

# Final Report



Exploring the toxic effects of mixtures of mycotoxins  
in infant food and potential health impact

**Project reference:**

PTDC/DTP-FTO/0417/2012

**Principal Investigator:**

Paula Cristina da Cruz Oliveira Soromenho de Alvito

Lisbon, 30th November 2015

## Contents

---

Task 1 - Multimycotoxin determination and legislation values .....	4
Task 2 - Evaluation of cytotoxic and genotoxic effects of mixtures of mycotoxins .....	14
Task 3 - Bioaccessibility and absorption of mycotoxin mixtures .....	22
Task 4 - Children exposure assessment .....	36
Task 5 - Children´s health and mycotoxin mixtures-global evaluation .....	50
Project output indicators .....	54

Within this final report a detailed description of all tasks of the MYCOMIX project including a global evaluation is enclosed. The present project aims to explore the toxic effects of mixture of mycotoxins in infant food and potential health impact and includes a 1<sup>st</sup> task on multimycotoxin determination and legislation values, a 2<sup>nd</sup> on the evaluation of toxic effects of mycotoxin mixtures, a 3<sup>rd</sup> on bioaccessibility and absorption studies, a 4<sup>th</sup> on children exposure assessment and a final evaluation, the 5<sup>th</sup> task, on children's health and mycotoxin mixtures.

All tasks include an initial background and aims, materials and methods, results, deviations/comments, conclusions, references and project indicators. A final description of the total project output indicators is also included.

## **Task 1 - Multimycotoxin determination and legislation values**

---

### Background and aims

There is growing concern with public health about mycotoxin involvement in human diseases particularly related to syndromes related to children's exposure through contaminated food. Mycotoxins are secondary metabolites of fungi that cause toxic and carcinogenic outcomes in humans exposed to them. Mycotoxins affect several commodities including cereal grains and their finished products, infant formula and baby foods (Alvito, 2014). Children diet comprises a variety of different commodities, and the possibility of human co-exposure to several mycotoxins is very likely to occur and few data are available on this issue. According to the proposed project the goals from task 1, titled "Multimycotoxin determination and legislation values" include: i) purchase and sampling of approximately 60 baby foods (i.e. processed cereal-based foods (flours), infant formulae (milk powder) and "ready to drink" milk) and infant cereals samples (breakfast cereals), ii) development and optimization of an LC-MS/MS analytical method for multimycotoxin analysis, iii) identification and quantification of 12 mycotoxins in samples and extracts from bioaccessibility studies and iv) comparison of mycotoxin samples contents with legislative values.

### Materials and methods

Breakfast cereal samples (n=26), including corn, wheat, oat, rice and multigrain, twenty cereal based baby foods (n=20), cookies (n=6), infant formula (n= 10) and ready to drink milk (n=7), in a total of 69 samples, both from conventional and organic origin, were purchased during 2014-2015 in supermarkets from Lisbon region. High performance liquid chromatography with fluorescence detection (HPLC-FD) was used for aflatoxins (AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, AFG<sub>2</sub>, AFM<sub>1</sub>) and ochratoxin A (OTA) determination, HPLC with ultraviolet detection (HPLC-UV) for patulin (PAT) determination, liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) for fumonisins (FB<sub>1</sub>, FB<sub>2</sub>) and zearalenone (ZEA) and gas-chromatography coupled to mass spectrometry (GS-MS) for trichothecenes (DON, FUS-X, NIV, NEO, 15-ADON, T2-Triol and T2-Tetraol, DAS, 3-ADON, VER, T2-Toxin and HT-2). Method performance conditions were also assayed for the analyzed mycotoxins.

### Results

Table 1 refers method performance for the determination of the 22 analyzed mycotoxins and metabolites using HPLC-FLD, UHPLC-MS/MS and GC-MS/MS (Assunção et al, 2015). 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 and showed that the analytical methods applied are adequate for mycotoxins determinations (EC 2006a).

Table 2 shows mycotoxins contents ( $\mu\text{g kg}^{-1}$ ) in 26 breakfast cereal samples, 20 cereal based baby foods, 5 infant formulae and 4 ready to drink milk. AFG<sub>2</sub>, NEO, DAS, FUS-X, DON, 15-ADON, 3-ADON, HT-2, T-2, VER, T-2 TETROL, T-2 TRIOL were not detected in breakfast cereal samples. DON, NIV, NEO, DAS, FUS-X, DON, 15-ADON,

3-ADON, HT-2, T-2, VER, T-2 TETROL, T-2 TRIOL analysis in cereal based baby foods are still in course (Martins et al, 2014, 2015, in press).

**Table 1** - Method performance for the determination of the analyzed mycotoxins by HPLC-FLD, HPLC-UV, UHPLC-MS/MS and GC-MS/MS

HPLC												
FLD							UV					
	AFM <sub>1</sub>	AFB <sub>1</sub>	AFB <sub>2</sub>	AFG <sub>1</sub>	AFG <sub>2</sub>	OTA	PAT					
<b>Linearity (<math>\mu\text{g kg}^{-1}</math>)</b>	0.100 – 1.000	0.040 – 0.400	0.030 – 0.300	0.045 – 0.450	0.030 – 0.300	0.200 – 2.000	3.2 – 40.0					
<b>LOD (<math>\mu\text{g kg}^{-1}</math>)</b>	0.011	0.003	0.001	0.006	0.010	0.006	0.9					
<b>LOQ (<math>\mu\text{g kg}^{-1}</math>)</b>	0.032	0.009	0.004	0.018	0.029	0.019	2.9					
<b>Recovery (%)</b>	83	73	57	87	57	71	80					
UHPLC-MS/MS												
	FB <sub>1</sub>	FB <sub>2</sub>	ZEA									
<b>Linearity (<math>\mu\text{g kg}^{-1}</math>)</b>	2.5 - 800	2.5 - 800	0.24 – 10.00									
<b>LOD (<math>\mu\text{g kg}^{-1}</math>)</b>	0.06	0.12	0.12									
<b>LOQ (<math>\mu\text{g kg}^{-1}</math>)</b>	0.18	0.36	0.40									
<b>Recovery (%)</b>	70	68	*									
GC-MS												
	DON	NIV	T-2	HT-2	NEO	DAS	FUS-X	15-ADON	3-ADON	VER	T2-Tetrol	T-2 Triol
<b>Linearity (<math>\mu\text{g kg}^{-1}</math>)</b>	15 - 360	25 - 360	25 - 360	25 - 450	15 - 360	15 - 360	15 - 360	15 - 360	75 - 360	75 - 360	35 - 450	15 - 360
<b>LOD (<math>\mu\text{g kg}^{-1}</math>)</b>	0.4	5.6	6.8	6.4	1.3	3.1	2.8	2.5	17.3	19.2	10.5	0.9
<b>LOQ (<math>\mu\text{g kg}^{-1}</math>)</b>	1.2	18.4	22.3	21.1	4.2	10.1	9.2	8.3	57.0	63.3	34.6	3.0
<b>Recovery (%)</b>	93	46	44	93	103	117	99	131	92	135	84	74

**Table 2** - Occurrence of mycotoxins ( $\mu\text{g kg}^{-1}$ ) in 26 breakfast cereal samples, 20 cereal based baby foods, 6 cookies, 5 infant formulae and 4 ready-to-drink milk purchased in supermarkets in Lisbon region. AFG<sub>2</sub>, NEO, DAS, FUS-X, DON, 15-ADON, 3-ADON, HT-2, T-2, VER, T-2 TETROL, T-2 TRIOL were not detected in breakfast cereals samples. FB<sub>1</sub>, FB<sub>2</sub>, ZEA, DON, NIV, NEO, DAS, FUS-X, DON, 15-ADON, 3-ADON, HT-2, T-2, VER, T-2 TETROL, T-2 TRIOL analysis in cereal based baby foods, infant formulae and ready-to-drink milk are still in course.

		AFM <sub>1</sub>	AFB <sub>1</sub>	AFB <sub>2</sub>	AFG <sub>1</sub>	AFG <sub>2</sub>	OTA	PAT	FB <sub>1</sub>	FB <sub>2</sub>	DON	NIV	ZEA
<b>Breakfast cereals (n=26)</b>	> LOD (n)	3	19	7	1	0	18		15	10	16	1	19
	> LOD (%)	12	73	27	4	0	69	NA	58	38	62	4	73
	Maximum ( $\mu\text{g Kg}^{-1}$ )	<b>0.024</b>	<b>0.130</b>	<b>0.011</b>	<b>0.014</b>	<b>ND</b>	<b>0.100</b>		<b>67.0</b>	<b>14.0</b>	<b>207.8</b>	<b>27.1</b>	<b>5.61</b>
<b>Cereal based Baby foods (n=20)</b>	> LOD (n)	8	0	1	2	0	10	14	7	0	*	*	3
	> LOD (%)	40	0	5	10	0	50	11	35	0	*	*	15
	Maximum ( $\mu\text{g Kg}^{-1}$ )	<b>0.190</b>	<b>ND</b>	<b>0.002</b>	<b>0.016</b>	<b>ND</b>	<b>0.263</b>	<b>3.460</b>	<b>0.860</b>	<b>ND</b>	*	*	<b>0.98</b>
<b>Cookies (n=6)</b>	> LOD (n)	0	0	0	0	0	6		0	0	3	0	0
	> LOD (%)	0	0	0	0	0	100	NA	0	0	50	0	0
	Maximum ( $\mu\text{g Kg}^{-1}$ )	<b>ND</b>	<b>ND</b>	<b>ND</b>	<b>ND</b>	<b>ND</b>	<b>0.134</b>		<b>ND</b>	<b>ND</b>	<b>73.3</b>	<b>ND</b>	<b>ND</b>
<b>Ready to drink milk (n=4)</b>	> LOD (n)	<b>3</b>	4	4	0	0	4		*	*	*	*	*
	> LOD (%)	<b>75</b>	100	100	0	0	100	NA	*	*	*	*	*
	Maximum ( $\mu\text{g Kg}^{-1}$ )	<b>0.025</b>	<b>0.009</b>	<b>0.004</b>	<b>ND</b>	<b>ND</b>	<b>0.026</b>		*	*	*	*	*
<b>Infant Formulae (n=5)</b>	> LOD (n)	0	0	0	0	0	0		*	*	*	*	*
	> LOD (%)	0	0	0	0	0	0	NA	*	*	*	*	*
	Maximum ( $\mu\text{g Kg}^{-1}$ )	<b>ND</b>	<b>ND</b>	<b>ND</b>	<b>ND</b>	<b>ND</b>	<b>ND</b>		*	*	*	*	*

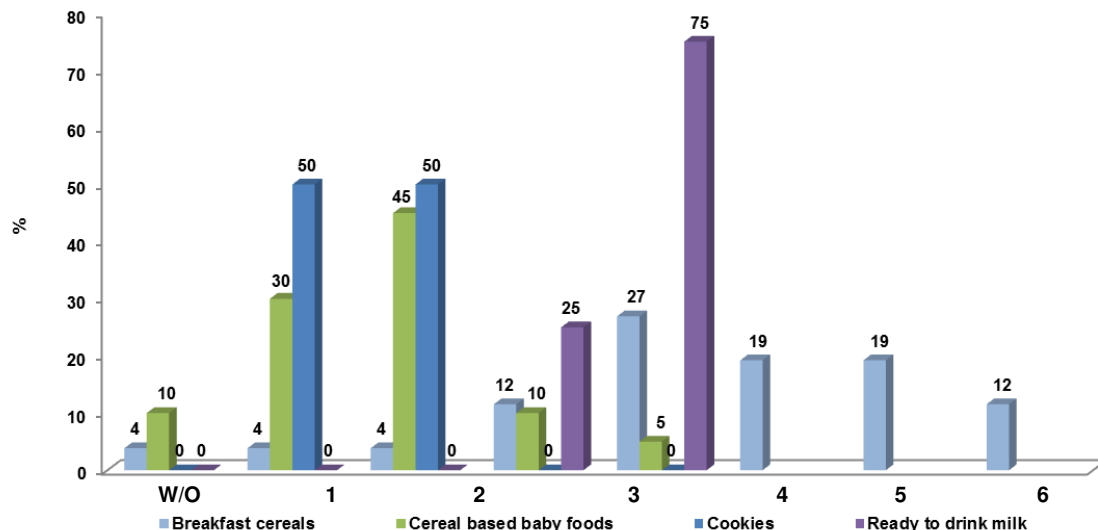
ND= not detected; NA= not applicable; \* = analysis in course

All samples presented levels below the maximum limits established by the Commission Regulation 1881/2006, when available (EC 2006b) but with values higher than the detection limits: 96 % of the breakfast cereals samples, 90 % of the cereal based baby food, 100% of the cookies samples and 100 % of ready to drink milk samples were contaminated with mycotoxins (values > LOD). Mycotoxins were not detected in infant formulae samples (values <LOD).

Cereal based baby foods with milk presented a maximum value of 0.190  $\mu\text{g AFM}_1 \text{ kg}^{-1}$ . There is no legislation for the presence of  $\text{AFM}_1$  in cereal based baby foods and the maximum admissible level for  $\text{AFM}_1$  in infant formulae is 0.020  $\mu\text{g kg}^{-1}$ . Breakfast cereals presented a maximum value of 0.130  $\mu\text{g AFB}_1 \text{ kg}^{-1}$ . There is also no legislation for the presence of  $\text{AFB}_1$  in breakfast cereals and it should be noted that the maximum levels admissible for  $\text{AFB}_1$  in cereal based baby foods is 0.100  $\mu\text{g kg}^{-1}$ .

ZEA,  $\text{AFB}_1$ , OTA, DON and  $\text{FB}_1$  were the most commonly detected mycotoxins in breakfast cereals, ranging between 58 % and 73 % of samples with contents above LOD. OTA,  $\text{AFM}_1$  and  $\text{FB}_1$  were the most commonly detected mycotoxins in cereal based baby foods with 50 %, 40 % and 35 % of the samples with contents above LOD, respectively. PAT, a target mycotoxin for children (due to their frequent consumption of fruit based products due to its sweet taste) was also detected in cereal based foods. OTA and DON were the most commonly detected mycotoxins in cookies samples with 100 % and 50 % of the samples with contents above LOD, respectively.  $\text{AFM}_1$ ,  $\text{AFB}_1$ ,  $\text{AFB}_2$  and OTA were the most commonly detected mycotoxins in ready to drink milk samples with 75 %, and 100 % for the remaining with contents above LOD, respectively.

Figure 1 presents the co-occurrence of mycotoxins in the analyzed samples. Co-occurrence of mycotoxins (2 to 7, simultaneously) was highly observed in the analyzed samples: 92 % in breakfast cereals, 60 % in cereal based baby foods, 50 % in cookies and 100 % in ready to drink milk.



7

**Figure 1** - Co-occurrence of mycotoxins in breakfast cereal, cereal based baby food, cookies and ready-to-drink milk samples. The X-axis represents the number of mycotoxins determined in the same sample (W/O = no mycotoxins detected) and the Y-axis the respective percentage.

Breakfast cereals revealed the presence of 22 different combinations of mycotoxins and the most frequent mixture was AFB<sub>1</sub>+AFB<sub>2</sub>+OTA+ZEA (n=2) and OTA+FB<sub>1</sub>+DON+ZEA (n=2). Cereal based baby foods revealed the presence of 10 different combinations of mycotoxins and the most frequent was OTA+FB<sub>1</sub> (n=3) although PAT (a target mycotoxin for children) was also present. In cookies samples the mixture of mycotoxins that was most detected was OTA+DON (n=3). In ready to drink milk samples the mixture of mycotoxins that was most detected was AFM<sub>1</sub>+AFB<sub>1</sub>+AFB<sub>2</sub>+OTA (n=3).

Occurrence data obtained at task 1 were combined with food consumption data from task 4 in order to perform exposure assessment studies therefore results are also included at task 4. More infant formula and ready to drink milk need to be purchased and analyzed to perform exposure assessment studies on these food groups since the planned sample size proposed under the project was already exceeded. Bioaccessibility extracts were also analyzed and results presented within task 3.

### Deviations

Deviations that occurred during the project execution include a i) first training at LC-MS/MS methodology by ABSciex for 4 team members (certificates already sent to FCT) that became necessary due to the complexity of mycotoxin mixtures analysis (1<sup>st</sup> year) and ii) a LC-MS/MS equipment failure enabling the progress of analytical studies during the 2<sup>nd</sup> year of project. This fact was responsible for a delay in the project progress and one of main reasons for the project prorogation and delays in the related tasks, namely tasks 4 and 5. This difficulty promoted the establishment of a national (LAQV-REQUIMTE, Laboratory of Bromatology and Hidrology, Faculty of Pharmacy, Porto University) and an international (Department of Food Engineering, School of


Animal Science and Food Engineering, University of São Paulo, Pirassununga, Brazil) collaborative studies on mycotoxin occurrence in order to overcome this difficulty and attain the planned task 1 goals. The exit of the task leader (Maria João Barreira) due to professional reasons (change of laboratory area) was compensated by an increase of % by two members of the task (Carla Martins and Paula Alvito) and Paula Alvito becomes the task leader.

### Conclusions

The present study evaluated for the first time the occurrence of 22 mycotoxins and their metabolites, instead of the proposed 12 toxins, in breakfast cereals, cereal based baby foods, infant formula and ready to drink milk marketed in Lisbon, Portugal. These results contribute to increase the knowledge on mycotoxin contents in these food matrices and highlight an urgent need for further studies in order to overcome the absence of legislated limits for mycotoxins in breakfast cereals other than DON and FB<sub>1</sub> and also the absence of legislated limits for mycotoxin mixtures in food. The last issue assumes particular importance considering the potential interactions that could occur between mycotoxins and its potential impact on human and, mainly, children health.

A book, the first in Portuguese concerning mycotoxins and human health, was elaborated to disseminate the information available in the literature concerning the main important issues on mycotoxins and human health. A publication on children risk assessment including mycotoxins determinations and occurrence data in breakfast cereals was performed and 3 communications on occurrence data were presented in scientific meetings, other presentations are included in tasks 3 and 4. One publication is in preparation concerning the assessment of mycotoxins in breakfast cereals available in Portuguese market that it is expected to be submitted until the end of the year.

All goals from Task 1 were attained and some additional tasks were performed beyond the proposed plan. Difficulties concerning LC-MS/MS analytical method and equipment failure were overcome with the establishment of collaborations with scientific laboratories allowing the determination of 22 mycotoxins and metabolites instead of the proposed 12 mycotoxins. Besides, a new LC-MS/MS method is now implemented at INSA, as planned, taking advantage of all acquired knowledge during this task. The developed analytical method has mass spectrometer and chromatography parameters already optimized and INSA had already participated in an interlaboratory study (as shown in figure 2) and is waiting for the results. Validation studies in food matrices are in course. Besides sample determinations, determination on extracts from task 3 were also performed and presented at that task.

 CODA-CERVA 2015 Proficiency Test on mycotoxins quantification, report form : (1) results

laboratory AC17

	BARLEY MALT FLOUR					Measurement Uncertainty (k=2) on av. res.	VIALS	
	reporting limits		results				value	unit
	low (µg/kg)	high (µg/kg)	result 1 (µg/kg)	result 2 (µg/kg)	average (µg/kg)			
1	0,8		4,1	4,5	4,3	N	11,2	2,4
2	0,8		0,6	0,5	0,55 (cIR)	N	11,2	3
3	0,8		2,4	0,9	1,65	N	10	1
4	0,8		ND	ND	ND (cLR)	N	14,3	1
5			7,1	5,9	6,5	N		
6	0,8		2,4	3,7	3,05	N	5,1	0,8
7	12		112,5	130,9	121,7	N	86,9	6,7
8	16		77,8	94,1	85,95	N	41,4	2
9								
10	4		715,8	712,4	714,1	N	72,5	8
11	22,4		18,9	26	22,45	N	122,1	4,2
12	14,4		751,1	1120	935,8	N	747	67,7
13			770	1146	958,2	N		
14	4		286,2	289,9	286,1	N	388	30,2
15	8		635,2	667,8	651,5	N	101,8	2
16			3498	3527	3512	N		
17								
18								
19								
20								
21								
22								

(\*) ng : equivalent to µg/L if the vial's content was reconstituted in 1 ml solvent

name PAULA ALVITO

date and signature 3/9/2015 PAULA ALVITO

Figure 2 - Participation in CODA CERVA Interlaboratory Study - 2015.

**References:**

Alvito P. Alterações do estado de saúde associados à alimentação - contaminantes químicos – micotoxinas (2014), INSA, Lisboa. <http://hdl.handle.net/10400.18/2283>

Commission Regulation. (2006a). European Commission Regulation. EC 401/2006, laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Official Journal of the European Communities* 5e24. L70/13.

Commission Regulation. (2006b). European Commission Regulation. EC 1881/2006, setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Communities* 5e24. L364.

Martins C, Assunção R., Cunha S., Jager A, Alvito P. (2014). Ocorrência de micotoxinas em produtos à base de cereais. 1º Simpósio Nacional Promoção de uma Alimentação Saudável e Segura - Alimentação infantil e contaminantes químicos (SNPASS), Lisboa, Portugal. Available at: <http://hdl.handle.net/10400.18/2682>

Martins C, Assunção R, Cunha S, Jager A, Alvito P. (2015) Assessment of mixtures of mycotoxins in cereal based foods available in Portuguese market, Abstracts Book RAFA2015, Prague, Czech Republic, pp 334. <http://hdl.handle.net/10400.18/3219>

Martins C, Assunção R, Cunha S, Jager A, Alvito P. (in press). Assessment of mixtures of mycotoxins in breakfast cereals available in portuguese market, Food Contaminants and Human Health - Challenges in chemical mixtures. Eds: Alvito, Assunção, Louro H, Silva MJ, Vasco E. Publisher: National Institute of Health Doutor Ricardo Jorge, Lisbon, Portugal <http://hdl.handle.net/10400.18/3214>

Assunção, R., Vasco, E., Nunes, B., Loureiro, S., Martins, C. and Alvito, P. (2015). Single-compound and cumulative risk assessment of mycotoxins present in breakfast cereals consumed by children from Lisbon region, Portugal. *Food and Chemical Toxicology*. 86, 274-28. doi: 10.1016/j.fct.2015.10.017

## PROJECT INDICATORS

### Books

Alvito P. Alterações do estado de saúde associados à alimentação - contaminantes químicos – micotoxinas (2014), INSA, Lisboa. <http://hdl.handle.net/10400.18/2283>

### Publications

In international papers

Assunção, R., Vasco, E., Nunes, B., Loureiro, S., Martins, C. and Alvito, P. (2015). Single-compound and cumulative risk assessment of mycotoxins present in breakfast cereals consumed by children from Lisbon region, Portugal. *Food and Chemical Toxicology*. 86, 274-28. doi: 10.1016/j.fct.2015.10.017

In preparation

Martins C, Assunção R, Cunha S, Fernandes J., Jager A, Oliveira C, Alvito P. (in preparation). Assessment of mixtures of mycotoxins in breakfast cereals available in Portuguese market

## Communications

### International meetings

Martins C, Assunção R, Cunha S, Jager A, Alvito P. (2015) Assessment of mixtures of mycotoxins in cereal based foods available in Portuguese market, Abstracts Book RAFA2015, Prague, Czech Republic, pp 334. <http://hdl.handle.net/10400.18/3219>

Martins C, Assunção R, Cunha S, Jager A, Alvito P. (in press). Assessment of mixtures of mycotoxins in breakfast cereals available in portuguese market, Food Contaminants and Human Health - Challenges in chemical mixtures. Eds: Alvito, Assunção, Louro H, Silva MJ, Vasco E. Publisher: National Institute of Health Doutor Ricardo Jorge, Lisbon, Portugal <http://hdl.handle.net/10400.18/3214>

### National meetings

Martins C, Assunção R., Cunha S., Jager A, Alvito P. (2014). Ocorrência de micotoxinas em produtos à base de cereais. 1º Simpósio Nacional Promoção de uma Alimentação Saudável e Segura - Alimentação infantil e contaminantes químicos (SNPASS), Lisboa, Portugal. Available at: <http://hdl.handle.net/10400.18/2682>

## **Task 2 - Evaluation of cytotoxic and genotoxic effects of mixtures of mycotoxins**

---

### 1. Background and objectives

Food and feed can be contaminated by several fungal species at once and most fungi are able to produce several mycotoxins simultaneously as secondary metabolites (Corcuera et al., 2011; Heussner et al., 2006). For this reason and the fact that human diet comprises a variety of different commodities, the possibility of human co-exposure to several mycotoxins is very likely to occur (Corcuera et al., 2011; Cano-Sancho et al., 2015). This is even more relevant when children dietary co-exposure is considered due to their increased vulnerability (Alvito et al., 2010). Although some predictions about the toxic effects of combinations of mycotoxins can be made based upon their individual toxicities, experimental data are still limited to allow a reliable hazard assessment. Thus, more studies are needed to allow new risk assessment strategies that take into account the toxicological interactions of mycotoxins in food and feed.

This task aimed at characterizing the cytotoxic and genotoxic effects of binary mixtures of mycotoxins identified in baby food, particularly in products consumed by Portuguese children (Martins et al., in press). To accomplish that objective, ochratoxin A (OTA), a mycotoxin frequently identified in cereals-based baby food was combined with aflatoxin M<sub>1</sub> (AFM<sub>1</sub>), fumonisin B<sub>1</sub> (FB<sub>1</sub>) or patulin (PAT), and the observed cytotoxic and genotoxic effects were compared to their individual effects in cell lines representative of their target organs. In addition, putative interactions were ascertained using two reference mathematical models, the concentration addition (CA) and the independent action (IA) models (Loureiro et al., 2010).

### 2. Materials and Methods

To assess the toxicity of binary mixtures of mycotoxins, the first approach consisted on establishing a dose-response curve based on cell viability and determining the concentration that inhibits 50% of cell growth, the IC<sub>50</sub> value. The second approach was directed to determine cells viability following exposure to binary mixtures of OTA and AFM<sub>1</sub>, OTA and FB<sub>1</sub> and OTA and PAT, using various concentrations combinations. For this evaluation, full factorial designs were used as exemplified in Figure 1 for the OTA and AFM<sub>1</sub> mixture (for detailed study designs please see Tavares et al., 2013 and Pinhão et al., submitted). Finally, genotoxicity was assessed for binary mixtures of OTA and FB<sub>1</sub> in HepG2 and HK-2 cells or OTA and PAT in Caco-2 cells.

Human cell lines were obtained from the American Type Culture Collection - the colonic carcinoma Caco-2 cell line (HTB-37), the hepatocellular carcinoma HepG2 cell line (HB-8065) and the proximal renal tubular epithelial HK-2 cell line (CRL-2190) - and were maintained according to standardized cell culture practices. To analyse cell viability, the neutral red (NR) or the MTT assay were carried out according to Repetto et al. (2008) and Monsmann et al. (1983), respectively, with slight modifications. For genotoxicity characterization the comet assay was carried out according to standardized conditions and the FPG-modified comet assay was used in order to assess the potential to induce oxidative damage in the DNA of the cells (Pinto

et al., 2014). The results of both assays were analyzed using a parametric One-Way ANOVA (followed by the Tukey post-hoc) or Student's *t*-test.

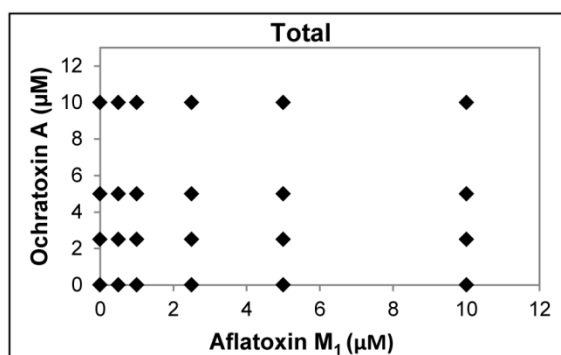


Figure 1 – Experimental design used for the cytotoxicity assessment of OTA combined with AFM<sub>1</sub> (Tavares et al., 2013).

### 3. Results and Discussion

#### 3.1. Single mycotoxin cytotoxicity and genotoxicity

A decrease in cell viability was noted after Caco-2 cells exposure to single treatments with AFM<sub>1</sub>, during 48h, resulting in an IC<sub>50</sub> value of 25.7 µM (please see Tavares et al., 2013). Patulin was more cytotoxic in the same cell line, with an IC<sub>50</sub> value of 15.27 µM for a 24h exposure period. OTA caused a time- and dose-related decrease of Caco-2 cells viability, with an IC<sub>50</sub> value of 186.94 µM for 24h and of 16.98 µM for 48h exposure. In HK-2 cells, both OTA and FB<sub>1</sub> revealed accentuated cytotoxic effects in the tested concentration range. From these data, an IC<sub>50</sub> value of 8.71 µM was estimated for OTA while FB<sub>1</sub> was moderately cytotoxic presenting an IC<sub>50</sub> of approximately 2118.29 µM. In liver cells, OTA caused a marked loss of viability and the IC<sub>50</sub> of this toxin was 31.50 µM; FB<sub>1</sub>, on the other hand, did not cause a significant reduction of cells viability (please see Pinhão et al., submitted). In summary, different cell lines displayed diverse sensitivities to OTA cytotoxicity, with the HK-2 cells, a renal cell line, showing clearly the highest sensitivity. This finding agrees with the known OTA nephrotoxic and hepatotoxic effects reported in humans and suggest that deleterious effects may also occur in intestine, especially for long-term exposures.

#### 3.2. Mixtures cytotoxicity and genotoxicity

In Caco-2 cells where the combined toxic effect of OTA and AFM<sub>1</sub> was studied, data modeling allowed us to infer that both toxins exerted interactive effects (Figure 2). The application of the CA conceptual model, which is the most conservative one, to the total data showed that the observed cytotoxic effects were lower than expected, i.e., an antagonistic pattern was identified (Tavares et al., 2013, Pinhão et al., submitted). In addition, the exposure of this cell line to combinations of OTA and PAT revealed a decrease in cell viability comparatively to individual effects of both toxins, pointing to an antagonistic effect, as well (unpublished data). Despite of the predominant antagonistic effects observed in this study in intestinal cells, it is important to retain that even

mycotoxins that mainly act through different mechanisms of action have the potential to interact with each other. This highlights the need for further toxicological studies on combined mycotoxins, rather than on the individual toxic effects, thus contributing to a more realistic scenario.

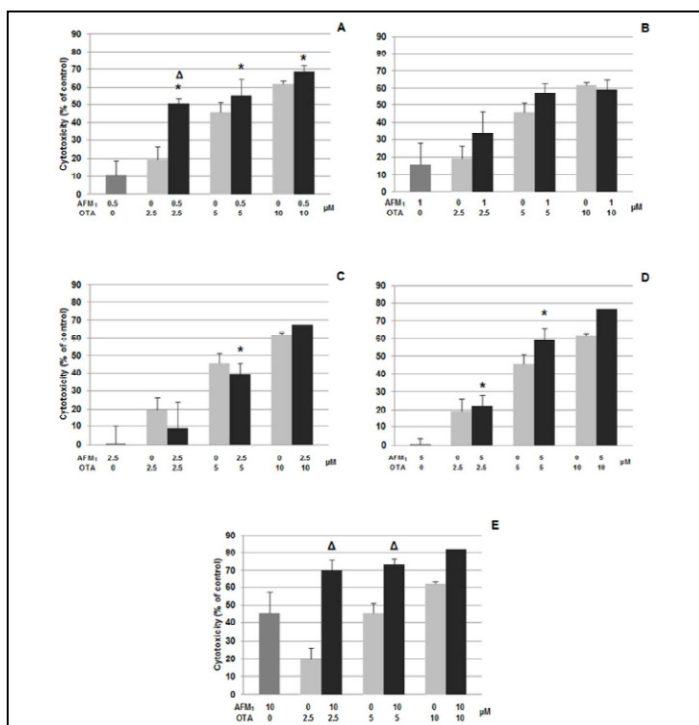


Figure 2 - Caco-2 cells cytotoxicity following exposure to mixtures of (A) 0.5 μM AFM<sub>1</sub>, (B) 1 μM AFM<sub>1</sub>, (C) 2.5 μM AFM<sub>1</sub>, (D) 5 μM AFM<sub>1</sub> and (E) 10 μM AFM<sub>1</sub> with 2.5, 5 and 10 μM OTA. Results (mean ± SE) were expressed as % of cytotoxicity (calculated as the reduction of viability of treated cells relative to the negative control). \* mixture effects significantly different from the single AFM<sub>1</sub> effects (P<0.05); Δ mixture effects significantly different from the single OTA effects (p<0.05) (Tavares et al., 2013).

In HK-2 cells, the cytotoxic effects of the mixtures increased with the concentration of both OTA and FB<sub>1</sub>. Besides, the cytotoxic effects of the mixtures are steadily greater than the effect of both individual toxins. In fact, the combination of OTA and FB<sub>1</sub> consistently caused a significantly higher cytotoxic effect comparatively to the same concentrations of OTA or FB<sub>1</sub> alone; all combinations analyzed caused a significant increase of cell death comparatively to the control values, as well. Data modeling pointed to a synergistic pattern of cytotoxicity in the combinations with lower doses of OTA, shifting to antagonism in the mixtures with higher OTA concentrations, irrespectively of the model applied (Figure 3). In HepG2 cells, the combination of the two mycotoxins caused significantly higher cytotoxic effects than the respective concentration of FB<sub>1</sub> individually in some cases. The comparison of the combined cytotoxicity results with the individual cytotoxicity of OTA, on the other hand, presented mostly lower results. Data modeling through the CA and IA models led to a similar conclusion as achieved in HK-2 cells, i.e., synergism at lower doses of OTA and FB<sub>1</sub>, changing to antagonism at a higher dose level (Tavares et al., 2015; Pinhão et al., submitted). Although both mycotoxins are able to induce the formation of reactive

oxygen species that can induce cell lethality, other modes of action (MoA) may contribute to the observed combined effects. Since human exposure to the studied mycotoxins co-occurs mostly at low doses and during lifetime, the results obtained to date point to a greater health hazard than expected, as these toxins appear to have a synergistic interaction at a low concentration range. This finding is even more relevant for children exposure through food, e.g. cereals-based products and baby food, due to their high vulnerability to toxic effects and should be taken into account in risk assessment (Assunção et al., 2015).

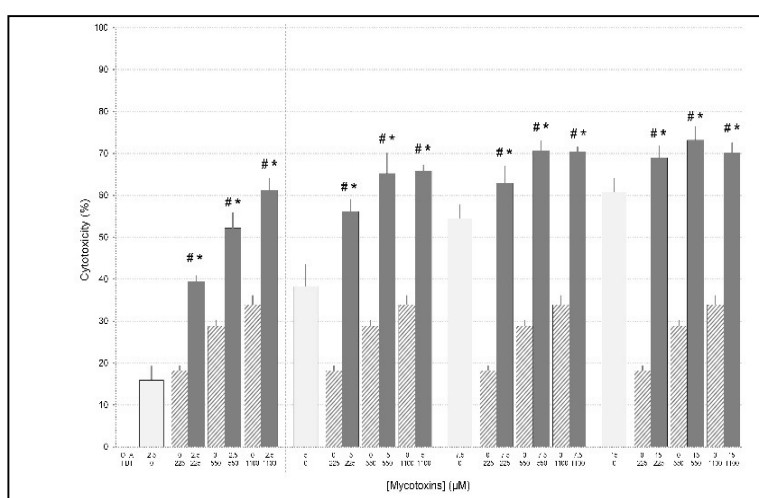


Figure 3 – HK-2 cells cytotoxicity following exposure to mixtures of OTA and FB<sub>1</sub>. Results (mean ± SE) were expressed as % of cytotoxicity (calculated as the reduction of viability of treated cells relative to the negative control). \*# mixture effects significantly different from the single OTA and FB<sub>1</sub> effects, respectively (P<0.05) (Pinhão et al., submitted).

The individual and combined genotoxic effects of OTA, FB<sub>1</sub> and PAT were assessed through quantification of DNA breaks and oxidative DNA damage induced in the several cell lines (comet assay and FPG-modified comet assay, respectively). Data analysis revealed that neither OTA nor FB<sub>1</sub> appeared to cause marked difference in the level of DNA breaks, comparatively to untreated cells. Concerning the genotoxicity results of the combination of the two toxins, no significant change in the amount of DNA damage was detected for the mixtures in comparison to the individual toxins. Lastly, comparing the results of the conventional comet assay and its modified version (i.e. DNA strand breaks vs. oxidative damage), it is possible to observe a modest increase in DNA damage that can be associated to an induction of oxidative DNA damage. As to the Pat genotoxicity, the highest concentrations tested were able to induce a moderate increase in the level of DNA breaks in Caco-2 cells. The results of the combined genotoxicity of OTA and PAT suggested an antagonistic effect, either in the conventional or in the modified comet assays (data under analysis).

The overall results obtained in this task are displayed in Table 1.

Table 1 – Summary of the results obtained for the individual and combined toxic effects of several mycotoxins in three human cell lines

		Caco-2			HepG2			HK-2		
		Cytotoxicity	DNA Damage	Oxidative DNA Damage	Cytotoxicity	DNA Damage	Oxidative Damage	Cytotoxicity	DNA Damage	Oxidative Damage
Individual effects	OTA	+	-	+/-	+	-	+/-	+	-	+/-
	AFM1	+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	FB1	n.d.	n.d.	n.d.	-	-	-	+	-	-
	Pat	+	+/-	+/-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Combined effects	OTA+AFM1	Antagonistic	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	OTA+FB1	n.d.	n.d.	n.d.	Synergistic LD/antagonistic HD	No interaction	No interaction	Synergistic LD/antagonistic HD	No interaction	No interaction
	OTA+Pat	Antagonistic	UA	UA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

AFM<sub>1</sub> – Aflatoxin M<sub>1</sub>, FB<sub>1</sub> – Fumonisin B<sub>1</sub>, OTA – Ochratoxin A, Pat – Patulin; n.d. – not done; UA – under analysis; LD – low dose; HD – high dose.

#### 4. Conclusions

The specific objective of this task was to disclose possible interactive effects between OTA and AFM<sub>1</sub> plus OTA and PAT in intestinal cells and OTA and FB<sub>1</sub> in liver and kidney cells, relatively to their individual cytotoxic and genotoxic potential. With regard to cytotoxicity, it was possible to conclude that binary mixtures of toxins interact differently, depending on the relative concentrations of each toxin. Within this task new evidences on interactive effects of AFM<sub>1</sub> and OTA were disclosed, revealing the predominance of an antagonistic pattern in an intestinal cell line. A similar pattern was found for the combined effect of OTA and PAT in the same cell line. As to the interactions between OTA and FB<sub>1</sub>, in a low concentration-range more realistic in terms of public health, the detected effect was predominantly synergistic, which is of utmost importance because it represents a considerable hazard for human health. In terms of the combined genotoxic effects of the same mixtures, in general no marked interactions were detected, although the combined effect of OTA and PAT should be further explored in a near future. Because the MoA of the studied toxins are not completely understood yet, mechanistic studies should be further performed, in order to clarify the main mechanism underlying the observed interactions.

Considering the potential health hazard related to mixtures of mycotoxins, these experimental studies using *in vitro* cell models together with multi-mycotoxins analysis in food can help preventing mycotoxins-associated adverse health effects. Indeed, especially for vulnerable populations as children, the present study addressed a pertinent and existing concern that impacts in risk assessment of mycotoxins.

## 5. Deviations and comments

All task goals of characterizing the toxicity of binary mixtures of mycotoxins were achieved. The effects were studied in three cell lines whereas only an intestinal cell line had been planned for these studies. Susana Loureiro, scientific consultant, gave support on modeling the interactive effects on binary mycotoxin mixtures and training to the fellows on the application of MIXTOX model. Two master fellows, Ana Tavares and Mariana Pinhão, both collaborated on this task development and one MSc Thesis was presented. Two papers were published, one was submitted and another one is in preparation (see Project indicators below). Several communications were performed in national and international scientific meetings.

## 6. References

Alvito, P.C., Sizoo, E.A., Almeida, C.M.M., van Egmond, H.P., 2010. Occurrence of Aflatoxins and Ochratoxin A in Baby Foods in Portugal. *Food Analytical Methods* **3**:22-30.

Assunção R, Vasco E, Nunes B, Loureiro S, Martins C, Alvito P (2015) Single-compound and cumulative risk assessment of mycotoxins present in breakfast cereals consumed by children from Lisbon region, Portugal. *Food And Chemical Toxicology*. **86**, 274-281.

Cano-Sancho G, González-Arias CA, Ramos AJ, Sanchis V, Fernández-Cruz ML. (2015) Cytotoxicity of the mycotoxins deoxynivalenol and ochratoxin A on Caco-2 cell line in presence of resveratrol. *Toxicology in Vitro*. **29** 1639–1646.

Corcuera LA, Arbillaga L, Vettorazzi A, Azqueta A, López de Cerain A. (2011) Ochratoxin A reduces aflatoxin B1 induced DNA damage detected by the comet assay in Hep G2 cells. *Food and Chemical Toxicology*. **49** 2883–2889.

Heussner AH, Dietrich DR, O'Brien E. (2006) *In vitro* investigation of individual and combined cytotoxic effects of ochratoxin A and other selected mycotoxins on renal cells. *Toxicology in vitro*. **20**:3 332-341.

Loureiro S, Svendsen C, Ferreira A, Pinheiro C, Ribeiro F, Soares A. (2010) Toxicity of Three Binary Mixtures to *Daphnia Magna*: Comparing Chemical Modes of Action and Deviations From Conceptual Models. *Environmental Toxicology and Chemistry*. **29**:8 1716–1726.

Martins C, Assunção R, Cunha S, Jager A, Alvito P. (in press). Assessment of mixtures of mycotoxins in breakfast cereals available in portuguese market, *Food Contaminants and Human Health: Challenges in Chemical Mixtures*, Eds: Paula Alvito, Ricardo Assunção, Henriqueta Louro, Maria João Silva, Elsa Vasco. Publisher: National Institute of Health Doutor Ricardo Jorge, Lisbon, Portugal

Mosmann T. (1983) Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *Journal of immunological Methods*. **65** 55-63.

Pfohl-Leskowicz and Manderville, (2007) Pfohl-Leskowicz A and Manderville RA (2007) Ochratoxin A: an overview on toxicity and carcinogenicity in animals and humans, *Molecular Nutrition and Food Research*, **51**, 61–99

Pinhão M, Loureiro S, Tavares A, Mendonça I, Louro H, Alvito P, Silva MJ. Combined cytotoxic and genotoxic effects of ochratoxin A and fumonisin B1 in human kidney and liver cell models (submitted)

Pinto M, Costa PM, Louro H, Costa MH, Lavinha J, Caeiro S, Silva MJ (2014) Determining oxidative and nonoxidative genotoxic effects driven by estuarine sediment contaminants on a human hepatoma cell line. *Science of the Total Environment*, 478: 25-35.

Repetto, G., del Peso, A., Zurita, J.L., 2008. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nature Protocols* 3:1125-1131.

Tavares AM, Alvito P, Loureiro S, Louro H, Silva MJ. (2013) Multi-mycotoxin determination in baby foods and in vitro combined cytotoxic effects of aflatoxin M1 and ochratoxin A. *World Mycotoxin Journal*. 6:4 375-388.

Tavares AM, Mendonça I, Alvito P, Loureiro S, Louro H, Silva MJ. (in press). Evaluation of combined cytotoxic effects of ochratoxin A and fumonisin B1 in human liver and renal cells. *Food Contaminants and Human Health: Challenges in Chemical Mixtures*, Eds: Paula Alvito, Ricardo Assunção, Henriqueta Louro, Maria João Silva, Elsa Vasco. Publisher: National Institute of Health Doutor Ricardo Jorge, Lisbon, Portugal.

## PROJECT INDICATORS

### Publications

Papers in international journals

Tavares AM, Alvito P, Loureiro S, Louro H, Silva MJ (2013) Multi-mycotoxin determination and in vitro combined cytotoxic effects of aflatoxin M1 and ochratoxin A. *World Mycotoxin Journal*. 6, 4, 375-388 <http://hdl.handle.net/10400.18/1792>

Tavares A, Mendonça I, Louro H, Pinhão M, Loureiro S, Alvito P, Silva M J A contribution to hazard assessment of combined exposure to mycotoxins using *in vitro* toxicity testing. *Toxicology Letters*, 238/2S, S352. <http://hdl.handle.net/10400.18/3182>

Submitted

Pinhão M, Loureiro S, Tavares A, Mendonça I, Louro H, Alvito P, Silva MJ. (submitted)

Combined cytotoxic and genotoxic effects of ochratoxin A and fumonisin B1 in human kidney and liver cell models, *Archives Toxicology*.

Papers in national journals

Tavares AM, Alvito P, Loureiro S, Louro H, Silva MJ (2013). Estudos da toxicidade de misturas de aflatoxina M<sub>1</sub> e ocratoxina A numa linha celular humana. *Boletim Epidemiológico Observações*, 2, 20-21, <http://hdl.handle.net/10400.18/2116>

In preparation

Assunção R, Pinhão M, Silva, MJ, Alvito P. Insights into individual and combined toxic effects of patulin and ochratoxin A in human intestinal cells.

### Communications

Communications in international meetings

Tavares AM, Mendonça I, Alvito P, Loureiro S, Louro H, Silva MJ. (in press). Evaluation of combined cytotoxic effects of ochratoxin A and fumonisin B1 in human liver and renal cells. Food Contaminants and Human Health - Challenges in chemical mixtures. Eds: Alvito, Assunção, Louro H, Silva MJ, Vasco E. Publisher: National Institute of Health Doutor Ricardo Jorge, Lisbon, Portugal <http://hdl.handle.net/10400.18/3214>

Communications in national meetings

Silva MJ. (2014). Genotoxicidade de micotoxinas e potenciais implicações para a saúde. SNPASS, FCG, Lisboa, Portugal. <http://hdl.handle.net/10400.18/2834>.

Tavares A. (2014). Efeito tóxico combinado de micotoxinas: ocratoxina A e aflatoxina M<sub>1</sub>, SNPASS, FCG, Lisboa, Portugal. <http://hdl.handle.net/10400.18/2680>.

### **Advanced training**

Master Thesis

Inês Mendonça (2014). Avaliação dos Efeitos Citotóxicos e Genotóxicos de Misturas de Micotoxinas. Dissertação de Mestrado em Biologia Humana e Ambiente, Faculdade de Ciências, Universidade de Lisboa, 16 Dezembro <http://hdl.handle.net/10451/15817>

## **Task 3 - Bioaccessibility and absorption of mycotoxin mixtures**

### **Background and aims**

In human health risk assessment, ingestion of food is considered a major route of exposure to many contaminants including mycotoxins, although the total amount of an ingested contaminant (external dose) does not always reflect the amount that is available to the body (internal dose). The amount of mycotoxin resisting to the digestion process and potentially absorbable by the systemic circulation is only a smaller part of that ingested. Considering this, task 3 aims to study the i) bioaccessibility and ii) intestinal absorption of single and combined mycotoxins, namely patulin (PAT), ochratoxin A (OTA) and aflatoxin M<sub>1</sub> (AFM<sub>1</sub>), mycotoxins found, individually and in combination, in the analyzed food samples: fruit juices (PAT), processed cereal based baby foods (PAT and OTA) and infant formula (AFM<sub>1</sub>), results from Task 1.

### **Material and methods**

#### *1. Bioaccessibility studies*

Two *in vitro* digestion (IVD) methods were used in order to study the bioaccessibility of mycotoxins in different foods intended for children consumption. Initially, a method developed by Versantvoort et al. (2005) was applied in order to gather data on patulin bioaccessibility present in fruit juices. Briefly, this model consists of a short incubation (5 minutes) of the sample with saliva, addition of gastric juice followed by a two hour incubation and addition of duodenal and bile juice followed by a two hour incubation. The experiments were performed in the presence or absence of a standard meal (commercial baby food “meat with spaghetti”) to simulate the matrix effect (Assunção et al., 2014; Ferreira, 2014). Patulin (PAT) bioaccessibility in fruit juices was assessed using this model. Seven commercial fruit juices of different brands containing apple or a mixture of apple with other fruit juices were previously checked not to be contaminated with PAT by HPLC with UV detection and then artificially contaminated at 200 µg/kg for the bioaccessibility assays, a concentration that allowed obtaining quantifiable patulin values after digestion dilution and under the working range applied.

In a second phase, under advice of Didier Dupont (scientific consultant, from INRA, France) a standardized static *in vitro* digestion (IVD) method was used to assess the

bioaccessibility of PAT, OTA and AFM<sub>1</sub> in two different food matrixes: processed cereal based baby foods (for PAT and OTA) and infant formula (for AFM<sub>1</sub>). The used standardized IVD method was developed under INFOGEST (a COST action that aimed to improve the scientific knowledge on how foods are disintegrated during digestion), and resulted from an international consensus concerning several aspects as fluids composition, enzymatic activities and sample and fluids amount. Briefly, standardized IVD method consists of three sequential stages: oral (oral fluid containing amylase - 75 U/mL, pH 7), gastric (gastric fluid containing pepsin - 2000 U/mL, pH 3) and intestinal phase (intestinal fluid containing a pancreatin-bile mixture - 100 U/mL and 0.6 mM, respectively, pH 7), with incubation times ranging from two minutes (oral phase) to two hours (gastric and intestinal phases) (Minekus et al., 2014).

PAT and OTA bioaccessibility was determined individually and in combination. For this, six different processed cereal based baby food samples (3 with fruit and 3 without fruit in their composition) were used. These samples were previously checked not to be contaminated with these mycotoxins and artificially contaminated to 1 µg/kg of OTA and 20 µg/kg of PAT. Mycotoxins quantification was performed according to methods described by Assunção and collaborators (Assunção et al, 2015a), namely by HPLC-FLD for OTA and HPLC-UV for PAT. The interference of fruit ingredients in the bioaccessibility of these mycotoxins was also evaluated (Assunção et al, in preparation).

AFM<sub>1</sub> bioaccessibility was determined using three infant formula samples selected from the same brand, intended for three different ages (1, 2 and 3 years). Each sample was previously checked not to be contaminated with AFM<sub>1</sub> and was contaminated with 0.5 µg/kg of AFM<sub>1</sub>. AFM<sub>1</sub> quantification was performed according to method described in Assunção et al (2015a), namely by HPLC-FLD. The interference of different protein content of the infant formula was also evaluated (Tavares et al, 2015).

## *II. Intestinal absorption studies*

Intestinal mucosa is the first biological barrier encountered by natural toxins and it could be exposed to high amounts of dietary mycotoxins. Under task 3, and using cell cultures as model, the effects of mycotoxins on barrier properties and function of the gut mucosa were evaluated to infer the potential amount of mycotoxins that reach the systemic circulation. PAT is one of the best known enteropathogenic mycotoxins able to alter functions of the intestine and it was used to evaluate the effects on barrier

properties and function of intestine. To achieve these objectives, viability (MTT assay), proliferation ( $^3\text{H}$ -thymidine incorporation assay), transepithelial electrical resistance (TER), SDS-PAGE and immunoblotting and flow cytometry methodologies were applied in order to characterize the effects of PAT on the intestinal cell model (Caco-2), human peripheral blood lymphocytes (PBMC) and human blood monocyte-derived dendritic cells (moDC) (Assunção et al, submitted).

## Results

### *I. Bioaccessibility results*

Table 3 summarizes the PAT bioaccessibility results obtained under Versantvoort et al. (2005) method. Bioaccessibility values in the absence of a standard meal ranged between 16 % and 48 % with a mean value of  $28 \pm 13.5$  %. Bioaccessibility values in the presence of a standard meal ranged between 5 % and 13 % with a mean value of  $8 \pm 4.0$  %. Bioaccessibility in the absence of a standard meal presented a significantly higher value than in the presence of a standard meal ( $p = 0.001$ ). The mean bioaccessibility value for clear fruit juices (25 %) was not significantly lower ( $p = 0.088$ ) than the one for cloudy juices (32 %), in the absence of a standard meal. On the contrary, the mean bioaccessibility value for clear fruit juices (10 %) was not significantly higher ( $p = 0.093$ ) than the one for cloudy juices (5 %), in the presence of a standard meal (Assunção et al, 2014).

**Table 3.** Bioaccessibility (%) results of PAT in seven selected fruit juices (clear and cloudy), in the absence or presence of a standard meal, artificially contaminated. Bioaccessibility values are expressed as mean  $\pm$  SD of two replicates.

Fruit Juice	Nature of juice	Bioaccessibility (%)	
		Absence standard meal	Presence standard meal
<b>Apple</b>	Clear juices	$16 \pm 1.7$	$10 \pm 3.8$
<b>Apple and other 3 fruits</b>		$19 \pm 1.0$	$13 \pm 0.4$
<b>Apple and other 8 fruits</b>		$16 \pm 0.5$	$8 \pm 9.5$
<b>Apple and other 8 fruits</b>		$48 \pm 0.1$	$8 \pm 2.0$
<b>Mean of clear juices</b>		$25 \pm 14.7$	$10 \pm 4.5$
<b>Apple</b>	Cloudy juices	$27 \pm 0.6$	$5 \pm 0.2$
<b>Apple and carrot</b>		$21 \pm 1.8$	$5 \pm 0.5$
<b>Apple and other 5 fruits</b>		$46 \pm 2.6$	$7 \pm 2.2$
<b>Mean of cloudy juices</b>		$32 \pm 11.8$	$5 \pm 1.4$

<b>Total</b>	28 ± 13.5	8 ± 4.0
--------------	-----------	---------

Table 4 shows PAT and OTA bioaccessibility results, in single form or in mixture. PAT presented bioaccessibility values ranging from 30 % to 77 % in single form and in mixture the bioaccessibility values ranged from 30 % to 69 %. Bioaccessibility values for OTA ranged between 95 % and 105 % in single form and were above 100 % in all assays in mixture with PAT (Assunção et al., 2015b; Assunção et al., in preparation).

**Table 4.** Bioaccessibility (%) results of PAT and OTA in cereal and fruit based baby 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, respectively.

	<b>Patulin</b>		<b>Ochratoxin A</b>	
	<b>Single</b>	<b>Mixture</b>	<b>Single</b>	<b>Mixture</b>
<b>F1</b>	70 ± 3.2	54 ± 0.9	95 ± 0.3	104 ± 1.2
<b>F2</b>	42 ± 1.2	69 ± 4.3	105 ± 1.5	103 ± 1.2
<b>F3</b>	56 ± 1.8	47 ± 4.2	97 ± 1.8	107 ± 0.7
<b>W/o 1</b>	77 ± 1.9	64 ± 1.5	98 ± 1.5	109 ± 0.1
<b>W/o 2</b>	39 ± 0.7	30 ± 1.9	102 ± 0.3	107 ± 1.5
<b>W/o 3</b>	30 ± 2.5	61 ± 0.6	102 ± 3.9	108 ± 0.6

Statistically significant differences were verified when compared single and mixture OTA bioaccessibility results (Wilcoxon Signed Rank test,  $p=0.001$ ). No significant differences were found when comparing single and mixture PAT bioaccessibility results (Wilcoxon Signed Rank test,  $p=0.777$ ).

Statistically significant differences were found when compared OTA in mixture, considering samples with and without fruit (Mann-Whitney test with  $p=0.02$ ). For the remaining test scenarios no significant differences were found [PAT, single ( $p=0.222$ ) and mixture ( $p=0.863$ ), and OTA, single ( $p=0.489$ )].

For the question if the number of fruits affected the mycotoxins bioaccessibility, comparison were made between samples without fruits and samples with 1, 2 and 5 fruits. Statistically significant differences were determined for 1 and 2 fruits, comparing to 0 fruits, for OTA in mixture (Mann-Whitney test with  $p=0.018$  and  $p=0.009$ , respectively). For the remaining no significant differences were found.

**Table 5.** Bioaccessibility (%) results of AFM<sub>1</sub> in infant formula samples, artificially contaminated.

Samples	Bioaccessibility (%)
	Mean ± Standard deviation
Infant formula age 1	104 ± 5.1
Infant formula age 2	86 ± 3.5
Infant formula age 3	92 ± 4.4

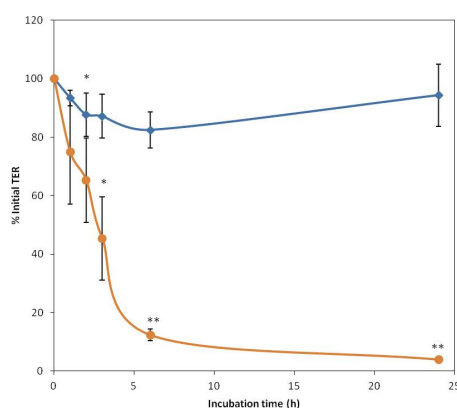
Table 5 shows AFM<sub>1</sub> bioaccessibility results in single form. AFM<sub>1</sub> presented bioaccessibility values ranging from 86 % to 104.

Infant formula age 1 showed bioaccessibility values significantly higher than those observed for age 2 ( $p= 0.002$ , Mann-Whitney) and age 3 ( $p= 0.024$ , Tukey HSD) and corresponds to the assayed sample containing the lowest casein percentage analysed.

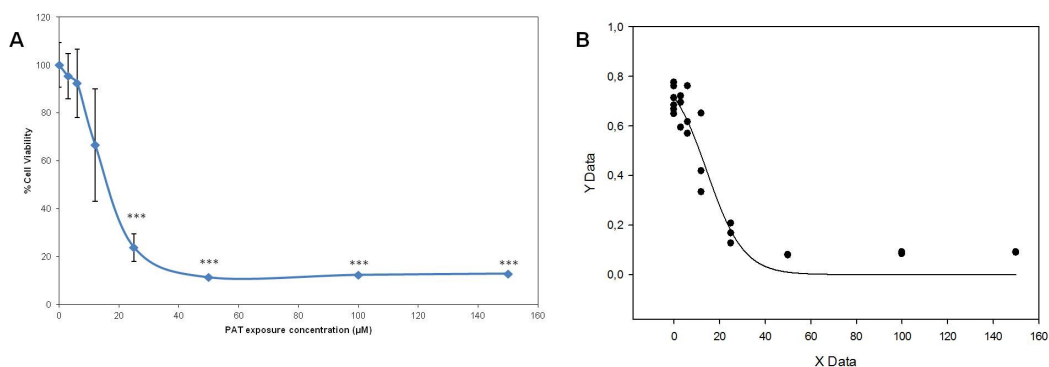
The results suggest a high bioaccessibility for AFM<sub>1</sub> in artificially contaminated infant formulae, more pronounced for lower casein content samples (Tavares et al., 2015).

## II. Intestinal absorption results

PAT exposure reduced Caco-2 cell viability at concentrations above 12  $\mu$ M. The integrity of the Caco-2 monolayer was affected by PAT exposure, as demonstrated by a decrease in TER values, becoming more pronounced at 50  $\mu$ M (Figure 3 and Figure 4) (Assunção et al., submitted)

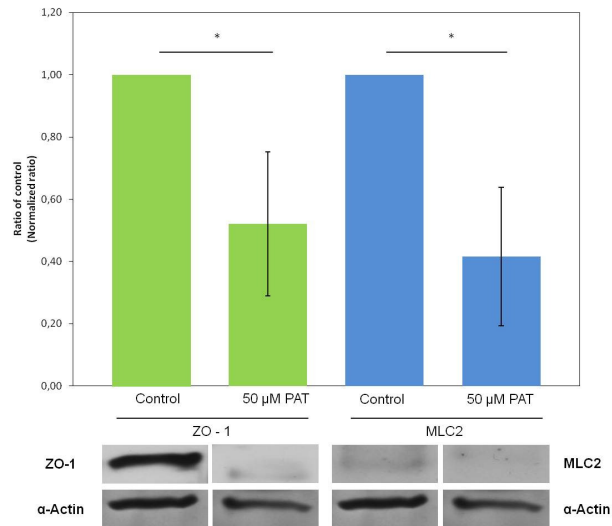


**Figure 3.** Effect of PAT treatment on Caco-2 cells trans-epithelial electrical resistance (TER) values at different incubation times. Cells were exposed to 50  $\mu\text{M}$  of PAT (—●—) in apical side during 24 hours. Negative control (—●—) received no patulin. Values are the mean  $\pm$  SD (n=4).  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) represent significant difference as compared to negative control. % Initial TER was calculated considering the initial TER values measured at 0 h of incubation and the respective incubation time.

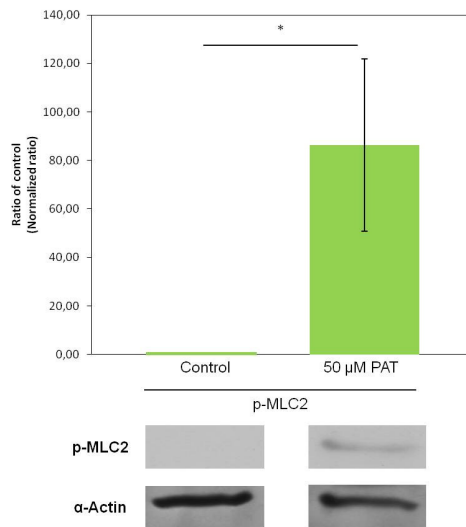


**Figure 4.** Effect of PAT treatment on Caco-2 cells viability after 24 hours of exposure to different PAT concentrations (1 to 150  $\mu\text{M}$ ). (A) Graphical representation of cell viability percentage for each concentration tested. Values are expressed as mean  $\pm$  SD (n=6).  $p < 0.001$  (\*\*\*) represent significant difference as compared to control values (negative control). (B) Dose-response curve best fitted to results obtained in MTT assay determined by regression analysis using SigmaPlot™ software.

No effects were detected on the expression levels of the tight junction proteins occludin, claudin-1 and claudin-3 at 50  $\mu\text{M}$ . However, the expression of zonula occludens-1 (ZO-1) and myosin light chain (MLC) declined and levels of phospho-MLC increased, after 24 h of exposure to 50  $\mu\text{M}$  of PAT (Figure 5) (Assunção et al., submitted).

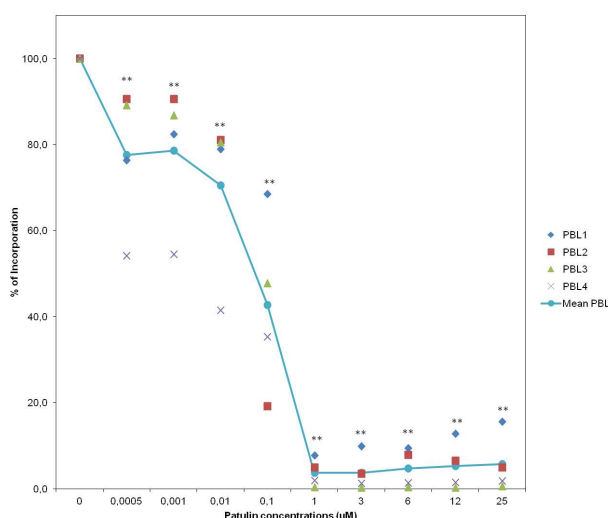


**Figure 5.** Immunoblot analysis showing the expression of ZO-1 (220 kDa) and MLC2 (18 kDa) in Caco-2 cells exposed to 50 μM of PAT for 24 hours. Data of densitometry and typical images for control (negative control) and 50 μM PAT treatment concentration are shown. Densitometry data were obtained using 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 4.** Immunoblot analysis to determine 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.

T cell (PBMC) proliferation was highly sensitive to PAT with the major effects for concentrations above 10 nM of PAT (Figure 6). The same conditions did not affect the maturation of moDC (Assunção et al., submitted).



**Figure 6.** Effect of PAT in the proliferation of PBMC cells using the incorporation of [<sup>3</sup>H]-Thymidine 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 [<sup>3</sup>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.

## Deviations

After performance of some assays with the proposed *in vitro* digestion model, a new harmonized *in vitro* model was developed within the INFOGEST Cost Action, to which some team members belong. Didier Dupont, the leader of this Cost Action and also scientific consultant of this project, had suggested to move to this new method since it is much more specific and accurate due to the possibility of enzymatic control activities, which doesn't happen in other published methods besides the fact that is the unique standardized. The team decided to follow the consultant suggestion and a first training was performed by the fellow Ana Tavares in Agroscope, Liebefeld, Switzerland, funded by FCT and INFOGEST. This training was crucial for the project progress although it had led to some delay in the planning schedule, one of the main reasons for the project prorogation. The replacement of this fellow by Mariana Pinhão (task 2 and 3) was due to the attribution of a PhD fellowship. Ricardo Assunção, a PhD student and project fellow in collaboration with Carla Martins had increased their % participation in the project in order to attain all task goals. The studies already published, submitted and in preparation by Ricardo, will be included in his PhD Thesis.

The intestinal absorption studies performed under this task used different methodologies than those previously planned. Following an INFOGEST training performed by Ricardo before its inlet in the project team on the Norwegian University of Life Sciences, new methodologies were acquired in order to evaluate the effects of mycotoxins on barrier properties and function of the gut mucosa. Thus, viability (MTT assay), proliferation (<sup>3</sup>H-thymidine incorporation assay), transepithelial electrical resistance (TER), SDS-PAGE and immunoblotting and flow cytometry methodologies were applied in order to characterize the effects of PAT on the intestinal cell model (Caco-2), human peripheral blood lymphocytes (PBMC) and human blood monocyte-derived dendritic cells (moDC). PAT is one of the best known enteropathogenic mycotoxins able to alter functions of the intestine and has a particular target the children group due to their frequent consume of apple based products (food group most often contaminated with this mycotoxin). Few studies are available on the specific mechanism of action of this toxin and the possibility to perform this study under the collaboration with one recognized international laboratory with expertise on this area was unique. Therefore a different approach to intestinal absorption studies was performed to infer the potential amount of mycotoxins that reach the systemic circulation.

### Conclusions

Results obtained within Task 3 showed that a significant portion of PAT, OTA and AFM<sub>1</sub> can reach the small intestine and thus, be available to cross the intestinal barrier and produce their toxic effects. The results demonstrated also that simultaneous presence of mycotoxins affects the bioaccessibility values. However, further and future studies with different mixtures of mycotoxins are needed to corroborate it. The presence/absence of fruit and the number of fruits in cereal and fruit based baby foods indicated a potential influence in mycotoxins bioaccessibility. Protein content of infant formula suggest also an influence in mycotoxin bioaccessibility.

In another way, results showed that mycotoxins, namely PAT, affect the intestinal mucosa through reduction of barrier function, mainly by perturbation of ZO-1 levels and phosphorylation of MLC and inhibition of T cell proliferation and this affect the potential amount of mycotoxins that reach the systemic circulation.

Task 3 results provide new information related with the bioaccessibility of mycotoxins in baby foods and offer a more comprehensive picture of what occurs during the digestion

of food contaminants in the gastrointestinal tract. These results provide also 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. All these data contributes to provide a more accurate risk assessment of single and multiple food contaminants.

All task goals were achieved and the deviations had promoted the use and implementation at INSA of the unique harmonized *in vitro* digestion method as well as the acquisition of knowledge concerning a new set of methodologies to evaluate the effects of mycotoxins on barrier properties and function of the gut mucosa. Two papers were already published, two were recently submitted and other is in preparation, 9 presentations in meetings, a PhD thesis in preparation including the project papers that is expected to be presented within the first six months, a MSc thesis and 6 videos produced by team members to disseminate INFOGEST new harmonized method corresponds to an important output for this project progress.

## References

Assunção, R., Alvito, P., Kleiveland, C. R. and Lea, T. E. (Submitted). Characterization of *in vitro* effects of patulin on intestinal epithelial and immune cells. *Toxicology in vitro*

Assunção, R., Martins, C., Dupont, D. and Alvito, P. (in preparation). Assessment of patulin and ochratoxin A bioaccessibility in cereal and fruit based baby foods using a harmonized *in vitro* digestion model.

Assunção, R., Vasco, E., Nunes, B., Loureiro, S., Martins, C. and Alvito, P. (2015a). Single-compound and cumulative risk assessment of mycotoxins present in breakfast cereals consumed by children from Lisbon region, Portugal. *Food and Chemical Toxicology*. 86, 274-28. doi: 10.1016/j.fct.2015.10.017

Assunção, R., Martins, C., Leclerc Duarte, E. and Alvito, P. (2015b). Bioaccessibility assessment of patulin and ochratoxin A in cereal and fruit based baby foods using a harmonized *in vitro* digestion model: contribution for the risk assessment of chemical mixtures, *Toxicology Letters*, 238/2S, S116. doi:10.1016/j.toxlet.2015.08.375

Tavares, A.M., Egger, C., Portmann, R., Martins, C., Assunção, R. and Alvito P. (2015). Bioaccessibility of aflatoxin M<sub>1</sub> in three artificially contaminated infant formula using a standardised static *in vitro* digestion method. Proc. 4<sup>th</sup> ICFD, Naples, Italy, 118 <http://hdl.handle.net/10400.18/3041>

Assunção, R., Ferreira, M., Martins, C., Diaz, I., Padilla, B., Dupont, D., Bragança, M. & Alvito, P. (2014). Applicability of *in vitro* methods to study patulin bioaccessibility and its effects on intestinal membrane integrity. *Journal of Toxicology and Environmental Health, Part A*, 77: 983–992.

Ferreira, M. (2014). Simulação *in vitro* do processo digestivo de patulina em sumos de fruta. Dissertação de Mestrado em Biologia Humana e Ambiente, Faculdade de Ciências, Universidade de Lisboa, 18 Dezembro <http://hdl.handle.net/10451/15578>

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T. et al. (2014). A Standardised Static *in Vitro* Digestion Method Suitable for Food - an International Consensus, *Food & Function*, 5 (2014), 1113-1124.

Versantvoort, C. H. M., Oomen, A. G., Van de Kamp, E., Rompelberg, C. J. M. and Sips, A. J. A. M. (2005). Applicability of an *in vitro* digestion model in assessing the bioaccessibility of mycotoxins from food. *Food and Chemical Toxicology*, 43: 31-40.

## **Project Indicators**

### Publications

In international papers

Assunção, R., Ferreira, M., Martins, C., Diaz, I., Padilla, B., Dupont, D., Bragança, M. and Alvito, P. (2014). Applicability of *in vitro* methods to study patulin bioaccessibility and its effects on intestinal membrane integrity. *Journal of Toxicology and Environmental Health, Part A*, 77: 983–992. [doi:10.1080/15287394.2014.911138](https://doi.org/10.1080/15287394.2014.911138)

Assunção, R., Martins, C., Leclerc Duarte, E. and Alvito, P. (2015). Bioaccessibility assessment of patulin and ochratoxin A in cereal and fruit based baby foods using a harmonized *in vitro* digestion model: contribution for the risk assessment of chemical mixtures, *Toxicology Letters*, 238/2S, S116. [doi:10.1016/j.toxlet.2015.08.375](https://doi.org/10.1016/j.toxlet.2015.08.375)

Assunção, R., Alvito, P., Kleiveland, C. R. and Lea, T. E. (Submitted). Characterization of *in vitro* effects of patulin on intestinal epithelial and immune cells. *Toxicology in vitro*

Egger, L., Ménard, O., Delgado-Andrade, C., Alvito, P., Assunção, R., et al (submitted). The harmonized INFOGEST *in vitro* digestion method: From knowledge to action. *Food Research International*.

In preparation

Assunção, R., Martins, C., Dupont, D. and Alvito, P. (in preparation). Assessment of patulin and ochratoxin A bioaccessibility in cereal and fruit based baby foods using a harmonized *in vitro* digestion model.

## Communications

### International meetings

Alvito, P., Vasco, E., Martins, C., Dupont, D. (2013). Applicability of an *in vitro* model to assess the bioaccessibility of food contaminants. 2nd International Conf on Occupational and Environmental Toxicology, 16-17 Setembro, Porto, Portugal, p.156. <http://hdl.handle.net/10400.18/1947>

Tavares, A., Egger, C., Portmann, R. and Alvito, P. (2014). The effects of matrix proteins on the aflatoxin M<sub>1</sub> bioaccessibility and the Caco-2 intestinal transport, Proceedings of the 3rd International Conference on Food Digestion, COST Action Infogest FA 1005, 11-13 March, p.141, <http://hdl.handle.net/10400.18/2282>

Assunção, R., Ferreira, M., Martins, C., Dupont, D. and Alvito, P. (2014) Mycotoxins and applicability of *in vitro* methods of digestion and absorption assessment - the patulin case study. Proceedings of the 3rd International Conference on Food Digestion, COST Action Infogest FA 1005, 11-13 March, p. 83, <http://hdl.handle.net/10400.18/2281>.

Assunção, R., Martins, C., Tavares, A. and Alvito, P. (2015). First bioaccessibility assessment of patulin in cereal and fruit based baby foods using the harmonized IVD model. Proc. 4<sup>th</sup> ICFD, Naples, Italy, 117 <http://hdl.handle.net/10400.18/3040>

Tavares, A.M., Egger, C., Portmann, R., Martins, C., Assunção, R. and Alvito P. (2015). Bioaccessibility of aflatoxin M<sub>1</sub> in three artificially contaminated infant formula using a standardised static *in vitro* digestion method. Proc. 4<sup>th</sup> ICFD, Naples, Italy, 118 <http://hdl.handle.net/10400.18/3041>

Assunção, R., Kleiveland, C. R., Lea, T. L. and Alvito, P. (2015). Interactions between dietary toxins and gut mucosa: patulin effects on the structure and function of intestinal and immune cells. Proc. 4<sup>th</sup> ICFD, Naples, Italy, 115 <http://hdl.handle.net/10400.18/3042>

Martins C, Tavares A, Assunção R, Alvito P (in press). Bioaccessibility of mycotoxins in baby foods using the harmonized *in vitro* digestion model. Food Contaminants and

Human Health - Challenges in chemical mixtures. Eds: Alvito, Assunção, Louro H, Silva MJ, Vasco E. Publisher: National Institute of Health Doutor Ricardo Jorge, Lisbon, Portugal <http://hdl.handle.net/10400.18/3214>

Assunção R, Kleiveland CR, Lea TL, Alvito P (in press). Insights of enteropathogenic effects of mycotoxins on the human intestinal gut mucosa. Food Contaminants and Human Health - Challenges in chemical mixtures. Eds: Alvito, Assunção, Louro H, Silva MJ, Vasco E. Publisher: National Institute of Health Doutor Ricardo Jorge, Lisbon, Portugal <http://hdl.handle.net/10400.18/3214> (BEST POSTER AWARD)

#### National meetings

Assunção R, Martins C, Tavares A, & Alvito P (2014). Método harmonizado de digestão *in vitro*: uma ferramenta para o estudo da bioacessibilidade de contaminantes alimentares. Comunicação oral apresentada no 1º Simpósio Nacional Promoção de uma Alimentação Saudável e Segura - Alimentação infantil e contaminantes químicos, Fundação Calouste Gulbenkian, Lisboa, Portugal, 26 novembro. <http://hdl.handle.net/10400.18/2681>.

#### Advanced training

##### *PhD Thesis*

Assunção, R. (*in preparation*). Infant food and mycotoxins: insights for risk assessment. Exposure assessment and toxic effects of single and multiple mycotoxins. PhD Thesis. Universidade de Évora, Portugal.

##### *Master thesis*

Mariana Ferreira (2014). Simulação *in vitro* do processo digestivo de patulina em sumos de fruta. Dissertação de Mestrado em Biologia Humana e Ambiente, Faculdade de Ciências, Universidade de Lisboa, 18 Dezembro <http://hdl.handle.net/10451/15578>

Other

*Audiovisual material*

Chymotrypsin Assay for *In Vitro* Food Digestion

<http://hdl.handle.net/10400.18/3052>

Pancreatic Lipase Activity Assay for *In Vitro* Food Digestion

<http://hdl.handle.net/10400.18/3053>

Static *In Vitro* Digestion Method for Food

<http://hdl.handle.net/10400.18/3054>

Pepsin Activity Assay for *In Vitro* Food Digestion

<http://hdl.handle.net/10400.18/3055>

Alpha Amylase Activity Assay for *In Vitro* Food Digestion

<http://hdl.handle.net/10400.18/3056>

Trypsin Assay for *In Vitro* Food Digestion

<http://hdl.handle.net/10400.18/3059>

## **Task 4 - Children exposure assessment**

---

### Aims

The main aim of this task “Children exposure assessment” was to perform the exposure assessment of children to multiple mycotoxins through baby foods and infant cereals consumption. To perform this study occurrence (see task 1), toxicological (see tasks 2 and 3) and consumption (task 4) data were used. The software “Open Portugal” was adapted to Portuguese language, food composition table and recipes in collaboration with Barbara Seljak (BS), Jožef Stefan Institute, Eslovénia, project consultant, and was used as a dietary recording tool.

### Materials and methods

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 2 and 36 months (generally referred as 0 and 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 (paper and in OPEN Portugal - [http://pt.opkp.si/pt\\_PT/cms/vstopna-stran](http://pt.opkp.si/pt_PT/cms/vstopna-stran); Vasco, 2014). 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 (INSA, 2014; Leal, 2014; Leal et al., 2015).

Two mathematical approaches, point evaluation (deterministic approach using Microsoft Excel 2007) and Monte Carlo simulation (probabilistic approach using software @Risk® for Microsoft Excel version 6) were used for the computation of the exposure assessment of mycotoxins (Assunção et al., 2014). 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) 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.

For the risk characterization, the outputs of exposure, namely the daily intake values, were compared with the reference dose values (PMTDI or PTWI and MoE).

## Results

### *Population study*

The children population was composed of approximately an identical number of boys and girls, and gathered in 3-11 months and 12-36 months groups according to Health Care Unit facilities. Thirty three percent of analyzed population presented overweight or obesity and 24% belonged to the population group with ages ranging from 12-36 months. Two percent of the analyzed boys presented cases of thickness. It was the first study performed at this Health Care Unit on children nutritional status and dietary habits and the results pointed out the importance to study these issues in order to avoid future children health risks (Leal et al, 2015). See developed food diaries for this task in 8.

### *Exposure assessment to Breakfast cereals foods (Assunção et al, 2015a)*

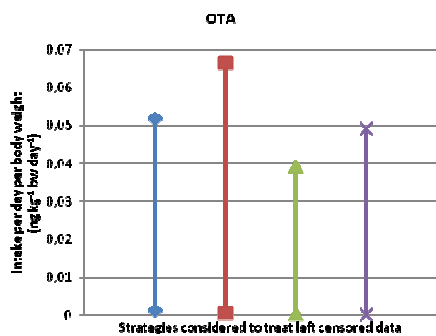
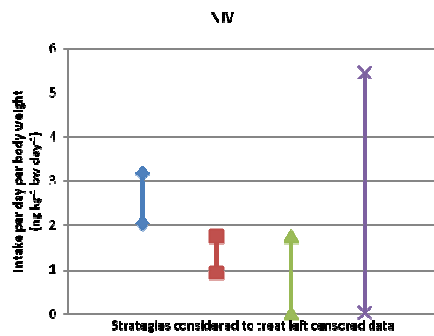
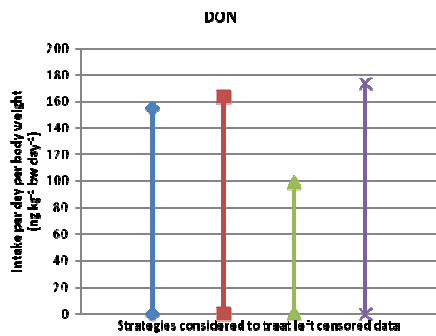
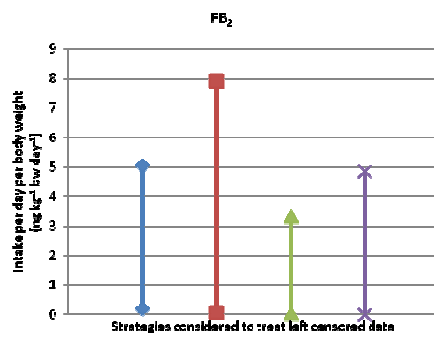
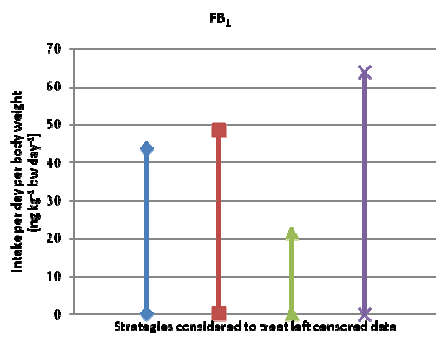
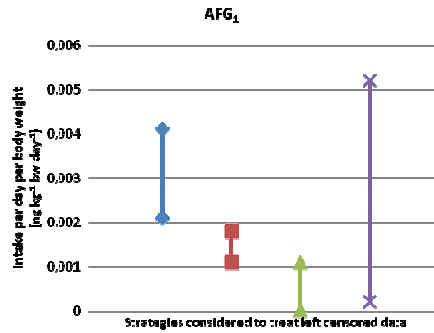
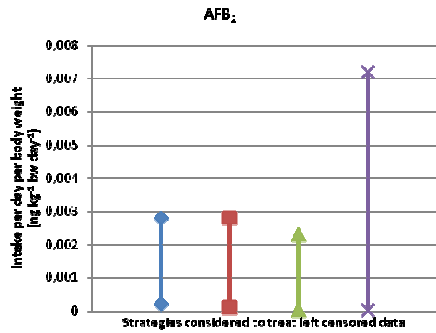
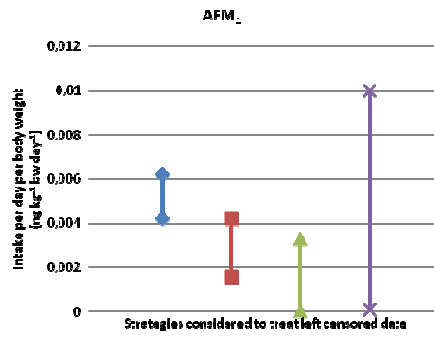
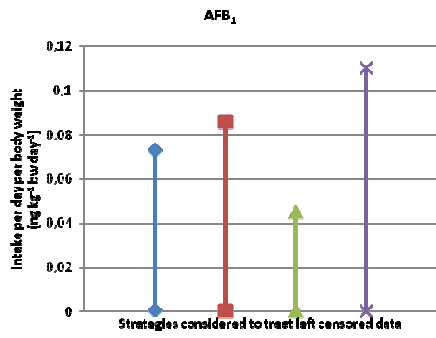
Forty percent of the studied children (1-3 years old) consumed breakfast cereals at least one time in three days as reported in food diary presenting a mean weight of 13.39 kg and a mean consumption of breakfast cereals of 5.62 g day<sup>-1</sup>. Breakfast cereals consumed by the studied children showed 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 1.

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

H1: $< \text{LOD} = \text{LOD}$		H2: $< \text{LOD} = 1/2 \text{ LOD}$		H3: $< \text{LOD} = 0$	
Mycotoxin content ( $\text{ng kg}^{-1}$ )	Daily intake ( $\text{ng kg bw}^{-1} \text{day}^{-1}$ )	Mycotoxin content ( $\text{ng kg}^{-1}$ )	Daily intake ( $\text{ng kg bw}^{-1} \text{day}^{-1}$ )	Mycotoxin content ( $\text{ng kg}^{-1}$ )	Daily intake ( $\text{ng kg bw}^{-1} \text{day}^{-1}$ )
12	0.005	7	0.003	2	0.001
28	0.012	27	0.011	27	0.011
2	0.001	2	0.001	1	0.001
6	0.003	4	0.001	1	0.0003
26	0.011	25	0.010	23	0.010
Mycotoxin content ( $\mu\text{g kg}^{-1}$ )	Daily intake ( $\text{ng kg bw}^{-1} \text{day}^{-1}$ )	Mycotoxin content ( $\mu\text{g kg}^{-1}$ )	Daily intake ( $\text{ng kg bw}^{-1} \text{day}^{-1}$ )	Mycotoxin content ( $\mu\text{g kg}^{-1}$ )	Daily intake ( $\text{ng kg bw}^{-1} \text{day}^{-1}$ )
13	5.461	13	5.390	13	5.319
3	1.033	2	0.929	2	0.826
59	24.827	59	24.797	59	24.767
6	2.681	4	1.559	1	0.438

Figure 1 showed the graphic representations for the probabilistic estimates of the intakes for individual toxins. These results 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 Table 2.



- ◆ H1: <LOD - LOD
- H2: <LOD = 1/2 LOD
- ▲ H3: <LOD = 0
- ✕ H4: <LOD = uniform distribution with min=0; max=LOD

**Figure 7.** Results of probabilistic approach to estimate children exposure to aflatoxins (AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFM<sub>1</sub>), fumonisins (FB<sub>1</sub> and FB<sub>2</sub>), trichothecenes (DON and NIV) and ochratoxin A, through ingestion of breakfast cereals (ng kg bw<sup>-1</sup> day<sup>-1</sup>), corresponding to 2.5 and 97.5 percentiles of intake.

**Table 2.** 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 <sub>1</sub>	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 <sub>1</sub>	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 <sub>2</sub>	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 <sub>1</sub>	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 <sub>1</sub>	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	+∞
	H1	RiskPearson5(1.8338;2.1344)	0.00	2.5598	+∞
FB <sub>2</sub>	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	+∞
	H1	RiskGamma(0.37205;159.05)	0.00	59.1746	+∞
DON	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	+∞
	H1	RiskLoglogistic(0;5.5872;16.403)	0.00	5.6215	+∞
NIV	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	+∞

Table 3 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 4 presents the MoE calculated for aflatoxins. Aflatoxins M<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> revealed a MoE above 10,000, which represent low risk for breakfast cereals children consumers. AFB<sub>1</sub>, which is considered the most potent aflatoxin, revealed a MoE below 10,000 for the higher percentiles of intake (P90, P95 and P99) suggesting a potential health concern.

**Table 3.** Risk characterization using HQ and HI derived from the estimates of ochratoxin A, fumonisins (FB<sub>1</sub>, FB<sub>2</sub>) 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 w ith min=0; max=LOD					
OTA <sup>c</sup>	FB <sub>1</sub>	FB <sub>2</sub> HQ <sup>a</sup>	DON	NIV	OTA <sup>c</sup>	FB <sub>1</sub>	FB <sub>2</sub> HQ <sup>a</sup>	DON	NIV	OTA <sup>c</sup>	FB <sub>1</sub>	FB <sub>2</sub> HQ <sup>a</sup>	DON	NIV	OTA <sup>c</sup>	FB <sub>1</sub>	FB <sub>2</sub> HQ <sup>a</sup>	DON	NIV	
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 <sup>b</sup> (FB <sub>1</sub> , FB <sub>2</sub> )		HI <sup>b</sup> (DON, NIV)			HI <sup>b</sup> (FB <sub>1</sub> , FB <sub>2</sub> )		HI <sup>b</sup> (DON, NIV)			HI <sup>b</sup> (FB <sub>1</sub> , FB <sub>2</sub> )		HI <sup>b</sup> (DON, NIV)			HI <sup>b</sup> (FB <sub>1</sub> , FB <sub>2</sub> )		HI <sup>b</sup> (DON, NIV)		
P5	0.00022		0.0018			0.00010		0.00083			0.00017		0.0014			0.00035		0.00065		
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<sup>-1</sup>; FB<sub>1</sub>, 0.8 µg kg<sup>-1</sup>; FB<sub>2</sub>, 0.8 µg kg<sup>-1</sup>; DON, 0.4 µg kg<sup>-1</sup>; NIV, 5.6 µg kg<sup>-1</sup>.

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

**Table 4.** 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 w ith min=0; max=LOD				
AFM <sub>1</sub>	AFB <sub>1</sub>	AFB <sub>2</sub>	AFG <sub>1</sub>	AFM <sub>1</sub>	AFB <sub>1</sub>	AFB <sub>2</sub>	AFG <sub>1</sub>	AFM <sub>1</sub>	AFB <sub>1</sub>	AFB <sub>2</sub>	AFG <sub>1</sub>	AFM <sub>1</sub>	AFB <sub>1</sub>	AFB <sub>2</sub>	AFG <sub>1</sub>	
MoE <sup>a</sup>				MoE <sup>a</sup>				MoE <sup>a</sup>				MoE <sup>a</sup>				
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 <sup>b</sup>				MoET <sup>b</sup>				MoET <sup>b</sup>				MoET <sup>b</sup>			
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<sub>1</sub>, 0.011 µg kg<sup>-1</sup>; AFB<sub>1</sub>, 0.003 µg kg<sup>-1</sup>; AFB<sub>2</sub>, 0.001 µg kg<sup>-1</sup>; AFG<sub>1</sub>, 0.006 µg kg<sup>-1</sup>.

<sup>a</sup>MoE (Margin of Exposure) = BMDL<sub>10</sub> / Exposure data; BMDL<sub>10</sub> (Benchmark Dose Lower Confidence Limit) = 0.00025 mg/Kg bw/day; <sup>b</sup>MoET (Combined Margin of Exposure) = 1/((1/MoE<sub>AFM1</sub>)+(1/MoE<sub>AFB1</sub>)+(1/MoE<sub>AFB2</sub>)+(1/MoE<sub>AFG1</sub>))

### Exposure assessment to Processed Cereal Based-foods (Assunção et al, 2015b)

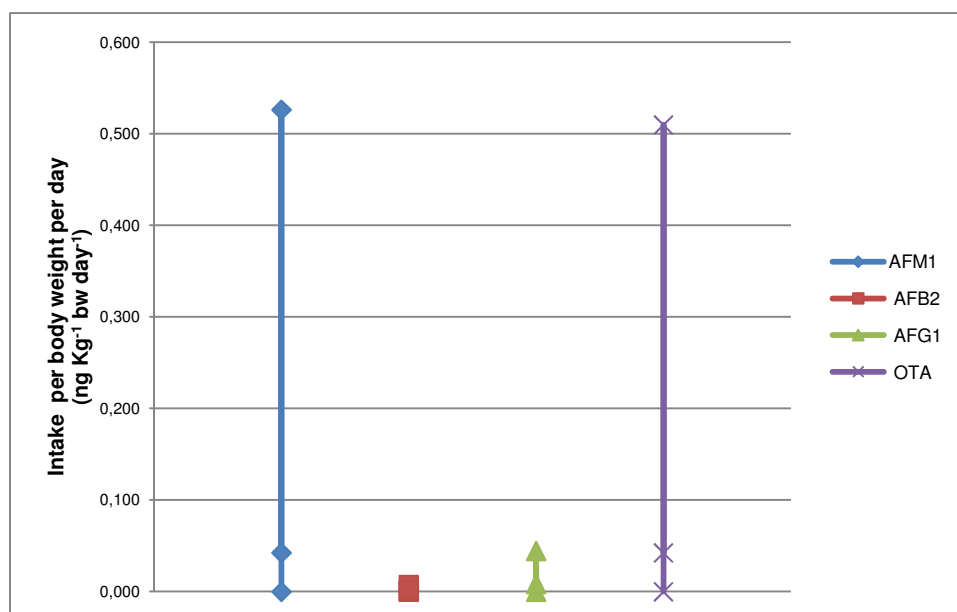
Approximately 47% of the studied children (1-3 years old) consumed processed cereal based-foods at least one time in these 3 days presenting a mean weight of 11.86 kg and a mean consumption of processed cereal based-foods of 14.96 g day<sup>-1</sup>. From the consumers 27% were aged < 1 year old and 73% aged between 1 and 3 years old.

The exposure assessment of mycotoxins through processed cereal based-foods consumption were first assessed using a deterministic approach as shown in Table 5.

**Table 5.** Deterministic approach to estimate children’s intake of mycotoxins present in processed cereal-based foods ( $\text{ng kg bw}^{-1} \text{day}^{-1}$ ) considering three different scenarios for non-detects ( $< \text{LOD}$ ). Mean values for mycotoxin content, consumption (14.96 g) and children weight (11.86 kg) were used for daily intake calculations.

Mycotoxin	H1: $< \text{LOD} = \text{LOD}$		H2: $< \text{LOD} = 1/2 \text{ LOD}$		H3: $< \text{LOD} = 0$	
	Mycotoxin content ( $\text{ng kg}^{-1}$ )	Daily intake ( $\text{ng kg bw}^{-1} \text{day}^{-1}$ )	Mycotoxin content ( $\text{ng kg}^{-1}$ )	Daily intake ( $\text{ng kg bw}^{-1} \text{day}^{-1}$ )	Mycotoxin content ( $\text{ng kg}^{-1}$ )	Daily intake ( $\text{ng kg bw}^{-1} \text{day}^{-1}$ )
AFM <sub>1</sub>	33,7	0,043	30,4	0,038	27,1	0,034
AFB <sub>2</sub>	1,05	0,001	0,575	0,001	0,1	0,000
AFG <sub>1</sub>	6,75	0,009	4,05	0,005	1,35	0,002
OTA	33,6	0,042	32,1	0,040	30,6	0,039

Figure 2 shows the results of deterministic (central mark, mean value) and probabilistic approaches (top and down marks for P1 and P99) to estimate children exposure to aflatoxins M<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and ochratoxin A, through ingestion of processed cereal based-foods ( $\text{ng Kg}^{-1} \text{bw day}^{-1}$ ). These results are from fourth scenario ( $< \text{LOD} = \text{uniform distribution with min}=0 \text{ and max}=\text{LOD}$ ). Remaining scenarios followed the same pattern.



**Figure 8.** Results of deterministic (central mark, mean value) and probabilistic approaches (top and down marks for P1 and P99) to estimate children exposure to aflatoxins M<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and ochratoxin A, through ingestion of processed cereal-based foods (ng Kg<sup>-1</sup> bw day<sup>-1</sup>).

AFM<sub>1</sub> revealed a margin of exposure (MoE) below 10000 suggesting potential health concern for the higher percentiles of intake ( $\geq$  P75). MoE of the remaining aflatoxins were above 10000 for all percentiles. OTA presented a hazard quotient (HQ) below 1 for all percentiles, suggesting no potential health concern. Considering the co-occurrence of aflatoxins, and applying the concentration addition concept, combined margin of exposure (MoET) was below 10000 for  $\geq$  P75 and this fact constitutes a potential health concern.

### Deviations

In order to perform the pilot study for children 0-3 years old food consumption data, it was necessary to submit a research protocol to the Ethics Committee of the National Institute of Health and to the National Data Protection Commission (CNPD). This protocol, titled "Evaluation of dietary habits, nutritional status and the probability of exposure to contaminants through infant feeding", was authorized by the CNPD (Resolution N<sup>o</sup>. 473/2014 ratifying the Authorization N<sup>o</sup>. 3367/2014) and included the payment of a tax to the CNPD in order to legalize the database of the research protocol, which was funded by the project. The pilot study in the Primary Health Care Unit of Cidadela Cascais with which it was established a cooperation agreement, replaced the Health Unit of Mafra that was not available at the moment of the study. In order to attract the participation of parents and their children in the pilot study and as an acknowledgement for the Family Health Unit collaboration, the elaboration of extra nutritional status and dietary habits evaluation reports for the participants is in course.

Foods identified as consumed by children were coded according to the FoodEx2 classification system, since it is the one actually recommended by international guidelines and on which the project team had recently gained expertise under other international ongoing project.

A delay on the exposure assessment study which contributed to the project prorogation was related with the failure of the analytical equipment and the need to establish unforeseen collaborations to overcome this difficulty (Task 1).

After a first trial with the available occurrence data concerning infant formula and ready to drink milk samples (Task 1), the team decided to purchase more samples from these food groups to perform a more accurate exposure assessment for further publication since the planned sample size proposed under the project was already exceeded.

### Conclusions

All goals from Task 4 were attained and several additional tasks were performed as the submission of a research protocol for the pilot study and nutritional and dietary habits evaluation protocols. A very good relationship was developed between health care professionals and team members namely with the fellowship Noélia Arruda. The health care unit professionals had greatly acknowledge the important help that this project gave to the knowledge and improving of children's health.

The present study evaluated for the first time the exposure assessment of mycotoxins mixtures of Portuguese children through the consumption breakfast cereals and cereal processed based-foods suggesting a potential health concern for the higher percentiles of intake. These are the first results available on this area in international literature. Exposure assessment of infant formula and ready to drink milk are in course for further publication.

Besides the studies already published and presented in scientific meetings (see project indicators below), more 2 manuscripts are in preparation which are expected to be submitted to international peer review journals within the next six months. A best poster award was attributed to the PhD student and fellowship Ricardo Assunção to the work developed in this area.

### References:

OPEN Portugal - [http://pt.opkp.si/pt\\_PT/cms/vstopna-stran](http://pt.opkp.si/pt_PT/cms/vstopna-stran)

Assunção, R., Vasco, E., Nunes, B., Loureiro, S., Martins, C. and Alvito, P. (2015a). Single-compound and cumulative risk assessment of mycotoxins present in breakfast cereals consumed by children from Lisbon region, Portugal. *Food and Chemical Toxicology*. 86, 274-28. doi: 10.1016/j.fct.2015.10.017

Assunção, R., Vasco, E., Nunes, B., Loureiro, S., Martins, C. and Alvito, P. (2015b). Risk assessment of portuguese children dietary exposure to co-occurring mycotoxins in processed cereal-based foods, 5<sup>th</sup> MoniQA International Conference, 16-18 September 2015, Porto, Portugal, 64, ISBN 978-3-9504109-0-7. (BEST POSTER AWARD)

Leal, S., Costa, C., Arruda, N., Vasco, E. and Alvito, P. (2015). Avaliação do estado nutricional, dos hábitos alimentares e da probabilidade de exposição a micotoxinas na alimentação infantil: contributo do estudo-piloto efetuado na USF Cidadela, Cascais. *Boletim Epidemiológico Observações*, 4 (Supl 5):28-29, INSA <http://hdl.handle.net/10400.18/3014>

Vasco, E. (2014). Consumo alimentar infantil: o contributo da OPEN – Plataforma Nutricional. Simpósio Nacional Promoção Alimentação Saudável e Segura (SNPASS), FCG, Lisboa, Portugal. <http://hdl.handle.net/10400.18/2638>.

INSA (2014). Avaliação dos hábitos alimentares, do estado nutricional e da probabilidade de exposição a contaminantes na alimentação infantil. Material de divulgação na Unidade de Saúde Familiar de Cidadela, Cascais. INSA, Lisboa. <http://hdl.handle.net/10400.18/2285>

Leal, S., Vasco, E. and Alvito, P. (2014). Porque é que a USF Cidadela, Cascais abraçou o projeto Mycomix?, 1º Simpósio Nacional Promoção de uma Alimentação Saudável e Segura - Alimentação infantil e contaminantes químicos (SNPASS), Lisboa. <http://hdl.handle.net/10400.18/2639>.

Assunção, R., Vasco, E., Nunes, B. and Alvito, P. (2014). Aplicação de uma ferramenta de análise probabilística da exposição. 1º Simpósio Nacional Promoção de uma Alimentação Saudável e Segura - Alimentação infantil e contaminantes químicos (SNPASS), Lisboa <http://hdl.handle.net/10400.18/2645>.

#### Project Indicators:

##### Publications

##### Papers in international journals

Assunção, R., Vasco, E., Nunes, B., Loureiro, S., Martins, C. and Alvito, P. (2015). Single-compound and cumulative risk assessment of mycotoxins present in breakfast cereals consumed by children from Lisbon region, Portugal. *Food and Chemical Toxicology*. 86, 274-28. doi: 10.1016/j.fct.2015.10.017

Alvito, P., Assunção, R., Borges, T., Leal, S., Loureiro, S., Louro, H., Martins, C., Nunes, B., Silva, M.J., Vasco, E., Tavares, A. and Calhau, M. A. (2015). Risk assessment of multiple mycotoxins in infant food consumed by Portuguese children –

the contribute of the MYCOMIX project, *Toxicology Letters*, 238/2S, S117.  
doi:10.1016/j.toxlet.2015.08.377

#### Papers in national journals

Leal, S., Costa, C., Arruda, N., Vasco, E. and Alvito, P. (2015). Avaliação do estado nutricional, dos hábitos alimentares e da probabilidade de exposição a micotoxinas na alimentação infantil: contributo do estudo-piloto efetuado na USF Cidadela, Cascais. *Boletim Epidemiológico Observações*, 4 (Supl 5):28-29, INSA  
<http://hdl.handle.net/10400.18/3014>

#### Papers in preparation (to submit at international journals)

Vasco, E., Leal, S. and Alvito, P. Assessment of the dietary intake of Portuguese children from 0 to 3 years old from a Primary Health Care Unit in Lisbon region.

Assunção, R., Vasco, E., Nunes, B., Loureiro, S., Martins, C. and Alvito, P. Risk assessment of Portuguese children dietary exposure to co-occurring mycotoxins in processed cereal-based foods, infant formula and milk.

#### Other publications

INSA (2014). Avaliação dos hábitos alimentares, do estado nutricional e da probabilidade de exposição a contaminantes na alimentação infantil. Material de divulgação na Unidade de Saúde Familiar de Cidadela, Cascais. INSA, Lisboa.  
<http://hdl.handle.net/10400.18/2285>

#### Communications

##### International meetings

Pereira C, Alvito P, Martins C, Seljak B, Vasco E (2013). Children Exposure assessment to patulin through the intake of apple based products. XVII Euroanalysis – Analytical chemistry for human well-being and sustainable development, 25-29 August, Warsaw, Polonia, p.648, <http://hdl.handle.net/10400.18/2284>

Alvito P, Arruda N, Assunção R, Barreira MJ, Borges T, Leal S, Louro H, Martins C, Martins V, Nunes B, Silva MJ, Vasco E, Tavares A, Calhau MA, 2014, Exploring the toxic effects of MIXtures of MYCOtoxins in infant food and potential health impact. Trends in Environmental Microbiology for Public Health (TEMPH2014), Lisboa, Portugal, 59. <http://hdl.handle.net/10400.18/2937>.

Assunção R, Vasco E, Nunes B, Alvito P (2014). Are Portuguese children exposed to mycotoxins through infant foods? A preliminary approach", TEMPH2014, Lisboa, Portugal, 60. <http://hdl.handle.net/10400.18/2937>.

Assunção R, Vasco E, Nunes B, Martins C, Alvito P. (2015) "Are breakfast cereals safe to consumption by children? A preliminary exposure assessment approach"; Euroanalysis XVIII, 6-10 September 2015, Bordeaux, France.

Assunção R, Vasco E, Nunes B, Loureiro S, Martins C, Alvito P. (2015). Risk assessment of portuguese children dietary exposure to co-occurring mycotoxins in processed cereal-based foods, 5<sup>th</sup> MoniQA International Conference, 16-18 September 2015, Porto, Portugal, 64, ISBN 978-3-9504109-0-7. (BEST POSTER AWARD)

Alvito P, Assunção R, Borges T, Dupont D, Leal S, Loureiro S, Louro H, Martins C, Nunes B, Pinhão M, Koroušić Seljak B., Silva M.J., Silva S., Vasco E., & Calhau M.A. (2015). MycoMix and risk assessment: a contribute to improve risk analysis, 5<sup>th</sup> MoniQA International Conference, 16-18 September, Porto, Portugal, 68, ISBN 978-3-9504109-0-7. <http://hdl.handle.net/10400.18/3155>

Assunção R, Vasco E, Nunes B, Loureiro S, Martins C & Alvito P (in press). Risk assessment of portuguese children exposed to single and multiple mycotoxins in breakfast cereals, Food Contaminants and Human Health - Challenges in chemical mixtures. Eds: Alvito, Assunção, Louro H, Silva MJ, Vasco E. Publisher: National Institute of Health Doutor Ricardo Jorge, Lisbon, Portugal <http://hdl.handle.net/10400.18/3214>

#### National meetings

Leal S, Vasco E., Alvito P (2014). Porque é que a USF Cidadela, Cascais abraçou o projeto Mycomix?, SNPASS, Lisboa, Portugal. <http://hdl.handle.net/10400.18/2639>.

Vasco E (2014). Consumo alimentar infantil: o contributo da OPEN – Plataforma Nutricional. SNPASS, Lisboa, Portugal. <http://hdl.handle.net/10400.18/2638>.

Assunção R, Vasco E, Nunes B & Alvito P (2014). Aplicação de uma ferramenta de análise probabilística da exposição. SNPASS, Lisboa, Portugal <http://hdl.handle.net/10400.18/2645>.

## **Task 5 - Children's health and mycotoxin mixtures-global evaluation**

---

### Background and aims

**There is growing concern within public** health about mycotoxin involvement in human diseases particularly related to children's exposure through contaminated food. Children diet comprises a variety of different commodities, and the possibility of human co-exposure to several mycotoxins is very likely to occur. Exposure to combined mycotoxins could be expected to exert greater toxicity and carcinogenicity than exposure to single mycotoxins and very few data is available in literature concerning this issue. Therefore MYCOMIX project, based on previous studies performed by the PI and her team, had proposed the hypothesis that "children are exposed to multiple mycotoxins through food and this could constitute a health risk" and tried to answer the three questions: 1) Are Portuguese children exposed daily to one or several mycotoxins through food? 2) Can this co-exposure affect children's health? and 3) Are there interaction effect between mycotoxins? Within task 5 it was also planned to organize a national and international meeting for dissemination of the project results.

### Global evaluation

Scarce data are available in the literature concerning the occurrence of multiple mycotoxins in infant food and their combined toxicity, and no data exists in Portugal concerning this issue. In order to contribute to fill this gap, the MYCOMIX project, funded by the Portuguese Foundation for Science and Technology, gathered a multidisciplinary team aiming at answering the three questions reported above. To address these questions, MYCOMIX project included 4 tasks and a final task for global evaluation and dissemination of results. Briefly, Portuguese children (< 3 years old, n=103) food consumption data were obtained using a 3 days food diary in a pilot study performed at a Primary Health Care Unit. The main declared infant foods were purchased from the Lisbon market along 2014-15 and analyzed by means of HPLC, UPLC-MS/MS and GC-MS, analytical techniques for multiple mycotoxins co-occurrence. Risk assessment of children (participating in the pilot study) to multiple mycotoxins in food were performed using a combination of food consumption and occurrence data. Toxicological studies including cyto and genotoxic interactions between detected mycotoxins and bioaccessibility and cell assays were also performed using *in vitro* approaches in order to contribute to a more accurate risk assessment.

Concerning the question 1) Are Portuguese children exposed daily to one or several mycotoxins through food? the results from Task 1 (multimycotoxin determination and legislative values) revealed that children were in fact exposed to several co-occurring mycotoxins (2 to 7, simultaneously): 92 % in breakfast cereals, 60 % in cereal based baby foods, 50 % in cookies and 100 % in ready to drink milk. Twenty two different combinations of mycotoxins were detected in breakfast cereals with the most frequent mixture including AFB<sub>1</sub>+AFB<sub>2</sub>+OTA+ZEA (n=2) and OTA+FB<sub>1</sub>+DON+ZEA (n=2). Ten different combinations of mycotoxins were detected in cereal based baby foods with the most frequent mixture including OTA+FB<sub>1</sub> (n=3), although PAT (a target mycotoxin for children) was also present. The mixture of mycotoxins that was most detected in cookies samples was OTA+DON (n=3) and in ready to drink milk, AFM<sub>1</sub>+AFB<sub>1</sub>+AFB<sub>2</sub>+OTA (n=3). All goals from Task 1 were attained and some aspects exceed the proposed plan (number of analyzed toxins). Difficulties concerning LC-MS/MS analytical method and equipment failure were overcome with the establishment of collaborations with two scientific laboratories allowing the determination of 22 mycotoxins and metabolites in spite of the proposed 12 mycotoxins. Besides, a new LC-MS/MS method is now implemented at INSA, taking advantage of all acquired knowledge and collaborative studies developed during this task. This study is one of first available in literature on the occurrence of mixtures of mycotoxins in infant food and it was particularly important since it had highlight an urgent need for further studies in order to overcome the absence of legislated limits for mycotoxins in breakfast cereals other than DON and FB<sub>1</sub> and also the absence of legislated limits for mycotoxin mixtures in food. The last issue assumes particular importance considering the potential interactions that could occur between mycotoxins and its potential impact on human and, mainly, children health. The binary mixtures of OTA+AFM<sub>1</sub>, OTA +FB<sub>1</sub> and OTA+PAT were chosen to perform toxicological evaluations because they were present in most common combinations in breakfast cereals and cereal based foods.

Concerning the question 2) Can this co-exposure affect children's health?, the results from Task 4 (children exposure assessment) revealed a potential health concern for the high consumers of breakfast cereals and processed cereal-based foods. This is particularly important because aflatoxins, the main responsible for these results, are the most potent hepatotoxic compounds known. These are the first results available on this area in literature. The use of a probabilistic approach and the purchase of the @RISK software as well as the training in risk assessment had revealed to be crucial for the development of the exposure and risk assessment studies. Face to the present results it is recommended to parents to make a diversified diet for children with ages

until 3 years old including a moderate consumption of cereal-based products and including different types of cereals since this food group is also an important source of minerals and vitamins and the first solid food to be introduced in children diet. The achievement of children's 0-3 years old food consumption data was crucial to perform exposure assessment since no data were available in Portugal at the moment of the study. The collaboration of the team project with a Primary Health Care Unit was a successful case allowing a connection of scientific research with clinical realities. The health care unit professionals became very interested to use in future the OPEN Portugal platform and its facilities.

Concerning the question 3) Are there interaction effect between mycotoxins?, the results from task 2 (evaluation of toxic effects of mycotoxins mixtures) presented new evidences on interactive effects of AFM<sub>1</sub> and OTA, revealing the predominance of an antagonistic pattern in an intestinal cell line. A similar pattern was found for the combined effect of OTA and PAT in the same cell line. As to the interactions between OTA and FB<sub>1</sub>, in a low concentration-range more realistic in terms of public health, the detected effect was predominantly synergistic, which is of utmost importance because it represents a considerable hazard for human health. In terms of the combined genotoxic effects of the same mixtures, in general no marked interactions were detected, although the combined effect of OTA and PAT should be further explored in a near future.

Results from task 3 (bioaccessibility and absorption of mycotoxin mixtures) showed that a significant portion of OTA, AFM<sub>1</sub> and PAT, detected in infant food, can reach the small intestine and thus, be available to cross the intestinal barrier and produce their toxic effects. The results demonstrated also that the simultaneous presence of mycotoxins affects the bioaccessibility values. However, further future studies with different mixtures of mycotoxins are needed to corroborate it. In another way, results showed that mycotoxins, namely PAT, affect the intestinal mucosa through reduction of barrier function, mainly by perturbation of ZO-1 levels and phosphorylation of MLC and inhibition of T cell proliferation (original results obtained for the first time). This result 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. All these data obtained from tasks 2 and 3 contribute to provide a more accurate risk assessment of single and multiple food contaminants. The interactive effects between mycotoxins could affect the amount of toxin that reach the systemic circulation and this possibility must be taken into account in a risk assessment process.

All collaborative studies developed within MYCOMIX including the 3 project consultants Didier Dupont (INFOGEST Cost Action), Barbara Seljak (OPEN platform) and Susana Loureiro (modelling interactive effects) and the 3 collaborations developed with the Primary Health Care Unit and the analytical laboratories (LAQV-REQUIMTE, Portugal and University of São Paulo, Brazil) had greatly contribute to the project progress. The obtained output indicators overcome largely those expected: 2 books (instead of 1 proposed), 9 publications in international journals (instead of the 4 proposed, and 5 in preparation), 3 national publications (none was planned), 22 communications in international meetings (instead of 5) and 9 in national meetings (instead of 2), 3 FCT project reports (instead of 2), 3 MSc Thesis (instead of 2), 1 PhD Thesis (gathering papers all obtained under MYCOMIX project) that was not planned and 1 software (OPEN Portugal) as expected. Additionally, 6 movies performed by MYCOMIX team on the new harmonized digestion method are available at YOUTUBE promoting this method dissemination (not planned in the project).

The dissemination of the project's aims and results were performed through the elaboration of 2 project flyers (one in Portuguese and other in English), 1 flyer on children diet diversification until 3 years old requested by the Primary Health Care Unit (not planned), and the achievement of a national and an international meeting as previously proposed, gathering health care professionals, students, industry and scientific researchers. This new national meeting was held in the 26th November 2014 at the Foundation Calouste Gulbenkian, Lisbon, and intended to become an annual national symposium for the promotion of a healthy and safe food dedicated to research projects, always promoted by INSA. This year the 2<sup>nd</sup> Symposium had already took place in the same date and it is already planned the 3<sup>rd</sup> one to 2016. The international meeting was held in 13-14 April 2015, at INFARMED, Lisbon, it was dedicated specially to research on chemical mixtures and gathered more than 130 scientists from 10 European countries and Brazil. It included 13 invited speakers from recognized scientific institutions as EFSA, INRA, RIVM, etc., several dozens of posters, and 12 contributed talks. The extended abstracts prepared by some invited speakers as well as the abstracts presented on this meeting were gathered in a book that is under publication by INSA. This new conference was intended to be repeated each two years, next to be held in Minho University, with the participation of INSA. Within MYCOMIX two new conferences were created in order to disseminate results from scientific research projects.

## Project output indicators

---

### A - Publications

#### Books

1. Alvito P. Alterações do estado de saúde associados à alimentação - contaminantes químicos – micotoxinas (2014), INSA, Lisboa. <http://hdl.handle.net/10400.18/2283>
2. Food Contaminants and Human Health - Challenges in chemical mixtures (in press). Eds.: Alvito P, Assunção R, Louro H, Silva MJ, Vasco E. Publisher: National Institute of Health Doutor Ricardo Jorge, Lisbon, Portugal <http://hdl.handle.net/10400.18/3214>

#### Papers in international journals

##### Published or submitted

1. Alvito P., Assunção R., Borges T., Leal S., Loureiro S., Louro H., Martins C., Nunes B., Silva M.J., Vasco E., Tavares A., & Calhau M.A. (2015). Risk assessment of multiple mycotoxins in infant food consumed by Portuguese children – the contribute of the MYCOMIX project *Toxicology Letters*, 238/2S, S117. <http://hdl.handle.net/10400.18/3155>
2. Assunção R, Ferreira M, Martins C, Diaz I, Padilla B, Dupont D, Bragança M & Alvito P (2014). Applicability of in vitro methods to study patulin bioaccessibility and its effects on intestinal membrane integrity. *Journal of Toxicology and Environmental Health, Part A*, 77:983-992 <http://www.tandfonline.com/doi/pdf/10.1080/15287394.2014.911138>
3. Assunção R, Vasco E, Nunes B, Loureiro S, Martins C, Alvito P (2015). Single-compound and cumulative risk assessment of mycotoxins present in breakfast cereals consumed by children from Lisbon region, Portugal. *Food and Chemical Toxicology*. 86, 274-281 <http://hdl.handle.net/10400.18/3217>
4. Assunção, R., Alvito, P., Kleiveland, C. R. and Lea, T. E. (Submitted). Characterization of *in vitro* effects of patulin on intestinal epithelial and immune cells. *Toxicology in vitro*
5. Assunção R, Martins C, Leclerc Duarte E, Alvito P. (2015). "Bioaccessibility assessment of patulin and ochratoxin A in cereal and fruit based baby foods using a harmonized in vitro digestion model: contribution for the risk assessment of chemical mixtures", *Toxicology Letters*, 238/2S, S116. <http://hdl.handle.net/10400.18/3156>
6. Pinhão M, Loureiro S, Tavares A, Mendonça I, Louro H, Alvito P, Silva MJ. (submitted). Combined cytotoxic and genotoxic effects of ochratoxin A and fumonisin B1 in human kidney and liver cell models, *Archives Toxicology*.
7. Lotti Egger, Olivia Ménard, Cristina Delgado-Andrade, Paula Alvito, Ricardo Assunção, Simon Balance, Reyes Barberá, Andre Brodkorb, Thomas Cattenoz, Alfonso Clemente, Irene Comi, Didier Dupont, Guadalupe Garcia-Llatas, María Jesús Lagarda, Steven Le Feunteun, Lonneke JanssenDuijghuijsen, Sibel Karakaya, Uri Lesmes, Alan R. Mackie, Carla Martins, Anne Meynier, Beatriz Miralles, Brent S. Murray, Anne Pihlanto, Gianluca

- Picariello, Claudia N Santos, Sebnem Simsek, Isidra Recio, Neil Rigby, Laurie-Eve Rioux, Helena Stoffers, Ana Tavares, Lucelia Tavares, Sylvie Turgeon, Ellen K. Ulleberg, Gerd E. Vegarud, Guy Vergères, Reto Portmann (submitted). The harmonized INFOGEST *in vitro* digestion method: From knowledge to action. *Food Research International*.
8. Tavares AM, Alvito P, Loureiro S, Louro H, Silva MJ (2013) Multi-mycotoxin determination and *in vitro* combined cytotoxic effects of aflatoxin M1 and ochratoxin A. *World Micotoxin Journal*. 6, 4, 375-388 <http://hdl.handle.net/10400.18/1792>
  9. Tavares A, Mendonça I, Louro H, Pinhão M, Loureiro S, Alvito P, Silva M J A contribution to hazard assessment of combined exposure to mycotoxins using *in vitro* toxicity testing, *Toxicology Letters*, 238/2S, S352. <http://hdl.handle.net/10400.18/3182>

#### In preparation (to be submitted in 3-6 months)

1. Assunção R, Pinhão M, Silva, MJ, Alvito P (in preparation). Insights into individual and combined toxic effects of patulin and ochratoxin A in human intestinal cells.
2. Assunção R, Vasco, Nunes B, Loureiro S, Martins C and Alvito P (in preparation). Risk assessment of Portuguese children dietary exposure to co-occurring mycotoxins in processed cereal-based foods, infant formula and milk.
3. Assunção R, Martins C, Dupont D and Alvito P (in preparation). Assessment of patulin and ochratoxin A bioaccessibility in cereal and fruit based baby foods using a harmonized *in vitro* digestion model.
4. Martins C, Assunção R, Cunha S, Fernandes J., Jager A, Oliveira C, Alvito P. (in preparation). Assessment of mixtures of mycotoxins in breakfast cereals available in Portuguese market.
5. Vasco E, Leal S, Alvito, P. (in preparation). Assessment of the dietary intake of Portuguese children from 0 to 3 years old from a Primary Health Care Unit in Lisbon region.

#### Articles in peer review national journals

##### Published or submitted

1. Tavares AM, Alvito P, Loureiro S, Louro H, Silva MJ (2013). Estudos da toxicidade de misturas de aflatoxina M<sub>1</sub> e ocratoxina A numa linha celular humana. *Boletim Epidemiológico Observações*, 2, 20-21, <http://hdl.handle.net/10400.18/2116>
2. Leal S, Costa C, Arruda N, Vasco E, Alvito P (2015). Avaliação do estado nutricional, dos hábitos alimentares e da probabilidade de exposição a micotoxinas na alimentação infantil: contributo do estudo-piloto efetuado na USF Cidadela, Cascais. *Boletim Epidemiológico Observações*, 4 (Supl 5):28-29, INSA <http://hdl.handle.net/10400.18/3014>

3. Assunção R, Martins C, Ferreira M, Alvito P (2015). Aplicação de modelo de digestão *in vitro* para a determinação da bioaccessibilidade de patulina em sumos de fruta Boletim Epidemiológico Observações, 4 (13):30-32, INSA

## B - Communications

### International scientific meetings

1. Alvito P, Vasco E, Martins C, Dupont D (2013). Applicability of an *in vitro* model to assess the bioaccessibility of food contaminants. 2nd International Conf on Occupational and Environmental Toxicology, 16-17 Setembro, Porto, Portugal, p.156. <http://hdl.handle.net/10400.18/1947>
2. Alvito P, Arruda N, Assunção R, Barreira MJ, Borges T, Leal S, Louro H, Martins C, Martins V, Nunes B, Silva MJ, Vasco E, Tavares A, Calhau MA (2014), Exploring the toxic effects of MIXtures of MYCOtoxins in infant food and potential health impact. Trends in Environmental Microbiology for Public Health (TEMPH2014), Lisbon, Portugal, 59 (oral communication). <http://hdl.handle.net/10400.18/2937>.
3. Alvito P., Assunção R., Martins C., Tavares A., Egger C., Lea T. and Dupont D. (2015). MYCOMIX and INFOGEST: a fruitful collaboration. Proc. 4<sup>th</sup> International Conference on Food Digestion (ICFD), Naples, Italy, 114 <http://hdl.handle.net/10400.18/3039>
4. Alvito P, Assunção R, Borges T, Dupont D, Leal S, Loureiro S, Louro H, Martins C, Nunes B, Pinhão M, Koroušić Seljak B., Silva M.J., Silva S., Vasco E., & Calhau M.A. (2015). MycoMix and risk assessment: a contribute to improve risk analysis, 5<sup>th</sup> MoniQA International Conference, 16-18 Setemb, Porto, Portugal, 68, ISBN 978-3-9504109-0-7. <http://hdl.handle.net/10400.18/3155>
5. Alvito P (2015). Syndromes associated with children exposure to mycotoxins and health risk assessment to multiple mycotoxins in infant foods. In "ASPOMM meeting - Local Mycology Meeting", *Trends in Medical Mycology*, 10 October, Lisbon Congress Center, Portugal (oral communication) <http://hdl.handle.net/10400.18/3218>
6. Alvito P (in press). MYCOMIX - Exploring the toxic effects of mixtures of mycotoxins in infant food and potential health impact – a case study. Food Contaminants and Human Health - Challenges in chemical mixtures. Eds: Alvito, Assunção, Louro H, Silva MJ, Vasco E. Publisher: National Institute of Health Doutor Ricardo Jorge, Lisbon, Portugal <http://hdl.handle.net/10400.18/3214>
7. Assunção R; Ferreira, M.; Martins, C.; Dupont, D.; Alvito, P. (2014) Mycotoxins and applicability of *in vitro* methods of digestion and absorption assessment - the patulin case study. Proceedings of the 3rd International Conference on Food Digestion, COST Action Infogest FA 1005, 11-13 March, p. 83, <http://hdl.handle.net/10400.18/2281>.
8. Assunção R, Vasco E, Nunes B, Alvito P (2014). Are Portuguese children exposed to mycotoxins through infant foods? A preliminary approach", TEMPH2014, Lisboa, Portugal, 60. <http://hdl.handle.net/10400.18/2937>.
9. Assunção R., Martins C., Tavares A., Alvito P (2015). First bioaccessibility assessment

- of patulin in cereal and fruit based baby foods using the harmonized IVD model. Proc. 4<sup>th</sup> ICFD, Naples, Italy, 117 <http://hdl.handle.net/10400.18/3040>
10. Assunção R., Kleiveland C.R., Lea TL, Alvito P. (2015). Interactions between dietary toxins and gut mucosa: patulin effects on the structure and function of intestinal and immune cells. Proc. 4<sup>th</sup> ICFD, Naples, Italy, 115 <http://hdl.handle.net/10400.18/3042>
  11. Assunção R, Vasco E, Nunes B, Martins C, Alvito P. (2015) "Are breakfast cereals safe to consumption by children? a preliminary exposure assessment approach"; Euroanalysis XVIII, 6-10 Setemb, Bordeaux, France.
  12. Assunção R, Vasco E, Nunes B, Loureiro S, Martins C, Alvito P. (2015). Risk assessment of portuguese children dietary exposure to co-occurring mycotoxins in processed cereal-based foods, 5<sup>th</sup> MoniQA International Conference, 16-18 Setemb, Porto, Portugal, 64, ISBN 978-3-9504109-0-7. <http://hdl.handle.net/10400.18/3153> (BEST POSTER AWARD)
  13. Assunção R, Kleiveland CR, Lea TL, Alvito P (in press). Insights of enteropathogenic effects of mycotoxins on the human intestinal gut mucosa. Food Contaminants and Human Health - Challenges in chemical mixtures. Eds: Alvito, Assunção, Louro H, Silva MJ, Vasco E. Publisher: National Institute of Health Doutor Ricardo Jorge, Lisbon, Portugal <http://hdl.handle.net/10400.18/3214> (BEST POSTER AWARD)
  14. Assunção R, Vasco E, Nunes B, Loureiro S, Martins C & Alvito P (in press). Risk assessment of portuguese children exposed to single and multiple mycotoxins in breakfast cereals, Food Contaminants and Human Health - Challenges in chemical mixtures. Eds: Alvito, Assunção, Louro H, Silva MJ, Vasco E. Publisher: National Institute of Health Doutor Ricardo Jorge, Lisbon, Portugal <http://hdl.handle.net/10400.18/3214>
  15. Martins C, Tavares A, Assunção R, Alvito P (in press). Bioaccessibility of mycotoxins in baby foods using the harmonized *in vitro* digestion model. Food Contaminants and Human Health - Challenges in chemical mixtures. Eds: Alvito, Assunção, Louro H, Silva MJ, Vasco E. Publisher: National Institute of Health Doutor Ricardo Jorge, Lisbon, Portugal <http://hdl.handle.net/10400.18/3214>
  16. Martins C, Assunção R, Cunha S, Jager A, Alvito P. (in press). Assessment of mixtures of mycotoxins in breakfast cereals available in portuguese market, Food Contaminants and Human Health - Challenges in chemical mixtures. Eds: Alvito, Assunção, Louro H, Silva MJ, Vasco E. Publisher: National Institute of Health Doutor Ricardo Jorge, Lisbon, Portugal <http://hdl.handle.net/10400.18/3214>
  17. Martins C, Assunção R, Cunha S, Jager A, Alvito P. (2015) Assessment of mixtures of mycotoxins in cereal based foods available in Portuguese market, Abstracts Book RAFA2015, Prague, Czech Republic, pp 334. <http://hdl.handle.net/10400.18/3219>
  18. Pereira C, Alvito P, Martins C, Seljak B, Vasco E (2013). Children Exposure assessment to patulin through the intake of apple based products. XVII Euroanalysis –

- Analytical chemistry for human well-being and sustainable development, 25-29 August, Warsaw, Polonia, p.648, <http://hdl.handle.net/10400.18/2284>
19. Tavares A, Egger , Portmann R., Alvito P. (2014). The effects of matrix proteins on the aflatoxinM<sub>1</sub> bioaccessibility and the Caco-2 intestinal transport, Proceedings of the 3rd International Conference on Food Digestion, COST Action Infogest FA 1005, 11-13 March, p.141, <http://hdl.handle.net/10400.18/2282>
  20. Tavares, A.M., Egger C., Portmann R., Martins C., Assunção R., Alvito P. (2015). Bioaccessibility of aflatoxin M<sub>1</sub> in three artificially contaminated infant formula using a standardised static *in vitro* digestion method. Proc. 4<sup>th</sup> ICFD, Naples, Italy, 118 <http://hdl.handle.net/10400.18/3041>
  21. Tavares AM, Mendonça I, Alvito P, Loureiro S, Louro H, Silva MJ. (in press). Evaluation of combined cytotoxic effects of ochratoxin A and fumonisin B1 in human liver and renal cells. Food Contaminants and Human Health - Challenges in chemical mixtures. Eds: Alvito, Assunção, Louro H, Silva MJ, Vasco E. Publisher: National Institute of Health Doutor Ricardo Jorge, Lisbon, Portugal <http://hdl.handle.net/10400.18/3214>

#### National scientific meetings

##### Oral presentations

1. Martins C, Assunção R., Cunha S., Jager A, Alvito P. (2014). Ocorrência de micotoxinas em produtos à base de cereais. 1<sup>o</sup> Simpósio Nacional Promoção de uma Alimentação Saudável e Segura (SNPASS) - Alimentação infantil e contaminantes químicos, Fundação Calouste Gulbenkian (FCG), Lisboa, Portugal. <http://hdl.handle.net/10400.18/2682>.
2. Silva MJ. (2014). Genotoxicidade de micotoxinas e potenciais implicações para a saúde. SNPASS, FCG, Lisboa, Portugal. <http://hdl.handle.net/10400.18/2834>.
3. Tavares A. (2014). Efeito tóxico combinado de micotoxinas: ocratoxina A e aflatoxina M<sub>1</sub>, SNPASS, FCG, Lisboa, Portugal. <http://hdl.handle.net/10400.18/2680>.
4. Leal S, Vasco E., Alvito P (2014). Porque é que a USF Cidadela, Cascais abraçou o projeto Mycomix?, FCG, SNPASS, Lisboa, Portugal. <http://hdl.handle.net/10400.18/2639>.
5. Vasco E (2014). Consumo alimentar infantil: o contributo da OPEN – Plataforma Nutricional. FCG, SNPASS, Lisboa, Portugal. <http://hdl.handle.net/10400.18/2638>.
6. Borges T (2014). Avaliação do risco de crianças expostas a micotoxinas através do consumo de cereais de pequeno-almoço. FCG, SNPASS, Lisboa, Portugal <http://hdl.handle.net/10400.18/3051>
7. Assunção R, Martins C, Tavares A, & Alvito P (2014). Método harmonizado de digestão *in vitro*: uma ferramenta para o estudo da bioaccessibilidade de contaminantes alimentares. SNPASS, FCG, Lisboa, Portugal, <http://hdl.handle.net/10400.18/2681>.

8. Assunção R, Vasco E, Nunes B & Alvito P (2014). Aplicação de uma ferramenta de análise probabilística da exposição. SNPASS, FCG, Lisboa, Portugal. <http://hdl.handle.net/10400.18/2645>
9. Leal S, Costa C, Arruda N, Vasco E, Alvito P (2014). Avaliação do Estado Nutricional de crianças dos 0-3 anos da USF Cidadela, Cascais. ACES Cascais, 19 Novembro. <http://hdl.handle.net/10400.18/2628>

### Advanced training

#### Master Thesis:

1. Ferreira M (2014). Simulação *in vitro* do processo digestivo de patulina em sumos de fruta. Dissertação de Mestrado em Biologia Humana e Ambiente, Faculdade de Ciências, Universidade de Lisboa, 18 Dezembro <http://hdl.handle.net/10451/15578>
2. Mendonça I (2014). Avaliação dos Efeitos Citotóxicos e Genotóxicos de Misturas de Micotoxinas. Dissertação de Mestrado em Biologia Humana e Ambiente, Faculdade de Ciências, Universidade de Lisboa, 16 Dezembro <http://hdl.handle.net/10451/15817>

#### PhD Thesis:

1. Assunção, R. (*in preparation*). Infant food and mycotoxins: insights for risk assessment. Exposure assessment and toxic effects of single and multiple mycotoxins. PhD Thesis. Universidade de Évora, Portugal.

### Other

#### Dissemination material:

INSA (2015). Estudo exploratório dos efeitos tóxicos de misturas de micotoxinas em alimentos para crianças e potencial impacto na saúde <http://hdl.handle.net/10400.18/3215>

INSA (2015). Exploring the toxic effects of mixtures of mycotoxins in infant food and potential health impact <http://hdl.handle.net/10400.18/2684>

INSA (2015). Diversificação alimentar <http://hdl.handle.net/10400.18/3216>

INSA (2014). Avaliação dos hábitos alimentares, do estado nutricional e da probabilidade de exposição a contaminantes na alimentação infantil (2014). Material de divulgação na Unidade de Saúde Familiar de Cidadela, Cascais. INSA, Lisboa. <http://hdl.handle.net/10400.18/2285>

#### Audiovisual material

1. Chymotrypsin Assay for In Vitro Food Digestion <http://hdl.handle.net/10400.18/3052>
2. Pancreatic Lipase Activity Assay for In Vitro Food Digestion <http://hdl.handle.net/10400.18/3053>
3. Static In Vitro Digestion Method for Food

<http://hdl.handle.net/10400.18/3054>

4. Pepsin Activity Assay for In Vitro Food Digestion

<http://hdl.handle.net/10400.18/3055>

5. Alpha Amylase Activity Assay for In Vitro Food Digestion

<http://hdl.handle.net/10400.18/3056>

6. Trypsin Assay for In Vitro Food Digestion

<http://hdl.handle.net/10400.18/3059>

#### Software

1. OPEN Portugal - [http://pt.opkp.si/pt\\_PT/cms/vstopna-stran](http://pt.opkp.si/pt_PT/cms/vstopna-stran)  
(not yet available for public)