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Toxicity of Major and Emerging Mycotoxins

Laura Soler-Vasco

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Université Toulouse III - Paul Sabatier

Mémoire présenté en vue de l'obtention de
L'Habilitation à Diriger des Recherches

Toxicity of Major and Emerging Mycotoxins

Laura SOLER VASCO

Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement

Équipe Biosynthèse et Toxicologie des Mycotoxines

UMR 1331 Toxicologie Alimentaire

Composition du Jury

Elodie MERLOT, PhD, DR

Doris MARKO, PhD Prof

Siska CROUBELS, PhD, Prof

Pascal FROMENT, PhD, DR

Nadia EVERAERT PhD, Prof

Isabelle P OSWALD, PhD, DREX

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SECTION 1

Curriculum Vitae

1.1. Personal information and career path

1.1.a. Personal information

SOLER VASCO, Laura PhD DVM, Chargé de recherche classe normale

Date and place of birth: 28 October 1982 in Murcia, Spain

Nationality: Spanish

Address: Biosynthesis and Toxicity of Mycotoxins (BioToMyc) team, UMR1331 Toxicologie Alimentaire, INRAe – Occitanie, Site de Saint Martin du Touch. 180, chemin de Tournefeuille - BP.93173. F-31027 TOULOUSE cedex 3, France

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1.1.b. Education and qualifications

Degree in Veterinary Medicine (DVM) at the University of Murcia (UMU; Spain), with honors. September 2005.

- *Minor dissertation complementary to DVM degree* entitled “Validation of an automated method for total sialic acid determination in porcine serum” presented at the Veterinary Faculty from the UMU, Spain qualified with acknowledged excellence in 2007.
- *Undergraduate Research Fellowship*. Academic year 2004-2005, at the Department of Animal Medicine and Surgery. Granted by the Spanish Ministry Science and Education (SMSE).
- *National Award as Outstanding Graduate of the Year in Veterinary Medicine* granted by the SMSE, 2006.

PhD Thesis in Veterinary Medicine entitled “New contributions on porcine Serum Amyloid A” June 2010. Summa cum laude and extraordinary mention by the UMU Veterinary School (Spain).

- Awarded as *Best PhD thesis of year 2010*, granted by the UMU Veterinary School (Spain).

1.1.c. Overview of previous positions held, research work and responsibilities in chronological order

- Post-doctoral position funded by the SMSE with the project “New stress biomarkers discovery in porcine saliva” from July 2010 to May 2011 at the Department of Animal Medicine and Surgery, Veterinary School, UMU (Spain).
- Post-doctoral position funded by the project “FoBoS: Sharing molecular techniques for food-borne pathogen detection” and the COST action FA1002 Farm Animal Proteomics. From September 2011 to December 2011 at the Department of Animal Pathology, Hygiene and Veterinary Public Health DIPAV, Veterinary School, University of Milan (Italy).
- Post-doctoral position funded by the Martin Escudero Private Foundation (Spain) with the project “Discovery and validation of intestinal health biomarkers and the effect of candidate immunomodulatory feed additives in the pig”. From January 2012 to January 2014 at the Division of Livestock-Nutrition-Health, Biosciences Engineering Faculty, KULeuven (Belgium).
- Post-doctoral position granted by Marie-Curie FP7 COFUND People Program, through the award of an “AgreenSkills” fellowship (under grant agreement n° 267196) and the French National infrastructure CRB-Anim with the project

“Advancing in domestic birds reproduction technology through-omics techniques” since February 2014 until July 2016 at the UMR PRC/URA, Agricultural Research National Institute (INRA)-Centre Val de Loire (France).

1.1.d. Current position

Chargée de Recherche Classe Normale (CRCN) in the team biosynthesis and Toxicology of Mycotoxins (BioToMyc), led by Dr Olivier Puel since November 1st 2016. The team is placed at the Research Centre in Food Toxicology (**Toxalim**), belonging to the Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAe), École Nationale Vétérinaire de Toulouse (ENVT), École d'Ingénieurs de Purpan (membre de l'Institut National Polytechnique de Toulouse-INPT; INP-Purpan) and Université Paul Sabatier-Toulouse III (UPS), in Toulouse, France. The main purpose of my work is evaluating the toxicity of major and emerging mycotoxins, evaluating their interaction and identifying biomarkers of their toxic effect.

1.1.e. Commissions of trust

I have been requested as a jury for the recruitment of three engineers at INRAE, participated in one PhD jury (Jeremy Grandhaye; University of Tours, France) and as scientific evaluator by different agencies including ANSES or the Polish National Academy of Sciences.

I have been invited as a guest editor for a special issue (Toxic Effect of Mycotoxins) of the journal “Toxins” and a topic editor (Factors Influencing Biomarker Range Intervals in Farm Animals) of the journal “Frontiers in Veterinary Science, Sec. Veterinary Experimental and Diagnostic Pathology”.

I regularly review manuscripts for several journal such as “Journal of Proteomics”, “Frontiers in Veterinary Science”, “Toxins”, “Molecules”, “Molecular and Cellular Endocrinology”, “Animal”, “Research in Veterinary Science”, “BMC Veterinary Research”, “Food and Chemical Toxicology”, “Proteomes” and “Journal of Clinical Medicine”, among others.

1.1.f. Major collaborators

National collaborations

Local collaborations (Toxalim)

- Metabolization of mycotoxins. Team “Metabolism of Xenobiotics” and Platform AXIOM UMR Toxalim; collaboration with Emilien Jamin, 2019. 1 joint publication
- Mycotoxins and transcription inhibition. UMR Toxalim and BAP. Collaboration with Yannick Lippi and Pascal Martin. 1 project granted.
- Role of mycotoxins as metabolic disruptors. Team “Metabolism of Xenobiotics” UMR Toxalim; collaboration with Nicolas Cabaton. 2 projects submitted.
- Effect of zearalenone in FGF21 signaling. Team “Integrative Toxicology & Metabolism” UMR Toxalim; collaboration with Anne Fougerat.
- Versicolorin A and genotoxicity. Team “Genotoxicity & Signaling” UMR Toxalim; collaboration with Julien Vignard and Gladys Mirey.

Local collaborations (Toulouse)

- Pig intestinal organoid production. UMR GEnPhySE; Collaboration with Martin Beaumont. 1 project granted and 1 joint publication.
- Proteomic analysis. IPBS; Collaboration with Odile Schiltz. 1 joint publication.

Collaborations elsewhere in France

- Use of a novel TLR4 KO intestinal cell model for the research of the pro-inflammatory effect of mycotoxins. Collaboration with Ignacio Caballero-Posadas UMR ISP. 1 joint publication
- Development of intact cell MALDI-TOF MS- based tools for mycotoxicity screening. Pôle d'Analyse et d'Imagerie des Biomolécules (PAIB) Plate-forme PIXANIM, INRAE Nouzilly. 1 project granted. 3 joint publications.
- Mycotoxin effect in adipokine signaling. Team "Métabolisme et reproduction" UMR PRC ; collaboration with Joëlle Dupont et Pascal Froment. 2 projects submitted.

International collaborations

- Occurrence of aflatoxin B1 and its precursors in food. Max Rubner-Institut, (Kiel Germany; Ronald Maul) and BOKU (Tulln Austria; Michael Sulyok).
- Study of the genotoxicity of aflatoxin B1 with its precursors. University of Navarra (Spain; Ariane Vettorazzi). 1 publication in preparation; exchange of PhD visiting students.
- Toxicity of Zearalenone. Biomin, (Tulln Austria; Veronika Nagl; Barbara Novak). 1 joint publication.
- Toxicity of NX. University of Carleton, (Canada; David Miller). 2 joint publications.
- Study of the intestinal secretome exposed to mycotoxins. Veterinary University Vienna (Austria; Ingrid Miller). 1 project acquired and 3 joint publication.
- Discovery and validation of salivary biomarkers of mycotoxin exposure in pigs. Veterinary Faculty University of Murcia (Spain; Ana María Gutiérrez Montes). 2017. 9 joint publications.
- Histopathological evaluation of the toxicity of mycotoxins in pigs. Veterinary Faculty University of Murcia (Spain; Juan José Pallarés and Juan Seva). 1 joint publication.
- Study of the intestinal effect of mycoestrogens. Veterinary Faculty, University of Liège (Belgium; Marie-Louise Scippo). Project funded through the Innovative Training Network (ITN) project Protected.
- Evaluation of the effect of emerging mycotoxins using CALUX® bioassays. BioDetectors Systems (The Netherlands; Bart van der Burg). Project funded through the Innovative Training Network (ITN) project Protected. 1 joint publication.

1.2. Academic activities

1.2.a. Teaching activities:

During doctoral studies: 36 teaching hours on Clinical Veterinary Propedeutics (2007-2009; Grade in Veterinary Science, UMU, Spain). Participation in three teaching training courses (177 training hours) organized by the Sciences of Education Institute, UMU, Spain. Student everyday supervision and lab training (6 PhD students). Nine invited seminars/workshops.

As a CRCN: teaching participation in a module in preclinical toxicology of the master 2 "Pharmacological innovation and pharmaceutical industry". Coordination of the organization and teaching on a 3-days seminar on Food Toxicology for PhD students funded by the ITN "ProtectED". Participation in different outreach activities organized in the unit such as the "Researcher's Night", as well as with the Society of Spanish Researchers in France (SIEF; I am co-delegate of the Toulouse section) in collaboration with the Cervantes Institute of Toulouse.

1.2.b. Supervision of students:

When working as a research assistant in the University of Murcia I was in charge of the everyday supervision and training two PhD students (Damián Escribano and María Fuentes) developing and validating diagnostic methods to evaluate stress using saliva samples. I also supervised the visiting PhD student Francesca Dilda from the University of Milan (Italy), developing antibodies to detect different health biomarkers in cow.

During my stay at Prof. Niewold's lab at KU Leuven (Belgium) I was also in charge of the supervision of three PhD students: I worked with Alireza Khadem in planning chicken feed trials and results interpretation, as well as some *in-vitro* assays. In the case of Sanne Otts, I assisted in planning stress trials in pigs, collecting saliva samples, selecting which biomarkers she should measure and interpreting results. I also helped the PhD student Els Willems in interpreting proteomics results and performing adequate validation tests.

During the last years I have been the responsible of the everyday training of four PhD students working in evaluating the toxic effect of mycotoxins, two M2 (Aurora Bertaccini, and Carine Al-Ayoubi) and one M1 (Aleksandra Andonova) students. Two of the PhD students were visiting from University of Turin (Shiva Gadhiri, Italy; 3 months in 2017) and the University of Amsterdam/BDS Biosystems (Clemence Budin, The Netherlands; 3 months in 2019). I co-directed the other PhD students: Tarek Lahjouji, who defended his thesis in January 2021 and Carine Al-Ayoubi, who defender her PhD in February 2023.

1.2.c. Support and management activities:

I am a member of the Animal Welfare Structure and the Animal Facility User's committee of the unit ToxAlim since 2019, and I participate with the Carnot Institute F2E as the contact person for my unit. I have also participated in different work groups within the Animal Health Department including one aiming at applying and innovative design approach (C-K) to propose new scientific strategies for the department in the upcoming years.

1.3. Grants projects and funding

1.3.a. Participation in Research Projects and Contracts:

As main researcher

Name and Involved Institutions	Funding body and period	Role
PhD Fellowship under the national Spanish competitive program FPU (Training for University Professors).	SMSE. 2006-2010	PhD national competitive grant, recipient
"Discovery and validation of intestinal health biomarkers and the effect of candidate immunomodulatory feed additives in the pig"	Martin Escudero Foundation. 2012-2014	Postdoctoral competitive grant, recipient
"AgreenSkills" fellowship (under grant agreement n° 267196) and the French National infrastructure CRB-Anim with the project "Advancing in domestic birds reproduction technology through-omics techniques" since February 2014	7th Framework programme. European Commission. 2014-2016	Postdoctoral competitive grant, recipient
"Enterocyte molecular phenotyping associated with the exposure to different mycotoxin mixtures (PhenoMyco)"	INRAE Animal Health Department starting grant, 2017	Principal investigator
"Characterization of biomarkers of mycotoxin effect"	Campus France, PHC Amadeus programme	Principal investigator
"Toxicity and mitigation of Versicolorin A, a new fungal mycotoxin"	ANR JCJC. 2019-2023	Principal investigator
« Caractérisation de l'impact sur la transcription de la mycotoxine Versicolorine A »	SA GOS SSD 2021-2022	Principal investigator

As participant

Name and Involved Institutions	Funding body and period	Role
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"Applications of ultrasensitive assays for acute phase proteins and immunoglobulins measurements in saliva of pigs". UMU.	SMSE. 2006-2009.	Participant
"Applications of acute phase proteins to health inspection systems at slaughter". UMU.	Seneca Institute. 2009-2010	Participant
"Proteome analysis in porcine saliva samples". University of Murcia and VetMed Vienna (Institute of Medical Chemistry).	SMSE. 2009-2010	Participant
"Protein profiles analysis in an experimental test in rats". UMU.	CEBAS-CSIC. 2007-2008.	Participant
"BIN-018 administration to dogs submitted to chemotherapy and evaluation of the effect in immune system parameters". UMU.	BioIberica S.A. 2008	Participant
"Veterinary clinical analysis for experimental studies". UMU.	IMASDE Agroalimentaria SL. 2009-2015	Participant
"Acute phase proteins determinations in pigs". UMU.	CEBAS-CSIC. 2009	Participant
"Salivary acute phase protein determination in pigs for stress monitoring".UMU.	University of Newcastle, 2010	Participant
"Sangrovit testing in poultry". KU Leuven	Phytobiotics Futterzusatzstoffe GmbH 2013	Participant
"Sangrovit testing in cattle". KU Leuven	Phytobiotics Futterzusatzstoffe GmbH 2013	Participant
"Butyrate testing in poultry". KU Leuven	Sanluc International. 2013	Participant
"Oxytetracycline as a growth promotor in pigs".KU Leuven	Huvepharma Inc. 2012	Participant
"The development of an automated method to monitor pig welfare and health". KU Leuven	IWT- Agentschap voor Innovatie door Wetenschap en Technologie. 2012-2013	Participant
"ValBioDi: Strategies and conservation issues of regional endangered poultry breeds". INRA-Val de Loire, SYSAAF, BioDom Centre.	AAD Région Centre and partenaires. 2014-2015.	Participant
"Centre de ressources biologiques pour les animaux domestiques CRB-Anim"	Investissements d'avenir", ANR-11-INBS-0003	Participant
Détermination d'une signature « omique » caractéristique de l'exposition du porc aux mycotoxines. INRAE Toulouse	ANR-F2E, 2018-2020	Co-coordinator WP
Les mycotoxines émergentes : un nouveau risque pour l'Homme et les animaux ? INRAE Toulouse	ANR, 2019-2023	Coordinator WP
Développement d'un modèle d'organoïdes intestinaux de porcelets pour tester des stratégies innovantes de préservation de la santé digestive . INRAE Toulouse	ANR-F2E, 2020-2023	Coordinator WP

SECTION 2

Publications

*Peer-reviewed research articles (N=33; 16 as first author and 4 as last author; *Shared first authorship)*

1. E. Mussard, C. Lencina, L. Gallo, C. Barilly, M. Poli, K. Feve, M. Albin, L. Cauquil, C. Knudsen, C. Achard, G. Devailly, **L. Soler**, S. Combes and M. Beaumont. (2022) The phenotype of the gut region is more stably retained than developmental stage in piglet intestinal organoids. *Front. Cell Dev. Biol.*, 2022, 10:983031. <https://doi.org/10.3389/fcell.2022.983031>.
2. K.L. Gützkow, C. Al Ayoubi, **L. Soler**, S. Rohn, R. Maul (2022) Analysis of ochratoxin A, aflatoxin B1 and its biosynthetic precursors in cheese – Method development and market sample screening. *Food Control*, 2022, pp.109241. <https://dx.doi.org/10.1016/j.foodcont.2022.109241>.
3. C. Al-Ayoubi, J. Oules, E. Person, S. Bruel, A. Bouville, P. Pinton, I.P. Oswald, E.L. Jamin, O. Puel, **L. Soler** (2022) Metabolism of versicolorin A, a genotoxic precursor of aflatoxin B1: Characterization of metabolites using *in vitro* production of standards. *Food and Chemical Toxicology*, 288, pp.113272. <https://dx.doi.org/10.1016/j.fct.2022.113272>.
4. **L. Soler**, I. Miller, C. Terziolo, K. Hummel, K. Nöbauer, M. Neves, I.P. Oswald (2022) Exposure of intestinal explants to NX, but not to DON, enriches the secretome in mitochondrial proteins. *Archives of Toxicology*, <https://dx.doi.org/10.1007/s00204-022-03318-x>.
5. V. Nagl, B. Grenier, P. Pinton, U. Ruczizka, M. Dippel, M. Bünger, I.P. Oswald, **L. Soler** (2021) Exposure to Zearalenone Leads to Metabolic Disruption and Changes in Circulating Adipokines Concentrations in Pigs. *Toxins*, 13 (11), 790, <https://dx.doi.org/10.3390/toxins13110790>.
6. **L. Soler***, S. Alves*, A. Brionne, A. Jacques, V. Guérin, M. Cherif-Feildel, L. Combes-Soia, S. Fouchécourt, A. Thélie, E. Blesbois, M.J. Mcgrew, V. Labas, M. Govoroun (2021) Protein expression reveals a molecular sexual identity of avian primordial germ cells at pre-gonadal stages. *Scientific Reports*, 11 (1), <https://dx.doi.org/10.1038/s41598-021-98454-2>.
7. C. Budin, H.Y. Man, C. Al-Ayoubi, S. Puel, B. van Vugt-Lussenburg, A. Brouwer, I.P. Oswald, B. van der Burg, **L. Soler** (2021) Versicolorin A enhances the genotoxicity of aflatoxin B1 in human liver cells by inducing the transactivation of the Ah-receptor. *Food and Chemical Toxicology*, 153, 112258, <https://dx.doi.org/10.1016/j.fct.2021.112258>.
8. A. Pierron, M. Neves, S. Puel, Y. Lippi, **L. Soler**, J.D. Miller, I.P. Oswald (2021) Intestinal toxicity of the new type A trichothecenes, NX and 3ANX. *Chemosphere*, 288, 132415 <https://dx.doi.org/10.1016/j.chemosphere.2021.132415>.
9. A. Vitorino Carvalho*, **L. Soler***, A. Thélie, I. Grasseau, L. Cordeiro, D. Tomas, A.P. Teixeira-Gomes, V. Labas, E. Blesbois (2021) Proteomic Changes Associated With Sperm Fertilizing Ability in Meat-Type Roosters. *Frontiers in Cell and Developmental Biology*, 9, 15 p., <https://dx.doi.org/10.3389/fcell.2021.655866>.
10. A. Gutiérrez, A. Bassols, **L. Soler**, M. Piñeiro (2020) Editorial: Factors Influencing Biomarker Range Intervals in Farm Animals. *Frontiers in Veterinary Science*, 7, <https://dx.doi.org/10.3389/fvets.2020.587741>.
11. D. Brea*, **L. Soler***, I. Fleurot, S. Melo, C. Chevalyre, M. Berri, V. Labas, A.P. Teixeira-Gomes, J. Pujo, N. Cenac, A. Bähr, N. Klymiuk, A. Guillon, M. Si-Tahar, I. Caballero (2020) Intrinsic alterations in peripheral neutrophils from cystic fibrosis newborn piglets. *Journal of Cystic Fibrosis*, 19 (5), 830-836, <https://dx.doi.org/10.1016/j.jcf.2020.02.016>.
12. T. Lahjouji, A. Bertaccini, M. Neves, S. Puel, I.P. Oswald, **L. Soler** (2020) Acute Exposure to Zearalenone Disturbs Intestinal Homeostasis by Modulating the Wnt/ β -Catenin Signaling Pathway. *Toxins*, 12 (2), 113, <https://dx.doi.org/10.3390/toxins12020113>.
13. **L. Soler**, A. Stella, J. Seva, F.J. Pallarés, T. Lahjouji, O. Burlet-Schiltz, I.P. Oswald (2020) Proteome changes induced by a short, non-cytotoxic exposure to the mycoestrogen zearalenone in the pig intestine. *Journal of Proteomics*, 224, 1-11, <https://dx.doi.org/10.1016/j.jpro.2020.103842>.
14. **L. Soler**, R. Hermes, T.A. Niewold (2016) *Macleaya cordata* extract reduces inflammatory responses of intestinal epithelial cells *in vitro*. *American Journal of Plant Sciences*, 07 (11), 1531-1537, <https://dx.doi.org/10.4236/ajps.2016.711144>.

15. **L. Soler**, I. Miller, K. Hummel, E. Razzazi-Fazeli, F. Jessen, D. Escibano, T. Niewold (2016) Growth promotion in pigs by oxytetracycline coincides with down regulation of serum inflammatory parameters and of hibernation-associated protein HP-27. *Electrophoresis*, 37 (10), 1277-1286, <https://dx.doi.org/10.1002/elps.201500529>.
16. **L. Soler**, V. Labas, A. Thelie, I. Grasseau, A.P. Teixeira-Mechin, E. Blesbois (2016) Intact cell MALDI-TOF MS on sperm: a molecular test for male fertility diagnosis. *Molecular and Cellular Proteomics*, 15, 1998-2010, <https://dx.doi.org/10.1074/mcp.M116.058289>.
17. **L. Soler**, I. Miller, K. Nöbauer, S. Carpentier, T. Niewold (2015) Identification of the major regenerative III protein (RegIII) in the porcine intestinal mucosa as RegIII γ , not RegIII α . *Veterinary Immunology and Immunopathology*, 167 (1-2), 51-56, <https://dx.doi.org/10.1016/j.vetimm.2015.07.006>.
18. F. Ceciliani, **L. Soler**, G. Grilli, A.T. Marques, C. Giudice, C. Lecchi (2015) The localization and differential expression of Serum Amyloid A in bovine liver and adipose tissue depots. *Veterinary Immunology and Immunopathology*, 168 (1-2), 35-39, <https://dx.doi.org/10.1016/j.vetimm.2015.08.004>.
19. S. Ott, **L. Soler**, C.P.H. Moons, M.A. Kashiha, C. Bahr, J. Vandermeulen, S. Janssens, A.M. Gutiérrez, D. Escibano, J.J. Cerón, D. Berckmans, F.A.M. Tuytens, T.A. Niewold (2014). Different stressors elicit different responses in the salivary biomarkers cortisol, haptoglobin, and chromogranin A in pigs. *Research in Veterinary Science*, 97 (1), 124-128, <https://dx.doi.org/10.1016/j.rvsc.2014.06.002>.
20. E. Willems, T.T. Hu, **L. Soler**, J. Buyse, E. Decuypere, L. Arckens, N. Everaert (2014). Embryonic protein undernutrition by albumen removal programs the hepatic amino acid and glucose metabolism during the perinatal period in an avian model. *PLoS ONE*, 9 (4), np, <https://dx.doi.org/10.1371/journal.pone.0094902>.
21. A. Khadem, **L. Soler**, N. Everaert, T.A. Niewold (2014). Growth promotion in broilers by both oxytetracycline and *Macleaya cordata* extract is based on their anti-inflammatory properties. *British Journal of Nutrition*, 112 (07), 1110-1118, <https://dx.doi.org/10.1017/S0007114514001871>.
22. **L. Soler**, A. Molenaar, N. Merola, D. Eckersall, A.M. Gutiérrez, J.J. Cerón, V. Mulero, T.A. Niewold (2013). Why working with porcine circulating serum amyloid A is a pig of a job. *Journal of Theoretical Biology*, 317, 119-125, <https://dx.doi.org/10.1016/j.jtbi.2012.10.011>.
23. D. Escibano, **L. Soler**, A.M. Gutiérrez, S. Martínez-Subiela, J.J. Cerón (2013). Measurement of chromogranin A in porcine saliva: validation of a time-resolved immunofluorometric assay and evaluation of its application as a marker of acute stress. *animal*, 7 (04), 640-647, <https://dx.doi.org/10.1017/S1751731112002005>.
24. A.M. Gutiérrez, K. Nöbauer, **L. Soler**, E. Razzazi-Fazeli, M. Gemeiner, J.J. Cerón, I. Miller (2013). Detection of potential markers for systemic disease in saliva of pigs by proteomics: a pilot study. *Veterinary Immunology and Immunopathology*, 151 (1-2), 73-82, <https://dx.doi.org/10.1016/j.vetimm.2012.10.007>.
25. **L. Soler**, A. Gutiérrez, A. Müllebner, J.J. Cerón, J.C. Duvigneau (2013). Towards a better understanding of salivary and meat juice acute phase proteins determination in pigs: An expression study. *Veterinary Immunology and Immunopathology*, 156 (1-2), 91-98, <https://dx.doi.org/10.1016/j.vetimm.2013.09.018>.
26. **L. Soler**, A. Gutiérrez, D. Escibano, M Fuentes, J.J. Cerón (2013) Response of salivary haptoglobin and serum amyloid A to social isolation and short road transport stress in pigs. *Research in Veterinary Sciences*, 95(1), 298-302. <https://dx.doi.org/10.1016/j.rvsc.2013.03.007>.
27. **L. Soler**, A. Gutiérrez, José J. Cerón (2012). Serum amyloid A measurements in saliva and serum in growing pigs affected by porcine respiratory and reproductive syndrome in field conditions. *Research in Veterinary Science*, 93 (3), 1266-1270, <https://dx.doi.org/10.1016/j.rvsc.2012.06.008>.
28. **L. Soler**, T. Luyten, A. Stinckens, N. Buys, J.J. Cerón, T.A. Niewold (2011). Serum amyloid A3 (SAA3), not SAA1 appears to be the major acute phase SAA isoform in the pig. *Veterinary Immunology and Immunopathology*, 141 (1-2), 109-115, <https://dx.doi.org/10.1016/j.vetimm.2011.02.019>.
29. **L. Soler**, A.M. Gutiérrez, S. Martínez-Subiela, J.J. Cerón (2011). Development and validation of a novel competitive ELISA for the detection of serum amyloid A in pigs. *Veterinary Journal*, 190 (2), e7-e11, <https://dx.doi.org/10.1016/j.tvjl.2011.02.016>.

30. **L. Soler**, A.M. Gutiérrez, S. Martínez-Subiela, J.J. Cerón (2011). Fast measurement of serum amyloid A in different specimens from swine by using a new one-step time-resolved fluorescent immunoassay. *Journal of Veterinary Diagnostic Investigation*, 23 (5), 902-908, <https://dx.doi.org/110.1177/104063870802000615>.
31. Gutiérrez, A.M., S. Martínez-Subiela, **L. Soler**, F.J. Pallarés, J.J. Cerón (2009) Use of saliva for haptoglobin and C-reactive protein quantifications in porcine respiratory and reproductive syndrome affected pigs in field conditions. *Veterinary Immunology and Immunopathology*, 93 (3), 118-223, <https://dx.doi.org/10.1016/j.vetimm.2009.06.013>.
32. **L. Soler**, S. Martínez-Subiela, F. Tecles, A.M. Gutierrez, P. Fuentes, J.J. Cerón (2008) Serum total sialic acid in pigs: new possibilities for an old inflammatory biomarker. *Journal of Veterinary Diagnostic Investigation*, 20 (6), 799-803, <https://dx.doi.org/10.1177/104063870802000507>.
33. F.H. González, F. Tecles, S. Martínez-Subiela, A. Tvarijonavičiute, **L. Soler**, J.J. Cerón (2008) Acute phase protein response in goats. *Journal of Veterinary Diagnostic Investigation*, 20 (5), 580-584, <https://dx.doi.org/10.1177/104063870802000507>.

Review articles in peer-review journals (N= 5; 3 as first author, one as last author)

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Invited:

Unmasking the toxicity of AFB1 precursors: the case of Versicolorin A. Presented at : 9th Mycotoxin days, Marseille, France (2022-09-21), <https://hal.inrae.fr/hal-03720586>

Versicolorin A and Aflatoxin B1: like father, like son?. Presented at : 1st International Electronic Conference on Toxins, Toulouse, France (2021-01-16), <https://hal.inrae.fr/hal-03354729>

Zearalenone and pigs : to reprotoxicity and beyond. Presented at : World Mycotoxin Forum. Virtual pre-conference on Animal Health, Parma, Italie (2021-11-30), <https://hal.inrae.fr/hal-03367033>

Oral presentations:

Molecular phenotyping of intestinal epithelial cells exposed to mycotoxins. Presented at : 34. Réunion annuelle du Club d'Etudes des Cellules Epithéliales Digestives (CECED), Toulouse, France (2019-03-22 - 2019-03-23), <https://hal.inrae.fr/hal-02791539>

A new molecular test for male fertility diagnosis: Intact cell MALDI-TOF MS on sperm. Presented at : 25. World's Poultry Congress, Beijing, Chine (2016-09-05 - 2016-09-09), conférence invitée. <https://hal.inrae.fr/hal-02741433>

Avancée dans les technologies de la reproduction aviaire à travers des méthodes peptido-protéomiques/Proteome differences between male and female chicken primordial germ cells (PGCs). Presented at : Annual Meeting of CRB-Anim WP 2.2, Nouzilly, France (2015-12 - 2015-12), <https://hal.inrae.fr/hal-02801921>

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An integrative study of the early immune response against ETEC. Presented at : 5. Management Committee Meeting and 4. Meeting of Working Groups 1,2 & 3 of COST Action FA 1002, Milan, Italie (2014-11-17 - 2014-11-18), <https://hal.inrae.fr/hal-01605757>

Reproductive cells phenotyping in domestic birds through proteomics/Proteomic and epigenetic analysis of chicken primordial germ cells (PGCs). Presented at : CRB ANIM - Réunion du Réseau de Centres de Ressources Biologiques pour les animaux domestiques, Rennes, France (2014-11-26 - 2014-11-27), <https://hal.inrae.fr/hal-02793999>

Intact Cell MALDI-TOF MS : a male chicken fertility phenotypic tool. Presented at : Final Showcase Meeting of Farm Animal Proteomics, Milan, Italie (2014-11-17 - 2014-11-18), <https://hal.inrae.fr/hal-02796244>

Small intestinal response to enterotoxigenic Escherichia coli infection in pigs as revealed by label free UPLC/HDMSE proteomics. Presented at : 3. Meeting of Working Groups 1, 2 and 3 of COST Action FA1002, Kosice, Slovaquie (2013-04-25 - 2013-04-26), <https://hal.inrae.fr/hal-01605759>

Shotgun proteomics or how to see pig intestinal innate immune responses in another light. Presented at : 10th International Symposium of Veterinary Immunology, Milan, Italie (2013 - 2013), <https://hal.inrae.fr/hal-01605758>

Identification of a promising pig intestinal health marker as regenerating islet-derived 3-gamma (REG3g). Presented at : 1. Symposium of the Belgian Proteomics Association, Ghent, Belgique (2012 - 2012), <https://hal.inrae.fr/hal-01605766>

Porcine SAA is not likely an apolipoprotein bound to circulating HDL3. Presented at : 8. European Colloquium on Acute Phase Proteins, Helsinki, Finlande (2010-08-26 - 2010-08-26), <https://hal.inrae.fr/hal-01607287>

Patents, declaration of invention and intellectual protection of results

Declaration of invention: **L. Soler**, O. Galland, A.P. Teixeira-Mechin, E. Blesbois, V. Labas (2016). Base de données protéomiques Top down en reproduction animale. (Brevet n°: Déclaration d'invention DI-RV-16-0009). France
<https://hal.inrae.fr/hal-01606433>

Declaration of invention: **L. Soler**, E. Blesbois, V. Labas, A. Thelie (2016). Phénotypage moléculaire par ICM-MS de la semence. (Brevet n°: Déclaration d'invention et de résultats valorisables DI-RV-16-0010). France
<https://hal.inrae.fr/hal-01606488>

SECTION 3

Summary of early achievements

In spite of my training as a veterinarian and my taste for clinical courses, I soon recognized a passion for basic science. I thus pursued a scientific carrier oriented at understanding the physiopathology of biological systems with the objective of finding practical solutions for veterinarians, breeders and consumers. This vision of research has led me to gain training in disciplines that are rare for a veterinarian, such as mass spectrometry-based proteomics, but always seeking to translate my findings into useful tools for the scientific community and society. A brief description of my previous positions is found below in chronological order. The publications associated with the activities presented here are indicated in blue.

Doctoral studies and works as Research Assistant. 2006 - 2012. Department of Animal Medicine and Surgery, Veterinary School, University of Murcia (Spain) and Department of Animal Pathology, Hygiene and Veterinary Public Health DIPAV, Veterinary School, University of Milan (Italy). Dissertation entitled “New contributions on porcine Serum Amyloid A”

I obtained my **Degree in Veterinary Medicine** in September 2005 with honors, and even if I started my studies of veterinary medicine with the objective of being a clinician, I soon recognized a call for science. I hence started collaborating with research activities before obtaining my diploma, first by doing internships as a student and after by performing my own project, financed by an Undergraduate Research Fellowship from the Spanish Ministry of Science and Education. I completed my degree with a minor dissertation on the possible use of Sialic Acid as a health marker in pigs (32). Soon after, I got the national award “Outstanding Graduate of the Year in Veterinary Medicine” and then won a **competitive national grant** to perform my **PhD** at the **Department of Animal Medicine and Surgery of the Veterinary School from the University of Murcia** (Spain) under the supervision of Prof. **Jose J. Cerón Madrigal**, Dr. **Silvia Martínez Subiela** and Prof. **Víctor Mulero Méndez**.

My dissertation was entitled “*New contributions on porcine Serum Amyloid A*”, since it was a compendium on information gathered throughout the years explaining the biochemistry and biology of the protein Serum Amyloid A in pigs, as well as describing the development of diagnostic tests to measure this protein. Serum amyloid A (SAA) is a well-known acute phase protein and health biomarker in pigs, but no reliable SAA determination methods were commercially available (only a multispecies ELISA with low precision in pigs), and thus its implementation as diagnostic biomarker was not possible. The reason for that is the difficulty in isolating this unstable, hydrophobic protein and its low antigenicity. In fact, it was cited in the literature as “the protein from hell”¹. The first objective of my Thesis was the development of an immunometric assay to measure SAA in pig serum samples. I started by trying to purify SAA from pig serum using conventional chromatographic protocols known to be useful for the purification of SAA in different species. However, pure pig SAA could only be obtained through over-expression of recombinant protein in *E.coli*. This protein (known as poorly antigenic) was used to produce (from scratch) different in-house rabbit and egg yolk polyclonal antibodies as well as rat hetero-hybridomas from which monoclonal antibodies were produced. These reagents were used to produce two types of in-house immunoassays: a competitive ELISA (29) and an ultrasensitive time-resolved immunofluorometric assay (TR-FIA; 30), which was adapted for alternative samples like saliva and meat juice (27). Both assays showed better analytical parameters than the commercial ELISA. They were used in different field studies, and confirmed the usefulness of SAA as a health biomarker in pigs. I also collaborated in my research team in different projects involving the discovery and application of other health biomarkers in pigs and goats (32,33). The second objective of my thesis was to investigate the particular

characteristics of pig SAA. It was evidenced that the biochemical proprieties of pig SAA were different of that from other species, and that the impossibility to purify this protein through traditional protocols as well as other particularities –described by other colleagues- could be finally explained (22, 28). In all, the results from this PhD project helped advancing in the knowledge of the biology of SAA in the pig, and provided with new tools to adapt the clinical use of this promising health biomarker.

After finishing my PhD in June 2010 I continued collaborating with my home research group until May 2011, working in a postdoctoral project entitled “New stress biomarkers discovery in porcine saliva”, funded by the Ministry of Science and Innovation of Spain. The objective of this project was to identify and validate new stress biomarkers in pig saliva (24, 26). Several proteins that could serve as stress biomarkers were identified, such as alpha-amylase and chromogranin A (23). Specific pig reagents (pure protein and antibodies) were produced in-house, and new diagnostic tests based in the use of time-resolved immunofluorometry were successfully developed and applied in pig saliva during stress trials. In parallel, I was also in charge of the supervision of two international PhD students, whose projects involved pig health biomarker research. I also studied the expression of health/stress biomarkers in the pig salivary gland compared with their salivary protein abundance, as well as with other tissues and body fluids in order to better interpret health biomarker measurements in alternative fluids (25).

Shortly after, in September 2011 I was invited to visit the team of **Dr Fabrizio Ceciliani** at the **Department of Animal Pathology, Hygiene and Veterinary Public Health DiPaV, Veterinary School, University of Milan (Italy)** for a period of four months thanks to the project “FoBoS: Sharing molecular techniques for food-borne pathogen detection” and the COST action FA1002 Farm Animal Proteomics. The first objective of this project was evaluating serum abundance and pulmonary mRNA expression of several acute phase proteins during pig pulmonary diseases. Samples from pigs naturally suffering from respiratory diseases were collected, and classified after complete anatomo-pathological analysis. Levels of several acute phase markers (C-reactive protein, Haptoglobin and SAA) were determined in serum and compared with their pulmonary and liver expression. This was completed with the investigation of the SAA isoform composition of serum and saliva from the same animals. The second objective of this study was producing two new antibodies to detect Serum Amyloid A and acid alpha-1 glycoprotein (a health biomarker) in goats and cows. The first antibody was later employed to describe the localization of SAA in tissue depots in cows (18).

Post-doctoral fellow. January 2012 - January 2014. Division of Livestock-Nutrition-Health, Biosciences Engineering Faculty, KU Leuven (Belgium). Project “Discovery and validation of intestinal health biomarkers and the effect of candidate immunomodulatory substances in the pig”.

In January 2012 I joined the laboratory of Prof. **Theo A. Niewold** at the Division of Livestock-Nutrition-Health, at the Biosciences Engineering Faculty in the **KU Leuven (Belgium)** with a two-years competitive grant from the Martin Escudero Private Foundation (Spain). The research project was entitled “**Discovery and validation of intestinal health biomarkers and the effect of candidate immunomodulatory feed additives in the pig**”. The objective of this project was to investigate the pig intestinal pathophysiology during infection with the enteric pathogen Enterotoxigenic *Escherichia coli* (ETEC), as well as evaluating the effect of different foodstuffs such as banned growth-promoting antimicrobial substances (e.g. oxytetracycline) and natural extracts with immunomodulatory and/or growth promoting activities (e.g. sanguinarine). It was performed in the KU Leuven (Belgium) under direct international collaboration with the laboratory of Mass Spectrometry of GIGA Liège (Belgium), the Lelystad Central Veterinary Laboratory / Wageningen UU (The Netherlands) as well as the Institute of Biomedical Chemistry, Veterinary School Vienna (Austria).

Prof. Niewold and his team had a wide experience in pig intestinal pathophysiology, and they were in possession of a huge collection of intestinal samples produced in different *in-vivo* ETEC infection experiments or farm sampling campaigns. These sample sets were very well characterized for their clinical outcome, and they were employed to produce a big amount of transcriptomics and RT-qPCR data. My first research experience was to compare these results with those obtained through high-throughput proteomic analysis in the same samples, in order to better understand the molecular processes happening in the intestinal mucosa shortly after infection (24h p.i.). I performed mass spectrometry-based proteomic analyses (more specifically using a technique named nLC-HDMS^E) on pig intestinal mucosa scrapings at the laboratory of mass spectrometry of GIGA Liège (Belgium; Prof. de Pauw and Dr. Mazzucchelli). Results showed that proteomics and transcriptomics are highly complementary, and that their joint interpretation allows for a better understanding of the early stages of ETEC infection. Following the same approach as above, I performed a complete proteomic analysis on samples derived from several ETEC infection experiments using the *ex-vivo* intestinal modeling system called SISP (Small Intestinal Segment Perfusion). I got a two-weeks travel grant awarded by the European COST action FA1002 Farm Animal Proteomics 2012 to visit the laboratory of Prof. Smits to (1) get some theoretical training on the SISP protocol, (2) to obtain the samples and associated data, as well as discussing objectives, and (3) to receive valuable training in data mining and systems biology. Samples were analyzed at GIGA Liège by mass spectrometry-based proteomics (nLC-HDMS^E) and results were interpreted together with existing microarray results, which were re-analyzed using new bioinformatic tools. Results allowed us describing the molecular events associated with ETEC infection. The host lab also had experience in investigating the molecular basis of pig resistance to ETEC infection (the presence of the F4-receptor in pig intestinal cells makes them more susceptible to ETEC colonization). Previous works using genomics and transcriptomic approaches contributed with valuable information, but were not sufficient. Some of the pig intestinal samples present in the lab collection that were characterized for F4-receptor presence/absence were selected. I evaluated the proteomic (nLC-MS/MS) differences between the epithelial cells recovered from F4-receptor-positive and F4-receptor-negative piglets. This work resulted in the discovery of several molecular features of ETEC resistance in the pig. During our proteomic analyses we consistently observed that the levels of the protein RegIIIg were related with ETEC infection and diarrhea, which was in accordance with previous mRNA expression data produced by RT-qPCR. No commercial antibodies with cross-activity with pig RegIIIg were available so specific reagents were produced and used to evaluate the abundance of this protein in different experimental conditions. This allowed us to understand the biology of this protein during infection, and to evaluate its possibilities as intestinal health biomarker (17).

An additional objective of my project was the evaluation of the effect of different feed additives, such as the banned antimicrobial growth promoters or AGPs. These substances were mostly antibiotics that were added daily to the feed of farm animals to promote their growth. Their recent ban in the European Union has brought the need of looking for new natural alternatives that might promote growth but being harmless to animals and men. However, the real mechanism of action of AGPs is not clear, and so we were interested in using proteomics to investigate this topic. The pig feed experiments using oxytetracycline were performed in the experimental farm of the KU Leuven, whereas the gel-based proteomic analysis (2-DE DIGE combined with mass spectrometry) was carried out during a training visit to the Institute of Biomedical Chemistry, Veterinary School Vienna (Austria). We found out that the differences in the serum proteome of pigs that were treated orally with low doses of oxytetracycline could be associated with changes in their immuno-metabolic status, and that it could help understanding how AGPs promote growth (15). This possible anti-inflammatory role of AGPs were in agreement with results produced in our laboratory using broiler chicken feed trials, and after *in-vitro* tests performed in IPEC-J2 cultured cells using acetylsalicylic acid and sanguinarine, two extra experiences in which I was also directly involved (14, 21).

Post-doctoral fellow. February 2014 - July 2016. UMR PRC, URA, PAIB2, Agricultural Research National Institute (INRA)-Centre Val de Loire (France). Project “Advancing in domestic birds reproduction technology through-omics techniques”.

In 2014 I got the competitive mobility fellowships known as AgreenSkills, funded by the Marie-Curie FP7 COFUND People Programme (under grant agreement n° 267196) and INRAE with the project entitled “**Advancing in domestic birds’ reproduction technology through -omics techniques**”. This project was performed under the supervision of **Dr Elisabeth Blesbois** at the UMR PRC, at the **INRAE-Centre Val de Loire (France)** and supported by the French National Infrastructure of Research “CRB-Anim”. This project proposed the use of advanced proteomic techniques to study the physiology of reproduction in birds. Beyond the actual application of the techniques proposed in the project (I had some training but no research experience in reproduction), I was attracted by the novelty of the approach, the possibility of mining deeper into proteomic techniques I never used before and the quality of the project. The proteomic experiences described below were performed in the platform PAIB2 (today PIXANIM) at the UMR PRC of the same center, under collaboration with Valérie Labas and Ana-Paula Teixeira.

This project had two main objectives: the proteomic characterization of (1) chicken sperm and (2) chicken primordial germ cells.

1. In the need of new molecular tools to define male fertility, we employed a proteomic approach known as Intact Cells MALDI-TOF Mass Spectrometry (ICM-MS). ICM-MS is used in microbiology to aid in phenotyping, but has been rarely applied in eukaryotic cells. In brief, with this technique we can obtain a MS profile representing low-molecular weight peptides and proteins contained in a cell and their relative abundance. Given that the ICM-MS profiles obtained from fertile and subfertile spermatozoa were significantly different, we were able to construct fertility-predictive mathematical models based on chicken sperm cells ICM-MS profiles. The developed method showed higher diagnostic accuracy than any other in-vitro traditional sperm quality test (16). This new diagnostic tool was the object of an Invention Disclosure statement (ref number DI-RV-16-0010). Besides, the test attracted the attention of different private breeding companies (ALLICE, Hubbard, Novogen, SYSAAF) and fellow researchers, and different collaborations were set up to apply this methodology beyond reproduction. Notably, I collaborated with Dr. Ignacio Caballero-Posadas (UMR ISP-Infectious diseases and public health) in the ICM-MS-based proteomic analysis of pig neutrophils in a model of cystic fibrosis (11), and with Dr. Svetlana Uzbekova and Dr. Xavier Druart (UMR PRC) for the application of the previously mentioned technique in female reproduction technology.

In order to complete the development of the ICM-MS methodology, it was necessary to identify the peptides and proteins represented in the ICM-MS profiles. The most adequate proteomic approach for this is called top-down High-Resolution Mass Spectrometry (HRMS) protein identification analysis. The objective of this technique is the identification of intact endogenous peptides (<15kDa) contained in a sample, along with some structural information (phosphorylation, acetylation, etc). Results revealed that ICM-MS spectra mainly comprise degradation products of proteins implicated in the most important functional pathways in sperm, and helped characterizing our diagnostic tool. Additionally, some fertility biomarker candidates were identified and validated. All the information related with the sperm peptide repository and potential biomarkers was gathered and included in a database, which was the object of an Invention Disclosure statement (ref number DI-RV-16-0009).

To complete these proteomics studies in chicken sperm samples, the whole proteome of chicken sperm cells (analyzed with nLC-MS/MS) with high and low fertility abilities was compared to ICM-MS results in order to provide with a better understanding of the molecular basis of fertility, in a project coordinated together with Dr Anaïs Vitorino-Carvalho (9).

2. In addition to semen storage, the use of primordial germ cells (PGCs) complete the conservation of genetic variability, as embryo cryopreservation is not feasible in birds. PGCs differentiate into gametes that propagate genetic information from parent to offspring. Avian PGCs are very promising for genetic conservation and manipulation, as they can be collected from early embryonic chicken blood, cultured and cryopreserved. The main advantage of preserving PGCs is the preservation of the female chromosome in birds, since males are the homogametic sex (ZZ), while females are the heterogametic sex (ZW). Cultured male and female PGCs are functionally different (for instance, they require different culture conditions) but the molecular mechanisms underlying these differences are poorly understood. In collaboration with the team of Dr. Marina Govorun, chicken PGCs were isolated from embryos, successfully cultured *in vitro* and sexed. I evaluated the proteome differences of male and female PGCs using a classical mass-spectrometry proteomics approach (nLC-MS/MS), and results show significant differences in carbohydrate and lipid metabolism, as well as in gene expression regulation (6).

SECTION 4

Current research activities

In 2016, I joined the Biosynthesis and Toxicity of Mycotoxins Team (BioToMyc) of the Food Toxicology Unit (UMR 1331 ToxAlim) as Junior Researcher (CR2, today CRCN) on the profile “New mycotoxins in food chains: toxicological assessment”. The main objectives of the position was to investigate the toxicity and the impact on (pig) intestinal pathophysiology of toxic fungal metabolites present in food and feed known as mycotoxins. This new position allowed me to combine my previous competences and knowledge in pig physiopathology and the use of omics to discover the effect of mycotoxins, a world unknown to me. With my new position I integrated a multidisciplinary, dynamic team with an international background and very implicated in applied sciences, where I have discovered that mycotoxins are quite a challenging, interesting field of study. A description of my research activities is presented, with publications associated indicated in blue. A short paragraph on specific perspectives is included after each section, which is completed with a general perspectives section (research project) at the end of this document

4.1 State of the art and objectives

Mycotoxins are fungal secondary metabolites produced by several fungal genera that commonly colonize agricultural products such as grains, cereals, fruits, spices and animal forages, and that are toxic to human and animals at low doses². Mycotoxins are, therefore, natural food and feed contaminants and so their presence in the food chain is relatively unavoidable nowadays. They represent a major issue in food safety and public health, as their incidence is high (recent surveys indicate that 70% of raw materials are contaminated) they are related with the development, exacerbation and/or aggravation of very distinct syndromes, diseases and dysfunctions. Different factors define the real danger associated with the presence of mycotoxins in the food chain, which represent at the same time the main challenges in myco-toxicological research:

Challenge one: The discovery of new mycotoxins as technology advances. Mycotoxins can be classified as major, emerging and modified mycotoxins. Major mycotoxins are those whose main mechanism of toxicity is known and for whom regulations regarding their maximum levels in food or feed have been set. These mycotoxins are deoxynivalenol (DON), zearalenone (ZEN), patulin (PAT), ochratoxin A (OTA), aflatoxins (AFs), ergot alkaloids and fumonisin B1 (FB1). Emerging mycotoxins are those toxic fungal metabolites that have been identified relatively recently thanks to the development of more sensitive detection methods and the improvement of the characterization of fungal metabolomes³. Their toxicity and occurrence is less characterized and so no regulations exist. Examples of these mycotoxins are the aflatoxins precursors sterigmatocystin (STC) and versicolorin A (VerA), and the type A trichothecene NX. A third group of toxins are referred as modified mycotoxins, since they represent alternative chemical forms of the parent mycotoxins (major or emerging) and are produced by plant/host metabolization or during technological process⁴. As for emerging mycotoxins, the toxicity is poorly characterized, and its presence in food and feed is not regulated. The discovery/characterization of the risk related with the exposure to these relatively unknown mycotoxins represents a huge challenge.

Challenge two: Mycotoxins interact with each other and with other contaminants. Mycotoxins of different chemical nature can co-contaminate the same food commodities, and different food commodities can be combined during the preparation of diets. These toxic molecules can hence be present combined in different proportions and concentrations. More often than not, there can be an interaction between all the contaminants present in food,

including mycotoxins, giving place to unexpected antagonistic or agonistic toxic effects^{5,6}. The interaction of food contaminants with each other and with other food components is poorly described and scarcely taken into account to define the legal limits of each contaminant alone.

Challenge three: Multiple targets: where to start? Mycotoxins often have several target molecules at the genomic, transcriptomic, proteomic and metabolomic level. In consequence, if we want to characterize the toxic effect of these molecules, we need to combine different techniques for screening the effect on several cellular functions. In view of this context, it seems clear that following an -omics approach can present several advantages in exploring the mechanistic aspects of mycotoxin-induced pathologies⁷. The advantage in combining omics in mycotoxicology is backed up by the complementarity of the biological information that they provide. Together with this pre-clinic approach, results from omics have the potential of providing with promising candidate biomarkers of response to mycotoxins, with a possible use at the clinical level^{8,9}. These “molecular signatures” of mycotoxin effect can be used thus to (a) estimate the toxicity of an emerging mycotoxin of unknown function, (b) assessing the impact of a complex mixture, (c) evaluating the exposure to these contaminants in a big population or (d) the efficacy of a remediation measure.

Given the abovementioned, the main objective of my research project is **the evaluation of the impact of mycotoxins in human and animal health**. More specifically, I am mostly interested in deciphering the mode of action and molecular effects of both major and emerging mycotoxins at the intestinal level in the pig (both as a target and model species) using integrative approaches including the use of -omics.

4.2 Scientific activities

The results of the scientific activities performed since my arrival in the lab will be described divided in three parts: (1) research on the unknown toxicity of the regulated mycotoxins, (2) research on the toxicity of the emerging mycotoxins and (3) development of new tools to investigate the toxicity of mycotoxins.

4.2.1 Research on the new effects of the regulated mycotoxins deoxynivalenol and zearalenone

I have participated in several studies involving major mycotoxins, notably AFB1, FB1, DON and ZEN. In this section, I will provide with a brief summary of my works on the toxicity of DON and a wider overview of my works on the toxicity of ZEN.

4.2.1.a Deoxynivalenol, a known danger for animal production

DON is one of the most prevalent mycotoxins present in food, produced by *Fusarium* species typically in temperate areas including Europe¹⁰. Because it is a high human exposure toxin, much effort has been spent to understand the mechanisms of toxicity of DON. At a cellular level, DON interacts with the peptidyl transferase region of the 60S ribosomal subunit¹¹. It induces ribotoxic stress, triggering an inflammatory cascade mediated by the activation of mitogen-activated protein kinases (MAPKs) and oxidative stress that leads to apoptosis¹². The toxic effects associated with DON exposure include alterations on protein synthesis, immune system function, and intestinal functions. DON also interacts with several appetite suppression systems¹⁰. The impact of feed contamination with DON in animal farming is high, and there is a clear industrial interest in testing products oriented at diminishing the toxicity of DON. Since my arrival to the team in 2016 **I have participated in planning, execution and results**

analysis of different *in vivo* and *ex vivo* pig feed trials aiming at validating remediation strategies against DON toxicity in collaboration with private companies such as Lallemand, Lesaffre, Adisseo and Olmix.

One example of the latest trials performed in our lab involved a collaboration with the Bari Institute of Food Production Science (ISPA, Italy) and the Laboratory of Animal Pathology from the Londrina State University (Brazil). The object of the study was the investigation of the effect of having secure vs unsecure feeds in the toxicity of DON at doses near EU recommended guidelines for pig feed (0.9 ppm) and a higher contamination level (3ppm). While both feeds were nutritionally equivalent, they differed in their ingredients: the secure feed was rich in ingredients known to be beneficial or neutral to the digestive system, and the unsecure feed included constituents not as digestive such as soy derivatives. Weaned piglets were exposed to contaminated secured and unsecured diets for 21 days, and then slaughtered. An immunization challenge was also included to investigate differential reactions in vaccine response. Performance parameters were recorded during the trial, blood samples were obtained in one-week intervals and urine and different tissues were collected at the end of the exposure period. Diet did not have a clear effect in performance nor in inflammation blood markers, vaccine response no lesion scores at the intestine. However, the unsecure diet promoted a significantly higher DON absorption at the lower level of DON contamination in feed (0.9 ppm; Fig. 1).

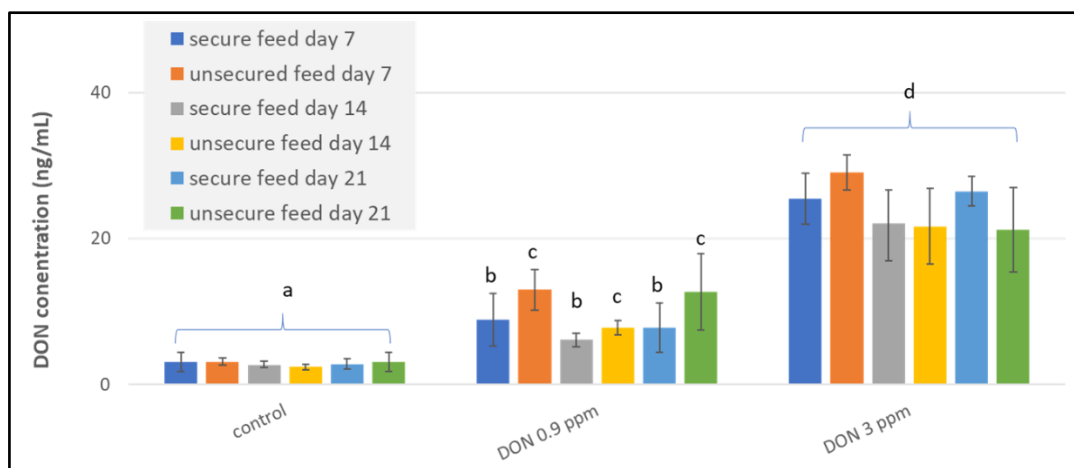


Figure 1. Effect of diet composition in DON concentration in serum of exposed pigs. A three-way ANOVA was performed considering DON concentration in the feed (CONTROL, DON-0.9 ppm and DON-3 ppm), feed type (secure/unsecure), and time (day 7, 14 and 21) as factors. DON concentration in plasma significantly differed depending on DON level in the feed ($p < 0.001$). DON concentration in plasma samples collected at days 7, 14 and 21 did not differ ($p = 0.43$). At 0.9 ppm, an interaction between two factors (DON concentration in feed and feed type) was observed. At moderate DON levels in the feed, the type of diet consumed by animals (secure/unsecure feeds) can affect DON absorption, being significantly higher when animals consume contaminated unsecure feeds ($p = 0.004$)

No differences were found at the higher level of contamination (3 ppm), probably because DON toxicity promotes its own absorption and hides the effects of the diets. These results underline that diet can have an effect on the absorption of toxins at a level approaching the EU-guidelines. These results warrant the study of the long-term effects of unsecured diets in mycotoxins absorption and consequent toxicity.

Perspectives: The presented and other results from our team indicate that the effect of mycotoxins can vary depending of the physiological and or sanitary status and the breeding/management conditions such as the diet composition. It remains to be established if other factors such as physical/psychological stress can also influence the toxicity of mycotoxins and if there is an interaction between these factors. As mentioned above these studies are particularly relevant in order to establish the adequacy of EU-guidelines, but also in order to identify “protecting” breeding conditions.

4.2.1.b Zearalenone, not only a reprotoxic mycotoxin.

ZEN is a mycotoxin synthesized by several *Fusarium* species that contaminate crops (cereals, dried fruits and spices) in the temperate areas of the world with a relatively high occurrence¹³. The structure of the mycoestrogen ZEN resembles the endogenous estrogen estradiol and is able to bind estrogen receptors. ZEN is hence considered an endocrine disruptor and its most known toxic effects are the functional and morphological alterations in reproductive organs that lead to multiple problems such as infertility, hormonal dysfunctions, reproductive tract hyperplasia and precocious puberty¹³. Due to its toxicity, a tolerable daily intake of 0.25 µg/kg body weight and maximum levels in food for human consumption (20-100 µg/kg) have been set by the European Commission by the Regulation EC No 1126/2007. ZEN is also toxic to other organs, especially those that are estrogen responding, such as the liver¹⁴, immune cells¹⁵ and the intestine¹⁶. The difficulty in characterizing the toxicity of this molecule is that it can change depending on the target organ (estrogen-responding or not) and the exposure conditions (low or high dose)¹⁷. **The aim of my research works was contributing to the characterization of the toxicity of ZEN outside the reproductive system. More specifically, my works have been oriented at studying the intestinal toxicity of ZEN and the metabolic disruption exerted by ZEN.**

Description of the intestinal toxic effect of the mycoestrogen ZEN.

The aim of this project was to investigate the biological response associated with the exposure to ZEN in the intestine. This was the object of the PhD thesis of Tarek Lahjouji, whom I co-directed with Isabelle Oswald, and completed his PhD on January 12th 2021. His project was funded by an Innovative Training Network funded by the EU under Horizon 2020 (ProtectED; 2017-2021), coordinated by Lisa Connolly (Queen's University Belfast, UK) involving several international research teams, whose main objective was to produce knowledge on endocrine disruptors.

There are two main estrogen receptors in the body, and their activation lead to different effects. Estrogen receptor α (ER α) signaling is the main regulator of estrogen-dependent genes leading to proliferative, anabolic effects, whereas estrogen receptor β (ER β), when co-expressed with ER α , restrain ER α activity and its activation inhibits cell proliferation and induces a catabolic effect. Both receptors have different spatiotemporal expression patterns and non-overlapping functions¹⁸. ZEN is a full agonist of ER α and a mixed agonist/antagonist of ER β . The estrogenic stimulation by ZEN, like that of estradiol, is therefore predominantly mediated by ER α ¹⁹. In consequence, the effect of ZEN will depend of the presence and ratio of ER receptors of each cell type and their physiological status (e.g. regarding the oestral cycle)¹⁹. However, at high concentrations the toxicity of ZEN is independent of estrogen signaling, and exerts a cytotoxic effect characterized by a high oxidative state, cell membrane disruption, DNA oxidative damage and apoptosis¹⁷.

The intestine is exposed to food contaminants like ZEN, and is a major site of xenobiotics metabolization. In some species like human and pig, ZEN is biotransformed in the small intestine into a more estrogenic metabolite, alpha-zearalenol (α -ZOL), which explains the high sensibility of these species to this toxin²⁰. Estrogen signaling is important for intestinal physiology, and a differential expression of ERs is present along the crypt-villi axis. In fact, ER α signaling predominates in the crypt, where the interplay with other signaling cascades such as Wnt/ β -catenin to promote cell proliferation, whereas ER β is important in the villi, where it interplays with signaling cascades as that of TNF- β to promote cellular differentiation²¹. The toxicity of ZEN in the intestine is known to involve changes in immune functions as well as signaling cascades linked with the development of cancer²². However, more research is still needed to fully understand the molecular mechanisms that lead to ZEN intestinal toxicity.

In a first series of experiments, our objective was to investigate the molecular mechanisms behind ZEN intestinal toxicity through the **analysis of the gene expression and protein abundance changes occurring in pig jejune explants exposed to ZEN (12)**. The toxic effect of ZEN in the intestine has been investigated in the past, mostly using pig as model for humans due to their similar sensitivity and toxicity. *In vivo*, some reports describe transient

morphological modifications in the small intestine during the first weeks of exposure, as well as an increase in the number of Paneth cells²³. Several reports have described changes in the expression of pro-inflammatory and pro-proliferative genes²². Taken together, these results suggest that ZEN could induce transient proliferation in the small intestinal crypt, which might be connected with pro-cancerogenic changes in Wnt/ β -catenin and TGF- β signaling pathways. We aimed at investigating if ZEN had an effect on the activation/repression of Wnt/ β -catenin and TGF- β signaling pathways in the intestine. Because ZEN is known to have an effect in the immune-metabolic status of exposed tissues, we also investigated the expression of several genes related with immunity and metabolism.

In agreement with previous results, we observed that ZEN increased significantly the abundance of ER α while had no effects on the abundance of ER β , which was also confirmed by immunohistochemistry (Fig.2).

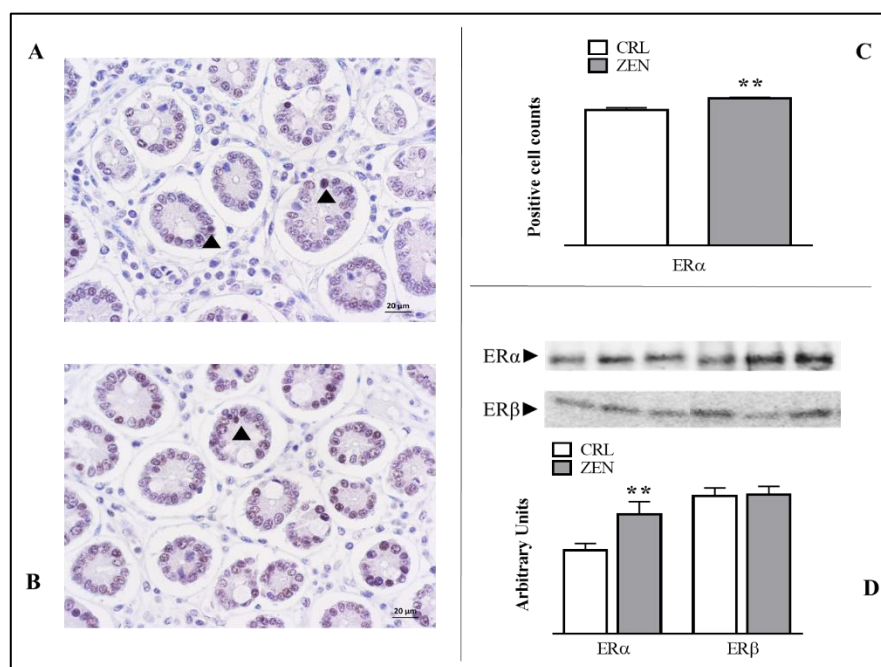


Figure 2. Immunoblotting and immunohistochemical analysis of ER α and ER β in paired pig jejunal explants exposed or not to 100 μ M ZEN (Z) (A) Representative image of positive immunostaining in a control sample and (B) treated sample from the same animal. Examples of positive nuclear staining is indicated by arrows. (C) Quantification of positive immunostained cells in paired control (CRL) and treated (ZEN) samples ($n = 6$) (D) Representative blots of 3 paired samples of ER α and ER β in control (C) and treated (Z) samples and relative quantification of normalized signal (arbitrary units). Values are means with standard errors of the mean represented by vertical bars ($n = 6$). Asterisks indicate statistical differences (* $p < .05$; ** $p < .01$, *** $p < .001$)

Gene expression and protein abundance results suggested that ZEN mediates an activation of Wnt signaling whereas TGF- β signaling is suppressed. This could be explained by the known ER α -mediated suppression of TGF- β -induced activation by interaction with Smad proteins and downregulation of the Wnt inhibitor DKK1. According to our results and those of and other authors, ZEN effectively inhibits the Wnt repression system through repression of DKK1. Results additionally indicate that ZEN does not remodel tight nor adherens junctions but is able to promote cytoskeletal changes related to enhanced migration, such as the regulation of the expression of α -catenin and CFN-1. Although our results were not significant, up-regulation/up-accumulation of the antimicrobial proteins lysozyme and RegIIIy produced by the Paneth cells was observed. Previous reports report a ZEN-induced increase in Paneth cells in pig²³. The homeostasis of Paneth cells depends on ER and Wnt/ β -catenin, and dysregulation in their function is related to the development of cancer and chronic inflammatory diseases. More in-depth research is required to investigate the influence of ZEN on the homeostasis of Paneth cells, and consequently on the crypt microenvironment. We observed no signs of inflammation, which could be linked to the ZEN-induced activation of ER α signaling, which has anti-inflammatory effects.

We observed that ZEN altered the expression of different genes linked with adipokines. Of note, the expression of adipokines receptors for adiponectin (AdipoR2), resistin (CAP1) and chemerin (CMKLR1) were significantly up-regulated. Adipokines are peptides that signal the functional status of adipose tissue to targets in different tissues, including the intestine. Their main roles are the regulation of inflammatory processes and energy balance, mainly glucose metabolism, as well as cell proliferation and differentiation, through the activation of ERK1/2, Akt, and/or PI3K signaling pathways. The effect of ZEN on the systemic levels and local signaling of adipokines merits further investigation.

In order to obtain additional data to understand the intestinal toxicity of ZEN, we aimed at exploring the **proteome changes induced by a short, non-cytotoxic exposure to ZEN in the intestine** using pig jejunal explants (13). This work was carried out in collaboration with the Toulouse Proteomics Infrastructure (IPBS, Toulouse) and the Service of Animal Pathology of the Murcia Faculty of Veterinary Medicine (Spain).

ZEN toxicity did not involve an extensive remodeling of the proteome from intestinal cells, as only 1% of identified proteins showed significantly different abundances. The functional analysis of results indicated an enrichment in GO terms associated with the NF- κ B signaling suggested a specific effect that was further investigated. Our results suggest that ZEN induces the activation of NF- κ B cascade, but blocks the pro-inflammatory transcriptional response associated with this activation. Indeed, the estrogenic activity of ZEN could be behind this effect, since estrogen can rapidly activate and regulate NF- κ B signaling, but also directly control its transcriptional response²⁴.

Functional analysis also suggested an activation of PI3K signaling by ZEN, which could be explained by the estrogenic activity of ZEN²⁵. In agreement with this hypothesis, the functional analysis predicted that ERK1/2, EGFR and the estrogen receptor beta were highly interconnected in the top scoring networks constructed with the list of differential proteins (Fig.3). To confirm this hypothesis, the activation of ERK1/2 signaling was investigated.

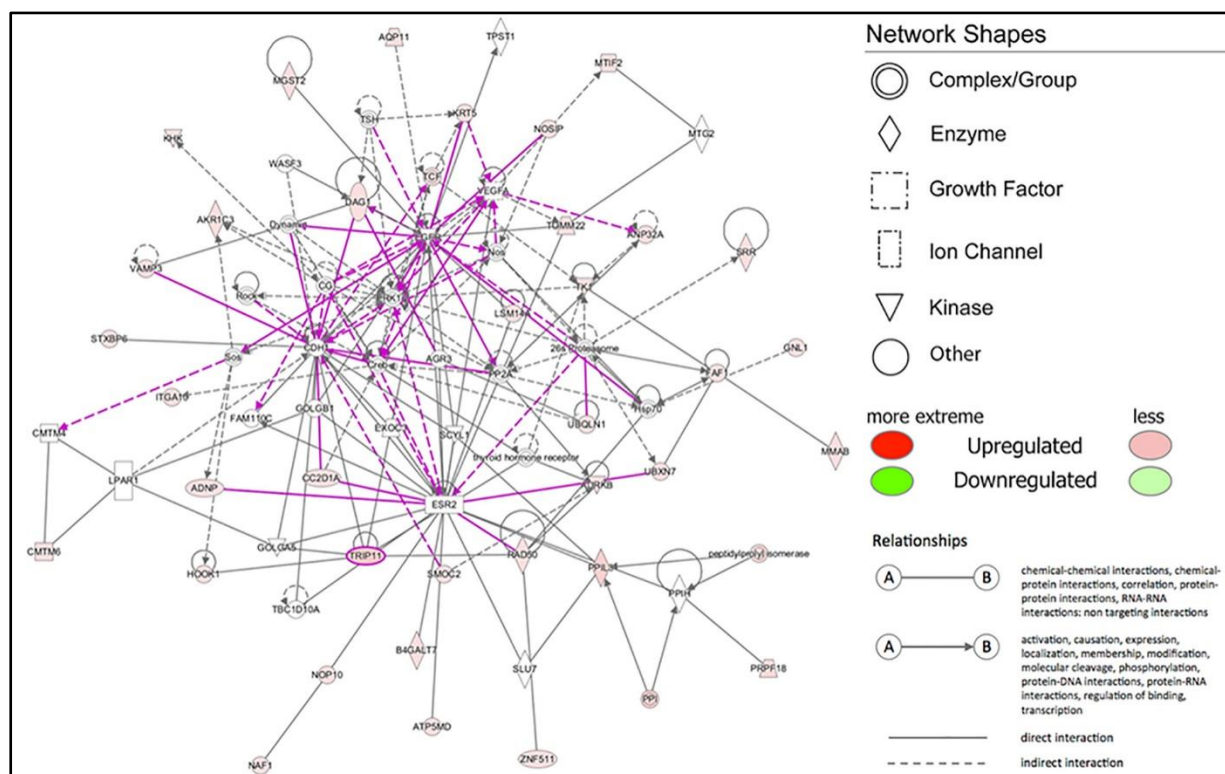


Figure 3. Graphical representation of the molecular relationships between molecules centered around the estrogen receptor beta (ER β), extracellular signal-regulated kinases 1/2 (ERK1/2) and epithelial growth factor receptor (EGFR) as a result of ZEN exposure to jejunal explants. Genes are represented by nodes with their shape representing the type of molecule/functional class, and the relationship between the nodes are indicated by edges. Nodes and arrows in pink represent proteins/interactions upregulated in ZEN-treated explants. White nodes are proteins not included in input set of proteins but having a strong connection to the proteins in the input list. The legend explaining node shape and edge type is given in the figure.

We were not able to see any significant differences in the relative phosphorylation of ERK1/2 in ZEN-exposed intestinal explants. That could be due to the rapid and transient activation of ERK by estrogen signaling. We thus investigated downstream events such as the changes in the signaling of two transcription factors CDX2 and HIF1 α that are targets of the two kinases PI3K and ERK. We observed a significant accumulation of CDX2 and MUCDHL, a protein whose expression is controlled by CDX2, confirming that ZEN activates CDX2-related signaling. CDX2 expression is controlled by ERK1/2 dynamics in intestinal cells and governs key processes in the intestinal epithelium including organization, renewal and differentiation. MUCDHL is an atypical cadherin important for brush border formation, epithelial cell differentiation and metabolism regulation. ZEN also prompted a significant increase in the protein levels of HIF1 α and its target SI (which is also a target of CDX2). HIF1 α is a transcriptional factor known for being the main regulator of the physiological response to hypoxia and the control of cellular metabolism, whose activity is controlled by ERK1/2, PI3k/Akt and NF- κ B signaling in non-hypoxic conditions. The activation of both CDX2 and HIF1 α signaling by ZEN is, therefore an indirect confirmation of the alteration of ERK1/2, PI3K and NF- κ B signaling in the intestine, and show that ZEN toxicity influences processes important for the regeneration of the intestinal epithelium.

Our results also indicated that ZEN can modulate the immune status of the intestine through the activation of the chemotactic axis CXCR4/ SDF-1, the increase of class I major histocompatibility complex (MHC-I) proteins and a depletion of class II major histocompatibility complex (MHC-II) proteins, together with the modulation of proteins linked with lipid antigen presentation by CD1. Moreover, the estrogenic activity of ZEN can also explain the morphologic changes observed in the intestinal tissues, namely the increased number of goblet cells and infiltrated lymphocytes in the lamina propria. These results indicate that ZEN can disturb the immune status of the intestine, increasing immune cells recruitment and modulating the immune sensing system.

In all, our results confirmed that the estrogenic activity of ZEN mediates a deregulation of the dynamics of several signaling cascades that are essential for the physiology of the intestine. Our results also show that ZEN can modulate the immune and energy sensing-related phenotype of intestinal tissues without inducing inflammation. These results warrants investigating the role of ZEN and other estrogenic substances as a factor in the development, progression or predisposition to intestinal diseases.

***Perspectives:** In order to confirm and complete our results, it would be advisable to investigate the effect of ZEN in other intestinal tissues, such as duodenum and colon, as well as in animals of different sex, age and physiological status (e.g. pre-puberal gilts, pregnant sows). In addition, the importance of estrogenic signaling in the described ZEN effects should be confirmed using ER inhibitors or KO cell lines. The effect of ZEN in the immune-related signaling suggest that the exposure to ZEN could influence the balance between anti-pathogen immunity and tolerance to innocuous antigens, and that metabolic-related signaling might be play an important role, which should be further investigated. The disruption of the physiology of Paneth cells is particularly interesting, not only because they are an important executor of the innate immune response of the intestine, but also because they play an important role in the development of chronic intestinal diseases like Crohn's disease. The investigation of the combined effect of ZEN with other xenoestrogens, particularly of natural origin such as soy isoflavones or mycotoxins (i.e. *Alternaria* spp. toxins) in intestinal toxicity would also be interesting.*

Role of ZEN as a metabolic disruptor.

The term endocrine disruptors (EDs) refers to those natural or synthetic chemicals that mimic, block, or interfere with hormones in the body's endocrine system. Exposure to EDs influences the regulation of body

homeostasis and contributes to promote a myriad of health issues²⁶. Recent epidemiological and experimental data indicate that exposure to specific EDs, known as metabolism disrupting chemicals (MDCs), can influence metabolism, contributing to the incidence of obesity as well as causing metabolic diseases such as diabetes or fatty liver disease²⁷. Most of the studied MDCs are synthetic chemicals such as polychlorinated bisphenols and some pesticides (dichloro-diphenyl-trichloroethane, hexachlorobenzene). Conversely, the role as MDCs of naturally occurring EDs such as mycoestrogens remains poorly understood. ZEN is an endocrine disruptor but the role of ZEN as a MDC is unexplored. Many evidences suggest that ZEN can alter metabolism. ZEN possess anabolic properties in farm animals²⁸, and actually the ZEN derivative zeranol is employed as an anabolic growth promoter for farm animals in some countries, e.g., for cattle in the US, and belongs to the list of substances prohibited in sports. Exposure to ZEN has an impact on different systemic metabolic processes, including cell membrane metabolism, protein biosynthesis, glycolysis, and gut microbiota metabolism in rats and pigs²⁹. Moreover, the toxicity of ZEN has been related with its ability to disrupt lipid metabolism and lipid-related signaling.

We were interested in **investigating the ZEN- mediated changes in energy balance and circulating molecules related with metabolism and immunity levels in response to different treatment diets (5)**. A collaboration with the industrial DSM/BIOMIN (Austria) and the National Veterinary School Vienna (VetMedUni, Austria) was established to this end. The experiment was carried out at facilities of the University Clinic for Swine, Vetmeduni Vienna with weaned, four-week-old, female crossbred piglets. Animals were exposed to different treatment diets (uncontaminated basal feed –control- or feed with a target contamination of 500 -ZENlow- and 1500 µg/kg ZEN –ZENhigh-; n = 10 for each group) for 28 days. The effects of both diets was evaluated by measuring the plasma abundance of a series of molecules related with metabolism on days 7 and 21.

Exposure to ZEN was not associated with hepatocellular damage but induced significant changes in the lipid profile such as significant increase of the lipoproteins LDL, HDL as well as cholesterol was observed at day 7. These results, together with AST concentration changes suggest a possible disruption of liver lipid metabolism by ZEN, which should be further investigated. Changes in glucose metabolism were also observed in animals exposed to ZEN, but they were different depending on the exposure conditions. A short exposure to a lower dose of ZEN increases serum glucose levels, whereas a longer exposure to a lower dose or exposure to a higher dose decrease glucose levels. This apparently contradictory effect has been identified before in pigs exposed to much lower ZEN concentrations²⁹, and can be explained by the dual effect of estrogens and xenoestrogens in glucose metabolism. These changes in metabolism markers often involve changes in the signaling of the protein hormones adipokines, which are also known to have important functions in the reproductive system. We obtained an overview of the changes in circulating adipokine concentrations using a protein array with sera pools of the ZENlow group. Results indicate that ZEN has a time-dependent effect on the abundance of multiple mediators of inflammation, coagulation, cell growth, and metabolism. The absolute quantification of the circulating levels of adiponectin, resistin and fetuin B using ELISA assays confirmed the trends in protein changes detected using the protein array (Fig 4). We observed that resistin was significantly accumulated and adiponectin was depleted, mostly after 7 days of exposure to ZENlow diet. The effect was not as clear in ZENhigh animals.

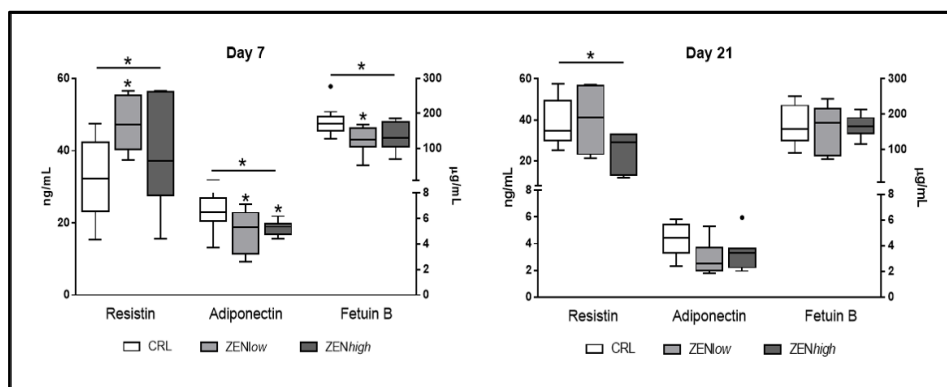


Figure 4. Concentrations of circulating adipokines resistin, adiponectin, and fetuin B in serum of pigs exposed to low concentrations of ZEN (ZENlow; n = 10), high doses of ZEN (ZENhigh; n = 10), and control (n = 10). The concentration of resistin is expressed in nanograms per milliliter (ng/mL) and plotted on the left axis. Adiponectin and fetuin B concentrations are expressed in micrograms per milliliter ($\mu\text{g/mL}$) and plotted on the right axis. The plot illustrates the median (line within box); 25th and 75th percentiles (box); 1.5 times the inter-quartile distance (Tukey; whiskers); and outliers (*). Asterisks over a bar indicate the level of significance of Kruskal–Wallis test, whereas an asterisk over the whisker of a group indicates a significant difference of that group with the control group according to Dunn's post-test. * $p < 0.05$.

Resistin and adiponectin are antagonist molecules, since resistin induces insulin resistance and is considered pro-inflammatory, whereas adiponectin induces insulin sensitivity and is considered protective against inflammation³⁰. In the pig, resistin is suspected to be involved in fattening, while adiponectin levels are negatively correlated with adiposity, both molecules have important roles in regulating sexual maturity, and their release is affected by sexual hormones including estrogen³¹. The results observed in our study are in agreement with the effect of other xenoestrogens, and suggest that ZEN is (at least transiently) able to disrupt glucose metabolism in a mechanism that might involve the regulation of circulating resistin and adiponectin abundance. The quantification of circulating fetuin B levels indicated a significant reduction only in ZENlow animals at day 7. Fetuin B is a hepatokine known to cause glucose intolerance, and its reduction is associated with an improvement of the body's ability to dispose of a glucose load³². Fetuin B is also known to have a role in reproduction, as fetuin-B deficiency renders female mice infertile³³. According to our results, the reduction of fetuin B levels coincide with an increase in glucose concentration, suggesting that fetuin B decrease might be a response to increased glycaemia. The regulation of fetuin B levels by xenoestrogens is mostly unknown but given the apparent important impact of fetuin B deletion in female reproduction, it would be essential to understand if changes in circulating fetuin B levels might be directly or indirectly regulated by ZEN.

In all, our results showed that ZEN is able to induce changes in metabolism that might be facilitated by a modulation of several adipokines known to have a pivotal role between metabolism and reproduction. These results suggest that ZEN might be a MDC and warrant further research.

Perspectives: the influence of ZEN in glucose metabolism in pigs observed in the present study seems especially relevant, because commercial pigs are resistant to the development of type II diabetes, even if porcine commercial breeding can be assimilated as a diabetogenic lifestyle in humans. Indeed, the current results warrant further research on the role of ZEN in insulin signaling and glucose tolerance. It would be interesting to check if the observed results are species-, sex- and age-dependent. The relationship with the expression/activity of circulating adipokines as well the role of adipose tissue signaling should be investigated. Further research is needed in order to identify ZEN as a MDC. The first step, and because the protein array analyses performed in our study were done in pooled samples, many of the results needs to be validated using quantitative assays. The serum quantification of several adipokines and pro-inflammatory molecules, as well as other markers involved in insulin signaling, together with the evaluation of the expression of relevant genes in target tissues such as the liver should be investigated more in detail. More perspectives regarding this topic are provided in the general perspectives section.

4.2.2 Research on the toxicity of the emerging mycotoxins NX and Versicolorin A

One of the main roles of my position in Toulouse is the study of the toxicity of emerging mycotoxins. I have participated in different projects involving emerging mycotoxins and in this section I will describe my works on the new type A trichothecenes NX and the AFB1 precursor VerA.

4.2.2.a NX, a new type of trichothecene. In temperate climates, infections of wheat and corn by the pathogen *F. graminearum* and related species are a chronic problem. The resulting food and feed is contaminated with the mycotoxin DON. A decade ago, a genetic population of *F. graminearum* was discovered that produced the novel trichothecene NX, an analogue of DON lacking the carbonyl moiety at the C8-position³⁴ (Fig. 5). NX is biosynthesized by new chemotypes of *F. graminearum* populations first identified in the Midwest United States³⁵. DON and NX have been demonstrated to co-occur. Analyses of corn samples that contained high amounts of DON have found NX at 1–7% of the DON concentration³⁵.

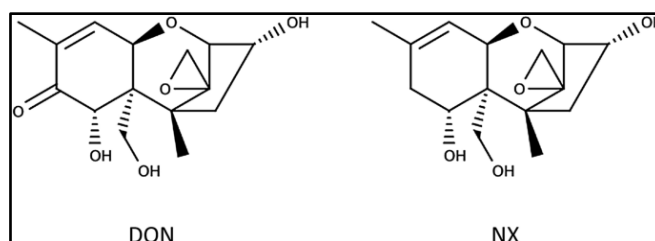


Figure 5. Structural formulas of the two mycotoxins in this study, DON (deoxynivalenol) and NX.

In the last years, we have performed a comparative study of the intestinal toxicity of NX and DON, and discovered that the high toxicity of NX recommends its inclusion in overall risk analysis linked to the presence of mycotoxins in our diet. In collaboration with David Miller (Carleton University, Canada), who provided the non-commercially available toxins NX and 3-NX, we aimed at obtaining a general view of the intestinal toxicity of these toxins using an explant model and combining histopathological analysis, transcriptomics and proteomics.

First, the effect of **DON, NX and the acetylated form of NX, 3-NX were compared using *in vitro* cytotoxicity assays, histological evaluation and transcriptional analysis (8)**. This work was performed in collaboration with the platform GET-Trix (Toulouse). As expected, the toxicity of acetylated NX was reduced compared to DON and NX, which is common in acetylated trichothecenes. Although analysis revealed common mechanisms of toxicity, the magnitude of toxicity was higher for NX than for DON. Indeed, Both DON and NX showed a similar impact in cell viability as assessed *in vitro* using intestinal cells. However, the severity of the lesions induced in exposed intestinal tissues and the magnitude in the changes induced in gene expression was higher for NX (Fig. 6). Functional analysis of transcriptomics results indicated that both toxins targeted the same molecular processes namely inflammation, immune response, cell proliferation, differentiation, apoptosis, and growth. These results were in agreement with previous results where no differences were found regarding the mechanism of action of both toxins, and NX was found to be more toxic than DON.

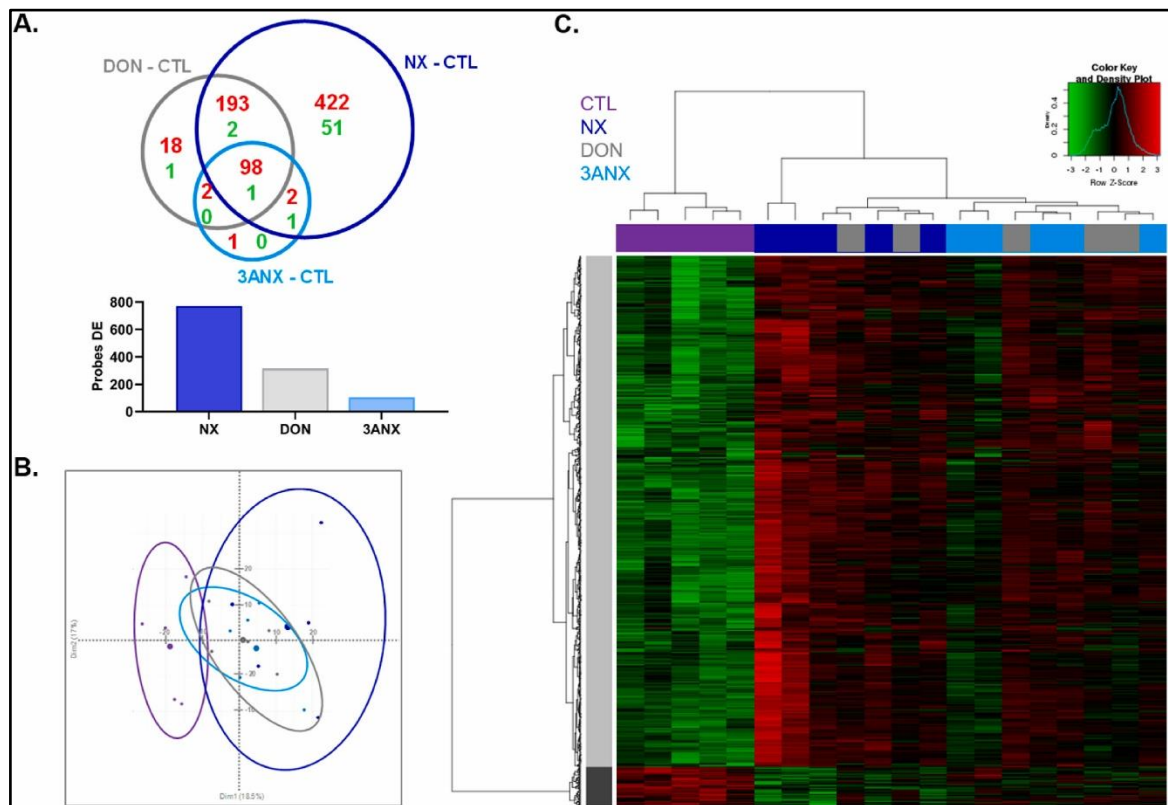


Figure 6. Gene expression profile of intestinal explants exposed to NX and 3ANX in comparison to DON. Jejunal explants from 5 animals were exposed for 4 h to diluent (purple), 10 μ M NX (dark blue), 3ANX (light blue) or 10 μ M DON (grey) and gene expression was analyzed with a 60 K microarray. (A) Venn diagram illustrating the overlaps between probes significantly up- or down-regulated (False Discovery Rate (FDR) p -value ≤ 0.05 , Fold Change (FC) ≥ 1) in response to NX, 3ANX or DON (upper panel). Number of differentially expressed genes in intestinal explants upon exposure to NX, 3ANX and DON (lower panel). (B) Principal component analysis of differential probes between NX, DON, 3ANX and control (with Benjamini-Hochberg (BH) adjusted p -value < 0.05). (C) Heat map representing differentially expressed probes in NX, DON, 3ANX and control explants. Red and green indicate values above and below the mean (average Z-score), respectively. Black indicates values close to the mean. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

We were interested in defining relevant mechanistic differences between DON and NX and their associated potential biomarkers. Our next goal was thus to **study of the tissue-specific leakage and secreted proteins (commonly referred to as the secretome) of intestinal explants exposed to DON and NX using proteomics (4)**. This study was the object of a grant I led as responsible scientist of a PHC Amadeus (started 2019) that allowed us to establish a collaboration with an Austrian network of farm animal proteomics scientists headed by Dr Ingrid Miller at the VetMedUni Vienna.

Our analysis showed that the secretomes of jejunal explants exposed to DON are enriched in markers of cell damage, loss of cell adhesion and altered metabolism, in agreement with the known toxicity of DON (Fig. 7).

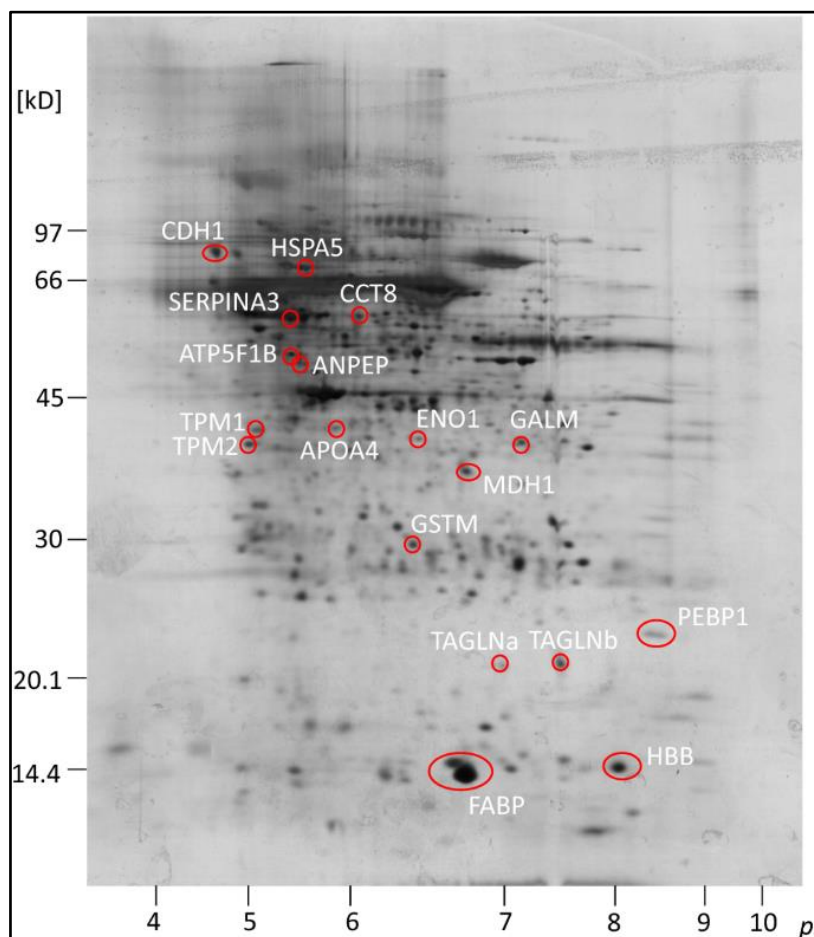


Figure 7. 2D separation of secretome, DIGE-gel after silver staining. Shown sample: mix of secretomes (equal amounts of CyDye-labeled control, NX-treated secretome and internal standard). Red circles indicate spots found differentially abundant in comparison to control sample and were subjected to LC-MS/MS analysis for protein identification. Labels according to gene names of the respective proteins (UniProt database)

In accordance with our previous results, changes in the secretomes of jejune explants exposed to NX reflected an alteration in cell shape, adhesion, and inflammation. In contrast to DON, most DAPs in NX secretomes were mitochondrial proteins, and enriched pathways were linked with mitochondrial functions. The enrichment in mitochondrial proteins of the jejune secretomes in response to NX is likely associated with the increased inflammation caused by this toxin compared to DON. Indeed, inflammation promotes the secretion of mitochondrial content³⁶. The presence of mitochondrial content in the extracellular space can mediate cell-to-cell communication and repair and function as an activator of the immune response. When contained in mitochondrial-derived vesicles, extracellular mitochondrial proteins reflect an enhanced oxidative stress and mitochondrial toxicity³⁷. In our conditions, it was not possible to distinguish if the identified DAPs are free in the surrounding medium or contained in vesicles or whole mitochondria. Further analyses are needed to investigate if NX is especially toxic to cell mitochondria, and if the secretion of mitochondrial-derived vesicles is important for the increased inflammation induced by NX compared to DON.

Our studies contribute with new information concerning the difference between DON and NX toxicity in terms of risk analysis. Both toxins induce inflammation and cell damage, being NX more potent than DON is. Moreover, NX toxicity is associated with the release of extracellular mitochondrial proteins in exposed cells. Quantification of this phenomenon, in comparison with common effects such as inflammation, would be useful to characterize the combined toxicity of DON and NX and their possible interactions.

Perspectives. Our results suggest that NX might be toxic for mitochondria and reveal a relevant role of mitochondrial proteins in the inflammatory signaling mediated by NX that should be further investigated. It would be interesting to study if these proteins are contained in extracellular vesicles derived from mitochondria, and their role in NX toxicity. This distinctive effect could be used to evaluate the combined toxicity of NX and DON, as well as with other type B trichothecenes. Our results also underline the interest in studying the secretomes of cells exposed to mycotoxins in search of new biomarkers of effect, mainly when comparing related compounds.

4.2.2.b Versicolorin A, a dangerous aflatoxin B1 precursor. Among mycotoxins, AFs represents the major threat to human and animal's health. The most toxic AF is Aflatoxin B1 (AFB1), known to be the most potent natural carcinogenic substance (classified as Group 1 by IARC; IARC, 1993). The presence of AFs in food and animal feed, in particular of AFB1, is regulated in most parts of the world (European Commission, 2006; US Food and Drugs Administration, 2021, 2019). In Europe, the maximum authorized levels of aflatoxins range from 0.1 to 15 µg/kg (European Union, 2006) depending on the foodstuff and their intended use.

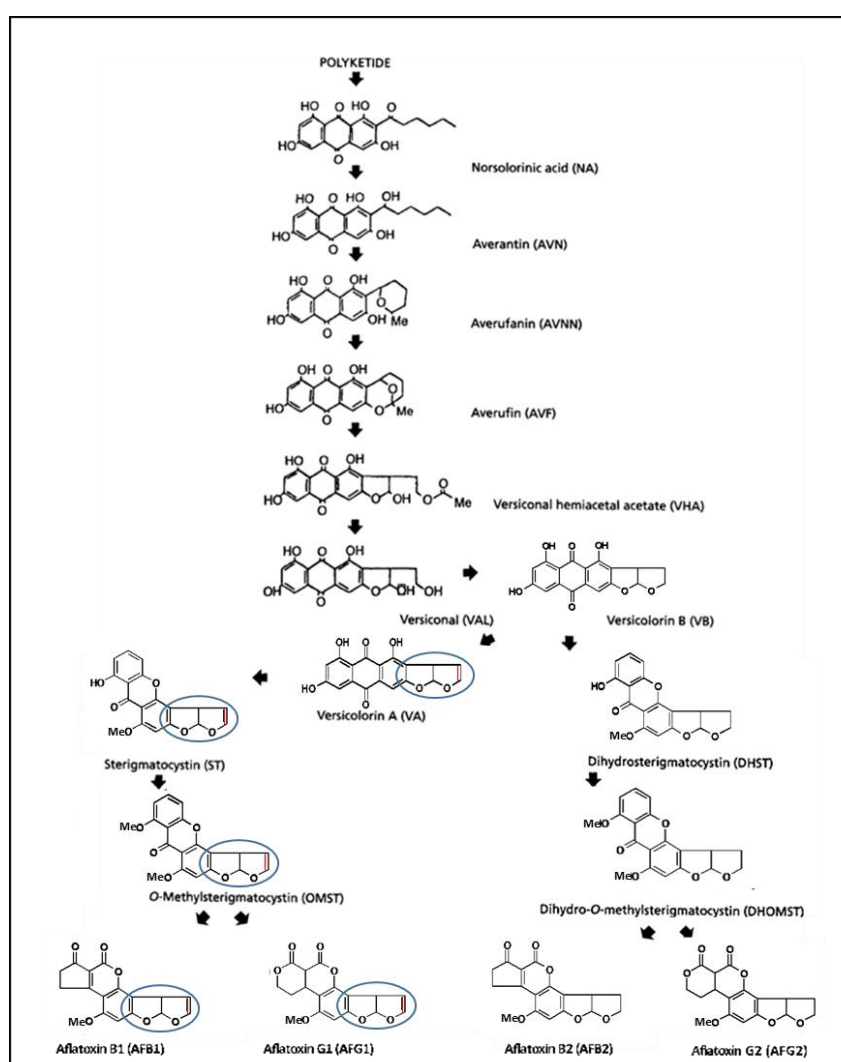


Fig. 8. Aflatoxin biosynthetic pathway Schematization of the main biosynthetic reactions leading to aflatoxins synthesis produced by *Aspergillus* species: *A.flavus* (B1, B2) and *A.parasiticus* (B1, B2, G1, G2) (Adapted from Trail et al. (1995)). The structure of dihydrobisfuran ring is circled and the double bonds are highlighted in red.

AF synthesis involves several enzymatic reactions and at least 15 compounds have been reported as intermediates of the aflatoxin biosynthetic pathway³⁸ (Fig. 8). Notably during the final steps of the synthesis of AFB1 and Aflatoxin G1 (AFG1) a characteristic unsaturated vinyl ether double bond of the terminal furan ring is formed, which is also present in three intermediate precursors: STC, O-methyl sterigmatocystin (OMST) and VerA (in red in

Fig.8). The mycotoxins containing this structure are known as bisfuranoids, acknowledged as mutagens and liver carcinogens³⁹⁻⁴¹. The involvement of this specific structure in the toxicity of AFB1 is well characterized, and is determined by epoxidation of the vinyl ether double bond mainly by the action of several cytochrome P450 (CYP450) enzymes in exposed tissues which metabolize AFB1 into the highly reactive aflatoxin-8,9-exo-epoxide (AFBO)⁴². AFBO reacts with critical biological nucleophiles, such as proteins and DNA, and causes point mutations, chromosomal aberrations, and genetic damage⁴².

The occurrence and exposure of VerA remains to be established, but human and animals are likely to be exposed to VerA for various reasons. First, because VerA accumulates considerably before inducing a large increase in AFB1 levels. Furthermore, the conversion rate is estimated only at 46%, so there is always some VerA that is not transformed in AFB1⁴³. This means that levels of VerA in samples contaminated with aflatoxigenic molds could be relatively high in absence of AFB1, and so exposure to VerA can take place when ingesting foods considered safe regarding its AFB1 content. A recent report indicates that levels of VerA are detectable in corn samples contaminated with aflatoxigenic fungal species with undetectable levels of AFB1, with concentrations reaching 46 µg/kg, and more than 100 µg /kg VerA in samples with more than 5 µg /kg of AFB1⁴³. Although no official surveys on the prevalence of VerA have been published, some data⁴⁴ indicate that around 30 % of maize samples are contaminated with VerA with concentrations reaching 315 µg/kg, whereas in some seeds the incidence raised up to 77.5 % of contaminated material. The second reason is that VerA can be synthesized also by other fungal species that do not contain the enzymes necessary for the conversion to AFB1, such as *A. versicolor* and *A. nidulans*.

First data on the toxicity and mutagenicity of VerA were published three decades ago, when it was found that it is a strong uncoupler of the oxidative phosphorylation in mitochondria⁴⁵ and that it is a genotoxin^{39,46}. No further studies on VerA geno- and cytotoxicity were performed later until very recently, when it was shown by members of the BioToMyc team that VerA has a genotoxic and clastogenic potential in respiratory and intestinal cells. The important toxicity observed warranted further characterization of the hazard associated with this compound.

I obtained an ANR project funding under the program JCJC that started in Mars 2019 with the objective of studying the toxicity of Versicolorin A. I have directed a M1 student (Aleksandra Andonova) and co-directed with Olivier Puel (Toxalim, INRAE Toulouse) a M2 student that became my PhD student (Carine Al-Ayoubi) that defended her PhD Thesis in February 2023 and has stayed in the lab working as an engineer. The works on VerA toxicity will be presented in sections.

Viability loss, cytotoxicity and proliferation inhibition in cells exposed to VerA

The ability of VerA to decrease cell viability has investigated in the past in different cell lines, showing a high toxicity and a variability depending on the cell line. Here I present some works that show that the **impact of VerA in the viability of the hepatic HepG2 and the intestinal epithelial IPEC-1** is different (7).

AFB1 and VerA induce similar cell viability loss in HepG2 cells. The time and concentration-dependent effects of AFB1 and VerA in cell viability were determined using the CellTiter-Glo® assay that measures viability through the evaluation of the metabolic status of cell (ATP content). For both AFB1 and VerA, the percentage of viable cells at 48h decreased at concentrations higher than 1µM in a dose-response manner. The maximal viability loss after 48h was obtained at 10µM for both toxins. Overall, our results show that in HepG2 cells, VerA and AFB1 have similar potency.

VerA induces cell viability loss at lower concentrations than AFB1 in IPEC1 cells. The time and concentration-dependent effects of AFB1 and VerA in cell viability were first determined using the CellTiter-Glo® assay (ATP

content). After 24h exposure, a significant concentration-dependent loss of cell viability was observed as from 3 μ M for VerA and 10 μ M for AFB1. At 48h exposure this effect was apparent in concentrations as low as 0.1 μ M for VerA and 3 μ M for AFB1.

The concentration-dependent response of IPEC1 cells to 24h exposure to VerA was then completed using complementary assays. Cell viability was evaluated using measures of the ATP content as explained above (CellTiter-Glo™ Cell Viability Assay), cell cytotoxicity was evaluated through the measurement of a protease leaked to medium if the membrane integrity of exposed cells is compromised (CytoTox-Fluor™ Cytotoxicity Assay). We also evaluated the incorporation of Bromodeoxyuridine (BrdU) in newly synthesized DNA of tested cells as a measure of cell proliferation (Fig. 9). We observed that cell membrane integrity and cell viability were compromised by 3 μ M or higher concentration. However, cell proliferation was significantly reduced at much lower concentrations, starting at 0.2 μ M (~5% reduction) and evident as from 0.3 μ M (~30% reduction).

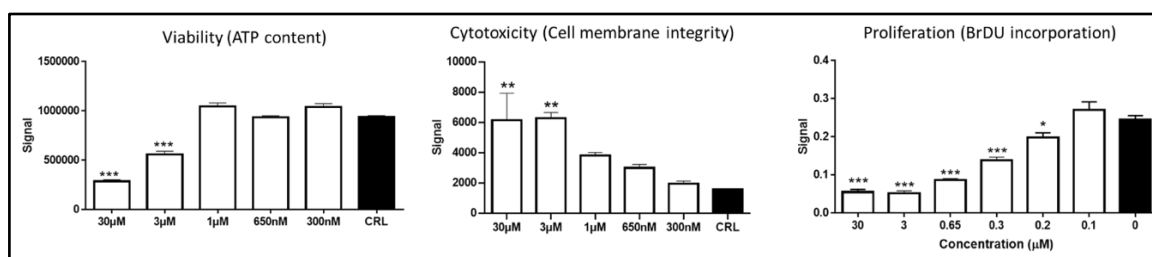


Figure 9. Effect of versicolorin A (VerA) in cell viability (ATP content of exposed cells), cytotoxicity and proliferation of IPEC1 cells after 24h of exposure. The plot illustrates the mean \pm SEM. Asterisk over a bar indicates a significant difference of that group with the control group according to Dunn's post-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

The study on cell viability was completed in order to understand the effect on viability time after a given exposure to VerA as an indirect measure of the effect of that exposure in the ability of cells to continue with their growth and functions. After 6h and 24h exposure to growing VerA concentrations, cells were washed and cultured in fresh cell medium for additional 48h. Cell viability related to solvent controls was determined immediately after the treatment and then 48h later of incubation in a fresh medium. Viability loss identified after toxin exposure was interpreted as a direct toxin effect while viability loss 48h after exposure was employed as an indirect measure of the toxin-mediated impairment of cells to proliferate and/or function normally. Our results show that 6h exposure resulted in viability loss at 20 μ M. However, 48h after this short exposure the viability of cells exposed to 3, 10 and 20 μ M was compromised compared to controls. In the case of 24h exposure, a significant loss in cell viability was evident for 10 and 20 μ M, whereas 48h after viability loss was evident as from almost 0.3 μ M and higher concentrations.

These results indicate that the exposure to a given concentration of VerA might not have an apparent direct effect in viability, but it would induce lesions leading to a relative loss of viability that is only visible after 48h. The identification of these effects are relevant to interpret other toxicity results presented in the next sections.

Mutagenicity and Genotoxicity of VerA

The complete **assessment of the mutagenicity and genotoxicity of VerA is necessary to evaluate the danger associated with VerA**. We contributed to this knowledge with some studies on this topic, paying particular attention to the evaluation of dose-response effect as well as low concentrations/short exposure, and the role of metabolic activation in mutagenicity, due to its relevance to the toxicity of the related toxins AFB1 and STC. This study involved a collaboration with Julien Vignard and Gladys Mirey (Team GS, Toxalim, France) as well as Arianne Vettorazzi and Amaia Azqueta (University of Navarra, Spain). This collaboration included the short stay of the PhD student María Alonso in our lab (3 months in 2022).

The mutagenicity of VerA depends on its metabolic activation. As explained in the introduction of this section, the mutagenicity of AFB1 depends on its bioactivation into a genotoxic metabolite. Some studies have reported that VerA is mutagenic, although the importance of metabolic activation was not sufficiently addressed. Because VerA is in-house purified and its availability is limited, we aimed at a toxin-effective selection of tester strains to perform a miniaturized Ames test, with and without external metabolization using human liver S9.

The tester strains TA98 and TA100 were chosen because it has been shown that this approach is sufficient to identify approximately 90% of the mutagens in a population containing about 35% mutagenic chemicals⁴⁷. Each of these tester strains revert following a different event, namely base-pair substitutions (TA100) and frameshifts (TA98). In the presence of metabolic activation, VerA induced a strong and dose-dependent mutagenic response in both strains. In turn, without metabolic activation equivocal results were observed, since a positive but non-dose-dependent response was observed at some concentrations. Our results are in agreement with other results⁴⁸, where a mutagenic activity was shown in the presence of S9 fraction at low doses in TA98 and TA100, respectively, while a weak but significant response was observed without metabolic activation in the same tester strains at high doses only. In all cases, no clear dose-response was evidenced in the absence of S9 fraction, making it difficult to confirm the mutagenicity of VerA without metabolic activation.

Because bioactivation was related with unequivocal mutagenicity, our results point at a possible biotransformation of VerA into a new compound that would be responsible of inducing direct DNA damage involving base-pair substitutions and frameshifts mutations.

The genotoxicity of VerA as measured by the SOS/Umu is dependent of metabolic activation. The SOS/Umu test is used for the detection of DNA-damaging agents that can be performed in the presence/absence of metabolic activation to detect protoxicants. This test was used as a screening of a wide range of VerA concentrations with or without standard liver S9 fraction from induced rats. We observed a positive response to this test ($FC > 2$; no cytotoxicity) in concentrations ranging from $1\mu\text{M}$ to $30\mu\text{M}$ only in the presence of metabolic activation, while the test was negative without metabolic activation ($FC < 2$) at all concentrations. According to the results of this test, the DNA damage induced by VerA in bacterial DNA is entirely dependent on its bioactivation.

VerA is highly genotoxic to the intestinal epithelial IPEC1 cells. We were interested in characterizing the genotoxicity of VerA in intestinal epithelial cells because they are very likely to be exposed to the toxin when contained in food. We evaluated the genotoxicity of VerA to IPEC1 cells using a combination of tests, namely the micronucleus test and the alkaline and Fpg-modified comet assay. Results were completed with the quantification of several mediators and effectors of the cell DNA damage response, namely the serine 139 phosphorylated Histone H2AX (γH2AX), the tumor suppressor P53-binding protein 1 (53BP1) and the Fanconi Anemia Complementation Group D2 (FANCD2) protein.

Our results indicate that VerA induces a significant genotoxic response at low concentrations, demonstrating the high danger associated with the exposure to this molecule. A short exposure (6h) of IPEC1 cells to VerA induced a significant increase in DNA DSBs as observed in the alkaline comet assay at $1\mu\text{M}$, as well as an increase in Fpg-sensitive sites and $\gamma\text{H2A.X}$ levels at $0.3\mu\text{M}$ (Fig. 10). At 24h exposure, only concentrations having an effect in cell viability/proliferation were associated with an increase of DNA DSBs, but Fpg-sensitive sites were significantly induced at much lower concentrations ($0.65\mu\text{M}$; Fig. 10). The presence of Fpg-sensitive sites could be related with DNA oxidation and the formation of 8-oxoguanine, since VerA is known to induce a strong oxidative stress in exposed cells^{44,49}. Alternatively, the increase in DNA damage after Fpg treatment at such low VerA concentrations can also be

explained by the formation of formamidopyrimidine adducts (VerA-FAPY), that can be recognized by Fpg enzyme, as described for AFB1⁵⁰.

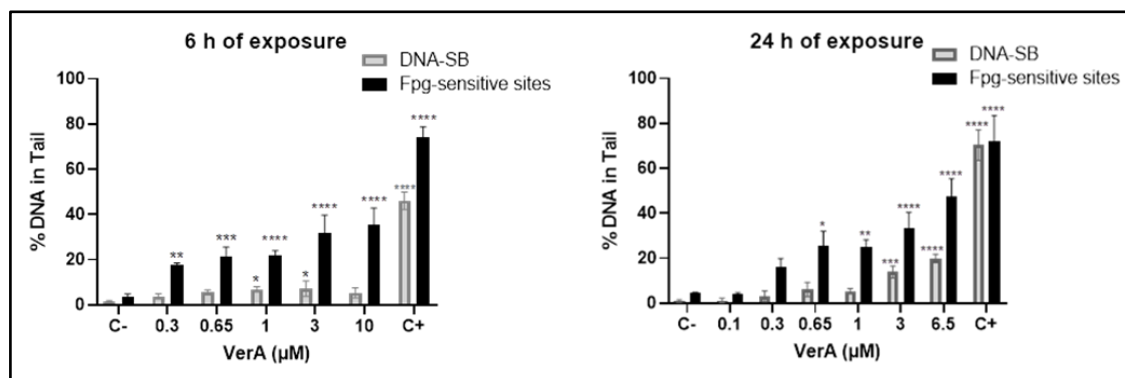


Figure 10. DNA damage evaluated by alkaline (-FPG) and Fpg-modified comet assay (+FPG) after treatment with increasing concentrations of VerA for 6 and 24 h. Positive controls (C+) were methyl methane sulfonate (MMS; 0.5 mM) and potassium bromate (KBrO₃; 2 mM) for each test, respectively. Negative control was vehicle (DMSO). Results are mean \pm SD of three independent experiments. (* p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001 versus CTRL. ANOVA followed by Dunnett's post-hoc test).

Likewise, a concentration as little as 0.1 μ M induced chromosomal instability (revealed by the micronucleus assay) and an activation of the DDR response characterized by an increase of nuclear foci of γ H2A.X, 53PB1 and the replication stress (RS) marker FANCD2. Taken together, these results show that VerA is capable of inducing RS in parallel with DSB formation at very low doses. The induction of RS might be important in the genotoxic activity of VerA, and could explain the VerA-mediated induction of γ H2AX in cell lines devoid of metabolic activity, such as ACHN cells⁵¹. Additional hints on how VerA induces RS in exposed cells is given in the next sections of this document. At some exposure conditions involving low VerA concentrations, the significant signs of DNA damage do not compromise cell viability. This indicates the possibility of propagating cell populations bearing mutations and suggesting a high carcinogenic potential for VerA that warrant further research.

VerA and AFB1 are genotoxic to liver HepG2 cells, but the mechanisms of genotoxicity seem to differ. The genotoxicity of VerA was compared to that of AFB1 in HepG2 cells through the evaluation of the induction of γ H2AX, serine15-p53 phosphorylation and p53 tumor repressor induction in HepG2 cells, and compared these results with p53 tumor repressor induction in cells devoid from phase I enzymes (U2OS cells). We observed that in HepG2 cells both AFB1 and VerA elicited a concentration-dependent increase of γ H2AX levels and p53 transcriptional activity. HepG2 cells exposed to 1 μ M of each toxin for 24h also showed a higher abundance of phosphorylated-p53 (Ser15). Additional experiments using the p53 CALUX U2OS cells, a cell line that does not show appreciable levels of CYP450s activity, showed that neither AFB1 nor VerA increased p53 transcriptional activity. This suggested that both AFB1 and VerA are genotoxic to human liver cells following cellular events dependent on the metabolic status of the cell.

The molecular mechanisms leading to these genotoxic effects seem to differ, as AFB1 induced greater phosphorylation of the key proteins H2AX and p53 while both toxins induced similar levels of p53 transcriptional activity. Indeed, VerA direct and indirect genotoxic effects were observed in cells with limited bioactivation capacity, suggesting that VerA may be genotoxic as a parent compound independent of its bioactivation by CYP450s⁵¹. As described in the previous section, VerA was able to induce an earlier and greater RS than AFB1 (8h for VerA vs 16h for AFB1), which in turn might be the trigger to DSB formation. Our results support these findings and suggest a similar mode of action in the liver emphasizing that despite sharing structural features, both toxins show some differences in their genotoxicity mechanisms. While AFB1 genotoxic effects are attributed to a bioactivated metabolite VerA genotoxic effects may be attributed to both a bioactivated metabolite and the induction of replication and/or oxidative

stress through its β -hydroxylated anthraquinone structure. Therefore, future experiments aiming at better understanding the toxicity of VerA in human liver cells should investigate such aspect.

***Perspectives:** According to our data, VerA is a cytotoxic, mutagenic and genotoxic mycotoxin, that share some effects with AFB1, but shows its own toxicity. Our results suggest that VerA is likely form DNA adducts, as well as oxidative DNA damage. Genotoxicity also seems related with the induction of replication stress, but the mechanism is still to be revealed. The elucidation of the importance of each of these possible DNA damage sources is essential to understand VerA toxicity.*

Our result also show that the effect of VerA in cell viability and genotoxicity is highly dependent on the cell type employed for the analysis. The pig intestinal cells IPEC1 (non-transformed, non-carcinogenic, p53/metabolically competent) cells appear as highly sensitive to the effects of VerA. The use of more complex intestinal models of research such as organoids would be useful to investigate if VerA targets a specific cell type. A collaboration with Martin Beaumont (GenPhySe, Toulouse) in the frame of the F2E Organopig project will aim at evaluating the cell-specific effect of VerA in pig intestinal organoids.

Moreover, and in order to complete and validate our results, human non-carcinogenic cell lines should be employed. Eventually, the use of co-cultures of human intestinal cells and liver cells using a transwell system will allow the study if the genotoxic effect of VerA is restricted to exposed cells or if VerA and its metabolites can exert a genotoxic effect after their passage of the intestinal barrier. Additional perspectives regarding carcinogenicity are detailed in the general perspectives section.

Metabolization of VerA *in vitro* and *in vivo*: a metabolomics study.

The formation of genotoxic metabolites of AFB1 and STC is described in the literature, along with their direct relationship with genotoxic effects⁵². The main metabolite of AFB1 with genotoxic activity is the epoxide AFB0, but the hydroxylated form Aflatoxin M1 is also genotoxic. In the case of STC, genotoxicity is associated with the biotransformation to both an epoxide and catechol metabolites. According to our results, the biotransformation of VerA is important for its mutagenicity and genotoxicity, but no data is available on the biotransformation of VerA.

The objective of this study was to **characterize the metabolites of VerA, especially in search of compatible forms with the formation of a genotoxic derivative (3)**. Human liver S9 fractions containing both microsomal and cytosolic enzymes were incubated with purified VerA and appropriate cofactors in order to provide a comprehensive list of metabolites produced *in vitro*, and to elucidate major metabolite structures. In a second step, we obtained a broader overview of the metabolites present in more complex samples using intestinal porcine epithelial cell line (IPEC1), porcine jejunum explants, and precision-cut liver slices exposed to VerA. Based on this workflow, we provide a first qualitative characterization of the metabolites derived from the metabolization of VerA (Fig. 11). This project was carried out in collaboration with the metabolomics platform AXIOM and the team MeX (Toxalim, INRAE Toulouse). Results have been disseminated in several (inter)national conferences and are the object of a paper published in the journal "Food and Chemical Toxicology" (3).

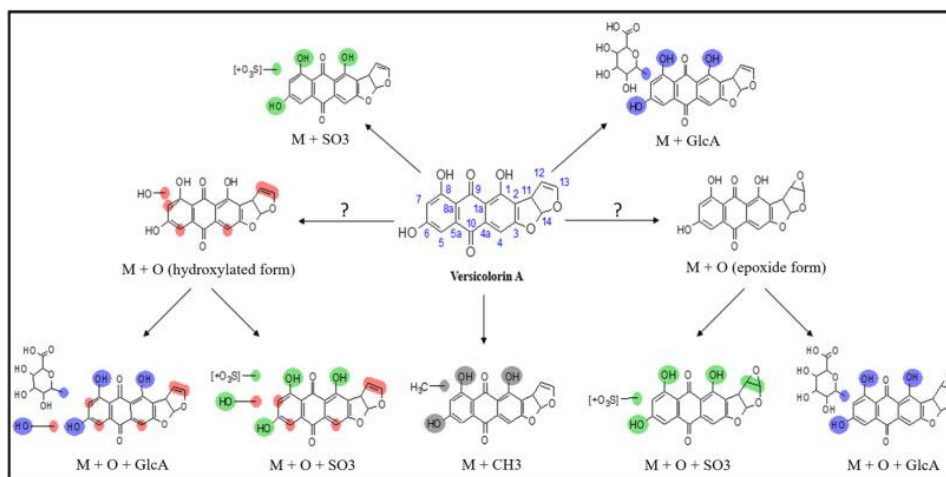


Fig. 11. Identified structures of VerA metabolites. Highlighted atoms correspond to possible bonding positions. Red positions correspond to hydroxylation (-OH), green to conjugation with sulfate (-SO₃), blue to conjugation with glucuronic acid, grey to methylation (-CH₃), and pink to double bond reduction. Question marks indicate the uncertain biotransformation of VerA into an epoxide and/or hydroxylated form. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

We identified one phase I metabolite M + O compatible with a hydroxylated and/or an endo- or exo- epoxide form of VerA. Unfortunately, the fact that only small amounts of toxin can be purified hinders the unambiguous elucidation of the chemical structure of this metabolite by NMR, and we can only speculate about the structural possibilities. However, the presence of the M+O metabolite strongly supports the idea that a genotoxic form is formed during the biotransformation of VerA, which is in line with the mutagenicity/genotoxicity results described previously. However, M+O the metabolite could represent an epoxide or a hydroxylated metabolite. Indeed, the aromatic ring of STC can be hydroxylated to form a genotoxic catechol⁵³. This suggests that catechol production, which can be formed by hydroxylation at C-7 in VerA and is equivalent to C-9 in STC, may represent an alternative genotoxic mechanism of VerA. A third possibility suggests that the hydroxylation at C-11 could lead to a hydroxylated metabolite homologous to aflatoxin M1 and 12c-hydroxy-STC, both also genotoxic⁵⁴.

Our analysis also provided with other elements useful to understand the detoxification of VerA. The phase II metabolism of AFB1 that lead to its detoxification involve conjugation reactions mainly to glutathione, glucuronic acid and sulphate. The conjugation of AFBO with glutathione (GSH) mediated by Glutathione-S-Transferase (GST) constitute the main detoxification pathway⁵⁵. In our present study, only glucuronide and sulphate metabolites were detected, representing the main potential detoxification routes and glutathione-conjugated forms were not detected. VerA induced the overexpression of GST enzymes in intestinal cells⁴⁴, so further research needs to be conducted using different incubation times and taking into account possible technical bias / sensitivity issues that might hinder the detection of GSH derivatives.

Overall, these results provide high-quality analytic evidence for further risk assessment of the harmful emerging mycotoxin VerA.

Perspectives: the current findings call for more research on VerA metabolism, including quantitative assessments of metabolization rates and transformation kinetics. Moreover, the identification of the M+O metabolite structure by NMR, the presence of VerA-DNA adducts and/or the identification of a GSH derivative is needed to understand the chemical nature of the possible genotoxic metabolite of VerA. In order to understand the metabolization route followed by VerA, the use of bacosomes expressing different p450 enzymes can be proposed.

VerA-mediated activation of AhR is important for the interaction between AFB1 and VerA.

This research was carried out in collaboration with BDS Biodetection Biosystems (The Netherlands) in the frame of the ITN ProtectED project described above, including a short stay of the PhD student Clémence Budin in our

lab, and were published in 2021 in the journal “Food and Chemical Toxicology” (7). The AhR is a latent cytoplasmic transcription factor involved in xenobiotic sensing, induction of CYP1A1/2 as well as other cellular processes such as cancer progression and control of the cell-cycle⁵⁶. A previous transcriptomic analysis on intestinal Caco-2 cells revealed significant up-regulation of AhR target genes in cells exposed to VerA, including several CYP450 enzymes, whereas AFB1 did not induce such changes⁴⁴. Moreover, typical ligands of the AhR, such as dioxins and certain dietary compounds, display a planar chemical structure, which further suggested that, **VerA could interact with the receptor and affect normal cell physiology through activation and/or disruption of normal AhR signaling**. AhR-responsive DR_{human} CALUX reporter cells were used to investigate this hypothesis. As showed in Fig. 12, VerA, but not AFB1, significantly induced AhR-transactivation in a concentration-dependent manner. The effects of AFB1 and VerA on AhR transactivation were confirmed in another (metabolically competent) AhR-responsive cell-line, the DR CALUX, in which we obtained similar results for both toxins. AhR activation was accompanied by a VerA-mediated induction of the expression of CYP1A1, in agreement with previous results⁴⁴.

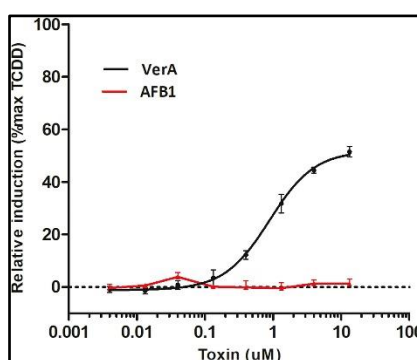


Fig. 12. Effects of versicolorin A (VerA) and aflatoxin B1 (AFB1) on the AhR-mediated luciferase induction in HepG2 DRhuman CALUX reporter cells after 4 h of incubation (mean \pm SD).

The **VerA-mediated induction of the AhR** also suggests a potential **enhancing effect of AFB1 genotoxicity** as the ligand-binding activation of the AhR results in increased expression of CYP450s that can catalyse the bioactivation of procarcinogens. As VerA and AFB1 may co-occur in food, we next studied the effect on cytotoxicity and genotoxicity of VerA in combination with AFB1, using increasing concentrations of AFB1 with 1 μ M of VerA. This concentration of VerA induces a significant activation of AhR and CYP1A1 expression over a 24h time-period of exposure but did not have a significant cytotoxic effect nor induced a significant increase of γ H2AX or phospho-p53 in HepG2 cells. Results showed that exposure to this mixture resulted in a stronger cytotoxic effect compared to that of 1 μ M VerA alone, as well as the effect of AFB1 alone, in particular after 48h of exposure.

Exposure of HepG2 cells to the mixture resulted in significantly higher levels of γ H2AX induction than the effect of 1 μ M of VerA alone and the effect of AFB1 alone from 0,1 μ M–3 μ M (Fig. 13A). The same effect was observed in the HepG2-p53 CALUX where the combination resulted in significantly greater levels of p53 transcriptional activity than 1 μ M of VerA alone and 0,1 μ M–1 μ M of AFB1 alone (Fig. 13B), as well as regarding Ser15-p53 phosphorylation at 1 μ M (Fig. 13C).

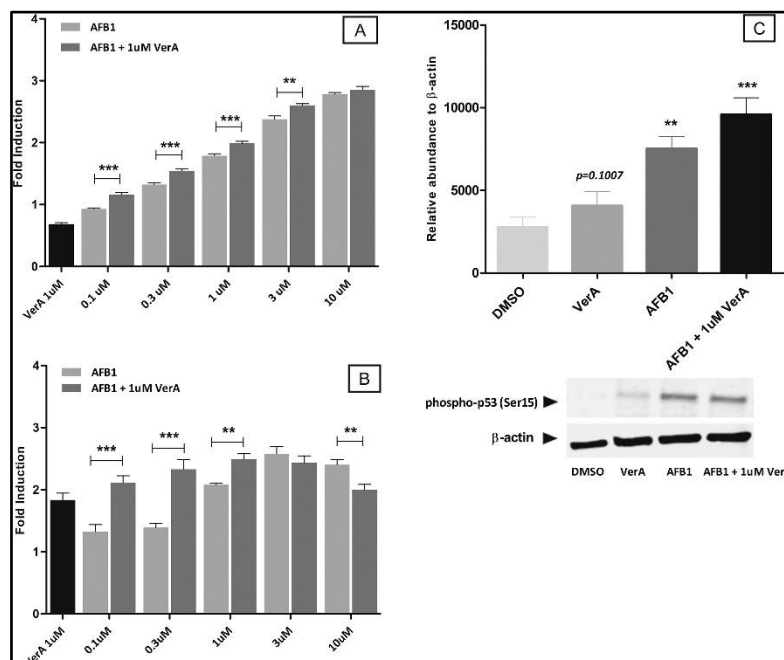


Fig. 13. Interaction between VerA and AFB1. Combined effects of aflatoxin B1 (AFB1) and 1 μ M of versicolorin A (VerA) on (A) H2AX phosphorylation in HepG2 cells measured using the yH2Ax-ICW assay and (B) p53 transcriptional activity in HepG2-p53 CALUX assay and (C) p53-Ser15 phosphorylation in wild-type HepG2 cells compared to their individual effects (mean \pm SD). *: p -value ≤ 0.05 ; **: ≤ 0.01 ; ***: ≤ 0.001 (t-test).

Interestingly, genotoxicity was enhanced especially in those mixtures containing relatively low AFB1 concentrations (0.1 μ M–1 μ M). The present results support the hypothesis that the AhR activation promoted by VerA could be associated with a greater bioactivation of AFB1. The present results pinpoint to a need of expanding the toxicological knowledge regarding mycotoxin biosynthetic precursors with the aim of identifying those who may pose a threat to food safety. Since the exposure to multiple mycotoxins is a rule rather than an exception, our results additionally contribute with valuable information to hazard identification and risk assessment in food safety.

Perspectives: The transactivation of AhR by VerA had a significant impact on AFB1 toxicity, and according to our results, it would also affect the own metabolism (and activation) of VerA, other bisfuranoid mycotoxins or other prototoxicants. Although the results presented here show an interaction between VerA and AFB1, a complete characterization of the interaction between these compounds as well as other related mycotoxins will be needed using multiple exposure conditions, mixtures composition and toxic endpoints. Because AhR signalling is particularly important in the intestine, it would be interesting to investigate the consequences of this activation at the intestinal level, as well as the possible ability of VerA to also activate other important transcription factors such as PXR and CAR.

4.2.3 Development of new tools to investigate the toxicity of mycotoxins

4.2.3.a Discovery and validation of biomarkers of mycotoxin effect. One of the common threads of my past and present research is the discovery and validation of biomarkers, usually with the pig as target and model species. I am interested in responding to the need in mycotoxin research of developing biomarkers linked with the presence and toxicity of mycotoxins.

A biomarker is a molecule that is consistently modified or present at abnormal concentrations in specific illnesses or other health conditions. In toxicology, a biomarker can be defined strictly as a biological response to a chemical but not the presence of the agent or its metabolites within the body⁸. Rather than biomarkers of exposure (usually represented by the xenobiotic of interest or their metabolites in the body) or biomarker of susceptibility (usually genetic traits that indicate a particular sensibility to a toxic compound), the main interest in mycotoxicology is finding biomarkers of effect, i.e. indicators of the molecular changes that result to the exposure to mycotoxins. The

main advantage of these biomarkers is that they reflect the toxic effect of mycotoxins even when the toxins are no longer present, neither in the body nor in the food. Among all types of biomarkers of effect, one group must be preferably sought: tissue leakage proteins⁵⁷. These are proteins that are liberated only or more abundantly from those tissue(s) specifically affected by the toxins and can be later found in body fluids (blood, urine, saliva, etc.). These biomarkers are more specific for the detection of the toxic effect and can be easily quantified from easy to collect sample types. The identification of these new tools to improve the management of porcine health regarding the effects of mycotoxins is therefore largely needed.

I have participated in a scientific project entitled ExpoMycoPig (ANR-17-Carn012, coordinated by Philippe Pinton) whose main objective was identifying and validating biomarkers of effect of mycotoxins in the pig. My contribution to the project was related to the toxicity of the mycotoxin ZEN in the intestine as well as the activity of this mycotoxin as a metabolic disruptor described above. Concerning biomarkers of effect, our results indicated that the toxicity of ZEN affected the immune-metabolic status of exposed tissues such as the intestine. We discovered that exposure to ZEN induced varying phenotypes in pigs regarding metabolic-related parameters and that such changes depended on the exposure conditions to ZEN (dose and time of exposure). These data also suggest that a more complete study of the metabolic disruption induced by ZEN will provide with a list of circulating molecules (e.g. adipokines, acute phase proteins, metabolites, etc) whose concentration might be related with the toxicity of ZEN, thus representing potential biomarkers of effect.

Perspectives: The research of molecular markers of the specific biological response that can be linked to mycotoxin intake is a hot topic in mycotoxicology, since they do not exist in spite of the efforts of the scientific community and the interest of the private companies developing remediation products. The use of omics, and more specifically proteomics, has been pinpointed as the most suitable tool to unravel the toxicity of mycotoxins and thus contribute to the identification of novel and applicable biomarkers of effect. The discovery of such molecules requires a specific workflow that prioritizes the use of ex vivo exposure of tissue explants and the secretome as analytical sample for biomarker discovery. This strategy has proven useful in the past, as described in a previous section of this document. It would be convenient to develop ex vivo exposure conditions mimicking different physiological status (i.e. adapting the hormones and metabolites media composition to simulate stress) or infectious conditions (use of bacteria or their bacterial toxins) with would also help screening candidate biomarkers using specific conditions.

4.2.3.b Development and use of a toxicity screening tool based in molecular phenotyping.

The risk associated with the exposure to major mycotoxins is highly characterized, and the specific mechanisms by which each of these molecules exert their toxic effects are mostly known. However, mycotoxins can have common toxic effects such as the induction of oxidative stress or inflammation. Moreover, the type of toxicity can change according to the toxin concentration and the length of the exposure. Consequently, *in vitro* toxicity assessment needs from the combination of results from multiple testing of single cellular functions, and in consequence the discovery of relevant toxic effects often rely on the existence of some a-priori information on the molecule's toxicity mechanism. Moreover, there are more than 300 potentially emerging mycotoxins identified, whose presence in food represents a potentially high risk that needs to be characterized. This awareness calls for the development of new analytical techniques that are able to provide rapid information on the toxicity of these metabolites and to identify the main biological processes altered by their toxicity.

With this objective, we evaluated the ability of the molecular phenotyping technique ICM-MS (laser desorption ionization time-of-flight (MALDI-TOF) Mass Spectrometry) to be used as toxicity screening tool applied to mycotoxins in a collaboration a collaboration between TOXALIM and the PIXANIM platform (INRAE Nouzilly) thanks to a starting

grant of the INRAE Animal Health Department. This method is an untargeted, high-throughput analytical tool that allows the identification of specific molecular phenotypes through the identification of specific peptide/protein MS fingerprints. The molecular phenotypes acquired using ICM-MS phenotyping have the ability to represent all the changes that derive from the multiple cell functions disrupted following an insult, and has been successfully applied in toxicology. To this end, the ICM-MS profiles of differentiated Caco-2 human intestinal cells exposed for 24h to 0.1 μM and 10 μM of regulated mycotoxins (deoxynivalenol, zearalenone, aflatoxin-B1, Fumonisin-B1, patulin, ochratoxin-A) were acquired and compared.

PCA was produced using only discriminant peaks ($p < 0.05$) showed a clear discrimination between control and treatment groups. Toxins were grouped two by two at 10 μM , namely AFB1 with PAT, ZEN with FB1 and OTA with DON. This grouping is also evident at 0.1 μM for AFB1 and PAT, but less clear for the other toxins, even in the trend is already visible.

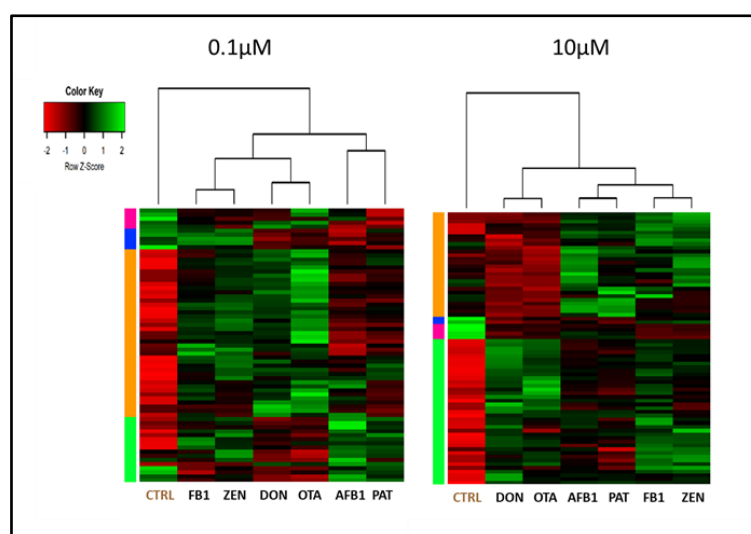


Figure 14. Hierarchical clustering analysis of differentially abundant masses (p -value < 0.05) in Caco-2 intestinal cells exposed to 0.1 μM and 10 μM of the mycotoxins Aflatoxin B1 (AFB1), patulin (PAT), zearalenone (ZEN), fumonisin B1 (FB1), Ochratoxin A (OTA) and deoxynivalenol (DON) and solvent control DMSO (CTRL). Heat maps represent differentially abundant masses in exposed cells. Red and green indicate values above and below the mean (average Z-score), respectively. Black indicates values close to the mean.

Hierarchical cluster analysis showed similar trends as PCA. Four mass clusters were identified, and controls were well differentiated from treated cells (Fig. 14). Moreover, toxins were paired together two by two in the same manner: AFB1-PAT, OTA-DON and FB1-ZEN.

ICM-MS profiles of Caco-2 cells are related with common mechanisms of toxicity. Our results indicate that at 10 μM , the cell phenotype as analyzed by ICM-MS is very similar between certain toxins that appear clustered by pairs, and distinct between these pairs of toxins. This clustering by pairs reflects the initiation of a similar response by the exposed cells. In the case of the pair of toxins AFB1 and PAT, both their structures are associated with a mutagenic effect and the induction of oxidative DNA lesions. PAT is a $\alpha, \beta, \gamma, \delta$ -unsaturated lactone. The α, β -unsaturated carbonyl group present in PAT is recognized as alert for mutagenicity, as it is highly reactive towards nucleophilic amines and thiols⁵⁸. PAT is hence a direct genotoxic able to modify DNA bases by the electrophilic α, β -unsaturated carbonyl. Additionally, PAT can also bind and deplete GSH from exposed cells, thus decreasing the anti-oxidant ability of cells. In consequence, oxidative DNA damage is facilitated⁵⁹. As explained above, AFB1 is a bisfuranoid toxin that is biotransformed into a reactive exo epoxide form that binds DNA inducing point mutations, and it is also able to use the GSH pool of exposed cells during its biotransformation, which is related with the induction of oxidative stress and DNA damage.

DON and OTA are both known to inhibit protein synthesis. OTA interferes with protein synthesis through the inhibition of the activity of phenylalanine t-RNA synthase and phenylalanine hydroxylase. The interaction with phenylalanine t-RNA synthase is related the isocoumarin structure of OTA, while the phenylalanine moiety of the toxin also plays a role as a competitor with the enzyme⁶⁰. In the case of DON (and other trichothecenes), the binding to the ribosome and interference with the action of peptidyl transferase are mainly responsible of the DON-mediated of the disruption of protein synthesis, although a PKR-dependent inhibition of eIF2 α -mediated translation initiation has been also described⁶¹.

The toxicity of the mycotoxins FB1 and ZEN is based in their structural similarity to endogen molecules, FB1 being similar to sphingosine and ZEN to estrogen. Because of its structural similarity with sphingosine, FB1 competitively inhibit ceramide synthase and, as a result, disrupt the de novo biosynthesis of ceramide and sphingolipid metabolism⁶². As detailed above, ZEN is an estrogen-mimetic mycotoxin, that disrupts ER-mediated cell signaling.

Because one of the possible uses of ICM-MS phenotyping in (myco)toxicology would be providing with an evaluation of the comparative toxicity of a molecule of unknown toxicity, we used the emerging mycotoxin, VerA to evaluate this hypothesis. Both PCA and HCA analysis (Fig. 15) show that the phenotype of cells exposed to VerA were clustered together with mycotoxins inducing direct DNA damage, in agreement with the results detailed in previous sections of this document. Indeed, VerA is mutagenic and genotoxic.

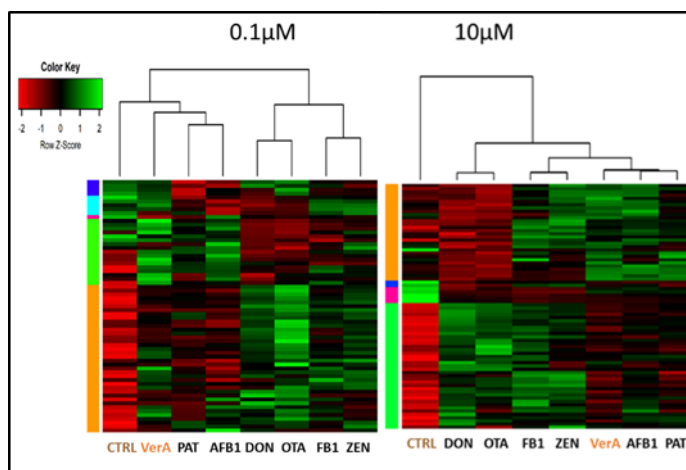


Figure 15. Hierarchical clustering analysis of differentially abundant masses (p -value < 0.05) in Caco-2 intestinal cells exposed to 0.1 μ M, 1 μ M and 10 μ M of the mycotoxins Aflatoxin B1 (AFB1), patulin (PAT), zearalenone (ZEN), fumonisin B1 (FB1), Ochratoxin A (OTA) and deoxynivalenol (DON), versicolorin A (VerA) and solvent control DMSO (CTRL). Heat maps represent differentially abundant masses in exposed cells. Red and green indicate values above and below the mean (average Z-score), respectively. Black indicates values close to the mean.

Results showed that the ICM-MS profile of intestinal cells exposed to mycotoxins was related to the main toxic mechanism of action of each mycotoxin, and was related with the toxicity of unknown mycotoxins. ICM-MS profiling, in conclusion, represents a promising toxicity-screening tool, and results allows for the screening of the toxicity of emerging mycotoxins. These results have been communicated in several national and international conferences, and the corresponding manuscript is currently being prepared.

Perspectives: The current findings indicate that the ICM-MS molecular phenotypes of cells exposed to different mycotoxins are able to identify common patterns of toxic effect. More data is needed to validate ICM-MS as a screening technique in toxicology including the use of more known mycotoxins and their precursors and metabolites. The use of HRMS and top-down databases will be also useful to identify the peptides/proteins that are the most discriminant within toxicity-specific phenotypes, thus revealing relevant specific cell responses. ICM-MS can also be used in cells isolated from

exposed animals. For instance, circulating immune cells are likely to bear ICM-MS signatures specific of a given mycotoxicosis and help in diagnostics. Molecular phenotyping can also be applied at the tissue level using MALDI imaging. This technique uses mass spectrometry for mapping the distribution of molecules in a tissue. The possible applications of this technique in mycotoxicology are varied, including the evaluation of the molecular composition changes in exposed tissues in search of new adverse outcome pathways (AOP) or biomarkers of effect, as well as the identification of the fate of a mycotoxin (and its metabolites) in its target organ(s).

SECTION 5

Research project and scientific perspectives

In this section, I will detail my research project and will provide with an overview of the global perspectives that can be distilled from the works detailed above. These will be divided in three parts regarding the identification of new effects of known mycotoxins, the identification of new mycotoxins and the use of new methodologies to evaluate mycotoxins toxicity.

5.1 Identification of new effects of known mycotoxins

5.2.1 Zearalenone as a metabolic disruptor and relationship with reprotoxicity. Our results showed that ZEN might function as a MDC and that the liver is a target for ZEN-mediated metabolic disruption, affecting lipid and glucose metabolism as well as insulin signaling without inducing hepatocellular damage. The main metabolic organ of the body is the liver. The use of omics in exposed liver cells would be valuable in order to understand what are the main affected pathways and metabolic networks. These changes could be used to identify the main ZEN targets regarding metabolism, identify relevant AOPs and key molecules involved in this toxicity (thus potential biomarkers of effect). Such results could be then use to design a list of potential molecular targets to be investigated in organ and serum samples from exposed animals in order to provide with an overview of the impact of ZEN exposure in metabolism, immunity and endocrine parameters.

ZEN exposure also seemed to promote a systemic pro-inflammatory status. These changes were associated (and probably facilitated) by a ZEN-mediated modulation of several proteins including several adipokines. Because the dysregulation of adipokines encompass fertility disorders, there is a possibility that they participate in the reproductive toxicity induced by ZEN, which would be interesting to investigate. The combination of *in vitro* studies using ovarian cells (i.e. granulosa cells) and exposed tissues *in vivo* would give an indication of the importance of metabolic disruption in reproductive functions, as well as hints on eventual pharmaceutical intervention strategies.

The disruption of metabolism, inflammation and endocrine status has certainly an effect in growth and development that is yet to be estimated in animal production. Moreover, ZEN seem to modulate intestinal and immune functions. In all, exposure to ZEN, especially at an early age, could have the potential to hinder the expression of the growth potential of an animal. It would be thus interesting to complete the evaluation of the cost of exposure to ZEN, alone or in combination with other xenoestrogens or MDCs in terms of growth impairment added that of reproductive losses, since it would help establishing recommendations of maximal exposure levels at each production phase.

5.2.2 Depicting the metabolic disruption exerted by ZEN as a source of biomarkers of effect. In female pigs, ZEN mycotoxicosis induce morphological alterations in the reproductive tract (hyperplasia, edema, hyperemia, prolapse, ovarian atrophy), precocious puberty, alterations of oestral cycle, decreased fertility and reduced litter size. In males, it is associated with testicular atrophy, decrease of sperm quality and poor libido. ZEN is also teratogenic, inducing uterine-vulvar hyperplasia and splayleg. ZEN toxicosis is presently difficult to diagnose because it depends on the availability of the contaminated feed for analysis: symptoms typically appear when feed is already consumed⁶³. The discovery of biomarkers of ZEN effect (BZEN) is hence largely needed to this end. In the case of ZEN, markers or exposure are not helpful, as circulating levels of ZEN do not reflect exposure levels nor the severity of toxicosis symptoms^{29,63}. In the case of BZEN, none of the parameters related to ZEN toxicity in reproduction, endocrine, immune nor intestinal toxicity showed a clear dose-response relationship with ZEN exposure. The scientific community consider that the evolving omics-technologies are the most suitable tools to unravel the toxicodynamics

of ZEN and thus contribute to the identification of novel and applicable biomarkers⁷. In this sense, the discovery of new AOPs for ZEN will provide with a new group of molecules candidates to be BZENs. A more complete study of the metabolic disruption induced by ZEN would provide with a list of circulating molecules (e.g. adipokines, acute phase proteins, metabolites, etc) whose concentration might be related with its toxicity, thus representing potential BZEN. The use of a list of biomarkers instead of single molecules would help increasing their diagnostic performance. In this sense, some mathematical approaches might be useful, in order to find non-linear relationships between the levels of BZEN that aid in the diagnostics of ZEN mycotoxicosis. The validation of these BZENs would thus require the evaluation of their dose-response and their performance, by using a panel of differential diagnostics including frequent reproductive pathogens, different patho-physiological conditions or exposure to other xenoestrogens.

I lead a consortium including private (Veronika Nagl and Barbara Novak; DSM Austria) and academic partners (Nicolas Cabaton; Team MeX, Toxalim INRAE Toulouse and Pascal Froment/Joëlle Dupont, Team Sensor; INRAE Val de Loire) that share an interest on this topic. Grant proposals on the characterization of zearalenone as a metabolic disruptor and its relationship with fertility and reproductive functions were submitted in the past and will still be submitted in 2023 (ANSES, F2E).

5.2 Toxicity of emerging mycotoxins

The results of the ANR JCJC project Versitox warrant further research on the toxicity of VerA. Indeed our data indicate that VerA is a highly toxic mycotoxin, which together with the emergence of the contamination of food and feed in southern Europe with Versicolorin A-producing *Aspergillus* species also illustrates the importance of continue advancing in this subject. My contribution will be centered in the following points:

5.2.1. Digging deeper in the toxicity of VerA. Data on the toxicity of VerA strongly suggest that it is more complex than that of AFB1, leaving many questions to be answered. In order to assess chemical hazards for compounds of unknown toxicity the use of omics is particularly helpful.

Transcriptomics and proteomics provides with an overview of the effect of mycotoxins at different molecular levels and functional analysis help identifying key toxic events. We have produced some preliminary data in collaboration with the transcriptomics platform GeT-TRIX (Toxalim, INRAE Toulouse) and the Toulouse Proteomics Infrastructure (IPBS; Toulouse) in the frame of the JCJC project VersiTox. First, transcriptomic analysis of differentiated IPEC-1 cells exposed to VerA was applied to follow the early changes (6h) in gene expression of intestinal cells exposed to VerA. Next, we aimed at following the early changes and kinetics of the proteome of differentiated intestinal cells exposed to VerA for 3h, 6h and 24h. In summary, omics analysis confirmed that the exposure of intestinal cells to VerA induces a DNA damage response, oxidative stress and cell shape/adhesion changes. Additional hints on the toxicity of VerA such as the disruption of RNA biology/processing, a permanent disruption of eIF2 signaling, the toxicity of mitochondria and the consequences in the promotion of an immune response were given. The elucidation of these mechanisms of toxicity will be essential to assess the hazard associated with VerA.

Mitochondria is one target of VerA toxicity, which was confirmed by the regulation of many molecular targets involved in mitochondrial function. We observed that ATP production was maintained in IPEC1 cells exposed for 6h to VerA at concentrations up to 1 μ M in the presence of glucose (Fig. 16A). However, when glucose was replaced by galactose, cells could not use glycolysis to compensate the inability to produce ATP by oxidative phosphorylation, and ATP dropped significantly at a concentration as low as 650nM compared to controls (Fig. 16B). This mitochondrial toxicity was associated with a significant activation of caspase 3/7 (Fig. 16C), effectors of the intrinsic apoptotic pathway.

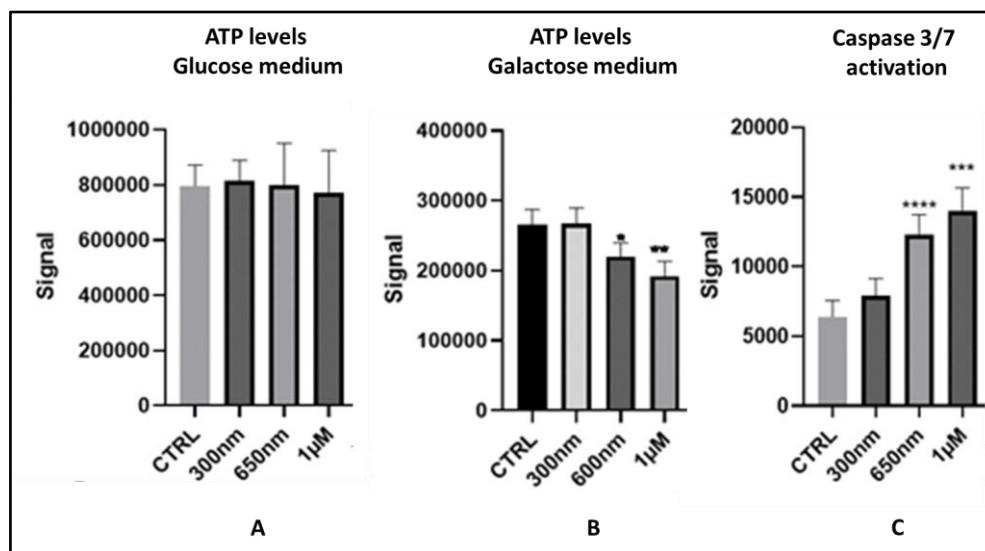


Figure 16. Evaluation of the mitochondrial toxicity of VerA in intestinal cells. ATP levels in intestinal cells exposed to different doses of VerA for 6 h in the presence of glucose (A) or galactose (B), measures of cytotoxicity (C), and caspase 3/7 activation (D). Values are means with standard errors of the mean represented by vertical bars ($n = 3$). Asterisks indicate statistical differences (* $p < .05$; ** $p < .01$, *** $p < .001$, **** $p < .0001$).

Mitochondria are the energy engines of cells and key to cellular adaptation to metabolic changes. Further studies should be oriented at evaluating the effect of VerA as an inhibitor of the mitochondrial respiratory chain enzyme activity, its role in inducing mitochondrial oxidative stress, its activity as an oxidative phosphorylation uncoupler and the VerA-dependent effect on the integrity of the mitochondrial membrane and mitochondrial biogenesis. Many cell responses can appear when mitochondrial functions are impaired such as the activation of an inflammatory status, inhibition of protein synthesis as well as epigenetic changes. Indeed, the mitochondria is much more than only a metabolic organelle, and mitochondrial status can influence many cell functions by interplaying with genomic DNA. Mitochondria are controlled by the coordination of two genomes: the mitochondrial and the nuclear DNA. As such, variations in nuclear gene expression as a consequence of mutations and epigenetic modifications can affect mitochondrial functionality. Conversely, the opposite could also be true. Because VerA is a genotoxin and our results point at a role as DNA replication/transcription repressor (discussed below), it will be essential to investigate how VerA affects mitochondrial DNA integrity, replication and transcription. Moreover, mitochondria function as central metabolic hub controls some of the main substrates involved in nuclear DNA methylation. Of note, the methionine degradation pathway seems to be disturbed in VerA-exposed cells, associated with an increase activity of sirtuins (a family of histone deacetylases) and the regulation of the expression on many histones. These data warrant further research on the role of VerA in the epigenetic reprogramming exposed cells.

According to our data, the mitochondrial toxicity induced by VerA might be responsible for the activation of PKR, which would regulate eIF2 signaling, altering protein synthesis. Protein synthesis would also be affected by DNA replication/transcription repression. It would be interesting to investigate the conditions leading to these effects. The role of VerA as a protein synthesis inhibitor should be confirmed. The study of eIF2 phosphorylation dynamics and its dependence of PKR signaling and mitochondrial toxicity will provide with an idea of the importance of these phenomena in the VerA-mediated protein synthesis.

Our omics analysis indicate that an evident disruption of different processes important for replication, transcription and protein synthesis. These findings are agreement with previous transcriptomic results obtained from proliferating intestinal cells, where VerA increased the abundance of sequences corresponding to small nucleolar RNAs (both box C/D and box H/ACA snoRNAs) that are generally encoded within introns and guide chemical modification on ribosomal RNAs. VerA exposure also affected the expression of a number of genes involved in RNA

processing both in Caco-2 and IPEC-1 cells altered the abundance of spliceosome proteins in IPEC-1 cells. In all, these data suggest that VerA could inhibit the splicing of introns that host small nucleolar RNAs, induce alternative mechanisms of transcription initiation or defaults in transcription termination control. The alteration of transcription by natural compounds is rare, and among the few molecules having this effect it is common to find fungal metabolites, such as alpha-amanitin or mycophenolic acid. Of note, transcription disruptors are known to be extremely toxic, with many effects leading to multi-organ failure and cancer but they also bear a high therapeutic potential as anti-cancer or anti-viral drugs. Because of their proprieties leading to an inhibition of cell division and their special mechanism of action, these substances are also widely employed tools in molecular biology and in pharmacology. The complete evaluation of the extent and the mechanisms by which VerA affects transcription is therefore essential to fully characterize the danger associated with the exposure to this toxin as well as to understand what future uses could be made of this specialized metabolite. In order to characterize the impacts of VerA on transcription and the kinetics of these changes we need to perform a complete study of RNA sequencing. The study of the whole transcriptome by RNAseq will allow to evaluate the impact of VerA on splicing (exon skipping or intron retention), on the use of alternative transcription initiation or termination sites, on transcript read-throughs. Moreover, we will be able to identify differential changes in gene expression or transcript structures over time in cells exposed to VerA, which, integrated to other omics data obtained previously will help to decipher the disturbed mechanisms behind the observed changes. The new information will contribute to the evaluation of the risk to public and animal health associated with the emerging presence of VerA in food and feed. In order to further investigate the role of VerA as an inhibitor of transcription, and in collaboration with Pascal Martin (BFP, INRAE Bordeaux) and Yannick Lippi (Toxalim, INRAE Toulouse), we aim to perform a complete RNA sequencing study. This project has been funded by the INRAE Animal Health Department under the call "Action incitative sur priorités SSD -2021-2025 Dept SA" and will start in 2023.

We observed a significant regulation of the expression of two topoisomerases TOP1 and TOP2B in exposed IPEC1 cells. The inhibition of topoisomerases by VerA might be one of the reasons leading to replication stress observed in our data. Actually, a preliminary *in silico* analysis in collaboration with Luca Dellaflora (University of Parma, Italy) predicted an inhibitory role of VerA for topoisomerases. Preliminary results of decatenation assays indicates that VerA can inhibit Topoisomerase-II activity in concentrations as low as 10 μ M. Ongoing research include *in vitro* assays in carcinogenic and non-carcinogenic intestinal cell lines using the ICE (in-vivo complex of enzymes) assay. In this assay protein-genomic DNA complexes are recovered and specific topoisomerases are detected. The analysis will be completed imaging and complementary genotoxicity measures.

VerA is a mutagenic and genotoxic mycotoxin, and our omics results suggest that induce cell shape and adhesion changes that are compatible with the initiation of several phenotypic changes compatible with the development of an EMT. We were not able to detect some of the EMT hallmarks in our exposure conditions, but the fact of finding changes compatible with this phenotypic transformation after transcriptomic and proteomic analyses justify further investigations. EMT is related, among other things, with malignant transformation. Indeed, one of the main research questions that rise from our data is to investigate if VerA is carcinogenic. Carcinogenesis refers to a complex multistage process in which normal cells transform into cancer cells. During carcinogenesis cells suffer many transformations including a block in cellular differentiation visualized as morphological changes in the cells, the acquisition of immortality, the acquisition of tumorigenicity (visualized through foci formation, anchorage independent growth in semi-solid agar and autocrine factor production) and eventually full malignancy when cells are injected into a suitable host. *In vitro* cell transformation refers to the induction of phenotypic alterations that have long been considered alterations associated with cells exhibiting neoplastic potential *in vivo*. Transformed cells with

the characteristics of malignant cells have the ability to induce tumors in susceptible animals; this supports the practice of using specific phenotypic alterations *in vitro* as criteria for predicting carcinogenic potential *in vivo*. The combination of cell transformation assays, multimodal imaging and quantification of selected biomarkers should be employed to estimate the carcinogenicity of VerA *in vitro* using chronic exposure conditions. Because the digestive system is a very likely target organ, known models for the *in vitro* study of esophagus, stomach and colorectal cancer should be foreseen.

The fact that VerA is purified in-house, has limited the design and execution of *in vivo* studies. Ideally, the use of mammal models would be desired. The follow-up and quantification of VerA and its metabolites in different fluids and body tissues would provide with a first evaluation of its metabolization sites and toxicokinetics. The combination of histo-anatomopathological analysis, omics and in-vivo genotoxicity assays would help identify the target organ of VerA. Ideally, if the *in vivo* toxicity of VerA was confirmed, it would be essential to evaluate the exposure conditions related with carcinogenesis. An *in vivo* trial is foreseen in the project "VerAfla" led by Olivier Puel and submitted to the ANSES in 2023, in which I participate.

5.2.2 Production of elements to characterize the risk for public and animal health related to the presence of VerA and other *Aspergillus* toxins in food and feed. One of the priorities in order to evaluate the VerA-associated risk is estimating its occurrence in food and feed as well as human exposure. However, as concluded from a collaboration with Ronald Maul (Max Rubner-Institut, Germany; 2), there is still much room for improvement in the development reliable VerA quantification methods. One collaboration from BioToMyc team established in the past with Michael Sulyok (BOKU, Austria) was able to provide with preliminary data on the presence of VerA in food contaminated with AFB1⁴⁴. Nevertheless, surveys including more food types are advised, since VerA is also produced by the STC-producing molds *A. nidulans* and *versicolor*. I am involved in collaborations that aim at producing data on VerA occurrence/exposure that will be useful to adapt toxicology studies, particularly if co-occurrence data with AFB1 and other toxic precursors and side metabolites becomes available. Indeed, this will improve interaction studies using concentrations and ratios based in real conditions. In parallel with the determination of the occurrence of VerA in feedstuffs, it would be also essential to evaluate human exposure to VerA. In this sense, new collaborations with Ariane Vettorazzi and Elena González (Universidad de Navarra, Spain) as well as Florence Forget (UMR MycSA, INRAE Nouvelle Aquitaine - Bordeaux) and Michael Sulyok (BOKU, Austria) are foreseen in order evaluate human exposure and food/feed occurrence, respectively.

Aflatoxigenic *Aspergillus* species are now also found in temperate regions like Europe, due to climate change. For example, the presence of AFB1 has been confirmed in samples of corn harvested in France. This problem is also likely to be exacerbated by changes in agronomic practices under social and economic pressure. The recent energy crisis has also led storage organizations to rethink the strategies of drying and storage of corn, to define less energy intensive and less costly methods but which may affect the presence and production of aflatoxins. The occurrence of AFB1 related compounds is much less characterized and no data is available for French maize. Moreover, the impact of the use of alternative agricultural practices in their production is not known. These compounds are likely to appear as complex mixtures in contaminated samples, so research into their interaction would be essential to establish the hazard associated with their presence, particularly with regard to the possibility of a synergistic effect with AFB1. A consortium including different INRAE units (Toxalim, Mycsa, Frise), food safety agencies (ANSES) and private partners (Syngenta, Maisadour) will work together regarding this perspective (AspeRisk project, submitted to the INRAE metaprogramme XRisques grant call). This project covers the evaluation how climate change and alternative agricultural practices affect the profiles of *Aspergillus* toxins in French maize, as well as evaluating the risk of enrichment with toxic *Aspergillus* metabolites in the present climate and agricultural context.

As mentioned earlier, VerA could contaminate foodstuffs together with AFB1, its precursors and side metabolites. The interaction between these compounds might result in enhanced toxicity (as detailed in part for AFB1 and VerA in hepatic cells in this document) and should be investigated in detail. Indeed, the enhancing role of bioactivation promoted by VerA could affect the toxicity of mixtures of bisfuranoid mycotoxins. Moreover, the mixed genotoxicity mechanisms described here for VerA might also result in different genotoxic outcomes when present with other genotoxic compounds such as AFB1. The study of the interaction between VerA and STC is particularly interesting according with our preliminary data. Results from a comparative microarray analysis of human intestinal Caco-2 cells exposed to AFB1 and its precursors VerA and STC suggest similarities in some toxic effects of VerA and STC as opposed to AFB1. While exposure to AFB1 include little transcriptome changes, VerA induced a strong differential expression of genes and STC. Several canonical pathways were regulated in a similar way for VerA and STC, including molecular mechanisms involved in cancer, oxidative phosphorylation and senescence. These findings showed the high toxicity of VerA and STC, as well as the existence of specific toxicity mechanisms for AFB1's precursors, and warrant further investigation. In this sense, I lead a WP of the collaborative ITN project entitled "BioToxDoc", submitted in November 2022, coordinated by Franz Berthiller (BOKU, Austria). This project will contemplate the training of a PhD student in a specific scientific program, the PhD project will be centered in the interaction of AFB1 and its precursors, and the description of the mechanisms involved.

The toxicity of many *Aspergillus* toxins can be related with their structure. On one hand, the dihydrobisfuran structure of bisfuranoid mycotoxins is responsible of the mutagenic and genotoxic activity of several metabolites such as AFB1, STC and VerA. On the other hand, anthraquinone mycotoxins such as VerA, Versicolorin B (VerB) and averufin (Avn) are linked with mitochondrial toxicity⁶⁴. This anthraquinone structure could also be behind the other distinctive effects identified for VerA, such as the ability to disturb transcription⁶⁵⁻⁶⁷. All these toxins are potentially found co-contaminating food and thus a complex co-exposure is expected. The toxicity of VerB and Avn is relatively unknown. Due to structural resemblance with VerA it would be interesting to investigate how exposure to these compounds affect nuclear and mitochondrial DNA integrity, DNA/RNA biology, topoisomerase activity, mitochondrial function and epigenetic signaling. These aspects will be investigated during the project "Precursors", submitted to the ANR joint grant call (PRCI). Results will provide with useful data to define the risk associated with the exposure to the relatively unknown but potentially dangerous AFB1 precursors.

5.2.3 Mycotoxins beyond food: Respiratory exposure to *Aspergillus* toxins. *Aspergillus nidulans* and *versicolor* are molds frequently present in the air in substantial quantities, colonizes air-conditioning equipment and is present in the carpet dust of damp, confined premises⁶⁸. They produce important toxic metabolites with cytotoxic, mutagenic, genotoxic and teratogenic properties such as VerA, VerB, and VerC and STC. The occupants of contaminated premises may therefore be exposed to these mycotoxins, primarily by inhalation. While the presence of STC has been investigated in some studies, data on the other toxic metabolites is scarce. A few studies have investigated the effect of respiratory exposure to STC and 5-methoxysterigmatocystin alone or in combination applied at naturally occurring concentrations detected in damp indoor areas, found a significant DNA damage in lung tissues, and VerA is also known to be toxic for lung cells. More research is needed to characterize the inhalation exposure to complex mixtures of these toxins and the effects for human health.

Moreover, it has been shown that poor air quality in a pig-confinement building is related with the presence of *Aspergillus versicolor*. While the occupational risk of pig farmers has been addressed⁶⁸, the effect of respiratory inhalation of *Aspergillus* toxins and its interaction with other causes of respiratory diseases in swine has not been addressed to date. Moreover, STC is known to be hepatotoxic to pigs, but no data is available for the other toxins. The

health impact of the combination of respiratory and oral exposure to this family of compounds in pig health remains to be investigated.

SECTION 6

General conclusion

Mycotoxin research will never get old. This is possibly the main conclusions of my first years working in this topic. It is surprising that, for instance, while the presence of natural endocrine disrupting substances has been known since at least the 1940s⁶⁹, and the identification of ZEN as a natural xenoestrogen date from the 1960s⁷⁰, the effect of natural estrogenic substances is far from being completely elucidated, and we still discover new effects nowadays. Moreover, advances in metabolomics provide with more in-depth characterization of fungal metabolomes, including many potentially toxic compounds. In addition to this, there is the question of those “forgotten” toxins that were identified as dangerous one day but due to lack of funding, interest or commercial availability were left behind in research. This is sometimes the case of mycotoxins showing a high toxicity such as VerA. This specific compound was identified as dangerous in the 70s, but largely forgotten until recently. Our recent findings also suggest that VerA is a molecule representing a challenge in terms of detection. This makes me wonder how many dangerous mycotoxins are present in our food without being acknowledged. The development of new detection techniques identify more complete profiles of mycotoxins contaminating food and feed. Moreover, climate change, agro-ecological transitions, use of new crops varieties and current changes in agricultural practices (i.e. the use of alternative energy- and cost-effective drying/stocking methods, the use of agricultural by-products as ingredients in animal rations to compensate the shortage of raw materials) delineate a new scenario of mycotoxins contamination in most parts of the world, where predictions have left room to a new reality. This situation is bringing changes in the mycotoxin exposure profiles, whose consequences are yet to be understood. Indeed, new challenges are constantly present in mycotoxin research.

A second conclusion is that it is time to see mycotoxins in the context of the exposome. This concept refers to ‘the totality of environmental exposures from conception onwards’. The exposome consists thus of all those non-genomic factors that may be related with different phenotypes and responsible of perturbation of biological processes (Wild, 2005). These factors include infectious and chemical agents, radiation, diet, lifestyle factors, occupation and medical interventions, but also the social, economic and psychological dimension for instance the education, the psychological and mental stress, or climate. The impact of all these aspects in mycotoxin toxicity and in consequence in the adequacy of regulations are mostly unknown, although they seem to have a significant impact. It is also important to consider the effect of prenatal exposure to mycotoxins and its transgenerational effect. In this sense, different studies underline the impact of the pre-/perinatal exposure to complex mixtures of toxicants, having effects that cannot be predicted otherwise.

The change in the paradigm in mycotoxin research brings the need of new tools. First, molecular tools to characterize exposures to complex mixtures and complex toxicity outcomes such as reliable biomarkers. This is, however, an old problem. We are in need of specific molecular tools for mycotoxicosis, but also still in need of objective measures of the health status of a herd, in particular when evaluating the effect of different intervention measures related with the improvement of intestinal health and productivity. In this sense, the use of mathematical models inspired on computer-learning algorithms for the integration of complex (meta)data represents a promising alternative to the more traditional reductionist approaches. Indeed, the democratization of the use of omics have allowed for the production of massive amounts of information, but has brought a new challenge: how to (re)use that much amount of data efficiently? A collaboration between researchers, clinicians, breeders and mathematicians is required. Second, in response to the growing identification of potentially toxic mycotoxins, there is a need of high-

throughput, simple techniques of toxicity screening that will provide with an interpretation of the relative potency and outcome of a given toxic challenge.

Mycotoxins research, mainly when applied to animal health and production, largely depends on *in vivo* trials. Many examples in this document illustrates the importance of such approach, as it the only way of confirming the danger of a toxin, the effect of breeding/dietetic interventions or the efficiency of remediation measures. However, as every other discipline, mycotoxin research also need to adapt its trials to be as much animal-free as possible. Luckily, the development of alternative research models such as those based in tissue engineering, predictive toxicology tools and non-invasive testing are starting to be useful in this sense, allowing reducing the numbers of *in vivo* trials required to evaluate the toxicity of a given compound.

In conclusion, mycotoxin research is always facing emerging challenges. In order to succeed in finding answers to mycotoxin-related questions a multi-disciplinary approach is often required, going from fundamental to applied sciences as well as from hypothesis-producing to validating experimental schemes. Mycotoxins represent primarily a sanitary problem due to their toxicity, but they impact many other aspects of society, because of the induction of direct and indirect economic losses (reduced crop yields, rendering a commodity unacceptable in national or international trade, reduced animal performance, need of use of costly remediating additives), that can ultimately have an effect in food security. It is my intention to continue to tackle these challenges in the future, following an approach centered in collaborative science and a transversal understanding of mycotoxin-related threats from a one-health perspective.

SECTION 7

Summary

I have engaged in different research activities during my career, from applied veterinary sciences to more fundamental research. My trajectory is characterized by a high mobility, which has allowed me to build a nice scientific network and grow an interest in collaborative research. I have had the opportunity to work with and train students from different horizons and origins, and I have received technical and scientific training on multiple disciplines, many of them uncommon for a veterinarian. I think that this particular curriculum has made me prone to be inclined to like global, dynamic fields of research such as mycotoxicology.

My research results show that, even when working with regulated mycotoxins, new effects can be always be detected, bringing new challenges. My research activities on the known reprotoxic and endocrine disruptor zearalenone indicate that the intestine is a target for its toxicity, where it disturbs estrogen-related signaling important for its physiology. My results also suggest that zearalenone might act as a metabolic disruptor, inducing changes in important signaling systems implicated in metabolism, immunity and reproduction. The characterization of the effect of this mycotoxin in metabolism and its relationship in the inflammatory/fertility status is part of my research project.

My research activities also show that the toxicity of many dangerous emerging mycotoxins are yet to be fully understood. My works on the aflatoxin B1 precursor versicolorin A show that this compound is has a complex toxicity. My research project is to continue to contribute to characterize the hazard that this compound represents, mostly because my results warrant further research on its carcinogenicity and ability to disrupt basic cell functions such as oxidative phosphorylation, transcription and protein synthesis.

I have also understood during these years that we are in constant need of discovering new tools to tackle our scientific challenges, such as new biomarkers, new technical approaches or new research models. My research project is centered in continuing to respond to these elements, and provide with useful data to help limiting the health effects of mycotoxins.

Résumé

J'ai participé à différentes activités de recherche au cours de ma carrière, des sciences vétérinaires appliquées à la recherche plus fondamentale. Ma trajectoire est caractérisée par une grande mobilité, ce qui m'a permis de construire un beau réseau scientifique et de développer un intérêt pour la recherche collaborative. J'ai eu l'occasion de travailler avec et de former des étudiants de différents horizons et origines, et j'ai reçu une formation technique et scientifique sur de multiples disciplines, souvent peu communes pour un vétérinaire. Je pense que ce cursus particulier m'a rendu enclin à aimer les domaines de recherche globaux et dynamiques tels que la mycotoxicologie.

Les résultats de mes recherches montrent que, même en travaillant avec des mycotoxines réglementées, il est toujours possible de détecter de nouveaux effets, ce qui entraîne de nouveaux défis. Mes activités de recherche sur la zéaralénone, un perturbateur endocrinien et reprotoxique connu, indiquent que l'intestin est une cible de sa toxicité, où elle perturbe la signalisation liée aux œstrogènes, importante pour sa physiologie. Mes résultats suggèrent également que la zéaralénone pourrait agir comme un perturbateur métabolique, induisant des changements dans d'importants systèmes de signalisation impliqués dans le métabolisme, l'immunité et la reproduction. La caractérisation de l'effet de cette mycotoxine dans le métabolisme et sa relation dans le statut inflammatoire/fertilité fait partie de mon projet de recherche.

Mes activités de recherche montrent également que la toxicité de nombreuses mycotoxines émergentes n'est pas encore totalement comprise. Mes travaux sur le précurseur de l'aflatoxine B1, la versicolorine A, montrent que ce composé est mutagène et génotoxique, et qui a une toxicité complexe. Mon projet de recherche est de continuer à contribuer à la caractérisation du danger que représente ce composé, notamment parce que mes résultats justifient des recherches plus poussées sur sa cancérogénicité et sa capacité à perturber les fonctions cellulaires de base telles que la phosphorylation oxydative, la transcription et la synthèse des protéines.

J'ai également compris au cours de ces années que nous avons constamment besoin de découvrir de nouveaux outils tels que la découverte des biomarqueurs d'effet, nouveaux modèles d'étude ou de nouveaux outils pour relever nos défis scientifiques. Mon projet de recherche vise à continuer à répondre à ces éléments, et à fournir des données utiles pour aider à limiter les effets des mycotoxines sur la santé.

SECTION 8

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