as wheat, barley, sorghum and rye throughout various countries of the world (EFSA, 2011a; Marin et al., 2013). ZEA is of major interest when it comes to the public health concern, because despite its low acute toxicity, it has proven to be hepatotoxic, immunotoxic, and carcinogenic to a number of mammalian species. Moreover, ZEA acts as an endocrine disruptor as some of its metabolites, and they have been shown to competitively bind to oestrogen receptors in a number of different species and are responsible for hyperoestrogenism and infertility in livestock (Ferrigo et al., 2016). Nevertheless, it was found that ZEA affinity is much lower than that of physiological concentration of 17β-estradiol. The estrogenic potential of α-zearalenol is higher than for ZEA and β-zearalenol, probably due to its greater binding affinity to estrogen receptors (EFSA, 2016; Ferrigo et al., 2016; Lee and Ryu, 2015; Marin et al., 2013; Nesic et al., 2014). ZEA is classified under Group 3, although carcinogenic, hepatotoxic, genotoxic, and immunosuppressive properties of this endocrine disruptor have been documented (Hueza et al., 2014; Lee and Ryu, 2015).

3.2. MYCOTOXINS AND CHILDREN HEALTH

3.2.1. Current situation of Mycotoxins in food and children food in Portugal

The presence of mycotoxins in food products available to be consumed in Portugal generally follows the occurrence pattern of these toxins in other European countries and elsewhere in the world, as recently reviewed by Abrunhosa et al. (2016). The characterization of mycotoxins occurrence in food consumed in Portugal, with focus on the main mycotoxins with public health concern, is presented in Table 5. Different food products were analysed in order to clarify the occurrence of different mycotoxins, including cereal-based products and food products of animal origin such as milk and dairy products. It is important to notice that some authors evaluated mycotoxins levels in foods intended to be consumed by children, a specific vulnerable population group (Alvito et al., 2010; Barreira et al., 2010; Cunha et al., 2009).

Table 5. Mycotoxins analysis in food products from the Portuguese market.*

Mycotoxins	**Food Products**	**References**
Aflatoxins	Infant and children food; Milk, yoghurt, cheese and other dairy products; Peanuts, corn, dried figs, and spices	(Alvito et al., 2010; Martins et al., 2007, 2005, 2001; Martins, 2004; Martins and Martins, 2000; Peito and Venâncio, 2004)
Ochratoxin A	Infant and children food; Cereals and cereal-based products; Coffee; Spices; Wine	(Alvito et al., 2010; Bento et al., 2009; Duarte et al., 2010; Juan et al., 2008a, 2008b, 2007; Lino et al., 2006; Martins et al., 2003; Miraglia and Brera, 2002; Paíga et al., 2013; Peito and Venâncio, 2004; Pena et al., 2010, 2005, Serra et al., 2006a, 2006b, 2004)
Patulin	Apples and apple-based products; Other fresh fruits	(Barreira et al., 2010; Cunha et al., 2009; Majerus and Kapp, 2002; Martins et al., 2002)
Fumonisins	Cereal-based products, specially corn and corn-based food products	(Lino et al., 2006; Lino et al., 2007; Martins et al., 2008)
Deoxynivalenol	Cereal-based products	(Cunha and Fernandes, 2010; Marques et al., 2008; Martins et al., 2008; Martins and Martins, 2001; Peito and Venâncio, 2004)
Zearalenone	Cereal-based products	(Cunha and Fernandes, 2010; Marques et al., 2008; Peito and Venâncio, 2004)

*The studies referred reflect the published reports presenting results on mycotoxins occurrence in food since 2000.

In fact, the results obtained, especially those by Alvito et al. (2010) highlighted the occurrence of mycotoxins, namely AFTs and OTA, in marketed baby foods in Portugal and the potential negative health impact resulting from the ingestion of contaminated foods by children. The authors suggested that the obtained data should be used to estimate Portuguese children exposure in the future, combining them with Portuguese data intakes of these food products by children. The results also highlighted the potential exposure of Portuguese children to more than one mycotoxin simultaneously. Gathering all these conclusions, the need for additional information about Portuguese children exposure to co-occurring mycotoxins through food consumption led to the development of MYCOMIX national funded project, "Exploring the toxic effects of mixtures of mycotoxins in infant food and potential health impact" (Alvito et al., 2015). MYCOMIX explored the toxic effects of mycotoxins mixtures in infant food and its potential health impact. This research project included different tasks in order to study the occurrence of multiple mycotoxins and related toxicity interactions in infant foods and cereals consumed by Portuguese children. Within this project, Portuguese children (<3 years old, n=103) food consumption data were obtained using a three days food diary in a pilot study performed at a Primary Health Care Unit (Leal et al., 2015). The main declared foods eaten by children were purchased in the Lisbon area along 2014 - 15 and analysed by chromatographic analytical techniques for multiple mycotoxins co-occurrence. Toxicological studies including cyto and genotoxic interactions (Tavares et al., 2013) and bioaccessibility between detected mycotoxins using *in vitro* approaches (https://www.youtube.com/watch?v=CsKaz3mt2J4) were also performed.

An important output of MYCOMIX was the publication of a book reviewing the main aspects of mycotoxins and their impact on human health (Alvito, 2014). This book, published by the National Institute of Health Dr. Ricardo Jorge, was the first written in Portuguese: one of the author's goal was to disclose the issue of mycotoxins and its impact on human health to the Portuguese speaking populations, namely to health-care professionals, food regulatory entities, industries, researchers and university students (Paula Alvito, 2016, Personal Communication). These include African countries, where mycotoxins exposure could assume a particular threat to human health. Reflecting the importance of multiple mycotoxins issue nowadays, an international conference was held for the first time in Portugal, including scientifically relevant lectures, oral and poster communications, focusing on different aspects of mycotoxins in foods (ICFC2015 – International Conference on Food Contaminants: challenges in chemical mixtures, held in Lisbon, 13th and 14th April 2015). A book, compiling the main conclusions and original articles produced by the invited speakers was published (Alvito et al., 2016).

All the above-mentioned aspects suggest the mycotoxin issue relevance in Portugal accompanying the international development of this research area, contributing to an improved understanding of specific aspects of mycotoxins human health impact.

3.2.2. Mycotoxins with relevance for children's health

The period of infancy and early childhood encompasses a special life stage of rapid growth, development, and maturation. Organ systems undergo rapid development during gestation and are still developing in infants and young children, resulting in potentially different sensitivities to chemical exposures compared to adults. Early adverse effects may have lifelong consequences, and chronic diseases, including neurological and immune-related conditions, often begin in infancy or childhood (Felter et al., 2015). Some of the physiological and exposure differences between infants/children and adults include: small size and large surface area in relation to weight; a higher metabolic rate; rapid growth; different body composition; and functional immaturity of the organs and other body systems (WHO, 2011). These features allow international institutions to assume that "children are not simply small adults but rather are a unique population for health risk assessment". Many factors contribute to these differences, and the key physiological parameters resulting in differences between infants and adults are summarized in Table 6. These factors could render an infant or child more or less susceptible than an adult (Felter et al., 2015).

Infants and children are considered to be more susceptible than adults to different toxins as mycotoxins. This fact results mainly of their lower body weight, higher metabolic rate, lower ability to detoxify and incomplete development of some organs and tissues such as the central nervous system (NAS, 1993; Peraica et al., 2014; Sherif et al., 2009). As previously referred, several mycotoxins could be present in unprocessed and processed food and feed and consequently can affect human and animal health. Although there are many species of toxigenic moulds, only a few mycotoxins are considered to be significant for humans. As presented in Table 7, mycotoxins of worldwide public health primary importance to children are aflatoxins, fumonisins, trichothecenes, ochratoxins, patulin and ZEA (Etzel, 2006; Sherif et al., 2009). After exposure (by ingestion, inhalation or skin contact), the toxicity of a mycotoxin is determined by a sequence of events (metabolism) involving the administration, absorption, transformation, toxicokinetics, molecular interactions, distribution and excretion of the toxin and its metabolites. In turn, the toxicity of a mycotoxin will be manifested by its effect on health and productivity of crops, humans and animals and these effects will influence the production of wealth associated with human endeavour and agricultural and livestock products (WHO, 2011).

Table 6. Comparison of key physiological parameters resulting in differences between infants and adults (adapted Felter et al., 2015).

Physiological parameter	Key differences and maturational timeline
GI absorption	Newborns and young infants have a higher gastric pH, reduced gastric emptying, and reduced GI motility compared to children and adults. Generally, the net impact is that GI absorption is generally lower in newborns and younger infants.
Dermal absorption	Barrier properties for full-term infant skin, which matures very quickly in the days following birth and is generally comparable to adult skin.
Volume of distribution	Infants have a higher water content than older children and adults (80 – 90% in young infants vs 55 – 60% in adults) and thus a higher volume of distribution.
Protein binding	Infants have lower levels of serum proteins. For compounds that bind to these proteins, infants have a correspondingly higher fraction of unbound compound.
Liver size	Per kg of body weight, infants have a larger liver than children and adults (5% of body weight at birth compared to 2% in adults)
Hepatic blood flow	Lower in infants; however, when normalized to either liver volume or body weight, it is the highest in neonates and comparable in infants, children and adults.
Liver enzyme content (CYP)	The level at birth and timeline for maturation of CYP enzymes is highly variable, with some approaching adult levels within a few weeks (e.g. CYP3A4/5) and others maturing more slowly over a period of years (e.g. CYP1A2).
Phase II metabolism	Infants have lower levels of glucuronidation, which approach adult levels by 3 – 6 months. In contrast, levels of sulfo conjugation already approach adult levels at birth.
Renal blood flow	Lower in young infants; increases fairly quickly and reaches adult levels by ~ 5 months.
Glomerular filtration rate (GFR)	GFR is reduced in infants, but matures fairly quickly. The literature suggests that GFR reaches adult levels at about 6 months of age; in children it generally exceeds that of adults.
Renal tubule secretion	Lower in infants; reaches adult levels by ~ 7 months but timeline for maturation is more variable than for GFR; in children, it can exceed that of adults.
Tubular reabsorption	Lower in infants; less known about maturation timeline. More important for lipid soluble compounds that are not metabolized.

GI: Gastrointestinal tract; GFR: Glomerular filtration rate; CYP: Cytochrome P450.

Acute mycotoxicoses in children are serious diseases, mostly diagnosed only when an epidemics breaks out, affecting several children. They should be suspected when symptoms cannot be explained by the infection with a known microorganism. Acute mycotoxicoses are more frequent in tropical regions but are no stranger to temperate climates as well. Chronic mycotoxicoses may appear all over the world and paediatricians should keep in mind that their clinical manifestations vary a lot (Peraica et al., 2014).

Considering that children have more future years of life than do most adults, they have more time to develop chronic diseases that may be triggered by early chemical exposures. Insults to health occurring during childhood may manifest themselves over a lifetime of growth to adulthood and senescence (Sherif et al., 2009). Comparing child to adult, many factors are considered: i) children consume more food (particularly for certain foods) and water compared to adults when expressed per kg of body weight (bw), resulting in relatively higher exposures to compounds; ii) specific dietary patterns of children may contribute to a higher exposure to contaminants present in food; iii) once exposure has occurred, the toxicokinetic handling of xenobiotics is likely to differ from that in adults with respect to their metabolism, clearance, protein binding and volume of distribution; and iv) toxicodynamic differences in which the sensitivity of rapidly developing tissues/systems in neonates and young children may differ from that in adults (Boon et al., 2009; NAS, 1993; Raiola et al., 2015; Sherif et al., 2009).

Table 7. Mycotoxin-producing fungi with relevance to children's health (adapted from Etzel, 2006).

Fungus	Mycotoxins	Associated health effects
Aspergillus flavus *Aspergillus parasiticus*	Aflatoxins	Vomiting, hepatitis Liver cancer
Fusarium verticillioides *Fusarium proliferatum* *Aspergillus ochraceus*	Fumonisins	Vomiting Neural tube defects Oesophageal cancer
Fusarium culmorum *Fusarium graminearum* *Fusarium cerealis*	Deoxynivalenol	Vomiting
Fusarium sporotrichiodes	T-2 toxin	Alimentary toxic aleukia Vomiting, haemorrhage
Aspergillus ochraceus *Aspergillus niger* *Aspergillus alliaceus* *Penicillium verrucosum*	Ochratoxins	Balkan nephropathy Renal cancer
Penicillium expansum	Patulin	Vomiting
Fusarium graminearum	Zearalenone	Estrogenic effects, cervical cancer (suspect)

Due to significant postnatal development of different organ systems during childhood, children may be more sensitive to neurotoxic, endocrine and immunological toxic effects up to four years of age. This is also true for children aged five to twelve years, however to a lesser extent for immunological toxic effects. Due to these differences between adults and children regarding exposure and physiology, it is important to address children as a separate subgroup in risk assessments (Boon et al., 2009). Still, the exercise of risk assessment for children and mycotoxins is faced with many difficulties, such as seldomly available reliable exposure data and the long latency of many mycotoxins induced diseases, confounding exposures from natural co-occurrences of mycotoxins or other toxins, or the effect of other dietary elements on toxicity (as vitamins, antioxidants or chlorophyllic ingredients) (as reviewed by Peraica et al., 2014; Sherif et al., 2009).

3.2.3. Regulatory limits concerning food for children

Nowadays, it is really important to adopt measures aimed at guaranteeing that unsafe food is not placed on the market and at ensuring that systems exist to identify and respond to food safety problems, ensuring the proper functioning of the internal market and human health protection (Stoev, 2015). Until the early 1960s mycotoxicoses remained "neglected diseases" (van Egmond et al., 2007). The first limits for mycotoxins were set in the late 1960s for aflatoxins. The subsequent recognition that mycotoxins were a significant health concern to both humans and animals conducted progressively to the development of regulations for mycotoxins in food and feed. They were established by developed countries, and limits often had an advisory or guideline character. As reviewed by van Egmond et al. (2007), over the years, the number of countries with known specific mycotoxin regulations has increased from 33 in 1981, to 56 in 1987, 77 in 1995, and 100 in 2003. In Europe, the EU has introduced comprehensive mycotoxin regulations for food to facilitate world trade and protect consumer health (Cheli et al., 2014). The European Commission Regulation 1881/2006 established the maximum permissible limits for aflatoxins, ochratoxin A, patulin and some other mycotoxins in specific products (EC, 2006). EU is also actively involved in considering additional mycotoxins that need regulation (Stoev, 2015). In addition to mycotoxin maximum contents, European Commission Regulation 401/2006 provides sampling plans according to nine different groups of food commodities taking into account the heterogeneous distribution of mycotoxins in agricultural commodities (EC, 2006b).

The regulation issue assumes a particular importance for groups of the population that are more vulnerable, as children. The potential health risk of children exposure to any contaminant in foods is set to be three times higher than for adults (EC, 1993). Consequently, EU payed a special attention to this

group and established regulatory and indicative limits for mycotoxins in foods for children. These limits, considering children vulnerability, are much lower than the limits set for all other regulated matrices (EC, 2006; EC, 2013), and are summarized in Table 8.

Table 8. Maximum permitted content and indicative levels of mycotoxins in food intended for children or potentially consumed by this population group (EC, 2013; EC, 2006) (adapted from Stoev, 2015).

Mycotoxins	Foodstuffs	Maximum content (µg/kg)
Aflatoxin B_1	Processed cereal-based foods and baby foods for infants or children	0.10
Aflatoxin M_1	Raw milk, heat-treated milk and milk-based products	0.050
	Dietary foods for special medical purposes and infant milk	0.025
Ochratoxin A	Processed cereal-based foods and baby foods for infants/children	0.50
Patulin	Baby foods other than processed cereal-based foods, apple juice and solid apple products as compote or apple puree for infants and children	10.0
Deoxynivalenol	Cereals for direct human consumption as flour, bran, pasta and germ	750
	Bread and bakery wares, pastries, biscuits, cereal snacks or breakfast	500
	Processed cereal-based foods and baby foods for infants and children	200
Zearalenone	Cereals for direct human consumption as cereal flour, bran and germ	75
	Bread and bakery wares, pastries, biscuits, cereal snacks or breakfast, excluding maize snacks and maize-based breakfast cereals	50
	Maize for direct human consumption as snacks and breakfast cereals	50
	Processed cereal-based foods and baby foods for infants and children	20
Fumonisins B_1 + B_2	Processed maize-based foods or baby foods for infants and children	200
T-2 toxin + HT-2 toxin*	Cereal products for human consumption:	
	Oat bran and flaked oats	200
	Cereal bran except oat bran, oat milling products other than oat bran and flaked oats, and maize milling products	100
	Other cereal milling products	50
	Breakfast cereals including formed cereal flakes	75
	Bread and bakery wares, pastries, biscuits, cereal snacks, pasta	25
	Cereal-based foods for infants and young children	15

* Indicative levels – Commission Recommendation 2013/165/EU

Although the regulation developments, other problems associated to mycotoxin issues are emerging, such as multiple mycotoxins exposure, which is an increasing area of concern.

3.3. MULTIPLE MYCOTOXINS PRESENT IN FOOD

Humans and animals are exposed on a daily basis to several contaminants, and food and feed are reservoirs where the presence of chemical contaminants is possible. There is an increased concern about the exposure health effects to combined chemicals present in food, air or water nowadays. However, the mechanism of action and effects of combined chemical contaminants are still poorly understood (Dorne and Fink-Gremmels, 2013; Pose-Juan et al., 2016; Smith et al., 2016). As schematically presented in Figure 8, several chemical contaminants can enter human body through the mouth, skin, ingestion and inhalation. After exposure, different fates can be associated to each compound. Consequently, each substance can have different targets, specific mechanisms of action and excretion routes.

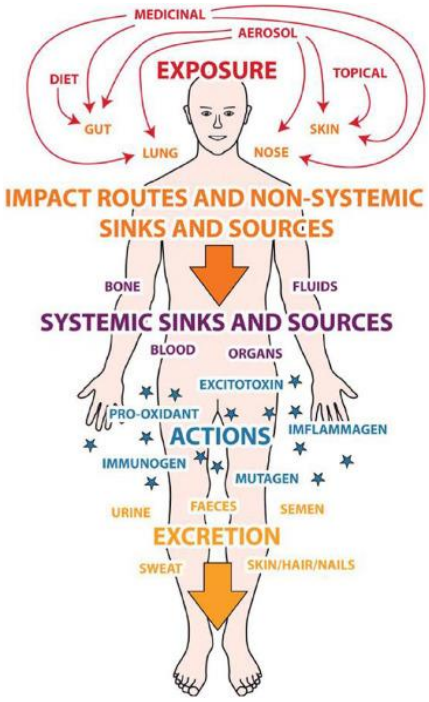


Figure 8. Human exposure to multiple chemical contaminants from different sources (Exley, 2013).

The issue on combined effects has particularly concerned policy makers because combined effects can be higher than the individual effects of each chemical contaminant (Alassane-Kpembi et al., 2016; Dorne and Fink-Gremmels, 2013; Pose-Juan et al., 2016; Smith et al., 2016). Currently, a significant number of studies have highlighted human and animal exposure to several chemical contaminants, but these studies were mainly developed with one type of chemical at a time. Relatively to the risk assessment,

historically, the health risk from human exposure to chemical contaminants has been evaluated on the basis of single-chemical and single-exposure pathway scenarios. In general, exposures to a chemical through food were assessed independently, and no concerted effort has been made to evaluate possible multiple exposures simultaneously. However, in the last years a tiered approach has been proposed by WHO and EFSA in order to assess the risk of multiple chemicals, including contaminants (EFSA, 2013; Meek et al., 2011). This hierarchical approach involves integrated and iterative considerations of exposure and hazard at all phases, with each tier being more refined (*i.e.* less conservative and uncertain) than the previous one, but more laborious and intensive. The framework comprises a tiered approach for exposure assessment, hazard assessment and risk characterization and requires at the higher tiers increasing knowledge about the group of chemicals under assessment. Briefly, the tiers can range from tier 0 (default values, data poor situation) to tier 3 (full probabilistic models) (EFSA, 2013; Meek et al., 2011).

Relatively to mycotoxins, a low level of these compounds in food is generally considered inevitable. Although several actions to manage fungal contamination have been realized, toxigenic fungi are ubiquitous in nature and therefore occur commonly in foodstuffs as a consequence of susceptible matrices infestation (Raiola et al., 2015). The co-contamination of foods and feeds with known or unknown mycotoxins is being reported at an increasingly higher rate. Although a limited information on the effects of specific mycotoxins or mycotoxin combinations in the course of various human/animal diseases is available (Stoev, 2015). As previously referred, the current regulations were established on toxicological data from studies taking into account only one mycotoxin exposure at a time, not considering the combined effects of mycotoxins. However, the natural co-occurrence of mycotoxins particularly in cereals grains is well-known, and can be supported by at least three reasons: i) most fungi are able to produce several mycotoxins concurrently (Table 2); ii) food commodities can be contaminated by several fungi simultaneously or in quick succession and iii) foodstuffs could be made up of multiple grain sources with different potential fungi contamination (Smith et al., 2016). Several surveys reported the natural co-occurrence of mycotoxins from all over the world. Smith et al. (2016) evaluated the relevant data from 1987 to present dealing with mycotoxin co-occurrence in different foods and feeds. The main mycotoxins combinations quoted in the evaluated data, depending on their geographic origin, are presented in Figure 9.

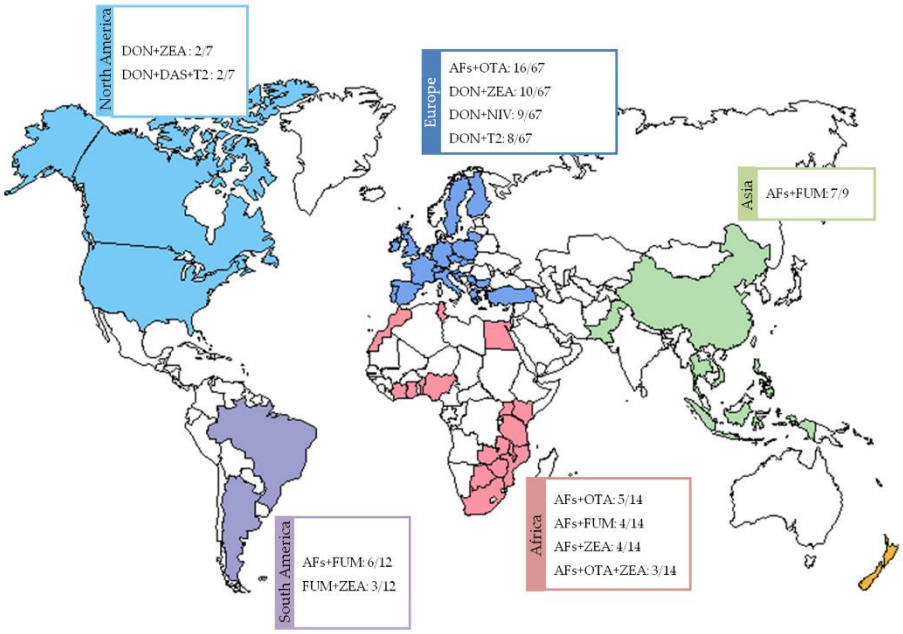


Figure 9. Main mycotoxin mixtures found by geographic origin reported by Smith et al. (2016). Data were generated through analysis of 107 articles (since 1987 to present).

Relatively to the European studies, Smith et al. (2016) reported that among 105 mycotoxin mixtures found, the most reported one was AFT + OTA. This mixture was also verified in African studies as the main mixture. In Asia and South America, AFT + fumonisins was the most observed mixture. Concerning North America, the main mycotoxin mixtures were DON + ZEA and DON + diacetoxyscirpenol (DAS) + T2 (Smith et al., 2016).

All this information reinforces that the risk of human co-exposure to multiple mycotoxins is real, raising a growing concern about their potential impact on human health. Consequently, the risk assessment of combined human exposure to multiple mycotoxins poses several challenges to all the stakeholders of this process. The main aspects of risk assessment of multiple mycotoxins in food will be presented following.

3.3.1. Hazard assessment

Particular attention has been dedicated in last years to the occurrence of multiple chemicals in food, leading to a change in the paradigm of the hazard assessment. The urgent need for a deep understanding of the potential effects of chemicals in mixture gave rise to the concept of combined toxicity which is defined as the "response of a biological system to several chemicals, either after simultaneous or sequential exposure" (EFSA, 2013; Loewe and Muischnek, 1926). Combined toxicity can take three possible forms: concentration addition (CA), independent action (IA) or interaction (Loewe and Muischnek,

1926). According to the CA model, the joint action of multiple chemicals is the summation of individual toxicities, assuming the same Mode of Action (MoA) and/or at the same target cell, tissue or organ. In the IA model, the combined effects are estimated assuming that chemicals act independently by dissimilar MoA or at different target cells, tissues or organs and considers that the probability of toxicity from exposure to one chemical is independent from the probability of toxicity from exposure to another chemical in the mixture (Bliss, 1939; Jonker et al., 2004; Meek et al., 2011). These two reference models have found successful application to the toxicological assessments of mixtures of similarly and dissimilarly acting compounds, both in ecotoxicology studies using a range of species (Backhaus et al., 2004; Faust et al., 2003; Loureiro et al., 2010) and in human toxicity studies using cell lines or animal models (Alassane-Kpembi et al., 2016; Grenier and Oswald, 2011; Mueller et al., 2013; Smith et al., 2016; Tavares et al., 2013). Interactions are inferred when a mixture of chemicals produces a biological response greater or lower than expected (Alassane-Kpembi et al., 2016; Grenier and Oswald, 2011) and include synergism (mixture effect greater than additive), antagonism (mixture effect less than additive) and more subtle interactions that depend on the actual doses of the mixture components (e.g. synergism at low doses and antagonism at higher doses) or on the concentration ratio between the compounds in the mixture (e.g. the extent of the synergism or the antagonism depends on the relative contribution of each compound in the mixture) (Grenier and Oswald, 2011; Jonker et al., 2005, 2004). Figure 10 schematically presents the potential interactions that can occur between chemical compounds. From a practical point of view, the application of a single model for all situations is desirable and, as the CA represents the most conservative model, EFSA recommended its use within the risk assessment of food contaminants, which includes mycotoxins (EFSA, 2013).

A particular attention must be dedicated to carcinogens in hazard assessment, including mycotoxins and other food contaminants (Jeffrey and Williams, 2005). Genotoxicity, *i.e.*, the capacity of exerting a damaging effect on cell's genetic material (DNA, RNA, chromosomes), affecting its integrity and/or function, is a major mechanism of the carcinogenic process. Considering that genetic events, such as gene mutations, structural or numerical chromosomal aberrations and recombination are closely related with carcinogenesis, genotoxic effects can be characterized in a faster, easier, and inexpensive way using standard *in vitro* and *in vivo* genotoxicity assays, instead of performing long-term carcinogenesis assays in animals (Dearfield and Moore, 2005; Louro et al., 2015). However, chemicals acting through a non-genotoxic mechanism, including induction of epigenetic events and mitogenesis, can be equally relevant to the carcinogenic process. Therefore, besides genotoxicity testing, mechanistic studies are also needed to clarify the MoA of carcinogenic agents. In this respect, it is generally assumed that a threshold of

exposure may be determined for non-genotoxic carcinogens, below which no biologically significant effect will be induced (Dybing et al., 2008). In contrast, from a conservative and health protection point of view, it has been assumed that genotoxic carcinogens act by a non-threshold mechanism, giving rise to linear dose-response curves. The decision on whether or not a chemical is genotoxic is thus of primary importance to select between a non-threshold or threshold risk assessment approach in the step towards hazard identification.

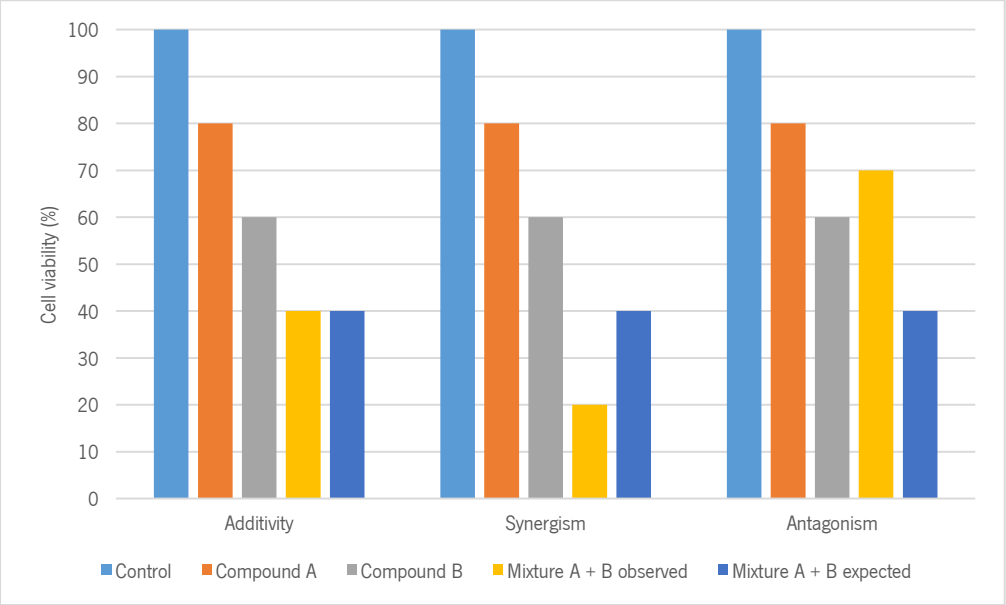


Figure 10. Potential interactive effects between mixture compounds (adapted from Grenier and Oswald, 2011; Smith et al., 2016).

The number of studies addressing the combined effects of mycotoxins using *in vitro* and *in vivo* models and several endpoints (e.g., cytotoxicity, immunotoxicity and genotoxicity) has been steadily increasing in the last decade, giving rise to a set of data that might greatly contribute to hazard assessment of multiple mycotoxins. Klarić (2012) and, more recently, Alassane-Kpembi et al. (2016) and Smith et al. (2016) have comprehensively reviewed combined toxicity studies involving regulated groups of mycotoxins. Cytotoxicity assays, using a diversity of cell lines, have been widely used considering that they are fast and economic assays that may help predicting the *in vivo* toxicity of combinations of mycotoxins co-occurring in food (Creppy et al., 2004; Tiemann and Dänicke, 2007). They additionally have the advantage of allowing a reduction in the number of animals under experimentation, in compliance with the European Union recommendations (EU, 2010).

An important aspect is related with the approaches that can be applied to measure the dose–effect relationships of single mycotoxins and its combinations, and to ascertain putative interactive effects. Classically, a two-step approach is recommended when analysing the toxicological interactions between the different compounds of a mixture. First, the expected effects of the combination in case of no interaction have to be predicted. This means a clear statement of the effect size to be expected if the compounds in the mixture do not interact. Then, data on the effects of the experimental combination have to be compared to the expected ones in order to classify the combination as additive (no interaction *i.e.* as expected), synergistic (*i.e.* interaction resulting in greater effect than expected) or antagonistic (*i.e.* interaction leading to lesser effect than expected) (Alassane-Kpembi et al., 2016). Among the approaches used to characterize the combination effects of multiple chemicals, the combination index (CI)-isobologram equation by Chou (2006) and Chou and Talalay (1984) is one of the most used, which is based on the median-effect principle (mass-action law). This index demonstrates the existence of an univocal relationship between dose and effect, independently of the number of substrates or products and of the mechanism of action or inhibition (Alassane-Kpembi et al., 2013; Bernhoft et al., 2004; Ruiz et al., 2011). This method involves plotting the dose-effect curves for each compound and their combinations in multiple diluted concentrations and is based on the assumption that when two compounds are combined and subjected to several dilutions, the combined mixture of the two compounds behaves as the third compound for the dose-effect relationship. The CI indicates not only the type of interaction (additivity, synergism or antagonism) but also its magnitude. In addition, the conceptual models of CA and IA also incorporate a set of deviation functions within a nested framework (Jonker et al., 2005, 2004). Several prerequisites are required to allow the application of these models, implicating a careful experimental design that will depend on the number of chemicals in the mixture and the degree of detail needed concerning the dose–effect relationship for single and combined toxicity; the single compounds in the mixture should be tested at a constant dose ratio, using a full factorial design. Whereas the well-known CA model assumes that both toxins have a similar MoA, the IA model assesses the probability of toxicity from exposure to one mycotoxin being independent from the toxicity of the other toxin in the mixture (Jonker et al., 2005, 2004). However, when dealing with a mixture of several chemicals, where the number of experimental groups increases exponentially, fractionated factorial designs can be used to identify interactions in a manageable way (Groten et al., 2004). Another possibility is to apply a tiered approach as suggested by Tajima et al. (2002) for *Fusarium* mycotoxins, starting from the study of the whole mixture effect, followed by a screening of interactions using a fractionated factorial design and, finally, the confirmation of interaction through a full factorial design.

In spite of the number of studies addressing the cytotoxicity of multiple toxins, genotoxicity is of a greater concern for most of the mycotoxins and their mixtures due to their association to carcinogenesis. However, the specific genotoxic properties of multiple mycotoxins are much more difficult to address comprehensively in complex combinatory experiments and thereby studies reporting combined genotoxic effects of mycotoxins are more limited.

3.3.2. Exposure assessment

As previously stated, dietary exposure assessment consists of combining deterministically or probabilistically food consumption figures with occurrence of a given chemical substance in a number of food categories (EFSA, 2013). This is a common process when it is aimed to estimate the exposure of a population to chemicals present in food, as mycotoxins.

Food consumption data reflect what individuals or groups consume in terms of solid foods, beverages, and dietary supplements. National food consumption surveys are the principal sources of information for determining real food consumption habits in a population of consumers. In addition to the general population, the risk assessments generally also consider the exposure of specific consumer groups, such as infants, children, and people following specific diets (e.g. vegetarians) (FAO/WHO, 2009). Food consumption can be estimated through food consumption surveys, including records/diaries, food frequency questionnaires and dietary recall (EFSA, 2011). The food records or food diaries require the report of all foods consumed during a specific period (usually ranging between 24 hours to 7 days). Food frequency questionnaires consist of a structured listing of individual foods or food groups where the respondent is asked to estimate the number of times the food is usually consumed per day, week, month or year (FAO/WHO, 2009).

Classical analytical methods for mycotoxins present in foodstuffs include thin layer chromatography (TLC), enzyme linked immunosorbent assays (ELISA), high-performance liquid chromatography (HPLC) coupled to diode array (DAD) or fluorescence (FD), single mass spectrometry (MS) or tandem mass spectrometry (MS/MS) detection, and gas chromatography (GC) coupled with electron capture (ECD), flame ionisation (FID), or MS detection (Köppen et al., 2010). Considering the increasing evidence of food matrices co-contamination, the proposal to quantify the simultaneous occurrence of multiple mycotoxins goes on the direction of developing multi-analyte methods, combining a generic sample preparation protocol with a highly selective method exhibiting sufficient detection capacity, such as LC-MS (Köppen et al., 2010; Krska et al., 2008; Malachová et al., 2014; Pereira et al., 2014; Turner et al., 2015, 2009).

EFSA suggested that Total Diet Studies (TDSs) provide the most accurate estimates of mean contamination by chemicals in the food consumed by the population or collective group of individuals (FAO/EFSA/WHO, 2011). As TDSs consider total exposure from whole diets and are based on food contamination "as consumed" rather than contamination from raw commodities, they are considered to ensure a more realistic exposure measure than exposure studies based on monitoring programs and surveillance data (Papadopoulos et al., 2015). Consequently, this approach could be very useful in the assessment of multiple mycotoxin exposure.

Within the general framework of chemical risk assessment, and consequently also in mycotoxins risk assessment, a difficult step in dietary exposure assessment is the handling of concentration data reported to be below the limit of detection (LOD) of the analytical method. These data are known as non-detects and the resulting distribution of occurrence values is left-censored. EFSA has so far mainly used substitution methods (EFSA, 2010). The most common approaches are the substitution methods that replace non-detects by LOD divided by 2 or producing an upper and lower bound by substitution of non-detects by LOD or 0, respectively (EFSA, 2010). Relatively to mycotoxin contamination, datasets are characterized by the presence of non-detects or none quantified values which constitutes an important issue for the exposure assessment studies (Cano-Sancho et al., 2013; Marin et al., 2013; Sirot et al., 2013). Therefore, a representative food sampling design (selecting the most susceptible foods, considering a large set of individual and/or composite samples), an accurate chemical analysis method (with low detection limits) and a suitable method to manage left-censored data will be decisive to obtain realistic exposure estimations with a low level of uncertainty.

The indirect approach obtained with the combination of data of mycotoxin occurrence in food and food consumption patterns is associated with some limitations for the mycotoxins exposure assessment, including the heterogeneous distribution of mycotoxins in food, the possible exposure through exposure routes other than ingestion, the presence of masked mycotoxins, the influence of food processing, inter-individual variation in ADME, and the under- and overestimation in food consumption data (Arcella and Leclercq, 2004; Heyndrickx et al., 2014). These limitations could lead to an under- and/or overestimation of the exposure, and biomarkers have been proposed as a suitable alternative. Human biomonitoring is considered a quite new frontier for establishing the real human exposure to mycotoxins. Recent results on this domain (Gerding et al., 2015, 2014; Heyndrickx et al., 2015; Warth et al., 2012a, 2012b) surprisingly revealed a level of exposure to mycotoxins above the widely accepted TDI values, especially to DON, highlighting the importance to perform mycotoxin biomonitoring studies. Typical biomarkers of exposure are the parent toxins themselves, protein or DNA adducts, and/or major phase I or phase II

metabolites (e.g. glucuronide conjugates), which are measured in biological fluids such as urine or plasma/serum, and are related to the actual intake of the toxin through contaminated food (Warth et al., 2013). The mainly methods used for the quantification of biomarkers contents in urine were LC/MS-MS. Urine is the biological fluid mostly chosen to determine mycotoxin biomarkers contents. The number of analytes studied simultaneously varied between four (Warth et al., 2012a) and 33 (Heyndrickx et al., 2015) compounds according to the available published studies.

3.3.3. Risk characterization

Risk characterization is the last step of the risk assessment process, integrating information obtained in hazard assessment and exposure assessment steps and consequently producing scientific advice for risk managers. As generally presented before, different approaches have been used for risk characterization of toxic compounds, according to their genotoxic and carcinogenic potential. In the risk characterization of non-genotoxic and non-carcinogenic substances, a HBGV is compared with estimates of dietary exposure. For mycotoxins, some HBGV was proposed, according to the availability of toxicological data. Table 9 presents the HBGV for the main mycotoxins. It should be highlighted that reference doses are only defined for the adult population, rendering difficult children and infants risk assessment, for which the available adult reference doses are not suitable.

For those substances that are genotoxic and carcinogenic, the traditional assumption is that some degree of risk may exist at any level of exposure and it is recommended that the exposure should be as low as reasonably achievable (ALARA). However, this approach presents limited value, since it does not allow risk managers to prioritize different contaminants or to target risk management actions. The Margin of Exposure (MOE) approach, which is the ratio between an amount of a substance producing a small but measurable effect in laboratory animals or humans and the estimated human exposure, has been proposed by WHO and EFSA as the methodology for risk characterization of compounds that are genotoxic and carcinogenic (EFSA, 2013; FAO/WHO, 2009).

Table 9. Health-based guidance values available for the main mycotoxins (Alvito, 2014).

Mycotoxins	Health-based guidance values			References
Ochratoxin A	PTWI	112	ng/kg bw/week	(JECFA, 2007)
Patulin	PMTDI	400	ng/kg bw/day	(JECFA, 1996)
Fumonisins (group value)	PMTDI	2	µg/kg bw/day	(JECFA, 2011)
Deoxynivalenol	PMTDI	1	µg/kg bw/day	(JECFA, 2011a)
T-2 + HT-2 toxins (group value)	TDI	100	ng/kg bw/day	(EFSA, 2011b)
Nivalenol	TDI	1.2	µg/kg bw/day	(EFSA, 2013)
Zearalenone	TDI	0.25	µg/kg bw/day	(EFSA, 2016)

PTWI: provisional tolerable weekly intake; PMTDI: provisional maximum tolerable daily intake; TDI: tolerable daily intake.

As referred previously, combination effects could occur as a result of different chemicals present in food and consequently leading to different combined effects. Diverse approaches have been used for multiple chemicals risk characterization, most of these are based on the concepts of CA and IA. Examples of risk characterization methods include the Hazard Index (HI), Point of Departure Index (PODI), Combined Margin of Exposure Index (MOET), Toxic Unit Summation (TUS) and Relative Potency Factors/Toxic Equivalency Factors (RPF/TEF) (Sarigiannis and Hansen, 2012; WHO, 2009). The HI, the mostly used for non-genotoxic and carcinogenic mycotoxins, is defined as the sum of the respective hazard quotients for individual mixture components, calculated as the ratio between exposure and a reference dose (e.g. TDI) and has been put forward as the preferred approach when extensive mechanistic information of the mixture components is not available. The HI does not predict the overall health effect of the mixture, but provides a measure of the total risk based on the individual risk of each component. Thus, the HI can be used also for identification of the largest contributors to the risk (EFSA, 2013; Sarigiannis and Hansen, 2012). The combined MOE is called the MOET, and is calculated as the reciprocal of the sum of the reciprocals of the individual MOEs (EFSA PPR, 2008). MOET is usually used for chemicals mixtures that have genotoxic and carcinogenic potential, and was applied for the characterization of risk associated to mycotoxins exposure (e.g. Assunção et al., 2015).

One of the main challenges posed to risk characterization of multiple mycotoxins is the absence of toxicological data that could be used to characterize the risk. The use of toxicological data is mandatory to risk characterization, and independently of the mechanisms of combined effects or interactions, data of multiple mycotoxins are not yet complete for all the toxins potential present in food.

3.4. INTESTINE – THE FIRST TARGET FOR INGESTED MYCOTOXINS

In human health risk assessment, ingestion of food is considered a major route of exposure to many contaminants, including mycotoxins (Versantvoort et al., 2005). It is well accepted that the total amount of an ingested contaminant (*i.e.* intake) does not always reflect the amount available to be absorbed into the body and only a certain amount will be available to reach the systemic circulation. This proportion of ingested contaminant that reaches the systemic circulation is known as bioavailability (Versantvoort et al., 2005). The oral bioavailability of a compound, such as a mycotoxin, depends on three different processes: i) the release of a mycotoxin from the food matrix during digestion in the gastrointestinal tract (GIT). This step corresponds to the mycotoxin bioaccessibility; ii) the absorption of the bioaccessible mycotoxin through the intestinal epithelial cells of the GIT and transport to the blood (or lymph) stream; and iii) mycotoxin metabolism prior to systemic circulation (namely its biotransformation and excretion by the intestinal epithelium or the liver) (González-Arias et al., 2013; Versantvoort et al., 2005). Figure 11 presents a schematic representation of a contaminant fate after ingestion.

Bioaccessibility assumes a considerable importance because it represents the amount of a mycotoxin that could reach the blood after intestinal absorption and consequently the mycotoxin bioaccessibility from its matrix can be seen as an indicator of the maximal oral bioavailability of the mycotoxin (González-Arias et al., 2013; Versantvoort et al., 2005). So, knowing the amount of an ingested mycotoxin may not be enough for exposure assessment. Knowing its bioaccessibility, is also advisable. Different factors such as pH changes, enzymatic activities, among others, play an important role during the gastrointestinal transit of mycotoxins and thus affect bioaccessibility (González-Arias et al., 2013).

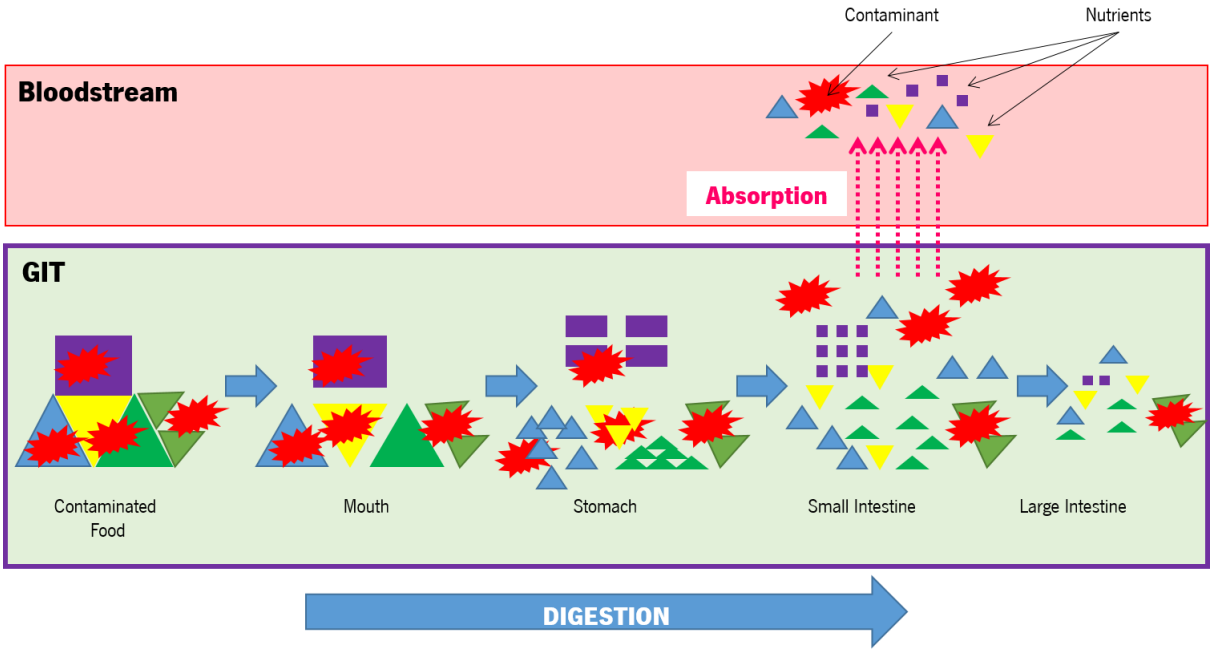


Figure 11. Schematic illustration of the fate of a contaminant after ingestion.

Another important aspect is the fact that the bioaccessible amount of a mycotoxin corresponds to the amount available to exert its potential toxic effects at the intestinal level. After ingestion, the intestinal mucosa is the first host defense barrier against mycotoxins (Figure 12). However, although cells from intestinal mucosa are the first to be exposed to mycotoxins and at higher doses than other tissue cells, studies on the effect of mycotoxin mixtures on the GIT are scarce and currently there is an increasing awareness of the adverse effects of various mycotoxins on vulnerable structures in the intestine and impairment of intestinal integrity (Akbari et al., 2016; Pinton and Oswald, 2014; Smith et al., 2016; Suzuki, 2013). A compromised barrier function is associated with an increased epithelial permeability and translocation of luminal allergens and pathogens, as well as a non-specific inflammatory response and an overstimulation of the gut-associated immune system (Figure 12) (Camilleri et al., 2012; Smith et al., 2016; Suzuki, 2013). Some authors hypothesized that human exposure to certain mycotoxins (e.g. DON) may play an important role in aetiology of various chronic intestinal inflammatory diseases, such as inflammatory bowel disease, and in the prevalence of food allergies, particularly in children (Bol-Schoenmakers et al., 2016; Maresca and Fantini, 2010). Due to increasing prevalence in food commodities, mycotoxins appear to be important, but often neglected substances that are able to affect the equilibrium and integrity of the intestinal barrier.

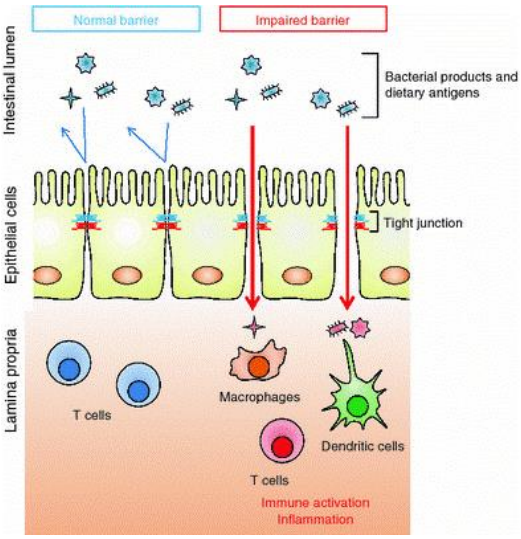


Figure 12. Schematic representation of the intestinal barrier function and the impairment of the barrier (Suzuki, 2013).

3.4.1. Brief description of anatomical, physiological and histological features

The GIT represents the largest interface between the body and the environment and, when functioning correctly, absorbs nutrients while providing protection from harmful components. The GIT extends from the mouth to the anus and can be divided into the upper and the lower tracts. The upper tract consists of the oral cavity, oesophagus, stomach, duodenum, jejunum and the ileum. The latter three represent the small intestine. The lower tract comprises the large intestine consisting of the cecum, colon, rectum and anal canal (Verhoeckx et al., 2015). Every part of the GIT has its own function as presented in Figure 13. The mouth is responsible for mastication and mixing of the food with saliva, which contains a complex array of components including amylase, an enzyme that catalyses the hydrolysis of starch into sugars. After the formation of a food bolus, the food is transported through the oesophagus to the stomach where it is further processed. The food bolus is mixed with enzymes like protease and lipase, which break down proteins and lipids, respectively. Acid is also secreted into the stomach and this will gradually lower the pH of the content and aid hydrolysis. The food bolus is broken down into chyme, which is gradually transported to the small intestine. In the duodenum, the low pH of the stomach is neutralised by bicarbonate. Digestive juices from the pancreas (trypsin and chymotrypsin) and the gall bladder (bile acids) are introduced. Proteins, lipids and starch are broken down by the digestive enzymes, while the bile acids help emulsify the products of lipid hydrolysis into micelles. The nutrients produced are mainly absorbed by the enterocytes of the jejunum and to a lesser extent in the ileum. Relatively to the large intestine, one of its main functions is the absorption of water. Additionally, microbial concentration is

greatest in the large intestine, although present through all the extent of the GIT. Many of these microorganisms contribute to the digestion of food components (Verhoeckx et al., 2015).

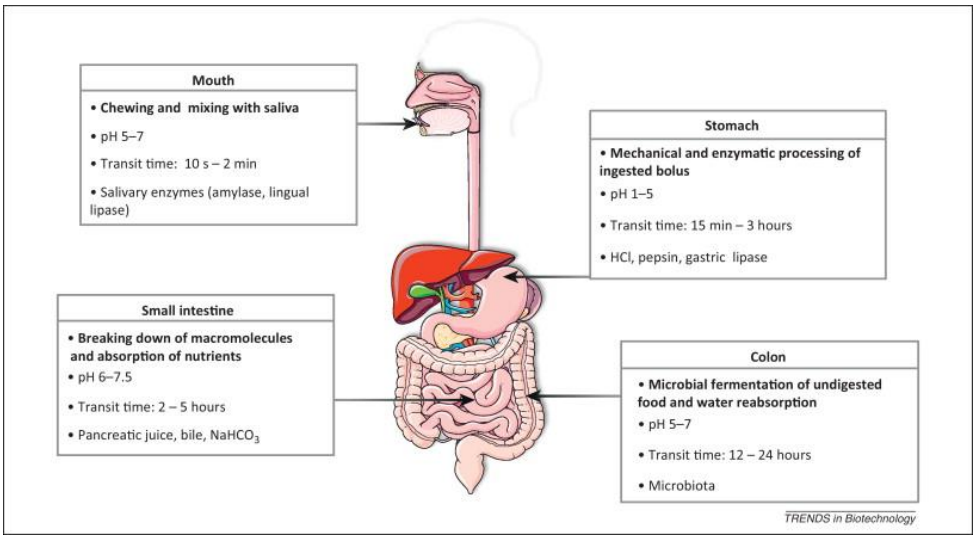


Figure 13. Human gastrointestinal tract components and its main physiological conditions (Guerra et al., 2012).

The GIT serves as an interface between the environment and the host immune system (Faria et al., 2013). This interface comprises a single epithelial layer, folded into crypts and villi, to increase the surface area of the gut, with the exception of the colon cells, that do not possess villi. The intestinal epithelial layer is composed of several cell types that are originated from the multipotent stem cells of the crypts (Figure 14).

The most abundant cells are the enterocytes that have an absorptive function. Interlaced between the enterocytes are mucin-secreting goblet cells and peptide hormone exporting enteroendocrine cells. During their migration to the top of the villi, enterocytes, goblet cells and enteroendocrine cells differentiate and eventually die (through apoptosis) when they reach the top of the villi. A fourth cell type, the Paneth cells, migrates downwards to the crypt base (Figure 14). The Paneth cells secrete digestive enzymes, growth factors and antimicrobial peptides (AMPs) such as cryptdins or defensins.

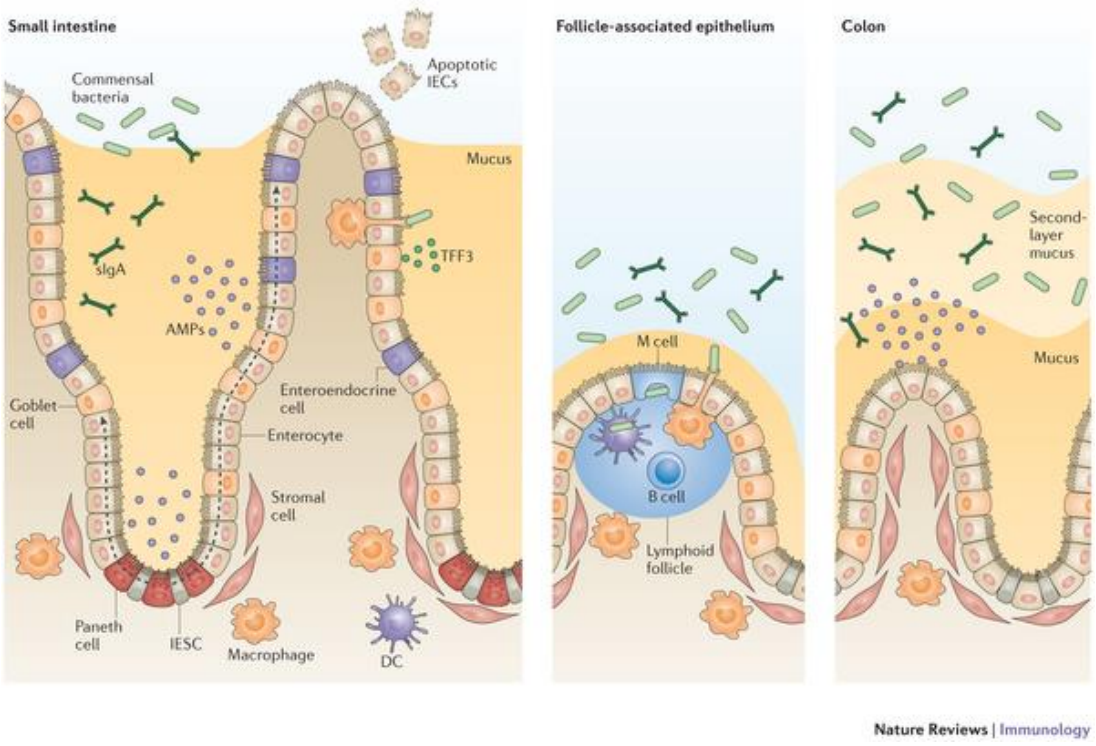


Figure 14. Intestinal cells and structures that maintains the physical barrier between the lumen and the mucosal immune system (Peterson and Artis, 2014).

IEC = Intestinal epithelial cells; IESC = Intestinal epithelial stem cell; DC = Dendritic cells; AMP = Antimicrobial proteins; M cells = Microfold cells.

Given its large surface area and the number of antigens and microorganisms to which the GIT is exposed, it is not surprising that it contains the highest number of lymphoid (immune) cells in the entire body (Faria et al., 2013). The gut immune system consists of inductive sites where antigen recognition and primary adaptive immune responses take place and effector sites that harbour, amongst other cells, activated T- and B-cells and memory cells. The main inductive sites are gut-associated lymphoid tissues (GALT) such as Peyer's patches (PP), isolated lymphoid follicles and the mesenteric lymph nodes. The lamina propria (LP) and the epithelium constitute the main effector sites. In the GALT and at the effector sites, a wide range of immune cells are present (Faria et al., 2013; Verhoeckx et al., 2015). Dendritic cells (DCs), which are recognised as an important link between innate and adaptive immunity, take up, process and present antigens to T-cells. DCs are found in all organised intestinal lymphoid tissues. In the sub-epithelial dome of PP, they capture antigens that are transported into the PP by specialised epithelial cells called microfold cells (M cells). Next to DCs a mixture of T and B lymphocytes, plasma cells and macrophages, a second type of antigen-presenting cells, are present. In the LP, a range of different immune cells can be found, typically DCs, macrophages, plasma cells, memory B- and T-cells, mast cells, eosinophils and cytotoxic natural killer (NK) cells (Peterson and Artis, 2014; Verhoeckx et al., 2015). Also, LP contains

additional innate immune cell populations that are absent in peripheral blood. These cells, called innate lymphoid cells, are potent cytokine producers, much like the classical T helper cell subsets. The only immune cells that are virtually absent in the healthy intestine are neutrophils and basophils. Both cell types, however, will infiltrate intestinal tissues in case of inflammation. Generally, the small intestine contains more immune cells than the colon (Verhoeckx et al., 2015).

The intestinal mucosa is considered a selective barrier. This selectivity is achieved by intercellular tight junction (TJ) structures, which regulate paracellular permeability. Disruption of the intestinal TJ barrier, followed by permeation of luminal noxious molecules, induces a perturbation of the mucosal immune system and inflammation, and can act as a trigger for the development of intestinal and systemic diseases. In this context, much effort has been taken to understand the roles of extracellular factors, including cytokines, pathogens, and food factors and contaminants (including mycotoxins), for the regulation or disruption of the intestinal TJ barrier (Akbari et al., 2016; Bouhet and Oswald, 2005; Camilleri et al., 2012; Grenier and Applegate, 2013; Jeon et al., 2013; Maresca, 2013; Maresca and Fantini, 2010; Pinton and Oswald, 2014; Shen, 2012; Suzuki, 2013).

The selective permeability of the intestinal barrier is created by two pathways: the transcellular and the paracellular pathway. The transcellular pathway is involved in the absorption and transport of nutrients, including sugars, amino acids, peptides, fatty acids, minerals, and vitamins. As the cell membrane is impermeable, this process is predominantly mediated by specific transporters or channels located on the apical and basolateral membranes (Shen, 2012; Suzuki, 2013). The paracellular pathway is associated with transport in the intercellular space between the adjacent epithelial cells. It is regulated by an apical junctional complex, which is composed of TJs and adherence junctions (AJs) (Figure 15). The AJ, along with desmosomes, provides strong adhesive bonds between the epithelial cells and also aids intercellular communication, but does not determine paracellular permeability (Suzuki, 2013). The TJs, which are multiple protein complexes, encircle the apical ends of the lateral membranes of intestinal epithelial cells as enterocytes, and determine the selective paracellular permeability to solutes (Figure 15) (Camilleri et al., 2012; Shen, 2012; Suzuki, 2013). In this regard, the TJs provide both a barrier to noxious molecules and a pore for the permeation of ions, solutes, and water when appropriate.

Four integral transmembrane proteins, occludin, claudins, junctional adhesion molecule (JAM), and tricellulin, have been identified. The extracellular domains of the transmembrane proteins form the selective barrier by homophilic and heterophilic interactions with the adjacent cells. The intracellular domains of these transmembrane proteins interact with cytosolic scaffold proteins, such as zonula occludens (ZO) proteins, which in turn anchor the transmembrane proteins to the perijunctional

actomyosin ring. The interaction of TJ proteins with the actin cytoskeleton is vital to the maintenance of TJ structure and function. In addition, the interaction of the TJ complex with the actomyosin ring permits the cytoskeletal regulation of TJ barrier integrity (Shen, 2012; Suzuki, 2013). The circumferential contraction and tension in the perijunctional actomyosin ring is regulated by myosin light chain (MLC) activity (through phosphorylation). Induction of MLC phosphorylation by kinases such as myosin light chain kinase and Rho-associated kinase causes the contraction of the actomyosin ring, resulting in the opening of the paracellular pathways (Cunningham and Turner, 2012; Suzuki, 2013).

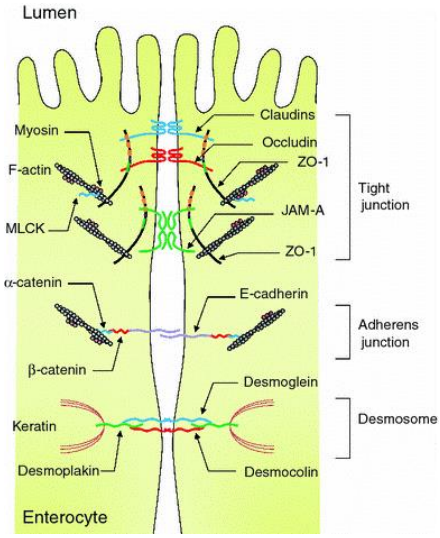


Figure 15. Molecular structure of the intercellular junction of intestinal epithelial cells (Suzuki, 2013). ZO = Zonula occludens; MLCK = Myosin light chain kinase; JAM = Junctional adhesion molecule.

Evidence from basic science and clinical studies indicate that the intestinal TJ barrier has a critical role in the pathogenesis of intestinal and systemic diseases (Camilleri et al., 2012; Suzuki, 2013). The modification of TJ barrier function and paracellular permeability is dynamically regulated by various extracellular stimuli and is closely associated with human health and disease susceptibility. TJ barrier disruption and increased paracellular permeability, followed by permeation of luminal pro-inflammatory molecules, can induce activation of the mucosal immune system, resulting in sustained inflammation and tissue damage.

3.4.2. Models to study mycotoxins bioaccessibility

As referred previously, within the science-based risk assessment, a precise evaluation of the health impact of mycotoxins present in food must consider their effects on the GIT, reflecting their entrance in the organism and the beginning of their journey from the gut to the internal organs. It was well stated that the amount of mycotoxins consumed via food does not always reflect the amount available to exert toxic action in a target organ of the body as only a part of the ingested compound will be bioavailable (Cheli et al., 2015; González-Arias et al., 2013; Versantvoort et al., 2005). The food matrix, type of mycotoxin contamination, presence of masked mycotoxins, food treatments, and additives influence the level of internal exposure to mycotoxins (Kabak et al., 2009). Consequently, bioaccessibility data are needed to evaluate the amount of a toxin that becomes available for absorption through the intestinal epithelium, the direct toxicological effects on intestinal mucosa and to subsequently estimate health effects correctly. For these purposes, *in vitro* digestion models have been widely used and validated for nutritional studies in both humans and animals (as reviewed by Hur et al., 2011). Within *in vitro* digestion models, two main groups were distinguished: static (e.g. RIVM model) and dynamic (e.g. TIM) models. Most of the *in vitro* tools are static, and these models simulate the transit through the digestive tract by sequential (compartmentalized) exposure, *i.e.* simulate the mouth, gastric and small intestinal conditions. These models are a good first approach to the problem, as they can be easily performed and generally allow rapid processing of a large number of samples. However, they represent to a lesser extent the GIT physiological reality. On the other hand, dynamic models mimic the gradual transit of ingested compounds through the simulated compartments of the GIT, giving a more realistic simulation. In these models, successive physiological conditions in the stomach and segments of the intestines of humans and animals are closely simulated. Dynamic models usually take into account factors such as gastric emptying patterns, transit times in combination with changing pH values, variable concentration of electrolytes, enzymes and bile salts, absorption of water and, in some cases, microbial activity during passage of the food through the entire GIT (González-Arias et al., 2013; Aurélie Guerra et al., 2012; Hur et al., 2011). The TIM models, extensively validated by data obtained from animal nutritional research, are multi-compartmental, dynamic computer-controlled models (Minekus et al., 1999, 1995). TIM-1 simulates the digestive processes of the stomach and small intestine of monogastric animals, and TIM-2 simulates the colon and includes a rich microbial gut-derived flora (Verhoeckx et al., 2015). The use of *in vitro* digestion models to assess mycotoxin bioaccessibility was described by some authors and Table 10 presents the main characteristics of the *in vitro* digestion models used to ascertain mycotoxins bioaccessibility.

The *in vitro* models used to evaluate mycotoxin bioaccessibility represent effective tools to perform simplified experiments under uniform and well-controlled conditions and may provide insights not achievable with animal studies. However, these methods present also some disadvantages, namely, they do not take into account important physiological factors such as the intestinal mucosa, enterohepatic cycle, or the immune system (González-Arias et al., 2013).

Table 10. Main characteristics of in vitro *digestion models used for determination of mycotoxins bioaccessibility (adapted from Cheli et al., 2015; González-Arias et al., 2013).*

	TIM-1	**Döll**	**RIVM**	**Gil-Izquierdo**	**INFOGEST**
Type of model	Dynamic	Static	Static	Static	Static
Simulated physiology	Pig	Pig	Human	Human	Human
Simulation					
Mouth	No	Yes	Yes	Yes	Yes
Stomach	Yes	Yes	Yes	Yes	Yes
Small intestine	Yes	Yes	Yes	Yes	Yes
Large intestine	No	Yes	No	No	No
First time assayed with mycotoxins	2003	2004	2004	2012	2016
Mycotoxins assayed	ZEA, AFB_1, DON, OTA, NIV, FB	DON, ZEA	AFB_1, OTA, PAT	ENN, BEA, PAT, DON	OTA, PAT

One of the main drawbacks associated to the use of different *in vitro* digestion models is the difficulty to compare the results obtained from different research groups, since different models present different properties and characteristics. For example, a large variety of enzymes from different sources have been used, differing in their activity and characterization. Differences in pH, mineral type, ionic strength and digestion time, which alter enzyme activity and other phenomena, may also considerably alter results (Minekus et al., 2014). Recently, to overcome this difficulty, a standardized static *in vitro* digestion model suitable for food was developed within the COST action INFOGEST (https://www.cost-infogest.eu/). This standardized digestion method (Egger et al., 2015; Minekus et al., 2014) is based on the current state of knowledge of *in vivo* digestion conditions and describes a detailed line-by-line protocol with recommendations and justifications of the applied experimental procedures. Figure 16 presents a flow diagram of the INFOGEST *in vitro* digestion model, summarizing the method. The harmonized method comprises three steps, namely an oral, gastric, and intestinal phase, similar to other models published earlier. However, in the past, a major source of results discrepancy among laboratories, even when applying the same method, was the differences in digestive enzyme activities used to simulate digestion. In most cases, digestive enzymes were added based on weight or non-standardized activity units. To overcome these problems, the INFOGEST harmonized method proposed a standardized assay for activity determination for each added enzyme and therefore is expected to improve the comparability of

experimental results between laboratories (Egger et al., 2015). Other important harmonized aspects included chemical constitution of the simulated fluids (salivary, gastric and intestinal) and digestion times for each phase (oral, gastric and intestinal).

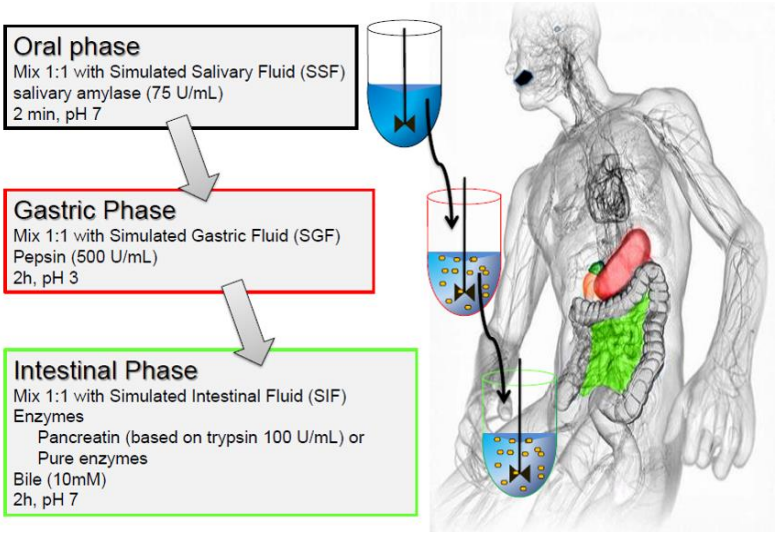


Figure 16. Overview of the INFOGEST in vitro *digestion model (Minekus et al., 2014).*

Despite their complexity, the *in vitro* digestion models described so far remain simplified compared to the *in vivo* situation: they do not include feedback mechanisms, resident microbiota, immune system, or specific hormonal controls. Further efforts and technological innovations are therefore needed to improve *in vitro* models and keep up with the growing interest of industry and researchers (Guerra et al., 2012).

3.4.3. Impact of mycotoxins on the intestinal function

The maintenance of a healthy GIT improves the welfare and health of humans and animals by ensuring that nutrients are absorbed at an optimum rate and by providing protection against pathogens through the organism's own immune system and gut microbial community. It has been reported that epithelial integrity, which is critical for the maintenance of a physical but selective barrier between external and internal environments, may be altered by several mycotoxins (Akbari et al., 2016; Bouhet and Oswald, 2005; Cheli et al., 2015; Grenier and Applegate, 2013; Maresca, 2013; Maresca and Fantini, 2010; Pinton and Oswald, 2014; Sergent et al., 2008). Over the past decade, mycotoxins interaction with intestine has received significant research interest for different reasons. First, most acknowledge that a healthy intestinal tract guarantees the welfare and the health of both humans and animals. Second,

rapidly dividing and activated cells and tissues with a high protein turnover are predominant in gut epithelium. Intestinal cells and tissues can become a main target of mycotoxins as many of these metabolites are inhibitors of protein synthesis. Third, the absorption of mycotoxins and their fate within the GIT suggests that the epithelium is repeatedly exposed to these toxics, and at higher concentrations than other tissues (Cheli et al., 2015; Grenier and Applegate, 2013). Grenier and Applegate (2013) reviewed the modulation of intestinal functions following mycotoxin ingestion. This review focused on several mycotoxins of concern and also on seven different intestinal processes, namely, nutrient digestibility, enzyme activity, nutrient uptake, digestive microflora interaction, barrier integrity, mucosal immunity and pathogen clearance. The authors concluded through a meta-analysis that mycotoxins compromise several key functions of the GIT, causing a decrease in the surface area available for nutrient absorption, modulation of nutrient transporters, or loss of barrier function. In addition, some mycotoxins facilitate intestinal pathogens persistence and trigger intestinal inflammation. Considering all these effects, the impact on the barrier function assumes particular importance. Figure 17 illustrates schematically the mycotoxin-induced intestinal epithelial barrier breakdown and summarizes the results of several studies reporting that various mycotoxins induce intestinal barrier breakdown through i) decrease in transepithelial electrical resistance (TEER), ii) increase in paracellular transport, and iii) modulation of the expression of the TJ proteins (Akbari et al., 2016).

Considering that epithelial cells from the intestine have been very difficult to cultivate *in vitro* as primary cells, some cell lines have been used to model the intestine environment *in vitro*. One of the preferred model of the intestinal epithelium is Caco-2 cell line (ATCC® number: HTB-37). Caco-2 cells, originally isolated from a human colon adenocarcinoma, have been used to evaluate the effects of mycotoxins on intestinal epithelium permeability as well as to investigate the effects on barrier function (Akbari et al., 2016; Grenier and Applegate, 2013). These cells are routinely cultivated as monolayers on permeable filters and during culturing, cells undergo spontaneous differentiation resulting in polarization and formation of the TJ proteins between adjacent cells. The differentiated Caco-2 cells show a polarized apical (mucosal) and basolateral (serosa) sides that are structurally and functionally similar to epithelial cells of the small intestine (Akbari et al., 2016; Artursson et al., 2001; Verhoeckx et al., 2015). In turn, the established cell monolayer can be challenged either from the apical site or the basolateral site with toxins, as well as other antigens, and allow a wide range of functional parameters to be measured. In addition, this Caco-2 cell system is a commonly used model to study mycotoxins absorption and excretion rates across the intestinal epithelium (Akbari et al., 2016; Grenier and Applegate, 2013).

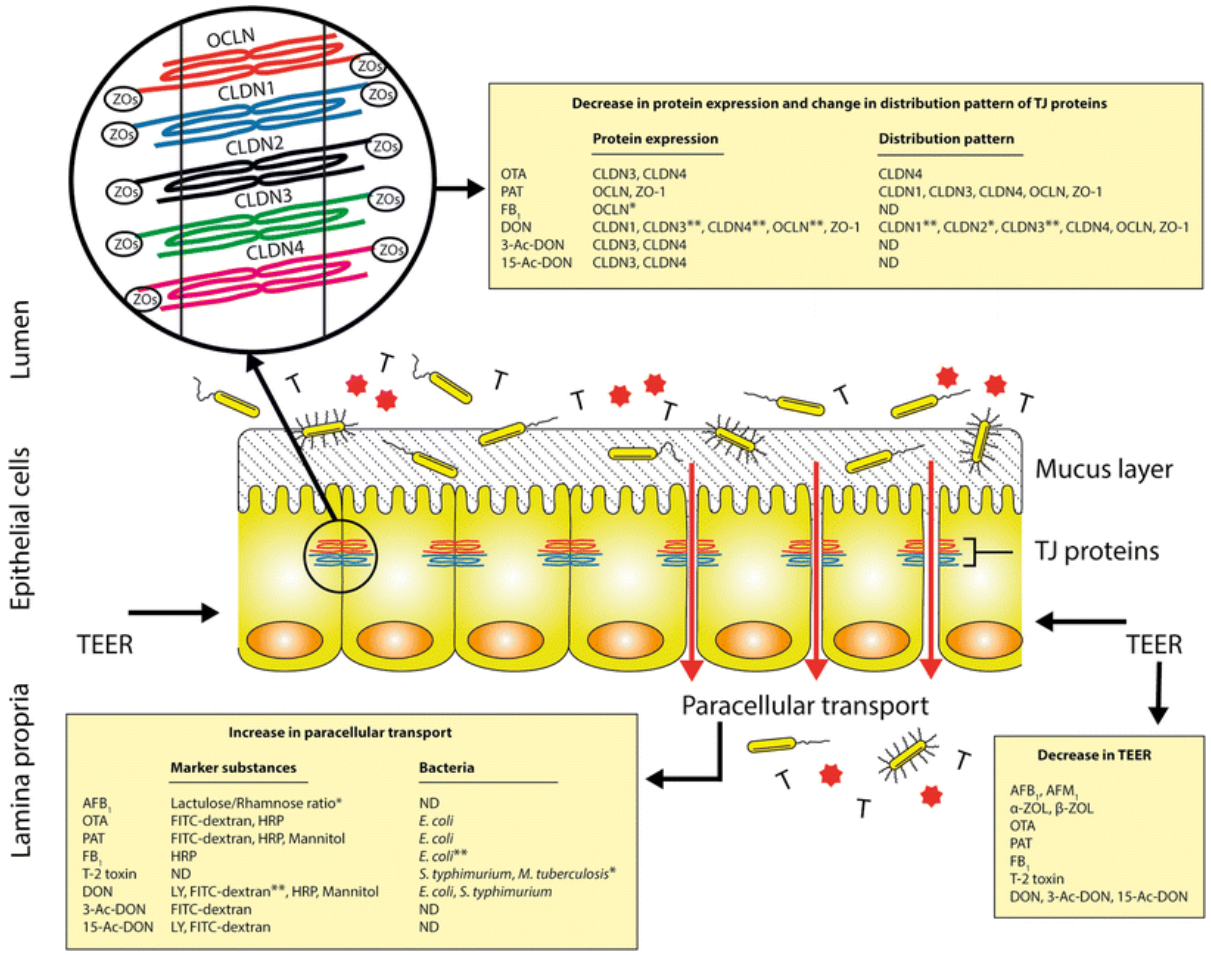


Figure 17. Schematic representation of the mycotoxin-induced intestinal epithelial barrier breakdown (Akbari et al., 2016).

3-Ac-DON = 3-acetyl deoxynivalenol; 15-Ac-DON = 15-acetyl deoxynivalenol; AFB_1 = aflatoxin B_1; AFM_1 = aflatoxin M_1; α-ZOL = alpha-zearalenol; β-ZOL = beta-zearalenol; CLDNs = claudins; DON = deoxynivalenol; E. coli = *Escherichia coli*; FB_1 = fumonisin B_1; FITC-dextran = fluorescein isothiocyanate-dextran; HRP = horseradish peroxidase; LY = lucifer yellow; M. tuberculosis = *Mycobacterium tuberculosis*; ND = not determined; OCLN = occludin; OTA = ochratoxin A; PAT = patulin; S. typhimurium = *Salmonella typhimurium*; T = food-borne toxins; TEER = transepithelial electrical resistance; TJ = tight junction; ZOs = zonula occludens; * = *in vivo* studies; ** = *in vitro* as well as *in vivo* studies.

One of the mostly used parameter to evaluate the integrity of the epithelial barrier in the Caco-2 cell model is the transepithelial electrical resistance (TEER) (Akbari et al., 2016). TEER measurement is a simple and convenient technique that provides information about the uniformity of the Caco-2 cell layer on the filter support, and the integrity of the TJs formed between the polarized cells (Bouhet and Oswald, 2005; Verhoeckx et al., 2015). This parameter is directly measured by a portable voltmeter like the Millicell-ERS Voltmeter (Millipore, Figure 18.A) or the EVOM2, Epithelial Voltohmmeter for TEER (World Precision Instruments Inc., Sarasota, FL, Figure 18.B). The electrode is placed in the medium of the upper chamber, and resistance is measured. Routine TEER measurement was used by different authors in order to evaluate mycotoxins effects on intestinal integrity, as reviewed by Akbari et al. (2016).

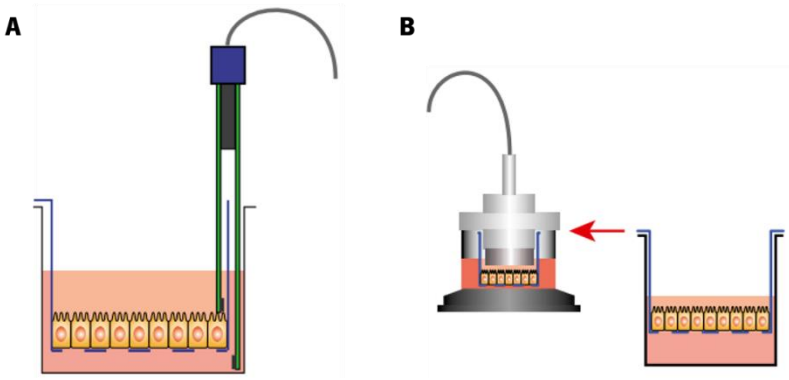


Figure 18. Schematic representation of two different portable voltmeters to measure transepithelial electrical resistance.

(A) Millicell-ERS Voltmeter; (B) EVOM2, Epithelial Volttohmmeter for TEER.

In addition to the TEER measurement, determination of the paracellular flux of marker substances across the cell monolayer can be monitored. Within this domain, at least four different transport mechanisms exist that allow selective uptake and transport of macromolecules across the epithelial cell layer, *i.e.* paracellular transport, passive diffusion of molecules from the apical to the basolateral side, vesicle-mediated transcytosis and carrier-mediated uptake and diffusion through the epithelial cell layer (Figure 19). Each of these transport mechanisms depends on the physicochemical properties of the compound, its ability to interact with the plasma membrane, its molecular weight and size, stability and charge distribution (Verhoeckx et al., 2015).

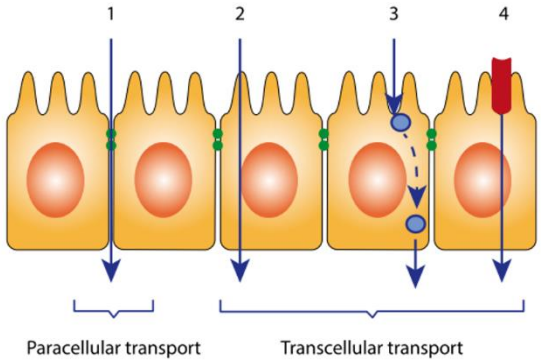


Figure 19. Modes of absorption and transport through the intestinal epithelium (Verhoeckx et al., 2015).

(1) Paracellular transport; (2) passive diffusion of molecules from the apical to the basolateral side; (3) vesicle-mediated transcytosis; and (4) carrier-mediated uptake and diffusion through the epithelial cell layer.

Transport of molecules through the epithelium by paracellular transport is regulated by complex intracellular processes that finely tune the permeability of the TJ complex. The paracellular flux of marker

substances across cell monolayer can be monitored in order to get additional information about the leakiness of the TJs. The most common paracellular markers used in *in vitro* models are fluorescent compounds (such as lucifer yellow, LY) or fluorescently labelled compounds (such as fluorescein isothiocyanate (FITC)-dextran and FITC-inulin). In particular, apical-to-basolateral flux of paracellular markers is used to identify a compromised intestinal barrier function (Akbari et al., 2016; Verhoeckx et al., 2015). As presented in Figure 17, increase of the paracellular transport is one of the consequences of the intestinal cells exposure to mycotoxins (Akbari et al., 2016).

From all mycotoxins, deoxynivalenol, ochratoxin A and patulin are the best known enteropathogenic mycotoxins able to alter intestinal functions resulting in malnutrition, diarrhoea, vomiting and intestinal inflammation *in vivo* (Maresca et al., 2008). Several authors studied the modulation of the intestinal barrier function by different mycotoxins and their metabolites using Caco-2 cells as a model of intestinal epithelial cells. Table 11, Table 12 and Table 13 summarize the main conclusions obtained from different authors relatively to the modulation effects of DON, OTA and PAT, respectively, on the intestinal barrier function of Caco-2 cells.

Table 11. Modulation of the intestinal barrier function of Caco-2 cells by deoxynivalenol (adapted from Akbari et al., 2016).

References	**Concentration and exposure time**	**Effects on barrier function**
Akbari et al., 2014	1.39–12.5 µM 24 h	Decrease in TEER values Decrease in horizontal impedance value Increase in permeability of LY and 4 kDa FITC-dextran Increase in transcript level of CLDN (3 and 4), OCLN and ZO-1 Decrease in protein expression of CLDN (1, 3 and 4) Distribution pattern of CLDN (1, 3 and 4), OCLN and ZO-1 compromising
De Walle et al., 2010	0.16–16 µM 24 h	Decrease in TEER values Increase in permeability of mannitol Increase in transcript level of CLDN (4) and OCLN Decrease in protein expression of CLDN (4)
Kasuga et al., 1998	0.16–0.67 µM	Decrease in TEER values
Manda et al., 2015	0.37–1.5 µM 6–120 h	Decrease in horizontal impedance value of undifferentiated cells
Maresca et al., 2008	1–100 µM 12 h	Decrease in TEER values Increase in permeability of HRP and 4 kDa FITC-dextran Increase in translocation of commensal *Escherichia coli* (strain k12)
Pinton et al., 2009	5–100 µM	Decrease in TEER values

	48 h	Increase in permeability of 4 kDa FITC-dextran Increase in translocation of pathogenic *Escherichia coli* (strain 28C) Decrease in protein expression of CLDN (4)

TEER = Transepithelial electrical resistance; CLDN = Claudin; OCLN = Occludin; ZO = Zonula occludens; LY = Lucifer yellow; FITC = Fluorescein isothiocyanate; HRP = Horseradish peroxidase.

Table 12. Modulation of the intestinal barrier function of Caco-2 cells by ochratoxin A (adapted from Akbari et al., 2016).

References	**Concentration and exposure time**	**Effects on barrier function**
Lambert et al., 2007	100 µM 24 h	Decrease in TEER values Decrease in protein expression of CLDNs (3 and 4)
Maresca et al., 2001	0.01–100 µM 48 h	Decrease in TEER values
Maresca et al., 2008	1–100 µM 12 h	Decrease in TEER values Increase in permeability of HRP and 4 kDa FITC-dextran Increase in translocation of commensal *Escherichia coli* (strain k12)
McLaughlin et al., 2004	100 µM 24 h	Decrease in TEER values Increase in permeability of 4 and 10 kDa FITC-dextran Decrease in protein expression of CLDNs (3 and 4)
Ranaldi et al., 2007	40–1000 µM 48 h	Decrease in TEER values Distribution pattern of CLDN (4) compromising
Romero et al., 2016	1–100 µM 7 days	Decrease in TEER values Decrease in transcript level of CLDN (3 and 4) and OCLN
Sergent et al., 2005	10 µM 3 h	Neither a significant decrease in TEER values nor an increase in permeability of [^{14}C]-mannitol

TEER = Transepithelial electrical resistance; CLDN = Claudin; OCLN = Occludin; FITC = Fluorescein isothiocyanate.

Table 13. Modulation of the intestinal barrier function of Caco-2 cells by patulin (adapted from Akbari et al., 2016).

References	**Concentration and exposure time**	**Effects on barrier function**
Katsuyama et al., 2014	50 µM 36 h	Decrease in TEER values Increase in permeability of 4 kDa FITC-dextran Increase in phosphorylation of CLDN (4) Distribution pattern of ZO-1 compromising
Kawauchiya et al., 2011	50 µM 72 h	Decrease in TEER values Decrease in protein expression of ZO-1 Increase in phosphorylation of ZO-1 Distribution pattern of CLDN (4), OCLN and ZO-1 compromising
Mahfoud et al., 2002	1–100 µM	Decrease in TEER values

Maresca et al., 2008	1–100 μM 12 h	Decrease in TEER values Increase in permeability of HRP and 4 kDa FITC-dextran Increase in translocation of commensal *Escherichia coli* (strain k12)
McLaughlin et al., 2009	100 μM 5 h	Decrease in TEER values Increase in permeability of 4, 10, 20 and 40 kDa FITC-dextran Proteolysis of OCLN Decrease in protein expression of ZO-1 Affect the distribution pattern of CLDN (1, 3 and 4), OCLN and ZO-1
Mohan et al., 2012	0.2–100 μM 72 h	Increase in plasma membrane permeability

TEER = Transepithelial electrical resistance; CLDN = Claudin; OCLN = Occludin; ZO = Zonula occludens; FITC = Fluorescein isothiocyanate; HRP = Horseradish peroxidase.

The impairment of the intestinal barrier leads to mucosal inflammation and has been suggested as a mechanism involved in the pathogenesis of various chronic inflammatory intestinal diseases as Crohn's disease, ulcerative colitis, celiac disease and irritable bowel syndrome. TJ proteins are one of the most important functional elements of the intestinal barrier, and consequently every potential factors that affect their abundance and/or distribution could contribute to the development of diseases (Camilleri et al., 2012; Jeon et al., 2013; Peterson and Artis, 2014; Suzuki, 2013). Considering the mycotoxins prevalence in different foodstuffs, these compounds present some important concerns. Mycotoxins were associated to affect TJ proteins and impair the integrity of the intestinal barrier. A continuing mycotoxins exposure through food consumption suggests a role of this mycotoxin in the aetiology of intestinal diseases (Akbari et al., 2016; Grenier and Applegate, 2013; Maresca and Fantini, 2010). The observation that even pathogenic bacteria are translocated from the intestinal lumen to the internal environment, when animals are challenged with mycotoxins confirms their significance in inflammatory reactions. Additionally, considering the apparent mycotoxins transfer from maternal plasma into milk (Muñoz et al., 2013), exposure of infants deserves special attention. Changes in the developing intestinal barrier can lead to exposure to antigens usually present in the intestinal lumen in early phases of life and may result in accelerated immunological responses and clinical manifestations, such as allergies in later stages of life (Akbari et al., 2016). As advocated by Akbari et al. (2016), better explanation of the impact of mycotoxins exposure on TJ proteins, and their effect on the intestinal barrier should be achieved and included in the overall risk assessment of mycotoxins in foods.

II. JUSTIFICATION AND AIMS

There is growing concern relatively to mycotoxin involvement in human and animal diseases. This fact assumes particular importance since vulnerable populations like children are exposed to mycotoxins through contaminated food, which is considered the main source of human exposure to these contaminants. Some foods are often contaminated by multiple mycotoxins simultaneously, but most risk assessment studies have been focused on the occurrence and toxicology of single mycotoxins. Scarce data are available in the literature concerning the co-occurrence of mycotoxins in foods intended to be consumed by children. In Portugal, similar situation is verified. Consequently, it could be expected that Portuguese children are exposed to multiple mycotoxins through food and this could constitute a health risk. Additionally, studies evaluating mycotoxins interactions were recently published, thus this issue is far from being well characterized.

Addressing these considerations, some important questions were highlighted and supported the aim of the present thesis, namely: 1) are Portuguese children exposed to multiple mycotoxins through food consumption? 2) are mycotoxin contents in foods for children above the legal limits? and is there a potential impact on children health? Considering that food ingestion is the main source of mycotoxins exposure, 3) are there toxic effects associated to intestinal exposure to multiple mycotoxins? and 4) do bioaccessibility and potential toxic effects interfere on risk associated to mycotoxins exposure?

In order to answer to these questions, the present thesis aimed to study the children exposure to multiple mycotoxins through food consumption applying a holistic approach for risk assessment. In order to achieve this aim, the following main objectives were considered:

1) To characterize Portuguese children exposure to multiple mycotoxins through mostly consumed foods, based on a pilot study results on food consumption;
2) To determine mycotoxins bioaccessibility in foods usually consumed by children;
3) To evaluate intestinal toxic effects associated to mycotoxins exposure, using different *in vitro* cell models of intestinal epithelial cells, dendritic cells and lymphocytes;
4) To characterize the risk associated to the consumption of foods usually eaten by children, using a holistic approach, considering data from: exposure assessment, bioaccessibility and intestinal toxicity assays.

The results obtained under experimental work were organized in different studies which were converted into manuscripts, submitted/published in international journals. These studies were organized in four different sections, as follows:

Section 1: Portuguese children exposure assessment to mycotoxins through food consumption

Assessment of multiple mycotoxins in breakfast cereals available in the Portuguese market (Manuscript 1)

Carla Martins, Ricardo Assunção, Sara C. Cunha, José O. Fernandes, Alessandra Jager, Tânia Petta, Carlos Augusto Oliveira, Paula Alvito

Single-compound and cumulative risk assessment of mycotoxins present in breakfast cereals consumed by children from Lisbon region, Portugal (Manuscript 2)

Ricardo Assunção, Elsa Vasco, Baltazar Nunes, Susana Loureiro, Carla Martins, Paula Alvito

Children exposure assessment to co-occurring mycotoxins present in cereal-based products in Portugal (Manuscript 3)

Ricardo Assunção, Carla Martins, Elsa Vasco, Alessandra Jager, Carlos Augusto Oliveira, Sara Cunha, José Fernandes, Baltazar Nunes, Susana Loureiro, Paula Alvito

Section 2: Bioaccessibility of mycotoxins present in foods intended for children consumption

Patulin and ochratoxin A co-occurrence and their bioaccessibility in processed cereal-based foods: A contribution for Portuguese children risk assessment (Manuscript 4)

Ricardo Assunção, Carla Martins, Didier Dupont, Paula Alvito

Section 3: Toxic effects of mycotoxins detected in foods intended for children consumption

A multi-endpoint approach to the combined toxic effects of patulin and ochratoxin A in human intestinal cells (Manuscript 5)

Ricardo Assunção, Mariana Pinhão, Susana Loureiro, Maria João Silva, Paula Alvito

Characterization of *in vitro* effects of patulin on intestinal epithelial and immune cells (Manuscript 6)

Ricardo Assunção, Paula Alvito, Charlotte Ramstad Kleiveland, Tor Erling Lea

Section 4: Challenges in risk assessment of multiple mycotoxins

Challenges in risk assessment of multiple mycotoxins in food (Manuscript 7)

Ricardo Assunção, Maria João Silva, Paula Alvito

III. EXPERIMENTAL WORK

SECTION 1: PORTUGUESE CHILDREN EXPOSURE ASSESSMENT TO MYCOTOXINS THROUGH FOOD CONSUMPTION

MANUSCRIPT 1: **ASSESSMENT OF MULTIPLE MYCOTOXINS IN BREAKFAST CEREALS AVAILABLE IN THE PORTUGUESE MARKET**

Carla Martins[a,b,c*], Ricardo Assunção[a,c,d], Sara C. Cunha[e], José O. Fernandes[e], Alessandra Jager[f], Tânia Petta[g], Carlos Augusto Oliveira[g], Paula Alvito[a,c]

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Manuscript submitted to Food Chemistry (under revision)

Highlights:

- Breakfast cereals from Portuguese market revealed a high incidence of mycotoxins
- None of the analysed samples presented contents above the maximum legislated limits
- Different mycotoxins (two to seven) occurred simultaneously in breakfast cereals
- Twenty two different combinations of mycotoxins were detected in breakfast cereals

ABSTRACT

Mycotoxins are secondary metabolites of fungi that cause toxic and carcinogenic effects. Human exposure to multiple mycotoxins constitutes an increasing health concern due to potential mycotoxins combined effects. The presence of mycotoxins mixtures in foodstuffs as cereals has been reported over the last years, but few studies are available concerning its occurrence in cereals primarily marketed for children, a particular vulnerable population group. The present study aims to assess the co-occurrence of twenty-one mycotoxins and metabolites present in breakfast cereals primarily marketed for children in Portugal. Results showed that 96% of the analysed breakfast cereal samples were contaminated with ten mycotoxins. Twenty two combinations were identified including two to seven different mycotoxins. Conclusions pointed out an urgent need to review legislative limits in food matrices consumed by children and to perform a more accurate risk assessment of children's exposure to mycotoxins mixtures in food.

Keywords: Mycotoxins mixtures, Breakfast cereals, Children, Human health

1. INTRODUCTION

Mycotoxins are secondary metabolites of fungi that cause toxic and carcinogenic outcomes in humans and animals exposed to them (Wu et al., 2014). The report on the co-occurrence of mycotoxins in food products, either from the same or from different fungal species is everyday more frequent (Pereira et al., 2014; Stoev, 2015) and their natural co-occurrence is an increasing concern due to the hazard of exposure of combined mycotoxins to humans, which could be expected to exert greater toxicity and carcinogenicity than exposure to single mycotoxins (Bouaziz et al., 2008; Grenier and Oswald, 2011).

Food matrices are very prone to contamination with fungi in the pre-harvest and at the moment of storage, especially when it is executed in poor conditions with excessive humidity (Piotrowska, 2013). These facts could lead to mycotoxin contamination of foodstuffs namely cereals and their products, including breakfast cereals. The presence of multiple mycotoxins (mycotoxin mixtures) produced by *Aspergillus* and *Fusarium* genus has been reported in breakfast cereals, over the last years, in different countries: aflatoxins, ochratoxin A (OTA), zearalenone (ZEA) and trichothecenes in Spain (Ibáñez-Vea et al., 2011b; Montes et al., 2012) and Pakistan (Iqbal et al., 2014); aflatoxin B_1 (AFB_1) and OTA in Greece (Villa and Markaki, 2009), fumonisins B_1, B_2, B_3 (FB_1, FB_2, FB_3) in Morocco (Mahnine et al., 2012); trichothecenes and ZEA in Italy (Romagnoli et al., 2010); trichothecenes, fumonisins and zearalenone in Canada (Roscoe

et al., 2008). In Portugal, deoxynivalenol (DON) and zearalenone were already reported in breakfast cereals by Cunha and Fernandes (2010) with contents ranging between 46 and 525 μg/kg and 28 and 69 μg/kg, respectively (Cunha and Fernandes, 2010). Gareis (2003) also reported the contamination of breakfast cereals marketed in Portugal with deoxynivalenol and zearalenone, with mean levels of 162 μg/kg and 5.1 μg/kg, respectively (SCOOP, 2003).

The ingestion of contaminated foodstuffs is one of the most common routes of exposure to mycotoxins including cereal grains and their finished products, nuts, dried fruits, spices, meat, milk, wine, beer, infant formula and baby foods (Turner et al., 2012). Cereals are among the first solid foods eaten by children and thus constitute an important food group of their diet (Schwartz et al., 2008) providing essential macronutrients and also supplying other important vitamins, minerals, and micronutrients for optimal health (Collins et al., 2010). Children are a vulnerable part of the population due to, in part, their physiology, a fairly restricted diet and a higher consumption relative to their body weight (Barreira et al., 2010) thus particular attention must be given to the exposure of this population group to co-occurring food contaminants. Children in many parts of the world are routinely exposed to many mycotoxins in the food chain and the three most common are aflatoxins, fumonisins, and deoxynivalenol (Etzel, 2014). For the main mycotoxin groups, maximum levels for regulatory purpose already exist for foodstuffs for adults and children consumption (European Commission, 2006a). Processed cereal-based foods, infant formulae and baby foods for infants and young children are regulated by Commission Regulation 1881/2006 with respect to mycotoxins contents (European Commission, 2006a). However, the available legislation for some toxins (AFB_1, sum of AFB_1, AFB_2, AFG_1 and AFG_2, DON, ZEA, sum of Toxin T-2 and Toxin HT-2, and sum of FB_1 and FB_2) does not consider breakfast cereals as products consumed by children, which usually presents lower limits than those considered for general population. This issue constitutes a legislative gap of special concern (European Commission, 2006a).

The occurrence of mycotoxins in breakfast cereals can be influenced by the sample composition such as the presence of chocolate or dried fruits. These products were referred as raw material largely susceptible for OTA contamination as stated by Molinié et al. (2005) and by the EU report SCOOP task 3.2.7 (Miraglia and Brera, 2002; Molinié et al., 2005). Some toxins can occur more frequently than other according to the producing area of food material (Cheli et al., 2014), thus the source of raw materials can influence mycotoxin contents in the marketed samples, which should be taken into account by the manufacturers and regulatory institutions.

The report on the co-occurrence of mycotoxins is increasing (De Ruyck et al., 2015; Stoev, 2015) and the exposure assessment of general population and, particularly, of specific vulnerable population groups

as children, to multiple mycotoxins is crucial. Therefore, there is a need to assess the risk associated with exposure of the population, namely the vulnerable groups, to the presence of these co-occurring compounds in foodstuffs. Mycotoxins occurrence data including their possible combinations found in foodstuffs are a key piece involved in the risk assessment procedure to accomplish this goal. Children's exposure to single mycotoxins with contents below legislated limits is considered safe to protect human health. However if the children are also protected against a combined exposure to mycotoxins is unknown. The co-occurrence of mycotoxins in breakfast cereals consumed by children gains particular relevance since recent consumption data gathered under the Portuguese project MYCOMIX (Alvito et al., 2015) revealed that 40% of the studied children (1 to 3 years old), consumed breakfast cereals at least one time in three days including different types (with a maximum of four) and various proportions (38 to 83%) of grains in their composition (Assunção et al., 2015). Within this context, the present study aims to assess the co-occurrence of 21 mycotoxins and their metabolites [AFB_1 (Aflatoxin B_1), AFB_2 (Aflatoxin B_2), AFG_1 (Aflatoxin G_1), AFG_2 (Aflatoxin G_2), AFM_1 (Aflatoxin M_1), OTA (Ochratoxin A), FB_1 (Fumonisin B_1), FB_2 (Fumonisin B_2), ZEA (Zearalenone), NIV (Nivalenol), NEO (Neosolaniol), DAS (Diacetoxyscirpenol), FUS-X (Fusarenon-X), DON (Deoxynivalenol), 15-ADON (15-acetyl-deoxynivalenol), 3-ADON (3-acetyl-deoxynivalenol), HT-2 (HT-2 toxin), T-2 (t-2 toxin), VER (Verrucarol), T-2 TETROL, T-2 TRIOL] in breakfast cereals available in the Portuguese market, to compare the results with the maximum limits (ML) established by the EU and to assess the combinations of multiple mycotoxins present in this food matrix.

2. MATERIALS AND METHODS

2.1. Sampling

Twenty six breakfast cereal samples were purchased from supermarkets in Lisbon region, in 2014. Samples composition included maize (n=5), wheat (n=7), rice (n=4) and multigrain (n=10). Nine samples also had chocolate in its composition (n=9). This classification was based on label information. The purchased samples were from six different generic brands (n=20) and three different name-brands (n=6). The sample size of each sample was at least of 1 kg, accordingly to Commission Regulation (EC) 401/2006 of 23 February 2006 (European Commission, 2006b). The samples were homogenized in a food homogenizer, saved in plastic bags and stored at 4 °C until further analysis.

2.2. Chemicals and Reagents

Methanol, sodium chloride, potassium bromide, PBS (suprapur grade), acetic acid, n-hexane, acetonitrile and methanol (HPLC grade), were supplied by Merck®, Fluka®, JT Baker® and Sigma®. Immunoaffinity columns AflaOchra were purchased from Vicam®. Dispersive-SPE sorbents for experiments including C_{18}-bonded silica were purchased from Waters®. Primary secondary amine (PSA; particle size 50 mm) and the derivatization reagent BSA (N,O-bis(trimethylsilyl) acetamide) + TMCS (trimethylchlorosilane) + TMSI (N-trimethylsilyimidazole) (3:2:3) (also known as Tri-Sil TBT) were from Supelco®. Ultra-pure water was produced on a Milli-Q Gradient A10 system, from Millipore® (Molsheim, France). Ultrahigh purity He (helium) for GC-MS and N_2 (nitrogen) for solvent evaporation were obtained from Gasin® (Maia, Portugal).

Standard solutions were purchased from Biopure® (OTA, AFB_1, AFB_2, AFG_1, AFG_2, AFM_1 and $^{13}C_{18}$-ZEA), Fluka® (DON, FUS-X, NIV, NEO, 15-ADON, α-chloralose, T2-Triol and T2-Tetraol) and Sigma® (DAS, 3-ADON, VER, T2-Toxin, HT-2 Toxin, FB_1, FB_2, ZEA,$^{13}C_{34}$-FB_1, $^{13}C_{15}$-DON).

From the OTA and aflatoxins standard solutions, a mycotoxins mixture stock solution was prepared in chloroform with 0.20 µg/mL of AFM_1, 0.080 µg/mL of AFB_1, 0.060 µg/mL of AFB_2, 0.090 µg/mL of AFG_1, and 0.060 µg/mL of AFG_2 and 0.40 µg/mL of OTA, and stored at -18 °C. From this stock solution, a working stock solution was prepared in water–methanol (85:15, v/v), with a 1000x dilution, followed by further dilutions to obtain the calibration solutions in water–methanol (85:15, v/v). Standard stock solutions of each trichothecene at 100 mg/L were prepared in acetonitrile (MeCN), with the exception of T2-triol and T2-tetraol prepared at 50 mg/L in MeCN. From these stock solutions, a mixture standard working solution at 20 mg/L was prepared in MeCN. All standard solutions were stored at -18 °C. Individual stock internal solutions of α-chloralose (at 100 mg/L) and $^{13}C_{15}$-DON (at 25 mg/L) were prepared in MeCN and stored at -18 °C. The individual working internal solutions were prepared by dilution of the stock internal solutions. From $^{13}C_{34}$-FB_1 and $^{13}C_{18}$-ZEA standards, individual stock solutions were prepared in acetonitrile:water (50:50, v/v) and stored in amber vials at -18 °C. Calibration curves were prepared with stock solutions dissolved in H_2O:MeOH (8:2, v/v).

2.3. Sample preparation

2.3.1. Aflatoxins and Ochratoxin A

The extraction of aflatoxins and ochratoxin A from breakfast cereals was performed according to the method described in EN15851 (EN ISO, 2010), with few modifications. Briefly, a test portion (10 g) is

extracted with 50 mL of methanol/water (80:20, v/v). The extract was filtered with folded filter paper, diluted with phosphate buffered saline (PBS) to 100 mL (1:9), filtered again with a glass microfiber filter and totally applied to an immunoaffinity column containing antibodies specific to aflatoxins and ochratoxin A (AflaOchra, Vicam®). Elution was performed with 1.6 mL of methanol (HPLC grade), with subsequent dilution with water to 10 mL (MeOH/H_2O, 16:84, v/v). A volume of 800 µL was injected into the HPLC system.

2.3.2. Trichothecenes

The extraction of trichothecenes from breakfast cereals was performed according to a procedure based on QuEChERS methodology, previously developed by (Pereira et al., 2014). Briefly, 2.5 g of homogenized sample after added with water and 250 µL of α-chloralose was extracted with 10 mL MeCN, 4 g $MgSO_4$ and 1 g NaCl. Then a d-SPE was performed with 1350 mg $MgSO_4$ and 450 mg PSA using 9 mL of MeCN extract. Finally, the extracted was added with 50 µL of the $^{13}C_{15}$-DON, evaporated to dryness under a stream of nitrogen and derivatized with 50 µL of BSA+TMCS+TMSI (3:2:3) at 80 °C for 20 min. The excess of derivatizing reagent was then removed with 300 µL of phosphate buffer 0.1 M pH 7.2 and the analytes extracted with 100 µL of n-hexane.

2.3.3. Fumonisins and Zearalenone

The extraction of fumonisins from breakfast cereals was performed according to a procedure described by (Ndube et al., 2011) with minor modifications. In brief, 25 g of homogenized sample was weighted in an Erlenmeyer flask and 100 mL of methanol/water (75:25, v/v) were added. The flask was placed in an orbital shaker (Tecnal®, Piracicaba, Brazil) for 30 min and a portion of the mixture was centrifuged at 5000 rpm for 5 minutes. After, 20 µL of the $^{13}C_{34}$-FB_1 working solution at 100 ng/mL were added to each sample. The supernatant was diluted with deionized water [1:3 (extract/water), v/v)] and transferred to a vial for injection into an ultra-performance liquid chromatographic (UPLC)-MS/MS system. For the determination of ZEA, 1 g of homogenized sample was weighted in 10 mL falcon polypropylene tubes and 4 mL of methanol/water (75:25, v/v) were added. After, 16 µL of the $^{13}C_{18}$-ZEA working solution at 100 ng/mL were added to each sample. The tubes were placed in an orbital shaker (Tecnal®, Piracicaba, Brazil) for 30 min and then centrifuged at 5000 rpm for 5 minutes. The supernatant was diluted with deionized water [1:3 (extract/water), v/v)] and transferred to a vial for injection into the UPLC-MS/MS system.

2.4. Instruments and analytical conditions

Aflatoxins and ochratoxin A were determined by reverse-phase high performance liquid chromatography with fluorescence detection (HPLC-FD) and with post column derivatization involving bromination, in conditions previously described by (Sizoo and van Egmond, 2005). The method was previously in-house validated for the analysis of these mycotoxins. HPLC analysis was performed using a Waters® Alliance 2695 equipped with fluorescence detector Waters 2475 (Waters®, USA) with Empower Chromatography Software. Post-column derivatization was carried out with electrochemically generated bromine (Kobra cell, R-Biopharm®). The chromatographic column was a Prodigy ODS 100 Å (5 µm, 150×4.6 mm, Phenomenex®, Torrance, CA). The mobile phases were a gradient comprised between phase A [KBr (175 mg/L)/MeOH/MeCN/$C_2H_4O_2$ (1650:465:390:50, v/v/v/v)] and phase B [KBr (175 mg/L)/MeOH/MeCN/ $C_2H_4O_2$ (140:1283:1073:50, v/v/v/v)], with a flow rate of 1 mL/min. Auto sampler and chromatography column temperatures were kept at 10 °C and 35 °C, respectively. Total run time was 59 minutes. The wavelength of the fluorescence detector was set at 365 and 322 nm (excitation) and 465 and 468 nm (emission), for aflatoxins and ochratoxin A detection, respectively. Trichothecenes were determined by GC-MS analysis performed on an Agilent® (Little Falls, DE, USA) gas chromatograph 6890 equipped with an electronically controlled split/splitless injection port and an inert 5973N mass selective detector with electron impact (EI) ionization chamber, in conditions previously described by (Pereira et al., 2014). The analytical separation was conducted in DB-5 MS (30 m x 0.25 µm x 0.25 mm film thickness; J&W Scientific, Folsom, CA, EUA) capillary column. The injection was made in splitless mode (60 s) at 270 °C. The oven temperature program was as follows: 140 °C held for 1.5 min, ramped to 280 °C at 10 °C/min and 300 °C at 20 °C/min, and held for 5.0 min. Total run time was 21.5 minutes. The MS transfer line temperature was held at 280 °C. Mass spectrometric parameters were set as follows: electron impact ionization, 70 eV energy; ion source temperature, 230 °C; MS quadrupole temperature, 150 °C and solvent delay 3.0 min. The MS system was routinely set in selective ion monitoring (SIM) mode and each mycotoxin was quantified based on peak area using one target and more than two qualifier ion(s). Complete SIM parameters and retention times of the analytes are shown in Table 14. Agilent Chemstation was used for data collection/processing and GC-MS control.

Fumonisins and zearalenone contents were determined in an UPLC-MS/MS system consisting of a Waters® Acquity I-Class UPLC coupled to a Waters® Xevo TQ-S mass spectrometer (Milford, MA, USA) and equipped with a BEH C¬18 column (2.1 × 50 mm, 1.7 µm, Waters®).

Table 14. GC-MS conditions, SIM parameters and retention times of trichothecenes analysis.

Mycotoxins [M]$^+$	Time windows (min)	t_R (min)	SIM ions (*m/z*)	
			Quantification	Identification
α-chloralose (IS1) [MW+216]$^+$		11.024	423	421, 423, 317, 245, 205
VER [MW+144]$^+$		11.231	173	173, 234, 277, 380
$^{13}C_{15}$-DON (IS2) [MW+216]$^+$	11.50	11.738	245	527, 245, 437
DON [MW+216]$^+$		11.743	235	235, 259, 422, 512, 497
FUS-X [MW+216]$^+$	12.50	12.727	480	480, 450, 555
DAS [MW+72]$^+$		12.815	378	378, 290
3-ADON [MW+144]$^+$		12.826	377	362, 377
15-ADON [MW+144]$^+$		12.826	407	422, 407, 325
NIV [MW+288]$^+$		12.970	289	379, 289
T2-tetrol [MW+288]$^+$		12.915	275	496, 275, 365
NEO [MW+144]$^+$	13.40	13.908	193	193, 252, 436, 371
T2-Triol [MW+216]$^+$	14.60	14.993	185	103, 185, 173, 275, 377, 496
HT-2 [MW+144]$^+$	15.50	15.650	347	466, 377, 347
T-2 [MW-72]$^+$		15.728	350	436, 244, 350

Electrospray interface in positive mode was used for detection in multiple reactions monitoring (MRM) mode. Auto sampler and chromatography column temperatures were kept at 15 °C and 40 °C, respectively. Ten microliters of each standard or sample extract were injected, and separation was carried out using a gradient elution with mobile phase composed by water (eluent A) and acetonitrile (eluent B), both containing 0.1 % of formic acid, at a flow rate of 0.6 mL/min. The main mass spectrometer parameters were optimized and established as follows: capillary voltage, 0.75 kV; source and dessolvation temperatures, 150 °C and 550 °C, respectively; dessolvation and gas flows, 800 L/h and 150 L/h, respectively. Intellistart program (Milford, MA, USA) of Acquity UPLC console was used to optimize cone voltage, collision energy and MRM transitions (major precursor ion > fragment ion) for each compound individually. Table 15 contains the optimized mass spectrometer conditions for FB_1 and FB. MassLynx software version 4.1 (Milford, MA, USA) was used for data collection and processing.

Calibrations were performed with external standards (aflatoxins and OTA) and with internal standards (trichothecenes, fumonisins and zearalenone) prepared in solvents. Matrix-matched calibration curves

were used in trichothecenes analysis, considering the significant suppression of the response for all analytes, verified after comparison between the slope of calibration curves obtained from standard solutions and from matrix-matched standards.

Table 15. Mass spectrometer analytical conditions for fumonisins and zearalenone analysis.

Mycotoxin	Precursor ion (*m/z*)	Product ions (*m/z*)	Cone Voltage (V)	Collision Energy (V)
FB_1	722	352[a]	30	35
		334[b]	30	40
FB_2	706	336[a]	30	35
		318[b]	30	35
ZEA	317	175[a]	40	23
		131[b]	40	33
$^{13}C_{18}$-ZEA	335	185[a]	40	31
$^{13}C_{34}$-FB1	756	375[a]	30	30

[a] Transitions used for quantification
[b] Transitions used for confirmation
V, voltage

ZEA, FB_1 and FB_2 levels were determined by internal calibration, using $^{13}C_{18}$-ZEA for quantification of ZEA and $^{13}C_{34}$-FB_1 for quantification of FB_1 and FB_2. The concentration of each analyte in samples was determined using calibration curves plotted by the relative response of the corresponding analyte as a function of the analyte concentration. The relative response was the ratio between the analyte peak area in sample, and the peak area of the corresponding internal standard. In fumonisins and zearalenone analysis, suppression or increase of the analyte signal caused by possible interferents of the extract was compensated by verifying identical signal variation of the internal standard.

Recoveries were evaluated through spiking of samples before extraction with mycotoxins solutions at one concentration level (0.5 µg/kg of AFM_1, 0.25 µg/kg of AFB_1, AFB_2, AFG_1 and AFG_2, and 1.25 µg/kg of OTA) and at two concentration levels (20 and 25 µg/kg of DON, 3-ADON, 15-ADON, NIV, VER, DAS, T2-Triol, NEO, FUS-X, and 160 and 200 µg/kg of T2, HT-2 and T2-Tetraol). Recovery was determined by extrapolation of the absolute responses obtained from the spiked samples in the calibration curve; the

calculated concentration was compared with the expected concentration for a 100% recovery. Considering the use of internal standards, fumonisins and zearalenone recoveries were not determined.

Limits of detection (LOD) and limits of quantification (LOQ) were determined according to: i) LOD = [b_0 + 3S(b_0)]/b_1, LOQ = [b_0 + 10S(b_0)]/b_1, where b_0 is the response of the blank (intercept of the calibration model), S(b_0) is the standard deviation of the blank and b_1 is the sensitivity (calibration model slope), for aflatoxins and ochratoxin A; ii) successive analyses of spiked blank samples with decreasing amounts of analytes until a signal-to-noise (S/N) ratio of 3:1 was reached, whereas the LOQ was determined considering a S/N of 10:1, for trichothecenes; and iii) the calibration curves in solvent, based on a signal-to-noise ratio of peaks corresponding to the qualifier MRM transitions (transitions used for confirmation) of 3:1 and 10:1, respectively, for fumonisins and zearalenone.

2.5. Statistical analysis

The statistical analysis was performed using SPSS 22.0 software. Median and maximum calculations were performed using Microsoft® Excel 2007. Results below LOD were assigned as the value of LOD to perform the statistical analysis in order to have a more conservative approach (EFSA, 2010). Statistical comparisons were made for mycotoxins presented in more than 50% of the analysed samples. The normality of distributions was evaluated with the Shapiro-Wilk test. Differences between sample groups (number of cereals in sample composition, type of brand, presence/absence of chocolate) were evaluated with the nonparametric tests of Mann-Whitney and Kruskal-Wallis. A level of significance of 5 % was used to evaluate the statistical analysis.

3. RESULTS AND DISCUSSION

3.1. Analytical performance

The results have shown good linear response with coefficient of determination (R^2) > 0.995 for all the analysed mycotoxins. Recovery values were 83% (AFM_1), 73% (AFB_1), 57% (AFB_2), 87% (AFG_1), 57% (AFG_2), 71% (OTA), 70% (FB_1), 68% (FB_2), 93% (DON), 103% (NEO), 117% (DAS), 99% (FUS-X), 131% (15-ADON), 74% (T-2 Triol), 46% (NIV), 44% (T-2), 93% (HT-2), 92% (3-ADON), 135% (VER) and 84% (T2-Tetrol).

Limits of detection (LOD) and limits of quantification (LOQ) were 0.011 and 0.032 µg/kg (AFM_1), 0.003 and 0.009 µg/kg (AFB_1), 0.004 and 0.012 µg/kg (AFB_2), 0.006 and 0.018 µg/kg (AFG_1), 0.010 and 0.029 µg/kg (AFG_2), 0.006 and 0.019 µg/kg (OTA), 0.060 and 0.180 µg/kg (FB_1), 0.120 and 0.360 µg/kg

(FB_2), 0.12 and 0.40 µg/kg (ZEA), 0.400 and 1.20 µg/kg (DON), 1.30 and 4.2 µg/kg (NEO), 3.1 and 10.1 µg/kg (DAS), 2.8 and 9.2 µg/kg (FUS-X), 2.5 and 8.3 µg/kg (15-ADON), 0.900 and 3.00 µg/kg (T-2 Triol), 5.60 and 18.4 µg/kg (NIV), 6.8 and 22.3 µg/kg (T-2), 6.4 and 21.1 µg/kg (HT-2), 17.3 and 57.0 µg/kg (3-ADON), 19.2 and 63.3 µg/kg (VER), 10.50 and 34.6 µg/kg (T2-Tetrol). All these results, with the exception of NIV, T-2, DAS and 15-ADON recoveries, were in agreement with the criteria mentioned in the Commission Regulation (EC) No. 401/2006 and showed that the analytical methods applied are adequate for mycotoxins determinations (European Commission, 2006b).

3.2. Occurrence of mycotoxins in breakfast cereals

The analysis of breakfast cereals primarily marketed for children in Portugal revealed the occurrence of ten mycotoxins from the twenty one mycotoxins and metabolites assessed. AFG_2, NEO, DAS, FUS-X, 15-ADON, 3-ADON, HT-2, T-2, VER, T-2 TETROL, T-2 TRIOL were not detected in the analyzed breakfast cereal samples. Samples that presented at least one mycotoxin above the respective LOD were considered positive. The overall incidence of positive samples for mycotoxins was 96% (25/26). ZEA presented the highest incidence (73%, 19/26) with decreasing values for the remaining toxins e.g. AFB_1 and OTA (both 69%, 18/26) >, DON (62%, 16/26) >, FB_1 (58%, 15/26) >, FB_2 (38%, 10/26) >, AFB_2 (27%, 7/26) >, AFM_1 (12%, 3/26) >, AFG_1 and NIV (both 4%, 1/26). Mycotoxin contents were all below the maximum levels established in the European legislation for breakfast cereals, when available (European Commission, 2006a).

Table 16 summarizes the results on mycotoxin contents determined in breakfast cereals related to the number of cereals in sample composition (one, two, three or more cereals including wheat, maize, rice, oat), presence or absence of chocolate and type of brand (generic and name brand). The limits of detection and quantification for each mycotoxin as well as the available legislation concerning the presence of mycotoxins in breakfast cereals are also reported. Mycotoxin contents reported in Table 16 are not corrected for recovery. None of the mycotoxins presented a normal distribution (p<0.05) thus median mycotoxin contents were calculated.

3.2.1. Aflatoxins and Ochratoxin A in breakfast cereals samples

AFB_1 and OTA were the second most frequently detected mycotoxins. All aflatoxins, except AFG_2, were detected in the analysed breakfast cereals revealing an incidence of positive samples from 69% (AFB_1) to 4% (AFG_1). Aflatoxins were detected in a range from 0.011 (LOD) to 0.024 µg/kg (AFM_1), 0.003 (LOD) to

0.130 µg/kg (AFB_1), 0.001 (LOD) to 0.011 µg/kg (AFB_2), 0.006 (LOD) to 0.014 µg/kg (AFG_1) with median values of 0.017 µg/kg (AFM_1), 0.013 µg/kg (AFB_1), 0.004 µg/kg (AFB_2) and 0.013 µg/kg (AFG_1). Within the group of aflatoxins, AFB_1 presented the maximum content in a sample from a name brand, with two cereals and no chocolate in its composition. Available legislation considers a maximum limit of 2.0 µg/kg and 0.100 µg/kg for AFB_1 in processed cereal products and processed cereal-based foods and baby foods for infants and young children, respectively (European Commission, 2006a). Since the analysed breakfast cereals were primarily marketed for children it should be noted that three samples over passed the maximum limit of 0.100 µg/kg for this mycotoxins (Table 16). The range of contamination determined for AFB_1 in this study was similar to those reported by Ibáñez-Vea et al. (2011) in breakfast cereal samples from the Spanish market (0.051 - 0.130 µg/kg). When comparing the present results with those reported by Villa and Markaki (2009) (0.05 - 4.3 µg/kg of AFB_1, with incidence of 56.3%) and with those reported by Iqbal et al. (2014) (0.04 - 6.9 µg/kg of AFB_1, with an incidence of 41%) in samples from the Greek and Pakistan markets, respectively, it can be concluded that breakfast cereals samples marketed in Portugal presented generally lower levels of mycotoxins, but with a higher aflatoxins incidence, particularly AFB_1.

The incidence of positive samples for OTA was 69%. This toxin was detected in a range from 0.006 (LOD) to 0.100 µg/kg, with a median value of 0.040 µg/kg. Maximum contents were determined in a sample from a generic brand, and with one cereal and chocolate in its composition. Available legislation considers a maximum limit of 3.0 µg/kg and 0.500 µg/kg for OTA in processed cereal products and processed cereal-based foods and baby foods for infants and young children, respectively (European Commission, 2006a), thus all analysed samples were below the legislated limits (Table 16). Villa and Markaki (2009) and Iqbal et al. (2014) had reported similar percentages of positive samples (60 and 48%, respectively) but in a range of contamination much higher (0.020 - 0.870 and 0.02 - 8.45 µg/kg of OTA) (Iqbal et al., 2014; Villa and Markaki, 2009).

Table 16 also revealed a statistically significant difference between the presence and absence of chocolate (p=0.016) and OTA contents in the analysed breakfast cereals. In fact, other studies had already reported an association between OTA presence in cereals and chocolate (Miraglia and Brera, 2002; Molinié et al., 2005), and between cocoa derived products and OTA (Brera et al., 2011; Copetti et al., 2013, 2012). Cocoa beans are produced mainly in West Africa, Asia, Oceania and Central and South America, regions with temperate and humid climate conditions that together with agricultural practices, may favour *Aspergillus* and *Penicillium* growth and OTA biosynthesis (Brera et al., 2011).

Table 16. Occurrence of mycotoxins in breakfast cereal samples primarily marked for children in Portugal.

			AFM$_1$	**AFB$_1$**	**AFB$_2$**	**AFG$_1$**	**OTA**	**FB$_1$**	**FB$_2$**	**DON**	**NIV**	**ZEA**
EC 1881/2006, breakfast cereals, maximum content (µg/kg)			NA	AFB$_1$ =2 µg/kg AFB$_1$ + AFB$_2$ + AFG$_1$ + AFG$_2$= 4 µg/kg (All cereals and all products derived from cereals, including processed cereal products)			3 µg/kg (All cereals and all products derived from cereals, including processed cereal products)	FB$_1$+ FB$_2$ = 200 µg/kg (Processed maize-based foods)		500 µg/kg (breakfast cereals)	NA	50 µg/kg (breakfast cereals) 50 µg/kg (maize breakfast cereals)
LOD (µg/kg)			0.011	0.003	0.001	0.006	0.006	0.06	0.12	0.4	5.6	0.12
LOQ (µg/kg)			0.032	0.009	0.004	0.018	0.019	0.18	0.36	1.2	18.4	0.40
Breakfast cereals (n=26)		**Incidence of positive samples, n, (%)**	3 (12 %)	18 (69 %)	7 (27 %)	1 (4 %)	18 (69 %)	15 (58 %)	10 (38 %)	16 (62 %)	1 (4 %)	19 (73 %)
			(µg kg^{-1})									
		Max	0.024	0.130	0.011	0.014	0.100	67.0	14.0	207.8	27.1	5.6
		Med	0.017	0.013	0.004	0.013	0.040	12.5	4.2	91.5	27.1	0.7
Number of cereals in sample composition	1 Cereal (n = 16)	Max	0.013	0.124	0.011	0.012	0.100	65.0	14.0	207.8	27.1	5.6
		Med	0.013	0.013	0.006	0.012	0.045	11.7[a]	5.4	103.1	27.1	1.0
	2 Cereals (n = 4)	Max	ND	0.130	0.008	ND	0.039	31.0	4.1	103.3	ND	2.1
		Med	ND	0.088	0.006	ND	0.036	20.0[a]	4.1	91.0	ND	0.4
	3 Cereals (n = 6)	Max	0.024	0.013	0.002	0.014	0.072	67.0	5.0	145.2	ND	1.3
		Med	0.021	0.009	0.002	0.014	0.028	19.0[a]	4.2	59.0	ND	0.7
Chocolate	W/O (n = 17)	Max	0.024	0.130	0.008	0.014	0.080	67.0	14.0	197.3	27.1	2.8
		Med	0.017	0.029	0.007	0.013	0.028[b]	15.8[c]	4.1	95.9	27.1	0.7
	W (n = 9)	Max	ND	0.065	0.011	ND	0.100	9.2	4.3	207.8	ND	5.6
		Med	ND	0.012	0.003	ND	0.051[b]	9.2[c]	4.3	60.8	ND	0.7
Type of Brand	Generic brand (n = 20)	Max	0.017	0.109	0.007	0.017	0.100	67.0	5.4	207.8	27.1	5.6
		Med	0.015	0.013	0.006	0.017	0.039	15.8	4.2	103.2	27.1	1.0
	Name brand (n = 6)	Max	0.024	0.130	0.008	ND	0.080	65.0	14.0	78.7	ND	0.6
		Med	0.024	0.016	0.008	ND	0.056	12.0	4.2	30.7	ND	0.4

W – With chocolate; W/O – without chocolate; ND – Not Detected; NA - Not available; Max – Maximum; Med – Median; [a] $p<0.05$ (Kruskal-Wallis); [b,c] $p<0.05$ (Mann-Whitney).

In the present study, 89% (8/9) of the breakfast cereals with chocolate presented OTA; this is of special concern since these breakfast cereals are primarily marketed for children who are more sensitive to mycotoxins toxic effects (Copetti et al., 2013).

3.2.2. Trichothecenes in breakfast cereals samples

DON has been reported as a common contaminant in cereal samples, and in the present study it was the third most frequently detected mycotoxin, revealing an incidence of positive samples of 62%. DON was detected in a range from 0.4 (LOD) to 207.8 µg/kg, with a median value of 91.5 µg/kg. Maximum contents were verified in a sample from a generic brand, with 1 cereal and chocolate in its composition. Available legislation considers a maximum limit of 500 µg/kg for DON in breakfast cereals (Table 16) and 200 µg/kg for DON in processed cereal-based baby foods for infants and young children (European Commission, 2006a), thus all samples were below the legislated limits for breakfast cereals; however one sample still over passed the maximum limit of 200 µg/kg for this mycotoxin (Table 16). The presence of DON in breakfast cereal samples was already reported by Cunha et al. (2010), Montes et al. (2012), De Boevre et al. (2013) and Romagnoli et al. (2010) who detected DON in 72.2%, 25.7%, 58% and 37% of the analysed samples, respectively. However, the range of contamination reported in the present study is lower than those reported by Cunha et al. (2010), Montes et al. (2012) and De Boevre et al. (2013) who referred ranges from 11 to 525, 11.4 to 468 and 9 to 718 µg/kg, respectively (Cunha and Fernandes, 2010; De Boevre et al., 2013; Montes et al., 2012; Romagnoli et al., 2010).

NIV had an incidence of positive samples of 4% (1/26), with a concentration of 27.1 µg/kgin a sample from a generic brand, with one cereal and without chocolate in its composition. There is no legislation concerning the presence of NIV in breakfast cereals or in processed cereal-based foods and baby foods for infants and young children (Table 16). NIV contents had been reported with a low incidence by Montes et al. (2012) that detected NIV in 2.7% (4/148) of breakfast cereal samples, with a median content of 15.1 µg/kg, and the maximum content detected (56.7 µg/kg) in a wheat based sample (Montes et al., 2012). Roscoe et al. (2008) also reported low incidence of NIV in the analysis of 156 breakfast cereal samples, with only one sample of multigrain presenting NIV in a concentration of 60 µg/kg (Roscoe et al., 2008).

3.2.3. Fumonisins and zearalenone in breakfast cereals samples

The incidence of positive samples for FB_1 was 58% with results detected in a range from 0.06 (LOD) to 67.0 μg/kg and a median value of 12.5 μg/kg. For FB_2, 38% of samples were positive, with results detected in a range from 0.12 (LOD) to 14.0 μg/kg and a median value of 12.5 μg/kg. The maximum contents were reported for FB_1 in a sample from a generic brand, with three or more cereals and no chocolate in its composition, and for FB_2 in a sample from a name brand, with one cereal and no chocolate in its composition. The available legislation sets maximum limits for the sum of FB_1 and FB_2 in maize-based breakfast cereals (400 μg/kg); however breakfast cereals composed by other cereals than maize are not referred in the regulation (European Commission, 2006a). The maximum contents of FB_1 and FB_2 were also lower than 200 μg/kg, the limit set for the sum of these mycotoxins in processed maize-based foods and baby foods for infants and young children (European Commission, 2006a). The presence of fumonisins was already reported by Mahnine et al. (2012) in breakfast cereals available in the Athens market, referring maximum contents of 152 μg/kg of FB_1 and 62.3 μg/kg of FB_2 in a sample composed of rice, maize and chocolate. These authors reported 37.5% (18/48) of breakfast cereals positive for fumonisins (Mahnine et al., 2012).

In the present study, a significant difference concerning FB_1 contents in samples with different number of cereals in their composition (p=0.003) and presence or absence of chocolate (p=0.001) were verified (Table 16) as well as the presence and absence of maize (p=0.000) (data not shown). Wu and co-authors referred that FB_1 and FB_2 are prone to contaminate maize samples and are frequently common co-contaminants (Wu et al., 2014). The fact that maize was present in 14 of the 26 analysed samples (alone or in mixture with other cereals), could have contributed to the significant difference verified for FB_1 contents and cereal number.

ZEA showed the highest incidence presenting 73% of positive samples for this mycotoxin, with detected contents in a range of 0.12 (LOD) to 5.6 μg/kg and a median value of 0.7 μg/kg. The maximum content was reported in a sample from a generic brand, with one cereal and chocolate in its composition. The maximum contents determined for ZEA were lower than the available legislation (Table 16) which refers maximum limits allowed for breakfast cereals (50 μg/kg), for maize based breakfast cereals (50 μg/kg) and for processed cereal-based foods and processed maize-based foods for infants and young children (20 μg/kg) (European Commission, 2006a). The presence of ZEA in cereal based samples, namely in breakfast cereals, has been previously reported by other authors. Ibañez-Vea et al. (2011), De Boevre et al. (2013) and Iqbal et al. (2014) reported a lower incidence of zearalenone (48%, 52% and 53%, respectively), although with higher maximum levels (38.61, 450.0 and 118.1 μg kg^{-1}, respectively) than

those reported in the present study. It should be pointed that the maximum value of 450 µg/kg reported by De Boevre et al. (2013) is 9 times the limit established by the European Commission (De Boevre et al., 2013; Ibáñez-Vea et al., 2011a; Iqbal et al., 2014).

ZEA and DON co-occur and this agrees with the fact that DON and ZEA were observed in 46% of the mixtures (11/24) in this study. Previous studies reported the simultaneous production of DON and ZEA by the same *Fusarium* species in the temperate climates of Europe, which could justify the simultaneous presence of both mycotoxins in the analysed samples (Kokkonen et al., 2010).

3.3. Assessment of mixtures of mycotoxins in breakfast cereal samples

Table 17 presents the results concerning the co-occurrence of mycotoxins in breakfast cereals including its number and combinations, the observed incidence and sample characterisation (number and cereal composition). A combination of *Aspergillus* and *Fusarium* mycotoxins co-occur with 92% of the analysed samples presenting two or more mycotoxins simultaneously

In the present study, the co-occurrence of mycotoxins in the same sample was highly observed, with 92% of the analysed samples presenting two or more mycotoxins simultaneously.

The number of mycotoxins in mixture ranged from two to seven with 22 different combinations detected, two of them repeated (AFB_1+AFB_2+OTA+ZEA and OTA+FB_1+DON+ZEA). The combination of four mycotoxins was the most commonly detected, with an incidence of 28% (7/26). Within these samples, five different associations of toxins were found (AFB_1+OTA+DON+ZEA, AFB_1+AFB_2+OTA+ZEA, OTA+FB_1+DON+ZEA, AFB_1+OTA+DON+ZEA, AFB_1+AFB_2+OTA+DON) and all have got OTA in common.

The co-occurrence of mycotoxins in cereals and processed cereals (*i.e.* breakfast cereals, processed cereal-based baby foods) has been widely reported in the last years. Alvito et al. (2010) reported the co-occurrence of AFM_1, AFB_1 and OTA in 40% (8/20) of cereal based baby foods marketed in Portugal (Alvito et al., 2010). Ibáñez-Vea et al. (2012) studied the co-occurrence of mycotoxins in 123 barley samples in Spain and observed an incidence of 28% of positive samples presenting OTA and ZEA as the most frequent mixture (92%) (Ibáñez-Vea et al., 2012). A 3-year survey in Canada, including the analysis of 156 breakfast cereals samples reported that 43% of the samples had multiple mycotoxins (Roscoe et al., 2008). The authors of the Canadian study also pointed out that samples presented levels far below the international guidelines (with only 3% (5/156) that surpassed the proposed maximum limits) but with high incidence, which are similar to results from the present study. In other cereal based foods, as infant flours, the co-

occurrence of mycotoxins was reported in 92% of the positive samples (Juan et al., 2014). In an official control of cereal products mycotoxins content in Slovenia, 116 out of 290 samples collected from 2008-2012, were positive. Within these positive samples, 35% contained two or three different mycotoxins (Kirinčič et al., 2015).

Table 17. Simultaneous occurrence of mycotoxins in breakfast cereals from Portuguese market.

Nº of mycotoxins	Co-occurrence of mycotoxins	Incidence (n=26) n, (%)	Number of cereals in sample
2	AFB_1+AFB_2	1 (4%)	1
Total		**1 (4%)**	
3	FB_1+FB_2+ZEA	1 (4%)	2
	FB_1+DON+ZEA	1 (4%)	1
	AFB_1+DON+ZEA	1 (4%)	1
Total		**3 (12%)**	
4	AFB_1+OTA+DON+ZEA	1 (4%)	1
	AFB_1+AFB_2+OTA+ZEA	2 (8%)	1,1
	OTA+FB_1+DON+ZEA	2 (8%)	1,2
	AFB_1+OTA+DON+ZEA	1 (4%)	1
	AFB_1+AFB_2+OTA+DON	1 (4%)	1
Total		**7 (28%)**	
5	AFB_1+AFB_2+OTA+DON+ZEA	1 (4%)	1
	OTA+FB_1+FB_2+DON+ZEA	1 (4%)	3
	AFB_1+AFB_2+OTA+FB_1+ZEA	1 (4%)	2
	AFB_1+AFB_2+OTA+DON+ZEA	1 (4%)	1
	AFM_1+AFB_1+OTA+FB_1+DON	1 (4%)	3
Total		**5 (20%)**	
6	AFB_1+OTA+FB_1+FB_2+DON+ZEA	1 (4%)	3
	AFB_1+AFG_1+OTA+FB_1+FB_2+DON	1 (4%)	3
	AFB_1+FB_1+FB_2+OTA+NIV+ZEA	1 (4%)	1
	AFB_1+AFB_2+FB_1+FB_2+DON+ZEA	1 (4%)	2
	AFM_1+AFB_1+AFB_2+OTA+FB_1+FB_2	1 (4%)	1
Total		**5 (20%)**	
7	AFM_1+AFB_1+AFB_2+OTA+FB_1+FB_2+ZEA	1 (4%)	3
	AFB_1+AFB_2+OTA+FB_1+FB_2+DON+ZEA	1 (4%)	3
	AFB_1+AFB_2+AFG_1+OTA+FB_1+FB_2+ZEA	1 (4%)	1
Total		**3 (12%)**	

Recent surveys worldwide highlight the fact that humans are more frequently exposed to multiple than to single mycotoxins (De Ruyck et al., 2015; Grenier and Oswald, 2011; Stoev, 2015). As a consequence, there has been an increasing concern about the health hazard from exposure to multiple mycotoxins in human and animals. Government and industry regulations exist to minimize the concentrations of individual mycotoxins allowed in food and feed products. However, these regulations are based on individual toxicities, and as such, do not take into account the complex dynamics of compounded risk from co-exposure to groups of mycotoxins, for even acute pathologies (De Ruyck et al., 2015; Zain, 2011). Results from Table 16 and Table 17 confirm the increasing report on the occurrence of multiple mycotoxins in cereals and emphasize the importance of urgently evaluate the interactive effects between co-occurring mycotoxins, especially due to potential synergistic effects. In fact, recent studies have identified synergistic effects between the main toxins detected in this study, namely aflatoxins, OTA and fumonisins. Aflatoxin B_1, ochratoxin A and fumonisin B_1 are contaminants which have been shown to regularly co-occur in a range of foods and tertiary combinations of these toxins, above the legislated limits, revealed synergistic effects with regard to mitochondrial integrity (Clarke et al., 2014). According to the same authors, the binary combination (OTA/FB_1) revealed synergistic interactions using high content analysis (Clarke et al., 2015a). All of these toxins are classified as group 1 or 2B by IARC which, corresponds to carcinogenic or possible carcinogenic compounds, thus a careful evaluation must be undertaken concerning these combinations. These toxins have been detected in multiple mycotoxins associations found in this study, revealing the great variety of possible co-exposure situations to which adults and children are exposed through contaminated breakfast cereals, so a multidisciplinary study must be urgently undertaken in order to evaluate the risk of children´s exposure to multiple mycotoxins in this food matrix. Recently, Assunção et al. (2015) performed for the first time a cumulative risk assessment on multiple mycotoxins present in breakfast cereals consumed by children suggesting that the combined margin of exposure (MoET) for the aflatoxins group could constitute a potential health concern with AFB_1 being the main contributor for MoET (Assunção et al., 2015). The present study highlights the importance to assess the presence of multiple mycotoxins in foods consumed by children as a main contribution to cumulative risk assessment studies and consequently, to the improvement of children's health.

4. CONCLUSIONS

The present study assessed for the first time the occurrence of mycotoxin mixtures in breakfast cereals marketed in Portugal, quantifying ten out of the twenty-one mycotoxins and their metabolites. Results showed high incidence of contaminated samples (96%) and also high incidence of co-occurrence of mycotoxins (92%) in breakfast cereals. These results agree well with those reported in the literature; however the observed data presented lower ranges of mycotoxin contamination than the other reported studies. This could probably be attributed to the fact that breakfast cereals analysed in this study were primarily marketed for children, which could be associated to a higher selection of raw materials by industry (Molinié et al., 2005).

This study contributes to increase the knowledge concerning the mycotoxin contents present in breakfast cereals and highlights several important issues related to the increasing evidence of co-occurring mycotoxins in cereals. It would be important that legislation also covers cereals other than maize relatively to the presence of fumonisins. The authors also suggest the need to revaluate the designation "infant food" since breakfast cereals primarily marketed for children are nowadays part of their diet, as pointed out in Assunção et al. (2015), but they are not considered as an infant food for legislative purposes. The present study generated reliable data regarding the simultaneous presence of mycotoxins in breakfast cereals primarily marketed for children, and these data are crucial to perform a more accurate children risk assessment through consumption of breakfast cereals, contributing to a more effective protection of children health.

MANUSCRIPT 2: **SINGLE-COMPOUND AND CUMULATIVE RISK ASSESSMENT OF MYCOTOXINS PRESENT IN BREAKFAST CEREALS CONSUMED BY CHILDREN FROM LISBON REGION, PORTUGAL**

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Highlights:

- An exposure assessment of mycotoxins present in breakfast cereals consumed by children from Lisbon region was undertaken.
- A risk characterization was developed to single and multiple mycotoxins.
- Potential health risks were associated with aflatoxins exposure.
- Aflatoxin B_1 was the main contributor for the health concern associated with the breakfast cereals consumption.
- Legal limits and control strategies regarding the presence of multiple mycotoxins in foodstuffs is an urgent need.

ABSTRACT

Humans can be exposed to multiple chemicals, but current risk assessment is usually carried out on one chemical at a time. Mycotoxins are commonly found in a variety of foods including those intended to consumption by children namely breakfast cereals. The present study aims to perform, the risk assessment of single and multiple mycotoxins present in breakfast cereals consumed by children (1-3 years old) from Lisbon region, Portugal. Daily exposure of children to ochratoxin A, fumonisins and trichothecenes showed no health risks to the children population considering individual mycotoxins, while exposure to aflatoxin B_1 (AFB_1) suggested a potential health concern for the high percentiles of intake (P90, P95 and P99). The combined exposure to fumonisins and trichothecenes are not expected to be of health concern. The combined margin of exposure (MoET) for the aflatoxins group could constitute a potential health concern and AFB_1 was the main contributor for MoET. Legal limits and control strategies regarding the presence of multiple mycotoxins in foodstuffs is an urgent need. To the best of our knowledge, this is the first time a cumulative risk assessment was performed on multiple mycotoxins present in breakfast cereals consumed by children.

Keywords: Cumulative risk assessment, Children, Multiple mycotoxins, Breakfast cereals, Probabilistic analysis

1. INTRODUCTION

Mycotoxins are toxic and carcinogenic metabolites produced by fungi that colonize food crops and they can occur in cereal based products as breakfast cereals. Cereals are among the first solid foods eaten by children and thus constitute an important food group of their diet (Schwartz et al., 2008). Several commercial brands provide breakfast cereals primarily marketed for this particular population group. Co-contamination of foodstuffs with known or unknown mycotoxins is being reported at an increasing high rate (Stoev, 2015) and there is a rising concern due to the hazard of exposure of combined mycotoxins to humans, which could be expected to exert greater toxicity and carcinogenicity than exposure to single mycotoxins (Bouaziz et al., 2008). Toxicological studies led the International Agency for Research on Cancer (IARC) to consider aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2) as human carcinogens (Group 1), aflatoxin M_1 (AFM_1), ochratoxin A (OTA) and fumonisins (FB_1 and FB_2) as possibly carcinogenic to humans (Group 2B) and the trichothecenes (T-2/HT-2, nivalenol or NIV and deoxynivalenol or DON) as not classifiable as to its human carcinogenicity (Group 3) (IARC, 2002). The European Union (EU) has set

maximum levels for certain mycotoxins as a risk management strategy and to achieve a high level of public health protection (European Commission, 2006a).

The risk characterization of food chemicals is based on the comparison between the dietary exposure and the relevant health-based guidance value. Dietary exposure assessment consists of combining deterministically or probabilistically food consumption figures with occurrence of a given chemical substance in a number of food categories. Within the general framework of chemical risk assessment, a difficult step in dietary exposure assessment is the handling of concentration data reported to be below the limit of detection (LOD) of the analytical method. These data are known as non-detects and the resulting distribution of occurrence values is left-censored. EFSA has so far mainly used substitution methods (EFSA, 2010).

Humans are naturally and frequently exposed to multiple mycotoxins, but health risk assessments are usually performed on individual mycotoxins, which may underestimate the total risks. A number of methods have been developed to predict the toxicity and risk of mixtures based on their chemical composition and knowledge of the toxicities of the mixture components. Most of these methods are based on the concepts of Concentration Addition (CA) and Independent Action (IA). CA assumes that the individual components act via a similar mode of action, only differing in their relative potency to elicit a toxic effect, whereas IA, assumes that the individual components act independently of each other (Backhaus et al., 2010; EFSA, 2013). Examples of cumulative risk assessment methods include the Hazard Index (HI) and the Combined Margin of Exposure Index (MoET) (Borg et al., 2013). The HI does not predict the overall health effect of the mixture, but provide a measure of the total risk based on the individual risk of each component. The MoE (margin of exposure) is proposed for the risk assessment of substances that have both genotoxic and carcinogenic properties and the MoET is usually used for the cumulative risk assessment (EFSA, 2013).

The few reports available in the Europe on the children dietary exposure to mycotoxins were mainly conducted to estimate the individual exposure to these toxins. In Spain, Catalonian infants (0–3 years) were identified as the most exposed population group to fumonisins through baby foods (Cano-Sancho et al., 2012b). In The Netherlands, a risk assessment of the dietary exposure of young children (2-6 years) to contaminants suggested that the health risk for FB_1, patulin (PAT) and DON was negligible (Boon et al., 2009).

Considering the scarce information on the risk assessment of children to multiple mycotoxins in breakfast cereals and the fact that they are the main food source of whole grains in children diet, the present study aims to perform, for the first time, the risk assessment of mycotoxins present in breakfast cereals consumed by children from Lisbon region, Portugal.

2. MATERIALS AND METHODS

2.1. Mycotoxins occurrence data

Twenty six breakfast cereals primarily marketed for children were purchased from supermarkets in Lisbon region, in 2014, including in their composition maize, wheat, rice and multigrain. Samples were homogenized in a food homogenizer, saved in plastic bags and stored in fridge at 4 °C until further analysis.

2.1.1. Aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1) and ochratoxin A (OTA)

Aflatoxins and ochratoxin A determination was performed according to the method described in EN15851, with few modifications (EN ISO, 2010). Mycotoxins were quantified by RP-HPLC with post column derivatization involving bromination followed by fluorescence detection. HPLC analysis was performed using a Waters® Alliance 2695 equipped with fluorescence detector Waters® 2475 (Milford, MA, USA) with Empower® Chromatography Software. Mycotoxin standard solutions were from Biopure® (Austria).

2.1.2. Trichothecenes (DON, NIV, T2-Toxin and HT-2 Toxin)

Trichothecenes extraction was performed according a procedure based on QuEChERS methodology previously developed by Pereira et al. (2015). GC-MS analysis was performed on an Agilent® (Little Falls, DE, USA) gas chromatograph 6890 equipped with an electronically controlled split/splitless injection port and an inert 5973N mass selective detector with electron impact (EI) ionization chamber. Standards of DON and NIV were purchased from Fluka® (West Chester, PA, USA), while T2-Toxin and HT-2 Toxin were purchased from Sigma® (St. Louis, MO, USA). The internal standards (IS) used were α-chloralose (IS1) and $^{13}C_{15}$-DON solution (IS2), purchased from Sigma® and Fluka®, respectively. Dispersive-SPE sorbents for experiments including C_{18}-bonded silica were purchased from Waters® (Milford, MA, USA) and primary secondary amine (PSA; particle size 50 mm) from Supelco® (Bellefonte, PA, USA).

2.1.3. Fumonisins (FB_1 and FB_2)

Fumonisins extraction was described by Ndube et al. (2011) with minor modifications. Extracts were analysed on a Waters® Acquity I-Class UPLC system (Milford, MA, USA) equipped with a BEH C_{18} column (2.1 × 50 mm, 1.7 µm) and coupled to a Waters® Xevo TQ-S mass spectrometer (Milford, MA, USA). The column was kept at 40 °C during analysis, and samples were maintained at 15 °C. The fumonisin B_1 and B_2 standards were purchased from Sigma® (St. Louis, MO, USA).

2.1.4. Performance of the analytical methods

Method performance was evaluated and limits of detection (LOD) and quantification (LOQ) (μg kg^{-1}), linearity range (μg kg^{-1}), coefficient of determination (R^2) and recoveries (%) were determined for all the studied mycotoxins.

2.2. Consumption data

The food consumption data were obtained from a pilot study performed between February and June 2014, in a Primary Health Care Unit in Lisbon region (Cidadela, Cascais, Portugal). This survey included a sample of 103 children, aged between 0 to 3 years old and selected from all children who were enrolled and attended the Primary Health Care Unit in the period of the survey. Information to the participant, with a standard explanation of why the survey was being carried out, was given to each parent before the interview. An informed consent was signed. The interview, in two parts, consisted of a brief personal history, followed by the explanation about how to fill the food diary. The brief personal history included among other gender, age, body weight (bw), height, birth date, food intolerances, physical activity and family data. The food diary was filled from three consecutive days for each child participant. This survey was conducted according to the guidelines laid down in the declaration of Helsinki and was approved by the Ethical Committee of the National Institute of Health Doutor Ricardo Jorge and by the Portuguese Data Protection Authority. Considering that children may begin to eat breakfast cereals after one year old, a subsample of 75 children aged between 1 to 3 years old was considered in the present study.

2.3. Exposure assessment

Two mathematical approaches, point evaluation (deterministic approach) and Monte Carlo simulation (probabilistic approach) were used for the computation of the exposure assessment of mycotoxins.

Four different scenarios were included for the mycotoxin dietary exposure assessment in relation to the data treatment of the non-detects (<LOD). Non-detects were considered as zero (H1), 1/2 LOD (H2), LOD (H3) (EFSA, 2010) and, for the probabilistic approach, a fourth scenario replacing the censored data by random samples from a uniform distribution with zero as minimum and LOD as maximum (H4) was also considered.

2.3.1. Deterministic exposure assessment

Calculations for the deterministic approach were executed using Microsoft Excel 2007. The mean estimated intakes of mycotoxins were determined by multiplying mean values of mycotoxin concentrations by mean consumption data and dividing by children's weight.

2.3.2. Probabilistic exposure assessment

Calculations were executed using the software @Risk® for Microsoft Excel version 6 (Palisade Corporation, USA). The mycotoxin exposure assessment was modelled by multiplying concentration by consumption data. Best fit function of @Risk software was applied in order to select best fitted probabilistic distribution of all mycotoxin concentrations and the respective consumption data, based on lowest Akaike's Information Criterion (AIC). Monte Carlo simulations were performed considering 100,000 iterations. By means of Monte Carlo simulation, the inherent variability associated with the mycotoxin contents of breakfast cereals and the food consumption patterns were considered. A bootstrap technique was applied in an attempt to characterize the uncertainty associated with the mycotoxin concentration data, considered as the parameter that would impute more uncertainty to the results.

2.3.3. Risk characterization

For the risk characterization, the outputs of exposure, namely the daily intake values, were compared with the reference dose values.

For aflatoxins, as carcinogenic compounds, the MoE was calculated as a ratio of $BMDL_{10}$ (benchmark dose lower confidence limit) and aflatoxin exposure. $BMDL_{10}$ value was derived from the study of Benford et al. (2010b). The magnitude of the MoE gives an indication of the risk level and the Scientific Committee of EFSA and WHO have concluded that a MoE of 10000 or more was of low concern for public health (EFSA, 2013).

For the remaining mycotoxins, the output of exposure was compared to the dose reference values (PMTDI or PTWI) in order to calculate the hazard quotients (HQ, ratio between exposure and a reference dose). If HQ <1 indicates a tolerable exposure level and a ratio of HQ >1 indicates a non-tolerable exposure level (Borg et al., 2013; EFSA, 2013).

For the cumulative risk assessment of multiple mycotoxins, the CA concept was used. Mycotoxins were grouped following the cumulative assessment group (a group of chemicals that could plausibly act by a common mode of action), as posted by EFSA (2013), namely by families (aflatoxins, ochratoxin A, fumonisins and trichothecenes).

The MoET were determined for aflatoxins and the HI for the remaining mycotoxins. The MoET was calculated as the reciprocal of the sum of the reciprocals of the individual margins of exposure (MoEs) and the HI, as the sum of the respective Hazard Quotients (HQs) for the individual mixture components of the same family. If HI > 1, the total concentration of mixture components exceeds the level considered to be acceptable (EFSA, 2013).

3. RESULTS AND DISCUSSION

3.1. Occurrence, consumption and exposure assessment

Performance results for all assayed mycotoxins showed a good linear response with coefficient of determination (R^2) > 0.995 for all the analysed mycotoxins. Linearity ranges used for quantification purposes were 0.100-1.000, 0.040-0.400, 0.030-0.300, 0.045-0.450, 0.030-0.300, 0.200-2.000, 15-360, 25-360, 25-360, 25-450, 2.5-8.0 μg kg^{-1} for AFM_1, AFB_1, AFB_2, AFG_1, AFG_2, OTA, DON, NIV, T2, HT-2, FB_1 and FB_2, respectively. Recovery values ranged from 57 to 101 % for all the mycotoxins, except nivalenol and T2 that presented a recovery of 46 % and 44%, respectively, suggesting a need for further improvement for these mycotoxins. For aflatoxins and ochratoxin A, the limits of detection ranged from 0.001 to 0.011 μg kg^{-1} and limits of quantification ranged from 0.004 to 0.032 μg kg^{-1}, which assured quantification at low ppb level for these mycotoxins usually present at low concentrations. For deoxynivalenol, nivalenol, T2 and HT-2 the limits of detection were 0.4, 5.6, 6.8 and 6.4 μg kg^{-1}, and the limits of quantification were 1.2, 18.4, 22.3 and 21.1 μg kg^{-1}, respectively. For fumonisin B_1 and fumonisin B_2, the limits of detection and quantification were 0.8 and 2.5 μg kg^{-1}, respectively, for both mycotoxins. All these results, with the exception of nivalenol and T2 recoveries, were in agreement with the criteria mentioned in the Commission Regulation (EC) No. 401/2006 (European Commission, 2006b) and showed that the analytical methods applied are adequate for mycotoxins determinations.

Mycotoxin mean contents in breakfast cereals ranged between 2 ng kg^{-1} for AFB_2 and 59 μg kg^{-1} for DON, considering the H1 scenario (<LOD = LOD). For the remaining scenarios, mean mycotoxin contents followed the same pattern, as shown in Table 18. AFG_2, T2 and HT-2 toxins were not detected in analysed samples. All samples presented levels below the legal maximum limits established, when available (EC, 2006a). The analysed breakfast cereals included in their composition maize, wheat, rice and multigrain however no cereal related mycotoxin contamination pattern was observed.

Forty percent of the studied children (1-3 years old) consumed breakfast cereals at least one time in three days as reported in food diary presenting a mean weight of 13.39 kg and a mean consumption of

breakfast cereals of 5.62 g day^{-1}. Breakfast cereals consumed by the studied children showed different types (with a maximum of four) and various proportions (38-83%) of grains in their composition.

The exposure assessment of mycotoxins through breakfast cereals consumption were first assessed using a deterministic approach as shown in Table 18. The results for aflatoxins revealed that AFB_1 presented the higher mean intake comparing to the remaining aflatoxins, with a mean value of 0.012 ng kg bw^{-1} day^{-1} for the H1 scenario (<LOD = LOD). OTA mean exposure for the same scenario was quantified as 0.011 ng kg bw^{-1} day^{-1}. For FB_1 and FB_2, DON and NIV the mean intake values for H1 scenario were, respectively, 5.461, 1.033, 24.827 and 2.681 ng kg bw^{-1} day^{-1}. The remaining scenarios followed the same pattern. These results showed that mean intake values for the studied population were lower than the health-based guidance values established for all mycotoxins, namely OTA (112 ng kg bw^{-1} $week^{-1}$), FB_1 and FB_2 (2 µg kg bw^{-1} day^{-1}), DON (1 µg kg bw^{-1} day^{-1}) and NIV (1.2 µg kg bw^{-1} day^{-1}) (EFSA, 2013; JECFA, 2007, 2011a, 2011b). Relatively to aflatoxins, as genotoxic carcinogens compounds, no exposure threshold is presumed and it is recommended that levels of such substances should be as low as technologically feasible or, as Joint FAO/WHO Expert Committee on Food Additives (JECFA) recommends, as low as reasonably achievable (ALARA) (EFSA, 2007). Previous studies reported that estimated mean dietary exposures to aflatoxins for the general population from all food sources were 0.93 ng kg bw^{-1} day^{-1} to 2.4 ng kg bw^{-1} day^{-1} in Europe (JECFA, 2007).

It is important to highlight that DON presented the highest daily intake of the studied mycotoxins. DON is one of the most prevalent mycotoxins encountered in grain fields and consequently, although it is not the most toxic one, it is considered to be one of the most important mycotoxins. The estimated intake of DON through breakfast cereals consumption, in the present case, represents around 2.5% of the tolerable daily intake established for DON, suggesting that the estimated individual DON intake values through breakfast cereals consumption do not imply a risk for the studied population (JECFA, 2011a).

The deterministic approach used to perform mycotoxin exposure assessment usually do not consider the variability and uncertainty of the food consumption and contamination level parameters, resulting, in some cases, in an unreal exposure (Han et al., 2014). The probabilistic approach, contrary to the deterministic one, takes into account every possible value that each variable can assume and the weight of each possible scenario for the probability of its occurrence, allowing a more accurate characterization of mycotoxin intake distribution. A full probabilistic model (Monte Carlo simulation) is therefore recommended in order to provide a more realistic exposure estimate (De Boevre et al., 2013; Han et al., 2014).

Table 18. Deterministic approach to estimate children's intake of mycotoxins present in breakfast cereals (ng kg bw^{-1} day^{-1}) considering three different scenarios for non-detects (< LOD). Mean values for mycotoxin content, consumption (5.62 g) and children weight (13.39 kg) were used for daily intake calculations.

Mycotoxin	H1: <LOD = LOD		H2: <LOD = 1/2 LOD		H3: <LOD = 0	
	Mycotoxin content (ng kg^{-1})	Daily intake (ng kg bw^{-1} day^{-1})	Mycotoxin content (ng kg^{-1})	Daily intake (ng kg bw^{-1} day^{-1})	Mycotoxin content (ng kg^{-1})	Daily intake (ng kg bw^{-1} day^{-1})
AFM_1	12	0.005	7	0.003	2	0.001
AFB_1	28	0.012	27	0.011	27	0.011
AFB_2	2	0.001	2	0.001	1	0.001
AFG_1	6	0.003	4	0.001	1	0.0003
OTA	26	0.011	25	0.010	23	0.010
	Mycotoxin content (µg kg^{-1})	Daily intake (ng kg bw^{-1} day^{-1})	Mycotoxin content (µg kg^{-1})	Daily intake (ng kg bw^{-1} day^{-1})	Mycotoxin content (µg kg^{-1})	Daily intake (ng kg bw^{-1} day^{-1})
FB_1	13	5.461	13	5.390	13	5.319
FB_2	3	1.033	2	0.929	2	0.826
DON	59	24.827	59	24.797	59	24.767
NIV	6	2.681	4	1.559	1	0.438

The graphic representations for the probabilistic estimates of the intakes for individual toxins presented at Figure 20 reinforce the results of the deterministic approach and confirm the idea that consumption of breakfast cereals is not associated with a health risk for the studied population since the computed single mycotoxins intakes did not exceed the established health-based individual guidance values, as previously referred. The best fit distributions obtained for mycotoxin occurrence and consumption data parameters, using @Risk software are presented in supplementary data (Table S1, Annex 1).

The present results are similar to those published by other authors. Sirot et al. (2013) reported intakes of 0.085 ng kg bw^{-1} day^{-1} for OTA and 0.020 ng kg bw^{-1} day^{-1} for AFB_1 in the second French total diet study, compared to 0.011 ng kg bw^{-1} day^{-1} and 0.012 ng kg bw^{-1} day^{-1}, for OTA and AFB_1, respectively, in the present study. Coronel et al. (2012) calculated a relatively higher mean exposure to OTA (0.37 ng kg bw^{-1} day^{-1}) through consumption of breakfast cereals by Spanish children (4 to 9 years old) than the present exposure assessment (child 1-3 years old) (Coronel et al., 2012). This intake difference could be attributed to distinct age groups and food consumption methodologies used. De Boevre et al. (2013) referred a mean DON intake of 20 ng kg bw^{-1} day^{-1} in exposure assessment of Belgian population (15 years or older) through cereal-based foods, including breakfast cereals, which was close to the present results (25 ng kg bw^{-1} day^{-1}).

3.2. Risk characterization

Table 19 presents the results concerning the risk characterization for OTA, fumonisins, and trichothecenes using HQ (individual mycotoxins) and HI (combined mycotoxins) derived from estimate of these mycotoxins exposure performed by the probabilistic approach. These results showed that all HQs were < 1, *i.e.* indicating no cause for concern for individuals exposed to mycotoxins through consumption of breakfast cereals. DON was the mycotoxin that presented the highest HQ however well below 1.

Table 20 presents the MoE calculated for aflatoxins. Aflatoxins M_1, B_2, G_1 revealed a MoE above 10000, which represent low risk for breakfast cereals children consumers. AFB_1, which is considered the most potent aflatoxin, revealed a MoE below 10000 for the higher percentiles of intake (P90, P95 and P99) suggesting a potential health concern.

As previously referred, humans are simultaneously exposed to multiple chemicals, although human health risk assessment of chemicals is normally performed on single substances, which may contribute to risk underestimation. The current results of the occurrence of mycotoxins in breakfast cereals confirm the idea that the studied population was exposed simultaneously to multiple mycotoxins. Considering the number of mycotoxins present in breakfast cereals and all possible mixtures, data of experimental tests that clarify all possible mycotoxins combinations are not available. Therefore, in the current study the different mycotoxins present in breakfast cereals were grouped considering their chemical class, *i.e.*, their similarity in chemical structure and biology activity (EFSA, 2013). Hence, the cumulative risk assessment was performed for aflatoxins (including AFB_1, AFB_2, AFG_1 and AFM_1), fumonisins (including FB_1 and FB_2) and trichothecenes (including DON and NIV). The method based on CA concept was applied for cumulative health risk assessment of congeners mycotoxins. The HI for fumonisins (FB_1, FB_2) and for trichothecenes (DON, NIV) in the P99 of the H4 scenario was 0.067 and 0.25, respectively (Table 19). Consequently, combined exposure to fumonisins or to trichothecenes through consumption of breakfast cereals are not expected to be of health concern. Trichothecenes presented the highest HI and DON was the main contributor with 97.7% (results not shown).

For the aflatoxin group, results on the combined margin of exposure (MoET) are summarized in Table 20 (EFSA, 2013). Aflatoxins MoET for the P90, P95 and P99 were all below 10000, indicating a potential health concern associated with the consumption of breakfast cereals, contrary to the remaining studied mycotoxins. AFB_1 was the main contributor for MoET, with 87.3% (results not shown). In contrast with

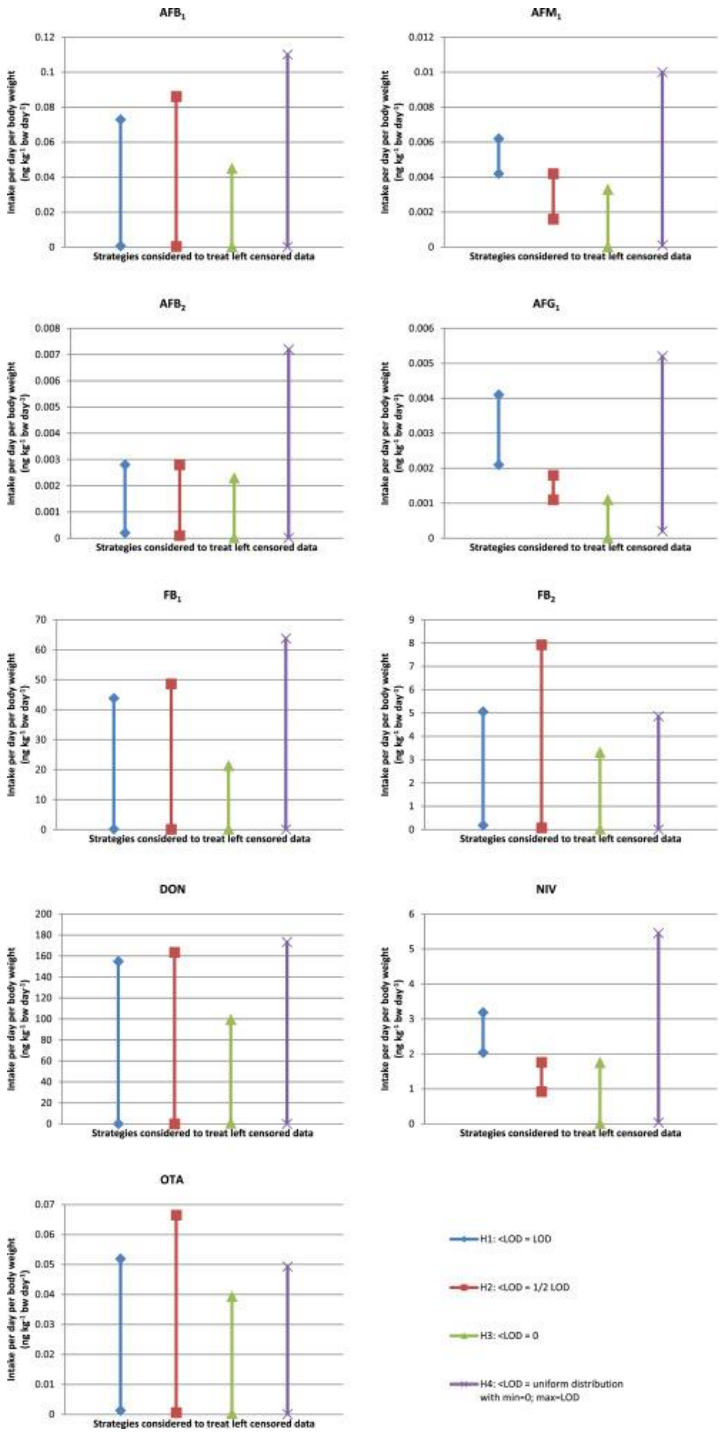

Figure 20. Results of probabilistic approach to estimate children exposure to aflatoxins (AFB_1, AFB_2, AFG_1 and AFM_1), fumonisins (FB_1 and FB_2), trichothecenes (DON and NIV) and ochratoxin A, through ingestion of breakfast cereals (ng kg bw^{-1} day^{-1}), corresponding to 2.5 and 97.5 percentiles of intake.

this study, previous published European studies rarely pointed out aflatoxins, namely AFB_1, as a potential health risk. This fact could be attributed to i) the small number of dietary exposure studies focused on this particular age range ii) children high consumption relatively to their body when compared to adults, and iii) the use of MoE to characterise the risk associated with aflatoxins. Although the potential uncertainties associated with the MoE calculation, it is important to underline that this methodology is considered the most scientifically credible approach for the formulation of advice because it takes into account both the dietary exposure and the available data on the dose-response relationship which should be used to support prioritisation for risk management action (Benford et al., 2010b).

To the best of our knowledge, this is the first time a cumulative risk assessment was performed on multiple mycotoxins present in breakfast cereals consumed by children. There are few reports on the risk characterization of multiple mycotoxins in foodstuffs. Han et al. (2014) proposed a model strategy for cumulative health risk assessment on the co-occurring hazards in the fields of food safety, namely multiple mycotoxins present in cereal grains (deoxynivalenol and its acetyl derivatives in wheat and maize). These authors applied the CA concept to perform the quantitative risk assessment by Monte Carlo simulation and compared the daily intake values with health based guidance values to evaluate the margin of safety (MOS). In the present study, the MoE and MoET approaches were used according to the international organisations recommendations for genotoxic and carcinogenic compounds as aflatoxins (EFSA, 2013).

3.3. Limitations associated with the exposure assessment study

There are always uncertainties associated with exposure assessments which need to be considered for the interpretation of results (De Boevre et al., 2013). In the present study the major uncertainty should be associated with the number of breakfast cereal samples (n=26) analysed and the pilot study children population (n=103) size. These facts may have influenced the estimate power of the obtained intake values. The number of breakfast cereal samples analysed corresponds to almost all different brands primarily marketed for children available in several supermarket chains with a good market share in Lisbon region. The children population was selected from all children who were enrolled and attended the Primary Health Care Unit in the period of the survey. The methodology chosen to collect consumption data, 3-day food diary, could contribute to reduce the uncertainty considering that consumptions were surely likely to vary with time. However, the present study was a snapshot of the studied population exposure to mycotoxins and changes over time could occur affecting the intake of mycotoxins. For the cumulative risk assessment, the present study grouped mycotoxins considering their chemical class and

not necessarily their mode of action. The reason for assumed this presupposition was the absence of reference points for the mycotoxins mixtures occurred in breakfast cereals. On the one hand, this assumption could contribute to an overestimation of the risk, although, on the other hand, this assumption could be considered as a more conservative prediction and contribute to a higher health assurance.

Table 19. Risk characterization using HQ and HI derived from the estimates of ochratoxin A, fumonisins (FB_1, FB_2) and trichothecenes (DON, NIV) exposure performed by the probabilistic approach.

	H1: <LOD = LOD					H2: <LOD = 1/2 LOD					H3: <LOD = 0					H4: <LOD = uniform distribution with min=0; max=LOD				
	OTA[c]	**FB_1**	**FB_2**	**DON**	**NIV**	**OTA[c]**	**FB_1**	**FB_2**	**DON**	**NIV**	**OTA[c]**	**FB_1**	**FB_2**	**DON**	**NIV**	**OTA[c]**	**FB_1**	**FB_2**	**DON**	**NIV**
			HQ[a]					**HQ[a]**					**HQ[a]**					**HQ[a]**		
P5	0.00010	0.00011	0.00011	0.000017	0.0018	0.000050	0.000055	0.000049	0.0000067	0.00082	0.000034	0.00015	0.000023	0.0014	0.000020	0.000010	0.000032	0.0000024	0.0000020	0.000063
P50	0.00044	0.00072	0.00032	0.0090	0.0021	0.00028	0.00041	0.00019	0.0076	0.0011	0.00046	0.0020	0.00031	0.019	0.00027	0.00040	0.00075	0.00023	0.0062	0.00085
P90	0.0017	0.0068	0.0011	0.077	0.0024	0.0017	0.0056	0.0011	0.078	0.0013	0.0015	0.0067	0.0010	0.062	0.00091	0.0018	0.0087	0.0013	0.080	0.0028
P95	0.0024	0.013	0.0017	0.11	0.0025	0.0028	0.013	0.0021	0.12	0.0014	0.0020	0.0087	0.0013	0.081	0.0012	0.0024	0.017	0.0019	0.12	0.0037
P99	0.0045	0.037	0.0043	0.21	0.0028	0.0063	0.048	0.0091	0.22	0.0016	0.0031	0.013	0.0021	0.12	0.0018	0.0040	0.064	0.0032	0.24	0.0057
		HI[b] (FB_1. FB_2)		**HI[b] (DON. NIV)**			**HI[b] (FB_1. FB_2)**		**HI[b] (DON. NIV)**			**HI[b] (FB_1. FB_2)**		**HI[b] (DON. NIV)**			**HI[b] (FB_1. FB_2)**		**HI[b] (DON. NIV)**
P5		0.00022		0.0018			0.00010		0.00083			0.00017		0.0014			0.000035		0.000065	
P50		0.0010		0.011			0.00060		0.0087			0.0023		0.019			0.0010		0.0070	
P90		0.0079		0.079			0.0067		0.080			0.0077		0.063			0.010		0.083	
P95		0.015		0.12			0.015		0.12			0.010		0.082			0.019		0.13	
P99		0.042		0.21			0.057		0.23			0.015		0.13			0.067		0.25	

H1, H2, H3 and H4 scenarios were considered for calculations regarding the data treatment of non-detects.

LOD = Limit of Detection: OTA, 0.006 µg kg^{-1}; FB_1, 0.8 µg kg^{-1}; FB_2, 0.8 µg kg^{-1}; DON, 0.4 µg kg^{-1}; NIV, 5.6 µg kg^{-1}.

[a]HQ (Hazard Quotient) = Intake values / reference values; [b]HI (Hazard Index) = sum of HQ for substances of the same family; [c]for OTA HQ calculation, the correspondence from weekly to daily was undertaken.

Table 20. Risk characterization using MoE and MoET derived from estimate of aflatoxin exposure performed by probabilistic approach.

	H1: <LOD = LOD				H2: <LOD = 1/2 LOD				H3: <LOD = 0				H4: <LOD = uniform distribution with min=0; max=LOD			
	AFM_1	**AFB_1**	**AFB_2**	**AFG_1**	**AFM_1**	**AFB_1**	**AFB_2**	**AFG_1**	**AFM_1**	**AFB_1**	**AFB_2**	**AFG_1**	**AFM_1**	**AFB_1**	**AFB_2**	**AFG_1**
			MoE[a]				**MoE[a]**				**MoE[a]**				**MoE[a]**	
P5	57498	268477	979506	115798	141278	457411	2169216	221642	5449111	399850	7946450	16356472	1790859	685317	15483806	1016470
P50	49261	48633	405623	88294	96303	71375	722894	182111	403195	29586	587952	1210245	132518	53808	1023193	177002
P90	43893	8211	161490	70173	72351	8522	208757	157281	121379	8906	176990	364327	39893	8058	134750	70920
P95	42204	5057	118229	65537	65647	4680	134251	149634	93296	6845	136041	280039	30663	4225	67631	57335
P99	38701	2277	61139	57418	52957	1769	51278	134037	60690	4453	88508	182166	19949	1015	14758	40120
		MoET[b]				**MoET[b]**				**MoET[b]**				**MoET[b]**		
P5		32496				70238				348258				326163		
P50		18297				31981				25768				30527		
P90		6060				7027				7757				5859		
P95		4079				4114				5962				3316		
P99		2005				1636				3879				886		

H1, H2, H3 and H4 scenarios were considered for calculations regarding the data treatment of non-detects. LOD = Limit of Detection: AFM_1, 0.011 µg kg^{-1}; AFB_1, 0.003 µg kg^{-1}; AFB_2, 0.001 µg kg^{-1}; AFG_1, 0.006 µg kg^{-1}.

[a]MoE (Margin of Exposure) = $BMDL_{10}$ / Exposure data; $BMDL_{10}$ (Benchmark Dose Lower Confidence Limit) = 0.00025 mg/Kg bw/day; [b]MoET (Combined Margin of Exposure) = $1/((1/MoE_{AFM1})+(1/MoE_{AFB1})+(1/MoE_{AFB2})+(1/MoE_{AFG1}))$

Relatively to aflatoxins, the $BMDL_{10}$ used to calculate the MoEs was determined for AFB_1 (Benford et al., 2010b) and, in the current study, it was also applied to the remaining aflatoxins, assuming a worst case perspective. This factor introduced bias in the MoEs and MoET estimates. However, as referred previously, AFB_1 was the aflatoxin that presented lower MoE and the main contributor for the MoET.

The multiple exposure assessments in European countries showed that small children were generally exposed to higher levels of mycotoxins than adults (Cano-Sancho et al., 2012b; Sirot et al., 2013). The average intakes for regions such as the Mediterranean area estimates that the exposure is generally below the levels deemed as tolerable. However, certain regions and mainly in the developing countries will encounter problems (e.g. climate, high humidity) for certain mycotoxins, particularly in subpopulations where the contamination of cereal-based food is extraordinary (Kearney, 2010). The discrepancies between studies could also be explained by the use of different methodologies, quantification strategies and food consumption surveys.

4. CONCLUSIONS

To the best of our knowledge, this is the first attempt to perform a cumulative risk assessment on multiple mycotoxins, including both genotoxic and carcinogenic compounds, present in breakfast cereals consumed by children (1-3 years old), a particularly vulnerable population group (Alvito et al., 2010).

Risk assessment is the process of evaluating the probable incidence of an adverse health effect to humans under various conditions of exposure with a description of the uncertainties involved. Deterministic and probabilistic approaches for OTA, fumonisins and trichothecenes indicate no health concern for individuals exposed to mycotoxins through consumption of breakfast cereals (hazard quotients below 1). Contrary to other aflatoxins, aflatoxin B_1 (AFB_1) revealed a margin of exposure (MoE) below 10000 suggesting potential health concern for the high percentiles of intake (P90, P95 and P99). The cumulative risk assessment for fumonisins and trichothecenes revealed, in both cases, a hazard index below 1 for P99 thus their combined exposure are not expected to be of health concern. Results on the combined margin of exposure (MoET) for the aflatoxins group were below 10000 for P90, P95 and P99 and this fact could constitute a potential health concern. AFB_1 was the main contributor. This fact assumes major importance since the present study only

concerns the risk associated with the children exposure to mycotoxins through breakfast cereals consumption and it is expected that the exposure resulting from the consumption of all foods present in a child diet could increase the intake of mycotoxins and, consequently, the inherent risk to children's health. These results corroborates the need for further studies in the domain of the mycotoxin cumulative risk assessment in view of establishing legal protective values to achieve an improvement of children's health.

MANUSCRIPT 3: **CHILDREN EXPOSURE ASSESSMENT TO CO-OCCURRING MYCOTOXINS PRESENT IN CEREAL-BASED PRODUCTS IN PORTUGAL**

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Highlights:

- Risk of children exposed to mycotoxins through cereal products was characterized
- 94% of cereal-based products were contaminated with at least one mycotoxin
- 75% of analysed samples were contaminated with two or more mycotoxins
- Aflatoxins exposure suggested potential adverse health effect for P50 or higher
- Mycotoxins present in food could constitute a risk for children's health

ABSTRACT

Mycotoxins are secondary fungi metabolites that induce acute and chronic toxic effects in humans and animals. Simultaneous contamination of cereal-based products by multiple mycotoxins has been increasingly reported and further studies need to be developed in order to evaluate the effects of human exposure to multiple mycotoxins through food, mainly for vulnerable population groups as children. To contribute to overcome this gap, the present study assessed the risk associated to the exposure to 13 mycotoxins in different cereal-based products (including breakfast cereals, processed cereal-based foods and biscuits) consumed by children (1 to 3 years old) from Lisbon region, Portugal. Results on mycotoxins occurrence showed that 94% of samples were contaminated with at least one mycotoxin, always bellow the legislated limits, when available. The co-contamination of samples was observed in 75% of the analysed samples. Aflatoxins exposure estimate suggested a potential adverse health effect for percentiles of intake above or equal to P50 (MoET < 10000). For the remaining mycotoxins (fumonisins, trichothecenes and zearalenone), no reasons for concern were verified (HI < 1). Bearing in mind the obtained results, the particular vulnerability of the studied population and the potential carcinogenic effects of some mycotoxins as aflatoxins, control strategies (e.g. restrictive limits, development of toxicological data, continuous monitoring of multiple mycotoxins children exposure) should be advocated and implemented by governments and industries, in order to warrant the children health.

Keywords: Cumulative risk assessment, Children, Multiple mycotoxins, Cereal-based foods

1. INTRODUCTION

Mycotoxins are secondary metabolites produced by certain fungi species and these toxins have become increasingly important worldwide, considering their impact on human and animal health (Borchers et al., 2010; Marin et al., 2013; Wu et al., 2014). These contaminants may be acutely toxic but currently, the main human and veterinary health burdens of mycotoxin exposure are related to chronic sequelae, such as carcinogenic, teratogenic, immunotoxic, nephrotoxic, and estrogenic effects. Human exposure to mycotoxins occurs directly through the intake of contaminated agricultural products (e.g. cereal-based products, fruits) or indirectly through the consumption of products of animal origin (e.g. meat, milk, eggs) prepared or obtained from animals

that were fed with contaminated material (Capriotti et al., 2012; Flores-Flores et al., 2015). The International Agency for Research on Cancer (IARC) have been evaluated several food-contaminating mycotoxins relatively to their impact on human carcinogenicity (IARC, 2016). IARC classified aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2) as carcinogenic to humans (Group 1), whereas ochratoxin A (OTA), aflatoxin M_1 (AFM_1), and fumonisins (FB_1 and FB_2) were classified as possible carcinogens (Group 2B). Trichothecenes [T-2/HT-2, nivalenol (NIV) and deoxynivalenol (DON)], zearalenone (ZEA) and patulin (PAT) were not classified as human carcinogens (Group 3). To ensure a high level of public health protection and as a risk management strategy, the European Union (EU) has established maximum levels for the major mycotoxins (*i.e.* aflatoxins, OTA, ZEA, PAT, DON, Fumonisins), considering their occurrence and toxicity (European Commission, 2006a).

The Food and Agriculture Organization of the United Nations (FAO) estimated that approximately 25% of the cereals produced in the world are contaminated by mycotoxins, but perhaps this value is closer to 50%, if one takes into account emerging mycotoxins of which so far have limited data (Rice and Ross, 1994). Cereals, namely cereal-based products as breakfast cereals, processed cereal-based foods (flours) and biscuits, are among the first solid foods eaten by children and thus constitute an important food group of their diet (Amezdroz et al., 2015; Rodrigues et al., 2007). Children are a vulnerable part of the population due to their physiology, a fairly restricted diet and a higher consumption relative to their body. Therefore, the significance and potential health risk of any contaminant in foods consumed by children is increased and meticulous attention must be paid to this particular issue (Alvito et al., 2010).

Food products are often contaminated simultaneously by numerous known or unknown mycotoxins, but most studies have focused on the risk assessment of single mycotoxins (Assunção et al., 2015; De Ruyck et al., 2015; Smith et al., 2016; Stoev, 2015). Hence, there is a rising concern relatively to human exposure to combined mycotoxins, which could exert different output toxicity and carcinogenicity than exposure to single mycotoxins (Bouaziz et al., 2008).

The dietary exposure assessment, one of the most important steps of the human risk assessment, consists in combining deterministically or probabilistically food consumption figures with occurrence data of a given chemical substance in a number of food categories. Several methods have been developed to predict the toxicity and risk of mixtures based on their chemical composition and knowledge of the toxicities of the mixture components [e.g. Hazard Index (HI) and the Combined Margin of Exposure Index (MoET)]. Most of these methods are based on the concepts

of Concentration Addition (CA) and Independent Action (IA) (Borg et al., 2013). The HI does not predict the overall health effect of a mixture, but provides a measure of the total risk based on the individual risk of each component. The MoE (margin of exposure) is proposed for the risk assessment of substances that have both genotoxic and carcinogenic properties and the MoET is usually used for the cumulative risk assessment (EFSA, 2013).

There are scarce reports in literature concerning the risk associated with human exposure to multiple mycotoxins in foods and especially for those intended for children consumption (Raiola et al., 2015; Sherif et al., 2009). The risk associated with Portuguese children exposure to multiple mycotoxins through the consumption of breakfast cereals (Assunção et al., 2015) as well as with the co-occurring presence of PAT and OTA in processed cereal-based foods (Assunção et al., 2016b) has been recently reported. Previous studies evaluated the occurrence of mycotoxins in different foodstuffs in Portugal, including cereal-based products (as reviewed by Abrunhosa et al., 2016), and also foods for infants and children (Alvito et al., 2010; Assunção et al., 2016b; Barreira et al., 2010; Cunha et al., 2009). However, to the authors' knowledge, no risk assessment study has been yet performed to estimate the risk associated with the ingestion of multiple mycotoxins present in different cereal-based products. Hence, the potential risk associated with the consumption of products that could be contaminated by co-occurring mycotoxins is far from being well characterized and should be urgently addressed. The present study aims to contribute to wane this gap, assessing for the first time, the risk associated to the exposure to multiple mycotoxins in different cereal-based products (including breakfast cereals, processed cereal-based foods and biscuits) consumed by children from Lisbon region, Portugal.

2. MATERIALS AND METHODS

In order to achieve the objective established in the present study, a stepwise approach was considered: i) the exposure assessment of 13 mycotoxins (aflatoxins, ochratoxin A, fumonisins, trichothecenes and zearalenone) to children, gathering occurrence and consumption data; followed by ii) the characterization of the risk resulting from the mycotoxins exposure associated to the consumption of cereal-based products by children aged between 1 to 3 years old.

2.1. Occurrence data

Samples

A total of 52 different cereal-based products primarily marketed for children [26 breakfast cereals (BC), 20 processed cereal-based foods (flours) (PC) and six biscuits (BIS)] were purchased from supermarkets in Lisbon region, in 2014 and 2015. The size of each sample was at least of 1 kg, accordingly to Commission Regulation (EC) 401/2006 of 23 February 2006 (European Commission, 2006b). The samples were homogenized in a food homogenizer, saved in plastic bags and stored at 4 °C until further analysis.

Analytical determination and method performance

The extraction and analytical methods applied for mycotoxins determination were performed according to the described in Assunção et al. (2015). Aflatoxins and ochratoxin A were determined by HPLC-FLD, with a previous purification by immunoaffinity columns (IAC). Trichothecenes were determined by GC-MS following an extraction based on QuEChERS procedure developed by Pereira et al. (2014). Fumonisins and zearalenone were determined by LC-MS/MS, with a previous extraction with methanol/water (75:25, v/v) as referred by Ndube et al. (2011). ZEA determination also used a labelled standard ($^{13}C_{18}$-ZEA) for spiking experiments. Method performance was evaluated and limits of detection (LOD) and quantification (LOQ) (μg/kg), linearity range (μg/kg), coefficient of determination (r^2) and recoveries (%) were determined for all the studied mycotoxins. Mycotoxin contents were expressed as mean, median and maximum, using Microsoft® Excel 2013.

2.2. Consumption data

The children food consumption data were obtained according to the procedure described in Assunção et al. (2015). Briefly, a pilot study was developed in a Primary Health Care Unit in Lisbon region (Cidadela, Cascais), between February and June 2014, where a food diary was applied to 103 children, aged between 0 and 3 years old. Participants enrolled in the study were attended in the Health Care Unit during the survey period, parents signed an informed consent and filled a brief health and socio-demographic questionnaire and a three days food diary. Considering that children may begin to eat all the cereal-based products mainly after one year old, a subsample of 75 children aged between 1 and 3 years old was considered in the present study.

2.3. Exposure assessment

2.3.1. Deterministic and probabilistic approaches

The exposure assessment was performed according to the procedure described by Assunção et al. (2015), considering two mathematical approaches (deterministic, with a point evaluation, and probabilistic, with a Monte Carlo simulation). For data treatment in relation to non-detects (<LOD), three scenarios were considered as recommended by EFSA [LOD=LOD (H1), LOD= ½ LOD (H2), LOD=0 (H3)] (EFSA, 2010). In the probabilistic approach, it was considered a fourth scenario replacing the censored data by random values from a uniform distribution with zero as minimum and LOD as maximum (H4). For each mycotoxin, an estimation of the exposure was evaluated, computing the intake of each mycotoxin from different food products. The exposure assessment was conducted only for mycotoxins which had been detected in analysed cereal-based products. Calculations for the deterministic and the probabilistic approach were executed using Microsoft® Excel 2013 and the software @Risk® for Microsoft Excel version 6 (Palisade Corporation, USA), respectively.

2.3.2. Risk characterization

For the risk characterization, performed according to the procedure described by Assunção et al. (2015), the outputs of exposure, namely the daily intake values, were compared with the reference dose values. Two different approaches were performed considering mycotoxins carcinogenicity. The MoE was applied for aflatoxins and was calculated as a ratio of $BMDL_{10}$ (benchmark dose lower confidence limit) and aflatoxin exposure. $BMDL_{10}$ value was derived from the study of Benford et al. (2010b). For the remaining mycotoxins, the output of exposure was compared to the dose reference value [provisional maximum tolerable daily intake (PMTDI) or provisional tolerable weekly intake (PTWI)] in order to calculate the hazard quotient (HQ, ratio between exposure and a reference dose). The generated outputs were evaluated according to different criteria. The magnitude of the MoE gives an indication of the risk level and the Scientific Committee of EFSA and WHO have concluded that a MoE of 10000 or more was of low concern for public health (EFSA, 2013). For the HQ, a tolerable or a non-tolerable exposure level was considered if HQ was below or above one, respectively (Borg et al., 2013; EFSA, 2013). For the cumulative risk assessment of multiple mycotoxins, the CA concept was applied. Mycotoxins were grouped by families as the cumulative assessment group (aflatoxins, ochratoxin A, fumonisins, trichothecenes and zearalenone) (EFSA, 2013). The MoET was determined for aflatoxins and the HI for fumonisins

and trichothecenes. The MoET was calculated as the reciprocal of the sum of the reciprocals of the individual margins of exposure (MoEs) and the HI, as the sum of the respective Hazard Quotients (HQs) for the individual mixture components of the same family (EFSA, 2013).

3. RESULTS AND DISCUSSION

3.1. Analytical performance

Table 21 summarizes the performance of analytical methods used for determination of the 13 mycotoxins in three different cereal-based products (breakfast cereals, processed cereal-based products (flours) and biscuits). All the analytical methods showed a good linear response over the working range, a coefficient of determination (r^2) > 0.995, with mean recoveries ranging between 44% (for T-2 toxin) and 93% (for DON), LOD values ranging between 0.001 (for AFB_2) and 6.8 (for T-2), and LOQ values ranging between 0.004 (for AFB_2) and 22.3 (for T-2). All the presented results were in agreement with the criteria mentioned in the Commission Regulation (EC) No. 401/2006 (EC, 2006a), with the exception of NIV and T-2 toxin recoveries. These performance results guaranteed the mycotoxins quantification at low level that are typically present in the foodstuffs usually intended for children consumption.

Table 21. Methods performance for mycotoxins determination by chromatographic methods.

Mycotoxins	Instrumentation	Linearity (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)	Recovery (%)
AFM_1	HPLC-FLD	0.100 – 1.000	0.011	0.032	83
AFB_1		0.040 – 0.400	0.003	0.009	73
AFB_2		0.030 – 0.300	0.001	0.004	57
AFG_1		0.045 – 0.450	0.006	0.018	87
AFG_2		0.030 – 0.300	0.010	0.029	57
OTA		0.200 – 2.000	0.006	0.019	71
FB_1	UPLC-MS/MS	2.500 – 800	0.060	0.180	70
FB_2		2.500 – 800	0.120	0.360	68
DON	GC-MS	15 – 360	0.400	1.20	93
NIV		25 – 360	5.60	18.4	46
T-2		25 – 360	6.8	22.3	44
HT-2		25 – 450	6.4	21.1	93
ZEA	UPLC-MS/MS	0.24 – 10.0	0.12	0.40	*

* use of internal standard.

AFM_1, aflatoxin M_1; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; OTA, ochratoxin A; FB_1, fumonisin B_1; FB_2, fumonisin B_2; DON, deoxynivalenol; NIV, nivalenol; T-2, T-2 toxin; HT-2, HT-2 toxin; ZEA, zearalenone. LOD, Limit of Detection; LOQ, Limit of Quantification.

3.2. Assessment of children exposure to mycotoxins

3.2.1. Overview of demographic data of participants

The participants enrolled in this study consisted of 75 children aged between one and three years old with the following distribution by gender and age: 18 male and 24 female (13-24 months), 9 male and 6 female (25-36 months), 7 male and 11 female (36-47 months). Parents of enrolled children were also characterized regarding age (mean ± Standard Deviation) and education level. Mothers were 32 ± 5 years old and fathers were 35±6 years old. Regarding the educational level, most of the parents received primary and secondary education (80%) and 20% received tertiary education. Within the children participants, 81% belonged to households until four persons and 19% to households with more than four persons.

3.2.2. Mycotoxins occurrence data

Table 22 shows the percentage of positive samples (mycotoxin contents above LOD) and mean (µg/kg), median (µg/kg) and maximum (µg/kg) contents of each mycotoxin in each food category. A total of 52 cereal-based products primarily marketed for children, including breakfast cereals, processed cereal-based foods (flours) and biscuits were analysed for determination of aflatoxins (AFTs), ochratoxin A (OTA), fumonisins (FMs), trichothecenes (TCs) and zearalenone (ZEA) contents.

Forty nine out of 52 samples (94%) evidenced at least one of the analysed mycotoxins (with values above the detection limit). All studied mycotoxins were present in the analysed samples, except AFG_2, T-2 and HT-2 toxins. Overall, OTA, ZEA and DON were the most commonly detected mycotoxins with 65%, 48% and 44% of analysed samples revealing values above the LOD, respectively. Mycotoxin mean contents ranged between 0.007 µg/kg of AFB_2 in a breakfast cereals sample and 95.9 µg/kg of DON in a breakfast cereals sample. The maximum contamination value was detected in a breakfast cereals sample with 207.8 µg/kg of DON. All analysed samples presented mycotoxin contents bellow the legislated limits, when available.

Aflatoxins and ochratoxin A

Regarding aflatoxins and ochratoxin occurrence, Alvito et al. (2010) reported a similar OTA contamination in 20 cereal-based baby foods from Portugal, with levels between 0.010 and 0.212 µg/kg. However, for AFB_1 and AFM_1 contamination, Alvito et al. (2010) reported lower levels (0.008

Table 22. Occurrence (%) of mycotoxins in cereal-based products primarily marketed for children in Portugal.

Toxins	Breakfast cereals (n = 26)				Processed cereal-based foods (flours) (n = 20)				Biscuits (n = 6)				Total Samples (n = 52)
	Positive samples (%)	Mean (µg/kg)	Median (µg/kg)	Maximum (µg/kg)	Positive samples (%)	Mean (µg/kg)	Median (µg/kg)	Maximum (µg/kg)	Positive samples (%)	Mean (µg/kg)	Median (µg/kg)	Maximum (µg/kg)	Positive samples (%)
AFB_1	19 (73%)	0.036	0.013	0.130	0	ND	ND	ND	0	ND	ND	ND	19 (37%)
AFB_2	12 (46%)	0.007	0.004	0.011	1 (5%)	NA	NA	0.002	0	ND	ND	ND	8 (15%)
AFG_1	1 (4%)	NA	NA	0.017	2 (10%)	0.014	0.014	0.016	0	ND	ND	ND	3 (6%)
AFG_2	0	ND	ND	ND	0	ND	ND	ND	0	ND	ND	ND	0
AFM_1	3 (12%)	0.017	0.013	0.024	8 (40%)	0.068	0.023	0.190	0	ND	ND	ND	11 (21%)
AFTs	19 (73%)				9 (45%)				0				28 (54%)
OTA	18 (69%)	0.047	0.043	0.100	10 (50%)	0.061	0.040	0.263	6 (100%)	0.086	0.091	0.134	34 (65%)
FB_1	15 (58%)	22.00	12.50	67.00	7 (35%)	0.44	0.31	0.86	0	ND	ND	ND	22 (42%)
FB_2	10 (39%)	5.10	4.20	14.00	0	ND	ND	ND	0	ND	ND	ND	10 (19%)
FMs	15 (58%)				7 (35%)				0				22 (42%)
ZEA	19 (73%)	1.20	0.69	5.61	6 (30%)	0.48	0.41	0.98	0	ND	ND	ND	25 (48%)
DON	16 (62%)	95.9	91.5	207.8	4 (20%)	41.8	37.5	71.0	3 (50%)	43.8	32.3	73.3	23 (44%)
NIV	1 (4%)	NA	NA	27.1	0	ND	ND	ND	0	ND	ND	ND	1 (2%)
T-2	0	ND	ND	ND	0	ND	ND	ND	0	ND	ND	ND	0
HT-2	0	ND	ND	ND	0	ND	ND	ND	0	ND	ND	ND	0

ND, not detected. NA, not applicable.

Positive samples, mycotoxin content ≥ LOD.

AFM_1, aflatoxin M_1; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; AFTs, aflatoxins; OTA, ochratoxin A; FB_1, fumonisin B_1; FB_2, fumonisin B_2; FMs, fumonisins; DON, deoxynivalenol; NIV, nivalenol; T-2, T-2 toxin; HT-2, HT-2 toxin; ZEA, zearalenone.

and 0.023 μg/kg) than the obtained in the present study. Other authors investigated also the occurrence of aflatoxins and OTA in cereal-based products intended for children consumption in Europe with similarities and dissimilarities between results (Araguás et al., 2005; Beltrán et al., 2011; Ibáñez-Vea et al., 2011b; Juan et al., 2014). All these results revealed heterogeneity in the contamination of the cereal-based products intended to be consumed by children which could be explained by the fact that different fungi can grow and produce mycotoxins in the same environmental conditions and food processing favours the production of several mycotoxins; consequently, different mycotoxins contamination, in type and contents, could be detected (Ibáñez-Vea et al., 2011b).

Fumonisins

Relatively to the present study, it is important to highlight that all samples contaminated with fumonisins presented in their constitution maize, being well recognized that fumonisins are the main mycotoxins found in maize overall when grown in warmer regions (Marin et al., 2013; Raiola et al., 2015). In Portugal, some previous studies evaluated the occurrence and the content levels of fumonisins in maize products (Lino et al., 2007), including in some cases breakfast cereals samples (Martins et al., 2008; Silva et al., 2007). None previous study evaluated fumonisins contents in food samples intended to be consumed by children. In other European studies, fumonisins were evaluated mainly in maize or in their products (Rubert et al., 2013; SCOOP, 2003).

Trichothecenes (DON, NIV, T-2, HT-2 toxins)

Regarding the presence of trichothecenes in cereal based samples, lower levels of DON were reported in the present work when compared with those described by Cunha and Fernandes (2010) and Martins and Martins (2001) for breakfast cereals. These authors stated a maximum DON content of 524.6 μg/kg and 6040 μg/kg in the positive samples, respectively. This situation could be explained by the fact that the present study only evaluated samples intended to be consumed by children, a type of products expected to present lower contents of contaminants than samples usually consumed by the general population. In the European Union, the SCOOP study (SCOOP, 2003) reported the occurrence of DON in 56%, 72% and 24% of breakfast cereals, baby food and biscuits samples, respectively. Other results were reported in Spain for trichothecenes (Castillo et

al., 2008; Montes et al., 2012; Serrano et al., 2012) and in Italy (Juan et al., 2014), for DON and NIV.

Zearalenone

The presence of ZEA is commonly associated with maize products but it can be also found in other crops such as wheat, barley, sorghum and rye (EFSA, 2011a). In this study, 47% and 50% of the analysed cereal samples (breakfast and processed based cereals, respectively) presented maize in their composition (results not shown).

In Portugal, Cunha and Fernandes (2010) reported the occurrence of ZEA in commercial breakfast cereals (67%) and flours (15%), with a maximum content of 69 μg/kg. These results revealed ZEA contents higher than those obtained in the present study which could be probably attributed to the different raw materials used for cereals preparation. The occurrence of ZEA in different flours for human consumption, including baby foods, from the Portuguese and Dutch markets, were evaluated by Aldana et al. (2014). These authors found that 37.5% of the samples were contaminated with ZEA and two of them (from Portugal) exceeded the maximum limit established by EU. A study performed by EFSA (2011) gathering analytical results from 19 European countries, revealed a frequency of occurrence of ZEA in maize of 33% with a mean content level of 15 μg/kg. ZEA was also detected in foods from Spain (Cano-Sancho et al., 2012a; Ibáñez-Vea et al., 2011b) and no data were reported from Italy cereal products (Juan et al., 2014; Serrano et al., 2012).

Co-occurrence of mycotoxins

The detected co-occurring mycotoxins, the number of mycotoxins included in the mixtures as well as their frequency of occurrence in the three cereal-based products analysed in the present study is presented in Table 23. The analysis revealed a co-occurrence of mycotoxins in 75% of the analysed samples, with two or more mycotoxins occurring simultaneously. The highest number of mycotoxins detected simultaneously was seven and the combinations of two (OTA and DON; OTA and fumonisins) and four (aflatoxins, OTA and ZEA) mycotoxins were the most commonly detected, with a percentage of occurrence of 6% for each combination. The present results contribute to the growing evidence related to the more frequent human exposure to multiple than to single mycotoxins (Alassane-Kpembi et al., 2016; De Ruyck et al., 2015; Grenier and Oswald, 2011;

Stoev, 2015). As a consequence, there has been an increasing concern about the health hazard from exposure to multiple mycotoxins in human and animals. In a recent review, Smith et al. (2016) verified that several surveys reported the natural co-occurrence of mycotoxins from all over the world, and most of them concerned the aflatoxins, OTA, ZEA, fumonisins and trichothecenes, especially DON. The same authors referred that among the 116 mycotoxin combinations found in cereal and derived cereal product samples, aflatoxins + fumonisins, DON + ZEA, aflatoxins + OTA, and fumonisins + ZEA were the most present. These results agree well with those obtained in the present study and point out the importance to develop more toxicity studies that consider the co-exposure to multiple mycotoxins simultaneously considering the potential impact for public health, especially in those cases of possible synergism and additive effects. The present data were crucial to perform a more accurate Portuguese children risk assessment through consumption of cereal-based products.

3.2.3. Consumption data and exposure assessment

Food consumption data analysis revealed that approximately 92% of the children aged between one and three years old consumed one or more cereal-based products, at least one time in three days, as reported in food diary (40%, 65% and 65% of the studied population, consumed breakfast cereals, processed cereal-based foods and biscuits, respectively) which represents a high consumption of cereal-based products by Portuguese children. The mean daily consumption of these food groups was 5.6 g, 25.3 g and 8.7 g for breakfast cereals, processed cereal-based foods and biscuits, respectively (Table 24). Guerra et al. (2012) that reviewed the knowledge and practices on infant feeding, suggested that for nutritional requirements and inherent neurosensory, motor and social infant development, foods other than milk and with less homogeneous texture should be progressively introduced, leading to entry in the family diet, which should occur around 12 months of age. Hence, the present results corroborate these practices, revealing that Portuguese children from early ages usually eat other foods than milk, including cereal-based products.

Table 23. Co-occurrence of mycotoxins in analysed cereal-based products.

Number of mycotoxins detected	Sample	Mycotoxins mixture	Number of samples contaminated with mixtures (%)
7	BC	AFTs, OTA, FMs, ZEA	1/52 (2%)
		AFTs, OTA, FMs, DON, ZEA	1/52 (2%)
		AFTs, OTA, FMs, ZEA	1/52 (2%)
6	BC	AFTs, OTA, FMs, DON, ZEA	1/52 (2%)
		AFTs, OTA, FMs, DON	1/52 (2%)
		AFTs, FMs, OTA, NIV, ZEA	1/52 (2%)
		AFTs, FMs, DON, ZEA	1/52 (2%)
		AFTs, OTA, FMs	1/52 (2%)
5	BC	AFTs, OTA, DON, ZEA	2/52 (4%)
		OTA, FMs, DON, ZEA	1/52 (2%)
		AFTs, OTA, FMs, ZEA	1/52 (2%)
		AFTs, OTA, FMs, DON	1/52 (2%)
4	**BC**	AFTs, OTA, DON, ZEA	1/52 (2%)
		AFTs, OTA, ZEA	**3/52 (6%)**
		OTA, FMs, DON, ZEA	2/52 (4%)
		AFTs, OTA, DON	1/52 (2%)
	PC	AFTs, OTA, FMs, ZEA	1/52 (2%)
3	BC	FMs, ZEA	1/52 (2%)
		FMs, DON, ZEA	1/52 (2%)
		AFTs, DON, ZEA	1/52 (2%)
	PC	AFTs, OTA, FMs	1/52 (2%)
		AFTs, OTA, ZEA	1/52 (2%)
2	BC	AFTs	1/52 (2%)
		AFTs, FMs	1/52 (2%)
		AFTs	2/52 (4%)
	PC	**OTA, FMs**	**3/52 (6%)**
		OTA, ZEA	1/52 (2%)
		AFTs, OTA	1/52 (2%)
		FMs, ZEA	1/52 (2%)
	BIS	**OTA, DON**	**3/52 (6%)**
		Total	39/52 (75%)

In the present study, the exposure assessment of children to multiple mycotoxins in cereal-based products was assessed through the estimation of the mycotoxins daily intake. Table 24 presents the estimated daily intake (ng/kg bw/day) by Portuguese children aged between one and three years old for the different cereal-based products, considering three different scenarios (according to the data treatment of the non-detects). For the H1 scenario (< LOD = LOD, worst case), the sum of daily intake through consumption of cereal-based products presented the highest value for DON

(57.22 ng/kg bw/day), followed by FB_1 (6.4 ng/kg bw/day), NIV (2.68 ng/kg bw/day), FB_2 (1.0 ng/kg bw/day), OTA (0.131 ng/kg bw/day), ZEA (0.86 ng/kg bw/day) and AFM_1 (0.069 ng/kg bw/day). The remaining scenarios followed approximately the same pattern. Considering the mycotoxins daily intake through the consumption of each cereal-based product considered, the highest mycotoxin intake was verified through the consumption of breakfast cereals for AFB_1, fumonisins, DON and NIV and through the consumption of processed cereal-based foods (flours) for AFM_1, AFB_2, AFG_1, OTA and ZEA.

The results for the probabilistic estimates of the daily intakes (mean and percentiles 50, 75, 90, 95, 99) for individual toxins in each cereal-based product and its sum in the three cereal based products considered are presented at Table 25.

Considering all the studied scenarios in probabilistic analysis (H1, H2, H3, H4), the worst case was reflected in H1 scenario, and the results reinforced the outcomes obtained with the deterministic approach. Considering the mean sum of daily intake through the consumption of the three groups of cereal-based products, the highest estimated value was presented for DON (53.93 ng/kg bw/day), followed by FB_1 (6.7 ng/kg bw/day), NIV (2.74 ng/kg bw/day), ZEA (0.89 ng/kg bw/day) and OTA (0.165 ng/kg bw/day). Within aflatoxins, AFM_1 presented the highest value for the mean sum of daily intake (0.058 ng/kg bw/day). Considering each analysed food group, the breakfast cereals was the highest contributor for the estimated daily intake of mycotoxins, revealing the highest values for fumonisins, trichothecenes, ZEA and AFB_1. On the other hand, processed cereal-based foods (flours) showed the highest contribute for estimated daily intakes for AFM_1, AFB_2, AFG_1 and OTA.

Table 24. Deterministic approach to estimate children's daily intake of mycotoxins present in cereal-based products (ng/kg bw/day) considering three different scenarios for non-detects (<LOD).

Toxins	Estimated daily intake (ng/kg bw/day)											
	Breakfast cereals			Processed cereal-based foods (flours)			Biscuits			Sum of estimated daily intake (ng/kg bw/day)		
	H1	H2	H3	H1	H2	H3	H1	H2	H3	H1	H2	H3
AFM_1	0.005	0.003	0.001	0.064	0.057	0.051	0.000	0.000	0.000	0.069	0.060	0.052
AFB_1	0.012	0.011	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.012	0.011	0.011
AFB_2	0.001	0.001	0.001	0.002	0.002	0.000	0.000	0.000	0.000	0.003	0.003	0.001
AFG_1	0.003	0.001	0.000	0.013	0.008	0.002	0.000	0.000	0.000	0.016	0.009	0.002
OTA	0.011	0.010	0.010	0.064	0.060	0.059	0.056	0.056	0.056	0.131	0.126	0.124
FB_1	6.0	5.4	5.3	0.4	0.3	0.3	0.0	0.0	0.0	6.4	5.7	5.6
FB_2	1.0	1.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0	0.8
DON	24.83	24.80	24.77	16.34	16.06	15.78	16.05	15.15	14.24	57.22	56.01	54.79
NIV	2.68	1.56	0.44	0.00	0.00	0.00	0.00	0.00	0.00	2.68	1.56	0.44
ZEA	0.42	0.41	0.41	0.43	0.36	0.28	0.00	0.00	0.00	0.86	0.77	0.69

H1: < LOD = LOD; H2: < LOD = 1/2 LOD; H3: < LOD = 0.

Mean consumption data: Breakfast cereals 5.6 g/day; Processed cereal-based foods (flours) 25.3 g/day; Biscuits 8.7 g/day.

Mean weight data: 13.4 kg.

AFM_1, aflatoxin M_1; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; OTA, ochratoxin A; FB_1, fumonisin B_1; FB_2, fumonisin B_2; DON, deoxynivalenol; NIV, nivalenol; ZEA, zearalenone.

Table 25. Probabilistic analysis of mycotoxins estimated daily intake [mean and percentiles 50 (P50), 75 (P75), 90 (P90), 95 (P95) and 99 (P99)] by 1 to 3 years old Portuguese children from different food products. Data for H1 scenario (H1: < LOD = LOD) is presented.

	Estimated daily intake (µg/kg bw/day)																							
Toxins	**Breakfast cereals**						**Processed cereal-based foods (flours)**						**Biscuits**						**Sum of estimated daily intake (ng/kg bw/day)**					
	Mean	**P50**	**P75**	**P90**	**P95**	**P99**	**Mean**	**P50**	**P75**	**P90**	**P95**	**P99**	**Mean**	**P50**	**P75**	**P90**	**P95**	**P99**	**Mean**	**P50**	**P75**	**P90**	**P95**	**P99**
AFM_1	0.005	0.004	0.007	0.013	0.017	0.027	0.053	0.022	0.054	0.117	0.186	0.458	0.000	0.000	0.000	0.000	0.000	0.000	0.058	0.026	0.062	0.130	0.203	0.485
AFB_1	0.013	0.003	0.011	0.030	0.055	0.160	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.013	0.003	0.011	0.030	0.055	0.160
AFB_2	0.001	0.000	0.001	0.002	0.004	0.010	0.002	0.001	0.003	0.005	0.006	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.002	0.004	0.007	0.010	0.020
AFG_1	0.003	0.002	0.004	0.007	0.010	0.016	0.012	0.008	0.017	0.029	0.038	0.062	0.000	0.000	0.000	0.000	0.000	0.000	0.015	0.010	0.021	0.036	0.048	0.078
OTA	0.019	0.009	0.023	0.047	0.069	0.126	0.091	0.018	0.05	0.131	0.246	0.951	0.056	0.029	0.072	0.142	0.202	0.356	0.165	0.056	0.145	0.321	0.517	1.433
FB_1	6.3	1.0	3.7	12.5	26.1	92.1	0.4	0.1	0.3	0.8	1.3	3.6	0.0	0.0	0.0	0.0	0.0	0.0	6.7	1.1	4.0	13.3	27.4	95.7
FB_2	1.2	0.5	1.2	2.6	4.3	11.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.2	0.5	1.2	2.6	4.3	11.7
DON	28.69	4.73	23.82	75.72	134.47	344.96	8.09	0.70	2.48	7.78	16.28	75.00	17.15	6.50	19.04	44.38	69.99	146.84	53.93	11.93	45.34	127.88	220.74	566.80
NIV	2.74	1.82	3.72	6.38	8.51	13.51	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.74	1.82	3.72	6.38	8.51	13.51
ZEA	0.44	0.14	0.41	1.05	1.82	4.65	0.44	0.23	0.52	1.04	1.53	3.15	0.00	0.00	0.00	0.00	0.00	0.00	0.89	0.37	0.94	2.09	3.34	7.80

3.3. Risk characterization

Figure 21 presents the results concerning the risk characterization for aflatoxins using MoE (individual aflatoxins) and MoET (combined aflatoxins). The results were obtained considering the estimates of aflatoxins exposure through the consumption of the three food categories considered (breakfast cereals, processed cereal-based foods (flours) and biscuits). The H1 scenario, as the worst case, was presented. The remaining scenarios followed the same pattern (data not shown).

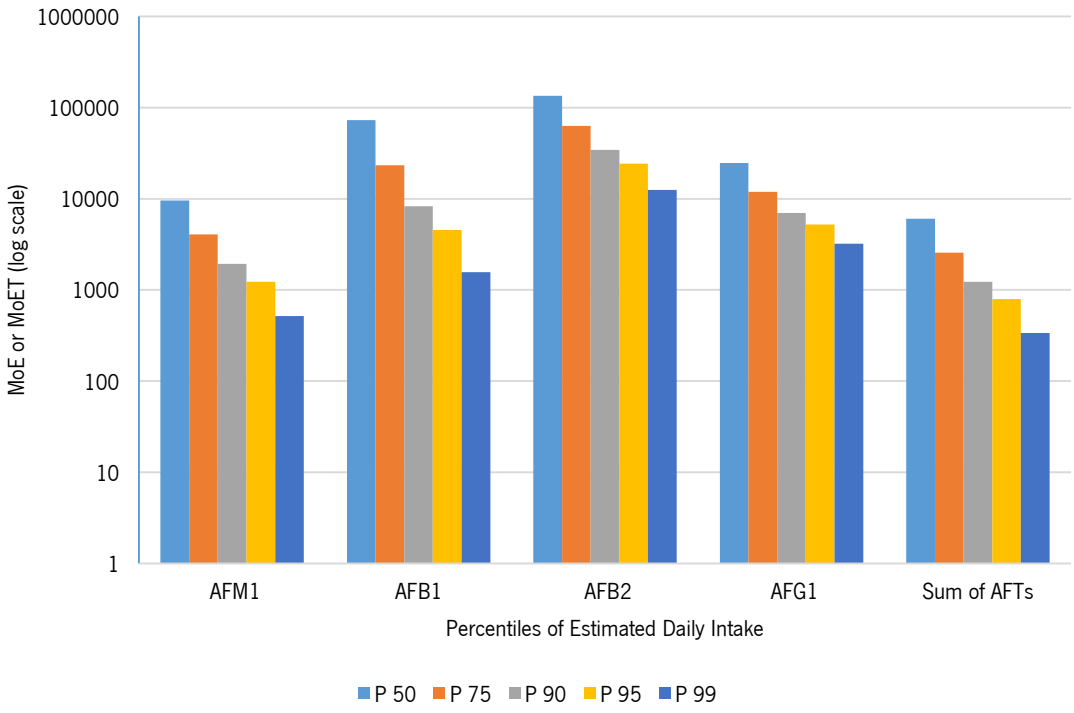

Figure 21. Risk characterization using MoE and MoET derived from estimates of aflatoxin exposure (percentiles 50 (P50), 75 (P75), 90 (P90), 95 (P95) and 99 (P99)) from different food products, performed by probabilistic approach. Data for H1 scenario (H1: < LOD = LOD), as the worst case scenario, is presented.

As presented in Figure 21, AFB_2 was the aflatoxin that revealed a MoE above 10000 for all percentiles of exposure, which represent low risk for children. However, for the remaining aflatoxins and for some percentiles of intake (AFB_1 and AFG_1, for percentiles 90, 95 and 99), the MoE values were below 10000, suggesting a potential health concern. When considered percentiles P50 or higher of AFM_1 intake, the MoE values were below 10000, suggesting also a potential health concern. When considered the simultaneous exposure to aflatoxins, MoET for percentiles P50 or higher revealed a potential health concern. Consequently, MoE/MoET below 10000 signifies that continuous exposure to such cereal-based products could pose serious adverse health effect to such susceptible groups of individuals, as young

children. Assunção et al. (2015) had already referred that a potential health concern could arise from the consumption of breakfast cereals, especially for high consumers (percentiles 90, 95 and 99), being AFB_1 the main contributor (87.3%) for the risk (MoET < 10000). As suggested by Serrano et al. (2012), one possible explanation for the higher risk associated with infants and children is the fact that they have an exceptionally high intake in relation to their body weight. Sherif et al. (2009) refereed that there is evidence that suggest increased susceptibility to cancer from early-life exposures, particularly for chemicals acting through a mutagenic mode of action, as aflatoxins. Additionally, Raiola et al. (2015) pointed out other potential effects of children exposure to aflatoxins, namely i) reduction of the efficiency of immunization in children with the consequent increase of susceptibility to infections; ii) children vulnerability to the risk of cancer from aflatoxin- contaminated milk since milk is an important constituent of their diet (the same could be proposed to aflatoxins present in cereal-based products); iii) young animals have been found to be more susceptible to AFB_1 and AFM_1 toxicity than adults and repeated exposures to aflatoxins *in-utero* and through childhood might predispose to liver cancer later in life. Considering all these data, more and deep information is needed in order to protect children health, ensuring a reduction on aflatoxins children exposure.

Relatively to the remaining mycotoxins, Figure 22 presents the results concerning the risk characterization for OTA, fumonisins, and trichothecenes using HQ (individual mycotoxins) and HI (combined mycotoxins). The outputs were derived from estimate of these mycotoxins exposure performed by the probabilistic approach and through the consumption of the three food categories considered. The H1 scenario, as the worst case, was presented. The remaining scenarios followed the same pattern (data not shown).

These results showed that all HQs were <1, *i.e.*, indicating no cause for concern for individuals exposed to mycotoxins through consumption of cereal-based products. The HQ for DON and HI for the simultaneous exposure to trichothecenes showed the highest values, however well below one.

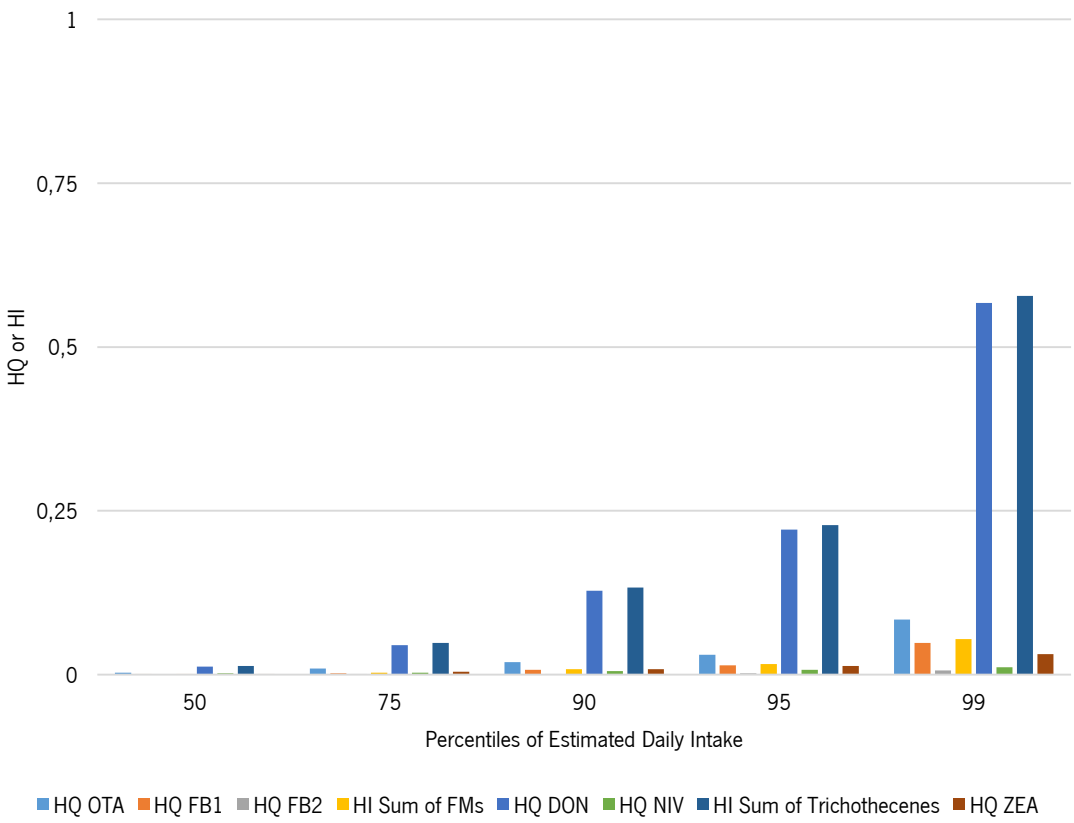

Figure 22. Risk characterization using HQ and HI derived from estimates of ochratoxin A (OTA), fumonisins (FB_1 and FB_2), trichothecenes (DON and NIV) and zearalenone exposure [percentiles 50 (P50), 75 (P75), 90 (P90), 95 (P95) and 99 (P99)] from different food products, performed by probabilistic approach. Data for H1 scenario (H1: < LOD = LOD), as the worst case scenario, is presented.

The risk associated with the children consumption of foods contaminated by mycotoxins is dependent of the magnitude, frequency of exposure as well as the hazard from each mycotoxin (Kuiper-Goodman, 2004). A simulation considering a quarter (1/4) of the aflatoxins daily intake previously estimated was performed, in order to evaluate the potential measures that could be suggested to reduce the aflatoxins MoET values. The results showed an improvement of the aflatoxins MoET, revealing that just percentiles of intake above P90 could be under health concern (data not shown). These results support the need for a reduction on the aflatoxins intake through consumption of cereal-based products by Portuguese children aged between 1 and 3 years old. This could be attained considering some measures as i) an improvement on the variety and diversity of cereal-based products consumed by children, ii) a reduction on the amount of daily ingestion of these products and iii) a reduction on the mycotoxins contaminations of food products usually consumed by children, using raw materials with an improvement in their quality. Relatively to the last measure, aflatoxin contamination of cereal-based products usually consumed by children is evident

and, due to their special role in children nutrition, more rigorous monitoring and quality control of the ingredients used in these products with a greater health hazard should be exercised.

The results on the present exposure assessment study were then compared to other reports on literature. Sirot et al. (2013) assessed the dietary exposure of the French population to mycotoxins through a total diet study. The authors reported that the highest mean concentrations were found in wheat and cereal-based products and only the exposure to DON and its acetylated derivatives was found to significantly exceed the reference values in adults and children. Hernández-Martínez and Navarro-Blasco (2010) evaluated the aflatoxins daily intake by 0-2 years old Spanish children through the consumption of infant cereals. The determined daily intake values ranged AFB_1, 0.01-29.06; AFB_2, 0.02-3.82, AFG_1, 0.005-3.91; AFG_2 0.0015-0.68 and AF total 0.08-37.47 ng/kg bw/day. The authors verified that apart from organically produced infant cereals with cocoa, which had an inadmissible risk to infant health, the other infant cereals were below 1 ng/kg bw/day, which was considered by authors as the reference value. More recently, Cano-Sancho et al. (2013) assessed the exposure to aflatoxins by Catalonian (Spain) population. The study included children aged above 4 years old. The authors reported a children daily intake of aflatoxins of 0.105 ng/kg bw/day with results for MoE of 8208 (mean) and of 2582 (P95), representing similarly to the present study a significant health risk. The authors concluded that the MoEs built in the study were in the line of those estimations reported for other European countries. The same researchers assessed also the exposure of Catalonian population to fumonisins (Cano-Sancho et al., 2012b), ZEA (Cano-Sancho et al., 2012a) and DON (Cano-Sancho et al., 2011). Relatively to fumonisins, the authors found that the most exposed group were infants (0-3 years old) with a mean fumonisins daily intake of 195.2 ng/kg bw/day, below the TDI (Cano-Sancho et al., 2012b). Considering ZEA, the age group with the highest estimated intake of this toxin was the group of infants (0-3 years old), with high consumers presenting estimated intakes in the range of 35.4–51.9 ng/kg bw/day (Cano-Sancho et al., 2012a). The authors assessed quantitatively the exposure of the Catalonian population to DON and verified that the Catalonian population was expected to be exposed at moderated levels of DON, and the infants and individuals with ethnic dietary patterns being the most exposed population groups. The mean DON estimated daily intake for infants was 900 ng/kg bw/day. The authors concluded that although the majority of the population does not exceed the TDI, there is still a large population exceeding this safety value. Another study, developed in Spain by Coronel et al. (2012), assessed the exposure of the Catalonian population to OTA by determining the contamination levels of certain foodstuffs sampled in Catalonia, and by considering data of consumption for infants (0-3 years old), children, adolescents and adults. The median estimated daily intake of OTA through the foodstuffs by each age group was below

the provisional TDI. Infant population was the most exposed group and revealed an OTA daily intake of 2.4 ng/kg bw/day, representing for the high quantiles 65% of the reference value. It is important to stress out that in Coronel et al. (2012) study, just the baby foods were included in the infant exposure assessment and consequently, this scenario could be altered if other food products would be considered. Rodríguez-Carrasco et al. (2013) assessed exposure to *Fusarium* mycotoxins (including, DON, NIV, T-2, HT-2 and ZEA) through cereals intake by Spanish population, including infants (0-3 years old). The authors showed that toxins might not pose a health risk for the average consumer, although mycotoxins intake by certain populations consuming high amounts of cereal-based products, such as infants, children or vegetarians, could exceed the safety limits. Generally, Spanish results agreed well with those obtained in the present study.

3.4. Strengths and limitations associated with the risk assessment

The present study is, to the authors' knowledge, the first risk assessment concerning the occurrence of multiple mycotoxins in food, including both genotoxic and carcinogenic compounds. The fact that this study was conducted in a children population from 1 to 3 years old was also important due to the particular vulnerability of this age group namely their high intake relatively to body weight. This study included, for the first time, the exposure to mycotoxins through consumption of various cereal-based products, thus reinforcing the importance of cumulative analysis of the risk of exposure to mycotoxins through food.

In the present study some sources of uncertainties were associated with the obtained results, namely i) the number of analysed samples, ii) the children sample size of the pilot study and iii) the used toxicological data. These facts may have influenced the obtained intake values estimates and the characterization of the risk. Also, the present study was a snapshot of the studied population exposure to mycotoxins and changes over time could occur affecting the intake of mycotoxins. Relatively to the toxicological data, it should be highlighted that reference doses are only defined for the adult population and this renders difficulty in the children and infants risk assessment for which the available reference doses are not suitable (Assunção et al., 2016c). Additionally, and regarding aflatoxins, the $BMDL_{10}$ used to calculate the MoEs was determined for AFB_1 (Benford et al., 2010b) and, in the current study, it was also applied to the remaining aflatoxins, assuming a worst case perspective. This factor introduced bias in the MoEs and MoET estimates.

Additionally, the present study estimated the children exposure through an indirect approach, combining data of mycotoxin occurrence in food and food consumption. This common approach is usually associated with some limitations for the mycotoxins exposure assessment, including the heterogeneous distribution of mycotoxins in food, the possible exposure through other exposure routes than ingestion, the presence of masked mycotoxins, the influence of food processing, inter-individual variation in absorption, distribution, metabolism and excretion (ADME), and the under- and overestimation in food consumption data (Arcella and Leclercq, 2004; Heyndrickx et al., 2014). These limitations could lead to an under- and/or overestimation of the exposure, and biomarkers have been proposed as a suitable alternative (Assunção et al., 2016c).

4. CONCLUSIONS

The combination of occurrence and consumption data allowed to assess, for the first time, the exposure of a children population group from Lisbon region to multiple mycotoxins in food and characterize the associated risk. Cereal-based products, including breakfast cereals, processed cereal-based foods (flours) and biscuits were analysed for mycotoxin contents revealing that 94% of samples were contaminated with at least one mycotoxin, always bellow the legislated limits, when available. The co-contamination of samples was observed in 75% of analysed samples. A high consumption (92%) of cereal-based products by children aged between one and three years old was observed. Aflatoxins exposure through consumption of cereal-based products revealed MoET below 10000 which correspond to a potential adverse health effect for susceptible groups of individuals (percentile of intake above or equal to P50). For the remaining mycotoxins, no reasons for concern were verified. Bearing in mind the particular vulnerability of the studied population, restrictive limits, especially for aflatoxins, should be assembled by Regulators to this age group. Additionally, the development of industry control strategies, from farm to fork, should be encouraged and established. Further studies need urgently to be performed concerning the development of toxicological data that could be used in children risk assessment and the monitoring of children exposure to multiple mycotoxins, especially those with carcinogenic or genotoxic effects as aflatoxins. Future biomonitoring studies assessing mycotoxins children exposure, considered as a quite new frontier for establishing the real human exposure to mycotoxins, should be set in motion in order to contribute to improve the risk assessment and consequently the protection of children health.

SECTION 2: BIOACCESSIBILITY OF MYCOTOXINS PRESENT IN FOOD INTENDED FOR CHILDREN CONSUMPTION

MANUSCRIPT 4: **PATULIN AND OCHRATOXIN A CO-OCCURRENCE AND THEIR BIOACCESSIBILITY IN PROCESSED CEREAL-BASED FOODS: A CONTRIBUTION FOR PORTUGUESE CHILDREN RISK ASSESSMENT**

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Highlights:

- PAT and OTA co-occurrence and bioaccessibility were evaluated in processed cereal-based foods
- PAT and OTA were detected in 75% and 50% of the processed cereal-based food samples
- PAT and OTA were present simultaneously in 40% of analysed samples
- A significant portion of PAT (52%) and especially of OTA (100%) can reach the small intestine
- Considering bioaccessibility and exposure results, PAT and OTA exposures are not expected to be of health concern

ABSTRACT

Patulin (PAT) and ochratoxin A (OTA) are well known enteropathogenic mycotoxins that are present in several foodstuffs. Processed cereal-based foods are among the first solid foods eaten by children, a particularly vulnerable population group. There is a lack of knowledge related to the co-occurrence of PAT and OTA in food intended for children consumption and their potential interactions during the digestion process. The present study aims to evaluate, for the first time, the co-occurrence of PAT and OTA in processed cereal-based foods for children consumption, the bioaccessibility of these two mycotoxins, and the contribution of the bioaccessibility data for human health risk assessment. PAT and OTA incidence were 75% and 50%, respectively. These mycotoxins co-occurred in 40% of analysed samples. Bioaccessibility assays revealed mean values of 52% and 56% for PAT, alone and combined with OTA; and 100% and 106% for OTA, alone and combined with PAT. Considering the human health risk assessment, and taking into account the co-occurrence and the bioaccessibility results, this study indicates a tolerable exposure to these mycotoxins representing a low risk for Portuguese children. The present work reinforces the importance of a holistic approach for risk assessment which gathers data from occurrence, exposure and bioaccessibility.

Keywords: Risk assessment, Bioaccessibility, Mycotoxins, Standardised *in vitro* digestion, Children

1. INTRODUCTION

Mycotoxins are biologically active secondary metabolites produced by fungi often found as contaminants in almost all agricultural commodities worldwide. The consumption of food or feed contaminated by mycotoxins represents a major risk for human and animal health (Del Regno et al., 2015). Mycotoxins are toxic compounds that exert multiple effects in humans and animals (Maresca, 2013), and the Food and Agriculture Organization of the United Nations (FAO) estimated that approximately 25% of cereals produced in the world are contaminated with, at least, one mycotoxin (Rice and Ross, 1994). Co-contamination of foodstuffs with mycotoxins is being reported at an increasing high rate (Stoev, 2015) and this issue constitutes a rising concern due to the hazard of exposure of combined mycotoxins to humans, which could be expected to exert different toxicity than exposure to single mycotoxins (Bouaziz et al., 2008). Considering the toxic effects of mycotoxins, their presence in the human diet is of great concern, especially when present in foods usually consumed by vulnerable populations such as children,

due to their physiological vulnerability and restrict diet (Alvito et al., 2010). Processed cereal-based foods constitute a type of foods usually eaten by children and used as part of a diversified diet, which help in the transition from a liquid diet based on breast milk or infant formula to a diet including solid foods. Cereals are among the first solid foods eaten by children and constitute an important food group of their diet (Schwartz et al., 2008) thus diligent attention must be paid to evaluate the potential health risk of mycotoxins in cereal-based foods consumed by children.

Upon ingestion, natural toxins present in food must first interact with the gastrointestinal tract. The intestinal mucosa, the first biological barrier encountered by these compounds, represents their first target and, consequently, it may be exposed to dietary toxins, including mycotoxins (Maresca and Fantini, 2010; Pinton and Oswald, 2014; Puel et al., 2010). Mycotoxins as patulin (PAT) and ochratoxin A (OTA), both produced by moulds of genera *Aspergillus* and *Penicillium*, are considered amongst the best known enteropathogenic mycotoxins and are able to alter functions of the intestine (Maresca et al., 2008). *In vivo* studies have demonstrated that PAT is rapidly absorbed and causes mucosal ulceration and inflammation (Speijers et al., 1988). These observations suggested that PAT affects gastrointestinal functions and it is one of the few mycotoxins which appears to have direct effects on the gut (Assunção et al., 2016a; McLaughlin et al., 2009). Relatively to OTA, although its principal target is the kidney, this mycotoxin is known to cause rapid inflammation, diarrhoea, and increased bacterial translocation, on interaction with the intestine (McLaughlin et al., 2004). Due to their health impact, PAT and OTA presence in foods for adults and child is regulated by the European Commission (EC) (European Commission, 2006a). It is important to highlight that nowadays the main importance associated to mycotoxins is related with chronic exposure to low levels of these compounds during long periods. This fact assumes especial importance to children considering that they have more time to develop chronic diseases since they have more future years of life than adults and consequently early adverse effects may have lifelong consequences (Felter et al., 2015; Raiola et al., 2015).

In human health risk assessment, the total amount of an ingested contaminant (intake) does not always reflect the amount that is available to the body. Only a certain amount of the contaminant will be bioavailable, and consequently, reaches the systemic circulation (Versantvoort et al., 2005). The oral bioavailability of a compound could result of three different processes: i) the release of the compound from the food matrix into digestive juice in the gastrointestinal tract, known as bioaccessibility; ii) the transport across the intestinal epithelium; and iii) the metabolism of the compound in the liver (Versantvoort et al., 2005). The amount of a contaminant that is bioaccessible represents the maximum amount of a toxic compound that could exert its effects at gastrointestinal level (Raiola et al., 2012b;

Versantvoort et al., 2004). Thus, a better insight in the bioaccessibility of a contaminant will lead to a more accurate health risk assessment. During the past decade, there has been an increasing interest in the use of *in vitro* methodologies, such as *in vitro* digestion models that simulate, in a simplified manner, the human digestion process, allowing the determination of bioaccessibility of food contaminants during transit in the gastrointestinal tract (Versantvoort et al., 2005). Several *in vitro* models of different complexities have been used to simulate food digestion and consequently, significant variations of *in vitro* parameters were verified (Hur et al., 2011), hampering the comparison of results across research-groups. A three-step standardised *in vitro* digestion method was recently published by Minekus et al., aiming to obtain more comparable data (Minekus et al., 2014).

In the scientific literature there is a lack of information regarding the i) co-occurrence of both PAT and OTA in foodstuffs, two enteropathogenic mycotoxins which could produce severe health effects, ii) bioaccessibility of co-occurring mycotoxins, iii) use of a standardised IVD model to evaluate mycotoxins bioaccessibility and iv) risk assessment of children exposure to mycotoxins in food including data on their bioaccessibility. Face to this situation, the present study aims to evaluate, for the first time, the co-occurrence of PAT and OTA in processed cereal-based foods, the bioaccessibility of co-occurring mycotoxins in this food matrix using a standardised IVD model, the assessment of the Portuguese children co-exposure to these two mycotoxins through consumption of processed cereal-based foods and the contribution of the bioaccessibility data for human health risk assessment.

2. MATERIALS AND METHODS

2.1. Sampling and general considerations

Twenty processed cereal-based foods samples were purchased in supermarkets in Lisbon region, in 2015. Samples contained in their composition one cereal type (n = 11) or multigrain (n = 9), including wheat, rice, corn, oat, barley and/or rye. Seventeen samples presented milk in their composition and 10 also included fruits (maximum of 40% of fruit), in a maximum of five different types, including apple, pear, orange, banana, pineapple, peach and apricot. This information was based on label contents. In order to represent the processed cereal-based foods available in Portuguese market, the purchased samples were from generic brands (n = 5) and name-brands (n = 15). The sample size was at least of 1 kg, accordingly to Commission Regulation (EC) 401/2006 of 23 February 2006 (European Commission, 2006b). The samples were homogenized in a food homogenizer, saved in plastic bags and stored in fridge at 4 °C until further analysis.

Good laboratory practices were applied, including material decontamination to prevent cross contamination, and specific safety handling of mycotoxins solutions. Different storing conditions were applied in order to guarantee mycotoxin stability in samples (room temperature), homogenized samples (4 °C), mycotoxin standard solutions (-20 °C) and digested extracts (-80 °C). All conditions are according to manufacturer's conditions and/or the reported method procedures (as in digestion assays).

2.2. Chemicals and reagents for mycotoxins determination

Ethyl acetate, n-hexane, acetonitrile, acetic acid and methanol (all HPLC grade), ethanol, acetic acid and anhydrous sodium hydrogen carbonate (all extra pure grade), methanol, sodium chloride, potassium bromide, PBS (suprapur grade), perchloric acid 60%, anhydrous sodium sulphate (p.a.) and sand (purified), all were purchased from Merck® (Darmstadt, Germany). Silica gel solid-phase (SPE) columns were purchased from Phenomenex® (Torrance, CA, USA). Immunoaffinity columns Afla–Ochra were purchased from Vicam® (MA, USA). Ultra-pure water was produced on a Milli Q Gradient A10 system, from Millipore® (Molsheim, France). The patulin standard was ordered from Sigma® (St. Louis, MO, USA). Stock solution with 200 mg/L of patulin was prepared in ethyl acetate. This solution was stored at -20 °C and when necessary appropriate volume was evaporated and diluted with ethanol for the preparation of an intermediate standard solution. The concentration of the patulin intermediate standard solution was determined by UV at 276 nm, against a solvent blank, using the molar extinction value (1460 m^2/mol in ethanol). Appropriate amounts of the intermediate standard solution were diluted with 0.1% acetic acid in order to get calibration solutions with concentrations between 8 and 100 μg/L. Standard solution of OTA (10.0 μg/mL, in acetonitrile) were from Biopure® (Austria). From the standard solution, a stock solution was prepared in chloroform with 0.40 μg/mL of OTA. From this stock solution, a working stock solution was prepared in water–methanol (85+15, v/v), with a 1000x dilution. This working stock solution was further diluted to get calibration solutions with concentrations from 40 to 400 ng/L.

2.3. Patulin determination

2.3.1. Sample preparation

For patulin extraction (processed cereal-based foods and digested samples), the clean-up procedure and preparation of stock standard solutions were performed according to conditions described by Arranz et al. (2005). Briefly, a test portion (10 g) was extracted with an ethyl acetate extraction solvent in the presence of sodium sulphate and sodium hydrogenocarbonate and was then cleaned up over an unconditioned silica gel solid-phase extraction (SPE) column (Phenomenex, Torrance, CA). The purified extract was evaporated to dryness, redissolved in 0.1% acetic acid (pH 4). A volume of 200 µL was injected into the HPLC system.

2.3.2. Apparatus and HPLC conditions

Patulin was quantified by reverse-phase high performance liquid chromatography (RP-HPLC) and quantitatively determined by ultraviolet (UV) detection at 276 nm. Analytical conditions used for patulin determination were as previously reported by Barreira et al. (2010). The HPLC apparatus was a Waters Alliance 2695 system (Waters®, Milford, MA, USA) equipped with a photodiode array detector, Waters 2998 (Waters®, Milford, MA, USA). Data collection and subsequent processing were performed using the Empower® Chromatographic Software. A stainless steel analytical column (250 x 4.6 mm i.d., 4 µm, Synergy Hydro-RP C18; Phenomenex®, Torrance, CA, USA) and a guard column (4 x 3 mm i.d.) with the same stationary phase were used. The mobile phase, eluting at a flow rate of 1 mL/min, consisted of an isocratic mixture of water–acetonitrile–perchloric acid (96:4:0.1) for 15 minutes followed by a 5 minutes gradient washing step which starts with a concentration of 100% acetonitrile and ends with a concentration of 65% acetonitrile in water.

2.4. Ochratoxin A determination

2.4.1. Sample preparation

The extraction of ochratoxin A from samples (processed cereal-based foods and digested samples) were performed according to the method described in EN15851 (EN ISO, 2010), with few modifications. Briefly, a test portion (10 g) was extracted with a mixture of methanol and water (80:20). The extract was filtered with folded filter paper, diluted with phosphate buffered saline (PBS) to a specified solvent concentration (1:9), filtered again with glass microfiber filter and applied to an immunoaffinity column containing antibodies specific to mycotoxins (AflaOchra, Vicam®). Mycotoxins were purified on the column

and elution was performed with methanol (HPLC grade), with subsequent dilution with water to MeOH/H_2O (15:85). A volume of 800 µL was injected into the HPLC system.

2.4.2. Apparatus and HPLC conditions

Ochratoxin A was quantified by reverse-phase high performance liquid chromatography (RP-HPLC) with post column derivatization involving bromination followed by fluorescence detection. The method was previously in-house validated for the analysis of aflatoxins and OTA, although in the present study only the OTA results were reported. HPLC analysis was performed using a Waters® Alliance 2695 equipped with fluorescence detector Waters 2475 (Waters®, Milford, MA, USA) with Empower Chromatography Software. Post-column derivatization was carried out with electrochemically generated bromine (Kobra cell, R-Biopharm®). The chromatographic column was Prodigy ODS 100 Å (5 µm, 150×4.6 mm, Phenomenex®, Torrance, CA). The mobile phases used for the analysis were a gradient comprised between phase A [KBr (175 mg/L)–MeOH–ACN–$C_2H_4O_2$ (1650:465:390:50, v/v/v/v)] and phase B [(175 mg/L)–MeOH–ACN–$C_2H_4O_2$ (140:1283:1073:50 v/v/v/v)], with a flow rate of 1 mL/min. The wavelength of fluorescence detector was set at 322 nm (excitation), and 468 nm (emission) for OTA detection.

2.5. In vitro digestion procedure

2.5.1. Chemicals and reagents

The following reagents were used to prepare the simulated digestion fluids: $CaCl_2(H_2O)$, KCl, $NaHCO_3$, NaCl, $MgCl_2(H_2O)_6$, NaOH (Merck, Darmstadt, Germany), $(NH_4)_2CO_3$ (Sigma-Aldrich, St. Louis, MO, USA), KH_2PO_4 and HCl (J. T. Baker, Center Valley, PA, USA). α-Amylase, pepsin, bile, pancreatin and Pefabloc® SC were purchased from Sigma-Aldrich® (St. Louis, MO, USA). OTA and PAT standard solutions used for samples spiking were from Biopure® (Austria) and Sigma-Aldrich® (St. Louis, MO, USA), respectively. Relatively to OTA, a stock solution was prepared from the standard solution with a final concentration of 50 µg/mL of OTA. From this solution, a working stock solution with 0.10 µg/mL was prepared in water–methanol (50+50, v/v). For PAT, a stock solution was prepared from the standard solution with 10 µg/mL of PAT. From this stock solution, a working stock solution with 2.0 µg/mL was prepared in aqueous acetic acid solution (pH 4).

2.5.2. Preparation of PAT and OTA artificially contaminated samples

Due to the low contamination of the analysed processed cereal-based foods and to the dilution obtained through the addition of the simulated digestive fluids, six samples (three with fruit and three without fruit

in their composition, previously checked not to be contaminated with PAT and OTA), were artificially spiked and used for the bioaccessibility assays. The samples were rehydrated according to manufacturer's recommendations described in the label, with ultra-pure water and spiked with 20 μg/kg for PAT and 1 μg/kg for OTA (concentrations that allow an expected concentration after *in vitro* digestion near the middle of the working range of each mycotoxin) enabling quantifiable mycotoxin levels. To evaluate the effectiveness of extraction of PAT and OTA from processed cereal-based foods in the presence of digestive juices, the same six samples free from PAT and OTA were digested without toxins and then spiked at 20 μg/kg for PAT and 1 μg/kg for OTA in order to perform the recovery assays (%).

2.5.3. In vitro digestion model

The digestion protocol used follows the standardised static *in vitro* digestion model suitable for food, proposed in a consensus paper, within the COST INFOGEST network (Minekus et al., 2014), slightly modified (use of 2 g of food sample instead of 5 g and the use of α-amylase from bacterial origin instead of α-amylase from human saliva) to enable the determination of mycotoxins bioaccessibility. The method includes three sequential steps: oral, gastric and intestinal phases. For the oral phase, a simulated salivary fluid (SSF) (pH 7) was used; for the gastric and intestinal phases, a simulated gastric fluid (SGF) (pH 3) and a simulated intestinal fluid (SIF) (pH 7) were used, respectively. Briefly, for the oral phase, 2 g of each rehydrated processed cereal-based foods were mixed with 2 mL of SSF with α-amylase (75 U/mL, pH 7). Concerning the gastric phase, 4 mL of SGF with pepsin (2000 U/mL, pH 3) were added and for the intestinal phase, 8 mL of SIF with pancreatin (100 U/mL of trypsin activity, pH 7) and bile (10 mM, pH 7) were added. Digestion tubes were placed on a mechanical shaker in an incubator at 37 °C for two minutes (oral phase) and 120 minutes (gastric and intestinal phases). After intestinal phase incubation time, reaction was stopped with addition of 1 mM of Pefabloc® (Sigma-Aldrich, St. Louis, MO, USA). Digests were immediately placed in liquid nitrogen and after that, samples were kept at -80 °C until further determinations. All experiments were conducted in triplicate. The applied *in vitro* digestion method indicates that the enzymes must be incorporated according to their enzyme activity (Minekus et al., 2014). This activity, as well as the bile salt concentration of the bile, were assessed according to supplementary material of the method reported by Minekus et al. (2014). Immediately before mycotoxin determination, the digests obtained in the end of the intestinal phase was centrifuged at 2500 rpm at 4 °C during 5 minutes and 10 g of the centrifuged supernatant were extracted according to the methods described in 2.3 and 2.4 to estimate bioaccessibility values. Mycotoxins bioaccessibility (%) was calculated using the following equation: (total amount of mycotoxin in supernatant obtained in the end of intestinal

phase/total amount in spiked processed cereal-based food) x 100, taking into account the different dilution processes during sampling preparation.

2.6. Analytical performance

PAT and OTA analytical method performance included the determination of the limits of detection (LOD) and quantification (LOQ) (µg/kg), linearity range (µg/kg), coefficient of determination (R^2) and recoveries (%). For PAT, the LOD was calculated using mean + 3 x standard deviation SD and the LOQ was calculated using mean + 10 x standard deviation SD. For OTA, the limits were calculated using residual standard deviation (Sx/y) and slope (b) of calibration curve, namely for LOD = 3 x ($S_{x/y}$)/b and for LOQ = 10 x ($S_{x/y}$)/b. For the mycotoxin determination studies, samples were considered positive for mycotoxin concentrations equal or above LOD value.

2.7. Estimated daily intake and risk characterization

The calculations of estimated daily intake (EDI) and risk characterization were performed according to the procedure described by Assunção et al. (2015) and were executed using Microsoft® Excel 2007. Briefly, food consumption and body weight (bw) data were obtained from a pilot study performed between February and June 2014, in a Primary Health Care Unit in Lisbon region (Cidadela, Cascais, Portugal) and include a sample of 103 children, aged between 0 and 3 years old. This survey was conducted according to the guidelines laid down in the declaration of Helsinki and was approved by the Ethical Committee of the National Institute of Health Dr. Ricardo Jorge and by the Portuguese Data Protection Authority. Relatively to PAT and OTA occurrence data, three different scenarios were included for the mycotoxin dietary exposure assessment in relation to the data treatment of the non-detects (<LOD) (EFSA, 2010). Non-detects were considered as LOD (H1), 1/2 LOD (H2), zero (H3). EDI were calculated multiplying mean values of mycotoxin concentrations by consumption data (mean, maximum and 50th, 75th, 95th and 99th percentiles, namely 24 g, 156 g, 12 g, 38 g, 95 g and 120 g, respectively) and dividing by mean children's weight (12 kg). For the risk characterization, the outputs of EDI were compared with the reference dose values, namely PAT provisional maximum tolerable daily intake [PMTDI, 400 ng/kg bw/day (JECFA, 1996)] and OTA provisional tolerable weekly intake [PTWI, 112 ng/kg bw/week (JECFA, 2007)], in order to calculate the hazard quotients (HQ, ratio between exposure and a reference dose). If HQ < 1 indicates a tolerable exposure level and a ratio of HQ > 1 indicates a non-tolerable exposure level

(EFSA, 2013). For the characterization of risk associated to PAT and OTA co-exposure, a hazard index (HI, sum of the respective HQ) was calculated. If HI > 1, the total concentration of mixture components exceeds the level considered to be acceptable (EFSA, 2013).

2.8. Statistical analysis

Statistical analysis was performed using IBM® SPSS Statistics 20 software (IBM, Armonk, NY). Data were expressed as means ± SE (standard error) of three independent experiments. To determine the significance in two group comparisons a Mann–Whitney test was applied. The level of $p \leq 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Analytical performance

Performance results for PAT and OTA showed a good linear response with coefficient of determination (R^2) > 0.995. Linearity ranges used for quantification purposes were 3.2–40.0 µg/kg and 0.200–2.000 µg/kg for PAT and OTA, respectively. Recovery values were 80% for PAT and 71% for OTA, suggesting matrix effects on the recovery. For PAT, the values of limit of detection (LOD) and limit of quantification (LOQ) of the chromatographic method were 0.9 and 2.9 µg/kg, respectively. For OTA, LOD and LOQ were 0.006 and 0.019 µg/kg, respectively. All these results, were in agreement with the criteria mentioned in the Commission Regulation (EC) No. 401/2006 and showed that the analytical methods applied are adequate for mycotoxins determinations (European Commission, 2006b).

3.2. Occurrence of PAT and OTA in processed cereal-based foods

Data on PAT and OTA contents in 20 processed cereal-based foods available at Portuguese market are reported in Table 26. PAT and OTA were detected in 75% (15/20) and 50% (10/20) of the analysed samples, respectively. For PAT, mean, median and maximum content were 2.33 µg/kg, 2.52 µg/kg and 4.50 µg/kg, respectively. For OTA, mean, median and maximum content were 0.061 µg/kg, 0.040 µg/kg and 0.263 µg/kg, respectively. Forty percent (8/20) of processed cereal-based food samples presented PAT and OTA simultaneously. None of the analysed samples exceeded the maximum levels established for OTA (0.50 µg/kg) for processed cereal-based foods and baby foods for infants and young children

(European Commission, 2006a). EU legislation only established a maximum level for PAT in baby foods other than processed cereal-based foods and apple juice and solid apple products, for infants and young children (10 μg/kg). Considering the potential occurrence of this mycotoxin in other foodstuffs it is important to set maximum limits regarding the occurrence of PAT in processed cereal-based foods.

Table 26. Incidence of patulin (PAT) and ochratoxin A (OTA) and their co-occurrence in processed cereal-based foods samples marketed in Lisbon, Portugal.

Mycotoxin	Positive samples n (%)	Mean (μg/kg)	Median (μg/kg)	Max. Level (μg/kg)	< LOD
PAT	75% (15/20)	2.33	2.52	4.50	5 (25%)
OTA	50% (10/20)	0.061	0.040	0.263	10 (50%)
PAT + OTA	40% (8/20)	—	—	—	—

The occurrence of PAT has been usually reported in fruits and their by-products. Different European research groups evaluated the occurrence of PAT in apple-based food samples for children consumption: in Spain, PAT incidence ranged from 5.2 to 42.3% (Cano-Sancho et al., 2009), with contents below the maximum levels (European Commission, 2006a); and in Italy, no PAT was detected in fruit-based baby foods (Juan et al., 2014). In Portugal, Barreira et al. (2010) determined the occurrence of PAT in apple-based foods including apple juices and homogenised apple purees. This mycotoxin was detected in 23% of samples with values ranging from 1.2 μg/kg to 42 μg/kg. In cereal-based food products, a Spanish study evaluated also the presence of PAT but no mycotoxin was detected (Rodríguez-Carrasco et al., 2014a).

The occurrence of OTA was mainly reported in cereal-based foods and, in last years, also in foods intended for children consumption. Cereal-based baby food samples were analysed in Spain (Araguás et al., 2005) and Italy (Juan et al., 2014) and showed similar OTA incidences (70% and 60%, respectively) to that observed in the present study (50%). In Turkey, identical food samples were evaluated by different authors (Kabak, 2009; Kara et al., 2015; Ozden et al., 2012), presenting OTA incidences ranging from 17% (Kabak, 2009) to 80% (Ozden et al., 2012). All the analysed samples revealed OTA contents below maximum levels established for OTA (European Commission, 2006a). In Portugal, Alvito et al. (2010) determined the occurrence of OTA in processed cereal-based foods and infant formulae available on the Portuguese market and detected 67% of positive samples for OTA with levels ranging between 0.034 and

0.212 μg/kg. More recently, Assunção et al. (2015) studied the exposure of Portuguese children to mycotoxins referring an OTA mean content of 0.026 μg/kg in breakfast cereals primarily marketed to be consumed by children.

To the extent of authors' knowledge, the present study reports for the first time the simultaneous occurrence of PAT and OTA in processed cereal-based food usually consumed by children. PAT occurrence was extensively studied in fruits and their by-products [as reviewed by some previous authors, (Abrunhosa et al., 2016; Marin et al., 2013; Raiola et al., 2015)], although no reports have explored the presence of PAT in cereal-based products usually consumed by children. The co-occurrence of mycotoxins in foods is expected. On the same food there are a heterogeneous population of fungi and several mycotoxins can be produced by one mould. For example, *Aspergillus* and *Penicillium* species can simultaneously produce several mycotoxins including PAT and OTA. Consequently, this mycotoxin mixture could occur in food products, including those consumed by children. No data are available concerning the interaction that could occur between PAT and OTA and their expected health effects. These mycotoxins could exert intestinal toxic effects individually, thus an increasing concern is expected relatively to the potential impact of the ingestion of co-contaminated foods especially by children, a vulnerable population group. This fact highlights the significance to consider PAT and OTA mixture in human health risk assessment, reinforcing the importance to develop toxicity studies to evaluate the potential impact of this mycotoxin mixture on human health and especially in the gut.

3.3. Bioaccessibility of PAT and OTA in processed cereal-based foods

PAT and OTA mean recoveries in artificially contaminated digested processed cereal-based foods were 100% and 66%, respectively. These values are in accordance with Commission Regulation (EC) No. 401/2006 (European Commission, 2006b) and showed that the extraction and quantification methods allowed an effective determination of mycotoxins contents in samples originated from the *in vitro* digestion (IVD) assays.

Patulin and ochratoxin A bioaccessibility results obtained after application of the standardised IVD model (oral, gastric and intestinal phases) are summarized in Table 27, corresponding to the amount of mycotoxins that reached the intestine. When PAT was assayed alone (single), bioaccessibility of artificially contaminated processed cereal-based samples ranged from 30 ± 2.5% to 77 ± 1.9%, with a mean value of 52 ± 4.2%. When this toxin was combined with OTA (mixture), bioaccessibility ranged from 33 ± 0.7% to 64 ± 1.5%, with a mean value of 56 ± 2.7%. No statistical significant differences were found when

comparing single and mixture PAT bioaccessibility results (p=0.356) and this fact could be attributed to the great variability between samples. When OTA was assayed alone (single), bioaccessibility results ranged from 95 ± 0.3% to 105 ± 1.5%, with a mean of 100 ± 1.1%. When this toxin was combined with PAT (mixture) bioaccessibility ranged from 103 ± 1.2% to 109 ± 0.1%. Bioaccessibility values higher than 100% could be attributed to possible interactions established between food matrix, mycotoxins and digestive fluids. Comparing the OTA bioaccessibility results, alone or combined with PAT, a statistical significant difference was found (p=0.001).

Figure 23 presents the bioaccessibility results of PAT and OTA in processed cereal-based food samples considering the presence or absence of fruit. A significant difference was verified between OTA bioaccessibility values concerning the presence or absence of fruit in the food matrix (p=0.02). For the remaining test scenarios reported in Figure 23, no significant differences were found [PAT, single (p=0.222) and mixture (p=0.546), and OTA, single (p=0.489)].

Nowadays, few studies on bioaccessibility of PAT and OTA have been carried out. Raiola et al. (2012) reported bioaccessibility values for PAT in artificially contaminated apple products (juices, nectars and purees) ranging from 25% to 71% (Raiola et al., 2012a). This study applied a two-step static IVD model developed by Gil-Izquierdo et al. (2002), simulating the physiological conditions in the stomach and small intestine. Brandon et al. (2012) studied the bioaccessibility of PAT in naturally contaminated apple sauce and spiked apple sauce and baby fruit reporting bioaccessibility values varying between 55 and 100% and used a value of 100% for risk assessment (Brandon et al., 2012). This study applied a three step static IVD model developed by Versantvoort et al. (2005), simulating the digestion in mouth, stomach and small intestine. This IVD model include a combination of the contaminated sample with a standard meal representative for the food mean intake of adults at a cooked meal to simulate the fed conditions. Assunção et al. (2014) reported a significant difference for the bioaccessibility values between artificially contaminated fruit juices (mean 27.65 ± 13.50%) and the same sample combined with a standard meal (mean 7.89 ± 4.03%) showing that the addition of a standard meal to the digestion procedure may affect bioaccessibility values.

Table 27. Bioaccessibility (%) results of PAT and OTA in processed cereal-based food samples (n=6), artificially contaminated. Bioaccessibility values are expressed as mean ± SE of three replicates. "F" and "W/o" samples represent samples with and without fruit in their content, respectively. (, $p \leq 0.05$ was considered statistically significant).*

	Patulin		**Ochratoxin A**	
	Single	**Mixture**	**Single**	**Mixture**
F1	70 ± 3.2	54 ± 0.9	95 ± 0.3	104 ± 1.2
F2	42 ± 1.2	63 ± 1.7	105 ± 1.5	103 ± 1.2
F3	56 ± 1.8	63 ± 4.0	97 ± 1.8	107 ± 0.7
W/o 1	77 ± 1.9	64 ± 1.5	98 ± 1.5	109 ± 0.1
W/o 2	39 ± 0.7	33 ± 0.7	102 ± 0.3	107 ± 1.5
W/o 3	30 ± 2.5	61 ± 0.6	102 ± 3.9	108 ± 0.6
Mean	52 ± 4.2	56 ± 2.7	100 ± 1.1*	106 ± 0.6*

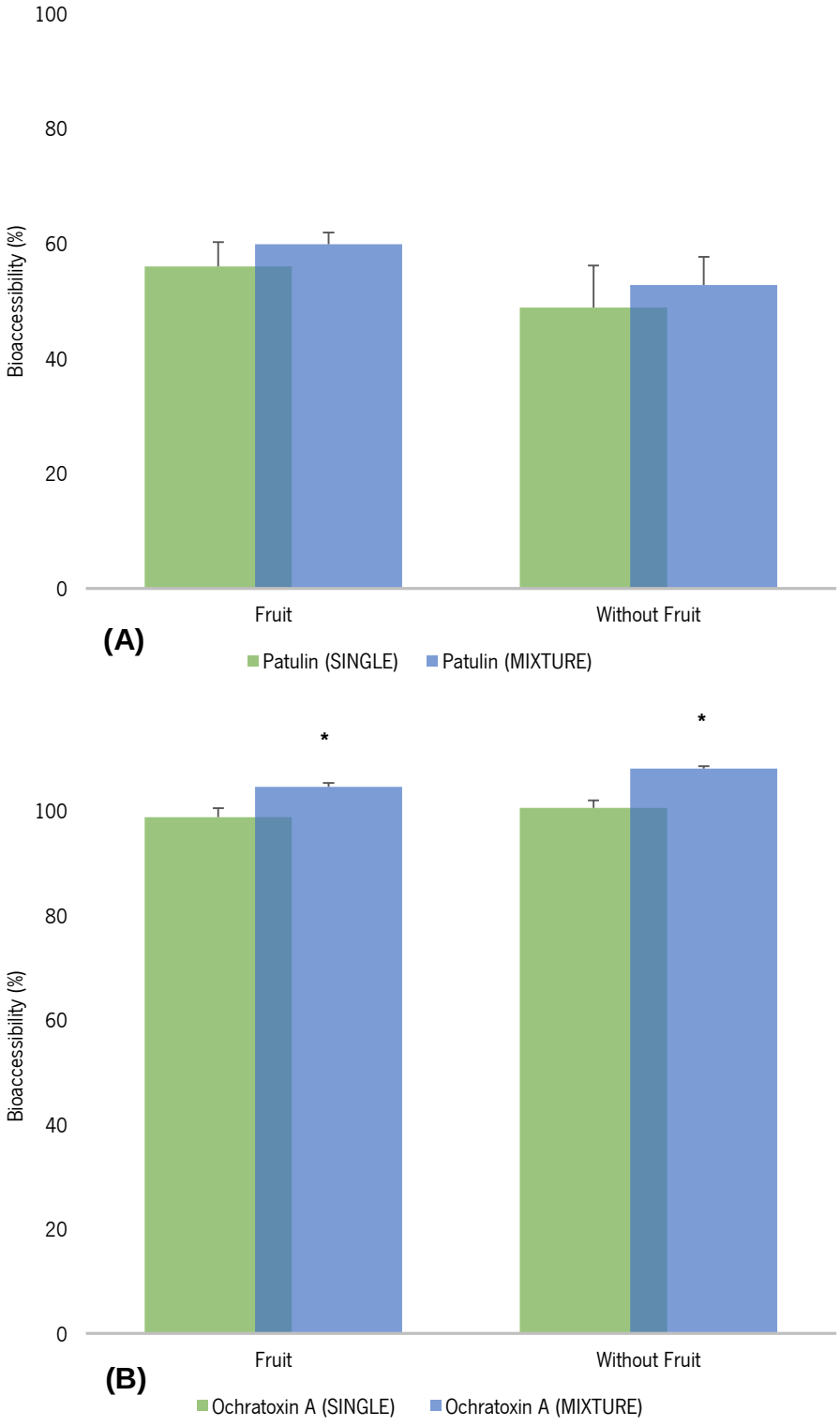

Figure 23. Bioaccessibility (%) results of PAT (A) and OTA (B) in processed cereal-based food samples (n=6) and its relationship with the presence of fruit. Statistically significant differences were determined when compared OTA in mixture, considering samples with and without fruit (, $p \leq 0.05$ was considered statistically significant).*

The Versantvoort et al. (2005) model was also applied to determine OTA bioaccessibility. Results revealed high bioaccessibility (100%) in naturally contaminated buckwheat (Versantvoort et al., 2005) and lower bioaccessibility in naturally (22%) and artificially contaminated infant food (29-32%) (Kabak et al., 2009). Versantvoort et al. (2005, 2004) suggested that bioaccessibility depends on several factors, such as food product, contamination level, compound and type of contamination (artificially *versus* naturally contaminated).

To the best of our knowledge, the present study is the first one reporting the bioaccessibility of PAT and OTA in artificially contaminated processed cereal-based foods and the first time that a standardised IVD model was applied to study mycotoxin bioaccessibility. Various digestion models have been proposed until now (González-Arias et al., 2013; Hur et al., 2011), often impeding the possibility to compare results across research teams. A large variety of enzymes from different sources have been used, differing in their activity and characterization. Differences in pH, mineral type, ionic strength and digestion time, which alter enzyme activity and other phenomena, may also considerably alter results (Minekus et al., 2014). The main feature of this IVD protocol, developed under the INFOGEST network, is the incorporation of enzymes on the basis of their activity, which must be determined in each of the reagents employed using assays described in an appended document (also available as easy to follow YouTube videos, at https://www.youtube.com/channel/UCdc-NPx9kTDGyH_kZCgpQWg). This document also include a procedure for assessing the bile salt concentration in the bile extract (Minekus et al., 2014). This protocol was also validated by three inter-laboratory IVD trials showing that the IVD INFOGEST method has led to an increased consistency that enables a better comparability of IVD studies in the future (Egger et al., 2015).

The present results constitute a first attempt to determine the impact of co-occurring mycotoxins in their bioaccessibility. OTA values revealed a significant difference when this toxin is alone or in mixture. However, further studies should be performed in order to corroborate these findings. Until now, few data is available on the bioaccessibility of co-occurring mycotoxins, namely for AFs (Kabak and Ozbey, 2012), AFB_1 and OTA (Kabak et al., 2009; Raiola et al., 2012b; Versantvoort et al., 2005), enniantins (Giuseppe Meca et al., 2012; Prosperini et al., 2013; Serrano et al., 2014), trichothecenes T-2 and HT-2 (Monaci et al., 2015). The lack of data on potential interactions between co-occurring mycotoxins contributes to an incomplete health risk assessment and thereafter, further studies with different combinations of mycotoxins need to be performed in order to corroborate the idea that the simultaneous presence of mycotoxins may affect their bioaccessibility. Relatively to the exposure to PAT and OTA through ingestion of processed cereal-based foods, the determination of PAT and OTA bioaccessibility values constitutes an

important step, considering that the knowledge of the real percentage of these mycotoxins that are available to be absorbed and to produce their toxic effects in the small intestine would enable an accurate approach to the exposure assessment and consequently, to mycotoxins risk assessment.

Previous studies demonstrated that the bioaccessibility of ingested mycotoxins strongly depends on the food constituents, namely the composition of food materials, which might affect the solubility of the mycotoxin, or its release in each food matrix (Kabak et al., 2009). In artificially or naturally contaminated food samples, mycotoxins are usually complexed to the food matrix. The formation of this complex is related to the amount of the micro and macronutrients contained in the food (Kabak et al., 2009), and the presence of fruits could constitute a factor that may interfere in the amount of nutrients contained in food and consequently induce some effects on the mycotoxins bioaccessibility values.

The aim of the present study is to determine mycotoxins bioaccessibility at intestinal level, corresponding to the amount of PAT and OTA that reaches intestine. With the generated data a contribution to a better understand of these mycotoxins impact at intestinal level was achieved. However, future studies should consider also the determination of mycotoxins release from food in mouth or stomach phases. The obtained results seems to be important for the mycotoxins risk assessment whereas different mycotoxins present different target organs and consequently influence the expected health effects. Some previous studies reported the mycotoxins mouth (De Angelis et al., 2014a, 2014b; Monaci et al., 2015) and gastric (De Angelis et al., 2014a, 2014b; Monaci et al., 2015; Raiola et al., 2012b) bioaccessibility. These studies revealed different bioaccessibility behaviours depending of the considered mycotoxin.

3.4. Estimated dietary intake of PAT and OTA and contribution of bioaccessibility for human risk assessment

Human risk assessment is a systematic process used to characterize the potential adverse effects resulting from exposure to hazardous agents (Lee and Ryu, 2015). Figure 24 shows the estimated daily intakes (EDI) of PAT and OTA through consumption of processed cereal-based foods by children, aged bellow three years old, from Lisbon region. The EDI values for PAT were 3.59 and 22.93 ng/kg bw/day, considering the mean and maximum (worst case) processed cereal-based foods consumption, respectively. The EDI values for OTA were 0.06 and 0.40 ng/kg bw/day, considering the mean and maximum (worst case) processed cereal-based foods consumption, respectively. The worst case EDI values represent approximately 7% of the PAT provisional maximum tolerable daily intake [PMTDI, 400

ng/kg bw/day (JECFA, 1996)] and 3% of the OTA provisional tolerable weekly intake [PTWI, 112 ng/kg bw/week (JECFA, 2007)].

For the characterization of risk, the hazard quotients (HQ) for PAT and OTA revealed values well below 1 (Table S2, Annex 2), suggesting no adverse health effects for consumers. For the mixture PAT and OTA, the concentration addition (CA) concept, one of the most frequently applied, was used to calculate the hazard index (HI). CA considers that the mixture act in the same way only differing in the concentrations for eliciting their toxic effects (EFSA, 2013). For the worst case scenario (H1 and maximum consumption), HI was also well below 1 and indicates a tolerable exposure level.

As previously referred, PAT is a mycotoxin mainly determined in fruit-based foods and consequently the food categories commonly selected to assess the exposure are those containing fruits and/or their by-products. Relatively to children exposure to PAT through consumption of food in Europe, some previous studies showed high EDI for these vulnerable groups but in all cases below the PMTDI, which are in accordance with the results obtained in the present study (Baert et al., 2007; Brandon et al., 2012; Cano-Sancho et al., 2009; González-Osnaya et al., 2007; Murillo-Arbizu et al., 2009; Piemontese et al., 2005; Piqué et al., 2013; Rodríguez-Carrasco et al., 2014a; Sirot et al., 2013). Relatively to OTA, exposure assessment studies have been mainly dedicated to estimate the ingestion through the consumption of target foods such as baby food or cereal-based food in the case of children and infants. Marin et al. (2013) reported that the most exposed population groups to OTA are expected to be infants and

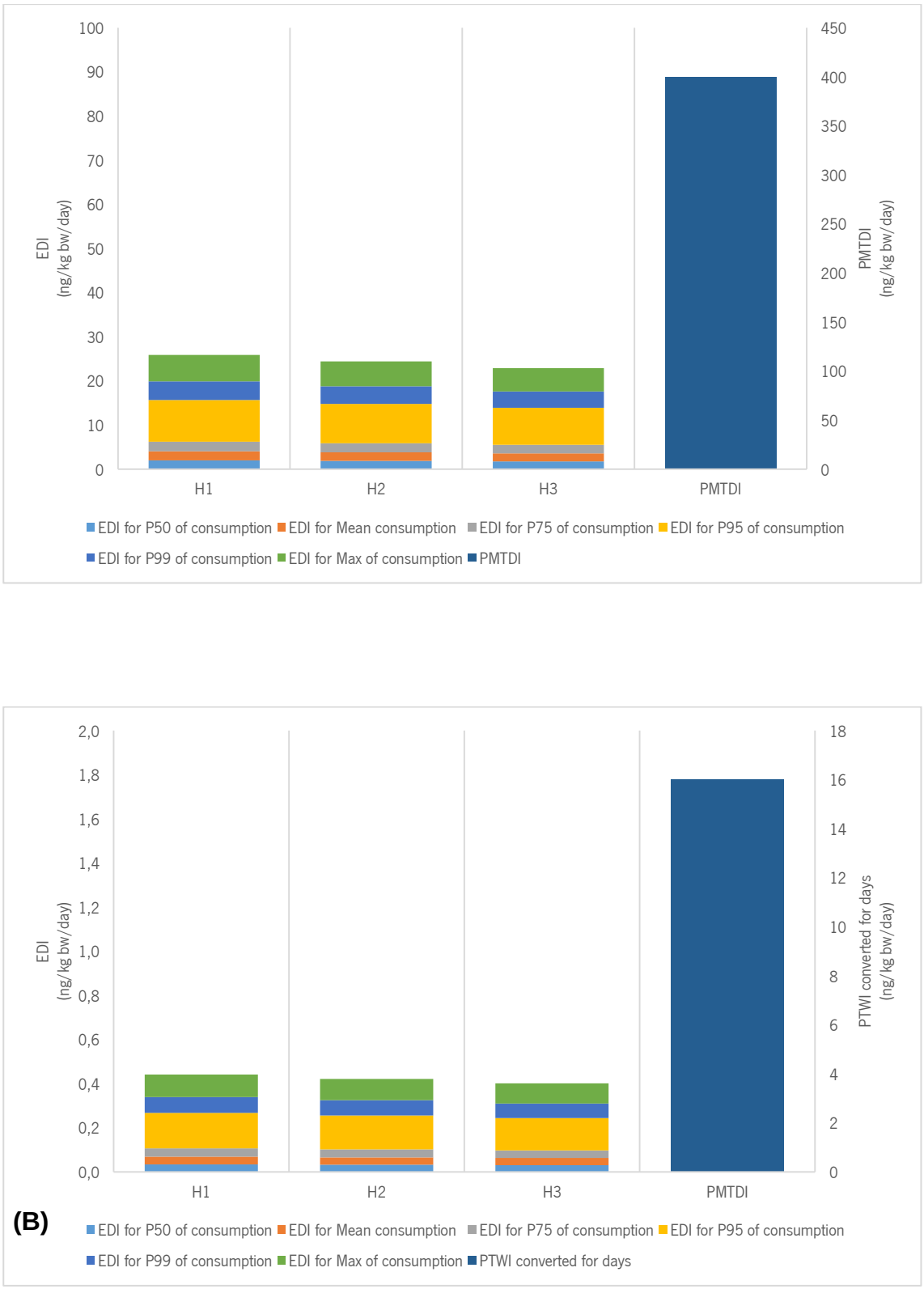

Figure 24. Estimated daily intake (EDI, ng/kg bw/day) of patulin (A) and ochratoxin A (B) through consumption of processed cereal-based foods by children under three years old. For calculations three different scenarios for non-detects (<LOD) were considered, H1: <LOD = LOD, H2: <LOD = ½ LOD and H3: <LOD = 0. Mean values for mycotoxin content, children weight (12 kg) and mean and percentiles (P) 50, 75, 90, 95 and 99 of consumption were used for daily intake calculations.

children. Previous studies evaluating the children exposure to OTA through consumption of food in Europe (Bakker et al., 2009; Brera et al., 2011; Coronel et al., 2012; Gauchi and Leblanc, 2002; Sirot et al., 2013; Thuvander et al., 2001; Villa and Markaki, 2009) showed that EDI were below the PTWI converted for days (16 ng/kg bw/day). In Portugal, a recent study assessed children (aged below 3 years old) exposure to OTA through consumption of breakfast cereals primarily marketed to be consumed by children and reported a OTA mean EDI of 0.011 ng/kg bw/day (Assunção et al., 2015) which is lower than the OTA mean EDI determined in the present study (0.069 ng/kg bw/day).

Taking into account the bioaccessibility results, OTA concentrations reach intestine almost unchanged; in contrast, PAT concentrations are significantly reduced during digestion process until reaching the intestine (Table 27). Considering the EDI (worst case, 0.402 ng/kg bw/day) and the mean bioaccessibility results (100%) of OTA, it is possible to conclude that the external and internal exposure are similar (0.402 ng/kg bw/day). Considering the EDI (worst case, 22.930 ng/kg bw/day) and the mean bioaccessibility results (52%) of PAT, the internal exposure (11.924 ng/kg bw/day) should be lower than the external one. These data are particularly significant considering that a bioaccessibility value of less than 100% implies that the internal exposure to the contaminant is lower than the external exposure, and consequently the internal exposure to the contaminant could be overestimated. Therefore, the use of the bioaccessibility values of a given contaminant as a maximum measure for oral bioavailability allow a convenient approach to assess the internal exposure and could be calculated by multiplying estimated mycotoxin daily intake by its bioaccessibility value, per product (Lei et al., 2015; Versantvoort et al., 2005).

Some limitations should be taken into account in the present risk assessment. The method used to determine mycotoxin bioaccessibilities is based in adult physiological parameters, which is expected to be different from children digestive parameters, especially for infants. Some authors reported the key parameters of gastroduodenal digestion in infants and young children with the aim of defining them for *in vitro* modelling purpose (Bourlieu et al., 2014; Havenaar et al., 2013). However, to achieve a better comparability of obtained results, a standardised method should be developed for these specific age groups. Additionally, the presence of mycotoxins in the intestinal fluid of a child could cause more significant damage to the intestinal enterocytes due to the reduced dimension of the intestinal epithelium compared to adult (Raiola et al., 2015). It is important to highlight that the present study only concerns the risk associated with the children exposure to mycotoxins through processed cereal-based foods consumption and it is expected that the exposure resulting from the consumption of all foods present in a child diet could increase the intake of mycotoxins in general, and of PAT and OTA in particular and,

consequently, the inherent risk to children's health. The present study was a snapshot of the studied population exposure to mycotoxins and changes over time could occur affecting the intake of mycotoxins.

4. CONCLUSIONS

Risk assessment is a complex process of evaluating the probable incidence of an adverse health effect to humans under various conditions of exposure. Co-occurrence of mycotoxins in foods poses several challenges for risk assessment and demanding a big effort from scientific community, risk assessors and managers in order to integrate knowledge from different scientific areas. With the present study a better understand of the risk of Portuguese children aged between 0 and 3 years old exposed to patulin and ochratoxin A through consumption of processed cereal-based foods, was achieved integrating occurrence, bioaccessibility, consumption and toxicological data. PAT and OTA co-occurred in processed cereal-based food samples available at Portuguese market and corroborate the idea that co-occurrence of mycotoxins represents the rule and not the exception. However, none previous studies determined the potential interaction between these mycotoxins, highlighting the need for further toxicological studies on their combined toxicity. These two mycotoxins are enteropathogenic and consequently a potential interactive effect could result in a higher impact on intestinal health. Within this domain, the generated bioaccessibility results, using for the first time the standardised *in vitro* digestion method, showed that a significant portion of PAT and especially OTA can reach the small intestine and thus, be available to produce their toxic effects and cross the intestinal barrier. Taking into account the co-occurrence and the bioaccessibility results, this study reported a tolerable exposure to these mycotoxins representing a low risk for Portuguese children, through consumption of processed cereal-based foods. The integration of all these data will support and prioritize the establishment of future regulatory measures that will contribute to protect children health.

SECTION 3: INTESTINAL TOXIC EFFECTS OF MYCOTOXINS DETECTED IN FOODS INTENDED FOR CHILDREN CONSUMPTION

MANUSCRIPT 5: **A MULTI-ENDPOINT APPROACH TO THE COMBINED TOXIC EFFECTS OF PATULIN AND OCHRATOXIN A IN HUMAN INTESTINAL CELLS**

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ABSTRACT

Humans and animals can be exposed to a complex and variable combination of mycotoxins co-occurring in food. These toxins may affect their health, especially if considered the long-term exposure. After mycotoxins ingestion, intestinal mucosa constitutes the first biological barrier and consequently it could be exposed to high concentrations of these toxins. Considering the natural mycotoxins co-occurrence, and the potential consequences of exposure to multiple mycotoxins, the present study aimed to characterize, for the first time, *in vitro* combined cytotoxic (through cell viability determination) and membrane integrity (through transepithelial electrical resistance, TEER) effects of patulin (PAT) and ochratoxin A (OTA), two enteropathogenic mycotoxins. Caco-2 cells were used as a model of intestinal epithelial cells and two conceptual mathematical models, concentration addition (CA) and independent action (IA), were applied for a multi-endpoint approach (cytotoxicity and barrier integrity) to ascertain putative interaction effects between mycotoxins. Relatively to cell viability, a dose-ratio deviation was verified, implying that OTA was mainly responsible for synergism when dominant in the mixture, while when PAT was at higher doses than OTA, this pattern was changed to antagonism (IA model). Regarding membrane integrity, evaluated through TEER, a potential synergism was attained at low levels of both mycotoxins, changing to antagonism at higher doses (CA model). Although the obtained results demand an accurate MoA clarification, they highlight a potential threat for public health, considering the potential mycotoxins-associated adverse health effects. Consequently, these results should be taken into account in future research studies in order to become reflected in future risk assessments and regulatory actions.

Keywords: Mycotoxin mixtures; Gastrointestinal toxicity; Interaction effects; Cytotoxicity; Barrier integrity

1. INTRODUCTION

Nowadays, different studies have demonstrated that humans and animals are daily exposed to multiple chemical contaminants. The potential combined effects of exposure to multiple chemicals can be higher than those caused by an individual chemical contaminant which constitutes a concern among policy makers (Alassane-Kpembi et al., 2016; Dorne and Fink-Gremmels, 2013; Pose-Juan et al., 2016; Smith et al., 2016).

Food is a potential reservoir of chemical contaminants and mycotoxins led the 2015-notifications by hazard category according to the annual report by the Rapid Alert System for Food and Feed (Pose-Juan

et al., 2016; RASFF, 2015). Mycotoxins are a problematic and toxic group of small organic molecules that are produced as secondary metabolites by several fungal species that colonise crops (Turner et al., 2015). The ingestion of these food contaminants may induce various chronic and acute effects on humans and animals, such as hepatotoxic, genotoxic, immunosuppressive, estrogenic, nephrotoxic, teratogenic, and/or carcinogenic effects (Marin et al., 2013; Rocha et al., 2014). Additionally to these toxic effects and following the ingestion of contaminated food, intestinal mucosa may be exposed to high concentrations of toxicants including mycotoxins, which could lead to disruption of intestinal functions. The intestinal mucosa constitutes the largest and most important barrier to prevent the passage from the external environment into the organism of harmful intraluminal substances, including foreign antigens, microorganisms and their toxins (Maresca and Fantini, 2010; Pinton and Oswald, 2014; Puel et al., 2010). In fact, the intestinal barrier was considered as an emerging target in the toxicological assessment of mycotoxins (Akbari et al., 2016). Several surveys have reported the natural co-occurrence of mycotoxins from all over the world (Smith et al., 2016). Their co-occurrence in food and feed is explained by three different reasons: i) some fungi produce several mycotoxins simultaneously, ii) food and feed commodities can be contaminated simultaneously by several fungi or in quick succession, and iii) the complete diet comprised different commodities (Alassane-Kpembi et al., 2016). Therefore, the risk of human co-exposure to multiple mycotoxins is real, raising a growing concern about their potential impact on human health (Assunção et al., 2016c). Some of the previous mycotoxin occurrence surveys reported the co-occurrence of mycotoxins in food, including food commodities usually consumed by specific vulnerable population groups, as children. First studies reporting the presence of mycotoxins in foods intended for children consumption in Portugal were described by Barreira et al. (2010) concerning the occurrence of patulin in apple based products and by Alvito et al. (2010), concerning the co-occurrence of aflatoxins and ochratoxin A (OTA) in baby foods. Recently, Assunção et al. (2016b) reported the co-occurrence of two mycotoxins, patulin (PAT) and OTA in a significant number of samples of processed cereal-based foods destined for children. These two mycotoxins, both produced by moulds of genera *Aspergillus* and *Penicillium*, jointly with deoxynivalenol, were considered amongst the best known enteropathogenic mycotoxins that are able to alter the intestinal functions (Maresca et al., 2008). Considering the natural mycotoxins co-occurrence, there is an increasing concern about the hazard of exposure to multiple mycotoxins. Consequently, the toxicity of multiple mycotoxins cannot always be predicted based on their individual toxicities (Assunção et al., 2016c; Speijers and Speijers, 2004). The theoretical models developed to explain the behaviour of chemical mixtures are based on two reference models that assume no interaction between chemicals, and are used to describe the joint toxicity based on the mode of action (MoA) of the single chemicals. The dose or concentration addition (CA) model (Loewe and Muischnek,

1926) assumes that chemicals have the same MoA. This model has a concentration-based summation of toxicity of similarly acting chemicals, scaled to reflect their relative toxicities. Alternatively, the model of independent action (IA) (Bliss, 1939) assumes that chemicals could induce toxic effects through different MoA, and their effects are therefore statistically independent of each other. Chemical mixtures can also be composed of chemicals whose MoA is unknown or ambiguous and, consequently in such cases, both CA and IA models are applied for actual effect prediction (Loureiro et al., 2010). For some combinations of chemicals, they deviate from the pattern of the reference models: the chemicals may have a greater (synergism) or smaller (antagonism) observed effects than predicted by the models, or the type of interaction identified by the model may change depending on the absolute (dose level) or relative (dose ratio) concentration of each chemical (Jonker et al., 2005; Loureiro et al., 2010; Tavares et al., 2013). The complete knowledge about the combined health risk from multiple mycotoxins exposure is far from being well characterized. Intestine is the first target organ for mycotoxins and consequently the potential direct effects between co-occurring mycotoxins should be described. Despite some mycotoxin mixtures were evaluated for the potential combined toxic effects in the last years, none previous work focused on the effects of simultaneous intestinal exposure to PAT and OTA. Considering the evidence on the co-occurrence of PAT and OTA in foods, and the inherent human exposure, the potential combined toxic effects of these two mycotoxins should be addressed. The present research study aimed to characterize *in vitro* the combined cytotoxic (through cell viability determination) and barrier integrity effects of the binary mixture of mycotoxins, PAT and OTA, on Caco-2 cells, used as a model of intestinal epithelial cells. To ascertain putative interactions, the two conceptual mathematical models, CA and IA models, were applied for the two different endpoints (cytotoxicity and barrier integrity).

2. MATERIAL AND METHODS

2.1. Toxins and cell culture

Patulin was ordered from Sigma-Aldrich (St. Louis, USA), dissolved in sterile Mili-Q water (18.2 MΩ cm) and stored at -80˚C. Ochratoxin A was purchased from Biopure (Tulln, Austria), dissolved in dimethylsulfoxide (DMSO) and stored at -80˚C. Working dilutions were prepared in cell culture medium. The highest DMSO concentration (0.6 % of DMSO in culture medium) of working dilutions was tested (MTT, TEER) and results were not significantly different from controls (cell exposure to culture medium).

Caco-2 human colon cancer cells (ATTC; Manassas, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acids

and 0.2% gentamycin, all from Sigma-Aldrich (St. Louis, USA). Cells were kept in a humidified incubator at 37 °C and 5% CO_2. The cells were passaged with 0.05% trypsin/0.02% EDTA (Sigma-Aldrich, St. Louis, USA) in DPBS when confluence reached around 70%. The medium was changed twice a week.

2.2. MTT assay

Caco-2 cells were cultured to confluence in 96-well tissue culture plates and then were exposed to serial dilutions of single or combination of mycotoxins or vehicle (DMSO in culture medium) for 24 h at 37˚C, in 5% CO_2. Mycotoxin solutions were diluted in serum-free medium (treatment medium). For OTA, the final toxin concentrations tested in the wells ranged from 1 μM to 200 μM. Relatively to PAT, individual results were previously reported (Assunção et al., 2016a). For the mycotoxin combinations, different toxin concentrations were tested, ranging between 0.7 μM to 18 μM for PAT and 6.7 μM to 180 μM for OTA (Figure 25A). After 24 h of cells exposure to mycotoxins, the treatment medium was removed and cells were washed twice with pre-heated DPBS. MTT solution (Calbiochem, Darmstadt, Germany) was reconstituted in DPBS, and then diluted in culture medium to a final concentration of 0.5 mg/mL; this solution was added to the cells. The plates were then incubated for approximately 3 h at 37 °C, in 5% CO_2, to allow MTT incorporation and subsequent formation of formazan crystals by the viable cells. After this incubation, the MTT solution was removed and DMSO was added. The plates were shaken for 15-30 minutes at room temperature protected from light to ensure adequate solubilisation of crystals; immediately after this step, the results were measured spectrophotometrically at 570 nm (reference filter: 690 nm) using a Multiskan Ascent spectrophotometer (Thermo Labsystems). This experiment was performed in triplicate and in each of them, three replicate wells were used for each treatment condition. Cell viability of treated cells was calculated relatively to the mean absorbance of the negative control (which is assumed to correspond to 100%). The results were expressed as the mean value (± SEM) of the three independent experiments.

2.3. TEER assay

Caco-2 cells were seeded on 0.4 μm pore size cell culture inserts (Falcon™ Cell Culture Inserts, Fisher Scientific, USA) at a density of $3.0x10^5$ cells/well. The experiments were performed after 18–21 days of cell monolayer growth. TEER values were monitored using a Millicell Voltohmmeter with chopstick electrodes (Millipore, USA). Only inserts with TEER values higher than 500 Ω cm^2 have been considered

acceptable and used. PAT (5, 25, 50 and 100 µM), OTA (10, 100 and 200 µM) and their combinations (Figure 25B) were applied at the apical side through 24 h of exposure. The TEER was measured at the beginning and the end of the experiment. TEER was normalized by the area of the monolayer and the background TEER of blank filter was subtracted from the TEER of the cell monolayer. Results were expressed as % Initial TEER which was calculated considering the initial TEER values measured at 0 h of incubation and after 24 h of incubation. The experiments were performed in triplicate and three replica were considered for each exposure condition.

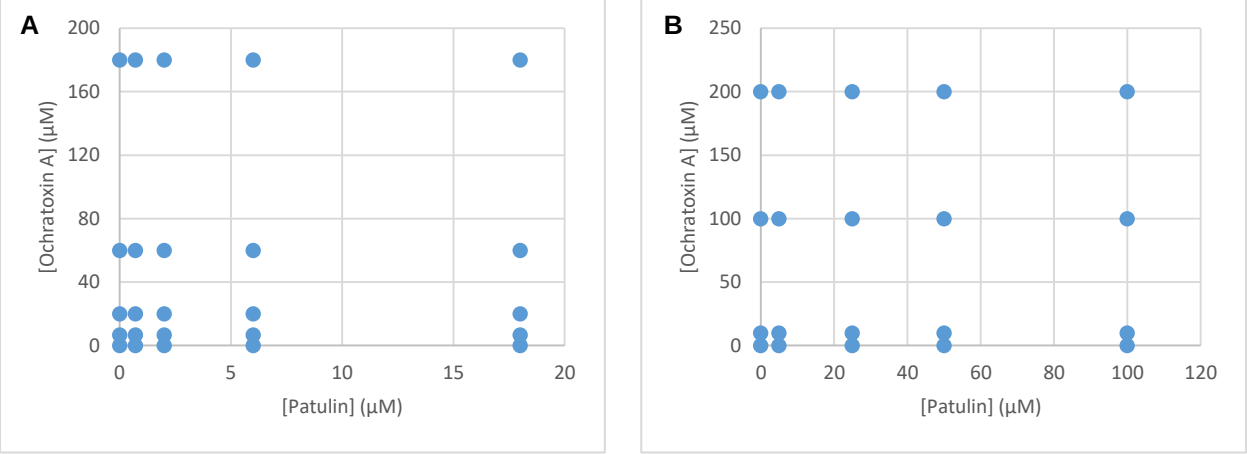

Figure 25. Full factorial experimental design for cell viability (A) and trans-epithelial electrical resistance (TEER) (B) assessment of patulin and ochratoxin A combinations.

2.4. Data analysis

Mycotoxin interactions were analysed by comparing the observed effects of each endpoint (cell viability and TEER) with the expected effects, calculated from single toxin obtained in the same experiments, and according to experimental design represented in Figure 25. CA and IA reference models were used to derive patterns for joint effects of mixtures (Bliss, 1939; Loewe and Muischnek, 1926). The CA model can be regarded as dilutions of one another and is mathematically described by Equation 1:

$$\sum_{n}^{i=1} \frac{C_i}{EC_{xi}} = 1$$

where C_i is the dose used for mycotoxin i in the mixture and EC_{xi} is the effective dose of mycotoxin i that produces the same effect (x%) as the whole mixture. The IA model is based on the hypothesis that the relative effect of a toxicant remains unchanged in the presence of another chemical (Jonker et al., 2005, 2004) and is mathematically expressed by Equation 2:

$$Y = u_{max} \prod_{i=1}^{n} q_i(C_i)$$

where Y denotes the biological response, C_i is the concentration of the mycotoxin *i* in the mixture, $q_i(C_i)$ the probability of non-response, u_{max} the control response for the selected endpoint and $\prod$ the multiplication function. In the second step of the data analysis, after fitting data to CA and IA models (using the method of maximum likelihood, by minimising the objective function (L) and statistically compared through likelihood testing), these models were extended according to Jonker et al. (2005), with deviation functions to describe synergistic/antagonistic interactions, dose-level, level, and dose-ratio dependency. For the deviation functions, the extra parameters needed (a and b, Table 28) form a nested framework. When a descriptive deviation model was identified, the parameter values reflected the effects pattern as in Table 28. The best fit was selected by a Chi-square test based on the minimisation of the objective function using the binominal log likelihood. The biological meaning of the additional deviation parameters was interpreted according to Table 28, which was developed based on Jonker et al. (2005).

Table 28. Data analysis for interpretation of mycotoxin interactions. Additional parameters (a and b) define the functional form of deviation pattern from the reference models: concentration addition (CA) and independent action (IA). Adapted from Jonker et al. 2005.

Deviation pattern	Parameter a (CA and IA)	Parameter b (CA)	Parameter b (IA)
Synergism/Antagonism (S/A)	a>0: antagonism a<0: synergism		
Dose-ratio dependent (DR)	a>0: antagonism except for those mixture ratios where negative b value indicates synergism a<0: synergism except for those mixture ratios where positive b value indicates antagonism	b_i>0: antagonism where the toxicity of the mixture is caused mainly by toxicant *i* b_i<0: synergism where the toxicity of the mixture is caused mainly by toxicant *i*	
Dose-level dependent (DL)	a>0: antagonism low dose level and synergism high dose level	b_{DL}>1: change at lower IC_{50} level b_{DL}=1: change at IC_{50} level	b_{DL}>2: change at lower IC_{50} level b_{DL}=2: change at IC_{50} level
	a>0: synergism low dose level and antagonism high dose level	0<b_{DL}<1: change at higher IC_{50} level b_{DL}<0: no change but the magnitude of S/A is DL dependent	1<b_{DL}<2: change at higher IC_{50} level b_{DL}<2: no change but the magnitude of S/A is effect level dependent

The obtained data (from cell viability and TEER measurements) were converted into toxic units (TUs), according to Equations 3 and 4:

$$z_i = \frac{TU_x}{\sum_{j=i}^{n} TU_{x_j}}$$

where $TU_x = \frac{c_i}{EC_{x_i}}$

The c_i/EC_{xi} corresponds to the dimensionless toxic unit (TU_{xi}) and in the present study, quantifies the relative contribution of each mycotoxin to the toxicity (z_i) in the mixture (Jonker et al., 2005).

2.5. Statistical analysis

Statistical analysis were performed using IBM® SPSS Statistics 20 software (IBM, Armonk, NY). Data were expressed as means ± SEM (standard error) of three independent experiments. Differences between mycotoxin treatments were analysed by the non-parametric Kruskal-Wallis test (all groups) and the Mann-Whitney (between groups) with a level of significance $p < 0.05$.

The existence of a dose-response curve was determined by regression analysis, and the IC_{50} (inhibitory concentration 50%) values for the individual mycotoxins were calculated from the curve equation that best fitted the experimental data, using Sigma Plot® (Systat Software Inc., San Jose, CA, USA).

3. RESULTS

Individual and combined cytotoxicity of PAT and OTA on Caco-2 cells

The cytotoxic effects of PAT and OTA on non-differentiated Caco-2 cells were evaluated, individually and in combination, through the MTT assay. Both mycotoxins showed a dose-dependent toxicity toward Caco-2 cells, as presented in Figure 26. When compared to negative control, a significant viability reduction was verified for concentrations equal or higher than 25 μM and 40 μM ($p < 0.05$), for PAT and OTA respectively (Figure 26). The IC_{50} values were derived for each mycotoxin considering the model that best fitted each dose-response curve (sigmoidal model). The IC_{50} for PAT was estimated as 15 μM. The IC_{50} for OTA was estimated as 145 μM. Based on their IC_{50}, it is possible to anticipate that non-differentiated Caco-2 cells are more sensitive to PAT than to OTA. The highest concentrations tested for single

mycotoxins decreased cell viability by almost 80% and 60% compared to control, for PAT and OTA respectively.

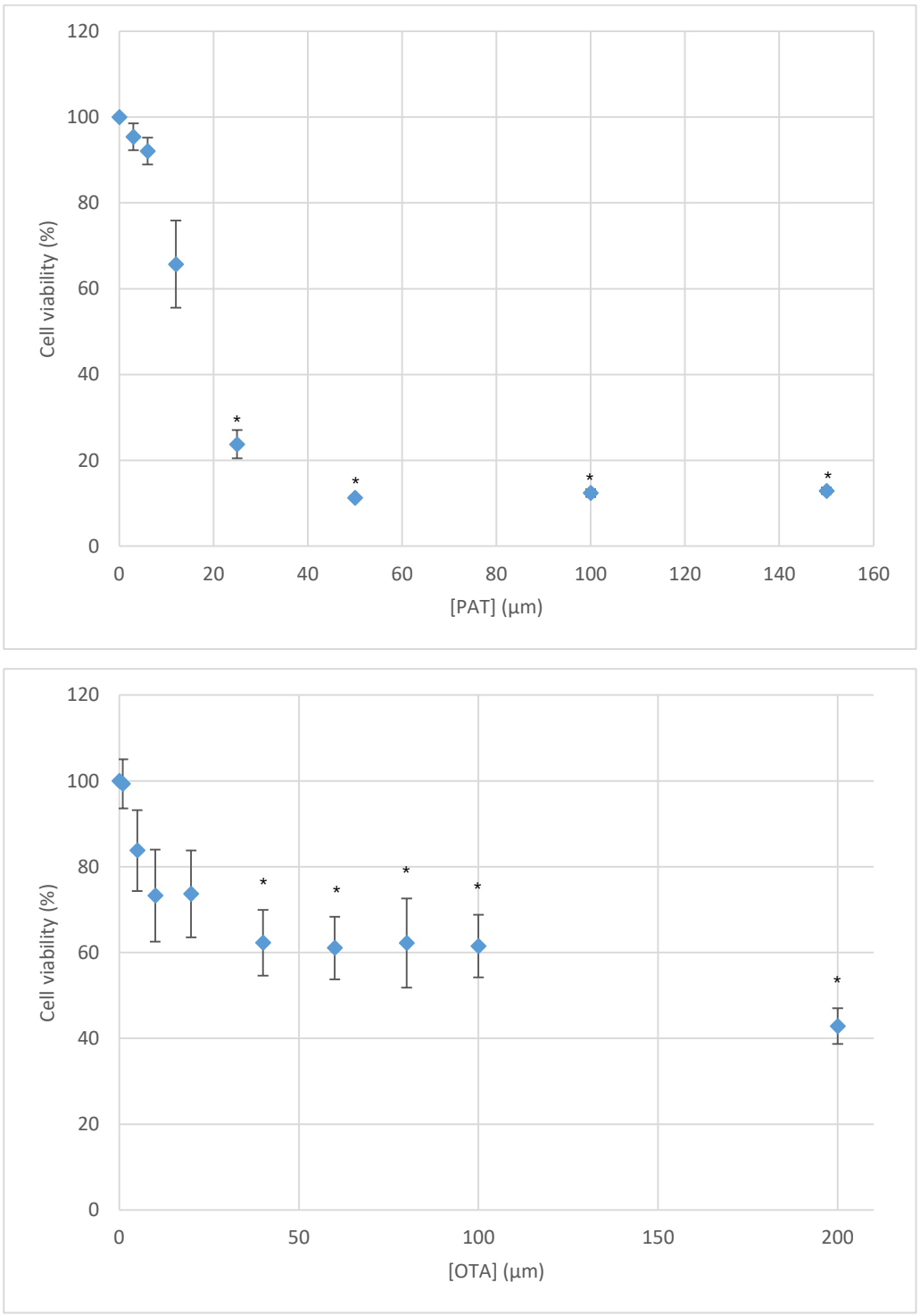

Figure 26. Effect of PAT and OTA treatment on Caco-2 cells viability after 24 h of exposure to different concentrations, using a MTT assay. Values are expressed as mean ± SEM of three independent experiments, performed in triplicate. (*) represent significant difference as compared to control values (negative control).

The results of the cytotoxic effects of single PAT and OTA and of several combinations in Caco-2 cells as assessed by the MTT assay are presented in Figure 27. The mycotoxins combined effects deviated from the effects obtained for each single toxin suggesting the existence of interactions. The lowest PAT concentrations tested in the mixture (0.7 μM, 2 μM and 6 μM) did not induce a marked decrease in cell viability while OTA alone was able to cause a concentration-dependent viability loss. Thus, it can be seen that for this concentration range of PAT the effect of OTA largely predominated. For the highest tested PAT concentration in mixture (18 μM), the combined cytotoxic effects were lower than those expected from the summation of the individual OTA and PAT effects suggesting an antagonistic effect.

Considering the application of both conceptual models, CA and IA, a good fit to the observed results was verified. By extending both models to improve data fitting, the CA model showed no further improvement (Table 29). On the other hand, the IA model extension to synergism or antagonism provided a better fit dependent on the mycotoxins ratio (SS=713.62; R^2=0.57). In fact, this dose-ratio deviation indicates that the ratio of each mycotoxin in the mixture influences their interactive effect. Data modelling thereby confirmed that OTA was mainly responsible for synergism when dominant in the mixture, while this pattern was changed to antagonism for the highest PAT concentrations.

Individual and combined effects of PAT and OTA on TEER of differentiated Caco-2 cells

The cell viability effects as a consequence of exposure to mycotoxins is likely to affect the maintenance of intestinal epithelial monolayer and therefore the intestinal barrier. The TEER value is generally accepted to reflect Caco-2 cell monolayer integrity (Assunção et al., 2016a). Figure 28 presents single PAT and OTA effects and their combined effects on TEER of differentiated Caco-2 cells. Comparing the observed combined effects (PAT + OTA), a statistical significance was detected between 5 μM and 25 μM of PAT and the observed effects after single exposure to PAT (Figure 28, *, $p < 0.05$). For the 50 μM PAT exposure, statistical significant differences were detected ($p < 0.05$) when comparing TEER reductions associated to combined exposure (for the three levels of OTA in combination with 50 μM of PAT) and single exposure to both mycotoxins (Figure 28, *, #). For PAT levels higher than 50 μM, statistical significant differences were only verified between combined effects and single OTA effects (Figure 28, #, $p < 0.05$).

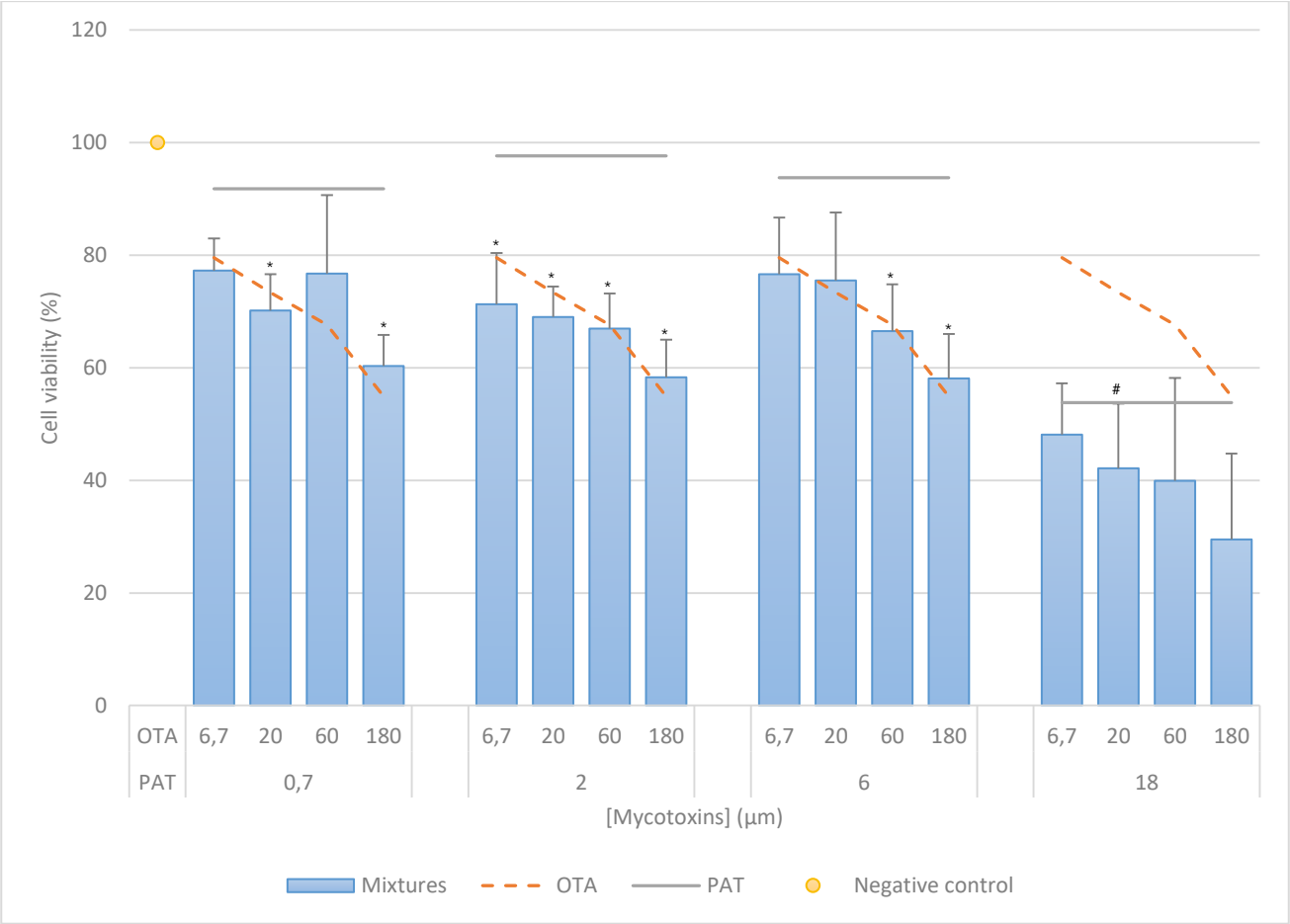

Figure 27. Comparative toxic effects of single mycotoxins (PAT and OTA, lines) and their binary combinations (bars) on cell viability of Caco-2 cells. Caco-2 cells were exposed for 24 h to serial dilutions of toxins alone or in combination and cell viability was assessed by the MTT assay. Data are expressed as mean ± SEM of three independent experiments, performed in triplicate. (*) represent significant differences ($p < 0.05$) between combined effect and single PAT effects. (#) represent significant differences ($p < 0.05$) between combined effect and single OTA effects.

For PAT concentrations of 5 and 25 µM no effect was observed, both for the individual and the combined effect of these mycotoxins, *i.e.*, PAT did not influence the single effect of OTA on TEER of Caco-2 cells. On the other hand, the mixtures that include the two highest PAT concentrations (50 and 100 µM) were able to markedly decrease the TEER values comparatively to single effects, accompanying those results observed for single PAT exposure. The observed combined effects were, however, milder than those that could have been predicted from the individual effects summation, suggesting an antagonistic effect at these dose levels. The results of data modelling showed that the best fit was observed by the CA model and a dose level deviation was noted (SS=379.07; R^2=0.80), where synergism was attained at low levels of both mycotoxins, changing to antagonism at higher concentrations (Table 29). This shift in pattern occurred at doses higher than the IC_{50} level of the mixture.

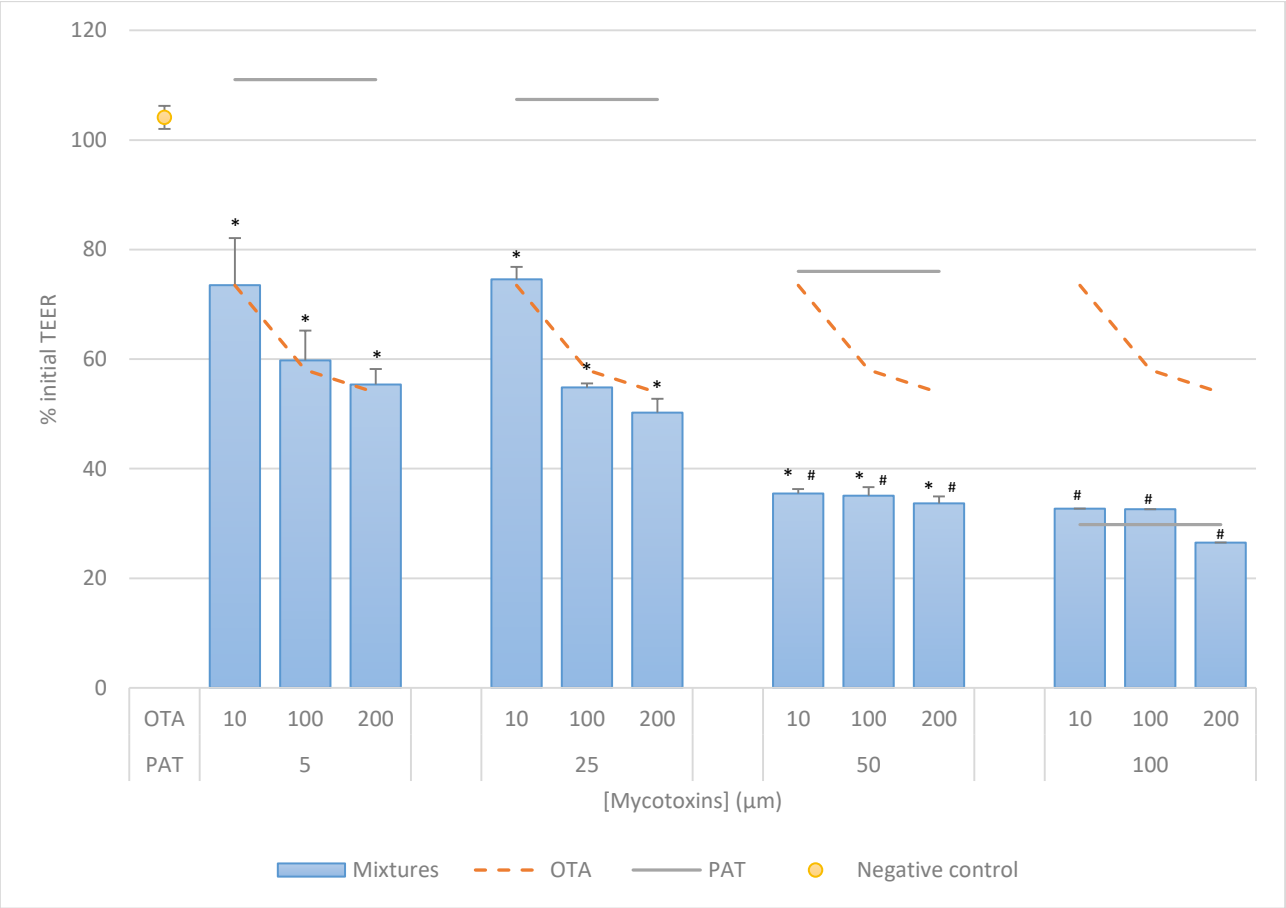

Figure 28. Comparative effects of single mycotoxins (PAT and OTA, lines) and their binary combinations (bars) on trans-epithelial electrical resistance (TEER) of differentiated Caco-2 cells. Caco-2 cells were exposed apically for 24 h to serial dilutions of toxins alone or in combination. % of initial TEER was calculated considering the initial TEER values measured at 0 h of incubation and after 24 h of exposure. Data are expressed as mean ± SEM of three independent experiments, performed in triplicate. (*) represent significant differences ($p < 0.05$) between combined effect and single PAT effects. (#) represent significant differences ($p < 0.05$) between combined effect and single OTA effects.

4. DISCUSSION

Individual and combined effects of PAT and OTA on Caco-2 cells

PAT and OTA were considered amongst the best known enteropathogenic mycotoxins and are able to alter functions of the intestine (Maresca et al., 2008). The present results demonstrated that PAT and OTA exposure produced toxic effects on Caco-2 cells, affecting the cell viability and membrane integrity.

Table 29. Summary of the analysis performed to evaluate the combined effects of patulin (PAT) and ochratoxin A (OTA) on cell viability and membrane integrity of Caco-2 cells.

Endpoint	Model	SS	R^2	$p(X^2)$	Parameter a	Parameter b	Pattern
Cell Viability	CA	721.20	0.57	$1.0x10^{-202}$	-	-	Additivity
	Deviation	713.62	0.57	$5.0x10^{-03}$	-0.99	2.18	**IA** Dose ratio: OTA is mainly responsible for synergism PAT is mainly responsible for antagonism (*i.e.* at high doses of OTA and low of PAT, the pattern is synergisms; at high doses of PAT and low of OTA, the pattern is antagonism)
	IA	721.61	0.57	-	-	-	
Membrane integrity (TEER)	CA	731.07	0.62	-	-	-	-
	Deviation	379.07	0.80	$3.7x10^{-77}$	-2.70	0.49	**CA** Dose level: Synergism low dose level and antagonism high dose level ($a<0$) and as $0<b_{DL}<1$, the change from synergism to antagonism occurs at higher IC_{50} level.
	IA	823.95	0.57	-	-	-	-

CA = concentration addition model; IA = independent action model; SS = sum of square residuals (objective function); R^2 = determination coefficient; $p(X^2)$ indicates the outcome of the likelihood ratio test; Parameters a and b were obtained from the modelling: a and b are the parameters obtained from the deviation functions ([a>0] antagonism or [a<0] synergism; [b>0] decreased or [b<0] increased toxicity where the first named chemical of the mixture dominates).

Cell viability results showed that both mycotoxins affected individually non-differentiated Caco-2 cells viability in a dose-dependent manner. Previous authors evaluated the cell viability impact on intestinal cell lines exposed to these two mycotoxins individually (Assunção et al., 2016a; Berger et al., 2003; Clarke et al., 2014; Creppy et al., 2004; Kawauchiya et al., 2011; Mahfoud et al., 2002; Maresca et al., 2001; McLaughlin et al., 2009, 2004; Romero et al., 2016; Tavares et al., 2013). For both mycotoxins, a wide variation in the obtained results was verified, among intestinal cell lines (Caco-2 cells, HT-29), different endpoints [MTT, neutral red uptake, trypan blue dye-exclusion or lactate dehydrogenase (LDH) release], time (24h or 48h of exposure) and concentrations (ranging from nM to μM) of exposure. Although PAT effects on kidney cells were mostly evaluated, the authors agreed that intestinal cells are sensitive to PAT in a dose-dependent manner (Assunção et al., 2016a; Speijers et al., 1988). In fact, *in vivo* studies revealed that PAT severely damaged the kidney, intestinal tissue and immune system (de Melo et al., 2012). The mechanisms of inducing PAT cytotoxicity are not completely understood. One of the most likely cellular targets of PAT is the sulfhydryl group of cysteine and glutathione (GSH) leading to depletion

of glutathione and subsequent increased generation of reactive oxygen species (ROS). Consequently, PAT is believed to induce cytotoxicity by forming covalent adducts with essential cellular thiols (organic compounds that contain a sulfhydryl group), by which it inhibits the activity of many enzymes (de Melo et al., 2012; Pfenning et al., 2016; Puel et al., 2010). Relatively to OTA, kidney is generally considered the main target organ for its toxicity. Additionally, although less toxic to intestinal cells than PAT, some authors considered the gastrointestinal tract as a possible target organ for OTA as well (Akbari et al., 2016; Bouhet and Oswald, 2005; Grenier and Applegate, 2013; McLaughlin et al., 2004), especially due to its capacity to inhibit cellular protein synthesis and to generate reactive oxygen as well as nitrogen species. In addition to these effects, OTA could also inhibit histone acetyltransferase, leading to disruption of mitosis and chromosomal instability formation of DNA adducts, particularly deoxyguanosine adducts (Akbari et al., 2016; Bennett and Klich, 2003; Hadjeba-Medjdoub et al., 2012; Pfohl-Leszkowicz and Manderville, 2012; Turner et al., 2009; Zepnik et al., 2001). The present study evaluated for the first time the combined effects on cell viability of Caco-2 cells as a result of simultaneous exposure to PAT and OTA. Several concentrations were tested in binary combinations to evaluate potential interactive effects. CA and IA conceptual models were used to identify potential combined toxic effects. It was verified a good fit to both conceptual models. As far as previously referred, the MoA of PAT is mainly associated to formation of covalent adducts with essential cellular thiols and consequently inhibition of enzyme activity. Different MoA was described for OTA. This toxin apart from inducing DNA damage, also disrupt protein synthesis, mitosis (through blockage of histone acetyltransferase) and cell death. These descriptions suggest apparently that these two mycotoxins act by distinct mechanisms and, then, the IA model would be mostly appropriate to describe the combined effects. However, it was also reported that both mycotoxins induce oxidative stress, which could suggest that a common MoA could occur. Consequently, two different perspectives should be taken into account to interpret data modelling results. On one hand, considering the IA model, a dose-ratio deviation was verified, implying that OTA was mainly responsible for synergism when dominant in the mixture, while when PAT was at higher doses than OTA, this pattern was changed to antagonism. On another hand, the CA model could also reflect the combined effects of these two mycotoxins and additive effects were showed on Caco-2 cells. Although using different cell lines, few previous authors evaluated the combined effects of these two mycotoxins. Heussner et al. (2006) investigated the interactive effects between PAT and OTA on a porcine renal cell line (LLC-PK1) and using the MTT assay as a cytotoxicity endpoint. The authors reported that the tested toxicity was higher than the predicted (simple additive) and the authors suggested that PAT and OTA simultaneously produced synergistic effects on the studied renal cells. The combined effects on immune cells were also evaluated. Bernhoft et al. (2004) determined the combined effects of PAT and OTA on proliferation of porcine

lymphocytes, obtained from piglets. The proliferation of lymphocytes were determined through thymidine incorporation assay. The authors described a combined effect on lymphocytes proliferation of less than additive as a result of PAT and OTA exposure. This combined effect is characterized by Bernhoft et al. (2004) as an effect significantly higher than independent and significantly less than additive effects, and may either be caused by an interactive mechanism, or not. Tammer et al. (2007) evaluated the combined effects of a quaternary mycotoxin mixture including PAT and OTA (and also gliotoxin and citrinin) upon the functional activity of immune cells, applying a model for immunotoxic studies using stimulated human peripheral blood mononuclear cells (PBMC). The authors suggested a response addition for the mixture combined effect. Despite the use of different experimental conditions and independently of the cell line or endpoint considered, all authors agreed on the fact that it is crucial to clarify the mechanisms of the combined effects of PAT and OTA, not well characterized until now.

The present study determined the effects of PAT and OTA and, for the first time, their binary mixture effects, on integrity of intestinal barrier, through the measurement of TEER values of differentiated Caco-2 cells. In fact, TEER consists in one of the most used parameter used to evaluate the integrity of the epithelial barrier in the Caco-2 cell model (Akbari et al., 2016). TEER is a simple and convenient technique that provides information about the uniformity of the Caco-2 cell layer on the filter support, and the integrity of the TJs formed between the polarized cells (Bouhet and Oswald, 2005; Verhoeckx et al., 2015). Individually, results showed that PAT and OTA induced a decrease on TEER values, suggesting a disruption of intestinal barrier. Similar results were also reported by previous studies for PAT (Assunção et al., 2016a, 2014; Katsuyama et al., 2014; Kawauchiya et al., 2011; Mahfoud et al., 2002; Maresca et al., 2008; McLaughlin et al., 2009; Mohan et al., 2012) and for OTA (Lambert et al., 2007; Maresca et al., 2008, 2001; McLaughlin et al., 2004; Ranaldi et al., 2007; Romero et al., 2016; Sergent et al., 2005). Most of these studies reported concomitant reduction in the expression levels of the TJs constituents associated to the decrease of TEER measurements. Akbari et al. (2016) reviewed recently the available evidences regarding direct effects of various mycotoxins in the intestinal epithelial barrier, including PAT and OTA. For both mycotoxins, the authors reported that mycotoxins-induced intestinal barrier effects were associated with specific alterations in the expression as well as distribution of TJs. For PAT, the affected TJs were zonula occludens-1 (ZO-1), occludin (OCLN) and claudins (CLDN) 1, 3 and 4. Relatively to OTA, the affected TJs were OCLN and CLDN 3 and 4. Evaluating the combined effects of PAT and OTA on integrity of intestinal barrier, a dose level deviation was verified, where synergism was attained at low levels of both mycotoxins, changing to antagonism at higher doses. However, the mechanism responsible for this pattern is not still comprehended. According to the individual effects of

these two mycotoxins on expression and distribution pattern of TJs, it is expected that the disruption of TJs could explain the obtained results. Consequently, an extension of the CA conceptual model, signifying that PAT and OTA could act through the same MoA to exert their effects on integrity of intestinal barrier, could be considered. OCLN and CLDNs determine the adhesion and permeability characteristics of the epithelium in terms of specificity and tightness (Fanning et al., 1999). Consequently, factors that affect these proteins could be involved in the development of intestinal diseases, as discussed on the next section. To the best of authors' knowledge, none previous study evaluated the combined effects of mycotoxins on intestinal integrity through TEER measurement, neither effects on TJs. Consequently, the present results reinforce the importance to understand the impact of multiple mycotoxins exposure on integrity of intestinal barrier and the involved mechanisms.

Relevance for mycotoxin mixtures risk assessment and impact for human health

Mycotoxins dietary exposure may involve several different compounds at the same time and during the last years, a progressive modification from single analyte to multi-target methods has allowed a better comprehension of the reality of mycotoxin co-contamination of foods and consequently affect the risk assessment process (Alassane-Kpembi et al., 2015). Recently Assunção et al. (2016b) reported the co-occurrence of PAT and OTA in processed cereal-based products usually eaten by children (40% of analysed samples) and highlighted the importance to consider PAT and OTA combined toxicity in the risk assessment process (Assunção et al., 2016b). The observed synergistic effects for the lower studied doses are of great importance since the studied mycotoxins may occur in the same diet, produce relevant cytotoxic activity and disrupt intestinal membrane integrity.

Nowadays, there is a growing evidence that some intestinal diseases are associated with a dysfunctional intestinal barrier through increased intestinal permeability, as inflammatory bowel diseases (König et al., 2016) and some authors suggested that mycotoxins could be involved on the onset and maintenance of some these intestinal diseases (Maresca and Fantini, 2010). Although it is difficult to explain how PAT and OTA interact due to an unknown MoA, as a matter of fact, synergistic combination effects related to this binary mixture could pose a significant threat to public health. Preserved intestinal barrier integrity prevent the passage from the external environment into the organism of harmful intraluminal substances, including toxins that are ingested through food (Maresca and Fantini, 2010; Pinton and Oswald, 2014). The disruption of the intestinal barrier could lead to permeation of luminal noxious molecules, inducing a perturbation of the mucosal immune system and inflammation. The present results relatively to PAT and

OTA impact on intestinal Caco-2 cells, contribute and reinforce the importance to deeply analyse the interactive effects between co-occurring mycotoxins, elucidating their interaction mechanisms in order to contribute to a more accurate risk assessment and thus providing a better human health protection.

5. CONCLUSIONS

The present study evaluated *in vitro* combined toxic effects on cell viability and barrier integrity of the binary mixture of mycotoxins, PAT and OTA, on Caco-2 cells and revealed, at certain conditions, potential additive or synergistic effects. Although the obtained results demand an accurate MoA clarification, they highlight a potential threat for public health, considering the potential mycotoxins-associated adverse health effects and, in this particular case, their potential contribution for the development of intestinal diseases. In fact, results from studies on combined effects are often difficult to interpret, due to complicated MoA for the various compounds that integrate the mixture or even because MoA seems to be not completely known. Future research studies should contribute to solve this problem, gathering data that explain the mechanisms behind the evidenced combined effects.

The present results disclose also an important question, what would be the intestinal effects considering a long-term real-world human exposure? Epidemiologic studies, including biomonitoring studies, are crucial to establish a relationship between exposure and development of (intestinal) diseases.

To finalize, these *in vitro* results deserve further *in vivo* confirmation, for better understanding of their mechanisms and their interactions in living organisms. Additionally, results should be addressed by regulatory bodies, integrating the risk assessment process, establishing regulatory standards about mycotoxin mixtures and contributing to prevent mycotoxins-associated adverse health effects.

MANUSCRIPT 6: **CHARACTERIZATION OF *IN VITRO* EFFECTS OF PATULIN ON INTESTINAL EPITHELIAL AND IMMUNE CELLS**

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Highlights:

- PAT affected Caco-2 barrier function by perturbation of ZO-1 levels
- Phosphorylation of MLC2 accompanied PAT barrier function perturbation
- Low doses of PAT inhibited T cell proliferation induced by a polyclonal activator
- No effects on the maturation of moDCs were detected

ABSTRACT

The intestinal mucosa is the first biological barrier encountered by natural toxins, and could possibly be exposed to high amounts of dietary mycotoxins. Patulin (PAT), a mycotoxin produced by *Penicillium* spp. during fruit spoilage, is one of the best known enteropathogenic mycotoxins able to alter functions of the intestine (Maresca et al., 2008). This study evaluated the effects of PAT on barrier function of the gut mucosa utilizing the intestinal epithelial cell model Caco-2, and scrutinized immunomodulatory effects using human peripheral blood mononuclear cells (PBMC) and human blood monocyte-derived dendritic cells (moDCs) as test systems. PAT exposure reduced Caco-2 cell viability at concentrations above 12 μM. As expected, the integrity of a polarized Caco-2 monolayer was affected by PAT exposure, as demonstrated by a decrease in TEER values, becoming more pronounced at 50 μM. No effects were detected on the expression levels of the tight junction proteins occludin, claudin-1 and claudin-3 at 50 μM. However, the expression of zonula occludens-1 (ZO-1) and myosin light chain 2 (MLC2) declined. Also, levels of phospho-MLC2 (p-MLC2) increased after 24 h of exposure to 50 μM of PAT. T cell proliferation was highly sensitive to PAT with major effects for concentrations above 10 nM of PAT. The same conditions did not affect the maturation of moDC. PAT causes a reduction in Caco-2 barrier function mainly by perturbation of ZO-1 levels and the phosphorylation of MLC. Low doses of PAT strongly inhibited T cell proliferation induced by a polyclonal activator, but had no effect on the maturation of moDC. These results provide new information that strengthens the concept that the epithelium and immune cells of the intestinal mucosa are important targets for the toxic effects of food contaminants like mycotoxins.

Keywords: Patulin, Intestinal mucosa, Tight junctions, Phosphorylation, Immune effects

1. INTRODUCTION

The gastrointestinal (GIT) tract is directly involved in the metabolism and transport of various endogenous and exogenous compounds, and the GIT epithelium constitutes an important barrier adapted to separate the internal body milieu from the external environment (Peterson and Artis, 2014). The intestinal barrier comprises different cells: (i) a monolayer of epithelial cells that includes enterocytes, goblet cells, Paneth cells and enteroendocrine cells, which constitute the first level of protection, and (ii) immune cells like T and B lymphocytes, intraepithelial lymphocytes, macrophages and dendritic cells (DC) with distinct phenotypic characteristics, forming a complex network that participate in the regulation of gut homeostasis and in orchestrating innate and adaptive immune responses to potential pathogens that could occur at GIT mucosa (Schenk and Mueller, 2008; Wershil and Furuta, 2008).

The enterocytes of the intestinal epithelium have three major functions: absorption and transport of nutrients, in some cases regulated by tight junctions (TJs); barrier functions; and signal recognition and transduction, which could include gut hormones and cytokines mediating interactions with immune cells (Shimizu, 2010). The TJs are intercellular, multiprotein complexes located at the apical ends of the lateral membranes of intestinal epithelial cells which provide both a barrier to noxious molecules and pores for the selective permeation of ions, solutes and water as appropriate (Qasim et al., 2014; Suzuki, 2013). TJs are comprised of transmembrane (occludin, claudins, junctional adhesion molecules and tricellulin) and peripheral membrane proteins, namely zonula occludens (ZO) family proteins, which comprises three members (ZO-1, -2 and -3) that anchor the transmembrane proteins to the perijunctional actomyosin ring. Activation of actomyosin contraction, as assessed by phosphorylation of myosin II regulatory light chain (MLC2), has been implicated in TJ regulation, namely by changing ZO-1 protein dynamics (Clayburgh et al., 2005; Cunningham and Turner, 2012; Shen, 2012; Shen et al., 2006; Suzuki, 2013).

The intestinal mucosa is the first biological barrier encountered by natural toxins in food. Consequently, it represents the first target for these compounds and could be exposed to high amounts of dietary toxins, including mycotoxins (Maresca and Fantini, 2010; Pinton and Oswald, 2014; Puel et al., 2010). Mycotoxins are toxic compounds produced by fungi, mostly by saprophytic moulds growing on a variety of foodstuffs that exert multiple effects in humans and animals (Maresca, 2013), including alteration of many intestinal functions (other than barrier function), comprising the production of mucus by goblet cells (Pinton et al., 2015), expression of inducible NO synthase (Graziani et al., 2015) and nutrient absorption (Maresca et al., 2002). Although hundreds of mycotoxins exist, the mycotoxins recognized as most important are the aflatoxins, ochratoxin A, patulin (PAT), fumonisins, zearalenone and the trichothecene group (Yang et al., 2014). Patulin (4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one) is an unsaturated heterocyclic lactone, mainly produced by *Penicillium* and *Aspergillus*, and is frequently detected in mouldy fruits and fruit products. PAT is highly reactive towards the thiol groups of proteins and glutathione, and exposure to this mycotoxin is associated with immunological, neurological and gastrointestinal outcomes (Puel et al., 2010). The acute toxicity of PAT is partly thought to be due to destruction of TJ proteins in GIT tissue (Mahfoud et al., 2002). PAT was found to interfere with the transepithelial electrical resistance (TEER) of human intestinal Caco-2 cells and to affect the expression of TJs proteins (Assunção et al., 2014; Katsuyama et al., 2014; Kawauchiya et al., 2011; Mahfoud et al., 2002; Maresca et al., 2008; McLaughlin et al., 2009). Programmed cell death, known as apoptosis, is an essential mechanism to eliminate cells during the homeostasis of multicellular organisms (Grütter, 2000). Some studies had previously reported the occurrence of apoptosis induced by PAT exposure in colon cancer (Katsuyama et

al., 2014; Kwon et al., 2012), kidney (Zhang et al., 2015) and skin cells (Saxena et al., 2009). Apoptotic cells death is dependent on caspase activity.

The intestine is a specialized immune site, where immunoregulatory mechanisms simultaneously defend the body against pathogens, but also preserve tissue homeostasis to avoid immune-mediated pathology in response to environmental challenges (Pinton and Oswald, 2014). The intestinal immune response involves the coordinated action of both immune (dendritic cells, macrophages, lymphocytes) and non-immune cells, including epithelial cells. Monocytes, macrophages, dendritic cells (DCs), as well as T- and B-lymphocytes can be cellular targets of ingested mycotoxins, as PAT. DCs are the most potent antigen-presenting cells of the immune system critically involved in the initiation of primary immune responses, and T lymphocytes have an essential role in the development of adaptive immunity. Few data are available on the potential adverse effects induced by PAT on human immune cells (Hymery et al., 2006; Luft et al., 2008; Özsoy et al., 2008; Stec et al., 2008). Several intestinal diseases are characterized by barrier defects (Qasim et al., 2014). These phenomena lead to increased intestinal permeability, and current literature clearly reveals a linkage between mycotoxin ingestion from contaminated food and human diseases, especially hepatic, gastrointestinal, carcinogenic, and teratogenic diseases (Maresca, 2013; Maresca and Fantini, 2010). The present study aimed to evaluate the PAT effects on the homeostasis of intestinal mucosa by evaluating the *in vitro* effects of PAT on (i) epithelial cytotoxicity, TEER, TJs expression and apoptosis, (ii) human T-cells proliferation and (iii) human DCs maturation.

2. MATERIALS AND METHODS

2.1. Chemicals and cell culture

Patulin was ordered from Sigma-Aldrich (St. Louis, USA), dissolved in sterile MiliQ water (18.2 MΩ cm) and stored at -80 °C. Caco-2 human colon cancer cells (ATTC; Manassas, USA) were maintained in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS), 100 µM non-essential amino acids, 1 mM sodium pyruvate, 50 µM thioglycerol and penicillin-streptomycin solution, all from Sigma-Aldrich (St. Louis, USA). Cells were kept in a humidified incubator at 37 °C and 5% CO_2. The cells were passaged with 0.05% trypsin/0.02% EDTA in PBS when confluence reached 70-90%. The medium was changed twice a week.

2.2. MTT assay

Caco-2 cells were cultured to confluence in 96-well tissue culture plates and then treated with fresh medium containing PAT at concentrations ranging from 1 to 150 µM and incubated for 24 h at 37 °C. The PAT exposure was followed by removal of the medium, and 10 µL of MTT solution [Cell Proliferation Kit I (MTT); Roche Life Science, USA] was added to each well. The plates were incubated for 4 h at 37 °C, and the formazan crystals formed were dissolved by the addition of 100 µL of solubilisation solution to each well, overnight at 37 °C (protected from light). The absorbance was measured at 570 nm using a 96-well ELISA plate reader (Tecan Sunrise; Tecan, Switzerland). The results were expressed in percentage of cell viability, calculated using the absorbance records of treated and untreated cells.

2.3. Measurement of TEER

Cells were seeded on 0.4 µm pore size 12-well plate cell culture inserts (Falcon™ Cell Culture Inserts, Fisher Scientific, USA) at a density of $3.0x10^5$ cells/well. The experiments were performed after 18–21 days of cell monolayer growth. TEER values were monitored using an Evometer (World Precision Instruments, Stevenage, UK) fitted with a chopstick electrode. TEER was normalized by the area of the monolayer and the background TEER of blank filter was subtracted from the TEER of the cell monolayer. Only inserts with TEER values higher than 500 $\Omega.cm^2$ have been considered acceptable and used in subsequent experiments. PAT solutions with concentrations ranging between 3 to 50 µM were used to expose cells through different times of exposure.

2.4. Cell extraction, SDS-PAGE and Immunoblotting

After TEER measurement assays, cells were extracted using 100 µL of lysis buffer containing NaCl (100 mM), Tris pH 7.5 (25 mM), NaF (20 mM), Na_3VO_4 (1 mM) and NP-40 (1%). The cell samples were incubated on ice for 30 minutes and then centrifuged for 15 minutes. The supernatants were used for SDS-PAGE assays. Proteins were electrophoretically transferred from gels onto PVDF membranes. The membranes were blocked in 5% skim milk (for occludin, claudin-1 and -3 and ZO-1) or bovine serum albumin (BSA; Sigma-Aldrich, USA for MLC2 and phospho-MLC2 (p-MLC2)). Blocked membranes were incubated with the primary antibodies [goat anti-occludin (1:500), goat anti-claudin-1 (1:500), rabbit anti-ZO-1 (1:1000); Santa Cruz Biotechnology, USA; rabbit anti-claudin-3 (1:500); Invitrogen, USA; rabbit anti-MLC2 (1:1000), rabbit anti-p-MLC2 (1:1000); Cell Signaling, USA]. After being washed, membranes were

incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies or rabbit anti-goat IgG as appropriate at a dilution of 1:4000 (Southern Biotech, USA). Luminata™ Crescendo Western HRP Substrate (Merck Millipore) was used as substrate for HRP-conjugated secondary antibodies, and membranes were exposed using X-Ray film (Pierce). Densitometric analysis was performed using the Image J 1.48 software. Normalized ratios of band densities were obtained by comparing the intensity of the band of interest with the intensity of the α-actin band (used as loading control) from the same sample.

2.5. Caspase 3 activity

Caspase-3 activity was evaluated in Caco-2 cells treated with PAT as described in Cell extraction, SDS-PAGE and Immunoblotting. The membranes were blocked in 5% skim milk. Blocked membranes were incubated with the rabbit anti-caspase-3 (1:500) antibody (Santa Cruz Biotechnology, USA).

2.6. DNA fragmentation assay

Caco-2 cells were cultured in 6-well tissue-culture plates in a concentration of $3.0x10^5$ cells per well during 24 h. Non-differentiated cells were exposed to 50 μM PAT for 24 h. After incubation, cells were harvested and the genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen, USA) as the protocol recommended by the manufacturer. Samples were run in a 1.5% (w/v) agarose gel. The gel was stained with GelRed™ and a gel photography was obtained (Gel Doc™ EZ System, Bio-Rad, USA).

2.7. Incorporation of [^{3}H]-Thymidine assay

Human peripheral blood mononuclear (PBMC) cells were prepared from buffy coats (obtained from Østfold Hospital Trust, Fredrikstad, Norway) of healthy donors. PBMCs were cultured using the previously described medium, and T cell proliferation was induced by incubating 10^5 PBMC per well with soluble anti-CD3 antibodies. Cells were exposed to PAT during 72 h in concentrations ranging between 0.5 nM and 25 μM. [^{3}H]-Thymidine (1 μCi; Perkin Elmer) was added and plates were incubated for 24 h. Cells were harvested onto glass-fiber filters and the incorporated radioactivity was determined by liquid scintillation counting (Packard). Results were given as counts per minute (cpm) as an indicator of rate of [^{3}H]-Thymidine incorporation.

2.8. Flow cytometry

CD14 MicroBeads (Miltenyi Biotech, USA) was used to positively select human monocytes from PBMCs. Monocytes were differentiated to immature dendritic cells in the presence of GM-CSF and IL-4 for seven days. The final maturation of these cells to antigen-presenting dendritic cells was done by stimulation with PGE_2, TNFα and LPS. The detection of CD80, CD83 and MHC class II markers was done by flow cytometry (MACS, Miltenyi Biotec).

2.9. Statistical analysis

Numerical values from individual experiments were pooled and expressed throughout as the mean ± standard deviation (SD) of the mean. To determine the significance in two group comparisons a Student's t-test was used. When normality failed, the Mann–Whitney test was applied. Multiple group comparisons were done by one-way ANOVA followed by Tukey's post hoc test. All statistics were computed using IBM SPSS Statistics 20 software.

3. RESULTS

3.1. Patulin induced a decrease in TEER values and viability of Caco-2 cells

The effects of PAT on the barrier integrity of differentiated Caco-2 cells monolayer was evaluated. For TEER assays, PAT was added to apical side of Transwell™ inserts at concentrations ranging between 3 µM to 50 µM at different incubation times (0 h, 1 h, 2 h, 3 h, 6 h and 24 h). Data showed a marked time-dependent decrease in TEER at a final PAT concentration of 50 µM. A statistically significant decrease at this concentration ($p < 0.05$) started after 2 h of treatment (Figure 29). For lower concentrations, TEER showed a numerically reduction (below 20%) after 24 h but had not showed a statistically difference relatively to the control.

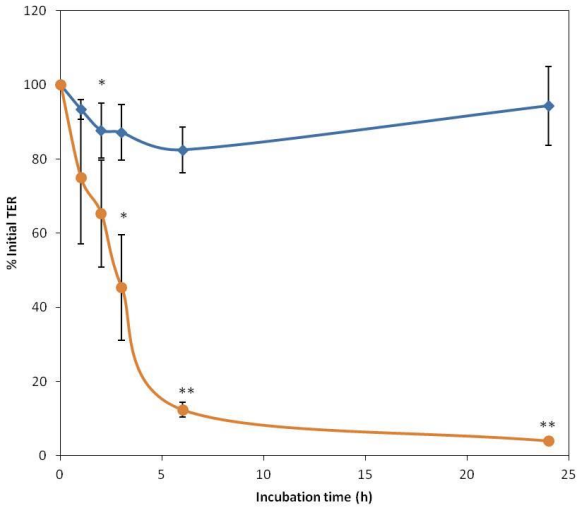

Figure 29. Effect of PAT treatment on Caco-2 cells trans-epithelial electrical resistance (TEER) values at different incubation times. Differentiated cells were exposed to 50 µM of PAT (—●—) in apical side during 24 hours. Negative control (—◆—) received no patulin. Values are the mean ± SD (n=4). p < 0.05 (*), p < 0.01 (**) represent significant difference as compared to negative control. % Initial TEER was calculated considering the initial TEER values measured at 0 h of incubation and the respective incubation time.

For viability assays (MTT assay), non-differentiated Caco-2 cells were incubated with PAT at concentrations ranging between 3 µM to 150 µM for 24 h. Results demonstrated a dose-dependent decrease in cell viability after 24 h of exposure to PAT for doses higher than 25 µM compared to the control ($p < 0.001$, Figure 30). The dose-response curve was fitted to a sigmoidal model ($R^2 = 0.92$) and an IC_{50} value (inhibitory concentration 50%) of 14.43 µM was estimated.

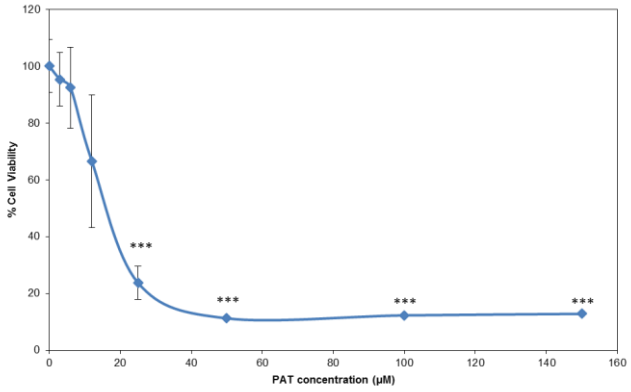

Figure 30. Effect of PAT treatment on Caco-2 cells viability after 24 hours of exposure to different PAT concentrations (1 to 150 µM), using a MTT assay. Graphical representation of cell viability percentage for each concentration tested. Values are expressed as mean ± SD (n=6). p < 0.001 (***) represent significant difference as compared to control values (negative control).

3.2. TEER reduction in Caco-2 cells was followed by changes in specific TJ proteins

Considering the TEER reduction obtained after exposure to PAT, it was investigated whether this phenomenon were associated with changes at TJ proteins level. The expression of five different TJ proteins occurring in Caco-2 cells was examined using immunoblotting technique: occludin, claudin-1, claudin-3, ZO-1 and MLC2. These proteins are expressed by intestinal epithelia and have crucial roles in maintenance and regulation of the TJ structure and functions (Suzuki, 2013). After 24 h, results showed that the intensity of the occludin, claudin-1 and claudin-3 bands did not decrease suggesting that their expression levels were unaltered by treatment with the different concentrations of PAT tested (3 μM to 50 μM). These results were confirmed by densitometry data revealing no significant differences relative to the control (data not shown). However, as shown in Figure 31, Caco-2 cells treated with 50 μM of PAT showed a decrease in the intensity of ZO-1 and MLC2 bands. A statistically significant difference was suggested by densitometry data for expression of both ZO-1 and MLC2 proteins. For PAT concentrations below 50 μM, the level of expression of these proteins remained unchanged when compared to controls.

As suggested by some authors, the phosphorylation of MLC2 is associated with epithelial tight junction modulation which leads to defective epithelial barrier function, and has been implicated in gastrointestinal diseases (Cunningham and Turner, 2012; Marchiando et al., 2010; Qasim et al., 2014). Therefore, in order to investigate whether phosphorylation of MLC2 could be involved in PAT-induced reduction of epithelial TEER values, the expression of p-MLC2 after Caco-2 PAT treatment was investigated. As shown in Figure 32, after 24 h of Caco-2 cells exposure to 50 μM of PAT, a specific band for p-MLC2 appeared, showing a statistically significant difference relatively to the control.

3.3. Pro-caspase 3 was expressed by Caco-2 cells after patulin exposure

To determine whether the decrease in TEER induced by PAT was due to cell apoptosis, caspase 3 expression by differentiated Caco-2 cells after exposure to PAT was studied. For a PAT concentration of 50 μM, only a band with a molecular weight near 32 kDa was detected, which is compatible with a pro-caspase 3 protein (Figure 33). No other bands, with lower molecular weight, were detected suggesting expression of caspase 3 subunits.

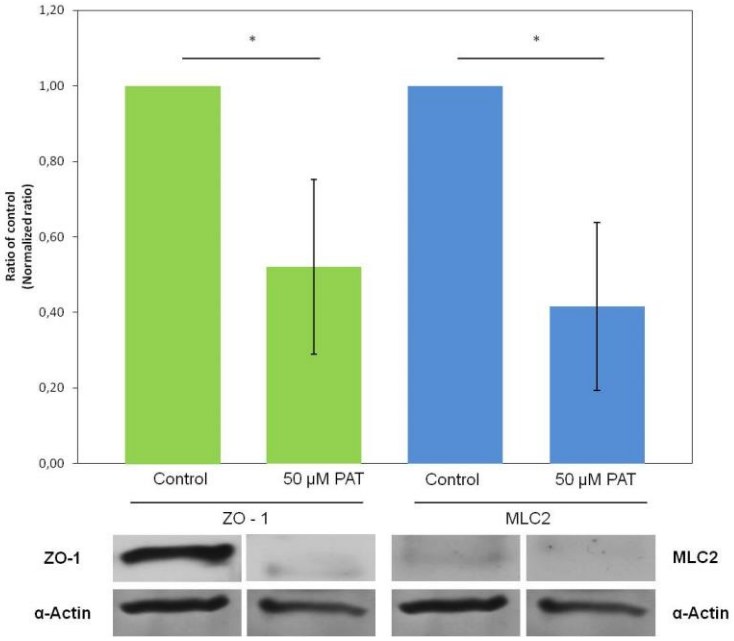

Figure 31. Immunoblot analysis showing the expression of ZO-1 (220 kDa) and MLC2 (18 kDa) in Caco-2 cells exposed to 50 µM of PAT for 24 hours. Data of densitometry and typical images for control (negative control) and 50 µM PAT treatment concentration are shown. Densitometry data were obtained using ImageJ 1.48 (http://imagej.nih.gov/ij/). The α-actin (43 kDa) was used as loading control. Values are the mean ± SD (n=4). p < 0.05 (*) represent significant difference as compared to control values.

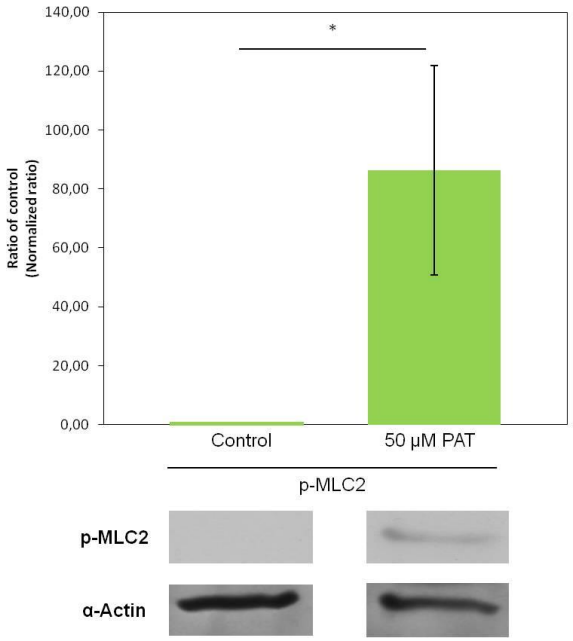

Figure 32. Immunoblot analysis showing the expression of p-MLC2 (18 kDa) in Caco-2 cells exposed to 50 µM of PAT for 24 hours. Data of densitometry and typical images for negative control and 50 µM PAT treatment concentration are shown. Densitometry data were obtained using ImageJ 1.48. The α-actin (43 kDa) was used as loading control. Values are the mean ± SD (n=4). p < 0.05 (*) represent significant difference as compared to control values.

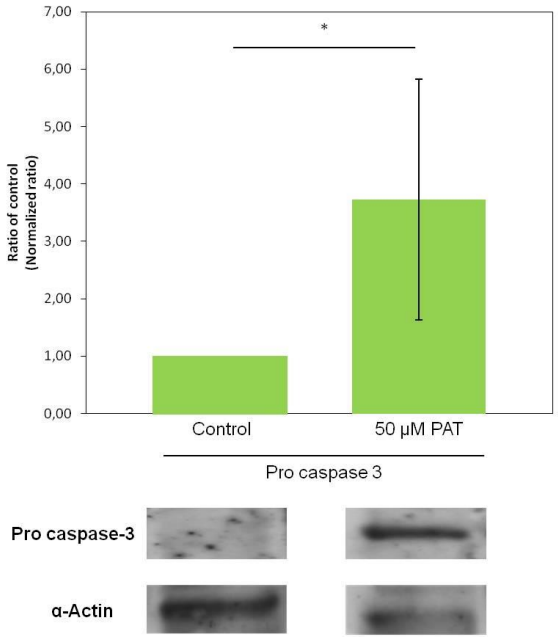

Figure 33. Immunoblot analysis to determine the expression of pro-caspase 3 (32 kDa) in Caco-2 cells exposed to 50 µM of PAT for 24 hours. Data of densitometry and typical images for negative control and 50 µM PAT treatment concentration are shown. Densitometry data were obtained using ImageJ 1.48. The α-actin (43 kDa) was used as loading control. Values are the mean ± SD (n=4). $p < 0.05$ (*) represent significant difference as compared to control values.

The above results indicate that PAT could induce apoptosis in Caco-2 cells. In order to improve the characterization of potential different effects of apoptosis, a DNA fragmentation assay was employed. However, DNA from treated Caco-2 cells did not exhibit DNA fragmentation patterns characteristic of apoptotic cells, as shown by positive controls (Figure 34; treated cells: lanes 3 and 4; positive controls: lanes 5 and 6). The absence of DNA fragmentation does not exclude the induction of apoptotic events in the cells since DNA fragmentation is a final step in the apoptotic process. It is important to consider that DNA fragmentation assay was developed using non-differentiated Caco-2 cells, while caspase-3 expression assay was developed using differentiated cells, and this fact could affect the cellular responses to PAT exposure.

3.4. Human T cell proliferation was highly sensitive to patulin exposure

To determine the effect of PAT on T cell proliferation, PBMC cells were incubated with soluble anti-CD3 antibodies and the incorporation of [^{3}H]-thymidine was assayed. As demonstrated in Figure 35, T cell proliferation was highly sensitive to PAT with the major effects for concentrations above 10 nM of PAT.

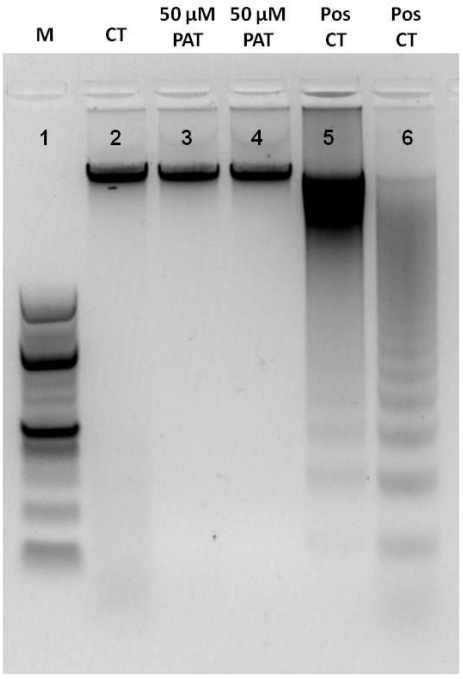

Figure 34. Effects on DNA fragmentation of Caco-2 cells exposed to 50 µM of PAT for 24 hours using agarose gel electrophoresis. DNA was extracted from patulin exposed Caco-2 cells (lanes 3 and 4) and negative control (lane 2), after 24 h. Electrophoresis was performed with 1.5% agarose gel and DNA was stained with GelRed. Lane 1 is a molecular size marker (M) and lanes 5 and 6 are positive controls (Pos CT).

Despite the findings that PAT affected T cell proliferation in the nM range, the same conditions did not affect the maturation of moDCs for PAT concentrations between 0.5 nM and 100 nM (Figure 36).

4. DISCUSSION

The GIT is directly involved in the metabolism and absorption of various endogenous and exogenous compounds. The intestinal mucosa is constantly challenged with microbes, nutrients, ions and liquids across the epithelium in the presence of a multitude of potentially harmful luminal compounds. The maintenance of intestinal homeostasis is achieved by a well orchestrated equilibrium for what the intestinal epithelial and immune cells contribute largely. Patulin is a mycotoxin, and its contamination of food has been reported by some authors to cause gastrointestinal inflammation, ulcers, and bleeding. This study intended to characterize the *in vitro* PAT effects at the intestinal level by: 1) evaluating the effects at intestinal epithelial cells, using a Caco-2 cell model; 2) studying the impact of PAT on T-lymphocyte proliferation and, 3) assessing PAT effects on maturation on monocyte-derived DCs.

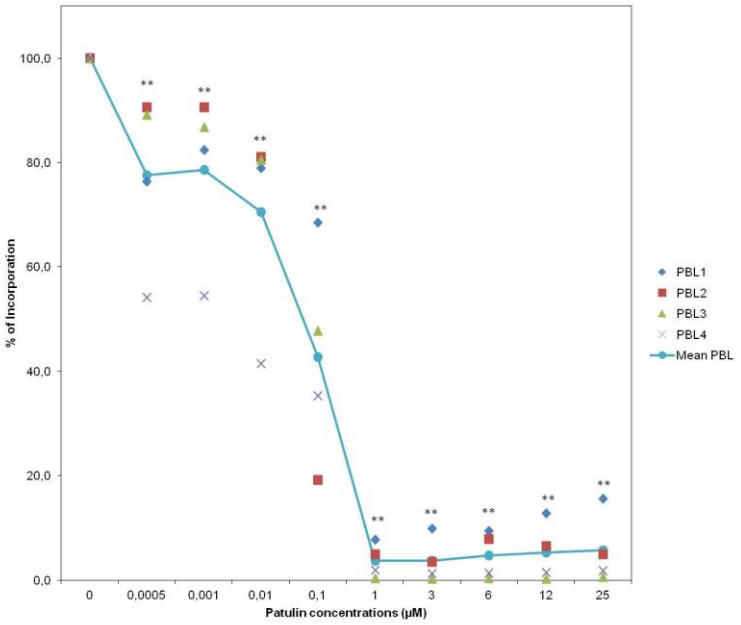

Figure 35. Effect of PAT on the proliferation of PBMC as demonstrated by [^{3}H]-Thymidine incorporation assay. PAT concentrations ranging between 5 nM and 25 µM for 72 h of incubation. Results of the % incorporation for 4 different donors (◆ , ■ , ▲ , ×) and the mean results (⟷) are shown. For each donor, 6 replicates of each PAT concentration were assayed. Counts per minute were used as an indicator of rate of [^{3}H]-Thymidine incorporation. % of Incorporation was calculated considering the cpm values obtained from each PAT concentration and negative control (0 µM of PAT). $p < 0.01$ (**) represent significant difference as compared to negative control values.

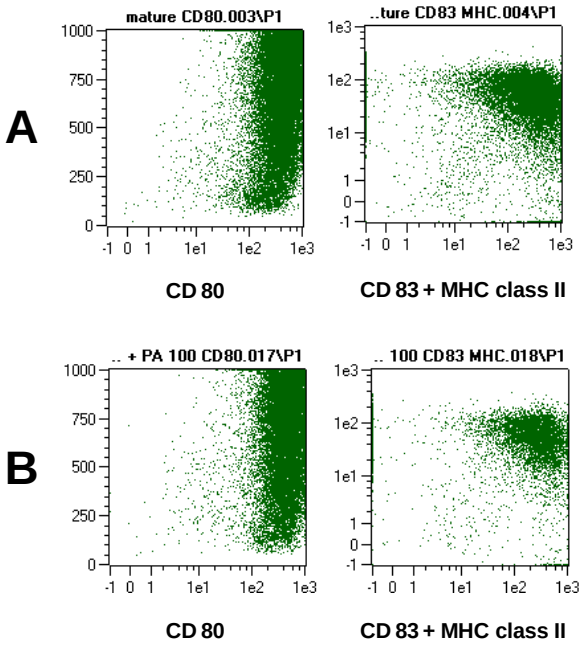

Figure 36. Flow cytometric analysis of the effect of PAT treatment on surface marker expression of human blood monocyte-derived dendritic cells. Cells were exposed to PAT during 72 hours. (A) Negative control received no PAT; (B) DCs treated with 100 nM of PAT, during 24 h.

Intestinal epithelial cells form a crucial physical and functional barrier, which regulates the movement of water, electrolytes, nutrients and xenobiotics. The toxicity of PAT is thought to be due to impairment of the intestinal barrier caused by the destruction of TJs in the epithelial cell layer. However, the mechanism of its toxicity has not been totally clarified (Assunção et al., 2014; Kawauchiya et al., 2011; McLaughlin et al., 2009). Some authors reported that PAT altered the barrier function of the intestinal epithelium, and TEER could be used as a marker of the integrity of the epithelial barrier function (Assunção et al., 2014; Katsuyama et al., 2014; Kawauchiya et al., 2011; Mahfoud et al., 2002; Maresca et al., 2008; McLaughlin et al., 2009). The present results confirm that PAT affects the integrity of the intestinal barrier by decreasing TEER values, mainly for 50 μM of PAT and with a statistically difference for exposures longer than 2 h. These results are in accordance with those obtained by Kawauchiya et al. (2011) that demonstrated a gradual decrease in TEER during the first 24 h of treatment with 50 μM of PAT. Different exposure conditions were also evaluated by some authors, with different exposure times, higher PAT concentrations or different intestinal cell lines showing that these conditions affected also the TEER values of *in vitro* epithelial monolayers (Assunção et al., 2014; Katsuyama et al., 2014; Mahfoud et al., 2002; Maresca et al., 2008; McLaughlin et al., 2009). Experiments performed using rat colonic mucosae showed that addition of 500 μM of PAT produced a sustained decrease in TEER, however the exposure to 100 μM of PAT did not alter TEER values, suggesting that just relatively high concentrations of PAT affect rat colonic mucosae permeability (Mohan et al., 2012). According to the present results, the decrease in TEER values of an epithelial cell monolayer by PAT exposure could be associated with three different mechanisms: necrosis, apoptosis and TJ proteins modulation. The present study revealed that PAT induces a dose-dependent decrease in cell viability and proliferation in Caco-2 cells. In turn, the present results suggested an IC_{50} value around 14 μM in Caco-2 cells. Mahfoud et al. (2002) evaluated the toxic effects of PAT on Caco-2 and HT-29-D4 cells and found that concentrations of PAT as high as 100 μM did not induce any release of lactate dehydrogenase (LDH) after 24 h of incubation for Caco-2 cells. However, for HT-29-D4, a higher sensitivity to PAT was found. McLaughlin et al. (2009) examined the effects on cell viability after 12 h exposure to 100 μM of PAT using the MTT assay and speculated that the effect of PAT on TEER was specific rather than due to general toxicity and disruption of the monolayer integrity. Kawauchiya et al. (2011) also evaluated the viability of Caco-2 cells after exposure to 50 μM of PAT using trypan blue dye-exclusion test and verified that this concentration had no effect on cell viability for up 24 h after treatment. All these data suggest that there is a wide variation in the results obtained among cell lines, time and concentrations of PAT exposure.

In order to investigate possible effects of PAT on Caco-2 cell apoptosis, caspase-3 activity was measured after 50 μM PAT exposure for 24 h. As previously referred, pro-caspase 3 shows high expression in Caco-2 cells. However, no cleavage products of pro-caspase 3 were detected after PAT exposure. Thus, it is not clarified whether the TEER decrease in Caco-2 cell monolayers exposed to PAT could be due to an apoptotic process. Some authors suggested that caspase-3 has important roles in tissue differentiation and regeneration in ways that are distinct and do not involve any apoptotic activity (Shalini et al., 2015), and this argument could explain the present results, considering the PAT-induced cell injury. Additionally, DNA fragmentation results obtained in the present study do not corroborate that apoptosis is induced by PAT exposure as previously suggested. Boussabbeh et al. (2015) provided new mechanistic insights in the signaling pathways of the cell death induced by PAT and demonstrated that PAT induces cytotoxicity through a reactive oxygen species-dependent (ROS) mechanism and activation of mitochondrial apoptotic pathway in human intestinal cells. Other studies suggested that PAT can induce apoptosis by different mechanisms in colon cancer (Katsuyama et al., 2014; Kwon et al., 2012), kidney (Zhang et al., 2015) and skin cells (Saxena et al., 2009). de Melo et al. (2012) evaluated for the first time the DNA damage in organs of mice treated with PAT and verified that PAT induces DNA damage in the brain, liver and kidneys in a dose-dependent manner. More studies are needed to clarify the possibility of PAT-induced Caco-2 cell apoptosis.

As suggested by Mahfoud et al. (2002), TEER measurements reflect the organization of TJs. Previously, Katsuyama et al. (2014), Kawauchiya et al. (2011) and McLaughlin et al. (2009) demonstrated that PAT reduce barrier properties of the intestinal cell line Caco-2 by altering the expression levels and/or distribution of several TJs proteins. Similar results were obtained in the present study. Caco-2 cells treated with PAT showed a markedly decreased expression of ZO-1, which agree with previous reported effects of PAT at TJs level. To our knowledge, PAT-mediated effects on MLC2 expression and phosphorylation have not been studied. The interaction of the TJ complex with the actomyosin ring permits the cytoskeletal regulation of TJ barrier integrity and activation of actomyosin contraction, as assessed by phosphorylation of MLC2, was referred by some authors with implications in TJ regulation, namely by changing ZO-1 dynamics. Considering the effects of PAT on TEER and ZO-1 expression, we hypothesized that these effects could be due to a disturbance of ZO-1 and MLC2 equilibrium. The present results showed, for the first time, that the expression levels of MLC2 protein decreased by PAT exposure. Interestingly, the levels of expression of p-MLC2 were evaluated and showed a significant increase after PAT exposure. The disruption of the intestinal barrier, suggested by a decrease in TEER values of Caco-2 cell monolayers, could be explained by these events, namely alterations in the function of TJs associated with MLC2

phosphorylation, contributing to an increase in intestinal permeability. In this respect, some authors suggest that MLC phosphorylation and subsequent cytoskeletal contraction constitute a common mechanism of barrier regulation in response to physiological and pathophysiological stimuli, including nutrient transport and parasitic and bacterial infections. These events could lead to the increased TJs permeability implicated in some barrier associated diseases (Clayburgh et al., 2005; Cunningham and Turner, 2012; Qasim et al., 2014; Shen, 2012; Shen et al., 2006). Until now, no other studies evaluated the phosphorylation of MLC as a mechanism of PAT toxicity. Shen et al. (2006) suggested that myosin light chain phosphorylation alone is sufficient to induce TJ deregulation and an increase in TJ permeability. This altered function is associated with structural redistribution of specific TJ proteins, namely ZO-1. These conclusions are in agreement with the present study indicating that PAT induce the phosphorylation of MLC2, an event that could also be associated with the decrease in ZO-1 levels. The phosphorylation of MLC2 is usually regulated by the MLC kinase (MLCK) which has emerged as a key regulator of TJ permeability (Cunningham and Turner, 2012). Further studies should be developed in order to examine whether this kinase is up-regulated in PAT pathophysiological processes and also to characterize its intervention on barrier loss.

Immunotoxicity is regarded as an important element when considering potential adverse effects of mycotoxin exposure, and immune cells have been found to be particularly sensitive to various mycotoxins. Past studies evaluated PAT immunotoxicity in animals and cells obtained from animals, and the results indicated variable effects of PAT on the immune system [reviewed in Bondy and Pestka (2000)]. These effects included changes in immune cell numbers, increased numbers of splenic monocytes and Natural Killer cells and increased numbers of splenic cytotoxic T lymphocytes. This study evaluated the effects of PAT on the proliferation of human T cells and on maturation of human moDCs. T cell proliferation was highly sensitive to PAT with major effects for concentrations above 10 nM. Luft et al. (2008) performed the only study available in literature until now to evaluate the effects of PAT on human T-cells and showed that 0.6 μM of PAT inhibited T-cell proliferation. These results are quite different from those obtained in the present study. As referred previously, a statistically significant inhibition was verified for concentrations above 10 nM of PAT. Some authors reported some divergences when evaluating the effects of PAT on immune cells of mice (Llewellyn et al., 1998). Despite these variations in results, the present study alerts for the risk of relatively low concentrations of PAT inhibiting T-cell proliferation and function. In addition, it is important to highlight that the range of PAT concentrations tested (concentrations > 0.5 nM of PAT) is close to those estimated as probable concentrations of human dietary exposure (Majerus and Kapp, 2002). These results points to a potential risk of toxic effects on the immune system when ingesting food

contaminated with PAT. Additionally, Stec et al. (2008) compared the potency of different mycotoxins and verified that PAT potency in inhibition of proliferation of lymphocytes was stronger than that of OTA.

DCs are an essential component of the immune system and intestinal mucosa contains numerous DCs, which induce either protective immunity and are crucial in the initiation of an immune response to stimulate naive T cells (Rescigno and Sabatino, 2009). This study presents data related to the characterization of PAT effects on the maturation of DCs, using three important activation and differentiation parameters: CD80, CD83 and MHC class II. The present results showed that maturation of DCs were not affected by PAT concentrations ranging between 0.5 nM and 100 nM. There are few studies about PAT effects on DCs, and no data are available until now on the evaluation of the potential adverse effects induced by PAT on human DCs. Özsoy et al. (2008) evaluated the effect of PAT on the interdigitating DCs of rat thymus and concluded that PAT change the ultrastructural characteristics of these cells, with nuclear and cytoplasmic degenerations and apoptotic bodies formation. Hymery et al. (2006) evaluated the *in vitro* effects of trichothecenes (T-2 and deoxynivalenol), a different family of mycotoxins, on human dendritic cells. These authors verified that DCs show different patterns of adverse effects when exposed to trichothecenes and these two mycotoxins partially affect the maturation process of DCs.

5. CONCLUSIONS

The intestinal mucosa is the first barrier encountered by natural toxins and consequently it could be exposed to high amounts of different dietary toxins, namely mycotoxins (Bouhet and Oswald, 2005). Effects of PAT, a mycotoxin produced by *Penicillium* spp. molds, on three different types of cells found at intestinal level, were evaluated in this study providing more and new information about how PAT may affect the intestinal epithelium and mucosal immune responsiveness. This study may explain also some of the described effects of PAT exposure on the human GIT. In this respect, further investigations should be performed in order to characterize the impact of exposure to mycotoxins as a contribution to the induction and/or persistence of human intestinal diseases, as for example, human intestinal inflammatory diseases (Maresca and Fantini, 2010).

SECTION 4: CHALLENGES IN RISK ASSESSMENT OF MULTIPLE MYCOTOXINS

MANUSCRIPT 7: **CHALLENGES IN RISK ASSESSMENT OF MULTIPLE MYCOTOXINS IN FOOD**

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ABSTRACT

Most fungi are able to produce several mycotoxins simultaneously and, consequently, to contaminate a wide variety of foodstuffs. Therefore, the risk of human co-exposure to multiple mycotoxins is real, raising a growing concern about their potential impact on human health. Besides, government and industry regulations are usually based on individual toxicities, and do not take into account the complex dynamics associated with interactions between co-occurring groups of mycotoxins. The present work assembles, for the first time, the challenges posed by the likelihood of human co-exposure to these toxins and the possibility of interactive effects occurring after absorption, towards knowledge generation to support a more accurate human risk assessment. Regarding hazard assessment, a physiologically-based framework is proposed in order to infer the health effects from exposure to multiple mycotoxins in food, including knowledge on the bioaccessibility, toxicokinetics and toxicodynamics of single and combined toxins. The prioritization of the most relevant mixtures to be tested under experimental conditions that attempt to mimic human exposure and the use of adequate mathematical approaches to evaluate interactions, particularly concerning the combined genotoxicity, were identified as the main challenges for hazard assessment. Regarding exposure assessment, the need of harmonized food consumption data, availability of multianalyte methods for mycotoxin quantification, management of left-censored data, use of probabilistic models and multibiomarker approaches are highlighted, in order to develop a more precise and realistic exposure assessment. To conclude, further studies on hazard and exposure assessment of multiple mycotoxins, using harmonized methodologies, are crucial towards an improvement of data quality and a more reliable and robust risk characterization, which is central for risk management and, consequently, to prevent mycotoxins-associated adverse effects. A deep understanding of the nature of interactions between multiple mycotoxins will contribute to draw real conclusions on the health impact of human exposure to mycotoxin mixtures.

Keywords: Mycotoxins mixtures, Human risk assessment, Combined toxicity, Food safety, Human health

1. INTRODUCTION

Food safety is threatened by numerous pathogens and toxins, including mycotoxins, which are associated to a variety of acute and chronic foodborne diseases. From an economic and public health standpoint, the foodborne mycotoxins that are considered as being relevant are aflatoxins (AFs), fumonisins (FBs),

certain trichothecene mycotoxins [including deoxynivalenol (DON) and T-2 and HT-2 toxins], ochratoxin A (OTA), patulin (PAT) and zearalenone (ZEA). Briefly, AFs are strong hepatocarcinogens and have also been implicated in child growth impairment and acute toxicoses; FBs have been associated with oesophageal cancer and neural tube defects; DON and other trichothecenes, display immunotoxic effects and cause gastroenteritis; OTA has been associated to nephrotoxicity and renal cancer; PAT induces gastrointestinal effects, as inflammation and ulcers; and ZEA affects the reproductive function (CAST, 2003; World Health Organization, 2011; Wu et al., 2014). These mycotoxins are able to naturally contaminate commodities at toxicologically relevant concentrations and are the focus of legislation and regulations in the European Union and beyond (Clarke et al., 2014; Wu et al., 2014). Maximum levels (MLs) are set in EU legislation to control these mycotoxin levels in food and feed (European Commission, 2002, 2006 and its amendments). Besides the parent forms, potential health effects of modified mycotoxins are also relevant due to their possibility to be hydrolyzed to the more toxic parent mycotoxins and thus lead to an increased health risk (Berthiller et al., 2013; Stoev, 2015).

All over the world, recent surveys highlight the fact that humans are more frequently exposed to multiple than to single mycotoxins (De Ruyck et al., 2015; Grenier and Oswald, 2011; Solfrizzo et al., 2014; Stoev, 2015) given the natural co-occurrence of mycotoxins in food and the globalization of food markets (McKean et al., 2006). Furthermore, climate changes towards an increase of temperature and humidity in certain European regions are expected to favour the growth of contaminating fungi, thereby increasing the likelihood of food commodities contamination with mycotoxins (Paterson and Lima, 2010). As a consequence, there has been an increasing concern about the health hazard from exposure to multiple mycotoxins (mycotoxin mixtures) in human and animals. Several studies have reported on the combined effects of mycotoxins (Corcuera et al., 2011; Klarić et al., 2013; Speijers and Speijers, 2004; Tavares et al., 2015, 2013) but the nature of the observed effect or the relative potencies of each mycotoxin in the mixture are not fully understood yet (Wan et al., 2013), thus limiting the actual health risk assessment to their single effects.

Human risk assessment is based on a 4-step process, namely i) hazard identification and ii) characterization (both also considered as hazard assessment) examining if, and the conditions by which, a certain mycotoxin has the potential to cause a particular adverse health effect or disease and the numerical relationship between the level of dietary exposure (dose) and the associated adverse effect (response); iii) exposure assessment, estimating the frequency, intensity, and duration of ingestion of a mycotoxin; and iv) risk characterization, integrating the results of the exposure assessment with those of hazard characterization to estimate the degree of concern (FAO/WHO, 1997).

Historically, the health risk from human exposure to chemical contaminants has been evaluated on the basis of single-chemical and single-exposure pathway scenarios. In general, exposures to a chemical through the food were assessed independently, and no concerted effort had been made to evaluate potential multiple exposures simultaneously. However, in the last years a tiered approach has been proposed by the World Health Organization (WHO) and by the European Food Safety Authority (EFSA) in order to assess the risk of multiple chemicals, including contaminants (EFSA, 2013; Meek et al., 2011). This hierarchical approach involves integrated and iterative considerations of exposure and hazard at all phases, with each tier being more refined (*i.e.* less conservative and uncertain) than the previous one, but more laborious and intensive. The framework comprises a tiered approach for exposure assessment, hazard assessment and risk characterization and requires at the higher tiers increasing knowledge about the group of chemicals under assessment. Briefly, the tiers can range from tier 0 (default values, data poor situation) to tier 3 (full probabilistic models) (EFSA, 2013; Meek et al., 2011).

Considering that risk assessment is intimately related to the establishment of regulatory guidelines, once the risk assessment is completed, an effort to reduce or manage the risk should be followed to protect public health (Renwick et al., 2003). The food safety legislation through mycotoxins risk assessment is in place to control mycotoxins in food and includes single and sum of maximum levels for some mycotoxins in foodstuffs (European Commission, 2006 and its amendments). Some approaches are now being reported for the first time in the literature concerning the risk assessment of co-occurring mycotoxins in foodstuffs (Assunção et al., 2015; De Boevre et al., 2013; Han et al., 2014) but far more research is required concerning the nature of interactions between multiple mycotoxins until real conclusions could be drawn concerning the health impact of mycotoxin mixtures.

Following the increasing interest of risk assessors, regulators and scientific community on the risk assessment of multiple mycotoxins in food, recent international meetings and research projects had pointed out the urgent need to address and discuss issues such as the co-occurrence of mycotoxins, their combined toxicity and cumulative risk assessment, namely: the "International Conference on Food Contaminants: challenges in chemical mixtures", 2015 (ICFC2015) (http://hdl.handle.net/10400.18/3214); a course and a symposium within the 51st Congress of the European Societies of Toxicology, 2015 (http://www.eurotox2015.com); the Portuguese project entitled "MycoMix, Exploring the toxic effects of mixtures of mycotoxins in infant food and potential health impact" (Alvito et al., 2015); and the EU research project entitled "EuroMix, A tiered strategy for risk assessment of mixtures of multiple chemicals" (https://www.euromixproject.eu/).

Risk assessment of combined human exposure to multiple mycotoxins poses several challenges to scientists, risk assessors and risk managers and open new avenues for research. This work aims to give a holistic overview of the main challenges and perspectives concerning the risk assessment of multiple mycotoxins in food, as a scientific evaluation process. For this purpose, the following sections include a general overview and report recent advances in mycotoxin research and main challenges for each risk assessment step.

2. HAZARD ASSESSMENT OF MULTIPLE MYCOTOXINS

2.1. An overview of hazard assessment of single and multiple mycotoxins

Information on the hazard assessment of a chemical to which humans are exposed to is a determinant step within the human health risk assessment. Particular attention has been dedicated in last years to the occurrence of multiple chemicals in food leading to a change in the paradigm of the hazard assessment. The urgent need for a deep understanding of the potential effects of chemicals in mixture gave rise to the concept of combined toxicity which is defined as the "response of a biological system to several chemicals, either after simultaneous or sequential exposure" (EFSA, 2013; Loewe and Muischnek, 1926). Combined toxicity can take three possible forms: concentration addition (CA), independent action (IA) or interaction (Loewe and Muischnek, 1926). According to the CA model, the joint action of multiple chemicals is the summation of individual toxicities, assuming the same Mode of Action (MoA) and/or at the same target cell, tissue or organ. In the IA model, the combined effects are estimated assuming that chemicals act independently by dissimilar MoA or at different target cells, tissues or organs and considers that the probability of toxicity from exposure to one chemical is independent from the probability of toxicity from exposure to another chemical in the mixture (Bliss, 1939; Jonker et al., 2004; Meek et al., 2011). These two reference models have found successful application to toxicological assessments of mixtures of similarly acting and dissimilarly acting compounds, both in ecotoxicology studies using a range of species (Backhaus et al., 2004; Faust et al., 2003; Loureiro et al., 2010) and in human toxicity studies using cell lines or animal models (Mueller et al., 2013; Tavares et al., 2013). Deviation from these models include synergism (mixture effect greater than additive), antagonism (mixture effect less than additive) and more subtle interactions that depend on the actual doses of the mixture components (e.g. synergism at low doses and antagonism at higher doses) or on the ratio of doses between the compounds in the mixture (e.g. the extent of the synergism or the antagonism depends on the relative contribution of each compound in the mixture) (Jonker et al., 2005, 2004). Because MoA

of chemicals in mixtures is often unknown or incompletely understood, a frequent option has been the application of both models of CA and IA for actual effect prediction, rather than making a theoretically based choice. However, from a practical point of view the application of a single model for all situations is desirable and, being the CA the most conservative model, EFSA recommended its use within the risk assessment of food contaminants that includes mycotoxins (EFSA, 2013).

Particular attention must be dedicated to carcinogens in hazard assessment, including mycotoxins and other food contaminants (Jeffrey and Williams, 2005). Genotoxicity, *i.e.*, the capacity of exerting a damaging effect on the cell's genetic material (DNA, RNA, chromosomes) affecting its integrity and/or function is a major mechanism that contributes to the carcinogenic process. Considering that genetic events, such as gene mutations, structural or numerical chromosomal aberrations and recombination are closely related with carcinogenesis, genotoxic effects can be characterized in a faster, easier, and inexpensive way using standard *in vitro* and *in vivo* genotoxicity assays, instead of performing long-term carcinogenesis assays in animals (Dearfield and Moore, 2005; Louro et al., 2015). However, chemicals acting through a non-genotoxic mechanism, including induction of epigenetic events and mitogenesis, can be equally relevant to the carcinogenic process. Therefore, besides genotoxicity testing, mechanistic studies are also needed to clarify the MoA of carcinogenic agents. In this respect, it is generally assumed that a threshold of exposure may be determined for non-genotoxic carcinogens, below which no biologically significant effect will be induced (Dybing et al., 2008). In contrast, from a conservative and health protection point of view, it has been assumed that genotoxic carcinogens act by a non-threshold mechanism, giving rise to linear dose-response curves. The decision on whether or not a chemical is genotoxic is thus of primary importance to select between a non-threshold or threshold risk assessment approach in the step of hazard identification.

A framework, similar to the one suggested by Spurgeon et al. (2010) to understand the effects of environmental chemical mixtures, may be proposed to investigate the combined toxicity of multiple mycotoxins in food and their potential impact on human health. The framework incorporates the concepts of external exposure or 'bioavailability' of the mixture in the environment together with its exposure in the target species associated with accumulation through toxicokinetics to the expression of toxicity as mediated via receptor interactions within toxicodynamics. A similar framework could be suggested to investigate the health effects of multiple mycotoxins in food reflecting physiological conditions occurring from ingestion of mycotoxins to their effects on target cells, tissues or organs and should include three sequential main concepts: i) bioaccessibility, release of mycotoxins from its matrix into digestive juice in the gastrointestinal tract (Versantvoort et al., 2005); ii) toxicokinetics and iii) toxicodynamics, taking into

account interactions between mycotoxins and/or any active metabolites and the target cells/tissues/organs.

2.2. A physiologically-based framework for hazard assessment of mycotoxins

In human risk assessment, ingestion of food is considered a major route of exposure to mycotoxins. Oral bioavailability, defined as the fraction of an orally ingested mycotoxin that reaches the systemic circulation and is distributed throughout the body to exert its toxic effects, can be seen as the resultant of three processes: i) release of the mycotoxin from its matrix into digestive juice in the gastrointestinal tract (bioaccessibility); ii) transport of the mycotoxin across the intestinal epithelium into the vena Portae (intestinal transport); and iii) biotransformation of the mycotoxin in the liver (and intestine) (metabolism) (González-Arias et al., 2013; Versantvoort et al., 2005). The concept of bioaccessibility of mycotoxins has become important in the risk assessment domain considering that the amount of mycotoxin consumed via food (external dose) does not always reflect the amount available to the body (internal dose) to produce its toxic effects on target cells, tissues or organs (González-Arias et al., 2013; Versantvoort et al., 2005). To determine the bioaccessibility, several models of different complexities have been proposed to simulate food digestion. Simulated digestion methods typically include the oral, gastric and small intestinal phases, and, occasionally, large intestinal fermentation. The majority of models reported in literature are the static ones (Gil-Izquierdo et al., 2002; Hur et al., 2011; Versantvoort et al., 2005). However, more sophisticated dynamic *in vitro* models are also available and although more realistic, they are complex and expensive (Avantaggiato et al., 2004; González-Arias et al., 2013). A recent review (González-Arias et al., 2013) on the bioaccessibility of single mycotoxins reported, in general, high bioaccessibility values for AFs and FBs, (70-100%), intermediate values for PAT and DON (30-70%) and lower values for ZEA. The bioaccessibility of OTA has proven to be very variable, including values near 100%, but also below 30%. Until now, few data on the bioaccessibility of co-occurring mycotoxins are available, namely for AFs (Kabak and Ozbey, 2012), AFB_1 and OTA (Kabak et al., 2009; Raiola et al., 2012b; Versantvoort et al., 2005), enniatin (G. Meca et al., 2012; Prosperini et al., 2013), trichothecenes T-2 and HT-2 (Monaci et al., 2015). A very recent preliminary study evaluated the possible interactions that could happen when PAT and OTA co-occur in cereal-based baby foods (Assunção et al., 2016b).

The *in vitro* digestion models could also be used in combination with intestinal models (e.g. Caco-2 cells) to address further mechanistic questions, such as intestinal transport, contributing to an accurate mycotoxin risk assessment, offering a more complete picture of what happens during digestion in the

intestinal tract (De Nijs et al., 2012; G. Meca et al., 2012; Prosperini et al., 2012; Versantvoort et al., 2005).

Regarding toxicokinetics, absorption occurs mainly through ingestion for chemicals in food and a central issue relates to the passage across the gut wall and entering into the blood circulation, although for some chemicals, uptake is restricted to the epithelium of the gastrointestinal tract (FAO/WHO, 2009). The sites of passive or active uptake across body barriers, depending on the chemical nature of the toxins, are potential points of interactions because one toxin might affect the efficiency of the uptake or elimination of other components of the mixture. In cases where toxins enter or are eliminated through selective transporters, competition between mycotoxins at relevant surface transporters can take place and can result in changes in uptake, bioaccessibility and toxicity, depending on the relative affinity of each toxin for the transporter.

Once (multiple) chemicals have entered the systemic circulation they can interact, inhibit or induce a range of metabolic pathways including phase I enzymes [e.g., cytochromes P450, (CYPs)], phase II enzymes (e.g., glutathione-S transferases) and antioxidant defence enzymes (e.g., superoxide dismutase or catalase) (Streetman et al., 2000). These systems provide a network of responses directed mainly to the detoxification of chemicals but in some cases lead the production of toxic metabolites. This is the case of AFB_1 biotransformation by CYP3A4 and 1A2 that results in the formation of an exo-epoxide and AFQ_1, whilst CYP1A2 can lead to the formation of some exo-epoxide but also a high proportion of endo-epoxide and AFM_1 (Dohnal et al., 2014). The exo-epoxide binds to DNA and forms a pre-mutagenic lesion that mediates AFB_1 mutagenicity and carcinogenicity (Bedard and Massey, 2006; Wild and Turner, 2002). It is plausible that interactive effects may occur when two or more mycotoxins are metabolized through the same pathway, so that one mycotoxin might substantially impact on the detoxification of the other ones. This may be mediated either by a competitive inhibition or by an over-induction of the metabolic system and a faster biotransformation of the mycotoxins. In the work by Corcuera et al. (2011) antagonistic genotoxic effects of OTA and AFB_1 combinations were observed in liver-derived HepG2 cells, concomitantly with an increase of intracellular reactive oxygen species (ROS). The authors hypothesized that competition between both toxins to the same CYP enzymes could have resulted in a lower amount of the mutagenic AFB_1 exo-epoxide molecules and thus in a lower level of DNA damage. Concerning interactions at the level of phase II metabolism, Tavares et al. (2013) proposed that the co-existence of OTA and AFM_1 in Caco-2 cells might have resulted in a competition for the glutathione molecules, decreasing the level of ROS produced by OTA and hence leading to an antagonistic cytotoxic effect.

A further source of mixture interactions relates to its toxicodynamics, *i.e.*, at the level of the dynamic contact of a toxicant with its biological target, possibly impacting on its biological effects at target cells/tissues/organs. For mycotoxins with a similar MoA, the assumption within the CA model is that both toxins are present at the target site, and each one is able to bind freely with no stimulatory or competitive influences. In mammalian systems, the toxicodynamic consequences of receptor-binding have been associated with a variety of effects, ranging from neurotoxicity, renal toxicity and cardiovascular toxicity (Dorne et al., 2007). Carefully designed experiments and informatics approaches can be used to investigate the mechanistic basis of mixture effects.

2.3. Evaluation of combined effects of multiple mycotoxins

The number of studies addressing the combined effects of mycotoxins using *in vitro* and *in vivo* models and several endpoints (e.g., cytotoxicity, immunotoxicity and genotoxicity) has been steadily increasing in the last decade giving rise to a set of data that might greatly contribute to hazard assessment of multiple mycotoxins. Šegvić Klarić (2012) and, more recently, Alassane-Kpembi et al. (2016) have comprehensively reviewed combined toxicity studies involving regulated groups of mycotoxins, particularly, OTA, aflatoxins, *Fusarium* toxins, and trichothecenes and emerging mycotoxins e.g., beauvericin and enniatins. Cytotoxicity assays, using a diversity of cell lines have been widely used because they are fast and economic assays that may help predicting the *in vivo* toxicity of combinations of mycotoxins co-occurring in food (Creppy et al., 2004; Tiemann and Dänicke, 2007), with the advantage of allowing the reduction of the number of animals under experimentation, in compliance with the European Union recommendations (European Union, 2010). Combinations involving AFB_1 or OTA are among the most frequently assessed, due to concerns related to their recurrent occurrence in several mixtures and their severe chronic adverse effects that can be even amplified if a synergistic effect is identified Table 30.

The potential of mycotoxins to elicit a cytotoxic response is transversal to many recent works, but some controversy still remains about the pattern that better describes mycotoxins combined effect. Table 30 compiles data from the joint effects of AFB_1 or OTA as components of several mixtures assessed *in vitro* and, more rarely, *in vivo*. For instance, combinations of OTA, AFB_1 and FB_1 in three different mammalian cell lines showed synergistic cytotoxic effects with regard to mitochondrial integrity although binary mixtures of the same mycotoxins followed the additivity pattern (Clarke et al., 2014). Noteworthy, no interactive effect was observed for mycotoxin mixtures tested at the EU regulatory limits, which highlights

the relevance of using an appropriate dose-range in the *in vitro* studies. For binary mixtures involving OTA, additive toxic effects were observed for combinations with AFB_1 in kidney cells (Golli-Bennour et al., 2010) and in hepatoma-derived cells (Corcuera et al., 2011) whereas synergistic effects were reported in rat brain glioma, Caco-2 and Vero cells (Creppy et al., 2004); antagonism was described for the combined toxicity of OTA and AFM_1 in Caco-2 cells (Tavares et al., 2013). In addition, synergistic effects between OTA and CIT were identified in renal cells *in vitro* (Bouslimi et al., 2008; Heussner et al., 2006) and *in vivo* (Pfohl-Leszkowicz et al., 2008). Recently, the effect of the ternary mixture of OTA, citrinin and sterigmatocystin was explored in a human hepatocellular cancer cell line (Hep3B), showing a synergistic effect at low toxin doses that shifted to antagonism at higher concentrations (Anninou et al., 2014). A similar dose-dependent interactive effect was found for the joint effects of OTA and FB_1 in human hepatoma and human renal cells (Tavares et al., 2015). Apart from mixtures involving OTA or AFB_1, the combined effects of a number of other mycotoxins combinations have been also addressed in the last years, including *Fusarium* and *Alternaria* toxins (Vejdovszky et al., 2016), DON, NIV and their acetylated derivatives (Alassane-Kpembi et al., 2015) or BEA, DON and T2-toxin (Ruiz et al., 2011), to name only some studies. Factors related to the experimental design, including the concentration range of the single toxins and relative concentration of each toxin in the mixture or the metabolic capacity of the target cell are central to the combined final effect and have to be carefully controlled. Another important aspect relates to the approaches that have been applied to quantitatively measure the dose–effect relationships of single mycotoxins and its combinations and to ascertain putative interactive effects. Among them, the usefulness of the combination index (CI)-isobologram equation by Chou (2006) and Chou and Talalay (1984), which is based on the median-effect principle (mass-action law) that demonstrates that there is an univocal relationship between dose and effect, independently of the number of substrates or products and of the mechanism of action or inhibition (Alassane-Kpembi et al., 2013; Bernhoft et al., 2004; Ruiz et al., 2011) (Table 30). This method involved plotting the dose-effect curves for each compound and their combinations in multiple diluted concentrations and is based on the assumption that when two compounds are combined and subjected to several dilutions, the combined mixture of the two compounds behaves as the third compound for the dose-effect relationship. The CI indicates not only the type of interaction (additivity, synergism or antagonism) but also the magnitude of the interaction found. In addition, the conceptual models of CA and IA incorporating also a set of deviation functions within a nested framework (Jonker et al., 2005, 2004) have been effectively applied to the analysis of cytotoxic effects of mycotoxins mixtures (Tavares et al., 2013)(Table 30). Several prerequisites are required to allow the application of these models, implicating a careful experimental design that will depend on the number of chemicals in the mixture and the degree of detail needed concerning the dose–effect

relationship for single and combined toxicity; the single compounds in the mixture should be tested at a constant dose ratio, using a full factorial design. This approach was employed by Tavares et al. (2013) to assess the combined effect of OTA and AFM_1 in an intestine cell line, following determination of the inhibitory concentration at 50% (IC_{50}) of the single toxins. Both CA and IA models were applied to derive potential interactions at concentrations below the individual IC_{50}. Whereas the well-known CA model assumes that both toxins have a similar MoA, the IA model assesses the probability of toxicity from exposure to one mycotoxin being independent from the toxicity of the other toxin in the mixture (Jonker et al., 2005, 2004). A fairly good agreement was obtained for both models, in that antagonism was found after the CA model fit, while a dose level deviation was observed after IA modeling, where antagonism was observed at low dose levels and synergism at high dose levels. However, when dealing with a mixture of several chemicals, where the number of experimental groups increases exponentially, fractionated factorial designs can be used to identify interactions in a manageable way (Groten et al., 2004). Another possibility is to apply a tiered approach as suggested by Tajima et al. (2002) for *Fusarium* mycotoxins, starting from the study of the whole mixture effect, followed by a screening of interactions using a fractionated factorial design and, finally the confirmation of interaction through a full factorial design.

In spite of the number of studies addressing the cytotoxicity of multiple toxins, genotoxicity is of a greater concern for most of the mycotoxins and their mixtures due to its association to carcinogenesis. However, the specific genotoxic properties of multiple mycotoxins are much more difficult to address comprehensively in complex combinatory experiments and thereby studies reporting combined genotoxic effects of mycotoxins are more limited. Interestingly, among the *in vitro* studies available, additivity or even antagonism are the predominant joint effects of mixtures containing AFB_1 or OTA (Table 30). A common drawback of *in vitro* studies is the use of dose-ranges that are much higher than those that have been found in biological fluids of exposed humans. Thus, further studies using realistic concentrations that follow a carefully planned experimental design are still needed. Moreover, for those mixtures showing interactive effects in *in vitro* assays, confirmatory *in vivo* assays should be used to better predict the effects on humans. For instance, the combined genotoxic effect of OTA and CIT was explored in a Chinese hamster lung cell line showing a synergism at low doses that changed to antagonism at higher doses (Föllmann et al., 2014). A synergistic effect was also observed through the analysis of chromosome aberrations in bone marrow cells from exposed mice (Bouslimi et al., 2008), confirming that low doses are more realistic and thus more relevant to predict *in vivo* effects. On the other hand, Corcuera et al. (2011) showed that the mixture of OTA and AFB_1 produced an antagonistic DNA damaging effect, comparatively to each single toxin. The observed antagonism was further confirmed in liver and bone

marrow cells of exposed rats using the comet and the micronucleus assays, respectively (Corcuera et al., 2015). These two examples suggest that carefully designed *in vitro* studies on combined genotoxic effects of mycotoxins may have a good predictive value for their *in vivo* joint effect, as advocated by Creppy et al. (2004). Nevertheless, more *in vivo* genotoxicity studies are urgently needed, using the oral route of exposure, realistic concentrations derived from human exposure data and allowing the quantification of several endpoints (e.g., DNA breaks, chromosome numerical and structural anomalies, gene mutations) to confirm the value of *in vitro* approaches. Furthermore, the application of mathematical models to ascertain genotoxic interactive effects, similarly to what has been done for combined cytotoxicity assessment, is highly relevant to have firm and reliable conclusions about genotoxic interactive effects. In this sense, Ermler et al. (2014) have already shown that the CA, IA and hybrid CA/IA models are applicable to data obtained for several model compounds with similar and dissimilar MoA, using the micronucleus assay.

Table 30. Combined toxicity and interactive effects concerning multiple mycotoxins studies

Mixture	Biological effects Methodologies	Experimental system	Combined effect	Data analysis/modelling	Reference
AFB_1-OTA-FB_1 OTA-FB_1	Cytotoxicity: High content analysis endpoints	MDBK cell line	Synergism (binary and tertiary mixtures)	Comparison between observed and expected additive effects	R. Clarke et al., 2015a
AFB_1-OTA	Genotoxicity: Comet assay Micronucleus assay	Rat kidney and liver bone marrow	Antagonism (liver and bone marrow)	Statistical comparison of data from single and combined effects	Corcuera et al., 2015
AFB_1-ZEA- DON AFB_1- ZEA AFB_1-DON	Cytotoxicity: MTT	BRL 3A rat liver cells	Synergism (binary mixtures)	Central composite design	Sun et al., 2015
AFB_1-OTA-FB_1 AFB_1OTA OTA-FB_1	Cytotoxicity: MTT NR	Caco-2 cell line MDBK Raw 264.7	Synergism (tertiary mixture) Additivity (binary mixtures)	Comparison between observed and expected additive effects	Clarke et al., 2014
AFB_1- ZEA AFB_1-DON AFB_1-ZEA- DON	Cytotoxicity: MTT, LDH Apoptosis: PI/Annexin Oxidative stress: Dichloro Fluorescein diacetate	PK15 cell line	AFB_1-ZEA or DON – Synergism AFB_1-ZEA – antagonism (apoptosis) AFB_1- ZEA (low doses) - Antagonism AFB_1-ZEA or DON (high doses) - Synergism	Central composite design; comparison between observed and expected dose-response curves	Lei et al., 2013
AFB_1-AFB_2 AFM_1- AFM_2	Cytotoxicity: MTT Immunotoxicity: Flow Cytometry NO2 assay	J774A .1 cell line	Synergism	Statistical comparison of data from single and combined effects	Bianco et al., 2012
AFB_1-OTA	Cytotoxicity: MTT Genotoxicity: Comet assay	HepG2 cell line	Additivity Antagonism	Statistical comparison of data from single and combined effects	Corcuera et al., 2011

AFB_1-OTA	Cytotoxicity: MTT Genotoxicity: Comet assay	Vero cell line	Additivity Additivity	Interactive index calculation	Golli-Bennour et al., 2010
AFB_1- T2-toxin	Cytotoxicity: WST-1 cell proliferation assay system	HepG2 cell line BEAS-2B	Additivity Synergism	Interactive index calculation	McKean et al., 2006
OTA-DON	Cytotoxicity: MTT Oxidative stress: Dichloro fluorescein assay	Caco-2 cell line	Additivity/ Synergism	Comparison between single and combined effects	Cano-Sancho et al., 2015
OTA-Sterigmatocystin-CIT-Sterigmatocystin,	Cytotoxicity: MTT Genotoxicity: SCE	Hep3B cell line	Additivity Additivity/ Antagonism	Calculation of the Coefficient of drug interaction	Anninou et al., 2014
OTA-CIT	Cytotoxicity: NR Genotoxicity: Micronucleus assay	V79 cell line	Additivity Synergism (low doses); Antagonism (High doses)	Comparison between observed and expected data	Föllmann et al., 2014
OTA- AFM_1	Cytotoxicity: NR	Caco-2 cell line	Antagonism	Full or partial factorial design; mathematical functions for CA/IA and dependent deviations	Tavares et al., 2013
OTA-BEA	Genotoxicity: Comet assay	PK15 cell line Human lymphocytes	Additivity/Synergism Additivity (lymphocytes)	Comparison between observed and expected additive effects	Klarić et al., 2010
OTA-FB_1-BEA	Genotoxicity: Micronucleus assay	PK15 cell line	Additivity	Comparison between observed and expected additive effects	Klarić et al., 2008
OTA-CIT	Cytotoxicity: MTT Genotoxicity: Comet assay Oxidative stress: Dichloro Fluorescein diacetate Apoptosis	HepG2 cell line	Synergism Synergism	Comparison of IC_{50} values between individual and combined effects	Gayathri et al., 2015
OTA-CIT	Cytotoxicity Genotoxicity: DNA fragmentation Chromosome aberrations *in vivo*	Vero cell line Mouse bone marrow cells	Synergism Synergism	Comparison of IC_{50} or LD_{50} between individual and combined effects	Bouslimi et al., 2008
OTA-PAT-CIT-Gliotoxin	Cytotoxicity: MTT Immunotoxicity: T cell function ELISA	Human Lymphocytes	Antagonism	Comparison between observed and expected dose-response curves using the response addition and CA models	Tammer et al., 2007
OTA-PAT OTA-CIT	Cytotoxicity: MTT	LLC-PK1 cell line	Synergism	Central composite design; comparison between observed and expected dose-response curves	Heussner et al., 2006
OTA-CIT OTA-PAT CIT-PAT	Cytotoxicity: Lymphocyte proliferation test	Porcine lymphocytes	OTA-CIT -synergism OTA-PAT - antagonism	Isobologram analysis	Bernhoft et al., 2004

			CIT-PAT - additivity		
OTA-FB_1	Cytotoxicity: NR	C6- glioma cell line Vero Caco-2	Synergism	Statistical comparison of data from single and combined effects	Creppy et al., 2004

2.4. Main challenges in hazard assessment of multiple mycotoxins

Knowledge of the real percentage of mycotoxins that can be absorbed in the small intestine would enable a more accurate risk assessment. Several factors may affect the bioaccessibility of single and multiple mycotoxins. Variability within mycotoxin bioaccessibility values depends on the compound, food product, contamination level and way of contamination (spiked or naturally contaminated) (Kabak et al., 2009). Additionally, the diversity of *in vitro* digestion models used to access the bioaccessibility of mycotoxins constitutes another important challenge. The individual static *in vitro* digestion models described in the literature exhibit significant variations in the *in vitro* digestion parameters as pH, mineral type, ionic strength and digestion time, which alter enzyme activity (González-Arias et al., 2013; Minekus et al., 2014). Consequently, this fact hampers the possibility to compare results across research-groups and to deduce general findings. To overcome this difficulty, recently a standardized static *in vitro* digestion model suitable for food was developed within the COST action INFOGEST (https://www.cost-infogest.eu/). This standardized digestion method (Egger et al., 2015; Minekus et al., 2014) is based on the current state of knowledge on *in vivo* digestion conditions and describes a detailed line-by-line protocol (https://www.youtube.com/channel/UCdc-NPx9kTDGyH_kZCgpQWg) with recommendations and justifications on the experimental procedures applied.

Mycotoxin absorption constitutes another challenge within risk assessment considering that toxins could reach intestine as the parent compound or as metabolites formed during the digestion, and methods for their detection could not be yet developed or currently available (González-Arias et al., 2013).

The level of effort required for hazard assessment of the combined effect of multiple chemicals to humans or to the ecosystem is quite high and should be initially weighted by the magnitude of potential risks, the objective (e.g. priority setting or screening for additional focus or risk management) and scope (e.g. local and national interest) (Meek et al., 2011). Since testing the combined effects of all possible combinations of mycotoxins is not feasible in a reasonable timeframe, priorities for hazard assessment need to be set in order to put more effort into the most relevant mixtures. The rational for priority setting may be based on the frequency of its co-occurrence in food, the hazardous potency of the single toxins, the structure-activity relationship pointing to a strong probability of interactions or on preliminary data suggesting synergistic effects. Even though the available data concerning combined toxic effects of mycotoxins have

been growing, a considerable degree of inconsistency is noted when comparing the outcomes of studies focused on similar mixtures and therefore, more studies are needed to allow firm conclusions. Given that these studies are laborious and time consuming, the future utilization of simpler and faster electrochemical biosensors (exemplified in Gu et al., 2015) and high content analysis that allows the simultaneous examination of a large set of endpoints with high sensitivity (Clarke et al., 2015b) are promising advances in combinatory toxicology. Studies on combined genotoxic effects of multiple mycotoxins are still scarce and more studies should be developed in order to go further on the risk assessment and provide information for risk assessors and, subsequently, for risk managers and regulators. In addition, exploring interactions at the mechanistic level remains a challenging issue and more studies are needed in order to clarify the biochemical, cellular or molecular mechanisms underlying the observed interactive effects. For this purpose, a systems toxicology approach, *i.e.*, a toxicogenomic approach can provide useful information about genes expression, proteins or biochemical pathways within a reasonable timeframe from which mechanisms of toxicity can be established (Altenburger et al., 2012).

3. EXPOSURE ASSESSMENT OF MULTIPLE MYCOTOXINS

3.1. Overview of methods for estimating dietary exposure

Exposure assessment is a key element for quantifying risk and constitutes one of the four steps included in the risk assessment process and is usually defined as the qualitative and/or quantitative evaluation of the likely intake of agents via food as well as exposures from other sources (FAO/WHO, 2009). Dietary exposure assessment consists of combining deterministically or probabilistically food consumption figures with occurrence of a given chemical substance in a number of food categories (EFSA, 2013).

Food consumption data reflect what individuals or groups consume in terms of solid foods, beverages, and dietary supplements. National food consumption surveys are the principal sources of information for determining real food consumption habits in a population of consumers. In addition to the general population, the risk assessments generally also consider the exposure of specific consumer groups, such as infants, children, and people following specific diets (e.g. vegetarians) (FAO/WHO, 2009). Food consumption can be estimated through food consumption surveys, including records/diaries, food frequency questionnaires and dietary recall (EFSA, 2011). The food records or food diaries require the report of all foods consumed during a specific period (usually ranging between 24 hours to 7 days). Food frequency questionnaires consist of a structured listing of individual foods or food groups where the

respondent is asked to estimate the number of times the food is usually consumed per day, week, month or year (FAO/WHO, 2009).

Considering the increasing evidence that co-contamination of food matrices is the rule, not the exception (Stoev, 2015), the proposal to quantify the simultaneous occurrence of multiple contaminants goes on the direction of developing multi-analyte methods combining a generic sample preparation protocol with a highly selective method exhibiting sufficient detection capacity, such as LC-MS (Malachová et al., 2014; Turner et al., 2015). EFSA suggested that Total Diet Studies (TDSs) provide the most accurate estimates of mean contamination by the chemicals in the food consumed by the population or collective group of individuals (FAO/WHO/EFSA, 2011). As TDSs consider total exposure from whole diets and are based on food contamination "as consumed" rather than contamination from raw commodities, they are considered to ensure a more realistic exposure measure than exposure studies based on monitoring programs and surveillance data (Papadopoulos et al., 2015). Within the general framework of chemical risk assessment, a difficult step in dietary exposure assessment is the handling of concentration data reported to be below the limit of detection (LOD) of the analytical method. These data are known as non-detects and the resulting distribution of occurrence values is left-censored. EFSA has so far mainly used substitution methods (EFSA, 2010). The most common approaches are the substitution methods that replace non-detects by LOD divided by 2 or producing an upper and lower bound by substitution of non-detects by LOD or 0, respectively (EFSA, 2010).

Food consumption and occurrence data are then combined to perform the dietary exposure assessment. A deterministic (point estimate) or probabilistic (stochastic) approach is generally applied to perform dietary exposure assessment studies. The structure of the probabilistic and deterministic approaches is similar and is based on the same basic equations whereby food consumption data are combined with concentration data to estimate dietary exposure. The fundamental difference is that at least one variable is represented by a distribution function instead of a single value and the model sample from each distribution is a distribution of potential dietary exposures generated using several thousand iterations. Monte Carlo simulation is the technique that has been applied to a wide variety of modelling scenarios in probabilistic dietary exposure assessment (FAO/WHO, 2009; Han et al., 2014).

3.2. Worldwide exposure assessment of co-occurring mycotoxins in food

Human exposure is a crucial element in the risk assessment of mycotoxins. Table 31 summarizes most recent reports on exposure assessment of worldwide populations to multiple mycotoxins in food,

published in the last six years. Children and adults are the main population groups considered in these studies. Cereal based products are important commodities prompt to mycotoxins contamination and as such were an obvious target in the reported exposure assessment studies (wheat and maize foods, breakfast cereals). Nuts and dried fruits were also assessed (Cano-Sancho et al., 2013; Cressey and Reeve, 2013; Van de Perre et al., 2015). AFs are the mycotoxin group most assessed (Assunção et al., 2015; Cano-Sancho et al., 2013; Cressey and Reeve, 2013; García-Moraleja et al., 2015; Signorini et al., 2012; Sirot et al., 2013; Van de Perre et al., 2015). Other evaluated mycotoxin groups include fumonisins, ochratoxins, patulin, trichothecenes and zearalenone and their respective metabolites, enniatins, beauvericin, sterigmatocystin and *Alternaria* toxins, with a maximum of 48 mycotoxins assessed simultaneously (Sirot et al., 2013; Sprong et al., 2016b). For the exposure assessment studies, food consumption data were mainly obtained from national food consumption surveys, including different data collection methodologies, namely 1-day (Cressey and Reeve, 2013; Han et al., 2014), 2-day (De Boevre et al., 2013; Sprong et al., 2016a; Van de Perre et al., 2015) and 3-day 24h recalls (Zhao et al., 2015), 3-day food diary (Assunção et al., 2015) and 7-day food diary (Sirot et al., 2013), all reported in Table 31.

LC/MS-MS was the most used technique for the quantification of multiple mycotoxins in foodstuffs although LC-FD is also used in several studies for aflatoxins quantification (Assunção et al., 2015; Cano-Sancho et al., 2013; López et al., 2016; Sirot et al., 2013). Recently, an LC–MS/MS "dilute and shoot" method for the determination of 295 fungal and bacterial metabolites was optimized and validated according to the guidelines established in the Directorate General for Health and Consumer Affairs of the European Commission (SANCO) document No. 12495/2011 (Malachová et al., 2014; SANCO, 2011). Based on this study, Malachová et al. (2014) considered that a quantitative determination of mycotoxins by LC–MS/MS based on a "dilute and shoot" approach is also feasible in case of complex matrices. TDSs were developed to estimate the exposure of populations to food contaminants, including mycotoxins. From the studies presented in Table 31, Sirot et al. (2013) and Sprong et al. (2016a) evaluated the exposure of French and Dutch populations, respectively, to mycotoxins. According to Lee et al. (2015), countries as Australia, France, Korea and China had developed their country-specific TDSs including mycotoxins but all countries are encouraged to conduct total diet studies to assess the safety and nutritional quality of diet of their population since TDS is not only a cost-effective tool but also a realistic tool for risk assessment of chemicals in foods. Actually, the TDS EXPOSURE project aims to provide guidance for future TDSs, as well as for new TDSs in countries that have no TDS experience (http://tds-exposure.eu/).

Despite the mentioned advantages, TDS also present some limitations (e.g. lack of harmonization on how to build up the list of foods or food categories) and other type of sampling procedures for food could be applied (e.g. duplicate portion). The substitution methods most used for the handling of non-detects are the replacement of non-detects by LOD values, half LOD values or 0 (EFSA, 2010). Although, some authors decided also to include the LOQ in the management of non-detects (García-Moraleja et al., 2015; Sirot et al., 2013). Until now, the assessment of mycotoxin exposure is mainly based on deterministic approaches however there are an increasing number of studies applying probabilistic models in last years, as shown in Table 31 (Assunção et al., 2015; Cano-Sancho et al., 2013; De Boevre et al., 2013; Han et al., 2014; Signorini et al., 2012; Van de Perre et al., 2015).

Table 31. Recent reports on worldwide exposure assessment to multiple mycotoxins in food products, published between 2010 and 2016.

Country	Population group (age, years old)	Samples	Number of analysed mycotoxins (toxin group)	Food consumption (data collection)	Analytical method: occurrence	Handling non-detects: substitution method	Exposure assessment	References
Argentina	adults (n.r.)	cow´s milk	3 (aflatoxins, trichothecenes, zearelenone)	Argentinean Ministry of Agriculture	ELISA	n.r.	probabilistic	(Signorini et al., 2012)
France	children (3-17) adults (18-79)	212 core foods (*as consumed*)	25 (aflatoxins, trichothecenes & metabolites, fumonisins, ochratoxins, patulin, zearalenone & metabolites)	Second National Individual Dietary Consumption Survey 2006-7 (7-day food record diary)	LC-FD; LC-MS/MS	0, LOD, LOQ	deterministic	(Sirot et al., 2013)
Spain	infants (0-3) children (4-9) adolescents (10-19) adults (20-65) elders (>65) immigrants (17-51) celiac sufferers (16-75)	cereal food baby food dried fruits	4 (aflatoxins)	Catalonia survey (food frequency questionnaire)	LC-FD	LOD/2	probabilistic	(Cano-Sancho et al., 2013)
Belgium	adults (> 15)	cereal-based foods	13 (trichothecenes & metabolites; zearalenone & metabolites)	Belgian National Food Consumption Survey 2004 (2-day 24h recall)	LC-MS/MS	0, LOD/2, LOD	deterministic & probabilistic	(De Boevre et al., 2013)
New Zealand	children (5-14) adults (>15)	maize-based foods, nuts & nuts foods, dried fruits, spice	4 (aflatoxins)	National Nutrition Survey 1997 (24h recall)	n.r.	LOD/2, 0	deterministic	(Cressey and Reeve, 2013)
China	children & adults (>7)	wheat & maize foods	3 (trichothecenes & metabolites)	Shangai Food Consumption Survey 2012-13 (24-h recall)	LC-MS/MS	LOD/2	deterministic & probabilistic	(Han et al., 2014)

Portugal	infants (1-3)	breakfast cereals	12 (aflatoxins, trichothecenes, fumonisins, ochratoxins)	Pilot survey 2014 (3-day food diary)	LC-FD; GC-MS, UPLC-MS/MS	0, LOD/2, LOD	deterministic & probabilistic	(Assunção et al., 2015)
Spain	adolescents & adults (n.r.)	Coffee	21 (aflatoxins, trichothecenes and metabolites, fumonisins, ochratoxins, enniantins, beauvericin, sterigmatocystin)	Spanish Agency for Food Safety Survey 2009	LC-MS/MS	0	deterministic	(García-Moraleja et al., 2015)
Belgium	adults (>15)	nuts, dried fruits	2 (aflatoxins, ochratoxins)	Belgian National Consumption Survey 2004 (2x24h recall)	n.r.	0	probabilistic	(Van de Perre et al., 2015)
China	children & adults (2-100)	wheat based foods	4 (*Alternaria* toxins)	China National Nutrient and Health Survey 2002 (3x24h recall)	UPLC-MS/MS	0, LOD/2, LOD	deterministic	(Zhao et al., 2015)
The Netherlands	children (2-6) & adults (7-69)	88 composite samples (as consumed)	48 (patulin, aflatoxins, ochratoxin A, fumonisins, zearalenone, trichothecenes, ergot alkaloids, *Alternaria* toxins, beauvericin and enniatins)	Dutch National Food Consumption Surveys: 2005/2006 and 2007/2010 (questionnaire, 2 day food diary, 2x24h recall)	LC-MS/MS, LC-FD	LOD, LOQ	Probabilistic	(Sprong et al., 2016a)

n.r. = not reported

3.3. Main challenges in exposure assessment to multiple mycotoxins

It is quite challenging to estimate the usual food consumption considering the limited amount of information from national surveys, since not all age groups (infant, toddlers, young children or older children, adolescents or adults) are included in each national dietary survey. Usually, they are conducted on a limited number of days (up to seven) and use different methodologies (24h dietary recall, food diary, food frequency questionnaire), as summarized in Table 31. This lack of harmonization compromises an accurate mycotoxin exposure assessment and does not allow the generation of European estimates of dietary exposure. To overcome this limitation a recent study was executed to assess how existing consumption data could be improved by developing a "Compiled European Food Consumption Database" (Vilone et al., 2014). According to this study, this database provides a fundamental tool to perform exposure assessments at the European level.

Recent surveys highlight the fact that humans are more frequently exposed to multiple than to single mycotoxins. LC-MS/MS has been the method mostly used for analysis and quantification of multiple mycotoxins and their metabolites in food (Berthiller et al., 2016; Malachová et al., 2014; Turner et al.,

2015). As mycotoxins comprise a wide range of chemical properties, the extraction and chromatographic conditions have to be compromised (Capriotti et al., 2012). The influence of matrix effects is the major challenge in developing reliable quantitative multi-analyte methods therefore, considerable efforts to control matrix effects should be carried out to obtain accurate results (Turner et al., 2015) namely, the inclusion of a sample clean-up step (e.g. using QuEChERS) and the compensation of the signal suppression/enhancement through the usage of matrix matched standards (Berthiller et al., 2016; Malachová et al., 2014; Turner et al., 2015).

Mycotoxin contamination datasets are characterized by the presence of non-detects or none quantified values which constitutes an important issue for the exposure assessment studies (Assunção et al., 2015; Cano-Sancho et al., 2013; Sirot et al., 2013). Therefore, a representative food sampling design (selecting the most susceptible foods, considering a large set of individual and/or composite samples), an accurate chemical analysis method (with low detection limits) and a suitable method to manage left-censored data will be decisive to obtain realistic exposure estimations with low level of uncertainty. This could be particularly important for the exposure assessment of vulnerable population groups such as small children that are generally exposed to higher levels of mycotoxins than adults (Alvito et al., 2010; Assunção et al., 2015; Cano-Sancho et al., 2013; Sirot et al., 2013).

In order to draw more sophisticated exposure scenarios an increasing number of authors applied information technology on probabilistic models instead of deterministic ones. One of the main drawbacks of the deterministic approach is that it does not allow calculating complicated statistics such as high quartiles. Defining high-level consumers is crucial for the outcome of risk assessment. In practice, it determines the proportion of the population that would exceed a health-based limit. Therefore, when refinements are required, simulation methods are proposed as the best approach, particularly for high quartiles (Marin et al., 2013).

The indirect approach obtained with the combination of data of mycotoxin occurrence in food and food consumption patterns is associated with some limitations for the mycotoxins exposure assessment, including the heterogeneous distribution of mycotoxins in food, the possible exposure through other exposure routes than ingestion, the presence of masked mycotoxins, the influence of food processing, inter-individual variation in absorption, distribution, metabolism and excretion (ADME), and the under- and overestimation in food consumption data (Arcella and Leclercq, 2004; Heyndrickx et al., 2014). These limitations could lead to an under- and/or overestimation of the exposure, and biomarkers have been proposed as a suitable alternative. Human biomonitoring is considered a quite new frontier for establishing the real human exposure to mycotoxins. Recent results on this domain (Gerding et al., 2015, 2014; Heyndrickx et al., 2015; Warth et al., 2012a, 2012b) surprisingly revealed a level of exposure to

mycotoxins above the widely accepted tolerable daily intake values, especially to DON, highlighting the importance to perform mycotoxin biomonitoring studies. Typical biomarkers of exposure are the parent toxins themselves, protein or DNA adducts, and/or major phase I or phase II metabolites (e.g. glucuronide conjugates), which are measured in biological fluids such as urine or plasma/serum, and are related to the actual intake of the toxin through contaminated food (Warth et al., 2013). Table 32 summarizes studies developed in different countries and performed using a multibiomarker approach to determine the human mycotoxin exposure. LC/MS-MS methods were the mainly used for the quantification of biomarkers contents in urine, the biological fluid mostly used to determined mycotoxin biomarkers contents (Table 32). The number of analytes studied simultaneously varied between four (Warth et al., 2012a) and 33 (Heyndrickx et al., 2015) compounds. The use of β-glucuronidase-assisted hydrolysis (in order to increase the levels of the parent toxins) (Ahn et al., 2010; Shephard et al., 2013; Solfrizzo et al., 2014, 2011; Wallin et al., 2015), immunoaffinity columns (IAC) (Ahn et al., 2010; Rubert et al., 2011; Shephard et al., 2013; Solfrizzo et al., 2014, 2011; Wallin et al., 2015) and solid-phase extraction (SPE) (Heyndrickx et al., 2015; Njumbe Ediage et al., 2012; Rodríguez-Carrasco et al., 2014b, 2014c; Shephard et al., 2013; Solfrizzo et al., 2014, 2011; Wallin et al., 2015) were applied by some authors as sample preparation and clean-up procedures. However, some studies have also successfully used the so-called dilute and shoot approach by omitting any cleanup procedure (Abia et al., 2013; Ezekiel et al., 2014; Gerding et al., 2015, 2014; Heyndrickx et al., 2015; Shephard et al., 2013; Warth et al., 2012a, 2012b). Although the analysis of mycotoxins in human urine is another important data source for exposure assessment, some challenges are posed to human mycotoxin multibiomarker approaches. These challenges include the lack of toxicokinetic data on mycotoxins in humans resulting in a lot of uncertainties that should be taken into account when perform a risk assessment based on urinary mycotoxin levels; difficulties to correlate human dietary habits, concurrent mycotoxin contamination of food and consequent presence of these mycotoxins in human urine; obstacles to comparison of obtained results between different studies, considering the differences in age, detection limits, number of subjects included in the study and the analytical performances of the used methods (Gerding et al., 2014; Heyndrickx et al., 2015; Rubert et al., 2011; Wallin et al., 2015; Warth et al., 2013). A more detailed information on mycotoxin multibiomarker approach was presented by de Nijs et al. (2016) within this WMJ special issue.

Table 32. Human mycotoxin exposure assessment using a multibiomarker approach.

Country	Nº of analytes	Analytes included	Sample preparation and cleanup	Methodology	Biological samples	Reference
Austria	4	DON, DON-3-GlcA, DON-15-GlcA, DOM-1	"Dilute and shoot"	LC-MS/MS	Urine	Warth et al., 2012a
Bangladesh	23	DON, DON-3-GlcA, T-2, HT-2, HT-2-4-GlcA, FB_1, FB_2, AFB_1, AFG_2, AFB_2, AFM_1, ZEA, ZAN, α-ZAL, β-ZAL, ZEA-14-GlcA, ZAN-14-GlcA, α/β–ZAL- 14-GlcA, OTA, Otα, enniatin B and DH-CIT	"Dilute and shoot"	LC-MS/MS	Urine	Gerding et al., 2015
Belgium	18	AFM_1, AFB_1, AFB1-N7-Gua, OTA, OTα, 4-OH-OTA, FB_1, HFB_1, DON, DON-3-GlcA, DOM-1, T-2, HT-2, ZEA, ZEA-14-GlcA, α-ZAL, β-ZAL, CIT	Liquid-liquid extraction + SPE	LC-MS/MS	Urine	Njumbe Ediage et al., 2012
	33	AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, FB_1, FB_2, FB_3, HFB_1, OTA, Otα, T-2, HT-2, DON, DON-3-GlcA, DON-15-GlcA, DOM-1, DOM-GlcA, 3ADON, 3ADON-15-GlcA, 15ADON, 15ADON-3-GlcA, DAS, FUS-X, ZEA, ZEA-14-GlcA, α-ZAL, α-ZAL-7-GlcA, α-ZAL-14-GlcA, β-ZAL, β-ZAL-14-GlcA, CIT, DH-CIT	"Dilute and shoot" or Liquid-liquid extraction + SPE	LC-MS/MS	Urine	Heyndrickx et al., 2015
Cameroon	15	AFM_1, OTA, FB_1, FB_2, DON, DON-3-GlcA, DON-15-GlcA, DOM-1, T-2, HT-2, NIV, ZEA, ZEA-14-GlcA, α-ZAL, β-ZAL	"Dilute and shoot"	LC-MS/MS	Urine	Abia et al., 2013; Warth et al., 2012b
Germany	23	DON, DON-3-GlcA, T-2, HT-2, HT-2-4-GlcA, FB_1, FB_2, AFB_1, AFG_2, AFB_2, AFM_1, ZEA, ZAN, α-ZAL, β-ZAL, ZEA-14-GlcA, ZAN-14-GlcA, α/β–ZAL- 14-GlcA, OTA, Otα, enniatin B and DH-CIT	"Dilute and shoot"	LC-MS/MS	Urine	Gerding et al., 2015, 2014
Haiti	23	DON, DON-3-GlcA, T-2, HT-2, HT-2-4-GlcA, FB_1, FB_2, AFB_1, AFG_2, AFB_2, AFM_1, ZEA, ZAN, α-ZAL, β-ZAL, ZEA-14-GlcA, ZAN-14-GlcA, α/β–ZAL- 14-GlcA, OTA, Otα, enniatin B and DH-CIT	"Dilute and shoot"	LC-MS/MS	Urine	Gerding et al., 2015
Italy	7	AFM_1, OTA, FB_1, DON, DOM-1, α-ZOL, β-ZOL	IAC + SPE + β-glucuronidase/sulphatase	LC-MS/MS	Urine	Solfrizzo et al., 2011
	8	DOM-1, DON, AFM_1, FB_1, β-ZAL, α-ZAL, ZEA, OTA	IAC + SPE + β-glucuronidase/sulphatase	LC-MS/MS	Urine	Solfrizzo et al., 2014
Korea	4	AFM_1, OTA, FB_1, FB_2	IAC + SIDA + β-glucuronidase	LC-MS/MS	Urine	Ahn et al., 2010
Nigeria	14	AFM_1, FB_1, FB_2, OTA, DON, DON-3-GlcA, DOM-1, NIV, T-2, HT-2, ZEA, ZEA-14-GlcA, α-ZAL, β-ZAL	"Dilute and shoot"	LC-MS/MS	Urine	Ezekiel et al., 2014
South Africa	15	AFM_1, OTA, FB_1, FB_2, DON, DON-3-GlcA, DON-15-GlcA, DOM-1, T-2, HT-2, NIV, ZEA, ZEA-14-GlcA, α-ZAL, β-ZAL	"Dilute and shoot" or IAC + β-glucuronidase/sulphatase or SPE	LC-MS/MS	Urine	Shephard et al., 2013
Spain	11	AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB^1, FB_2, DON, T-2, HT-2, ZEA	IAC	LC-MS/MS	Urine	Rubert et al., 2011
	15	DOM-1, DON, 3ADON, FUS-X, DAS, NIV, NEO, HT-2, T-2, ZAN, α-ZAL, ß-ZAL, ZEA, α-ZOL, ß-ZOL	Liquid-liquid extraction + SPE	GC-MS/MS	Urine	Rodríguez-Carrasco et al., 2014b, 2014c
Sweden	10	AFM_1, DON, FB_1, FB_2, NIV, OTA, ZEA, α-ZOL, β-ZOL, DOM-1	IAC + SPE + β-glucuronidase/sulphatase	LC-MS/MS	Urine	Wallin et al., 2015

Abbreviations: 15ADON, 15-acetyldeoxynivalenol; 15ADON-3-GlcA, 15-acetyldeoxynivalenol-3-glucuronide; 3ADON, 3-acetyldeoxynivalenol; 3ADON-15-GlcA, 3-acetyldeoxynivalenol-15-glucuronide; 4-OH-OTA, hydroxylated form ochratoxin A; AFB_1, aflatoxin B_1; AFB_1-N7-Gua, aflatoxin B_1-N7Guanine; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; AFM_1, aflatoxin M_1; CIT, citrinin; DAS, diacetoxyscirpenol; DH-CIT, dihydrocitrinone; DOM-1, de-epoxy deoxynivalenol;

DOM-GlcA, deepoxy-deoxynivalenol-glucuronide; DON, deoxynivalenol; DON-15-GlcA, deoxynivalenol-15-glucuronide; DON-3-GlcA, deoxynivalenol-3-glucuronide; FB_1, fumonisin B_1; FB_2, fumonisin B_2; FB_3, fumonisin B_3; FUS-X, fusarenone X; HFB_1, hydrolyzed fumonisin B_1; HT-2, HT-2-toxin; HT-2-4-GlcA, HT-2-toxin-4-glucuronide; NIV, nivalenol; OTA, ochratoxin A; OTα, ochratoxin alpha; T-2, T-2-toxin; ZAN, zearalanone; ZAN-14-GlcA, zearalanone-14-glucuronide; ZEA, zearalenone; ZEA-14-GlcA, zearalenone-14-glucuronide; α-ZAL, α- zearalanol; α-ZAL-14-GlcA, α- zearalanol-14-glucuronide; α-ZAL-7-GlcA, α- zearalanol-7-glucuronide; α-ZOL, α- zearalenol; β-ZAL, β-zearalanol; β-ZAL-14-GlcA, β-zearalanol-14-glucuronide; β-ZOL, β-zearalenol. IAC, immunoaffinity column. SIDA, stable isotope dilution assays. SPE, solid phase extraction.

4. RISK CHARACTERIZATION OF MULTIPLE MYCOTOXINS

4.1. Different approaches for risk characterization of toxic compounds

Risk characterization is the last step of the risk assessment process, integrating information obtained in hazard assessment and exposure assessment steps. Risk characterization aims to produce scientific advice for risk managers and has been defined as the qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterization and exposure assessment (FAO/WHO, 2009). Within the risk characterization step, a comparison between the dietary exposure and the relevant health-based guidance value is performed. It should be highlighted that reference doses are only defined for the adult population and this renders difficulty in the children and infants risk assessment for which the available reference doses are not suitable.

Different approaches have been used for risk characterization of toxic compounds, according to their genotoxic and carcinogenic potential. In the risk characterization for non-genotoxic and carcinogenic substances, a health-based guidance value is compared with estimates of dietary exposure. The Hazard Quotient (HQ) is derived by comparing their respective reference dose (RfD, e.g. tolerable daily intake (TDI)) with the exposure to evaluate whether the exposure level is tolerable or not, and a ratio of HQ <1 indicates a tolerable exposure level and a ratio of HQ >1 indicates a non-tolerable exposure level (EFSA, 2013). For those substances that are genotoxic and carcinogenic, the traditional assumption is that some degree of risk may exist at any level of exposure and it is recommended that the exposure should be as low as reasonably achievable (ALARA). However, this approach presents limited value, because it does not allow risk managers to prioritize different contaminants or to target risk management actions. The Margin of Exposure (MOE) approach, which is the ratio between an amount of a substance producing a small but measurable effect in laboratory animals or humans and the estimated human exposure, has been proposed by WHO and EFSA as the methodology for risk characterization of compounds that are genotoxic and carcinogenic (EFSA, 2013; FAO/WHO, 2009). The Scientific Committee of EFSA considers that MOE values of 10,000 or more, when based on a benchmark dose lower confidence limit 10 ($BMDL_{10}$) from an animal study and taking into account overall uncertainties in the interpretation are considered "of low concern from a public health point of view". Benchmark dose lower confidence limit corresponds

to the lower boundary of the confidence interval on the benchmark dose. EFSA's Scientific Committee notes that the magnitude of a MOE only indicates a level of concern and does not quantify risk (EFSA, 2012; EFSA, 2013).

Given the number of chemicals to which humans are potentially exposed, the risk characterization to this exposure should be also addressed. As referred in the hazard assessment section, combination effects could occur as a result of different chemicals present in food and consequently different combined effects could happen. Diverse approaches have been used for multiple chemicals risk characterization, most of these are based on the concepts of concentration addition (CA) and independent action (IA). Examples of risk characterization methods include the Hazard Index (HI), Point of Departure Index (PODI), Combined Margin of Exposure Index (MOET), Toxic Unit Summation (TUS) and Relative Potency Factors/Toxic Equivalency Factors (RPF/TEF) (Sarigiannis and Hansen, 2012; WHO, 2009). The Hazard Index, the mostly used for non-genotoxic and carcinogenic compounds, is defined as the sum of the respective Hazard Quotients (HQs) for individual mixture components, calculated as the ratio between exposure and a reference dose and has been put forward as the preferred approach when extensive mechanistic information of the mixture components is not available. The HI does not predict the overall health effect of the mixture, but provide a measure of the total risk based on the individual risk of each component. Thus, the HI can be used also for identification of the largest contributors to the risk (EFSA, 2013; Sarigiannis and Hansen, 2012). The combined MOE is called the MOET, and is calculated as the reciprocal of the sum of the reciprocals of the individual MOEs (EFSA PPR, 2008). MOET is usually used for the mixtures of chemicals that have genotoxic and carcinogenic potential. According to the PPR Panel of EFSA, no established criteria has been set yet to define the magnitude of an acceptable MOE for mixtures of chemicals with a threshold effect. However, it is widely accepted that for MOEs above the uncertainty factor of 100, the combined risk is considered acceptable (EFSA PPR, 2008; Sarigiannis and Hansen, 2012).

4.2. Characterization of risk from exposure to multiple mycotoxins in food

Several studies were performed to evaluate the dietary exposure to mycotoxins (Table 31), although the characterization of risk resulting from that exposure it is not usually done. In the last years, few works were published applying methodologies of evaluation of risk using approaches that consider the simultaneous exposure to different mycotoxins. Assunção et al. (2015) performed a risk assessment of single and 12 mycotoxins present in breakfast cereals consumed by children (1–3 years old) from Lisbon region (Portugal). The daily exposure of children to AFs, OTA, FBs and trichothecenes were determined

using deterministic and probabilistic approaches. For the non-carcinogenic mycotoxins, the authors used the hazard index to characterize the risk of mycotoxins from the same family group. For the AFs, as carcinogenic compounds, MOET was determined to characterize the risk. García-Moraleja et al. (2015) studied the presence of 21 mycotoxins in coffee and calculated the daily intake of mycotoxins from coffee consumption using deterministic approach at various scenarios of food consumption in Spanish adolescents and adults. The risk was characterized via comparison with the TDI or the provisional tolerable weekly intake (PTWI) proposed by The Joint FAO/WHO Expert Committee on Food Additives (JECFA). Han and collaborators (2014) assessed the cumulative health risks of concomitant exposure via dietary intake to multiple mycotoxins, namely deoxynivalenol and its acetyl derivatives of 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol. Sirot et al. (2013) evaluated the exposure to 25 mycotoxins of the general French population within the second French total diet study. The health risk assessment was performed via comparison of the dietary exposure with international health-based guidance values (TDI, PMTDI, or PTWI), and the population rate exceeding the health-based guidance value was also estimated for adults and children. Cano-Sancho et al. (2013) assessed the exposure of Catalonian (Spain) population to aflatoxins (AFB_1, AFB_2, AGB_1 and AFG_2) and individual aflatoxin risk characterization was calculated by estimating the MoEs, dividing the $BMDL_{10}$ by the average and percentile 95 of the exposure estimates. De Boevre et al. (2013) assessed the quantitative dietary exposure of mycotoxins and their modified or masked forms (13 in total) through consumption of cereal-based food products of the Belgian population. The output of exposure (individual and family groups) was compared to the mycotoxins TDI.

4.3. Main challenges in risk characterization of multiple mycotoxins

As referred previously, humans are naturally and frequently exposed to a multitude of mycotoxins, but health risk assessments are usually performed on individual mycotoxins, which may underestimate the total risks. This could be explained by the fact that evaluation of all possible combinations of mycotoxins that can occur in food and consequently their potential combined toxic effects are virtually impossible (FAO/WHO, 2009). One of the main challenges posed to risk characterization of multiple mycotoxins is the absence of toxicological data that could be used to characterize the risk. The use of toxicological data is mandatory to risk characterization, and independently of the mechanisms of combined effects or interactions, data of multiple mycotoxins are not yet complete for all the toxins potential present in food.

The use of harmonised terminology is an important step for a common understanding of the key terms and concepts that are used when dealing with combined exposure to multiple chemicals for risk assessment purposes (EFSA, 2013). This fact constitutes another challenge for risk characterization of multiple mycotoxins in food. It is visible that researchers used similar approaches, however not always using the same terminology. An improvement in the quality of the obtained results is expected if a harmonized approach and methodology for risk characterization is achieved.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

In the context of food safety, risk assessment is a conceptual framework that aims to estimate the risk of occurrence of adverse health effects after exposure to mycotoxins present in food. Risk assessment of multiple mycotoxins is a very challenging domain integrating knowledge from different scientific areas and demanding a big effort from scientific community, risk assessors and managers. The challenges posed to risk assessment of multiple mycotoxins were reviewed in the present work, and Figure 37 reflects the interrelation between different risk assessment steps including the identified challenges for each step.

This study gathers, for the first time, an overview of the main challenges associated to the human health risk assessment of multiple mycotoxins present in food. Additionally, a physiologically-based framework for the hazard assessment of multiple mycotoxins in food is proposed. Hazard assessment pointed challenges related to the prioritization of mixtures for risk assessment purposes; harmonization of the experimental approaches for toxicity testing and mathematical models to analyze interactions, among others. Future *in vitro* and *in vivo* studies on combinatory toxicology are still needed and should be directed to cover: i) mixtures of mycotoxins that are more likely to occur; ii) realistic low concentrations, considering the range of human exposure measured, e.g., in biomonitoring studies; iii) prolonged exposure times to better mimic long-term human exposure and iv) several relevant endpoints, including genotoxicity and immunotoxicity using high throughput methodologies.

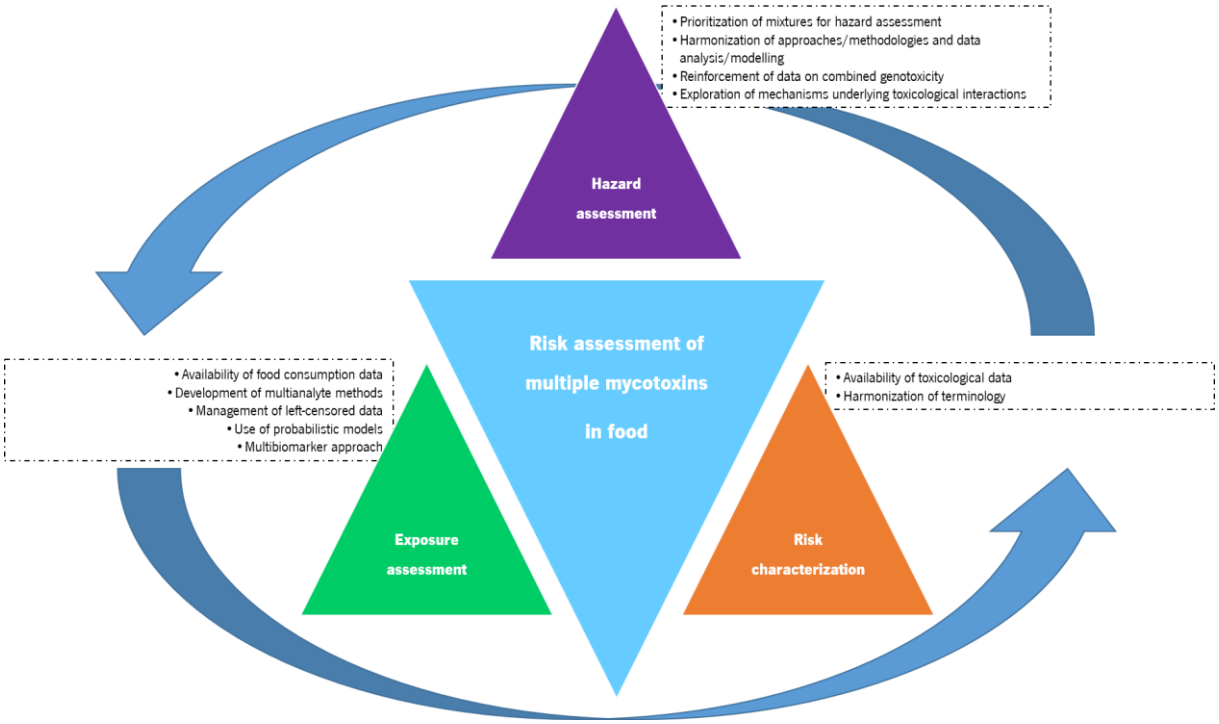

Figure 37. Holistic overview reflecting the interrelation between different steps of multiple mycotoxins health risk assessment and respective challenges.

Furthermore, modelling of data generated in such studies using adequate mathematical models is central to uncover interactions. On the other hand, exploring genotoxic effects and interactions at the mechanistic level remains a challenging issue because information on the mechanism of action of single mycotoxins within a mixture may be somewhat limited, making it difficult to understand their interactions. Such data can be generated from toxicity studies using predictive and high throughput methodologies, including toxicogenomic approaches. Exposure assessment highlights the importance concerning the availability of harmonized food consumption data to perform exposure assessments as well as the development of multianalyte methods for multiple mycotoxins quantification in different food matrices. The management of left-censored data is crucial for the quantification of low levels of mycotoxins as well as the use of probabilistic methods allowing a more realistic risk assessment and considering different exposure scenarios. Considering that risk characterization is the last step of the risk assessment process, the quality of data obtained in the hazard characterization and exposure assessment steps are crucial to evaluate properly the risk associated to the exposure to multiple mycotoxins. At this level, the availability of toxicological data and the use of harmonized terminology pose the main challenges for an accurate risk characterization.

A multidisciplinary effort should be developed to perform the human health risk assessment of multiple mycotoxins present in food, considering that the information obtained from the risk assessment process will be used by risk managers to prioritize risk and to develop actions towards disease prevention. The present work reinforces the urgent need to perform more research studies to clarify the nature of interactions among mycotoxins and derive new health-based guidance values for grouped mycotoxins and/or co-occurring mycotoxins in order to protect human health.

IV. GENERAL DISCUSSION

Mycotoxins are important and well-known food contaminants. Their toxic effects associated to human single exposure are characterized, especially for the main mycotoxins with public health impact. In the last years, the issue of chemical contaminants mixtures in food and their health effects arose and scientific community has been involved in the development of strategies that could contribute to a better approach to study their combined effects and related risk assessment. Within this framework, the present thesis aimed to contribute to the risk assessment of multiple mycotoxins through food consumption. An especial emphasis was dedicated to a specific vulnerable population group: children under three years old. The present thesis applied a holistic perspective to the risk assessment, considering the increased evidence that the attainment of a more accurate risk assessment of multiple mycotoxins exposure implies the need to adopt a holistic approach (Clarke et al., 2015b). The concept "holistic" intends to express the whole of something or the total system instead of just its parts. In this work, the holistic approach was achieved through the integration of generated data from deterministic and probabilistic tools for the calculation of mycotoxin daily intake values, children food consumption (3-days food diary), mycotoxins occurrence (in cereal-based products), bioaccessibility (using a standardized *in vitro* digestion model) and toxicological data (from *in vitro* evaluation of cytotoxicity, genotoxicity and intestinal impact), in order to obtain, as far as possible, a complete picture of the risk associated to the children exposure to multiple mycotoxins. The experimental work was supported by MYCOMIX project, a Portuguese funded project, which explored the toxic effects of mycotoxins mixtures in infant food and its potential health impact. The experimental work chapter was divided in four different sections. Section 1 addressed the Portuguese children exposure to mycotoxins through the consumption of some foods, usually consumed by this population group. Section 2 determined the bioaccessibility of PAT and OTA, two enterotoxins, when present in a specific food type usually consumed by children. Section 3 characterized the toxic effects of these two mycotoxins on intestinal level, considering that intestine is the first biological barrier to mycotoxins present in food. And finally, section 4 reviewed the main challenges associated to the risk assessment of multiple mycotoxins in food. The main results from each section will be described and integrated within the frame of the present general discussion.

Portuguese children exposure to multiple mycotoxins through food

The proper and safe nutrition of young children contributes to their optimal growth and development. Cereal-based products are among the first solid foods eaten by children and thus constitute an important food group of their diet (Schwartz et al., 2008) providing essential macro and micronutrients for optimal health (Collins et al., 2010). Food matrices like cereals are very prone to contamination with fungi in the

pre-harvest and in the storage phase, especially in poor conditions, with excessive humidity (Piotrowska, 2013). The co-occurrence of mycotoxins in food products, and consequently the multiple exposure of humans, either from the same or from different fungal species, is everyday more frequently reported (De Ruyck et al., 2015; Stoev, 2015). Additionally, children, a specific vulnerable population group, are routinely exposed to many mycotoxins through food in many parts of the world (Etzel, 2014).

In the present work, a Portuguese children population group exposure to multiple mycotoxins, through the consumption of food intended for children, was assessed using contamination and consumption data. Three different food matrices, breakfast cereals, processed cereal-based products and biscuits, were evaluated for their mycotoxin contents, including aflatoxins, ochratoxin A, fumonisins, trichothecenes and zearalenone. For the processed cereal-based products, patulin contents were also evaluated. Considering all the analysed food matrices, detected levels of mycotoxins were found in 94% of products (for at least one mycotoxin). Ochratoxin A presented the highest number of positive samples (65%, 34/52) with decreasing values for the remaining toxins, namely ZEA (48%, 25/52) > DON (44%, 23/52) > FB_1 (42%, 22/52) > AFB_1 (37%, 19/52) > AFM_1 (21%, 11/52) > FB_2 (19%, 10/52) > AFB_2 (15%, 8/52) > AFG_1 (6%, 3/52) > NIV (2%, 1/52). Relatively to the occurrence of PAT in processed cereal-based products, this mycotoxin was detected in 75% (15/20) of the analysed samples. When available, mycotoxin contents were all below the maximum admissible levels established by European legislation (European Commission, 2006a). Moreover, the present work demonstrated that foods usually eaten by children were simultaneously contaminated by more than one mycotoxin, as 75% of analysed samples presented two or more mycotoxins. The highest number of mycotoxins detected simultaneously was seven and the combinations of two (OTA and DON; OTA and fumonisins) and four (aflatoxins, OTA and ZEA) mycotoxins were the most common, with an occurrence of 6% for each combination (Manuscripts 1 and 3). These results concur to the growing evidence, also reported by other authors (Alassane-Kpembi et al., 2016; De Ruyck et al., 2015; Grenier and Oswald, 2011; Stoev, 2015) that humans, and more specifically children, are exposed to multiple mycotoxins. As a consequence, an increasing concern has been drawn upon the potential health consequences of the simultaneous exposure to multiple mycotoxins, especially in cases of possible additive and synergistic effects. This aspect will be discussed in detail later on this section, however it is important to highlight that the co-occurrence data were crucial to perform a more accurate risk assessment for children exposed to mycotoxins via food.

An important aspect within the framework of the determination of mycotoxins contamination levels is the handling of concentration data reported to be below the limit of detection (LOD) of the analytical method. These data are known as non-detects and the resulting distribution of occurrence values is left-censored.

With the present results, a significant number of none quantified values of mycotoxins was verified, which led to the implementation of suitable methods to manage left-censored data in order to obtain realistic exposure estimations, with a low level of uncertainty. As advocated by EFSA, substitution methods were applied (EFSA, 2010), namely the replacement of non-detects by LOD divided by 2 and an upper and lower bound by substitution of non-detects by LOD or 0, respectively (EFSA, 2010).

The unavailability of legal limits for some of the mycotoxins detected in cereal-based products usually consumed by children constitutes another important issue. In Europe, specific mycotoxins regulation (European Commission, 2006a) are available in order to control a number of mycotoxins in food. In fact, government and industry regulations exist to minimize the concentrations of individual mycotoxins allowed in food and feed products. However, in one hand, the available regulatory limits do not cover all mycotoxins that can contaminate food, and children foods in particular. In another hand, the available regulations are based on individual toxicities, and as such, do not take into account the complex dynamics of compounded risk from co-exposure to groups of mycotoxins (De Ruyck et al., 2015; Zain, 2011).

The present work also demonstrated that the three analysed cereal-based products were highly consumed by the Portuguese children included in the study. In fact, approximately 92% of the children aged between one and three years old consumed one or more cereal-based products, at least one time in three days (Manuscripts 2 and 3). These results are in accordance with the usual practices on infant feeding reviewed by Guerra et al. (2012), showing that for nutritional requirements and inherent neurosensory, motor and social infant development, foods other than milk and with less homogeneous texture as cereal-based products should be progressively introduced, leading to the progressively rapprochement to the family diet, which should occur around 12 months of age.

The daily intake estimate for each considered mycotoxin was addressed, gathering the contamination and consumption data, allowing the possibility to characterize the exposure magnitude of the studied population. Considering the worst case scenario (H1 of the probabilistic approach), the highest mean sum of daily intake through the consumption of the three groups of cereal-based products was 53.93 ng/kg bw/day for DON, followed by FB_1 (6.7 ng/kg bw/day), NIV (2.74 ng/kg bw/day), ZEA (0.89 ng/kg bw/day) and OTA (0.165 ng/kg bw/day). Within aflatoxins, AFM_1 presented the highest value for the mean sum of daily intake (0.058 ng/kg bw/day). Considering each analysed food group, the breakfast cereals was the highest contributor for the estimated daily intake of mycotoxins, revealing the highest values for fumonisins, trichothecenes, ZEA and AFB_1 (Manuscripts 2 and 3). The estimated mean daily intake values for the studied population were lower than the health-based guidance values established for all mycotoxins, namely OTA (112 ng/kg bw/week), FB_1 and FB_2 (2 μg/kg bw/day), DON (1 μg/kg

bw/day), NIV (1.2 μg/kg bw/day) and ZEA (250 ng/kg bw/day) (EFSA, 2011b, 2013, JECFA, 2007, 2011a, 2011b). Relatively to aflatoxins, as genotoxic carcinogenic compounds, no exposure threshold is presumed and it is recommended that levels should be as low as technologically feasible or, as Joint FAO/WHO Expert Committee on Food Additives (JECFA) recommends, as low as reasonably achievable (ALARA) (EFSA, 2007).

In addition to the characterization of the exposure magnitude, the results from the exposure assessment also highlighted probabilistic approaches importance. Although the estimated mean daily intake through both probabilistic and deterministic approaches are quite comparable, the probabilistic one generated a distribution function instead of a single value estimation. The probabilistic approach, contrary to the deterministic one, takes into account every possible value that each variable can assume and weights each possible scenario for the probability of its occurrence, allowing a more accurate characterization of mycotoxin intake distribution. Consequently, the probabilistic results correspond to a distribution of potential dietary exposures, permitting the definition of high percentiles of dietary intake, a crucial step for the outcome of risk assessment. In practice, it allows the determination of the proportion of the population that would exceed a health-based limit (FAO/WHO, 2009; Marin et al., 2013). In the present work, the determination of the higher percentiles of intake through the probabilistic methods allowed to estimate the percentiles of intake that exceeded the health-based guidance values (as presented in Manuscripts 2 and 3 and discussed below), which are, in the limit, the proportion of the population more exposed and consequently, under increased risk.

Mycotoxins bioaccessibility and their intestinal toxic effects

As previously referred, the total amount of an ingested mycotoxin does not always reflect the amount further available to the body (Versantvoort et al., 2005).

Mycotoxins bioaccessibility determination constitutes an important step, considering that the knowledge of the real percentage of mycotoxins that are available to be absorbed and to produce their intestinal toxic effects would enable an accurate approach to the exposure assessment and consequently, to a more accurate mycotoxins risk assessment. For instance, bioaccessibility values below 100% implies that the internal exposure to the contaminant is lower than the external exposure, and if bioaccessibility values were not taken into account, the internal exposure to the contaminant could be overestimated. Consequently, within the framework of mycotoxins risk assessment, bioaccessibility results could be used in two ways: i) as a maximum measure of oral bioavailability, allowing a convenient approach to assess the internal exposure, that could be calculated by multiplying estimated mycotoxin daily intake by its

bioaccessibility value, per product (Lei et al., 2015; Versantvoort et al., 2005); and ii) as a maximum amount of mycotoxins that reaches intestine after ingestion, contributing to comprehend the level of toxins that could induce toxic effects at intestinal level. In fact, the intestinal mucosa is the first biological barrier encountered by mycotoxins and it represents their first target.

The present work evaluated, *in vitro*, the bioaccessibility of two mycotoxins, PAT and OTA. These two mycotoxins showed different behaviours. Contrary to OTA, that evidenced almost unmodified levels during the digestion process, PAT bioaccessibility results revealed that its levels were reduced by almost 50% during the digestion before reaches the intestine (Manuscript 4).

Moreover, we demonstrated that intestinal cells could be affected by PAT and OTA, individually or in mixture, when studied different endpoints, namely cell viability and intestinal membrane integrity (Manuscript 5). In fact, Maresca et al. (2008) considered PAT and OTA amongst the best known enteropathogenic mycotoxins and their capacity to alter intestine functions. No previous study reported the intestinal effects of simultaneous exposure to these two mycotoxins.

Cell viability results, obtained *in vitro* using Caco-2 cells as an intestinal model, showed that intestinal cells are more sensitive to PAT than to OTA. PAT and OTA produced combined effects higher than those observed for each individual mycotoxin, which is in favour of a probable interaction between these two mycotoxins. Also, when OTA was dominant in the mixture a synergism was verified, but when PAT was dominant in the mixture, this pattern was changed to an antagonism.

PAT and OTA individual and combined effects on membrane integrity were also evaluated through the measurement of TEER. Obtained results showed that these two mycotoxins affected the membrane integrity of Caco-2 cells. Additionally, a dose level deviation was verified and synergism was attained at low levels of both mycotoxins, changing to antagonism at higher doses.

The present work also characterized the *in vitro* effects of PAT on barrier function of the gut mucosa utilizing the intestinal epithelial cell model Caco-2, and scrutinized immunomodulatory effects using human peripheral blood mononuclear cells and human blood monocyte-derived dendritic cells as test systems. Results showed that the expression of zonula occludens-1 and myosin light chain 2 declined and the levels of phospho-myosin light chain 2 increased after exposure to PAT. Relatively to the immune cells, T cell proliferation was highly sensitive to PAT, however, the maturation of dendritic cells was unaffected. These results provided additional knowledge that reinforces the concept that epithelium and immune cells of the intestinal mucosa are important targets for PAT (Manuscript 6).

Considering all these results, some important aspects were uncovered and should be highlighted in a risk assessment perspective, namely: i) intestinal cells constitute a potential target for PAT and OTA; ii) combined exposure to PAT and OTA resulted in synergistic effects, especially at low doses of mycotoxin exposure; iii) children under three years old constitute a potential exposed population; and iv) other mycotoxin mixtures could occur, presenting more than two toxins in the mixture.

Firstly, the present results indicated that intestinal cells constitute a target for PAT and OTA and these mycotoxins affected intestinal barrier function. However, whether this intestinal barrier disruption is an epiphenomenon, an early manifestation of disease, or a critical step in disease pathogenesis remains unknown and has been the subject of much debate (Camilleri et al., 2012). To date, the key work has been done in animal models and *in vitro*, and little is known about the equivalent processes in humans (König et al., 2016). Some previous authors suggested that mycotoxins could be involved on the onset and maintenance of some intestinal diseases, as inflammatory bowel diseases. Maresca and Fantini (2010) reviewed the literature on mycotoxin-induced intestinal dysfunctions and compared these perturbations to the impairments of intestinal functions typically observed in human chronic intestinal inflammatory diseases, based on various cellular and animal studies. Moreover, existing data was sufficient to support a possible association between mycotoxins and the induction and/or persistence of human chronic intestinal inflammatory diseases in genetically predisposed patients, even though additional epidemiologic evidence is required. The present results relatively to PAT and OTA impact on intestinal integrity, contribute and reinforce these previous suspicions. Although human intestinal simultaneous exposure to PAT and OTA is proven, no previous data evaluating their combined intestinal effects was reported. Even though intestine is recognized as a potential target, studies on mycotoxins combined effects usually stick to immune, hepatic and renal cells, as recently reviewed by Alassane-Kpembi et al. (2016). Future studies should be developed to understand the real impact of mycotoxin mixtures on intestine and to further clarify the role of mycotoxins exposure in the development of intestinal diseases.

As demonstrated by the present results, PAT and OTA in combination could affect membrane integrity synergistically, for the lower studied doses. This situation presents an especial impact considering that human exposure is usually characterized by low levels of mycotoxins. Consequently, synergistic effects could be a threat to consumer health, especially for more vulnerable consumers such as children. In fact, the children included in this study, aged under three, showed a reduced level of exposure to these two mycotoxins. It is important to highlight that, nowadays, the main concern associated rather to chronic exposure to low levels of these compounds, during extended periods. This fact assumes an especial

importance in children, considering the superior time they have to develop chronic diseases in comparison to adults and, consequently, that early adverse effects may have lifelong consequences (Felter et al., 2015; Raiola et al., 2015). Particular attention should be dedicated by Regulators to the risk characterization of children exposed to mycotoxins in order to prevent these long-term consequences of early-life exposure, especially for those mycotoxin mixtures with potential synergistic effects.

Herein, only the combination of PAT and OTA was considered. However, according to the occurrence results (Manuscripts 1 and 3), a maximum of seven mycotoxins was detected simultaneously in the same matrix (including AFTs, OTA, FMs, ZEA; AFTs, OTA, FMs, DON, ZEA; AFTs, OTA, FMs, ZEA). Additionally, the available toxicological data was restricted to the evaluation of binary or ternary mixtures of mycotoxins. Thus, the question regarding the combined effects of a simultaneous exposure to seven different mycotoxins remains open. As it is expected that different combinations of mycotoxins should present different toxicities, this issue should be addressed.

The need to generate relative potency factors is another important aspect. In fact, within a mixture, each mycotoxin could evidence different potencies, as demonstrated in the case of PAT and OTA combinatory effects (Manuscript 5). The nature of combined effects or the relative potencies of the mycotoxins are still not fully known. Recently, EFSA proposed relative potency factors for ZEA and its modified forms (EFSA, 2016). As far as possible, the same approach should be followed for different groups of mycotoxins in order to obtain estimates of risk presenting the lowest uncertainties possible.

Finally, another important aspect that should be enhanced is related to the fact that results (Manuscripts 4, 5 and 6) were generated *in vitro*. Two different perspectives should be acknowledged: in one hand, as generally agreed, *in vitro* data should be interpreted with caution, since experimental settings greatly influence the outcome of a certain experiment (Vejdovszky et al., 2016). However, *in vivo* studies on combinatory effects are not available for all mycotoxin combinations and endpoints needed for risk assessment. Therefore, urgent *in vivo* data are required. Future *in vivo* studies on combined effects of mycotoxins should be directed to cover: i) mixtures of mycotoxins that are more likely to occur; ii) realistic low concentrations, considering the range of human exposure measured, e.g., in biomonitoring studies; iii) prolonged exposure times to better mimic long-term human exposure and iv) several relevant endpoints, including genotoxicity and immunotoxicity using high-throughput methodologies (Manuscript 7). In another hand, there is a growing scientific evidence about the need for more efficient methods and strategies to assess the hazards, exposures and risks in face of the wide array of chemicals to which humans are exposed. This fact led in the last years to an effort to develop and replace as much as possible the *in vivo* testing by reliable and robust experimental and computational tools, in order to be more

efficient, economical, less animal intensive, and more relevant to human health. Envisaged testing strategies are anticipated to increasingly rely on *in vitro* data, as a basis to characterize early key events for toxicity at relevant dose levels in species of interest, and quantitative *in vitro* to *in vivo* extrapolations (Meek and Lipscomb, 2015). As a matter of fact, over the last three decades, *in vitro* toxicology has developed into an important sub-discipline of toxicology. The application of these techniques in toxicology opened the possibility to study the effects of chemicals at the cellular or tissue level (Blaauboer, 2015). However, a further important milestone is the realization that *in vitro* data cannot stand alone. The physiological networking and feedback loops might not exist in the isolation of an *in vitro* system. The development of perfusion systems and body-on-a-chip techniques are therefore interesting and could contribute to overcome these limitations (Blaauboer, 2015). To conclude, despite *in vitro* data limitations, it is clear that *in vitro* results framed in a physiological perspective could contribute to obtain important clues about the toxicological effects of chemicals exposure through food consumption to human health. Although *in* vitro, the present results represent a realistic exposure scenario, and consequently, should contribute to a better understanding of the impact of combined effects of simultaneous exposure to mycotoxin through food consumption, integrating the risk assessment as an attempt to obtain a holistic approach.

Characterization of the risk associated to the consumption of foods usually eaten by children, using a holistic approach

Risk characterization is the final step of the risk assessment process. At the end of the risk characterization a risk estimative was generated. The results obtained established an estimate relatively to Portuguese children exposure to multiple mycotoxins through food consumption, considering a population group of children aged between one and three years old. Two different perspectives might be discussed relatively to the presented risk characterization: i) the characterization of risk resulting from the consumption of three different food products by children under three years old (Manuscripts 1, 2 and 3); and ii) the characterization of risk resulting from the consumption of processed cereal-based foods (flours), with special emphasis for the intestinal consequences of simultaneous exposure to PAT and OTA (Manuscripts 4 and 5).

Within the first perspective, it was demonstrated that children exposure to OTA, fumonisins, trichothecenes and ZEA indicated no health concern for individuals exposed to these mycotoxins through consumption of breakfast cereals, processed cereal-based products and biscuits. Contrary to this,

aflatoxins exposure suggested a potential health concern for the high percentiles of intake (Manuscripts 2 and 3). In this case, the concept of margin of exposure (MOE) was applied to characterize the risk of aflatoxins children exposure, according to EFSA recommendations relatively to the characterization of risk of genotoxic and carcinogenic compounds (EFSA, 2013). According to this concept, the MOE was calculated as a ratio of $BMDL_{10}$ and aflatoxin exposure. The magnitude of the MOE gives an indication of the risk level and the Scientific Committee of EFSA and WHO have concluded that a MOE of 10000 or more was of low public health concern (EFSA, 2013). The obtained results revealed that AFB_2 was the aflatoxin that showed a MOE above 10000 for all percentiles of exposure, which represents a low risk for children. However, for the remaining aflatoxins and for some percentiles of intake (AFB_1 and AFG_1, for percentiles 90, 95 and 99), the MOE values were below 10000, suggesting a potential health concern. When percentiles P50 or higher of AFM_1 intake were considered, the MOE values were below 10000, suggesting also a potential health concern. In contrast with the present study, previous published European studies rarely pointed out aflatoxins, as a potential health risk. This fact could be attributed to i) the small number of dietary exposure studies focused on this particular age range; ii) children high consumption relatively to their body weight when compared to adults; and iii) the use of MOE to characterize the risk associated with aflatoxins. In Europe, previous reported studies estimated mean dietary exposures to aflatoxins for the general population from all food sources ranging from 0.93 ng kg bw^{-1} day^{-1} to 2.4 ng kg bw^{-1} day^{-1} (JECFA, 2007b). In order to compare these estimations with those obtained in the present study, the MOE concept was applied for the European estimates of dietary exposure to aflatoxins (Table 33).

Table 33. Risk characterization for the estimates of aflatoxin exposure reported in Europe by JECFA.

Estimated mean dietary exposure (ng/kg bw/day)[a]	**$BMDL_{10}$ (ng/kg bw/day)[b]**	**MOE[c]**
0.93	250	269
2.4	250	104

[a]JECFA, (2007b); [b]Benford et al., 2010b; [c] MOE (Margin of Exposure) = $BMDL_{10}$/Exposure data.

As presented in Table 33, MOE for the mean dietary exposure of European population revealed values well below 10000, and applying the same principle advocated by EFSA (2013), the MOE magnitude corresponds to a potential health concern. Although the uncertainties associated with the MOE calculations, it is important to underline that this methodology is considered the most scientifically credible approach for advice formulation because it takes into account both the dietary exposure and the available data on the dose-response relationship which should be used to support prioritisation of risk management actions (Benford et al., 2010b). Relatively to the potential consequences for children health, Sherif et al. (2009) suggested evidence of increased susceptibility to cancer from early-life exposures, particularly for chemicals acting through mutagenesis, as aflatoxins. Additionally, Raiola et al. (2015) pointed out other potential effects of children exposure to aflatoxins, namely i) reduction of immunization efficiency in children with a consequent increase of susceptibility to infections; ii) children vulnerability to cancer derived from aflatoxin-contaminated milk, since milk is an important constituent of their diet (the same could be proposed to aflatoxins present in cereal-based products); iii) young animals have been found to be more susceptible to aflatoxin toxicity than adults, and repeated exposures to aflatoxins *in-utero* and through childhood might predispose to liver cancer later in life (Chawanthayatham et al., 2015; Woo et al., 2011). Considering all these facts, additional and comprehensive information is required to protect children health, ensuring aflatoxins exposure reduction.

The second perspective is related with risk characterization results associated to the consumption of processed cereal-based foods (flours), especially for the intestinal consequences of simultaneous exposure to PAT and OTA (Manuscripts 4 and 6). The estimated daily intake values for PAT and OTA were 22.93 ng/kg bw/day and 0.40 ng/kg bw/day, respectively, considering the worst case (maximum processed cereal-based foods consumption) (Manuscript 4). Considering the estimated daily intake (worst case, 22.930 ng/kg bw/day) and the mean bioaccessibility results (52%) of PAT, the internal exposure (11.924 ng/kg bw/day) should be lower than the external one. Considering the estimated daily intake (worst case, 0.402 ng/kg bw/day) and the mean bioaccessibility results (100%) of OTA, external and internal exposure are similar (0.402 ng/kg bw/day). In both cases, the exposure values were well below the health-based guidance values (PMTDI of PAT = 400 ng/kg bw/day and PTWI of OTA = 112 ng/kg bw/week) indicating a tolerable exposure through the consumption of processed cereal-based foods (flours). However, mycotoxins present in the intestinal fluid of a child could cause increased damage to enterocytes due to the reduced extension of the intestinal epithelium compared to adults (Raiola et al., 2015). Additionally, the health-based guidance values were generated for adults and do not take into

account the children specific vulnerabilities. Consequently, despite tolerable, the estimated exposure should not be neglected in risk management strategies.

Considering the potential intestinal effects resulting from the simultaneous exposure to PAT and OTA, the observed synergistic pattern associated to the intestinal membrane integrity was detected for the lower tested doses. However, the tested doses were higher than the estimated daily intake values of PAT and OTA. This situation suggests that intestinal acute synergistic effects through the consumption of processed cereal-based foods (flours) it is not expected. Nevertheless, as stated before, chronic exposure to low levels of these compounds during long periods should be reminded.

Taking into account the co-exposure, bioaccessibility and toxicological results, the present study reported a tolerable exposure to PAT and OTA representing a low risk for Portuguese children, through consumption of processed cereal-based foods.

Challenges for risk assessment of mycotoxin mixtures

In the context of food safety, risk assessment is a conceptual framework that aims to estimate the risk of occurrence of adverse health effects after exposure to mycotoxins present in food. Risk assessment of multiple mycotoxins is a very challenging domain, as illustrated in the discussion of Manuscript 7, and integrates knowledge from different scientific areas, demanding a large effort from the scientific community, risk assessors and managers.

Hazard assessment pointed challenges related to the prioritization of mixtures for risk assessment purposes; harmonization of the experimental approaches for toxicity testing and mathematical models to analyze interactions, among others. On the other hand, exploring genotoxic effects and interactions at the mechanistic level remains a challenging issue, because information on single mycotoxins mechanism of action within a mixture may be somewhat limited, making it difficult to understand their interactions. Considering that risk characterization is the last step of the risk assessment process, the quality of data obtained in the hazard characterization and exposure assessment steps are crucial to properly evaluate the risk associated multiple mycotoxins exposure. At this level, the availability of toxicological data and the use of harmonized terminology pose the main challenges for an accurate risk characterization.

A multidisciplinary effort should be developed to perform the human health risk assessment of multiple mycotoxins present in food, considering that the information obtained from the risk assessment process will be used by risk managers to prioritize risk and to develop actions towards prevention. The present work reinforces the urgent need to perform supplementary studies to clarify the nature of interactions

among mycotoxins and derive new health-based guidance values for grouped mycotoxins and/or co-occurring mycotoxins in order to protect human health.

V. CONCLUSIONS AND FUTURE PERSPECTIVES

The present thesis aimed at answering several questions previously formulated:

1) Are Portuguese children exposed to multiple mycotoxins through food consumption?

Portuguese children included in the present study were exposed to multiple mycotoxins through food consumption (Manuscripts 1, 2 and 3).

For the three analysed food products, 75% of the samples were contaminated with two or more mycotoxins. The highest number of mycotoxins detected simultaneously was seven and the combinations of two (OTA and DON; OTA and fumonisins) and four (aflatoxins, OTA and ZEA) mycotoxins were the most common.

2) Are mycotoxin contents in foods for children above the legal limits? Is there a potential impact on children health?

None of the analysed samples presented contents above the maximum limits established for children food, when available (Manuscripts 1 and 3). However, gathering consumption and occurrence data, mycotoxins present in food could constitute a risk for children's health, especially regarding aflatoxins exposure for P50 or higher (Manuscripts 2 and 3).

3) Are there toxic effects associated to intestinal exposure to multiple mycotoxins?

Intestinal cells could be affected by patulin and ochratoxin A, individually or in mixture, when studied different endpoints, namely cell viability and intestinal membrane integrity (Manuscript 5). Patulin affected barrier function trough disruption of tight junctions. T cell proliferation was highly sensitive to patulin (Manuscript 6).

Using Caco-2 cells as an intestinal model, it was possible to conclude that PAT and OTA, two mycotoxins detected in foods usually consumed by children, affected these cells, when two different endpoints were evaluated. Additionally, simultaneous exposure revealed synergistic patterns, which constitute a special concern from a public health point of view. PAT was proven to disturb the intestinal barrier through zonula ocludens-1 disruption and through phosphorylation of myosin light chain 2. Considering immune effects, T cell proliferation was highly affected by PAT (Manuscript 6). These two mycotoxins could contribute to

the development of intestinal and immune toxic effects, which could assume a particular importance in young children.

4) Do bioaccessibility and potential toxic effects interfere on risk associated to mycotoxins exposure?

Digestion process affected the amount of mycotoxins that reached intestine, especially in the case of patulin (Manuscript 4). The consumption of processed cereal-based foods contaminated by patulin and ochratoxin A presented low risk, considering its co-exposure, bioaccessibility and toxicity results (Manuscripts 4 and 5). A holistic approach was highly recommended to obtain a more accurate multiple mycotoxins risk assessment (Manuscript 7).

Using an *in vitro* digestion model, PAT and OTA bioaccessibility values were determined. It was verified that a significant portion of PAT (52%) and especially of OTA (100%) can reach the small intestine (Manuscript 4). When characterized, risk associated to processed cereal-based foods (flours) consumption, comprising co-exposure, bioaccessibility and toxicity results, showed that Portuguese children were under low risk (Manuscripts 4 and 5). Altogether, these results consubstantiate the importance of considering a holistic approach for the multiple mycotoxins risk assessment.

Risk assessment of mycotoxin mixtures is a very complex field, since it is composed by different inputs obtained from different disciplines. Therefore, this field still needs additional examination. Considering all the aspects previously discussed and the conclusions obtained through analysis of the results, some important clues were generated relatively to the impact of multiple mycotoxins on human health in general, and on children's health in particular. At the same time, risk assessment of combined human exposure to multiple mycotoxins poses several challenges to scientists, risk assessors and risk managers and open new avenues for future research. Some of these topics are presented bellow as potential future directions within the multiple mycotoxins risk assessment:

- Intestinal impact of multiple mycotoxins

As referred previously, intestine is the first biological barrier encountered by ingested mycotoxins. The characterization of the intestinal consequences of multiple mycotoxins exposure is far from being well-

known. Consequently, future scientific efforts must include this topic as an important aspect to be covered.

- Biomarkers to evaluate children exposure to multiple mycotoxins

The present work uncovered the potential risk associated to food consumption by children. However, as considered by some authors, the determination of human exposure through consumption and contamination data is an indirect approach and could include some uncertainties. In the future, children exposure should be characterized using direct approaches as the determination of biomarkers of exposure.

- Potential relationship between mycotoxins exposure and development of diseases

As referred previously, there is a growing suspicion that mycotoxins exposure can be involved in the development of diseases, as intestinal inflammatory diseases and cancer. This issue should be correctly evaluated gathering experimental (mechanistic), but also epidemiological data to allow the establishment of a causality (cause/effect).

Finally, this thesis highlighted the importance of providing increasing knowledge on risk assessment of children exposure to multiple mycotoxins in food and its health impact, in order to provide data to governmental regulatory bodies, with approaches that contemplate human and, particularly, children exposure to chemical hazards in food. An accurate understanding of children's exposure to mycotoxins and the associated impact on children's health are crucial to support national and international actions suggested and implemented by risk managers and regulatory bodies in the future. In fact, the protection of children against multiple mycotoxins present in food products requests fundamental and broad revisions of the current approaches usually applied to assess risk. These actions should be founded on reliable scientific achievements, such as those presented in this thesis, integrating data from exposure, bioaccessibility and toxicity domains, contributing to a more accurate risk assessment and, consequently the safeguard of children's health.

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VII. ANNEXES

ANNEX 1

Table S1. Best fit distributions, mean, minimum and maximum determined for all scenarios of mycotoxin occurrence in breakfast cereals and for breakfast cereals consumption applied for probabilistic approach.

Mycotoxin	Scenarios	Function	Min	Mean	Max
AFM_1	H1	RiskLoglogistic(0;0.011147;19.044)	0.00	0.0112	+∞
	H2	RiskLoglogistic(0;0.005691;7.6835)	0.00	0.0059	+∞
	H3	RiskExpon(0.0019615)	0.00	0.0020	+∞
	H4	RiskExpon(0.0059694)	0.00	0.0060	+∞
AFB_1	H1	RiskInvgauss(0.027538;0.0089281)	0.00	0.0275	+∞
	H2	RiskInvgauss(0.027135;0.00497)	0.00	0.0271	+∞
	H3	RiskExpon(0.026731)	0.00	0.0267	+∞
	H4	RiskLoglogistic(0;0.010184;1.1572)	0.00	0.0668	+∞
AFB_2	H1	RiskPearson5(2.8213;0.0033711)	0.00	0.0019	+∞
	H2	RiskPearson5(1.7811;0.001109)	0.00	0.0014	+∞
	H3	RiskExpon(0.0013462)	0.00	0.0013	+∞
	H4	RiskLoglogistic(0;0.00053613;1.0838)	0.00	0.0065	+∞
AFG_1	H1	RiskPearson5(33.827;0.20814)	0.00	0.0063	+∞
	H2	RiskLoglogistic(0;0.0030161;14.989)	0.00	0.0030	+∞
	H3	RiskExpon(0.00065385)	0.00	0.0007	+∞
	H4	RiskGamma(1.6899;0.0022619)	0.00	0.0038	+∞
OTA	H1	RiskInvgauss(0.026154;0.018182)	0.00	0.0262	+∞
	H2	RiskInvgauss(0.024769;0.007705)	0.00	0.0248	+∞
	H3	RiskExpon(0.023385)	0.00	0.0234	+∞
	H4	RiskGamma(0.67087;0.036644)	0.00	0.0246	+∞
FB_1	H1	RiskInvgauss(13.015;1.9097)	0.00	13.0150	+∞
	H2	RiskLevy(0;0.88549)	0.00	N/D	+∞
	H3	RiskExpon(12.677)	0.00	12.6770	+∞
	H4	RiskLognorm(20.518;126.42)	0.00	20.5180	+∞
FB_2	H1	RiskPearson5(1.8338;2.1344)	0.00	2.5598	+∞
	H2	RiskPearson5(1.1183;0.68677)	0.00	5.8053	+∞
	H3	RiskExpon(1.9692)	0.00	1.9692	+∞

	H4	RiskGamma(0.52195;4.1423)	0.00	2.1621	+∞
DON	H1	RiskGamma(0.37205;159.05)	0.00	59.1746	+∞
	H2	RiskGamma(0.33033;178.92)	0.00	59.1026	+∞
	H3	RiskExpon(59.031)	0.00	59.0310	+∞
	H4	RiskGamma(0.2891;204.32)	0.00	59.0689	+∞
NIV	H1	RiskLoglogistic(0;5.5872;16.403)	0.00	5.6215	+∞
	H2	RiskLoglogistic(0;2.7996;11.413)	0.00	2.8353	+∞
	H3	RiskExpon(1.0435)	0.00	1.0435	+∞
	H4	RiskExpon(3.2426)	0.00	3.2426	+∞
Breakfast cereals consumption		RiskExpon(5.7627)	0.00	5.7627	+∞

ANNEX 2

Table S2. Risk characterization using hazard quotient (HQ) and hazard index (HI) derived from the estimated of patulin (PAT) and ochratoxin A (OTA) daily intakes. Three different scenarios were applied for treatment of non-detects (<LOD).

PAT				**OTA**		
	H1	H2	H3	H1	H2	H3
HQ for P50 of consumption	0.005	0.005	0.004	0.002	0.002	0.002
HQ for Mean consumption	0.010	0.010	0.009	0.004	0.004	0.004
HQ for P75 of consumption	0.016	0.015	0.014	0.007	0.006	0.006
HQ for P95 of consumption	0.039	0.037	0.035	0.017	0.016	0.015
HQ for P99 of consumption	0.050	0.047	0.044	0.021	0.020	0.019
HQ for Max of consumption	0.065	0.061	0.057	0.028	0.026	0.025

PAT + OTA			
	H1	H2	H3
HI for P50 of consumption	0.007	0.007	0.006
HI for Mean consumption	0.014	0.014	0.013
HI for P75 of consumption	0.022	0.021	0.020
HI for P95 of consumption	0.056	0.053	0.050
HI for P99 of consumption	0.071	0.067	0.063
HI for Max of consumption	0.092	0.087	0.082

HQ = Estimated daily intake/reference value (PMTDI or PWTI)
HI = sum of HQ of PAT and OTA
For OTA HQ calculation, the correspondence from weekly to daily was undertaken