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# Risk estimates of mycotoxin mixtures in the diets of our children


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**June 20th, 2017**

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# Outline

1. Multiple mycotoxins in foodstuffs – a reality
2. Risk assessment - change of paradigm
3. Challenges in the risk assessment of multiple mycotoxins (mycotoxin mixtures) in food:
   - ✓ Hazard assessment
   - ✓ Exposure assessment
   - ✓ Risk characterization
4. Risk estimates in the diet of our children:

   A Portuguese case study – MYCOMIX project

# 1. Multiple mycotoxins in food – a reality

**Ingestion of food** is considered a major route of exposure to many contaminants, namely **mycotoxins**

**Natural co-occurrence of mycotoxins in foods**

↓

**Increasing concern**

↓

**Combined mycotoxins**

↓

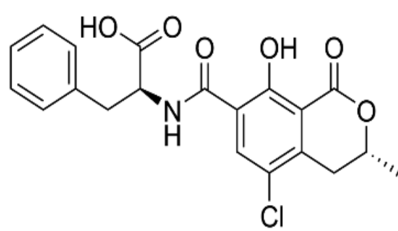
**Expected to exert greater toxicity than exposure to single mycotoxins**

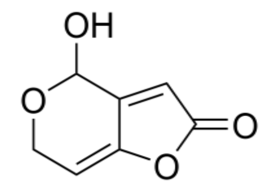
# 2. Risk assessment – change of paradigm

Historically, the health risk from human exposure to mycotoxins has been evaluated on the basis of single-chemical and single-exposure pathway scenarios.

In general, exposures to mycotoxins through the food were assessed independently, and no concerted effort had been made to evaluate potential multiple exposures simultaneously.

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.





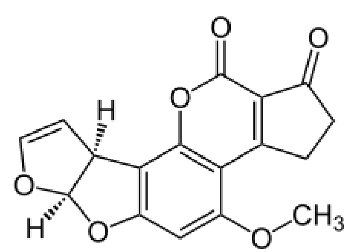
Mycotoxin co-occurrence

Toxikinetics
Metabolism
Toxicodinamics

Mechanism of action in the cell

Mycotoxin interactions

**ANTAGONISM?**
**SYNERGISM?**



# Combined toxicity and interactive effects concerning multiple mycotoxins studies

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

Assunção , Silva & Alvito, 2016. Challenges in risk assessment of multiple mycotoxins in food, World Mycotoxin Journal , 9 (5), 791-811

# Recent reports on worldwide exposure assessment to multiple mycotoxins in food

| 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 |
|---|---|---|---|---|---|---|---|---|
| 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.*, 2015b |
| 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 |

Assunção , Silva & Alvito, 2016. Challenges in risk assessment of multiple mycotoxins in food, World Mycotoxin Journal , 9 (5), 791-811

The assessment of mycotoxin exposure is often based on calculations combining mycotoxin occurrence data in food with population data on food consumption. This indirect approach is associated with some limitations and uncertainties:

- the heterogeneous distribution of mycotoxins in food.
- the limited accuracy of food consumption data (can lead to an under- and overestimation of the exposure).
- the individual variation in absorption, distribution, metabolism and excretion (which is not integrated).

**Human biomonitoring using biomarkers of exposure has been proposed as a suitable alternative. to perform an accurate mycotoxin exposure assessment at individual level.**

# Human mycotoxin exposure assessment using a multibiomarker approach

| Country | N° 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.*, 2014, 2015 |

Assunção , Silva & Alvito, 2016. Challenges in risk assessment of multiple mycotoxins in food, World Mycotoxin Journal , 9 (5), 791-811

# 3. Challenges in the risk assessment of multiple mycotoxins in food

## Risk Assessment

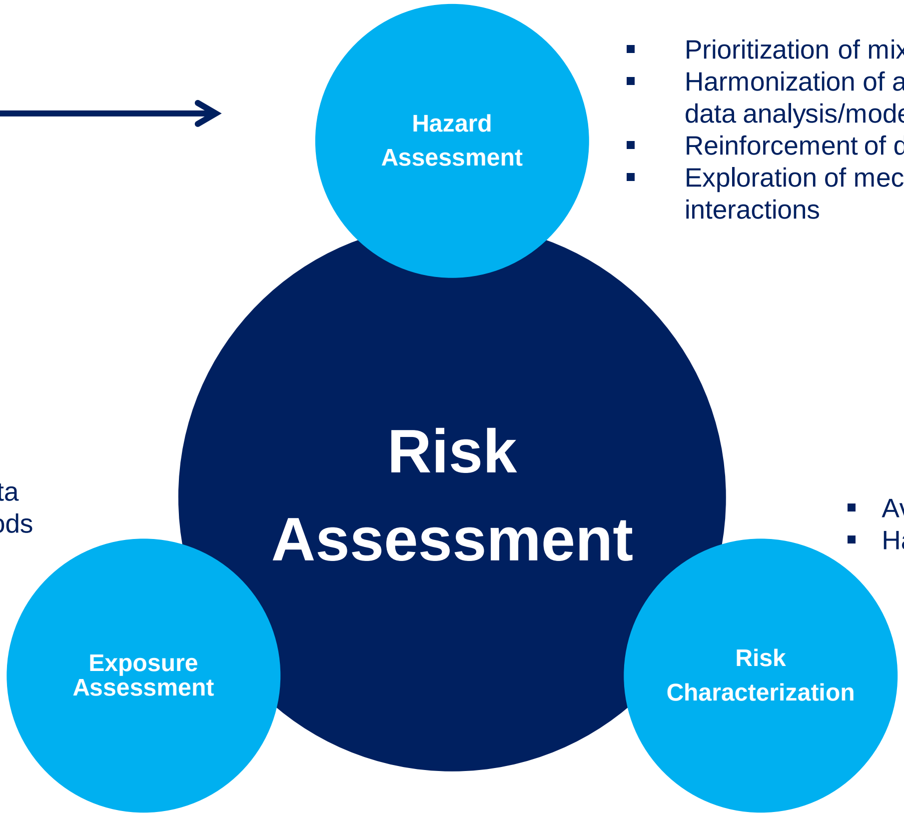
Science based

- Hazard identification
- Hazard characterization
- Exposure assessment
- Risk characterization

World Health Organization. (2014). *About risk analysis in food*. Available at: http://www.who.int/foodsafety/micro/riskanalysis/en/

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

- Prioritization of mixtures for hazard assessment
- Harmonization of approaches/methodologies and data analysis/modelling
- Reinforcement of data on combined genotoxicity
- Exploration of mechanisms underlying toxicological interactions




- Availability of food consumption data
- Development of multianalyte methods
- Management of left-censored data
- Use of probabilistic models

- Availability of toxicological data
- Harmonization of terminology

Assunção , Silva & Alvito, 2016. Challenges in risk assessment of multiple mycotoxins in food, World Mycotoxin Journal , 9 (5), 791-811

# Challenges on the hazard assessment

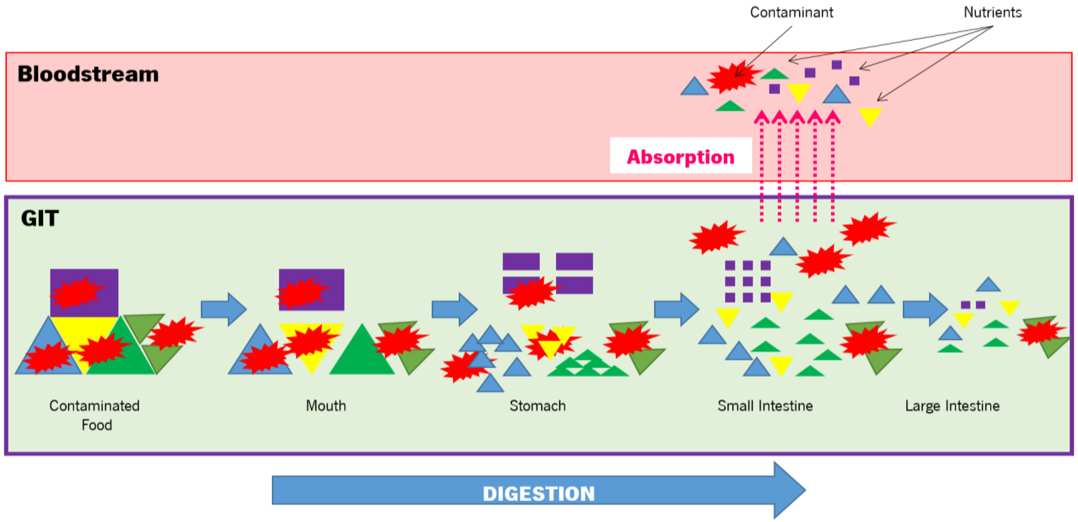
- Exploration of mechanisms underlying toxicological interactions
- Reinforcement of data on combined genotoxicity
- Harmonization of approaches/methodologies and data analysis/modelling
- Harmonization of methods to address bioavailability
- Prioritization of mixtures for hazard assessment

Bioavailability:

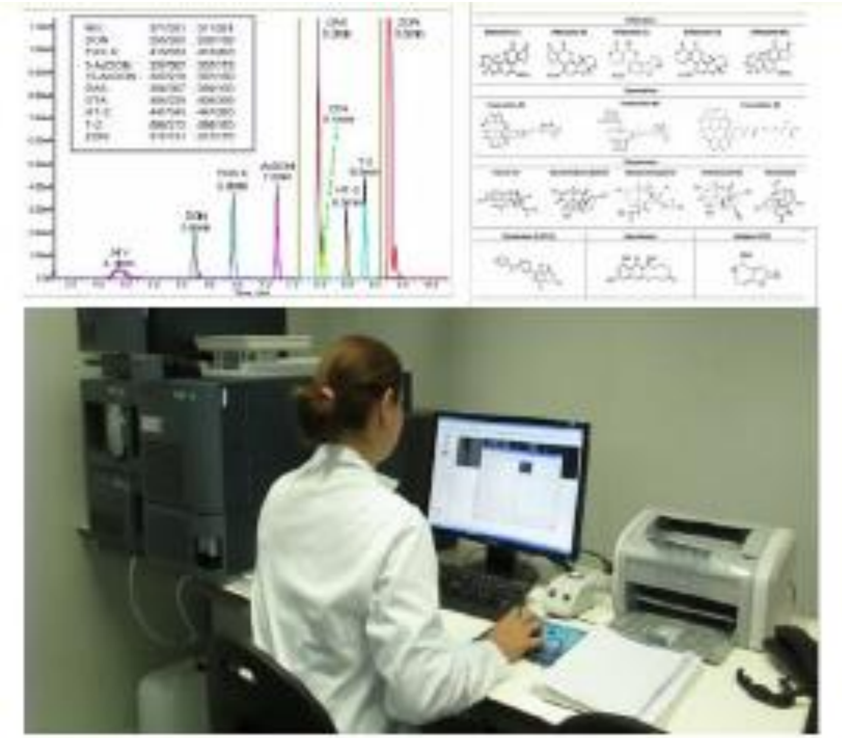
knowing the amount of an ingested mycotoxin may not be enough for exposure assessment. Only a certain amount of the contaminant will be available to reach the systemic circulation.

The amount of the contaminant that is available for the absorption in the gut after digestion corresponds to bioaccessibility (simulation by IVD models).

The fraction of the ingested contaminant that is present in the bloodstream and is available to exert its toxic effects on the target organs corresponds to the bioavailability.



# Challenges on the exposure assessment



- Availability of food consumption data
- Development of multianalyte methods
- Management of left-censored data
- Use of probabilistic models
- Multibiomarker approach

# Challenges on the risk characterization

- Availability of toxicological data
- Harmonization of terminology

Regulatory Toxicology and Pharmacology 60 (2011) S1–S14

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

ELSEVIER

Risk assessment of combined exposure to multiple chemicals: A WHO/IPCS framework ☆

M.E. (Bette) Meek [a], Alan R. Boobis [b], Kevin M. Crofton [c], Gerhard Heinemeyer [d], Marcel Van Raaij [e], Carolyn Vickers [f,*]

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[b] Department of Experimental Medicine and Toxicology, Division of Investigative Science, Imperial College London, London, England, United Kingdom
[c] National Health and Environmental Effects Research Laboratory, Office of Research and Development, Environmental Protection Agency, Research Triangle Park, NC, USA
[d] Federal Institute for Risk Assessment (BfR), Berlin, Germany
[e] National Institute of Public Health and the Environment (RIVM), Bilthoven, Netherlands
[f] International Programme on Chemical Safety, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland

efsa European Food Safety Authority

EFSA Journal 2013;11(7):3313

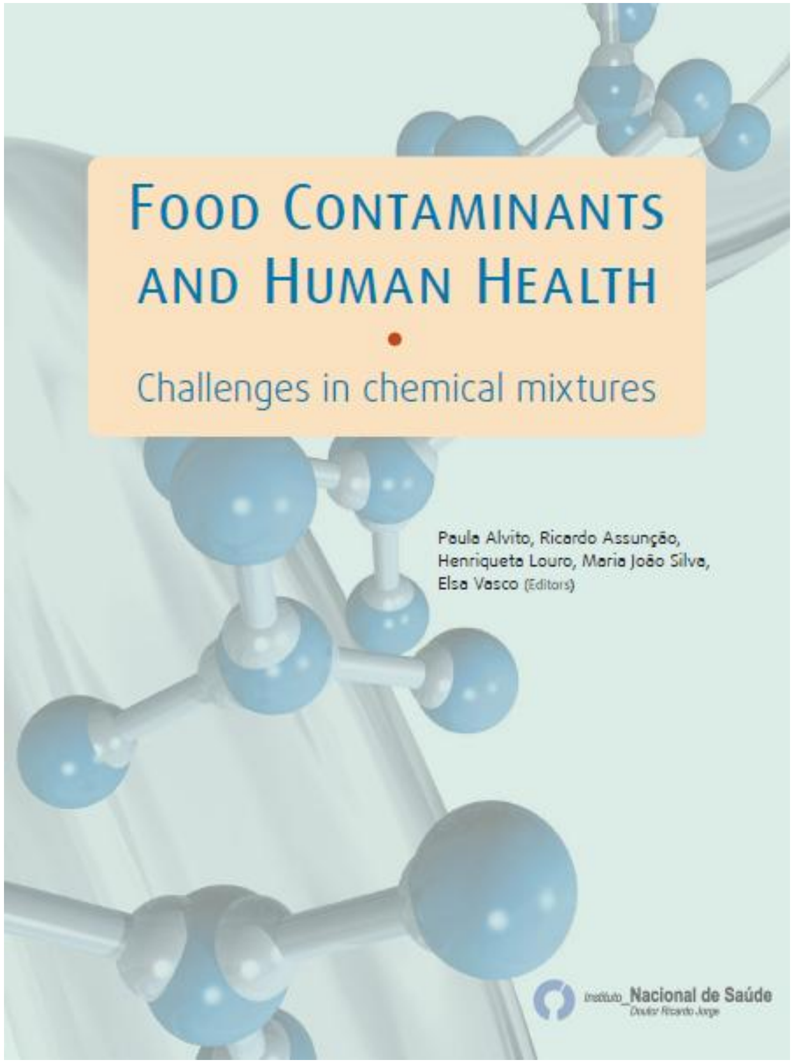
SCIENTIFIC REPORT OF EFSA

International Frameworks Dealing with

Human Risk Assessment of Combined Exposure to Multiple Chemicals[1,]

European Food Safety Authority[2,3]

European Food Safety Authority (EFSA), Parma, Italy




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 chemical mixtures including mycotoxins, their combined toxicity and cumulative risk assessment.

INSA repository
http://hdl.handle.net/10400.18/3214

# 4. Risk estimates of mycotoxin mixtures in the diets of our children

- **Children are constantly growing**. They breathe more air, consume more food, and drink more water than adults do, in proportion to their weight.
- **Children are still developing.** Exposure to toxicants (as mycotoxins) can lead to irreversible damage.

The **significance** and **potential health** risk of any **contaminant in foods** consumed by children is **increased** and diligent **attention** must be paid to this particular area.

# A Portuguese Case Study – MYCOMIX Project

MYCOMIX

“Exploring the toxic effects of **MIX**tures of **MYCO**toxins

in infant food and potential health impact”

(PTDC/DTP-FTO/0417/2012)

FCT Fundação para a Ciência e a Tecnologia MINISTÉRIO DA EDUCAÇÃO E CIÊNCIA

# A Portuguese Case Study – MYCOMIX Project

Are children exposed to mycotoxins through diet?

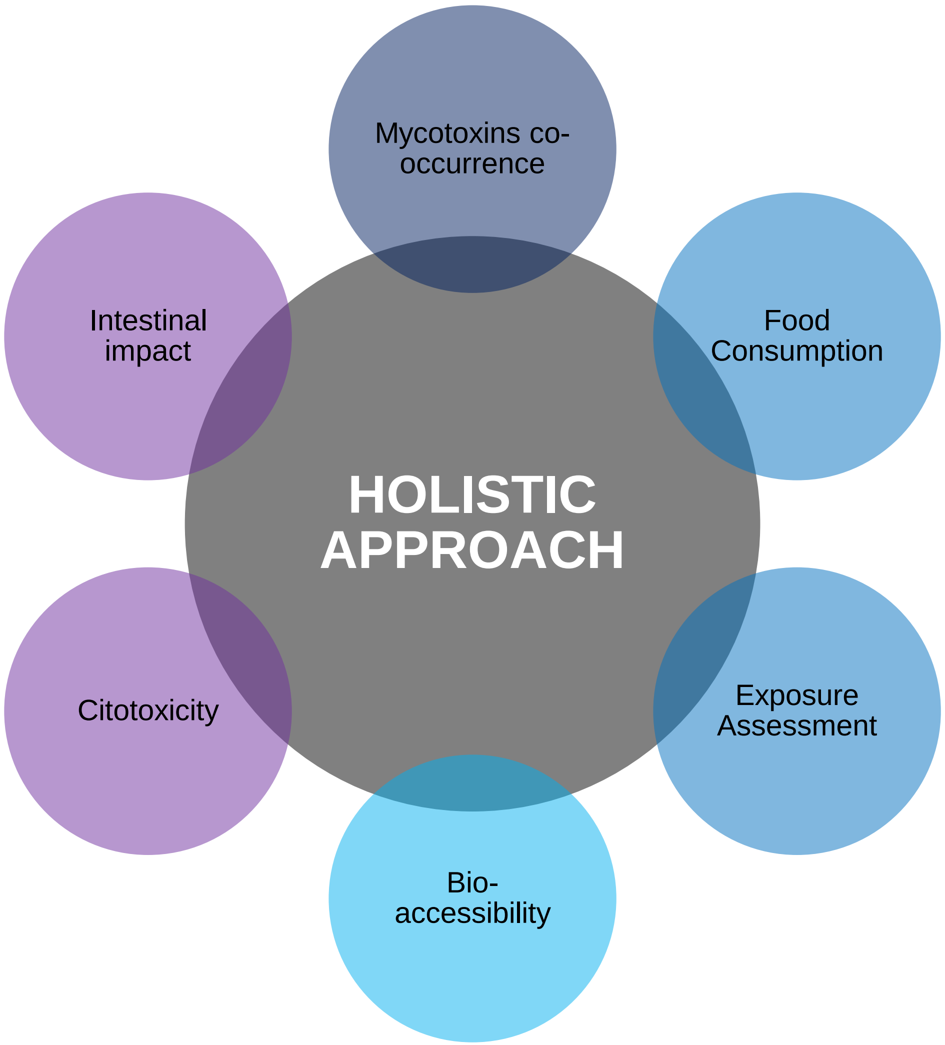
Are there interactive effects in toxicity of mixtures of mycotoxins?

**Children are exposed to mycotoxin mixtures through their diet and this constitutes a health threat**

Could this exposure be a health threat to children?

# Case Study

## To assess the risk using a holistic approach




**Portuguese children exposure to multiple mycotoxins in food**

I. Co-occurrence
II. Assessment of children exposure
III. Bioaccessibility using a standardized IVD model
IV. Potential interactive toxic effects with different endpoints

# I - Co-occurrence

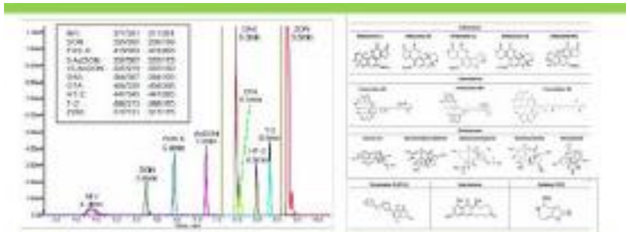
Carla Martins
PhD student
Public Health, PT

**Aim**:
Quantification of multiple mycotoxins (10) in cereal-based products primarily marketed for children in Lisboa region, PT (2014-15; n=52: BC-26, PC-20, BIS-6; maize, wheat, rice, multigrain)

**Methods**:

- HPLC-FD (AFTs and OTA)
- GC-MS (DON, NIV, T-2, HT-2)
- UPLC-MS/MS (FBs, ZEA)

Analytical method performance according to Reg (CE) 401/2006 (linearity, recoveries, LOD, LOQ)



**Colaborations**:
Alessandra Jager, Carlos Oliveira - University of S. Paulo, BR
Sara Cunha, José Fernandes - University of Porto, PT

## II - Assessment of children exposure

Ricardo Assunção,
PhD student Veterinary Sciences, PT

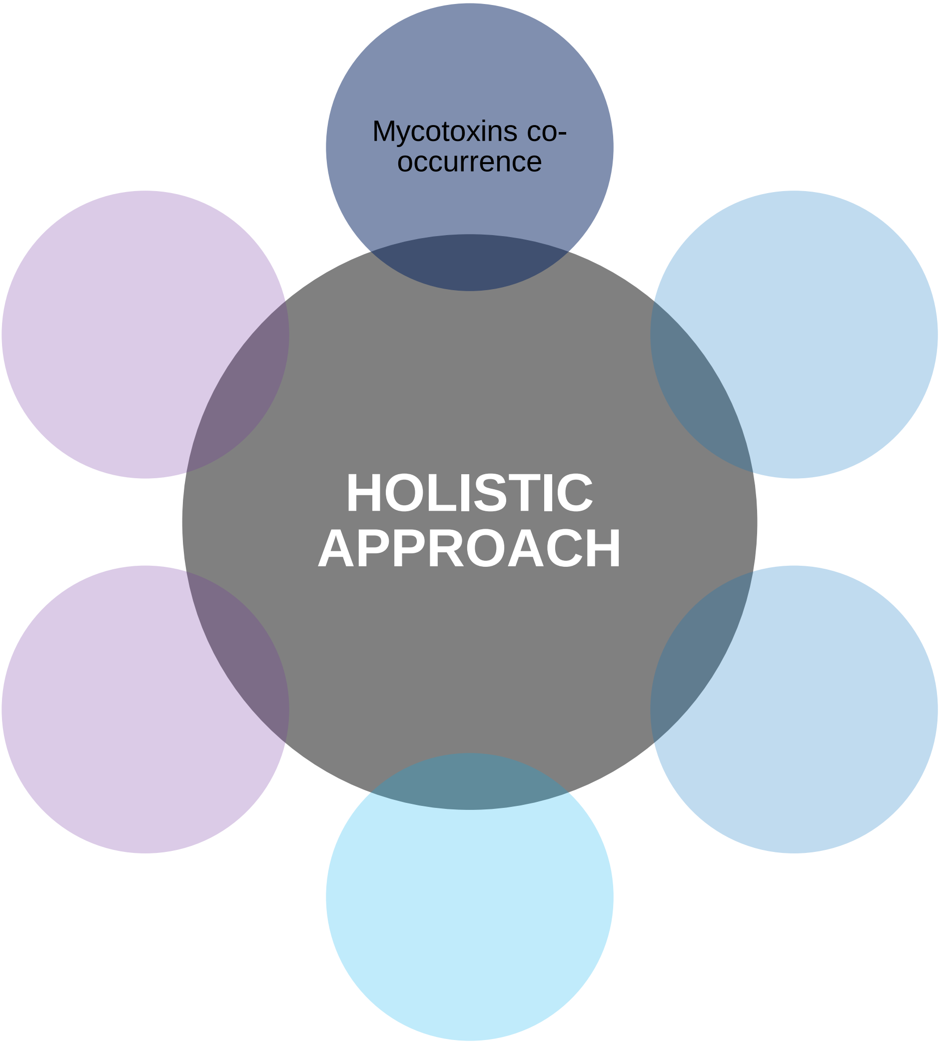
**Aim:**
Assess the children exposure to multiple mycotoxins through consumption of cereal-based foods primarily marketed for children in Lisboa region, PT

**Methods**:

- Consumption data: 3-days food diary, Primary Health Care Unit near Lisbon, to characterize of the consumption pattern of children from 1 to 3 years old (n=75), approved by Ethical Commission, Port. Data Prot Authorithy
- Food consumption data included in a plataform OPEN Portugal
- Multiplying consumption data with occurrence data
- Deterministic and Probabilistic approaches for exposure assessment
- Different strategies to treat the left censored data


**Colaborations**
Sonia Leal, Cidadela Primary Health Care Unit, Cascais, PT
Barbara Seljak, Josef Stephen Institute, SLO
Elsa Vasco and Baltazar Nunes, INSA, PT

# Case Study
To assess the risk using a holistic approach



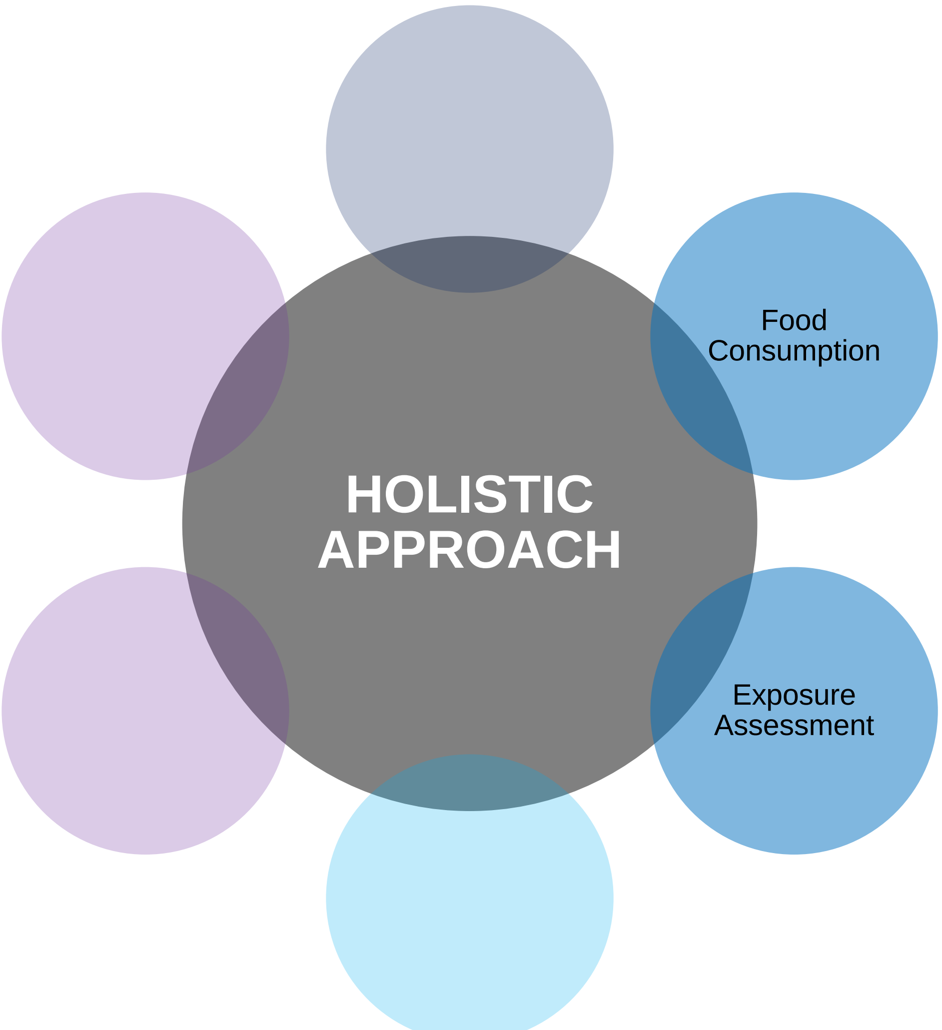

**Assessment of multiple mycotoxins in breakfast cereals available in the Portuguese market**

Carla Martins, Ricardo Assunção, Sara Cunha, José Fernandes, Alessandra Jager, Carlos Oliveira, Paula Alvito
(*in press* Food Chemistry, 2017)

- Breakfast cereals from Portuguese market revealed a high incidence of mycotoxins (below legislation levels)
- Different mycotoxins (two to seven) occurred simultaneously in breakfast cereals
- Twenty two different combinations of mycotoxins were detected in breakfast cereals

# Case Study

## To assess the risk using a holistic approach




Food and Chemical Toxicology 86 (2015) 274–281

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

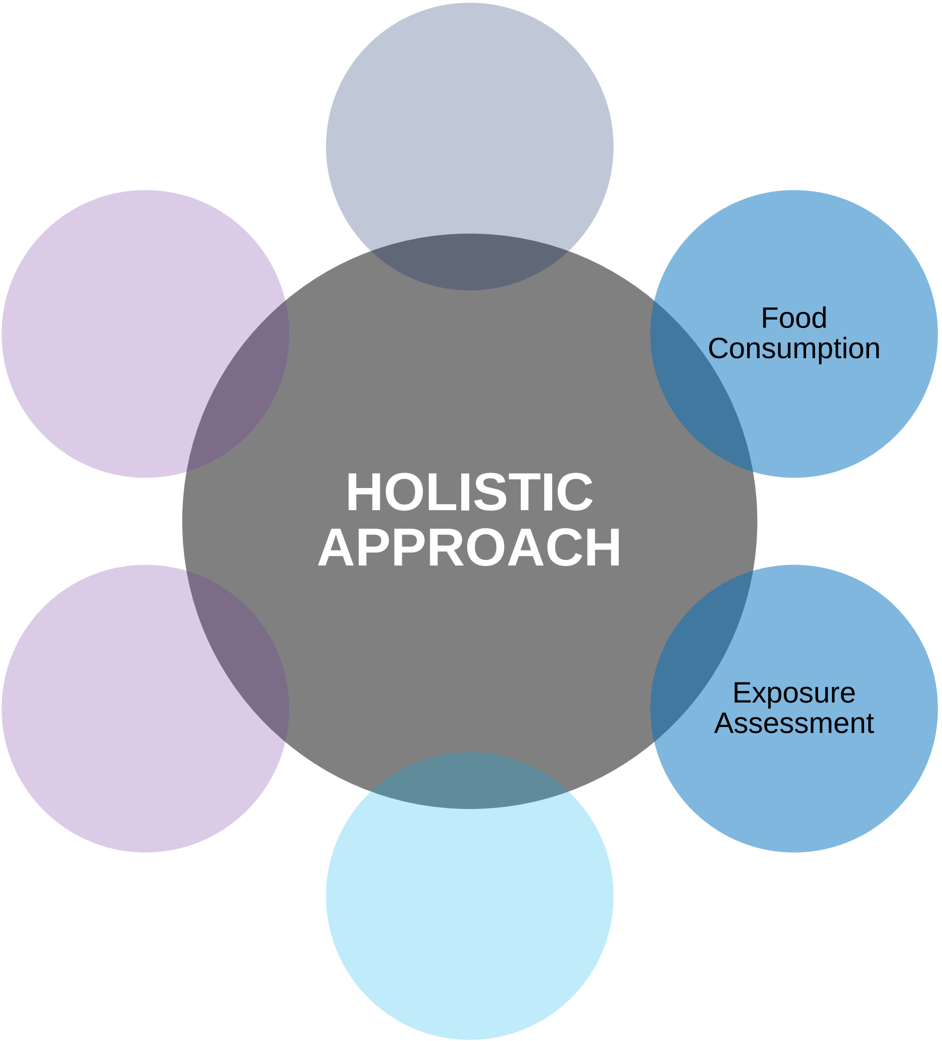
ELSEVIER

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

Ricardo Assunção [a, b, c], Elsa Vasco [a], Baltazar Nunes [d, f], Susana Loureiro [e], Carla Martins [a], Paula Alvito [a, c, *]

- Daily exposure of children to ochratoxin A, fumonisins and trichothecenes showed no health risks to the children population considering individual mycotoxins.
- Exposure to aflatoxin $B_1$ ($AFB_1$) suggested a potential health concern for the high percentiles of intake (P90, P95 and P99).
- 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.

# Case Study
## To assess the risk using a holistic approach




**Risk assessment of Portuguese children exposure to multiple mycotoxins through <u>cereal-based products</u>**

Ricardo Assunção, Carla Martins, Elsa Vasco, Alessandra Jagere, Carlos Oliveira, Sara Cunha José Fernandes, Baltazar Nunes Susana Loureiro and Paula Alvito (submitted Food Addit & Contam 2017)

Different mycotoxins (two to seven) occurred simultaneously in cereal-based products.

Estimated aflatoxins exposure suggested a potential adverse health effect for percentiles of intake above or equal to P50.

The obtained results suggested that future research actions should be set in motion in order to protect children health/ biomarkers.

**Table 1.** 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 | 13 (25%) |
| $AFG_1$ | 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 $M_1$, LOD = 0.011 µg/kg; $AFB_1$, aflatoxin $B_1$, LOD = 0.003 µg/kg; $AFB_2$, aflatoxin $B_2$, LOD = 0.001 µg/kg; $AFG_1$, aflatoxin $G_1$, LOD = 0.006 µg/kg; $AFG_2$, aflatoxin $G_2$, LOD = 0.010 µg/kg; AFTs, aflatoxins; OTA, ochratoxin A, LOD = 0.006 µg/kg; $FB_1$, fumonisin $B_1$, LOD = 0.08 µg/kg; $FB_2$, fumonisin $B_2$, LOD = 0.08 µg/kg; FMs, fumonisins; DON, deoxynivalenol, LOD = 0.37 µg/kg; NIV, nivalenol, LOD = 5.56 µg/kg; T-2, T-2 toxin, LOD = 6.8 µg/kg; HT-2, HT-2 toxin, LOD = 6.4 µg/kg; ZEA, zearalenone, LOD = 0.12 µg/kg.



- **OTA, ZEA and DON were the most commonly detected mycotoxins with 65%, 48% and 44% of analysed samples revealing values above the LOD, respectively.**

Table 3. Co-occurrence of mycotoxins in cereal-based products marketed in Portug

| Number of mycotoxins detected | Sample | Mycotoxins mixture | Number of samples contaminated with mixtures (%) |
|---|---|---|---|
| 7 | BC | AFTs, OTA, FMs, ZEA | 2/52 (4%) |
| | | AFTs, OTA, FMs, DON, 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%) |
| | PC | AFTs, FMs | 1/52 (2%) |
| | | AFTs | 2/52 (4%) |
| | | **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%) |

BC, Breakfast cereals; PC, Processed cereal-based products (flours); BIS, Biscuits. AFTs, Aflatoxins; OTA, Ochratoxin A; FMs, Fumonisins; DON, Deoxynivalenol; NIV, Nivalenol; ZEA, Zearalenone.
Bold values indicate most frequent mycotoxins combinations detected in each food group.



- **highest number of mycotoxins detected simultaneously was seven.**
- **combinations of two (OTA and DON; OTA and fumonisins) and four (aflatoxins, OTA and ZEA) mycotoxins were the most commonly detected.**

Table 4. 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[a] | 0.003[a] | 0.001[a] | 0.064 | 0.057 | 0.051 | 0.000 | 0.000 | 0.000 | 0.069 | 0.060 | 0.052 |
| $AFB_1$ | 0.012[a] | 0.011[a] | 0.011[a] | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.012 | 0.011 | 0.011 |
| $AFB_2$ | 0.001[a] | 0.001[a] | 0.001[a] | 0.002 | 0.002 | 0.000 | 0.000 | 0.000 | 0.000 | 0.003 | 0.003 | 0.001 |
| $AFG_1$ | 0.003[a] | 0.001[a] | 0.000[a] | 0.013 | 0.008 | 0.002 | 0.000 | 0.000 | 0.000 | 0.016 | 0.009 | 0.002 |
| OTA | 0.011[a] | 0.010[a] | 0.010[a] | 0.064[b] | 0.060[b] | 0.059[b] | 0.056 | 0.056 | 0.056 | 0.131 | 0.126 | 0.124 |
| $FB_1$ | 6.0[a] | 5.4[a] | 5.3[a] | 0.4 | 0.3 | 0.3 | 0.0 | 0.0 | 0.0 | 6.4 | 5.7 | 5.6 |
| $FB_2$ | 1.0[a] | 1.0[a] | 0.8[a] | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 1.0 | 0.8 |
| DON | 24.83[a] | 24.80[a] | 24.77[a] | 16.34 | 16.06 | 15.78 | 16.05 | 15.15 | 14.24 | 57.22 | 56.01 | 54.79 |
| NIV | 2.68[a] | 1.56[a] | 0.44[a] | 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 children weight data: 13.4 kg.
[a]Previously reported by Assunção et al., 2015; [b]Previously reported by Assunção et al., 2016a.
$AFM_1$, 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.

- **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), ZEA (0.86 ng/kg bw/day), OTA (0.131 ng/kg bw/day) and $AFM_1$ (0.069 ng/kg bw/day).**

**Table 5.** Probabilistic analysis of mycotoxins estimated daily intake [mean and percentiles 50 (P50), 75 (P75), 90 (P90), 95 (P95) and 99 (P99)] for 1 to 3 years old Portuguese children through different food products. Data for H1 scenario (H1: < LOD = LOD) is presented.

| Toxins | Estimated daily intake (µg/kg bw/day) 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 |

$AFM_1$, 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.



- **results reinforced the outcomes obtained with the deterministic approach.**

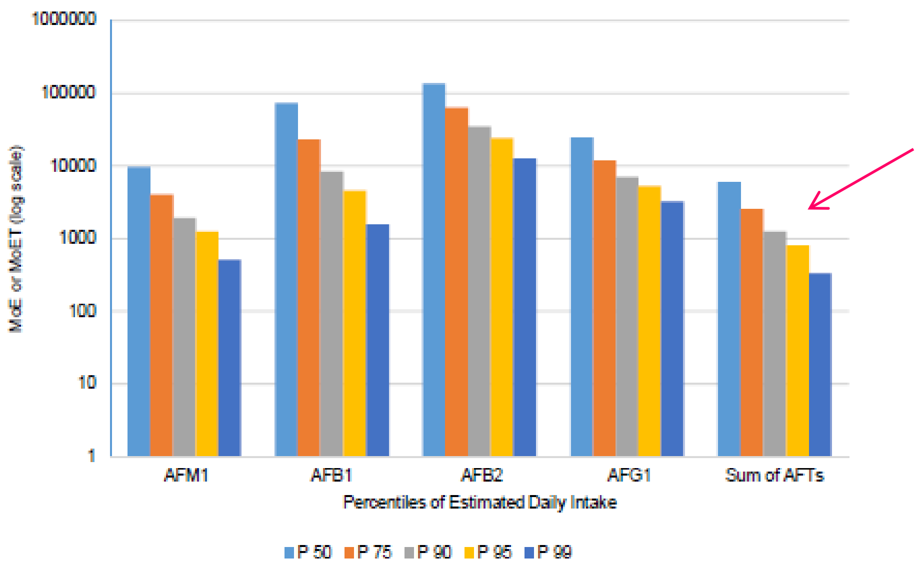



**Figure 1.** Characterization of risk associated with the exposure to aflatoxins through consumption of the three food products considered (breakfast cereals, processed cereal-based foods and biscuits). MoE and MoET were derived from estimates obtained by the probabilistic approach [percentiles 50 (P50), 75 (P75), 90 (P90), 95 (P95) and 99 (P99)]. Data for H1 scenario (H1: < LOD = LOD), as the worst case scenario, is presented.

- **When considered the simultaneous exposure to aflatoxins, MoET for percentiles P50 or higher revealed a potential health concern.**

- **A simulation considering a quarter (1/4) of the aflatoxins daily intake reduce the aflatoxins MoET values revealing that just percentiles of intake above P90 could be under health concern.**

Carcinogenic:
Values of MoE (ratio $BMDL_{10}$/exposure) or 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.

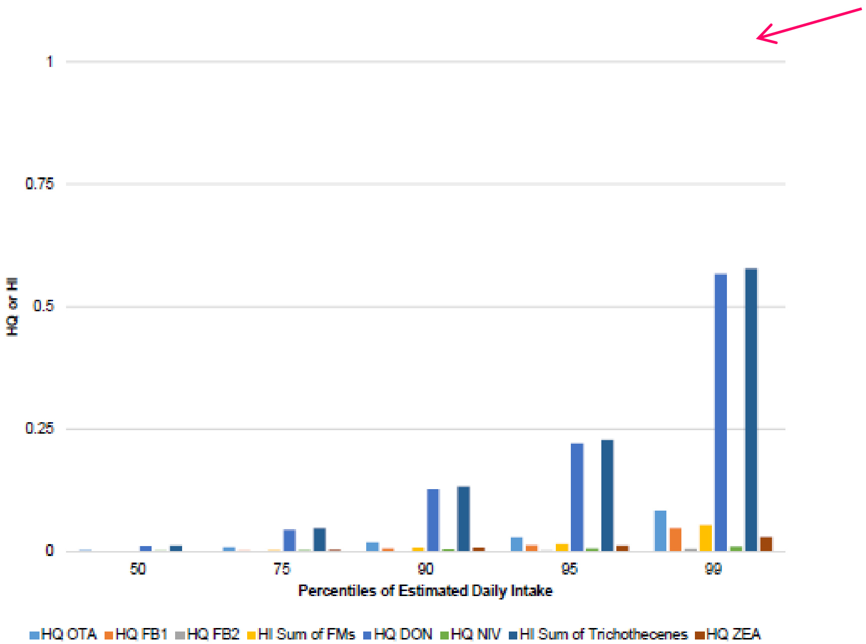



**Figure 2.** Characterization of risk associated with the exposure to ochratoxin A (OTA), fumonisins ($FB_1$ and $FB_2$), trichothecenes (DON and NIV) and zearalenone (ZEA) through the consumption of the three food products considered (breakfast cereals, processed cereal-based foods and biscuits). HQ and HI were derived from estimates obtained by the probabilistic approach [percentiles 50 (P50), 75 (P75), 90 (P90), 95 (P95) and 99 (P99)]. Data for H1 scenario (H1: < LOD = LOD), as the worst case scenario, is presented.



- all HQs were below one, *i.e.*, indicating no cause for concern for individuals exposed to mycotoxins through consumption of cereal-based products.

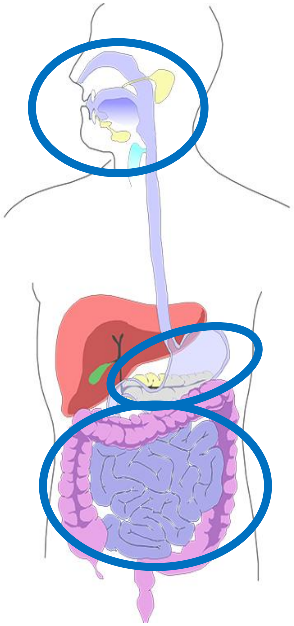
non-carcinogenic:
For the HQ (hazard quotients; exposure/reference dose=PTWI), a tolerable or a non-tolerable exposure level was considered if HQ was below or above one, respectively .

# III. Bioaccessibility using a standardized IVD model

**Aim:**
Determine the bioaccessibility of mycotoxins in cereal-based foods

**Methods:**
Standardized *in vitro* digestion model
Minekus et al (2014) Food and Function 5:1113-1124.

- Simulated digestion fluids composition (saliva, gastric and intestinal)
- Enzyme activities in each fluid
- pH and incubation time at different digestion stages



**Colaboration:**
Didier Dupont, INRA, Cost Action INFOGEST (WG2, Short term scientific mission)

## Improving Health Properties of Food by Sharing our Knowledge on the Digestive Process, *COST FA 1005 INFOGEST (2011-2015)*

Didier Dupont,
French National Institute for Agricultural Research, INRA, FR

Food & Function

PAPER

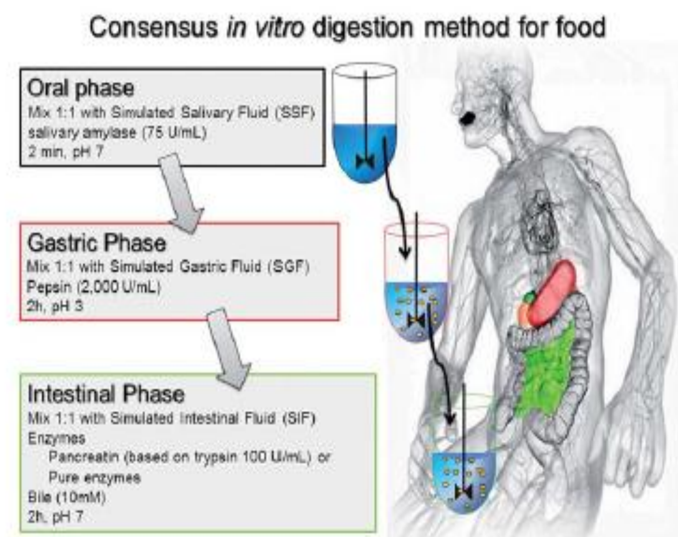
View Article Online
View Journal

Cite this: DOI: 10.1039/c3fo60702j

### A standardised static *in vitro* digestion method suitable for food – an international consensus†

M. Minekus,‡[a] M. Alminger,‡[b] P. Alvito,‡[c] S. Ballance,‡[d] T. Bohn,‡[e] C. Bourlieu,‡[fh] F. Carrière,§[g] R. Boutrou,‡[fh] M. Corredig,‡[i] D. Dupont,§[fh] C. Dufour,‡[j] L. Egger,‡[k] M. Golding,||[l] S. Karakaya,‡[m] B. Kirkhus,§[n] S. Le Feunteun,‡[o] U. Lesmes,‡[p] A. Macierzanka,‡[q] A. Mackie,‡[r] S. Marze,§[s] D. J. McClements,||[t] O. Ménard,‡[fh] I. Recio,‡[u] C. N. Santos,‡[vw] R. P. Singh,||[x] G. E. Vegarud,‡[y] M. S. J. Wickham,‡[z] W. Weitschies‡[aa] and A. Brodkorb‡*[ab]

Minekus et al (2014) Food and Function 5:1113-1124.



# **Standardized** *in vitro* digestion method:

## Oral phase

2 g of sample

+

2 mL of oral fluid

with:

1.5 mM $CaCl_2$

Amylase (75 U/mL)

At pH 7.0

2 min incubation in a rotation wheel, at 37 C

## Gastric phase

Oral sample

+

4 mL of gastric

fluid with

2000 U/mL Pepsin,

0.15 mM $CaCl_2$

At pH 3.0

2 h incubation in a rotation wheel, at 37 C

## Intestinal phase

Gastric sample

+

8 mL of

intestinal fluid

with

100 U/mL of

pancreatin, 0.6

mM $CaCl_2$, 10

mM Bile.

At pH 7.0

2 h incubation in a rotation wheel, at 37˚C

Digestion arrest with 1 mM of Pefabloc

and liquid $N_2$.

Samples kept at -80 C until analysis.

Minekus et al (2014) Food and Function 5:1113-1124

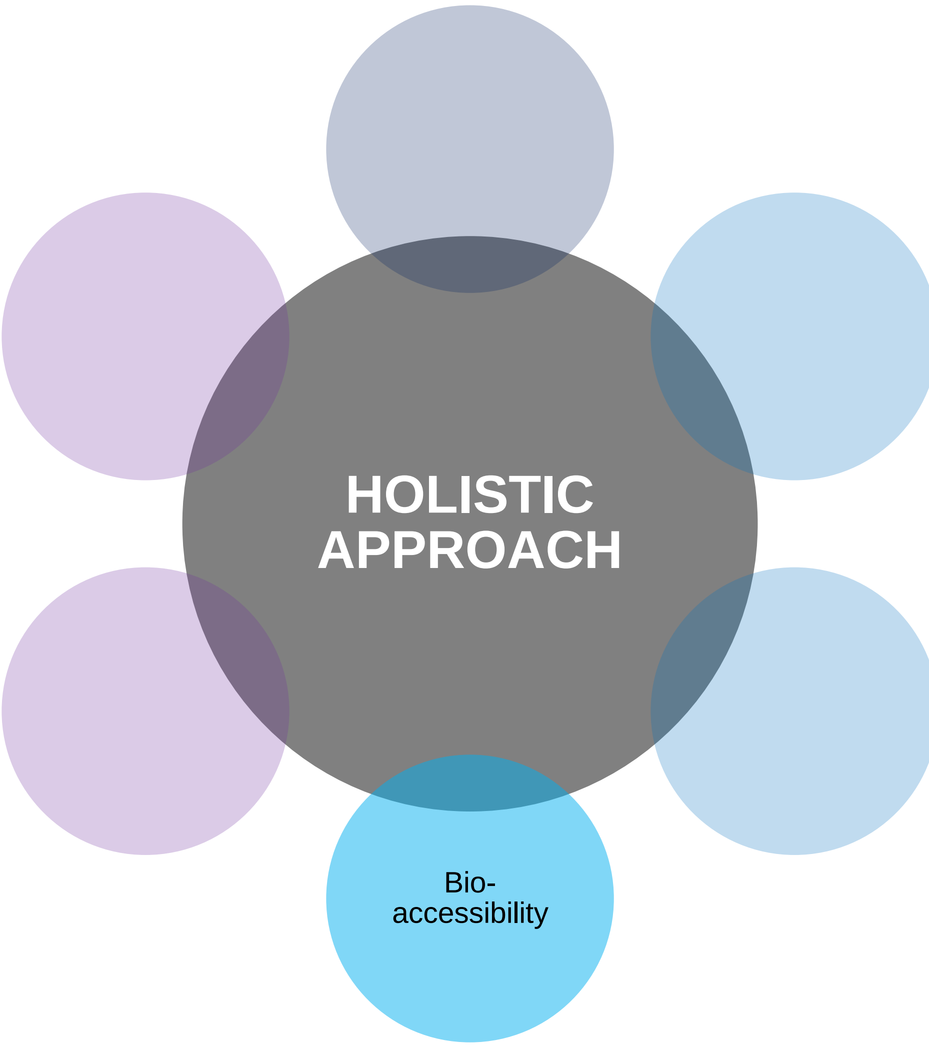
Harmonization of IVD models:

- Simulated digestion fluids composition (saliva, gastric and intestinal)
- Enzyme activities in each fluid
- pH and incubation time at different digestion stages

**Standardised**


YouTube

# Case Study
## To assess the risk using a holistic approach





Contents lists available at ScienceDirect
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Patulin and ochratoxin A co-occurrence and their bioaccessibility in processed cereal-based foods: A contribution for Portuguese children risk assessment

Ricardo Assunção [a, b, c], Carla Martins [a, b, d], Didier Dupont [e, f], Paula Alvito [a, b, *]

- 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 analyzed 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.

| | Patulin | Ochratoxin A |
|---|---|---|
| F1 | 70 ± 3.2 | 95 ± 0.3 |
| F2 | 42 ± 1.2 | 105 ± 1.5 |
| F3 | 56 ± 1.8 | 97 ± 1.8 |
| W/o 1 | 77 ± 1.9 | 98 ± 1.5 |
| W/o 2 | 39 ± 0.7 | 102 ± 0.3 |
| W/o 3 | 30 ± 2.5 | 102 ± 3.9 |
| **Mean** | **52 ± 4.2** | **100 ± 1.1** |

Table 3 - Bioaccessibility (%) results of PAT and OTA in processed cereal-based food samples (n=6), artificially contaminated.
“F” and “W/o” samples represent samples with and without fruit in their content, respectively.

- PAT concentrations **are significantly reduced** during digestion process until reach intestine (internal exposure overestimated if not using bioacessibility values)
- OTA concentrations reach intestine almost **unchanged**

# IV - Potential interactive toxic effects with different endpoints

**Aims:**

Determine the potential interactive effects of mycotoxins in cells, using different endpoints: cytotoxicity and intestinal membrane integrity.

**Methods:**

MTT using Caco-2 cells

Transepithelial electrical resistance (TEER)

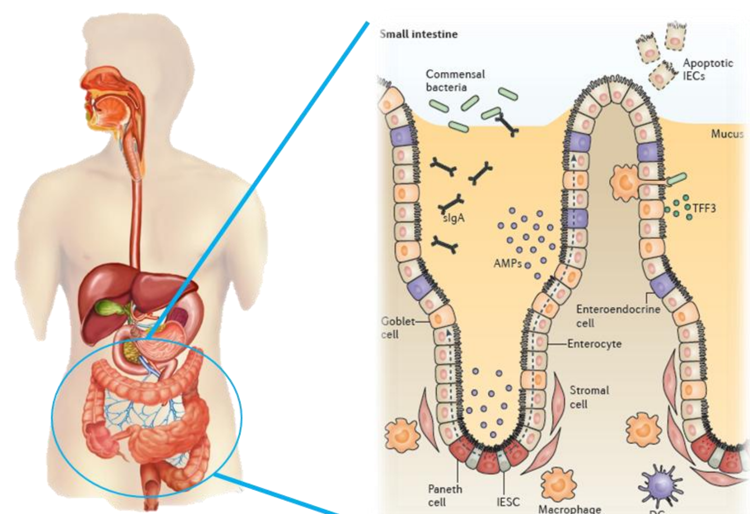
**Collaborations:**

Susana Loureiro, Aveiro University, PT

Tor Lea, Charlotte Kleiveland, NMBU, NO

MJ Silva, H Louro, A Tavares, INSA, PT

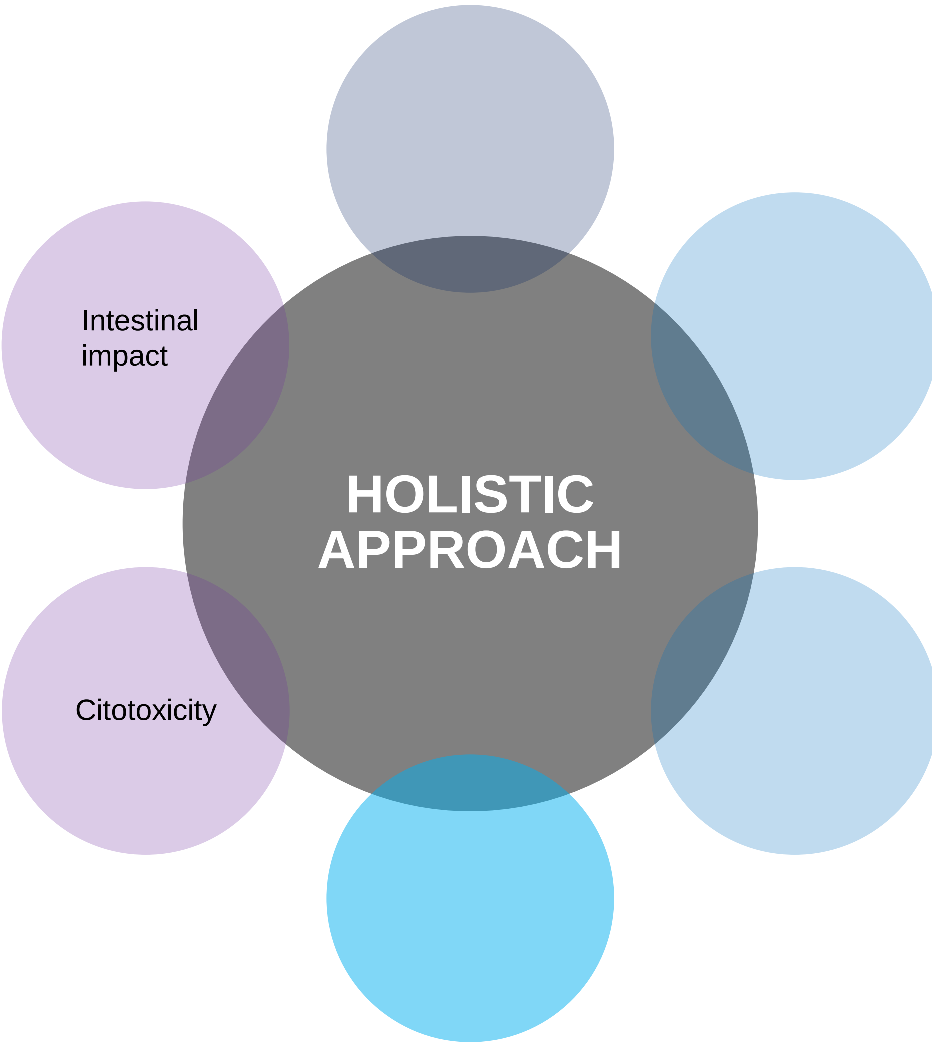
Intestinal mucosa is the first biological barrier encountered by natural toxins in food - Caco 2 cells




Addapted from Peterson and Artis, 2014[6].

# Case Study

## To assess the risk using a holistic approach



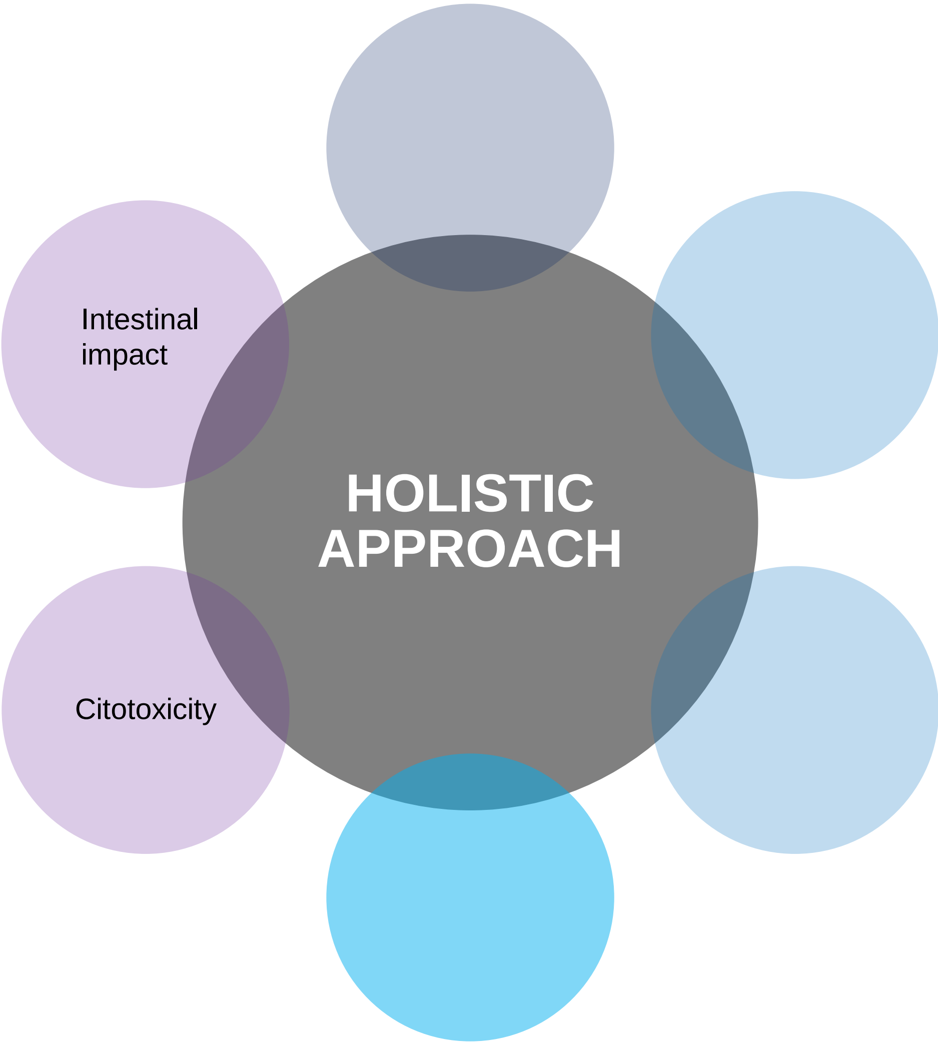

Contents lists available at ScienceDirect
Toxicology Letters
journal homepage: www.elsevier.com/locate/toxlet
ELSEVIER

Characterization of *in vitro* effects of patulin on intestinal epithelial and immune cells

R. Assunção[a,b,c], P. Alvito[a,c,*], C.R. Kleiveland[d], T.E. Lea[d]

- 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.
- Epithelium and immune cells of the intestinal mucosa are important targets for the toxic effects of mycotoxins

# Case Study
## To assess the risk using a holistic approach

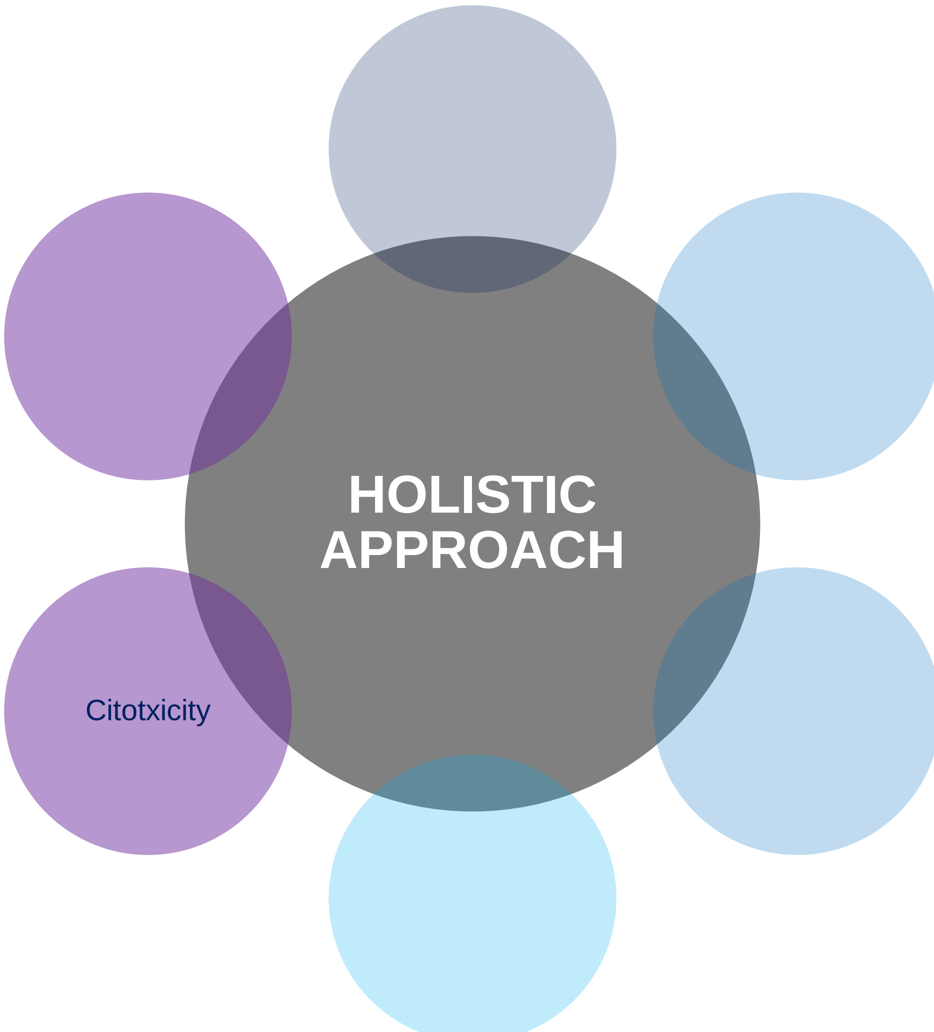



**Insights into individual and combined toxic effects of patulin and ochratoxin A on human intestinal cells**
(in preparation)

PAT ($IC_{50}$=16 uM) is significatly more toxic to Caco-2 cells, compared to OTA ($IC_{50}$=145 uM).

Combined effect to these two mycotoxins suggests an additive effect according to citotoxicity data, however synergism at low doses by TEER data (health concern).

# Case Study
## To assess the risk using a holistic approach



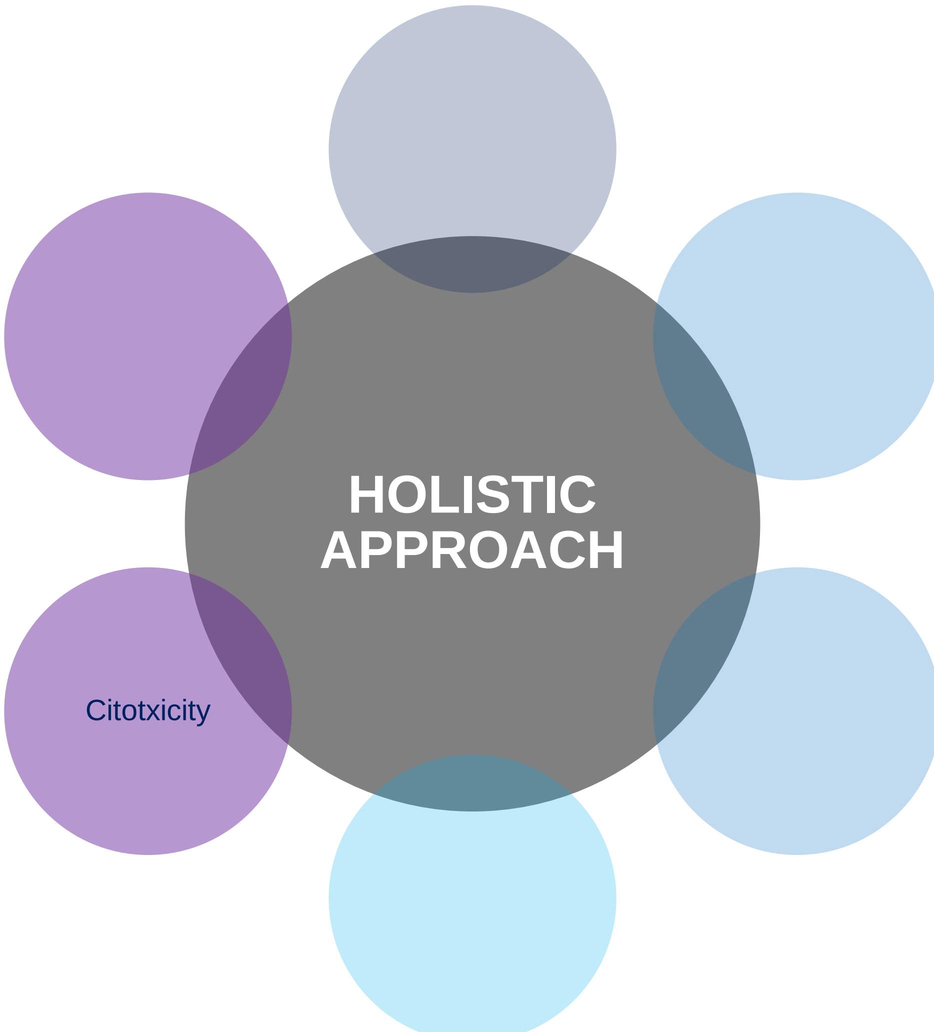

World Mycotoxin Journal, November 2013; 6 (4): 375-388
Wageningen Academic Publishers

**Multi-mycotoxin determination in baby foods and *in vitro* combined cytotoxic effects of aflatoxin $M_1$ and ochratoxin A**

A.M. Tavares[1,4], P. Alvito[1,2], S. Loureiro[3], H. Louro[4] and M.J. Silva[4]

- Existence of antagonistic toxic effects between OTA and $AFM_1$ in a human cell line representative of the primary site of contact of both toxins, i.e., the intestine;
- Both components, food monitoring and interactions characterization in *in vitro* models are complimentary and contribute to prevent mycotoxins-associated diseases, particularly, on the long-term (e.g., cancer).

# Case Study
## To assess the risk using a holistic approach

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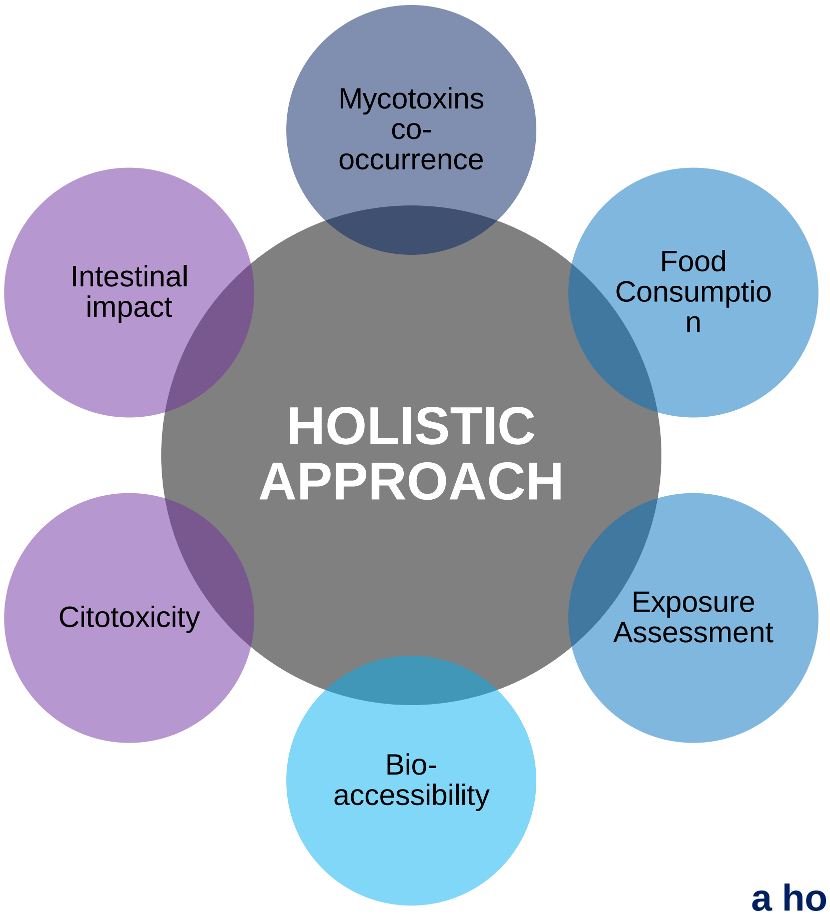



The present work underlines the need to **adopt a holistic approach for multiple mycotoxins risk assessment** integrating data from exposure, bioaccessibility and toxicity domains, in order to contribute to a **more accurate risk assessment.**

**CHILDREN EXPOSURE TO MULTIPLE MYCOTOXINS THROUGH FOOD CONSUMPTION:**
**a holistic approach for risk assessment**
**(PhD Thesis, July 2017)**

UNIVERSIDADE DE ÉVORA

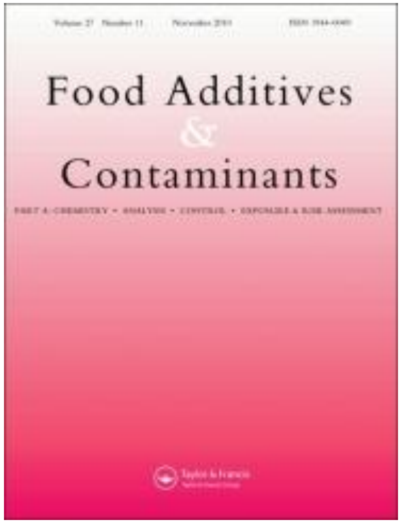
The National Institute of Health Dr. Ricardo Jorge (INSA) and the University of Minho invite you to join the

**2nd International Conference on Food Contaminants (ICFC2017) in Braga, 13-14 July 2017.**

ICFC 2017

International Conference on Food Contaminants

Climate change and food safety: challenges in the near future




http://www.icfc2017.uminho.pt/

# Acknowledgements


Maria Antónia Calhau
Head Food and
Nutrition Department

- MYCOMIX project (PTDC/DTP-FTO/0417/2012)
- CESAM (UID/AMB/50017/2013)
- ToxOmics Portugal

Fundação para a Ciência e Tecnologia (FCT), Portugal

**Thank you for your attention!**