VNIVERSITAT DE VALÈNCIA

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ESTUDIO TRANSCRIPCIONAL Y PROTEÓMICO DE MICOTOXINAS

TRANSCRIPTIONAL AND PROTEOMIC STUDY OF MYCOTOXINS

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La Graduada en Ciència i Tecnología dels Aliments **Dña. Alessandra Francesca Cimbalo** ha realitzat baix la seua direcció el treball **"Estudio transcripcional y proteómico de micotoxinas"**, i autoritzen la seua presentació per a optar al títol de Doctor per la Universitat de València.

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Aquest treball ha sigut plasmat en 4 articles publicats o que es publicaran en les següents revistes:

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Alla mia famiglia.

Table of contents

Tabla de contenidos

List of contents

List of tables XV

List of figures XIX

List of abbreviations XXV

RESUMEN 1

SUMMARY 4

1. INTRODUCTION 7

1.1 Micotoxinas: generalidades 9

1.1.1 Aflatoxinas 11

1.1.2 Ocratoxina A 13

1.1.3 Micotoxinas emergentes de Fusarium 14

1.2 Ciencias -ómicas 15

1.2.1 Transcriptómica 18

1.2.1.1 Estudios transcriptómicos de toxicidad *in vitro, in vivo* y *ex vivo* tras la exposición a micotoxinas 23

1.2.2 Proteómica 31

1.2.1.1 Estudios proteómicos de toxicidad *in vitro* e *in vivo* tras la exposición a micotoxinas mediante LC-MS-MS. 37

2. OBJECTIVES **45**

3. RESULTS **49**

3.1. Toxicity of mycotoxins *in vivo* on vertebrate organisms: A review 51

3.2. Proteomics evaluation of enniatins acute toxicity in rat liver 173

3.3. Transcriptional Changes after Enniatins A, A1, B and B1 Ingestion in Rat Stomach, Liver, Kidney and Lower Intestine 199

3.4. Protective role of fermented whey and pumpkin extract against AFB_1 and OTA toxicity in Jurkat T-cells 239

4. GENERAL DISCUSSION **268**

4.1 Toxicidad de las micotoxinas en vertebrados 270

4.2 Estudio proteómico de las ENs *in vivo* 273

4.3 Estudio transcripcional de las ENs *in vivo* 277

4.4 Evaluación del efecto beneficioso del suero fermentado y del extracto de calabaza frente a la toxicidad de la AFB1 y la OTA en linfocitos T Jurkat. 283

4. CONCLUSIONS **290**

6. REFERENCES **296**

List of tables

1. INTRODUCCIÓN

1.2.1.1 Estudios transcriptómicos de toxicidad *in vitro, in vivo* y *ex vivo* tras la exposición a micotoxinas

Tabla 1. Estudios transcriptómicos realizados con micotoxinas, dosis, tiempo exposición, organismo biológico, técnica, principales efectos, toxicidad y referencia. 27

1.3.1.1 Estudios proteómicos de toxicidad *in vitro* e *in vivo* tras la exposición a micotoxinas mediante LC-MS-MS.

Tabla 2. Estudios proteómicos realizados con micotoxinas, dosis, tiempo exposición, organismo biológico, técnica, principales efectos y referencia. 41

3. RESULTS

3.1 Toxicity of mycotoxins *in vivo* on vertebrate organisms: A review

Table 1. AFB1 toxicity studies: dose and administration route, exposure time, animal species, sex and age, biological sample, technique, damage and reference. 62

Table 2. OTA toxicity studies: dose and administration route, exposure time, animal species, sex and age, biological sample, technique, damage and reference. 77

Table 3. PAT toxicity studies: dose and administration route, exposure time, animal species, sex and age, biological sample, technique, damage and reference. 88

Table 4. CIT toxicity studies: dose and administration route, exposure time, animal species, sex and age, biological sample, technique, damage and reference. 93

Table 5. FBs toxicity studies: dose and administration route, exposure time, animal species, sex and age, biological sample, technique, damage and reference. 97

Table 6. DON toxicity studies: dose and administration route, exposure time, animal species, sex and age, biological sample, technique, damage and reference. 103

Table 7. ZEA toxicity studies: dose and administration route, exposure time, animal species, sex and age, biological sample, technique, damage and reference. 116

Table 8. AOH toxicity studies: dose and administration route, exposure time, animal species, sex and age, biological sample, technique, damage and reference. 128

Table 9. Emerging mycotoxins toxicity studies: dose and administration route, exposure time, animal species, sex and age, biological sample, technique, damage and reference. 131

Table 10. Other mycotoxins toxicity studies: dose and administration route, exposure time, animal species, sex and age, biological sample, technique, damage and reference. 136

3.2. Proteomics evaluation of enniatins acute toxicity in rat liver

Table 1. Medium and high doses of ENNs B, B1, A1, A (μg/ml) administered to rats. 185

Table 2. Functional annotation chart of the 5 most significant terms using DAVID resources of 46 proteins selected in the liver proteomics analysis after enniatins exposure of rats in the categories UniProt Keyword, UniProt Sequence Feature and Gene Ontology Molecular Function. 186

Table 3. Reactome's pathways results using 46 proteins selected in the liver proteomics analysis after enniatins exposure of rats by entities p value and false discovery rate (FDR). 187

3.2 Transcriptional Changes after Enniatins A, A1, B and B1 Ingestion in Rat Stomach, Liver, Kidney and Lower Intestine

Table 1. Target organ, samples: control (C), medium dose. 209

Table 2. Gene symbol, forward (F) and reverse (R) primers, efficiency and linearity of the selected genes plus reference genes β-actin and 18S rRNA. 212

3.3 Protective role of fermented whey and pumpkin extract against AFB_1 and OTA toxicity in Jurkat T-cells

Table 1. Concentration (μg/mL) and profile of carotenoids identified from gastrointestinal digests of pumpkin (PID) and PID with fermented whey (WF).. 247

Table 2. AFB1 and OTA Concentrations of each condition employed: A) mycotoxin standard in organic solvent (DMSO), B) intestinal digest of bread with pumpkin (PID), C) intestinal digest of bread with pumpkin and whey fermented (PID+WF). 248

List of figures

4. **INTRODUCTION**

1.1.1 Aflatoxinas

Figura 1. Estructura química de AFB1, AFB2, AFG1 y AFG2 12

1.1. Ciencias -ómicas

Figura 2. Representación grafica de las ciencias -ómicas básicas. 16

1.1.1. Transcriptómica

Figura 3. Pasos clave de la transcripción genética. 20

1.2.2. Proteómica

Figura 4. Representación del proceso de tres tapas de la traducción del ARNm en proteína. 32

4. **RESULTS**

3.1 Toxicity of mycotoxins *in vivo* on vertebrate organisms: A review

Fig. 1. Graphic representation of total number of articles screened throughout the bibliographic research. 59

Fig. 2. Percentage of revised publications according to the main toxicological effect of AFB1. 68

Fig. 3. Percentage of the revised publications according to the main toxicological effect of OTA. 81

Fig. 4. Percentage of revised publications according to the main toxicological effect of PAT. 90
Fig. 5. Pie chart of revised scientific articles according to the main toxicological effect of CIT. 94
Fig. 6. Percentage of publications according to the main toxicological effect of Fumonisins. 99
Fig. 7. Percentage of revised publications according to the main toxicological effect of DON. 108
Fig. 8. Percentage of revised publications according to the main toxicological effect of ZEA. 115
Fig. 9. Pie chart of scientific articles according to the main toxicological effect of emerging mycotoxins. 132
Fig. 10. Pie chart of the percentage of studies found depending on the mycotoxin studied. 137
Fig. 11. A. Percentage of the studies according to the animal species. B. Percentage of articles according to the organ studied. 139
Fig. 12. Pie chart of the percentage of the papers according to the main purpose of the study. 140
Fig. 13. Percentage of scientific articles according to the techniques used. 141

3.3 Transcriptional Changes after Enniatins A, A1, B and B1 Ingestion in Rat Stomach, Liver, Kidney and Lower Intestine

Figure 1. Box plot showing relative expression of mitochondrial and nuclear encoded genes in stomach when compared to control C1 (Log2RQ=0) to medium treatment (M) and high treatment (H) by qPCR. RQ, relative quantification. The box plots show the median value (horizontal line), the top

and bottom edges of the boxes (75% and 25% percentiles) and whiskers which are the furthest values away from the boxes that are not considered outliers. *$p < 0.05$; **$p < 0.01$ significantly different from the control. 215

Figure 2. Box plot showing relative expression of mitochondrial and nuclear encoded genes in liver when compared to control C1 (Log2RQ=0) to medium treatment (M) and high treatment (H) by qPCR. RQ, relative quantification. The box plots show the median value (horizontal line), the top and bottom edges of the boxes (75% and 25% percentiles), (★) extreme cases, (o) outliers (atypical values) and whiskers which are the furthest values away from the boxes that are not considered outliers. **$p < 0.01$; ***$p < 0.001$ significantly different from the control. 216

Figure 3. Box plot showing relative expression of mitochondrial and nuclear encoded genes in kidney when compared to control C1 (Log2RQ=0) to medium treatment (M) and high treatment (H) by qPCR. RQ, relative quantification. The box plots show the median value (horizontal line), the top and bottom edges of the boxes (75% and 25% percentiles), (★) extreme cases and whiskers which are the furthest values away from the boxes that are not considered outliers. **$p < 0.01$; ***$p < 0.001$ significantly different from the control. 217

Figure 4. Box plot showing relative expression of mitochondrial and nuclear encoded genes in lower intestine when compared to control C1 (Log2RQ=0) to medium treatment (M) and high treatment (H) by qPCR. RQ, relative quantification. The box plots show the median value (horizontal line), the top and bottom edges of the boxes (75% and 25% percentiles), (★) extreme case

and whiskers which are the furthest values away from the boxes that are not considered outliers. *p < 0.05; ***p < 0.001 significantly different from the control. 219

Figure 5. Box plot showing relative expression of oxidative stress genes in lower intestine when compared to control C1 (Log2RQ=0) to medium treatment (M) and high treatment (H) by qPCR. RQ, relative quantification. The box plots show the median value (horizontal line), the top and bottom edges of the boxes (75% and 25% percentiles), (★) extreme cases, (o) outliers (atypical values) and whiskers which are the furthest values away from the boxes that are not considered outliers. **p < 0.01; ***p < 0.001 significantly different from the control. 220

Figure 6. Box plot showing relative expression of apoptotic genes in lower intestine when compared to control C1 (Log2RQ=0) to medium treatment (M) and high treatment (H) by qPCR. RQ, relative quantification. The box plots show the median value (horizontal line), the top and bottom edges of the boxes (75% and 25% percentiles), (★) extreme cases, (o) outliers (atypical values) and whiskers which are the furthest values away from the boxes that are not considered outliers. *p < 0.05; **p < 0.01; ***p < 0.001 significantly different from the control. 222

Figure 7. Box plot showing relative expression of Il-1β in lower intestine when compared to control C1 (Log2RQ=0) to medium treatment (M) and high treatment (H) by qPCR. RQ, relative quantification. The box plots show the median value (horizontal line), the top and bottom edges of the boxes (75% and

25% percentiles) and whiskers which are the furthest values away from the boxes that are not considered outliers. *p < 0.05; **p < 0.01; ***p < 0.001 significantly different from the control. 223

List of abbreviations

A431	Celulas de carinoma epidermoide
AA	Aminoácidos
ADN	Ácido desoxirribonucleico
ADNc	ADN complementario
AFB1	Aflatoxina B1
AFM1	Aflatoxina M1
AFM2	Aflatoxina M2
AFs	Aflatoxinas
AOH	Alternariol
ARN	Ácido ribonucleico
ARNm	ARN mensajeros
ARNt	ARN de transferencia
ARNt-Met	ARN de transferencia cargado de metionina
BEA	Beauvericina
CHS	Bio-organoarcilla
DON	Deoxinivalenol
ECV304	Células de la barrera hematoencefálica
EFSA	European Agency of Food Security
EM	Espectrometría de masas
ENA	Eniatina A
ENA1	Eniatina A1
ENB	Eniatina B
ENB1	Eniatina B1
ENs	Eniatinas
FB1	Fumonisina B1

FB2	Fumonisina B2
FBs	Fumonisinas
HEK293	Células de riñón embrionario humano
HepG2	Celulas de carcinoma hepatocelular humano
HK-2	Celulas epiteliales del túbulo proximal humano
IARC	International Agency for Research on Cancer
IPEC-J2	Celulas intestinalis porcinas
LC	Cromatografía liquida
LC-MS	Cromatografía liquida acoplada a espectrometría de masas
LMH	Células hepáticas de pollo
MIX	Material de lignocelulosa
NF-κB	factor nuclear kappa de linfocitos B
NGS	Next Generation Sequencing
OTA	Ochratoxina A
PAT	Patulina
PI	Punto isoeléctrico
PTM	Modificaciones postraduccionales
R2C	Tumor de células de Leydig
ROS	Especies reactivas del oxigeno
RP	Cromatografía de fase inversa
RT	Transcripción inversa
RT-qPCR	PCR cuantitativa en tiempo real
SH-SY5Y	Células neuronales
T-2	Toxina T-2
THP-1	Macrófagos derivados de THP-1
ZEA	Zearalenona

RESUMEN

Las micotoxinas son compuestos producidos por mohos que pueden crecer sobre alimentos o materias primas en condiciones de temperatura y humedad determinadas. Para contextualizar el conocimiento generado en los últimos años sobre efectos tóxicos de micotoxinas *in vivo*, se realiza una revisión bibliográfica sobre los efectos de estos compuestos. Las enniatinas pertenecen a las micotoxinas sin normativa, por lo que su detección en alimentos a base de cereales es frecuente. Para conocer los mecanismos de toxicidad de este grupo de micotoxinas emergentes, se investigaron los efectos a nivel molecular provocados por una exposición aguda a las enniatinas A, A1, B y B1 *in vivo* en ratas Wistar. En cambio, entre las micotoxinas legisladas por la Unión Europea se encuentran la aflatoxina B1 (AFB1) y la ocratoxina A (OTA), cuya toxicidad se ha investigado en profundidad durante las dos últimas décadas. Con la intención de paliar estos efectos tóxicos, se propuso el estudio de la ingesta conjunta de AFB1 y OTA con calabaza y suero de leche fermentado en un mismo producto, por las propiedades biológicas y físico-químicas de estos ingredientes funcionales.

La revisión bibliográfica puso de manifiesto que en los ensayos *in vivo* recopilados, las micotoxinas más estudiadas fueron la AFB1, deoxinivalenol (DON), zearalenona (ZEA), OTA y también, las combinaciones de AFB1, DON y ZEA. En cuanto a las micotoxinas emergentes, destacaron eniatinas (ENs), beauvericina (BEA) y moniliformina (MON). Los animales de laboratorio más empleados fueron los roedores y los cerdos, mientras que el

órgano más analizado fue el hígado, seguido de sangre, riñón y bazo. Los objetivos principales de los estudios de toxicidad fueron la immunotoxicidad, genotoxicidad, estrés oxidativo, hepatotoxicidad, citotoxicidad, teratogenicidad y neurotoxicidad. Para llevar a cabo estos estudios, se empleó una amplia gama de técnicas diferentes, entre las cuales las más utilizadas fueron la qPCR, ELISA e inmunohistoquímica.

Los resultados del análisis transcripcional en estómago, hígado, riñón y colon de ratas Wistar demostraron que la exposición aguda a una mezcla de ENs causó cambios de expresión en genes de los complejos I, II, IV y V de la cadena de transporte de electrones. Más allá de los cambios mitocondriales, se alteró significativamente la expresión de tres marcadores esenciales implicados en la respuesta celular al estrés oxidativo en tejidos intestinales y renales. Asimismo, hay una alta probabilidad de pérdida de homeostasis en la barrera intestinal por el desequilibrio producido entre los genes pro- y anti-apoptóticos y también por la sobreexpresión detectada de la ocludina, proteína estructural.

El estudio proteómico en tejidos hepáticos de las mismas ratas mostró cambios de expresión en proteínas relacionadas con la acetilación, la región de unión a fosfato de nucleótidos:NAD y la actividad catalítica. Algunas de estas proteínas se encuentran en la mitocondria y otras relacionadas con la actividad oxidoreductasa. Tras el análisis de rutas de señalización y procesos biológicos, se observó que el metabolismo fue la más representada. Además, el daño hepático inducido por las ENs se confirmó con la sobreexpresión de la proteína mitocondrial carbamoil fosfato sintasa-1 (CPS-1), siendo uno de los principales marcadores de daño hepatocelular.

Por último, el ensayo proteómico *in vitro* en una línea celular de linfocitos T humanos expuestos a un digerido gastrointestinal de pan preparado con suero fermentado de leche de cabra y calabaza liofilizada contra la citotoxidad inducida por las micotoxinas AFB1 y OTA, mostró un incremento en la expresión de proteínas relacionadas con la gluconeogénesis, actividad antioxidante y estabilidad cromosómica frente a la exposición solo con micotoxinas. En cuanto a las vías metabólicas, la relacionada con el fenotipo secretor asociado a la senescencia fue la más alterada, destacando la disminución de la expresión de la ciclina A2 en presencia de los ingredientes funcionales.

Se necesitan más estudios *in vitro* e *in vivo* sobre la toxicidad de las micotoxinas mediante la utilización de las tecnologías -ómicas, las cuales representan un gran avance en la evaluación del riesgo en la seguridad alimentaria.

SUMMARY

The present work focused on the toxicological effects which mycotoxins can cause on human health, by examining *in vivo* studies in the last decade. The capacity of the emerging mycotoxins Enniatins (ENs) to cause changes in the expression of genes related to the electron transport chain, apoptosis, inflammation and structural processes in the liver, stomach, kidney and colon of Wistar rats was also evaluated by a transcriptional approach. In addition, the proteomic profile in liver was studied by LC-MS/QTOF technique. Likewise, the study allowed the identification of differentially expressed proteins in Jurkat lymphocyte T cells exposed to an intestinal digest of bread prepared with pumpkin and fermented whey, in combination with aflatoxin A (AFB1) and ocratoxin A (OTA) at different concentrations.

The literature reviewed revealed that in the *in vivo* assays collected, the most studied mycotoxins were AFB1, Deoxynivalenol (DON), Zearalenone (ZEA), OTA and also combinations of AFB1, DON and ZEA. As regard emerging mycotoxins, the most important were ENs, beauvericin (BEA) and moniliformin (MON). On the other hand, rodents and pigs were the main laboratory animals employed, whereas the most analyzed organ was the liver, followed by blood, kidney and spleen. Moreover, the main purpose of the toxicity studies was immunotoxicity, followed by genotoxicity, oxidative stress, hepatotoxicity, cytotoxicity, teratogenicity and neurotoxicity. As for the techniques used, the most common were real time PCR (qPCR), ELISA and immunohistochemistry (IHC).

The results of transcriptional analysis in Wistar rats' liver showed that an acute exposure to a mixture of ENs caused changes in the expression of genes involved in complex I (CI), complex II (CII), complex IV(CIV) and complex V(CV) of the electron transport chain (ETC). In addition to mitochondrial dysregulation, the activity of three essential markers implicated in cellular response to oxidative stress in intestinal and renal tissues was altered. Likewise, the homeostasis of the intestinal barrier was modified by the imbalance between pro- and anti-apoptotic genes and also by the overexpression of the structural protein Occludin.

Proteomic study in liver tissues from the same rats showed expression changes in proteins related to acetylation, nucleotide:NAD phosphate-binding region and catalytic activity. Some of these proteins were located in the mitochondria and others were related to oxidoreductase activity. Analyzing metabolic pathways, metabolism was the most significant and the most enriched pathway. In addition, ENs-induced liver damage was confirmed by overexpression of the mitochondrial protein carbamoyl phosphate synthase-1 (CPS-1), which is an important tumor marker of the liver.

In vitro assay in Jurkat cells exposed to a gastrointestinal digest of bread prepared with fermented goat milk whey and lyophilized pumpkin against AFB1 and OTA mycotoxin-induced cytotoxicity showed increased expression of gluconeogenesis-related proteins, antioxidant activity and chromosomal stability. In terms of metabolic pathways, the one related to the secretory phenotype associated with senescence was the most altered, being cyclin A2 overexpressed in all conditions analyzed.

Further toxicological studies on mycotoxins toxicity employing -omics technologies are needed. In fact, these innovative techniques, represent a major advance in food safety risk assessment.

1. INTRODUCTION

1. Introducción

1. INTRODUCCIÓN

1.1 Micotoxinas: generalidades

El término "micotoxina" define compuestos químicos tóxicos de bajo peso molecular (0,3-0,7 kDa), secretados como metabolitos secundarios por algunos hongos que pertenecen principalmente a los géneros *Fusarium*, *Penicillium*, *Aspergillus* y *Alternaria*. Estas toxinas se producen naturalmente y pueden contaminar los alimentos básicos de la dieta, en particular los cereales a lo largo de toda la cadena de producción, especialmente en condiciones de pre y postcosecha. El grado de contaminación por las micotoxinas, depende estrictamente de las condiciones climáticas durante la cosecha y el almacenamiento, pero también, de la composición y contaminación del aire, la humedad, el daño mecánico de los granos, la temperatura y la zona geográfica. Además, la disponibilidad del factor nutricional, los requisitos genéticos, la presencia de fungicidas y la interacción entre las especies de hongos toxigénicos colonizadores pueden causar el desarrollo del moho (Yang et al., 2020).

Aunque se hayan considerado posibles intervenciones para prevenir brotes en el campo en varios cultivos de todo el mundo, las micotoxinas siguen representando una importante carga económica y de salud pública. El Sistema de Alerta Rápida para Alimentos y Piensos de la Unión Europea (RASFF) en su ultimo informe, ha reportado un total de 400 notificaciones de contaminación por micotoxinas en alimentos y piensos. Entre las notificaciones de alimentos, el 89% se atribuyeron a la contaminación por

Aflatoxinas (AFs) (367 notificaciones), que se detectaron sobre todo en higos secos de Turquía (58 notificaciones), seguidos por cacahuetes de Estados Unidos (29 notificaciones). La ocratoxina A (OTA), el deoxinivalenol, las fumonisinas, la zearalenona y la patulina se notificaron en el 10%, 1,01%, el 0,71%, el 0,23% y el 0,09% de las notificaciones, respectivamente (Annual Report RASFF, 2020).

Debido a sus efectos toxicológicos, la presencia de micotoxinas en los alimentos tiene graves consecuencias para la salud humana y animal, incluso en concentraciones muy bajas. Pueden causar varias enfermedades agudas o crónicas: los episodios agudos se producen al consumir concentraciones que van desde altas a moderadas y se caracterizan por un inicio rápido y una respuesta tóxica evidente que incluye la muerte rápida mientras que las exposiciones crónicas se producen por la ingestión a dosis más bajas durante un largo periodo de tiempo, causando enfermedades crónicas, incluido el cáncer. Sin embargo, estas toxinas no son igualmente tóxicas, su toxicidad cambia durante el metabolismo, mientras que la susceptibilidad de los animales y los seres humanos varía también con la edad, la especie, la nutrición y otros factores (Barac et al., 2019).

Teniendo en cuenta los aspectos agroeconómicos y el impacto en la agricultura mundial, así como las posibles implicaciones en la salud pública, la Agencia Internacional para la Investigación sobre el Cáncer (IARC) inició un programa de Monografías en el que se examinó toda la información pertinente para evaluar las pruebas relacionadas con la exposición a las micotoxinas, que podrían alterar la incidencia del cáncer en los seres

humanos (Ostry et al., 2017). Las micotoxinas como agentes tóxicos se clasifican en cinco grupos (Grupo 1, 2A, 2B, 3 y 4) en función de la evidencia de su carcinogenicidad. Entre ellos, las aflatoxinas (AFs) fueron clasificadas por la IARC como carcinógenas para los humanos (Grupo 1), y reconocidas como uno de los carcinógenos genotóxicos hepáticos más potentes. Las fumonisinas B1 y B2 (FB1, FB2) y la OTA fueron clasificadas en el Grupo 2B, compuestos considerados cancerígenos para los animales y posiblemente cancerígenos para los humanos. Recientemente, la IARC también asoció la exposición dietética a las AFs y las FBs con altos niveles de retraso en el crecimiento y deficiencia en el crecimiento de los niños (IARC, 2012).

1.1.1 Aflatoxinas

Las AFs son derivados químicos de la difuranocumarina producidos principalmente por dos especies de hongos del genero *Aspergillus*, *A. flavus* y *A. parasiticus*. Las AFs contaminan ampliamente varios tipos de cultivos en todo el mundo, en particular el maíz, los cacahuetes, el trigo, la cebada y el arroz. Además, pueden contaminar productos comerciales como la leche, el aceite y la crema de cacahuete (Nazhand et al., 2020).

De las especies presentes, se han identificado aproximadamente 20 aflatoxinas, incluyendo la AFB1, B2 (AFB2), G1 (AFG1) y G2 (AFG2) (Figura 1). Entre ellas, la AFB1 es la más tóxica, ya que tiene propiedades hepatotóxicas, inmunotóxicas, mutagénicas, carcinogénicas y teratogénicas en los seres humanos y en los animales. También, se han identificado hidroximetabolitos de las AFs, como la aflatoxina M1 (AFM1) y la M2

(AFM2) en la leche y en los productos lácteos, como resultado de la ingestión de piensos contaminados por AFB1 y AFB2 (Caceres et al., 2020)

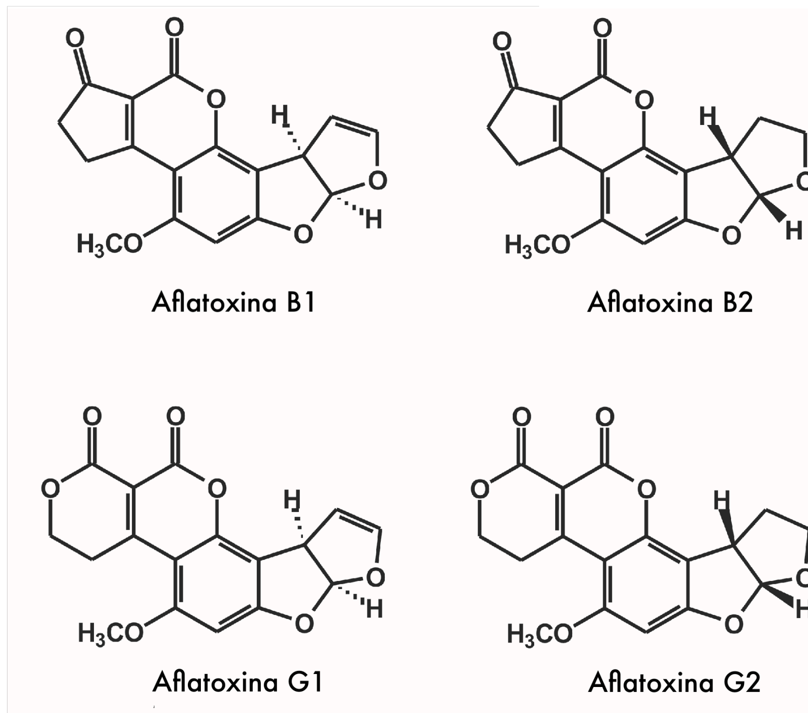


Figura 1. Estructura química de AFB1, AFB2, AFG1 y AFG2

La ingestión de AFs procedentes de alimentos y piensos contaminados provoca graves complicaciones de salud tanto en humanos como en animales. La opinión científica de la EFSA sobre los riesgos para la salud publica relacionados con la presencia de AFs en alimentos, ha reportado que la exposición dietética crónica a la AFB1 en todas las clases de edad, a lo largo de la vida causa inhibición del crecimiento, daños hepáticos y renales, así como alteraciones sostenidas de la microbiota intestinal y genotoxicidad. También se ha visto, que la AFB1 afecta a los parámetros reproductivos y de

desarrollo (desarrollo cerebral, acortamiento del tiempo hasta el parto, bajo peso al nacer y efectos adversos sobre la espermatogénesis y la foliculogénesis) a dosis bajas (≥ 4 µg/kg de peso corporal por día) mientras que el conjunto de AFs, perjudica la respuesta inmunitaria, especialmente a nivel celular. Los niveles sin efecto adverso observable (NOAEL) se sitúan alrededor de 30 µg/kg de peso corporal al día en roedores (EFSA, 2020).

Aunque la ingesta dietética de AFs se haya asociado con el cáncer primario de hígado, también se ha visto que otros órganos, como el riñón, el páncreas, la vejiga, los huesos y las vísceras, pueden desarrollan cáncer tras la exposición a estas micotoxinas (Fouad et al., 2019). La exposición crónica a las AFs provoca una serie de otras enfermedades graves, como inmunosupresión, teratogenicidad, mutagenicidad, citotoxicidad y efectos estrogénicos en los mamíferos (Klvana & Bren, 2019). Se ha observado también, que están implicadas en trastornos nutricionales y retraso del crecimiento, probablemente al interferir con la absorción de micronutrientes, con la síntesis de proteínas y con las actividades enzimáticas metabólicas (Benkerroum, 2020).

1.1.2 Ocratoxina A

La OTA es un metabolito secundario derivado de un policétido que contiene una fracción de dihidrocumarina acoplada a una l-β-fenilalanina (Phe), procedente de la vía del ácido shikímico. Las principales especies productoras de OTA pertenecen a los generos *Aspergillus* y *Penicillium*, en particular *A. Circumdat, A. Nigr, P. Verrucosum y P. nordicum.* Este metabolito se encuentra generalmente en todo tipo de cereales y productos derivados,

pero también en el café, cacao, uvas, soja, especias, legumbres, frutos secos, regaliz, vino y cerveza (EFSA, 2020).

La OTA suele ser causa de toxicidad crónica, debido a la ingesta prolongada de pequeñas cantidades contenidas en los alimentos y se manifiesta principalmente a nivel del riñón, que es el principal órgano diana (Tao et al., 2018). Sin embargo, puede dar lugar a diversos efectos toxicológicos, incluyendo la alteración de la homeostasis de la microbiota intestinal, teratogenicidad, carcinogenicidad, mutagenicidad, hepatotoxicidad, genotoxicidad, inmunotoxicidad, embriotoxicidad, toxicidad testicular y daños en la barrera hematoencefálica (Chen et al., 2018).

1.1.3 Micotoxinas emergentes de Fusarium

Las especies de Fusarium producen micotoxinas legisladas (fumonisinas, zearalenona y tricotecenos) y otros compuestos bioactivos menos conocidos y estudiados denominados micotoxinas "menores" o "emergentes". Este último grupo de micotoxinas incluye las eniatinas (ENs), la fusaproliferina, la beauvericina y la moniliformina, las cuales disponen de poca información sobre su toxicidad y presencia en los alimentos.

Entre ellas, las ENs son hexadepsipéptidos cíclicos metilados producidos por varias especies como *F. avenaceum*, *F. oxysporum*, *F. poae* o *F. tricinctum*, que crecen principalmente en los cereales. Hasta la fecha, se han aislado y caracterizado 29 especies de ENs y, entre ellos, las ENs A, A1, B y B1 son las que se detectan con mayor frecuencia en alimentos y piensos.

La toxicidad de las ENs se basa en sus propiedades ionóforicas. En efecto, la presencia de pares de electrones libres proporciona a estas moléculas unas características electrofóricas peculiares que les permiten coordinarse con cationes, formando enlaces químicos débiles principalmente con metales como el K^+, el Na^+ y el Ca^{2+}. Una vez formados estos complejos lipofílicos, los iones pueden ser transportados a través de las membranas celulares y el resultado es un aumento de la permeabilidad iónica que altera las concentraciones intracelulares de iones induciendo alteraciones de la función celular (Gruber-Dorninger et al., 2017).

La toxicidad de las ENs se ha asociado también a la capacidad de estos compuestos para desencadenar disfunciones mitocondriales (modificaciones del potencial de membrana mitocondrial), alteraciones lisosomales, interrupción del ciclo celular y producción intracelular de ROS (Bertero et al., 2020).

1.2 Ciencias -ómicas

La -ómica es una ciencia que engloba las tecnologías y enfoques colectivos para explorar las acciones y funciones de diversas moléculas que constituyen las células (iones, ácidos nucleicos, proteínas, enzimas, etc.) dentro de los organismos vivos. En las últimas décadas, la "-ómica" se ha extendido, desde la "genómica" inicial, a un amplio abanico de disciplinas biomoleculares dirigidas al estudio de aspectos específicos considerados en su conjunto (Calabrò et al., 2020). En general, el objetivo principal de las ciencias -ómicas es identificar, caracterizar y cuantificar todas las moléculas

biológicas que están involucradas en la estructura, función y dinámica de una célula, tejido u organismo. La tecnología -ómica puede clasificarse en varios tipos según su función: genómica, transcriptómica, prteómica y metabolómica (Figura 1).

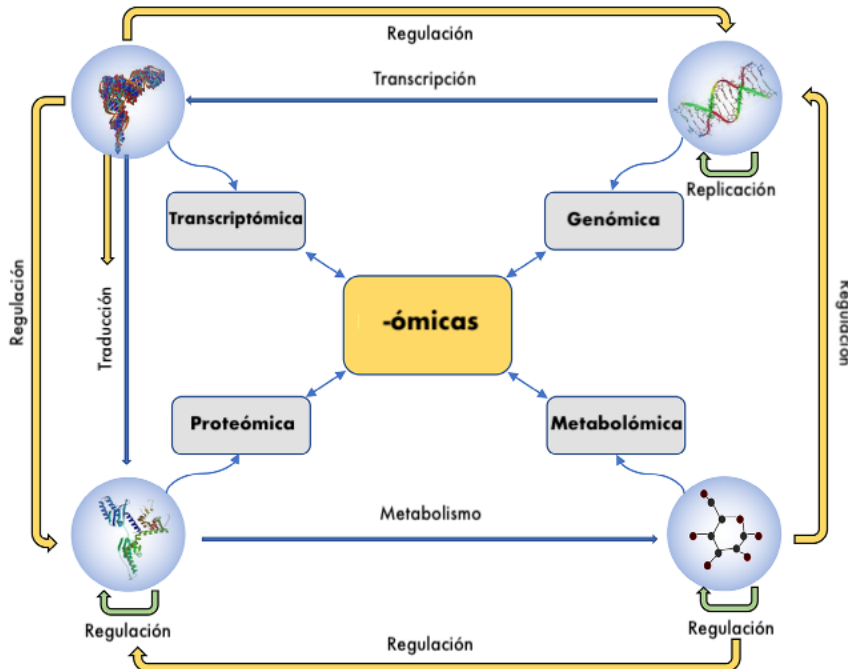


Figura 2. Representación grafica de las ciencias -ómicas básicas.

La genómica es el estudio interdisciplinario basado en la evaluación de la estructura y la función de los genes contenidos en el ADN y sus múltiples funciones. Las áreas posibles y altamente investigadas bajo la genómica incluyen la genómica funcional, la metagenómica y la epigenómica. La transcriptómica se ocupa del producto de transcripción del ADN-ARN, incluye la cantidad o concentración de cada molécula de ARN, que refleja el nivel de abundancia del gen correspondiente (expresión génica). La proteómica evalúa las proteínas

codificadas por el ADN a través del ARN, así estudia las proteínas en relación con sus propiedades bioquímicas y roles funcionales, y cómo cambian sus cantidades, modificaciones y estructuras durante el crecimiento y en respuesta a estímulos internos y externos. Finalmente, la metabolómica analiza todos los procesos químicos que involucran metabolitos. Más específicamente, es el estudio de las huellas químicas que los procesos celulares específicos establecen durante su actividad (Ferranti et al., 2017). Dentro de los objetivos de las ciencias -ómicas está también estudiar las conexiones e interacciones recíprocas entre el conjunto de moléculas biológicas (interactómica) y entre estas moléculas y los microorganismos de la flora intestinal (microbiómica), alimentos y/o nutrientes (nutribiómica). El aspecto básico de estos enfoques es que un sistema complejo puede comprenderse más a fondo si se considera como un todo (Vailati-Riboni et al., 2017).

Así las tecnologías -ómicas son aplicables para probar las conexiones e interrelaciones entre los muchos aspectos de un estado fisiológico complejo, y descubrir nuevas piezas en el conocimiento actual en los campos de la salud, la alimentación y agricultura y la seguridad alimentaria (Calabrò et al., 2020).

1.2.1 Transcriptómica

Las células contienen un gran grupo de moléculas similares al ácido desoxirribonucleico (ADN) llamadas ácido ribonucleico (ARN). Mientras que el ADN almacena y conserva la información, el ARN influye en la forma en que las células utilizan y regulan esa información. Por ello, la regulación de las cantidades de los distintos ARN es una parte fundamental de la forma en que las células sobreviven, crecen, se adaptan y responden a los cambios. Por ejemplo, los ARN mensajeros (ARNm) transportan la información genética del ADN que la célula transcribe para producir proteínas en los ribosomas. Los ARN que no son necesarios pueden ser degradados y eliminados de la célula por las proteínas de degradación del ARN (Dhanoa et al., 2018).

El control de la expresión de los genes en los eucariotas está organizado por una variedad de pasos reguladores que operan en diferentes puntos durante el ciclo de vida de un ARN, desde el inicio de la transcripción hasta los eventos de maduración posteriores que finalmente influirán en el procesamiento, la estabilidad, la localización y, en el caso de los ARNm, la traducción del mensaje en proteína. Estos acontecimientos están muy interrelacionados y a menudo tienen lugar en diferentes zonas de la célula, desde las subregiones del núcleo donde se almacena la información genómica y los ARN se sintetizan por primera vez como transcritos inmaduros nacientes, y tras la maduración, hasta los confines del citoplasma, o incluso más allá de los límites de la célula en el caso de los ARN que se secretan en el espacio extracelular. Así, todos los ARNm tienen que ser transportados desde el núcleo a través de los poros nucleares hasta el citoplasma (Oeffinger & Zenklusen, 2019). Por tanto, la distribución subcelular de las moléculas de ARN no sólo es clave para garantizar la correcta

coordinación de los eventos de maduración de los transcritos, sino que también es crucial para modular las funciones celulares de las distintas clases de ARN codificantes y no codificantes transcritos del genoma (Chin & Lécuyer, 2017).

La expresión de la información genética de una célula comienza con la transcripción. Este proceso biológico fundamental, conservado y extremadamente complejo, cataliza la síntesis de una molécula de ARN a partir del ADN que contiene la información genética, mediado por enzimas llamadas ARN polimerasas. Para iniciar la transcripción, la ARN polimerasa reconoce la región promotora de ADN al principio del gen. A continuación, la enzima abre el dúplex de ADN, comienza a sintetizar ARN y se separa del promotor. La posterior apertura del ADN convierte el complejo promotor cerrado en el complejo promotor abierto, que contiene la cadena molde de ADN en el sitio activo de la polimerasa. La síntesis de ARN dependiente del ADN genera entonces un complejo inicialmente transcriptor. Cuando el ARN crece hasta una longitud crítica, la polimerasa escapa del promotor y forma un complejo de elongación que puede unir factores de elongación. Finalmente, el complejo de elongación resultante extiende la cadena de ARN hasta que alcanza una señal de terminación y libera el ADN y el ARN (Figura 6) (Cramer, 2019).

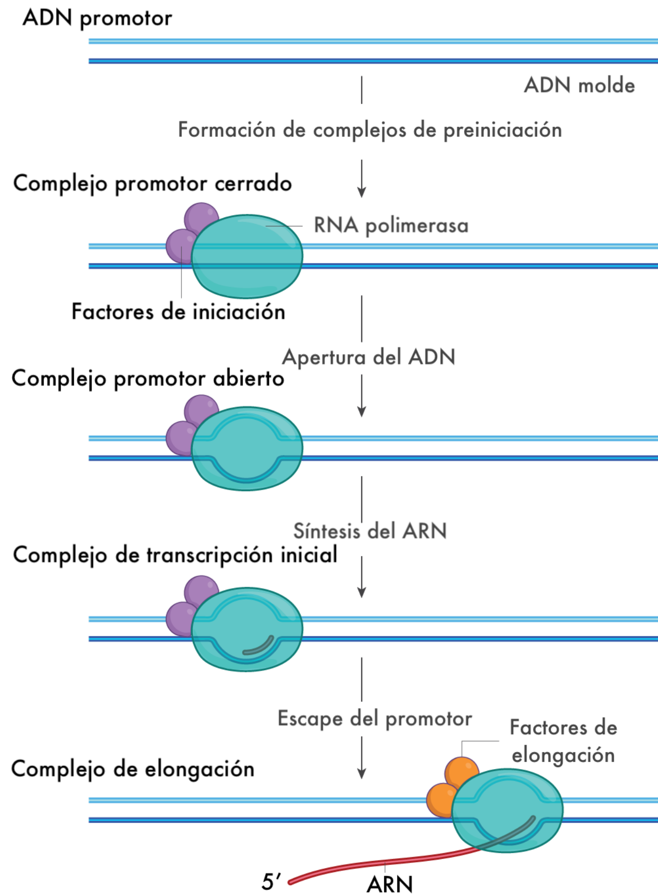


Figura 3. Pasos clave de la transcripción genética.

El transcriptóma se refiere al conjunto completo de transcripciones de genes o especies de ARN transcritas por el genoma de un determinado tipo de célula, tejido u organismo para una condición fisiológica o patológica específica. Comprende tanto el ARN codificante que se traduce en proteínas, como el ARN no codificante que participa en el control post-transcripcional, que influye además en la expresión de los genes (Chambers et al., 2019). La transcriptómica

es la ciencia que se centra en la expresión de los genes a nivel del ARN y ofrece información sobre su estructura y función en todo el genoma para revelar los mecanismos moleculares implicados en procesos biológicos específicos (Hasin et al., 2017).

Los estudios de los transcritos individuales se realizaron varias décadas antes de que se dispusiera de enfoques transcriptómicos. A finales de la década de 1970 se recogieron bibliotecas de ARNm de la polilla de la seda y se convirtieron en ADN complementario (ADNc) para su almacenamiento mediante transcriptasa inversa. El descubrimiento de las transcriptasas inversas y su potencial para generar ADNc a partir de ARN en 1971 permitió adaptar los métodos existentes para el análisis del ADN a la investigación concordante de las especies de ARN. En la década de 1980, Kary Mullis desarrolló lo que fue una innovación revolucionaria en el mundo de la transcriptómica, el método de reacción en cadena de la polimerasa (PCR). Desde entonces, se ha extendido su uso en la investigación biomédica, ya que puede detectar y cuantificar pequeñas cantidades de secuencias específicas de ácidos nucleicos. Por ejemplo, se pueden cuantificar pequeños niveles de ARNm mediante la combinación de la transcripción inversa (RT) para producir ADN complementario (ADNc) y la amplificación por PCR para producir niveles exponencialmente mayores de estas cadenas de ADNc (Hawkins & Guest, 2017).

Al igual que en el caso del ADN, estos esfuerzos de secuenciación se limitaron inicialmente al análisis de un solo gen, mientras que el desarrollo de la Next generation sequencing (NGS) en 2005 permitió finalmente el análisis global del transcriptoma (Chambers et al., 2019).

Todos los métodos transcriptómicos requieren que se aísle primero el ARN del organismo experimental antes de poder registrar los transcritos. Aunque los sistemas biológicos son increíblemente diversos, las técnicas de extracción de ARN son ampliamente similares e implican lo siguiente: disrupción mecánica de las células o tejidos, disrupción de la RNasa con sales caotrópicas, disrupción de macromoléculas y complejos de nucleótidos, separación del ARN de biomoléculas no deseadas, incluyendo el ADN, y concentración del ARN mediante precipitación desde la solución o elución desde una matriz sólida. El ARN aislado puede ser tratado adicionalmente con DNasa para digerir cualquier rastro de ADN. Es necesario enriquecer el ARN mensajero ya que los extractos de ARN total suelen tener un 98% de ARN ribosómico (Lowe et al., 2017).

Las estrategias transcriptómicas han tenido una amplia aplicación en diversas áreas de la investigación biológica, vegetal, médica, clínica y farmacéutica para el descubrimiento de biomarcadores y el diagnóstico y pronóstico de enfermedades. La transcriptómica es útil para identificar biomarcadores de enfermedades, así como respuestas biológicas a diversos estímulos y tensiones, y desempeña un papel clave en el avance de la investigación genómica y de biología molecular (Raghavachari & Garcia-Reyero., 2018). Entre las posibles aplicaciones de la transcriptómica destaca la toxicología, con especial énfasis en la prevención de los efectos adversos para la salud derivados de la exposición humana a xenobióticos y la detección de los mecanismos moleculares de la toxicidad de los órganos diana, con referencia a la prevención de los efectos adversos para la salud derivados de la exposición a tóxicos (Joseph, 2017).

1.2.1.1 Estudios transcriptómicos de toxicidad *in vitro, in vivo* y *ex vivo* tras la exposición a micotoxinas

La toxicidad de las micotoxinas se ha evaluado ampliamente en varios estudios *in vitro* e *in vivo* a través del perfil transcriptómico de los organismos biológicos. En concreto, se ha estudiado el efecto tóxico de DON, OTA, ZEA, ENs, BEA, AOH y AFB1, utilizando células epiteliales intestinales porcinas (IPEC-J2), células Sertoli de *Equus asinus,* células epiteliales del túbulo proximal humano (HK-2), células foliculares de la granulosa porcina, células de carcinoma hepatocelular humano (HepG2), Linfocitos T Jurkat, células de la barrera hematoencefálica (ECV304), Macrófagos derivados de THP-1 (THP-1), Celulas neuronales (SH-SY5Y), células hepáticas de pollo (LMH) y también explantes intestinales de cerdo, hígado de cerdo, hígado de pollo y embriones y larvas de Zebrafish. En la tabla 2 se muestran los estudios ordenados por micotoxina y tiempo de exposición.

El efecto beneficioso de *Lactobacillus rhamnosus* sobre la toxicidad intestinal del DON (10 μM) se ha investigado *ex vivo* en explantes de cerdo analizando el perfil de expresión génica relativo a la permeabilidad intestinal durante 4 h (García et al., 2018). De igual manera, se ha observado la activación de genes involucrados en los mecanismos de inflamación intestinal en una línea celular epitelial intestinal porcina (IPEC-J2) tras 24 h a diferentes concentraciones (0.25, 0.5, 1, 2, 4 μM). Además, se ha visto que la capacidad del pretratamiento con inhibidores de las rutas metabólicas involucradas (U0126, SP600125, SB203580) y los lipopolisacaridos pueden disminuir la respuesta inflamatoria (Zhang et al., 2020; Yu et al., 2021). El tratamiento agudo combinado de DON y ZEA, cambió notablemente la expresión de genes

asociados a procesos inflamatorios y genes relacionados con andrógenos y estrógenos en células Sartoli de *Equus* asinus (Song & Zhang, 2021). Por otro lado, se ha evaluado la inmunotoxicidad de este mix de micotoxinas en hígado de cerdo durante 4 semanas, remarcando la alteración de genes asociados a procesos del sistema inmune (Reddy et al., 2018).

En cuanto a la ZEA, se ha reportado que la exposición aguda a concentraciones de 10 y 30 μM en células foliculares de granulosa porcina, indujo la activación de procesos apoptóticos y la sobreexpresión de genes relacionados con el daño y reparación del ADN (γ-H2AX, BRCA1, RAD51 y PRKDC), el cual se redujo al añadir el antagonista de los estrógenos tamoxifeno (Liu et al., 2018). Exponiendo células HepG2 a concentraciones similares de ZEA y su metabolito α-ZOL (1, 10, 50 μM), se observó un aumento significativo de los niveles globales de genes implicados en la metilación del ADN, de las histonas (H3K27me3, H3K9me3, H3K9ac), de las enzimas modificadoras de la cromatina (EHMT2, ESCO1, HAT1, KAT2B, PRMT6 y SETD8), y relacionados con los receptores nucleares (AhR, LXRα, PPARα, PPARγ, L-fabp, LDLR, Glut2, Akt1 y HK2) (Karaman et al., 2020). Además, el impacto negativo de la ZEA en el sistema reproductivo del pez cebra se ha confirmado por afectación de los genes involucrados en función reproductiva asociada al eje hipotálamo-hipófisis-gónadas mediante RT-qPCR (Muthulakshmi et al., 2018).

La neurotoxicidad en embriones y larvas de pez cebra, se ha investigado también tras el tratamiento con AFB1 durante 7 días, confirmada por la alteración de marcadores relacionados con la neurogénesis (Wu et al., 2018). Al

contrario, utilizando células neuronales SH-SY5Y expuestas a una combinación de AFB1 y FB1 durante 24h, no se ha observado ninguna variación en la expresión de marcadores de estrés oxidativo del retículo endoplasmatico (IRE1 y Gadd153) (Kara & Oztas, 2020). Por otro lado, al combinar la AFB1 y la OTA en células hepáticas LMH, se han observado efectos citotóxicos de forma dependiente de la dosis a las 48h de exposición, identificados a nivel transcriptómico mediante RNA-Seq y confirmación por RT-qPCR (Choi et al., 2020).

Siendo la OTA un importante nefrotóxico, se han estudiado los cambios a nivel del perfil de expresión génica en células epiteliales tubulares (HK-2) durante 72 y 48h, a diferentes concentraciones (0.2,5,10,20,25,30,40 μM) (Celik et al., 2020, Pyo et al., 2021). Con el fin de reducir este efecto toxico a nivel renal, se ha evaluado la capacidad de una bio-organoarcilla (CHS) y una mezcla de una Na-esmectita tri-octaédrica con un material basado en lignocelulosa (MIX) en riñones de pollos tratados con OTA, demostrando su papel beneficioso (Andretta et al., 2020).

En cuanto a las micotoxinas emergentes, se ha confirmado la toxicidad mitocondrial de la ENB, individual o en combinación con BEA, en células Jurkat a diferentes concentraciones durante 24h, evidenciando la alteración de genes involucrados en el metabolismo, respiración y mecanismos antioxidantes de las mitocondrias (Alonso-Garrido et al., 2018; Escrivá et al., 2019). Además, el efecto beneficioso de los carotenoides sobre la toxicidad mitocondrial, se ha confirmado tras la exposición de células ECV304 a un extracto de calabaza (500nM) durante 2h (Alonso-Garrido et al., 2021). Por otra parte, Kollarova y

colaboradores (2018) han evaluado el impacto del Alternariol (AOH) en la vía del factor nuclear kappa B (NF-κB) en macrófagos derivados de THP-1, detectando una variación en la expresión de citoquinas pro- y antinflamatorias.

Tabla 1. Estudios transcriptómicos realizados con micotoxinas, dosis, tiempo exposición, organismo biológico, técnica, principales efectos, toxicidad y referencia.

	Dosis	Tiempo de exposición	Organismo biológico	Técnica	Principales efectos	Referencia
DON	10 μM + *Lactobacillus rhamnosus* RC007	4h	Explantes intestinales de cerdo	RT-qPCR	Aumento de la permeabilidad intestinal tras la sobreexpresion de CCL20, IL-1b, TNFa, IL-8, IL-22, IL-10. La pre-incubacion con *L. rhamnosus* disminuye la respuesta pro-inflamatoria	García et al., 2018
	0.25, 0.5, 1, 2, 4μM + U0126 (10μM) + SP600125 (20 μM) + SB203580 μM	24 h	IPEC-J2	RNA-seq + RT-qPCR	Sobreexpresión de genes proinflamatorios (TNF-a, IL-6). Activacion de p38 y Erk1/2	Zhang et al., 2020
	1 μM+ LPS (100 μM)	24 h	IPEC-J2	RT-qPCR	Sobreexpresión de inos, cox-2, claudin 3, occludin. Fosforilación de P38, ERK, JNK	Yu et al., 2021

	+ ZEA (10, 30 μM)	72 h	Células Sertoli de *Equus asinus*	RT-qPCR	Alteración de genes androgenos y extrogenos	Song & Zhang, 2021
	+ ZEA (8 mg/Kg de pienso; 0.8 mg/Kg)	4 semanas	Hígado de cerdo	RT-qPCR	Genes asociados a procesos del sistema inmune	Reddy et al., 2018
	10, 30 μM	72 h	Células foliculares de la granulosa porcina	RT-qPCR	Alteracion de genes apoptoticos, sobreexpresión de genes reparadores (γ-H2AX, BRCA1, RAD51 y PRKDC	Liu et al., 2018
ZEA	0.5, 1, 5, 10 μg/L	21 días	Zebrafish	RT-qPCR	Alteración de genes asociados a la función reproductiva del eje hipotálamo-hipofisario-gonadal (receptor de estrógenos (RE) α y CYP19a1b en el cerebro, del REα y de la vitelogenina (Vtg) en el hígado y del receptor de la hormona folículo-estimulante, del receptor de la hormona luteinizante, del REα, de la proteína reguladora aguda de la esteroidogénesis, de la 3β-hidroxiesteroide deshidrogenasa (HSD),	Muthulakshmi et al., 2018

					de la 17-βHSD y de los genes CYP19a1 en el ovario)	
	+ α-ZOL (1, 10, 50 μM)	24 h	HepG2	RT-qPCR	Alteración de genes involucrados en la metilación de ADN y modifcaciones de histonas	Karaman et al., 2020
	12.5 + FB1 (100 μM)	24 h	SH-SY5Y	RT-qPCR	Ninguna variación en la expresión de Gadd153 y IRE1-a (marcadores de estrés del ER)	Kara & Oztas, 2020
AFB1	0-3.0 μmM +OTA(0-20 μM)	48h	LMH	RT-qPCR	Alteración de genes asociados a respuesta inflamatoria, adhesión celular y apoptosis	Choi et al., 2020
	15, 30, 75, 150 ng/mL	7 días	Embriones y larvas de Zebrafish	RT-qPCR	Represión de markers neurotoxicos (gfap, huC, ngfa, atp1b1b), sobreexpresión de prtga	Wu et al., 2018
	5,10,20,25, 30,40 μM	72h	HK-2	RT-qPCR	**Detención** del ciclo celular en las fases G1 y G1/S mediante p53	Celik et al., 2020
OTA	0,2 μM	48h	HK-2	RNA-seq + RT-qPCR	genes implicados en mecanismos de hipoxia, transicion epitelial-mesenquimal, apoptosis y vias del metabolismo de xenobioticos	Pyo et al., 2021
	0.3 mg/kg pienso + CHS (5 g/Kg)	10 días	Riñón de pollo	RT-qPCR	Sobreexpresion de los genes NOX y p47-phox. CHS reduce la toxicidad de OTA, ripristinando los niveles de NOX.	Andretta et al., 2020

	ENB (1.5, 3, 5 μM)	24 h	Jurkat	NGS+RT-qPCR	Alteración de genes involucrados en el metabolismo y respiración de las mitocondra	Alonso-Garrido et al., 2018
Micotoxinas emergentes	ENs+ BEA+OTA+ZEA (0.1 μM) + extracto de calabaza (0.5 μM)	2h	ECV304	RT-qPCR	Represión de genes del comlplejo I y IV. Sobreexpresión de la Tioredoxina. Los carotenoides revirtieron la expresión de estos genes.	Alonso-Garrido et al., 2021
	BEA+ENB (0.1, 0.5, 1.5 μM)	24 h	Jurkat	RT-qPCR	Represion de genes antioxidantes	Escriva et al., 2019
AOH	1, 20 μM	2 h	THP-1, monocitos THP1-Lucia™ NF-κB	RT-qPCR	Alteración de citoquinas proinflamatorias (IL-8, IL-6, TNF-a); sobreexpresión de IL-10 (antiinflamatorio)	Kollarova et al., 2018

1.2.2 Proteómica

Las proteínas son los efectores críticos de las funciones y fenotipos celulares, ya que pueden captar mejor el estado funcional y las propiedades dinámicas de una célula. Dependiendo de su localización, pueden estar presentes en mezclas muy complejas que incluyen incluso más de 10.000 proteínas en una sola población celular (Labib & Kelley, 2020). El dogma central de la biología describe el flujo de información codificado en la secuencia de nucleótidos del ADN hacia la secuencia de residuos de aminoácidos (AA) que componen la estructura primaria de una proteína. La información fluye primero a través de la transcripción del ADN en ARN y luego, tras la transformación del ARN en ARNm, por la traducción del ARNm en proteína. En los seres humanos, la traducción tiene lugar en el citosol y en las mitocondrias, que son responsables del mantenimiento del equilibrio energético celular mediante la síntesis de proteínas que intervienen en la fosforilación oxidativa (Aibara et al., 2020). La traducción del ARNm a proteína es un paso crucial hacia la estructura final de la proteína, ya que las mutaciones en la secuencia codificadora y la variabilidad del sitio de inicio de la traducción, junto con las modificaciones postraduccionales, constituyen materiales para la secuencia proteica, que es el elemento clave que transmite la información sobre la estructura y la química de las mismas.

La síntesis de las proteínas es un proceso que consta de tres etapas principales: iniciación, elongación y terminación (Figura 2). La iniciación de la traducción implica el ensamblaje de ribosomas competentes para la elongación que contienen tres sitios de unión de ARNt denominados sitios aminoacil (A),

peptidil (P) y de salida (E) en los que un ARN iniciador de transferencia cargado de metionina (Met-ARNt) se empareja con el codón de iniciación en el sitio P ribosomal. Las tasas de elongación están reguladas por la disponibilidad de ARNt, la secuencia de codones del mensaje y las modificaciones de la secuencia codificante. Finalmente, la terminación de la traducción se produce cuando el ribosoma ha alcanzado un codón de parada y se detiene en esta posición (Knight et al., 2010).

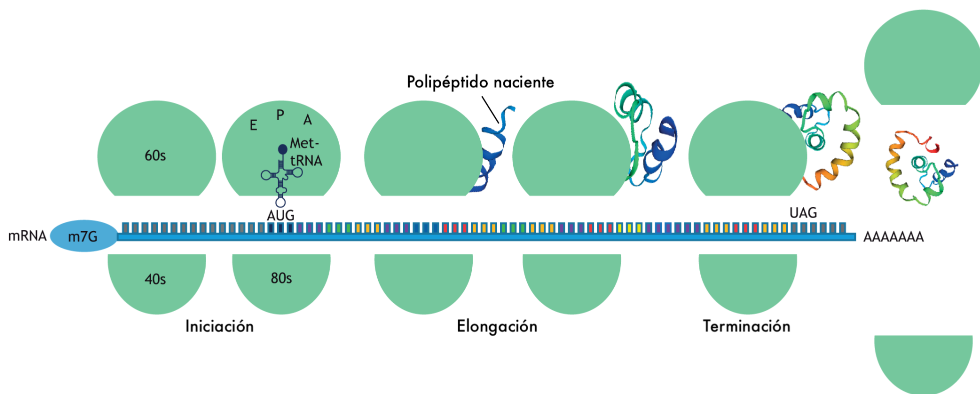


Figura 4. Representación del proceso de tres etapas de la traducción del ARNm en proteína.

El término proteóma se acuñó por primera vez para describir el contenido total de proteínas codificadas por el genoma. En la actualidad, el estudio del proteóma, denominado proteómica, se basa en el empleo de distintas tecnologías para la identificación y cuantificación no sólo de todas las proteínas de una célula determinada, sino también el conjunto de todas las isoformas y modificaciones proteicas, las interacciones entre ellas, la descripción estructural de las proteínas y sus complejos de orden superior y, en definitiva, el cuadro

postgenómico. El proteóma refleja el contexto biológico de un sistema biológico particular; es muy dinámico y está en constante cambio en respuesta a diferentes estímulos. La proteómica no solo incluye el conocimiento estructural y funcional de las proteínas, sino también el estudio de sus modificaciones e interacciones, el estudio de su localización intracelular, y la cuantificación de su abundancia (Piñeiro et al. 2015). Así, complementa a las otras tecnologías -ómicas, tales como la genómica y la transcriptómica para exponer la identidad de las proteínas de un organismo y conocer la estructura y funciones de una proteína en particular (Aslam et al., 2017).

Hasta principios de los años 90, la secuenciación de proteínas se realizaba principalmente mediante un proceso cíclico de reacciones químicas, conocido como degradación de Edman, para determinar la secuencia de aminoácidos de los péptidos. Este proceso, consiste en el acoplamiento de isotiocianato de fenilo a grupos amino accesibles, seguido de la liberación del aminoácido N-terminal derivado de la cadena peptídica, generando un nuevo N-terminal. Así, se identifica el aminoácido liberado y se repite el proceso para establecer la secuencia del péptido. Sin embargo, el proceso de Edman es lento y requiere grandes cantidades de péptidos altamente purificados (Doerr, 2019). En la década de 1990, la espectrometría de masas (EM) se convirtió en el método de elección para la secuenciación de proteínas, dejando la degradación de Edman en el ámbito de la historia de la ciencia. Además, hoy en día se ha incrementado el tratamiento bioinformático de los datos, lo que representa una poderosa estrategia para una identificación de péptidos y proteínas de alto rendimiento y una correcta cuantificación (Collins & Aebersold, 2018).

Los métodos modernos de proteómica basados en la EM se dividen generalmente en dos categorías: proteómica "ascendente" y proteómica "descendente". La proteómica ascendente difiere de las técnicas descendentes en el requisito de la digestión de proteínas antes de la separación y el análisis por espectrometría de masas acoplada a cromatografía liquida (LC-MS). Normalmente, los métodos de proteómica ascendente utilizan proteasas individuales o mezclas de proteasas para escindir selectivamente las proteínas en múltiples sitios de aminoácidos para producir una mezcla de péptidos pequeños. Se considera que la longitud óptima de los péptidos para la EM ascendente es de 6 a 50 residuos de aminoácidos para un análisis computacional eficaz. Por tanto, se utiliza la digestión con tripsina, ya que produce péptidos con una longitud media de 14 aminoácidos. La digestión de las proteínas en pequeños fragmentos de péptidos ofrece múltiples ventajas, como una mayor eficiencia de separación, un número limitado de cargas en cada péptido y una mayor homogeneidad de la muestra, lo cual es beneficioso para la detección por EM. De hecho, durante gran parte de la historia, la proteómica basada en la espectrometría de masas sólo ha sido posible utilizando métodos de análisis ascendentes debido a las limitaciones de la instrumentación de la EM, que no era capaz de alcanzar la resolución de los picos isotópicos necesaria para calcular las masas precisas de las proteínas. Por ello, se han desarrollado muchas técnicas de EM para cuantificar péptidos. Sin embargo, la proteómica ascendente tiene una limitación clave: cuando las proteínas se digieren en péptidos pequeños, se puede perder información sobre la proteoforma que tiene actividad biológica, como la ubicación de las modificaciones postraduccionales (PTM), el número de PTM y la proteólisis endógena (Cupp-Sutton & Wu, 2020).

Dentro del enfoque proteómico ascendente, el método 2DE se basa en un gel para la separación de las proteínas en muestras biológicas. Así las proteínas son inicialmente separadas generalmente en una matriz de gel de poliacrilamida, en base a su punto isoeléctrico (pI) y peso molecular, posteriormente estas se visualizan siguiendo diferentes procedimientos de tinción para ser finalmente cuantificadas. Las localizaciones de interés se extirpan e hidrolizan *in situ* mediante proteasas, como la tripsina, dando lugar a un conjunto específico de péptidos que finalmente se identifican mediante EM. Estos métodos basados en gel tienen algunas limitaciones, como la separación de compuestos hidrofóbicos y proteínas poco solubles. La sensibilidad está limitada por los métodos de detección disponibles (Lee et al., 2020).

En los enfoques sin gel también conocidos como escopeta, la mezcla de proteínas se digiere con proteasas y los péptidos de la mezcla resultante se separan por LC, comúnmente por cromatografía de fase inversa (RP). Posteriormente, los péptidos eluidos se analizan en línea mediante un espectrómetro de masas. Sin embargo, la separación cromatográfica de péptidos en estas mezclas se vuelve muy compleja y la capacidad máxima de la cromatografía unidimensional no es suficiente para identificar todos los péptidos individuales. Por lo tanto, la LC multidimensional acoplada a detección de masas se utiliza ampliamente. Estos enfoques sin gel tienen las desventajas de que la información cualitativa y cuantitativa sobre isoformas de proteínas a menudo se pierde y la identificación de especies cuyo genoma no está secuenciado completamente es más difícil (Piñeiro et al., 2015).

Sin embargo, entre todas las técnicas utilizadas en proteómica, la LC-MS ha demostrado ser una técnica de separación exitosa por su excelente rendimiento y buena reproducibilidad. El análisis multidimensional del proteoma ahora es posible gracias a importantes mejoras en la sensibilidad y resolución de la instrumentación de espectrometría de masas y los avances asociados a las tecnologías empleadas para la preparación de las muestras y análisis de los datos. Así, la proteómica basada en espectrometría de masas permite analizar una gran fracción de la población de proteínas endógenas no etiquetadas en células y organismos, evitando el tiempo, el coste y las limitaciones de las técnicas que son inherentes a la construcción y análisis de grandes cantidades de proteínas marcadas o la generación de anticuerpos específicos de proteínas. La espectrometría de masas proporciona rendimiento combinado, sensibilidad, rango dinámico y alta velocidad de adquisición de datos. Además, un espectrómetro de masas puede mejorar la precisión cuantitativa al identificar y cuantificar muchos péptidos de cada proteína en un solo experimento (Larance & Lamond, 2015).

En la actualidad, el campo de la proteómica abarca diversos temas de investigación, que van desde la elaboración de perfiles de expresión proteica hasta el análisis de vías de señalización y el desarrollo de sistemas de ensayo de biomarcadores de proteínas. Así la proteómica se utiliza en diversas áreas tales como, la detección de varios marcadores de diagnóstico, la búsqueda de candidatos para la producción de vacunas, la comprensión de los mecanismos de patogenicidad, la alteración de los patrones de expresión en respuesta a diferentes señales y la interpretación de rutas de proteínas funcionales en diferentes enfermedades (Aslam et al., 2017).

1.2.1.1 Estudios proteómicos de toxicidad *in vitro* e *in vivo* tras la exposición a micotoxinas mediante LC-MS-MS.

Entre otras aplicaciones, la proteómica es una herramienta útil para investigar el modo de acción y los efectos de los contaminantes, entre ellos las micotoxinas, ya que estos tóxicos a menudo alteran la síntesis y degradación de las proteínas, además de inducir cambios de importantes modificaciones postraduccionales (Soler & Oswald, 2018).

Hasta la fecha la LC-MS (con o sin gel) ha sido aplicada en varios estudios de proteómica *in vitro* e *in vivo* para evaluar el efecto toxico de diferentes micotoxinas tales como Aflatoxina B1 (AFB1), Ocratoxina A (OTA), 2-Amino-14,16-dimethyloctadecan-3-ol (AOD), Beauvericina (BEA), Eniatinas (ENs), Deoxinivalenol (DON), Patulina (PAT), Zearalenona (ZEA) y Toxina T-2 (T-2), utilizando como modelo biológico líneas celulares de hepatocarcinoma humano (HepG2), linfocitos T Jurkat, carcinoma epidermoide (A431), riñón embrionario humano (HEK293), tumor de células de Leydig (R2C), y células obtenidas de explantes de yeyuno de cerdo, granulosa bovina, hepatocitos primarios de pollo; plasma y ovarios de rata, hígado de rata y pollo, musculo de camarón y suero de pato. La tabla 1 resume los estudios en función de la micotoxina y del tiempo de exposición.

En concreto, se ha reportado la alteración significativa de un amplio espectro de proteínas plasmáticas tras la exposición aguda de ratas Sprague

Dawley a una dosis letal de AFB1 (0.5–1.0 mg/kg), demostrando la capacidad de la terapia de purificación de la sangre con perlas de polímero poroso de mejorar su supervivencia a través de la identificación de proteínas implicadas en el daño mediado por la micotoxina (Ruggerbeg et al., 2020). Así mismo, se han evaluado cambios en el proteóma de líneas celulares humanas de hígado (HepG2) y riñón (Hek29) tras la administración de AFB1 con o sin sulfato de zinc durante 24 h (Zhu et al., 2020; Dlamini et al., 2021). También, se ha estudiado la toxicidad sub-crónica de AFB1 a diferentes concentraciones (0.025-0.25 mg/kg) *in vivo*, reportando diferencias en la expresión de proteínas involucradas en el sistema inmunológico en suero de pato (Tansakul et al., 2019), en el daño muscular en camarón atenuado por el extracto de polifenoles de té (Huang et al., 2021) y en hígado de pollo con la suplementación beneficiosa de selenio (Zhao et al., 2021). La alteración de la homeostasis de proteínas implicadas en crecimiento, muerte celular y sistema inmunitario se ha observado en hepatocitos primarios de pollo bajo la exposición al efecto sinérgico de AFB1, ZEA y OTA (Sun et al., 2019), así mismo la combinación de AFM1 y OTA (4 µg/mL), produjo un sinergismo en la respuesta inflamatoria intestinal en células Caco-2 tras 48 h de exposición (Gao et al., 2020).

ZEA ha sido estudiada en explantes de yeyuno de cerdo en base a su immunotoxicidad (Soler et al., 2020) y en células R2C mediante la identificación de rutas metabólicas esenciales para el sistema reproductivo tras la exposición aguda (Wang et al., 2020). Mediante LC-MS se han identificado alteraciones en las proteínas implicadas en el metabolismo químico, la reparación del ADN y la reproducción en ovarios de ratones obesos, evidenciando modos de acción adversos para los ovarios inducidos por ZEA y sugiriendo que la obesidad es

consecuencia de la ovotoxicidad inducida por ZEA (González-Alvarez., 2021). En un estudio realizado por Smith y colaboradores (2018) se investigó los cambios proteómicos que se produjeron después de exposiciones cortas (24h) de ZEA (20 µM) y DON (individualmente y en combinación) a bajos niveles de citotoxicidad en la proliferación de células HepaRG utilizando LC-MS, mostrando que la respuesta celular es dependiente del tiempo y de la micotoxina o de la mezcla. Por otro lado, el mecanismo de respuesta toxica de concentraciones más altas de ZEA (200 µM) en combinación con la toxina T-2 sobre la función ovárica, ha evidenciado la acumulación de ROS, la producción de esteroides y la apoptosis celular en el proteóma de células foliculares de la granulosa bovina (CG) (Li et al., 2020).

Del Favero et al. (2018) tras realizar el análisis proteómico mediante UHPLC-Orbitrap-MS, observaron que la exposición aguda a DON (10 µM) en células A431 reguló significativamente varias proteínas que gobiernan la adhesión celular, las que organizan la matriz extracelular y las que regulan la dinámica del citoesqueleto. De manera similar, la disfunción de la barrera intestinal inducida por el DON en un modelo celular Caco-2 se ha confirmado mediante la expresión de proteínas de adhesión celular, la unión celular y el ensamblaje de la unión celular. Además, el pretratamiento con kaempferol ha mejorado la integridad de la barrera de la monocapa celular, afectando la expresión y el ensamblaje de las proteínas de la unión estrecha y de la unión adherente a través de la vía PKA y de la vía MAPK/ERK (Wang & Zhang., 2020).

La nefrotoxicidad de la PAT (2.5-10 µM) se ha evaluado en células HEK293 durante 10 h, mostrando cambios drásticos de expresión en genes y proteínas relacionados con la apoptosis, la fosforilación oxidativa del ribosoma y el ciclo celular, especialmente en la activación de la caspasa 3, UQCR11, la forma de transporte activo y la endocitosis (Han et al., 2020). Concentraciones similares de la micotoxina AOD (10 µM) indujeron una acumulación transitoria de vacuolas en células HepG2, afectando varias partes del sistema de transporte vesicular, resultando en una degradación de proteínas (Solhaug et al. 2020).

Siendo un contaminante habitual de los piensos para animales acuáticos, el efecto de la toxina T-2 en las proteínas musculares de las gambas, se estudió utilizando la espectrometría de masas con desorción/ionización láser asistida por matriz (MALDI-TOF/TOF MS), determinando cambios en la composición de las proteínas musculares, las actividades de las ATPasas y el contenido de sulfidrilos (SH) y la hidrofobicidad de la actomiosina (AM) (Huang et al., 2020).

Finalmente, el proteóma de linfocitos T- Jurkat expuestos a ENB en combinación con BEA a bajas concentraciones (0.01, 0.1, 0.5 µM) mostró cambios a nivel mitocondrial tras 24h de exposición (Alonso-Garrido et al., 2020).

Tabla 2. Estudios proteómicos realizados con micotoxinas, dosis, tiempo exposición, organismo biológico, técnica, principales efectos y referencia.

Micotoxina	Dosis	Tiempo exposición	Organismo biológico	Técnica	Principales efectos	Referencia
AFs	AFB1 (0.5-1.0 mg/kg)	4 h	Plasma de rata	LC-MS	Cambios en los niveles de proteínas plasmáticas	Ruggerbeg et al., 2020
	AFB1 (32 μM) + ZnSO4 (50 μM)	24 h	HepG2	MALDI-TOF/TOF	Citotoxicidad - El Zn alivia el cambio en el nivel de ROS intracelular inducido por la AFB1 a través de la regulación de la expresión de la peroxidasa	Zhu et al., 2020
	AFB1 (100 μM)	24 h	Hek293	LC-MS	Nefrotoxicidad	Dlamini et al., 2021
	AFB1 (0.025, 0.1, 0.25 mg/kg)	10 días	Suero de pato	SDS PAGE + HTC-ultra-LC-MS	Disminución de proteínas séricas, albúmina, imunotoxicidad (Aflatoxicosis)	Tansakul et al., 2019
	AFB1 (1.2, 1.8, 2.7, 4, 6 mg/kg) +	20 días	Musculo de camarón	LC-MS	Daños en el tejido muscular, cambios en la composición	Huang et al., 2021

	polifenoles de té (0.04-0.16% pienso)				proteica. Efecto significativo de TP contra el efecto tóxico de AFB1 a 1,2 y 2,7 mg/kg.	
	AFB1 1 mg/kg + Se 0.3 mg/kg	21 días	Hígado de pollo	HPLC-MS-MS	Hepatotoxicidad	Zhao et al., 2021
	AFM1+ OTA (10 μM)	48 h	Células Caco-2	LC-MS-MS	Inmunotoxicidad Intestinal	Gao et al., 2020
	AFB1 (32 μM) + ZEA (377 μM) + OTA (25 μM)	48 h	Embrión (hepatocitos primarios de pollo)	LC-MS-MS	Citotoxicidad (crecimiento y muerte celular, sistema inmunitario)	Sun et al., 2019
ZEA	100 μM	4 h	Explantes de yeyuno de cerdo	LC-MS-MS (Orbitrap)	Toxicidad intestinal, inmunotoxicidad	Soler et al., 2020
	30 μM	24 h	R2C	LC-ESI-MS/MS	Toxicidad en el sistema reproductor	Wang et al., 2020
	0.04 mg/kg	15 days	Ovarios de ratón obeso	LC-MS-MS	Toxicidad ovárica	González-Alvarez et al., 2021

	200 µM+ T-2(0.2 µM)	24 h	GC (Células de granulosa bovina)	LC-MS-MS	Toxicidad en el sistema reproductor y toxicidad hormonal	Li et al., 2020
	20 µM + DON (0.2 µM)	24 h	HepaRG	LC-MS-MS	Citotoxicidad (efecto sinérgico más proteínas que las micotoxinas individuales) para el ciclo celular, la proliferación y el proceso de desarrollo	Smith et al., 2018
DON	0.1, 1, 10 µM	24 h	A431	LC-MS-MS	Alteración de las proteínas implicadas en la adhesión celular y la modulación del citoesqueleto (Dermotoxicidad)	Del Favero et al., 2021
	100000 µM + pretratamiento con kaempferol	24 h	Caco-2	HPLC-MS-MS	Disfunción de la barrera intestinal	Wang & Zhang., 2020
PAT	2.5, 5, 7.5, 10 µM	10h	HEK293	LC-ESI-MS/MS	Nefrotoxicidad	Han et al., 2020
T-2	1.2, 2.4, 4.8, 12.2 mg/kg	20 días	Musculo de camarón	MALDI-TOF/TOF MS + 2D gel	Cambios en la composición y la calidad de las proteínas del músculo del camarón	Huang et al., 2020

					(degradación de las proteínas miofibrilares, sacroplásmicas y del estroma)	
AOD	5, 10 μM	6 h	HepG2	LC-MS-MS	Vacuolización inducida de lisosomas o endosomas tardíos	Solhaug et al., 2020
Micotoxinas emergentes	BEA, EN B (0.01, 0.1, 0.5 μM)	24 h	Jurkat	LC-UDMS	Toxicidad mitocondrial	Alonso-Garrido et al., 2020

2. Objetivos

2.OBJECTIVES

El objetivo general de la presente Tesis Doctoral es profundizar en el conocimiento de los mecanismos de acción y los efectos tóxicos de las micotoxinas mediante un enfoque transcriptómico y proteómico *in vivo* e *in vitro*. Para llevar a cabo este objetivo se plantean los siguientes objetivos específicos:

1. Recopilar los resultados de la investigación sobre los efectos toxicológicos que las micotoxinas pueden inducir en la salud humana, mediante el examen de estudios *in vivo*.

2. Estudio de los cambios transcripcionales en genes relacionados con fosforilación oxidativa, inflamación, apoptosis y estructura tras exposición aguda en ratas de eniatinas A, A1, B y B1 en estómago, hígado, riñón y colon de rata por PCR cuantitativa en tiempo real.

3. Evaluación del daño en hígado de rata tras exposición aguda a eniatinas A, A1, B y B1 mediante análisis proteómico mediante LC-MS/QTOF.

4. Análisis proteómico del efecto in vitro de un digerido gastrointestinal de pan preparado con suero fermentado de leche de cabra y calabaza liofilizada contra la citotoxidad inducida por las micotoxinas aflatoxina B1 y ocratoxina A.

2.OBJECTIVES

The overall objective of this Doctoral Thesis delves into the mechanisms of action related to toxicological effects of mycotoxins through a transcriptomic and proteomic approach *in vivo* and *in vitro*. In order to achieve this purpose, the following specific objectives are proposed:

1. To review the bibliographic research about the toxicological effects that mycotoxins induce on human health by focusing on *in vivo* studies.

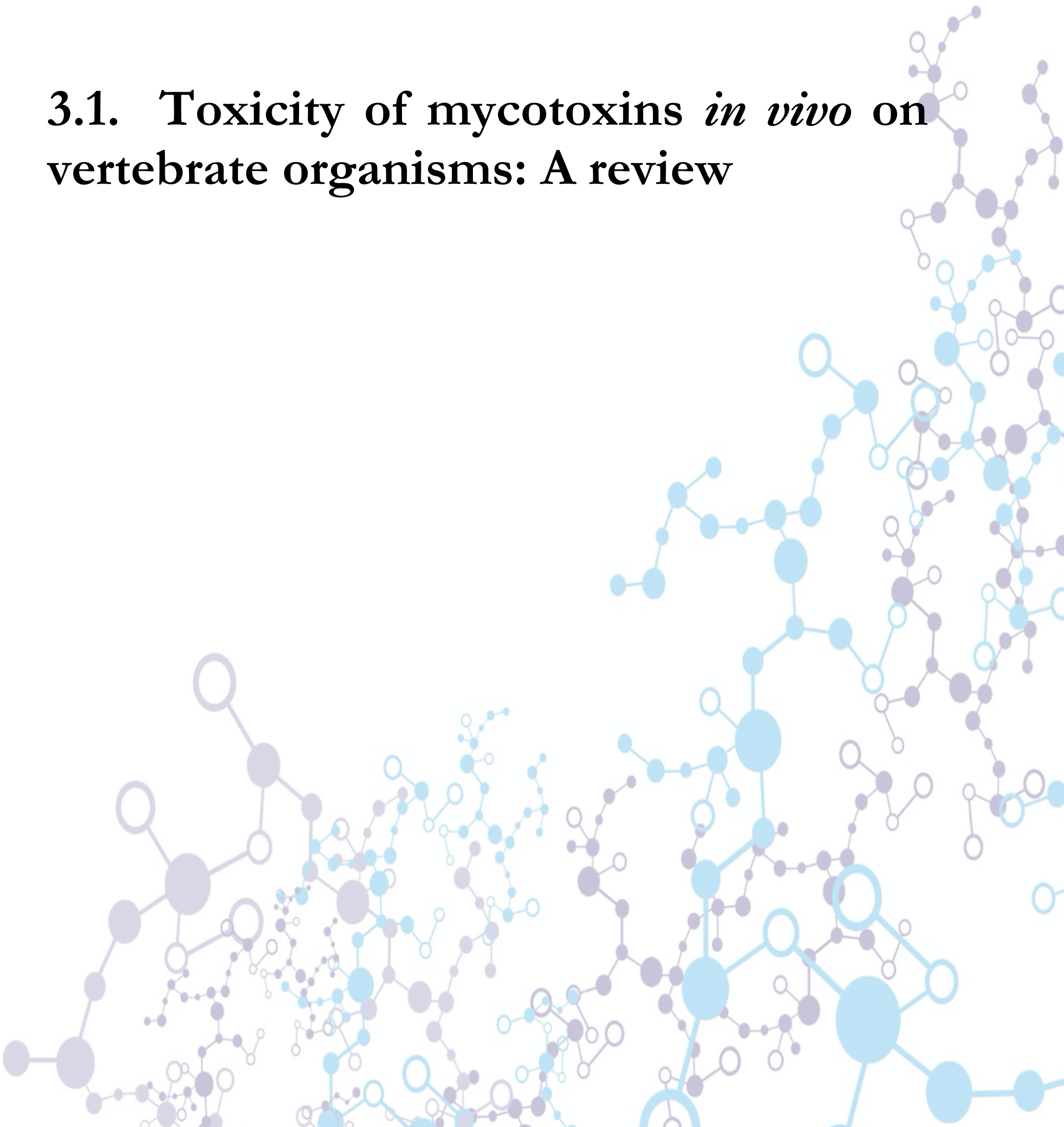
2. Study of transcriptional changes in genes related to oxidative phosphorylation, inflammation, apoptosis and structure after acute exposure in rats of eniatins A, A1, B and B1 in rat stomach, liver, kidney and colon by real-time quantitative PCR.

3. Evaluation of rat liver damage after acute exposure to enniatins A, A1, B and B1 through proteomic analysis by LC-MS/QTOF.

4. To perform a proteomic analysis *in vitro* on the effect of a gastrointestinal digest of bread prepared with fermented goat milk whey and lyophilized pumpkin against cytotoxicity induced by aflatoxin B1 and ochratoxin A mycotoxins.

3. Resultados

3.1. Toxicity of mycotoxins *in vivo* on vertebrate organisms: A review



Food and Chemical Toxicology

Toxicity of mycotoxins *in vivo* on vertebrate organisms: A review

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Abstract

Mycotoxins are considered to be a major risk factor affecting human and animal health as they are one of the most dangerous contaminants of food and feed. This review aims to compile the research developed up to date on the toxicological effects that mycotoxins can induce on human health, through the examination of a selected number of studies *in vivo*. AFB1 shows to be currently the most studied mycotoxin *in vivo*, followed by DON, ZEA and OTA. Scarce data was found for FBs, PAT, CIT, AOH and *Fusarium* emerging mycotoxins. The majority of them concerned the investigation of immunotoxicity, whereas the rest consisted in the study of genotoxicity, oxidative stress, hepatotoxicity, cytotoxicity, teratogenicity and neurotoxicity. In order to assess the risk, a wide range of different techniques have been employed across the reviewed studies: qPCR, ELISA, IHC, WB, LC-MS/ MS, microscopy, enzymatic assays, microarray and RNA-Seq. In the last decade, the attention has been drawn to immunologic and transcriptomic aspects of mycotoxins' action, confirming their toxicity at molecular level. Even though, more *in vivo* studies are needed to further investigate their mechanism of action on human health.

Keywords: Mycotoxin Molecular biology Spectrometry Bioinformatics Mechanism of action

1. Introduction

In vivo studies in Europe are limited because of their complexity and most importantly, the new rules in care and use of live animals for scientific purposes governed by internationally established principles of replacement, reduction and refinement. Furthermore, ethical and animal welfare considerations require that animal use is limited as much as possible. In this sense, Directive 2010/63/EU on the protection of animals used for scientific purposes was implemented into national laws in each EU Member State in 2013 and supports the application of 3R principle when considering choice of methods to be used. Nonetheless, animal usage represents the only way to corroborate toxicity in humans (Clark, 2018). World organization for animal health (OIE) in the Terrestrial Animal Health Code, recognizes the vital role played by the use of live animals in research and education (OIE, 2019). In fact, laboratory animals, particularly rats and mice which are the most frequently used species, have been employed successfully for the detection of several diseases. However, they are actually considered poor models for the majority of human diseases (Baumans, 2016).

In last years, the use of zebrafish in scientific research is expanding rapidly in a variety of biological investigations (Keller and Keller, 2018). Due to its stability and the ease of stable genetic manipulations, zebrafish has been positioned as an ideal vertebrate model for in vivo studies compared to other vertebrates, especially mice (Bercier et al., 2019). However, the choice of a certain animal model in research depends on their susceptibility to mycotoxins, which results different for each of them.

The definition of mycotoxins includes any non-enzymatic poisonous metabolites harmful to other organisms produced by fungi. They belong to the phylum Ascomycota, among which are genera *Aspergillus, Penicillium* and *Fusarium* (Marin et al., 2013). Studies have revealed the existence of at least 400 different mycotoxins, but scientific attention has mainly focused on those that have been proven to be toxic to human and animals such as: aflatoxins (AFs), ochratoxin A (OTA), citrinin (CIT), deoxynivalenol (DON), fumonisin B1 (FB1), patulin (PAT), T-2 toxin (T-2), zearalenone (ZEA) and the emerging mycotoxins species. Emerging mycotoxins are a group of *Fusarium* metabolites defined as neither routinely determined, nor legislatively regulated, but evidence of their print is rapidly increasing (Vaclavikova et al., 2013). Among them, the most frequently detected are beauvericin (BEA), enniatins (ENs), fusaproliferin (FP) and moniliformin (MON) (Huffman et al., 2010). These metabolites are structurally a diverse group of mostly small molecular weight compounds that vary from simple C4 to com- plex chemical substances (Alshannaq and Yu, 2017).

Mycotoxin production is susceptible to temperature, moisture, water activity, pH and oxygen concentration, same environmental factors that affect the growth of toxigenic fungi with climate re- presenting the key agro-ecosystem driving force of fungal colonization (Zinedine and El Akhdari, 2019). The Food and Agriculture Organization of the United Nations (FAO) estimated that approximately 25% of the cereals produced in the world are contaminated by mycotoxins (FAO, 2013). Furthermore, other authors have reported that 72–79% of feed samples from different parts of the world contained mycotoxins (Eskola et al., 2019; Streit et al., 2013; Kovalsky et al., 2016).

Due to their toxicological effects, the presence of mycotoxins in foods has severe implications on human and animal health even at very low concentrations. If ingested, they may cause acute or chronic dis- ease: a) acute episodes are characterized by a rapid onset and an obvious toxic response including rapid death; b) low-dose chronic exposures to mycotoxins over a long period of time reported toxic responses, including cancer. However, these toxins are not equally toxic, their toxicity changes during metabolism, whereas susceptibility of animals and humans varies with species age, nutrition and other factors as well.

The majority of mycotoxins currently known are grouped according to their toxicity (under chronic conditions) as mutagenic, carcinogenic and teratogenic. Considering the agroeconomic aspects and the impact on global agriculture, as well as the possible implications on public health, IARC initiated a *Monographs* program in which all relevant information was examined in order to assess the evidence related to mycotoxin exposure, which could alter the incidence of cancer in hu- mans (Ostry et al., 2017). The toxic agents are classified into five groups (Group 1, 2A, 2B, 3 and 4) depending on the evidence of their carcinogenicity (IARC, 2012). Carcinogenic properties were recognized for AFs and fumonisins (De Ruyck et al., 2015; Voss et al., 2017).

Nowadays various technologies can be used to determinate the toxicological mode of action of mycotoxins in organisms. Among them, molecular biology techniques allow to understand the basic structure of nucleic acids and other molecules and help to mimic their natural function in both *in*

vivo and *in vitro* studies. A major tool involved in the above-mentioned research field is represented by the *-omics* sciences like genomics, transcriptomics, proteomics, and metabolomics (Soler and Oswald, 2018). They are employed to assess various aspects of cytotoxicity, cell responses, gene expression and activation of specific signaling pathways and transcription factors. For example, several techniques can be used for isolation and quantification of nucleic acids and transcripts such as electrophoresis, DNA/RNA blotting, quantitative real time polymerase chain reaction (qPCR), microarray analysis or NGS (Next Generation Sequencing) (Rapley and Whitehouse, 2015).

The analysis of the proteome allows to identify and quantify protein content and evaluate their specific interaction inside an organism (Larance and Lamond, 2015). Proteomics also show how proteome can be perturbed by toxicants and reveal changes which could induce alterations in cellular pathways and networks (Rabilloud and Lescuyer, 2015). Common techniques used in proteomics analysis are mass spectrophotometry (MS), flow cytometry (FCM), ELISA, western blot- ting and microarrays (Aslam et al., 2017; Jia et al., 2019). Metabolomics involves the analysis of low molecular weight components of cells, tissues or biological fluids, focusing on the detection of com- pounds with less than 2000 molecular weight. Frequently used technologies in metabolomics, but also in proteomics, are nuclear magnetic resonance (NMR), gas chromatography (GC), high performance liquid chromatography (HPLC) or ultra-performance liquid chromatography (UPLC) coupled to quadrupole-time-of-flight mass spectrometer detector (QTOF-MS) or Orbitrap MS (Krska et al., 2017; Gamliel et al., 2017; Romera et al., 2018; Tamura, 2016).

The goal of the present review is to better understand mycotoxins mechanisms of action that lead to loss of homeostasis (LOH) by con- ducting bibliographic research regarding the published data on mycotoxins toxicity *in vivo* on vertebrate organisms, focusing on the techniques used.

2. Material and methods

Extensive bibliographic research was conducted using the scientific databases Web of Science, PubMed and Scopus by selecting articles and reviews in the last decade (2009–2019). The research was carried out by using the following keywords: *in vivo,* mycotoxin, vertebrate, animal, mechanism. To facilitate the screening, the research was refined by using the terms: tissue, molecular biology, proteomics, metabolomics, metabolome, qPCR, cytometry, microscopy, transcriptomics, transcriptome, transcription, RNA, gene expression, gene, nucleic acid, genome. A number of 214 articles, which met the criteria to be included into the study, were analyzed. Reports obtained were evaluated by screening abstracts to discard unnecessary, incomplete or irrelevant literature. A total of 100 articles were analyzed in full text and classified by groups based on specie of mycotoxin, duration of the analysis, type of biological sample, analytical determination techniques and damage (Fig. 1).

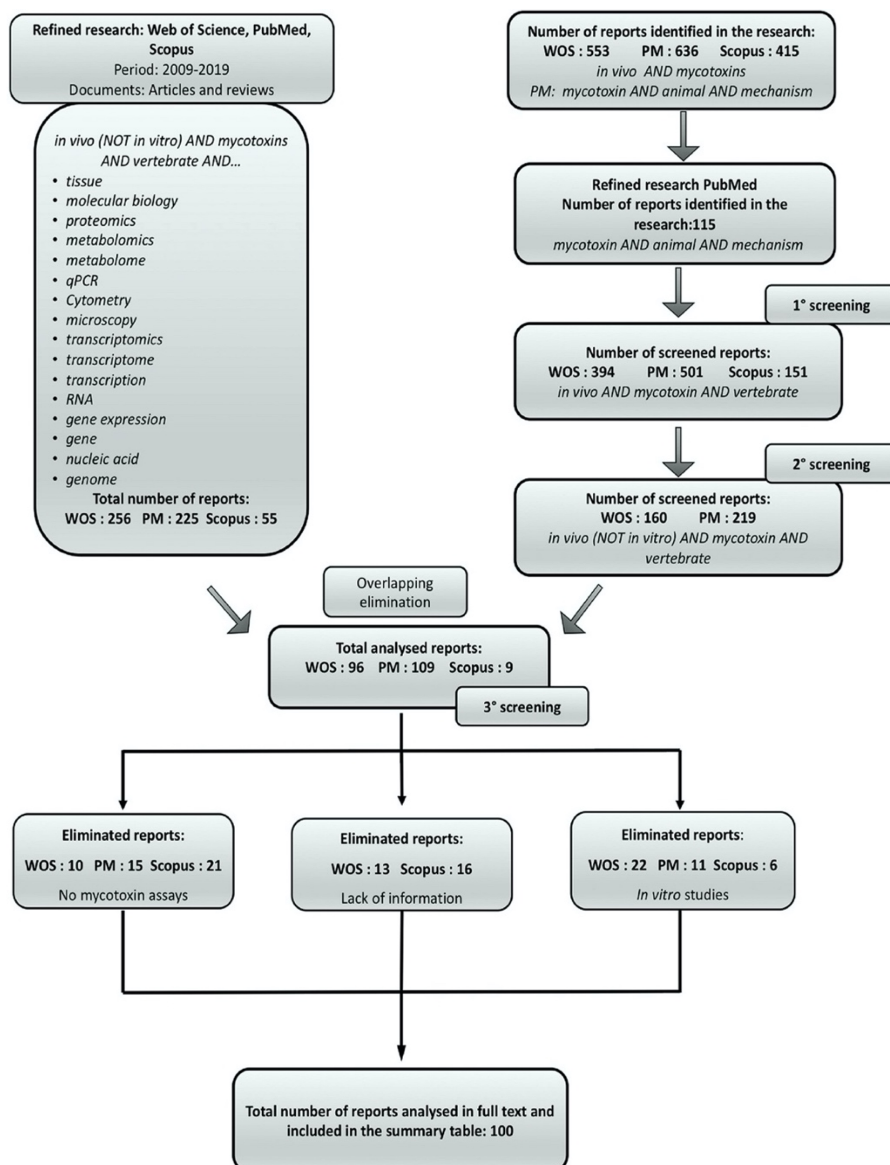

Figura 1. Graphic representation of total number of articles screened throughout the bibliographic research.

3. Results

3.1. Aflatoxins

AFs are the best known and most studied mycotoxins. They are polyketide compounds synthesized by a secondary metabolic pathway in *Aspergilli*. In particular, the common fungus *A. flavus* and the related species *A. parasiticus* are the two majors AFs productors. *A. flavus* produces only B aflatoxins and sometimes the mycotoxin cyclopiazonic acid (CPA), while *A. parasiticus* produces all of them B1 and B2 and G1 and G2, but not CPA. These species have been found in a variety of agricultural commodities, nut the most pronounced contamination has been encountered in cereal crops like wheat, walnut, corn, cotton, peanuts and tree nuts. Aflatoxin M1 and M2 is a metabolite of aflatoxin B1 and it can occur in milk and dairy products (Ren et al., 2020).

During the years, it has been demonstrated that they are highly genotoxic, immunosuppressive, mutagenic and carcinogenic. More especially, aflatoxins B1, B2, G1, G1 and M1 have been linked to human primary liver cancer, being classified by IARC as carcinogens in humans in Group 1 (IARC, 2012). Mechanistically, AFB1 is metabolized into an aflatoxin-8,9-exo-epoxide by p450 enzymes, which can react with DNA, RNA and proteins to form adducts, especially with the *p*53 tumour suppressor gene (Dai et al., 2017). In particular, AFB1-8,9-exoepoxide can bind to DNA forming predominantly 8,9-dihydro-8(N7-guanyl)-9- hydroxy-AFB1 (AFB1–N7-Gua) adduct which is suggested to be re- sponsible for the mutagenic properties of AFB1 (Coskun et al., 2018).

In AFB1 studies, the predominant specie studied was the mouse in acute and sub-acute studies, with doses ranged from 0.01 to 50 mg/kg. Rats and chicken's studies, are principally focused on gene expression analysis and determination of oxidative stress parameters. In general, the main toxicological effects of AFB1 reported in the bibliography for all the species are hepatotoxicity, immunotoxicity, oxidative stress, cytotoxicity, genotoxicity and neurotoxicity (Fig. 2). Therefore, the most used technique was qPCR, followed by microarray, FCM, ELISA and IHC. Only one study was found for turkeys and fish respectively, centred in changes in gene expression in sub-acute toxicity duration. Moreover, biological compounds and non-pathogenic microorganisms have been studied to ameliorate AFB1 toxicity, such as β-1,3-Glucan, lycopene, melatonin, curcumin, chlorophyllin, fermented milk, Selenium, bentonite, *Chromolena odorata* and lactic acid bacteria. The summary of the studies found for AFB1 are shown in Table 1, ordered by exposure time, from the shortest to the longest.

Table 1. AFB1 toxicity studies: dose and administration route, exposure time, animal species, sex and age, biological sample, technique, damage and reference.

AFB$_1$						
Dose/administration route	**Specie**	**Duration**	**Biological sample**	**Technique**	**Damage**	**Reference**
AFB1 (10 mg/kg)/ intraperitoneal administration		48h	Liver	IHC, IF	Aberrations in hepatocyte nuclear morphology and ploidy with RB loss	Reed *et al.,* 2009
AFB1 (0.240 mg/kg) / intraperitoneal injections	Mice	16 weeks	Liver, lung, tumors	IHC, microscopy	Hepatocellular carcinoma	Li *et al.,* 2011
AFB1 (50 mg/kg / oral administration)		2h	Lung, liver	PCR	Outbreak of adenomas, osteosarcomas, hepatocellular carcinoma; ogg1 don't have significant effect on AFB1-induced oxidatively damaged DNA or tumourigenesis, but increase susceptibility to other aspects of its toxicity.	Mulder et al., 2015
AFB1 (50 mg/kg / oral administration)		2h	Lung, liver	PCR	Outbreak of adenomas, osteosarcomas, hepatocellular carcinoma; ogg1 don't have significant effect on AFB1-induced oxidatively damaged DNA or tumourigenesis, but increase susceptibility to other aspects of its toxicity.	Mulder et al., 2015

AFB1 (0,08 mg/kg)+ FB1 (0,1 mg/kg) + LAB (2 mg/kg)/gavage	14 days	Spleen, thymus	qPCR, enzimatic assays, flow cytometry	Increase of ROS in spleen, down-regulation of cytokine mRNA gene expression, increasing of apoptosis ampg thymocytes	Abbès *et al.,* 2016
AFB1 (1.25, 150 mg/kg /oral administration)	7 days	Spleen	qPCR, FC, ELISA, WB	Induction of immune suppression	Bakheet et al., 2016
AFB1 (6 mg/kg) / feed	72 weeks	Liver	PCR, DNA-Seq, HCC	Hepatocellular carcinoma	Chawanthayatham *et al.,* 2017
AFB1 (5 mg/kg) intragastric + lycopene	30 days	Kidney	qPCR, WB, IHC	LYC potentially protected male mice from AFB_1-induced kidney injury and this protective effect may be associated with enhancing the renal antioxidant capacity, which may be caused by the activation of the Nrf2 antioxidant signaling pathway.	Yu et al., 2018
AFB1 (10, 20, and 40 µg/kg b.w.) i.p. Daily + influenza virus	15 days	Lung and spleen	qPCR, IHC, WB, ELISA	AFB_1 promotes SIV replication and SIV-induced lung damage by activating TLR4-NFκB signaling	Sun et al., 2018
AFB1 (0.417, 0.065 mg/kg) / oral administration	40 days	Liver, kidney	Microscopy	Histopathological changes	Luo *et al.,* 2018

AFB1 (0.5 mg/kg)+AFM1 (3.5 mg/kg) gavaged once per day (0.2 mL/ mice)		28 days	Kidney	qPCR, orbitrap, ELISA	AFB1 and AFM1 caused kidney toxicity by activating oxidative stress through altering expression of PRODH and L-proline levels, which then induced downstream apoptosis.	Li et al., 2018
AF, DON, ZEA (0.597, 0.729, 3.1 mg/kg / oral administration)		4 weeks	Blood, liver, spleen, kidney	LC-MS/MS	Induction of oxidative stress	Hou et al., 2013
AFB1 (0.5 mg/kg)+AFM1 (3.5 mg/kg) gavaged once per day (0.2 mL/ mice)		28 days	Kidney	qPCR, orbitrap, ELISA	AFB1 and AFM1 caused kidney toxicity by activating oxidative stress through altering expression of PRODH and L-proline levels, which then induced downstream apoptosis.	Li et al., 2018
AFB1 (0.5, 1 mg/kg / oral administration)	Rats	72 h	Heart, liver, spleen, lung, kidney, brain	LC-MS/MS	Carcinogenicity	Han et al., 2012
AFB1 (0.450 mg/kg / intraperitoneal injections) + fermented milk +		6 weeks (twice	Liver	qPCR	Expression of oncogenes, tumour suppressor gene, DNA damage and histopathological results in all groups; fermented milk and CHL: decrease oncogene expression and genotoxicity, increase of antioxidant status	Kumar et al., 2011

chlorophyllin (250 mg/kg / oral intubation)		a week)				
AFB1 (2.5 mg/kg) +FE (50,100 mg/kg) / intraperitoneal injection		7 days	Blood, liver, small itestine	ELISA, HP	Alterations in pro-inflammatory cytokines, histologic changes in liver and small intestine; FE protect tissue integrity	Akinrinmade *et al.,* 2016
AFB1 (0.05, 0.75 mg/kg) / oral gavage		4 weeks	Colon	RNA-Seq	Changes in the composition of gut microbial communitites	Wang et al., 2016
AFB1 (0,125 mg/kg) + Hgs (0,5 mL/rat) + Cur-NPs-Hgs (100-200 mg/kg)		3 weeks	Blood, femur bone marrow, liver	Comet assay, qPCR, microscopy	Disturbance in biochemical parameters, increasinf of chromosomal aberrations in bm, DNA fragmentation in liver and spleen	Abdel-Wahhab *et al.,* 2016
AFB1 (0.1-0.2 mg/kg) /		28 days	Liver	RNA-Seq, qPCR	Genotoxicity and hepatotoxicity	Liu *et al.,* 2018
AFB1 (1, 2mg/kg /oral administration)	Chicken	21 days	Blood, liver	qPCR, Microarray	Altered gene expression in liver at concentration of 2 mg/kg	Yarru *et al.*, 2009

AFB1 (1 mg/kg / oral administration)		42 days	Liver (30 mg)	qPCR	Icrease of hepatic biotransformation genes expression CYP1A1, CYP2H1	Sridhar et al., 2014
AFB1 (1,2 mg/kg) / contaminated diet		21 days	Blood, liver, cecum, spleen, duodenum	Microscopy	Reduction of body weight, BWG. FI	Galarza-Seeber *et al.,* 2016
AFB1, OTA (0.1,0.2, 0.6 mg/kg, 0.15, 0.3, 1.0 mg/kg) + BN (0.075 mg/kg)		42 days	Thymus, bursa de Fabricius	Antibody assays	Immunotoxicity, BN ameliorate AFB1 effects	Bhatti *et al.,* 2017
AFB1 (5mg/kg day) / oral contaminated feed + curcumin (300 mg/kg day)		28 days	Liver, serum	qPCR, ELISA, IHC, WB	AFB1 adducts contributes to liver damage as biomarker and curcumin can be supplemented into feed as a prophylactic measure	Li et al., 2019
AFB1 (0.6 mg/kg) + Se		21 days	Spleen	qPCR, flow cytometry	Se could partially ameliorate the AFB$_1$-caused excessive apoptosis of chicken splenocytes through downregulation of endoplasmic reticulum and death receptor pathway molecules	Fang et al., 2019
AFB1 (15, 30, 75, 150 ng/mL)	Fish	7 days	Embryo/larvae	qPCR, microarray, IF	AFB1 disrupted the neural morphology, altered genes in synaptic areas and functionally related to neurogenesis or neuroactive ligand-receptor interaction	Wu et al., 2018

AFB1 (0.320 mg/kg) / contaminated diet	Tur key s	14 days	Liver, blood	RNA-Seq, qPCR	Changes in expression of genes involved in cellular regulation, modulation of apoptosis and inflammatory responses	Reed *et al*., 2018

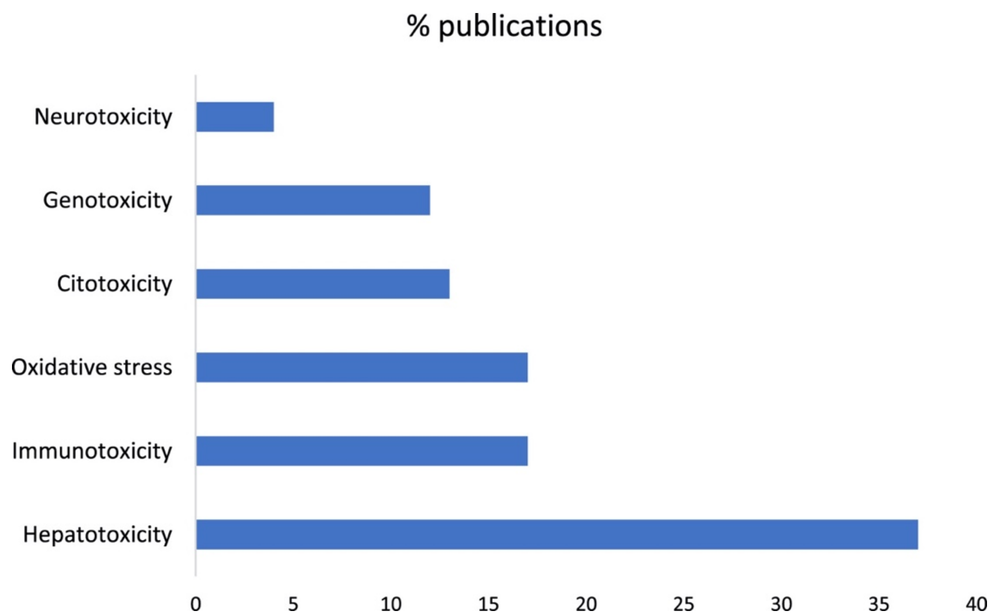

Figura 2 Percentage of revised publications according to the main toxicological effect of AFB1.

3.1.1. Mice

In vivo studies using AFB1 in mice had variable duration, from 2h until 72 weeks. Doses ranged from 0.01 to 50 mg/kg and the administration route was intraperitoneal/oral in contaminated diet or by gavage. The most frequent organ used was liver as biological sample, followed by kidney, spleen and lung. It has been also tested the efficiency of natural and biological compounds to counteract AFB1 toxicity such as lactic acid bacteria (LAB), β- glucane and lycopene.

Acute exposure to a tumorigenic dose of AFB1 (50 mg/kg) caused exacerbated weight loss and mortality in 8-oxoguanine glycosylase Acute exposure to a tumorigenic dose of AFB1 (50 mg/kg) caused exacerbated weight loss and mortality in 8-oxoguanine glycosylase (*ogg1*) deficient mice liver and lung, but did not increase sensitivity to AFB_1-induced oxidative DNA damage and tumorigenesis (Mulder et al., 2015). In contrast, retinoblastoma tumour (RB) suppressor deficiency influenced the acute response to AFB1 (10 mg/kg) and contributed to liver tumorigenesis by producing an aberrant proliferative response to the toxin (Reed et al., 2009). AFB1 exposure exacerbated swine influenza virus (SIV) infection, inflammation and lung damage by activating TLR4-NFB signalling. In contrast both TAK242 and TLR4 knockout, TLR4-specific antagonist, attenuated the AFB1-promoted SIV replication, inflammation and lung damage (Sun et al., 2018).

In a sub-acute immunotoxicity study, it has been shown that AFB1 significantly decreased the percentages of lymphocytes subsets and reduced the IL-2, TNF-α, IL-17 and IFN-γ production in spleen and serum. qPCR also showed AFB1 general immune suppression by downregulation of Th1, Th2, Th17 and Treg genes related to immune responses. Furthermore, in the same study it has been demonstrated that -1,3-Glucan enhances the responses of lymphocyte subsets, including cytokine production (Bakheet et al., 2016). Similarly, AFB_1 alone or in combination with FB_1, caused oxidative stress by increasing caspase-3 activity, LP, and IL-10 and IL-4 mRNA levels, while decreasing gluthatione (GSH) content and down-regulating expression of gluthatione peroxidase (GPx) and superoxide dismutase (SOD). LAB induced

protective effects against the oxidative stress and immunotoxicity of both mycotoxins (Abbés et al, 2016).

Yu et al. (2018) demonstrated that lycopene dissolved in olive oil owned has a protecting role against AFB1-induced renal damage after 30 day-exposure by activating the nuclear factor erythroid 2- related factor 2 (Nrf2) antioxidant signalling pathway. Moreover, ozone treatment of CC, was able to remove high quantities of AFB1 in ACC, alleviate its harmful effects and improve growth performance in mice fed with AFB_1 contaminated corn (ACC) during 40 days. They manifested the typical toxicity symptoms such as slow weight growth, abnormal serum enzyme indexes, and damaged liver and kidney (Luo et al., 2018).

Regarding long lasting exposures, hepatic oval cells (HOC) implanted in mice produced tumours in liver, including hepatocellular carcinoma cells (HCC) and mesenchymal cells, following transfection with HBx gene and intraperitoneal treatment with AFB1 (0.240 mg/kg) for 16 weeks (Li et al., 2011). In the longest study, after AFB1 exposure for 72 weeks with high concentrations in contaminated diet, DNA sequencing was used to reveal high-resolution mutational spectra (HRMS) in histopathological normal liver by identifying changes sequence context dependence of mutagenic processes during hepatocarcinogenesis. Interestingly, AFB_1 exposure showed remarkable similarity to the mutational spectra of an important subset of human liver tumours (Chawanthayatham et al., 2017).

Combined administration of AFB1 and AFM1 caused kidney toxicity by producing oxidative stress through altering expression of proline dehydrogenase (PRODH) and L-proline levels, which induced apoptosis (Li et al., 2018). Oxidative stress was also detected in liver, kidney and spleen of mice fed with a multimycotoxin contaminated maize diet (AF, ZEA, DON) for 4 weeks, as indicated by the increase of GPx activity and malondialdehyde (MDA) level and decreased of CAT and SOD activity in tissues (Hou et al., 2013).

3.1.2. Rats

In rat studies, the exposure time ranged between 72h and 6 weeks with concentrations of 0.1–2.5 mg/kg. As well as in mice studies, the use of natural compounds to reduce AFB1 damage was widespread (melatonin, *Chromolena odorata,* fermented milk, chlorophyllin and curcumin nanoparticles) loaded into hydrogels. Various were the purposes of the studies, such as genotoxicity, immunotoxicity, carcinogenicity, disturbance in biochemical parameters and changes in the composition of gut microbial communities.

Rats intraperitoneally treated with AFB1 (2.5 mg/kg) for 7 days have shown alterations in serum levels of pro-inflammatory cytokines interleukin 1 beta (IL-1β), tumour necrosis factor alpha (TNF-α) and histological changes. In contrast, it has been demonstrated that melatonin and the flavonoid-rich extract *Chromolena odorata* can produce considerable protection for hepatic tissues, although melatonin was not quite effective in protecting the intestinal lesions (Akinrinmade et al., 2016). Low-dose (0.1–0.2 mg/kg AFB1) exposure during 28 days increased the miRNA profile in liver tissue by p53 activation and

led to cell cycle arrest inhibiting cell cycle-related genes (CCND1, CCNE2 and MET) and affecting the micronuclei (MN) formation induced by AFB1 (Liu et al., 2015).

AFB1 induced disturbances in biochemical parameters, changes in the histological and histochemical picture and increased DNA fragmentation in liver. To counteract these toxic effects, it was proved that loaded hydrogel curcumin nanoparticles (Cur-Nps-Hgs) protect against AFB1-induced genotoxicity and histological disturbances (Abdel-Wahhab et al., 2016). The tumour incidence and AFB_1-induced molecular alterations in hepatic cells during carcinogenesis was also decreased by employing fermented milk and chlorophyllin during a 6 week-study (Kumar et al., 2011).

Furthermore, AFB1 can modify the gut microbiota of rats in a dose-dependent manner as shown in RNA-seq analysis which revealed that control samples had a phylogenetically diverse community, and that increasing AFB1 doses decreased this diversity but increased evenness of community composition (Wang et al., 2015).

High concentrations of AFB1 (1.34–0.76 µg/kg) and low levels of T-2 toxin were found in liver and small intestines of male rats after oral administration of respectively 0.5 and 1 mg/kg by using an isotope dilution LC-MS/MS method with a fast sample preparation using homemade clean-up cartridges (Han et al., 2012).

3.1.3. Chicken

Every study employing chicken as experimental model, focused on subacute and subchronic toxicity with exposure periods of 21, 28 and 42 days. qPCR was the most used technique in order to analyse changes in gene expression. Other techniques employed were FCM, IHC, microarray, ELISA and antibody assays to assess the immunotoxicity, hepatic biotransformation and changes in body's parameters. Furthermore, it has been confirmed that supplements with curcumin and bentonite in the diet can ameliorate AFB1 effects.

Selenium treatment played an important protective effect against cell apoptosis induced by AFB1, confirmed by FCM and TUNEL assays. The mRNA expressions of endoplasmic reticulum and death receptor pathway molecules CASP-3, CASP-8 and CASP-10, GRP78, GRP94, TND-α, TNF-R_1, FAS AND FALS of splenocytes were significantly decreased (Fang et al., 2019).

In a 21 days study, AFB1 (1 and 2 mg/kg) increased the total number of Gram-negative bacteria of the intestinal barrier, reduced body weight, body weight gain, feed intake and feed conversion, while at the same time increased relative weights of liver, spleen, and bursa of Fabricius (Galarza-Seeber et al., 2016). In the same experimental conditions, microarray analysis was used to identify candidate genes and analyse its expression, subsequently confirmed by qPCR, showed changes in the expression of genes coding for detoxification, fatty acid metabolism, oxidative phosphorylation, energy production, cell proliferation, immune response, metabolism, growth and development, coagulation, and antioxidant activities related pathways (Yarru et al., 2009).

The ingestion of AFB1-contaminated feed during 42 days caused hepatotoxicity by inducing an increase in biotransformation genes CYP1A1 and CYP2H1 (Sridhar et al., 2014). The addition of curcumin (300 mg/kg) to feed alleviated AFB1 – induced hepatotoxicity and oxidative stress by inhibiting the generation of ROS, 8-OHdG and AFB1 adducts, and activated Nrf2 signalling pathway (Li et al., 2019).

The combined action of AFB1 and OTA significantly reduced immune responses and histological changes in the bursa of fabricius and thymus, antibody responses to SRBC, lympho-proliferative responses to Phytohemagglutinin-P (PHA-P) and phagocytic function. The addition of bentonite significantly ameliorated the immunotoxicity of AFB_1 (Bhatti et al., 2017).

3.1.4. Turkeys

The only study found on turkeys was developed by using RNA-seq and qPCR techniques. Differences in gene expression were observed in wild and domesticated turkeys' liver by using RNA-Seq and qPCR that identified 4621 genes with significant differential expression, associated with cell regulation, modulation of apoptosis and inflammatory responses. As shown by Sridhar et al. (2014) in chicken, but employing lower concentrations (0.320 mg/kg), AFB1 caused the increase of the expression of GSTA3 biotransformation gene which was higher in wild birds and in treated birds for both genetic groups (Reed et al., 2018).

3.1.5. Fish

AFB1 at four different concentrations (15–150 ng/mL) induced neurotoxicity in zebrafish embryos and larvae during 7 days exposure time. Also, the lowest concentrations of AFB1, compared to other animal models, were used. Exposure to the toxin, resulted in an aberrant morphology of trigeminal ganglion and hindbrain neurons in transgenic embryos (huc: egfp). Additionally, AFB1 altered levels of neurotoxic markers, including gfap and huc and qPCR analysis verified that the expression of ngfa and atp1b1b genes was decreased, while treatment increased that of prtga gene (Wu et al., 2019).

3.2. Ochratoxin A

OTA is an organic soluble mycotoxin produced by *Aspergillus* and *Penicillium* fungi. It has been detected in a large number of food and feed like cereals, wine, coffee, beer, cacao, dried fruits, meat and spices (Heussner and Bingle, 2015). A few studies showing an increase in the incidence of hepatocellular tumours in mice and of renal-cell tumours in mice and rats led to the reclassification by IARC in Group 2B (*Possibly carcinogenic to humans*) on the basis of sufficient evidence in experi- mental animals (Ostry et al., 2017). Exposure to OTA causes fetotoxi- city, renal lesions and changes in haematological and morphological kidney parameters (Bondy et al., 2018). Moreover, it has been shown that this metabolite is neurotoxic, nephrotoxic, teratogenic, im- munotoxic and cytotoxic by causing the increase of NADPH and P450 enzyme, activating the caspase signalling pathway and inducing

apoptosis. Also, OTA-induced oxidative stress in mitochondria and en- doplasmic reticulum, inhibited cell cycle, lipid and nucleotide meta- bolism (Tao et al., 2018; Lee et al., 2019).

For OTA, the predominant animal model employed has been the rat, followed by mice, pigs and chicken. According to the main target organ related to OTA's toxicity, which is the kidney, the majority of the stu- dies focused on genotoxicity and nephrotoxicity in sub-acute exposure time, followed by immunotoxcity, hepatotoxicity and oxidative stress (Fig. 3). Various compounds have been used to mitigate its toxicity, such as astragalus polysaccharide, resveratrol, L-carnitine and vitamin E. Results are summarized in Table 2 in order of exposure time.

Table 2. OTA toxicity studies: dose and administration route, exposure time, animal species, sex and age, biological sample, technique, damage and reference.

OTA							
Dose/administration route	**Exposure time**	**Specie**	**Sex/age**	**Biological sample**	**Technique**	**Damage**	**Reference**
0.5, 1, 10 mg/kg + ŐR16 / gavage	72 h, 21 days	Mice	Male/ 7-9 weeks	Blood, spleen, kidney	qPCR, ELISA	Decrease of kidney weight, malformations in renal cortex tissue, ŐR16 degradate OTA in spleen.	Ferenczi et al., 2014
75 µg/kg Intraperitoneally + astragalus polysaccharide (200 mg/kg) / gavage	20 days		NR	Spleen, serum	qPCR, ELISA, WB	Astragalus polysaccharide can attenuate immune stress *in vitro* and *in vivo* induced by OTA *via* the activation of AMPK/SIRT-1 signalling pathway.	Liu et al., 2018
0, 1, 5 mg/kg / gavage administration	4 weeks		Male	Kidney	PCR, GE	Mutagenicity, apoptosis, and karyomegaly.	Hibi et al., 2013
0.5, 1, 2, 10, 15, 40 mg/kg / oral administration	26 weeks		Male/ 5-7 weeks	Kidney	FCM, ELISA	Carcinogenicity and genotoxicity.	Bondy et al., 2015

2 mg/kg + AA (10 mg/kg) / intraperitoneal injection	5 days		Male/ 5 weeks	Liver, kidney	IHC, WB	DNA adduct formation.	Stiborová et al., 2015
0.5 mg/kg / gavage administration)	7, 21 days		Male/ 10 weeks	Urine, blood, kidney, liver	HPLC-FLD, microarray	Changes in gene expression in renal and hepatic tissues.	Arbillaga et al., 2008
0.1, 0.2 mg/kg / oral administration	20 days	Rats	Male	Blood, liver, heart, spleen, lung, kidney and brain.	LC-TOF-MS	Carcinogenicity, hepatotoxicity, teratogenicity and immunosuppressivity.	Han et al., 2013
0.125-0.25 mg/kg + CIT (20 mg/kg) + resveratrol (20 mg/kg) / contaminated diet.	21 days		Male/ 10 weeks	Blood, liver, kidney	CA, IHC, HPLC, enzymatic assays	Induction of oxidative stress; resveratrol restores depleted GSH in tissues.	Rašić et al., 2018
0.7, 2.1, 6.3 mg/kg / gavage	4 weeks		Male/ 5 weeks	Kidney	WB, IHC, CA, qPCR	DNA double-strand breaks and large deletion mutations.	Kuroda et al., 2013a
5 mg/kg / oral administration	4-13 weeks		Male/ 5 weeks	Kidney	LC-EC, PCR	Hepatotoxicity.	Hibi et al., 2011

0.7, 2.10 mg/kg bw / gavage	13 weeks		Male/ 6-7 weeks	Feces, kidney	DNA- seq, RNA-seq	Changes in functional genes of gut microbiota.	Guo et al., 2014
0.21 mg/kg + AA (1 mg/kg) / oral gavage	3,6 months		Male, Female /7-8 weeks	Kidney	IHC, HP, microarray, qPCR	Preneoplastic lesions, carcinogenity.	Stemmer et al., 2009
0.021, 0.070, 0.210 mg/kg / gavage administration	2 years		Male, Female	Kidney epithelial cells and tubule	LC-MS/MS	Uptake into the proximal tubule epithelium, inhibition of histone acetyltransferases, disruption of mitosis, cell proliferation, genetic instability (renal tumour).	Mally, 2012
1.0-2.0 mg /kg + L-carnitnine (1.0 g/kg) +Vitamin E 0.2 g/kg) / gavage	42 days	Chicken	Male/ 1 day	Thymus, bursa de Fabricius	Antibody assays	Immunotoxicity, L-carnitine and Vitamin E suppress OTA immunotoxicity.	Bhatti et al., 2018
0.05 mg/kg / oral administration	30 days	Pigs	Female / 4 weeks	Plasma, kidney	Microarray, qPCR	Renal toxicity correlated with the activation of immune response pathways, oxidative stress response and early carcinogenic events.	Marin et al., 2017

0.4 mg/kg / oral admin feed + PCV infection	42 days	NR	Kidney	qPCR, FCM, LC-MS/MS	PCV2 infection aggravates ochratoxin A-induced nephrotoxicity via autophagy involving p38 signalling pathway.	Gan et al., 2018

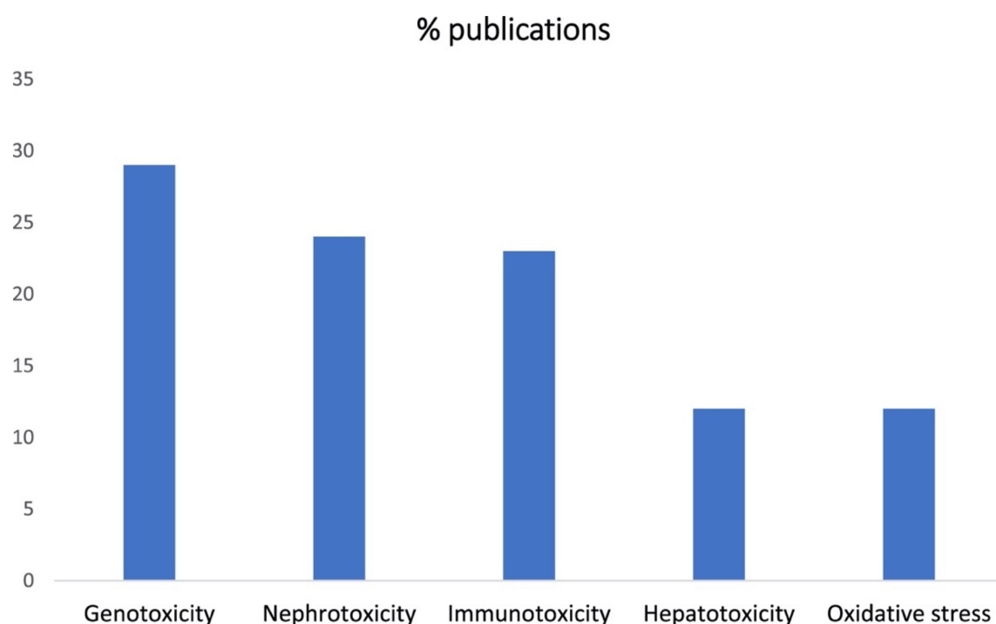

Figura 3. Percentage of the revised publications according to the main toxicological effect of OTA.

3.2.1. Mice

In mice examined studies, exposure time ranged between 72h and 26 weeks with low and high concentrations of OTA (0.1–40 mg/kg). In addition, it has been shown the effect of astragalus polysaccharide to alleviate immune stress induced by OTA and it has been proposed an OTA biodegradation strategy using Cupriavidus basilensis ŐR16 strain. In an acute and subacute study, the administration of OTA in male mice resulted in significant elevation of OTA levels in blood, histopathological alterations and transcriptional changes in OTA-dependent genes (annexinA2, clusterin, sulphotransferase and gadd45 and gadd153) in the renal cortex. On the other hand, no changes were observed

in animals treated with OTA incubated with Cupriavidus basilensis ŐR16 strain. Moreover, HPLC and ELISA methods identified ochratoxin α as the major metabolite of OTA in ŐR16 cultures, which efficiently degrade the toxin without producing toxic adventitious metabolites (Ferenczi et al., 2014). OTA toxicity in spleen was confirmed by induced damages and the promotion of the expression of apoptosis-related proteins and pro-inflammatory cytokines to counteract OTA's effects, it has been demonstrated that astragalus polysaccharide significantly alleviated OTA-induced immune stress and caused significant enhancement of AMPK/SIRT-1 and inhibition of NFB (Liu et al., 2018).

In kidney, the transcriptional regulator p53 contributed to DNA damage reparation by means of arresting cell cycle progression trough activation of the cell-cycle checkpoint and inducing the transcription of DNA damage repair factors (Hibi et al., 2013). Moreover, it has been shown that an OTA dose-dependent increased in hepatocellular necrosis, but no tumorous lesions in p53 heterozygous and homozygous mice. Renal changes have also been observed in OTA treatment, including increase in cellular proliferation, apoptosis, karyomegaly and tubular degeneration in proximal tubules (Bondy et al., 2015).

3.2.2. Rats

Rats were the most used species in subacute, subchronic and chronic studies, ranging from 5 days to 2 years. The main targets analysed were kidney and liver. The positive effect of resveratrol, aristolochic acid (AA), L-carnitine and Vitamin E against OTA has been evaluated by administrating different

concentrations together with the mycotoxin. A wide range of techniques were used, such as HPLC-FLD, LC-TOF-MS, LC-EC, DNA-Seq, RNA-Seq, microarray, CA and the antibody assays.

Exposure to OTA in combination with the nephrotoxin and carcinogenic aristolochic acid, leaded to DNA adducts formation in liver and kidney. Moreover, NQO1 protein expression resulted to be increased in liver, but no increase was found in kidney after the combined treatment. Furthermore, CYP1A1 protein levels were higher in hepatic than in renal microsomes while CYP1A2 was expressed only in liver (Stilborová et al, 2015). Likewise, OTA's toxicity and accumulation were determined in liver and kidney. Protein synthesis, annexin genes, calcium homeostasis and cell proliferation were up-regulated, while oxidative stress, cellular metabolism and transport, were down-regulated. Moreover, HPLC-FLD revealed that OTA accumulation was similar in target and non-target organs (Arbillaga et al., 2008). In a similar study, a combinatorial platform of LC-MC/MS and LC-TOF-MS confirmed the accumulation of high concentration of OTA in lung, liver, heart and kidney. Moreover, three less toxic metabolites than OTA were identified: Ochratoxin ß (OTß) and ochratoxin B (OTB) methyl ester were found in kidney and spleen, respectively, while phenylalanine was detected in heart and kidney (Han et al., 2013).

In a 4 week-study, a comet assay demonstrated that OTA was capable of inducing double-strand breaks (DSBs) at the target sites of the renal outer medulla (ROM) of rats treated with OTA at three different concentrations for 4 weeks. Furthermore, mRNA and/or protein expression levels of homologous

recombination repair-related genes (RAD51, RAD18 and BRIP1) were increased in response to OTA in a dose-dependent manner. WB and IHC staining also demonstrated that OTA increased γ-H2AX expression specifically at the carcinogenic target site and the expression of genes involved in G2/M arrest (CHEK1 and WEE1) and S/G2 phase (CCNA2 and CDK1), suggesting that DSBs induced by OTA were repaired predominantly by HR repair, consequently producing large deletion mutations at the carcinogenic target site (Kuroda et al., 2013a).

In a 13-week assay, exposure to OTA at a carcinogenic dose, reported an induced karyomegaly and apoptosis at the outer stripe of the outer medulla of the kidney, but failed to affect the reporter gene mutations in DNA extracted from whole kidneys (Hibi et al., 2011). A similar duration study revealed changes in functional genes of gut microbiota including signal transduction, carbohydrate transport, transposase, amino acid transport system, and mismatch repair were observed in male rats. Thin-layer chromatography showed that a strain of Lactobacillus could absorb but not degrade OTA, which agreed with the metagenomics result that no genes related to OTA degradation increased (Guo et al., 2014).

Histopathology and cell proliferation analysis demonstrated a compound and sex specific onset of preneoplastic lesions in rats' kidney treated with OTA and AA. In contrast, analysing gene expression profiles by microarray and qPCR of laser-microdissected preneoplastic lesions from carcinogen-treated rats, a reduced expression of genes involved in carcinogen uptake and metabolism was observed (Stemmer et al., 2009).

In the longest study, a mode of action for renal carcinogenity of OTA was developed, that involves a combination of genetic instability and increased proliferative drive as consequences of OTA-mediated disruption of mitosis, whereby the organ and site-specificity of tumour formation was determined by selective renal uptake of OTA into the proximal tubule epithelium (Mally, 2012).

A modified comet assay revealed rats' kidneys and liver oxidative DNA damage in a combined OTA and CIT treatment, which was not reversed by the use of resveratrol. CIT did not reduce GSH or increase MDA concentration in any tissue, while OTA reduced kidneys GSH and increased kidneys and liver MDA. Resveratrol increased GSH concentrations in all tissues and decreased MDA only in the liver (Rašić et al., 2018).

3.2.3. Chicken

The only study with chicken focuses on immunologic response during a subchronic exposure. When chicks were fed with 1 or 2 mg/kg OTA, total antibodies production and phagocytic potential of macrophages was suppressed, due to degenerative changes and depletion of lymphoid cells in the lymphoid organs. Moreover, dietary addition of L-carnitine and vitamin E effectively overcame OTA induced immunosuppression (Bhatti et al., 2018).

3.2.4. Pigs

In pigs' studies, similar doses, exposure time and biological sample were employed. Piglets subchronic exposure to 0.05 mg/kg OTA on immune response and oxidative stress parameters showed effects on gut and kidney even at such low concentration. A microarray analysis conducted on the genome expression in the kidney revealed that approximately 105 different transcripts were significantly altered. The transcripts were validated using qPCR for the genes CAMK1D, IKBKB, CYP1A1, CHD1L, AQP5, FGG (Marin et al., 2017). Nephrotoxicity of OTA was also confirmed by Masching et al., 2016 during a 42 days exposure. Porcine circovirus type 2 (PCV2) infection aggravated the OTA-induced nephrotoxicity, p38 phosphorylation and autophagy by altering Atg5, LC3 II and p62 protein expression, depended on the time and concentration of the treatment.

3.3. Patulin

PAT is a naturally occurring mycotoxin biosynthesized through a polyketide pathway by a variety of toxigenic fungal species belonging to the genera Aspergillus and Penicillium. However, Penicillium expansum remains as the major contributor to PAT production so far identified (Tannous et al., 2018). This mycotoxin has been described in numerous foodstuffs and is common in fruit products, especially in apples and apple-based products, which are more susceptible to infection by P. expansum (Sajid et al., 2019). According to the IARC, PAT is not carcinogenic and belongs to group 3 as "not classifiable as to its carcinogenic to humans" (Saleh and Goktepe, 2019). Nevertheless, several studies have demonstrated its cytotoxicity, immunotoxicity, embryotoxicity, chromosomal aberration, DNA damage, and MN formation (Jayashree et al.,

2017; Vidal et al., 2019). Damage to vital organs and systems including liver, kidney, intestinal tissues and immune system, by increasing ROS production and oxidative stress in mitochondria and activating the unfolded protein response in the endoplasmic reticulum, has also been reported (Ramalingam et al., 2019).

PAT studies focused on cardiotoxicity, immunotoxicity and the induction of oxidative stress in mice and fish in acute and sub-acute toxicity, followed by teratogenicity, nephrotoxicity, embryotoxicity and hepatotoxicity (Fig. 4). Administrated doses ranged from 0.005 to 3.75 mg/kg through intraperitoneal injections or immersion in egg water. Teratogenicity, genotoxicity and carcinogenicity has been demonstrated in liver, heart and embryos. Nevertheless, the antioxidant activity of green tea polyphenols and preventive effect of crocin (CRO) against PAT was confirmed in mice, while the synergic effect of CIT and PAT was proved in fish embryos by using qPCR. All the studies are reported in Table 3, in order of exposure time.

Table 3. PAT toxicity studies: dose and administration route, exposure time, animal species, sex and age, biological sample, technique, damage and reference.

PAT							
Dose/administration route	**Exposure time**	**Specie**	**Sex/age**	**Biologica l sample**	**Technique**	**Damage**	**Reference**
3.75 mg/kg intraperitoneal injections) + CRO (50, 100, 250 mg/kg)	24 hours	Mice	Female	Heart	WB, GE, enzymatic assays	Teratogenicity and carcinogenicity	Boussabbe h et al., 2015
0.005, 0.02 50 mg/kg / intraperitoneal injections)	20 days (every 2 days)		Female	Liver	WB, GE	Induction of apoptosis in tumoral cells	Boussabbe h et al., 2016
1 mg/kg) + green tea polyphenols (25, 50, 100 mg/kg)	PAT: 24 days, green tea polyphenols: 7 days		Male	Blood, liver, bone marrow	DCFH-DA	Oxidative damage (increase of ROS, decrease of GSH level), hepatotoxicity, genotoxicity, micronucleus and chromosomal aberration formation; green tea polyphenols has dose-	Song et al., 2014

						dependent antioxidative and antigenotoxic effect against PAT-induced hepatotoxicity and genotoxicity.	
0.2 mg/kg, CIT (0.015, 0.05 mg/kg) / egg water immersion)	NR	Fish	18h	Embryo	qPCR	Changes in renal histological structures of embryos, impairment of renal function.	Wu et al., 2012

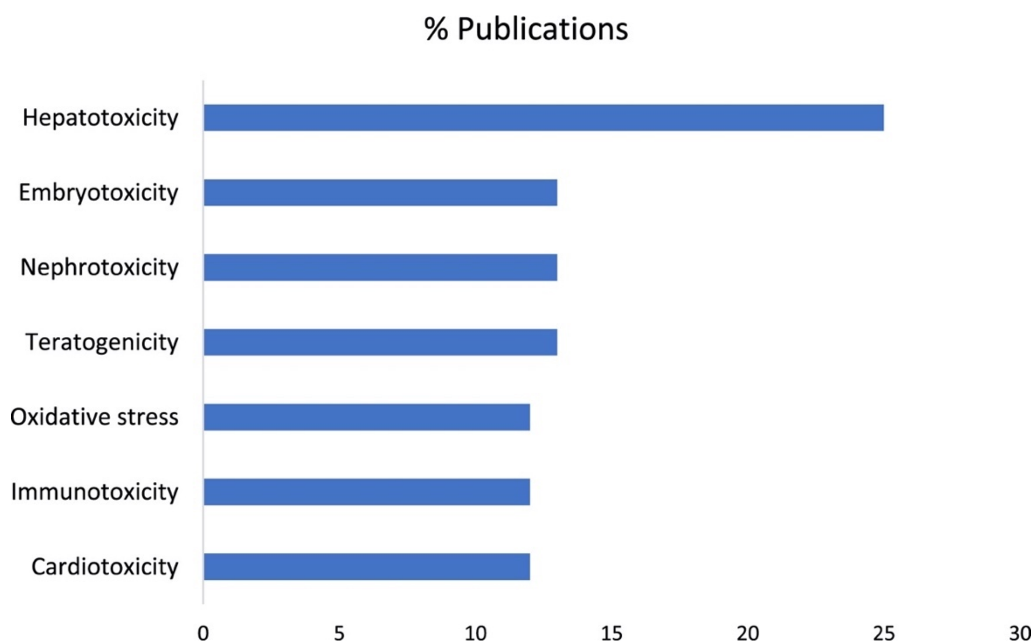

Fig. 4. Percentage of revised publications according to the main toxicological effect of PAT

3.3.1. Mice

Mice studies using PAT, had acute and subchronic duration, by using concentrations from 0.005 to 3.75 mg/kg. Exposure period lasted for 24h, 20 days and 24 days in female and male mice, respectively. The techniques used were GE followed by WB, enzymatic assays and DCFH-DA in carcinogenity and oxidative stress studies.

In the 24h study, PAT increased creatinine phosphokinase (CPK) level, induced lipoperoxidation, protein oxidation and triggered the antioxidant enzyme such as SOD and catalase (CAT) activities. Furthermore, PAT triggered

apoptosis via p53 overexpression and caspase 3 activation, while CRO prevents cardiac impairment by reducing CPK levels and suppressing apoptosis (Boussabbeh et al., 2015).

The administration of PAT for 20 days significantly induced tumour regression in B16F10 cell-implanted mice, evidenced by the activation of apoptosis which is supported by the increase in p53 expression, downregulation of the protein levels of Bcl2, and the increase in caspase-3 activity (Boussabbeh et al., 2016). During 24 day-exposure, hepatotoxicity and genotoxicity of PAT was determined by DCFH-DA assay and Ellman's. Lipid peroxidation was confirmed by the elevation of TBARS, aspartate transaminase (AST) and alanine transaminase (ALT) activities. On the other side, green tea polyphenols administration reported dose-dependent antioxidative and antigenotoxic effect against PAT-induced genotoxicity (Song et al., 2014).

3.3.2. Fish

The only study that used fish as experimental model focused on nephrotoxicity in histological structure and biological function of zebrafish embryos during an acute exposure time, in combination with CIT. qPCR analysis showed CIT induced the expression of proinflammatory genes on zebrafish embryonic kidneys, including COX2, TNF-α and IL-1β, but both CIT and PAT failed to modify the levels and distribution of Wilm tumor gene (wt1a) transcript and Na+/K+-ATPase protein, which plays a primary role in maintaining electrolyte/glucose homeostasis (Wu et al., 2012).

3.4. Citrinin

CIT is a polyketide compound produced by several species of the genera Aspergillus, Penicillium and Monascus. The presence of this toxin is common in grains of the harvested plants, under typical storage conditions and also in foodstuffs like beans, fruit juices, herbs and spices (Doughari, 2015). In vitro and in vivo studies have shown that this toxin has nephrotoxic, hepatotoxic and teratogenic effects and reproductive toxicity. Also, CIT provokes a clear cytotoxic effect, manifested in cells' elongation, flattening and swelling. Moreover, it causes alterations in the mitochondria by increasing ROS production and apoptosis (De Oliveira Filho et al., 2017). Despite its toxicity, it has been reported that components such as calcium chelator BAPTA-AM could prevent or reduce apoptosis, necrosis, and genotoxicity of CIT (Klarić et al., 2012).

CIT toxicological effect was investigated in rats and alpacas in sub-acute studies. The doses were administrated by gavage for rats (20,40 mg/kg) and in contaminated diet for alpacas (0.005, 0.1, 0.5 mg/kg). The main toxicological effects reported in the bibliography were equally nephrotoxicity, immunotoxicity and cytotoxicity (Fig. 5). Its toxicity was confirmed by qPCR, WB, IHC, ELISA and HPLC techniques. Results are shown in Table 4, in order of exposure time.

Table 4. CIT toxicity studies: dose and administration route, exposure time, animal species, sex and age, biological sample, technique, damage and reference.

CIT							
Dose/ administration route	**Duration**	**Specie**	**Sex/age**	**Biological sample**	**Technique**	**Damage**	**Reference**
20, 40 mg/kg / oral gavage	2 days	Rats	Male	Kidney	qPCR, WB, IHC, HPLC, CA	Renal carcinogenesis, induced cell cycle progression.	Kuroda et al., 2013b
0.005, 0.1, 0.5 mg/kg/ contaminated diet	15 days	Alpacas	NR	Blood	qPCR, ELISA	Immunotoxicity.	Xu et al., 2015

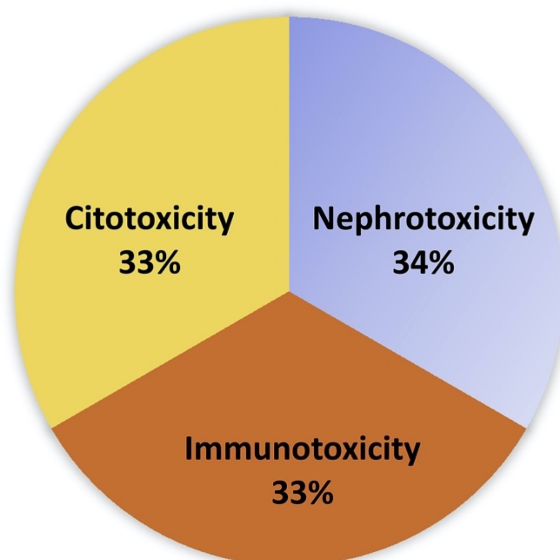

Fig. 5. Pie chart of revised scientific articles according to the main toxicological effect of CIT.

3.4.1. Rats

In rats, a two day-study was conducted by using a high-dose of CIT (20–40 mg/kg) to confirm its renal carcinogenicity. Increases in the mRNA expression level of Ccna2, CCnb1, Ccne1 and its transcription factor E2F1 were detected, suggesting induction of cell cycle progression at all tested doses. Histopathological changes were found only in rats treated with the higher dose, which was consistent with increases in the mRNA expression levels of mitogenic factors associated with tissue damage/regeneration, such as Hgf and Lcn2, at the same dose. Moreover, WB analysis showed that ERK phosphorylation was

increased at all doses, implying that cell cycle progression may be mediated by activation of the ERK pathway (Kuroda et al., 2013b).

3.4.2. Alpacas

The study of CIT toxicity using alpacas was subchronic and tested three different concentrations. A simple straightforward strategy was developed to isolate an anti-CIT monoclonal antibody in a β type AI alpaca heavy chain single domain antibody (β-AI VHH) from a naïve phage display VHH library. The obtained β-AI VHH was used as a competing antigen in V-ELISA to detect CIT (Xu et al., 2015).

3.5. Fumonisins

Fumonisins are mycotoxins whose structure resembles that of the sphingoid bases, sphinganine (Sa) and sphingosine (So). They are mainly produced by Fusarium verticilloides and Fusarium proliferatum, species commonly found on maize (Bryla et al., 2016). In mammals, they can cause hepatotoxicity, nephrotoxicity or cytotoxicity. Fumonisins having a primary amino group competitively inhibit de novo ceramide biosynthesis by binding to ceramide synthase and interfering with CoA-dependent acylation of Sa (Smith, 2018). Among the numerous fumonisin analogues, FB1 is the most abundant and has been classified as possibly carcinogenic to humans in Group 2B (Zain, 2011).

Single and combined studies of FBs in mice, chicken, pigs and turkeys were analysed. Doses ranged from 0.1 to 150 mg/kg with exposure time from 72h to 26 weeks. A wide variety of biological samples were analysed, such as femora bone marrow, blood, serum, liver, kidney, spleen, blood and jejunum. The main toxicological effects reported were hepatotoxicity, oxidative stress, immunotoxicity and cytotoxicity (Fig. 6). Different techniques like microscopy, LC-MS/MS, HPLC-MS/MS, FCM, PCR, MLR and ELISA were used. Nonetheless, it has been demonstrated that compounds and microrganisms such as phytic acid and Lactobacillus plantarum ameliorate Fumonisins-induced toxicity (Gerez et al., 2019; Deepthi et al., 2017). Results are summarized in Table 5, in order of exposure time.

Table 5. FBs toxicity studies: dose and administration route, exposure time, animal species, sex and age, biological sample, technique, damage and reference.

FBs							
Dose/administration route	**Exposure time**	**Specie**	**Sex/age**	**Biological sample**	**Technique**	**Damage**	**Reference**
0.1, 1.0,10 mg/kg / Intraperitoneal injections	72 h (every 24h)	Mice	Male/Female	Femora bone marrow	Microscopy	Cytotoxicity, reduced PCE/NCE ratio.	Karuna et al., 2013
5, 50, 150 mg/kg / oral administration	26 weeks		Male/Female	Liver, Kidney, Spleen	LC-MS/MS	Genotoxicity and carcinogenicity.	Bondy et al., 2012
18,6 mg/ kg / oral administration	17 days	Chicken	Male	Kidney, proventriculus, ventriculus, bursa of Fabricius, heart, lungs	qPCR	Alteration of intestinal microbial homeostasis.	Antonissen et al., 2015a
100 mg/kg / oral administration	21 days		Male	Blood, liver	Enzymatic assays	Liver oxidative stress concomitantly with SA/SO accumulation.	Poersch et al., 2014
1 mg/kg / gavage	10 days	Pigs	Male	Jejunum	FCM, PCR, MLR, APC	Immunotoxicity.	Devriendt et al., 2009

10 mg/kg /oral administration	4 weeks		Male	Jejunum, liver	qPCR, microarray, ELISA	Decrease in ceramide levels, disturbance of PI3-AKT pathway (inhibition of the integrin-mediated cell-matrix adhesion).	Régnier et al., 2017
FB1+FB2 (15 mg/kg), FB1+FB2 (2 mg/kg)/ oral administration	14 days	Turkeys , Pigs	Male/Female	Serum	HPLC-MS/MS	Disruption of sphingolipid metabolism.	Maschin g et al., 2016

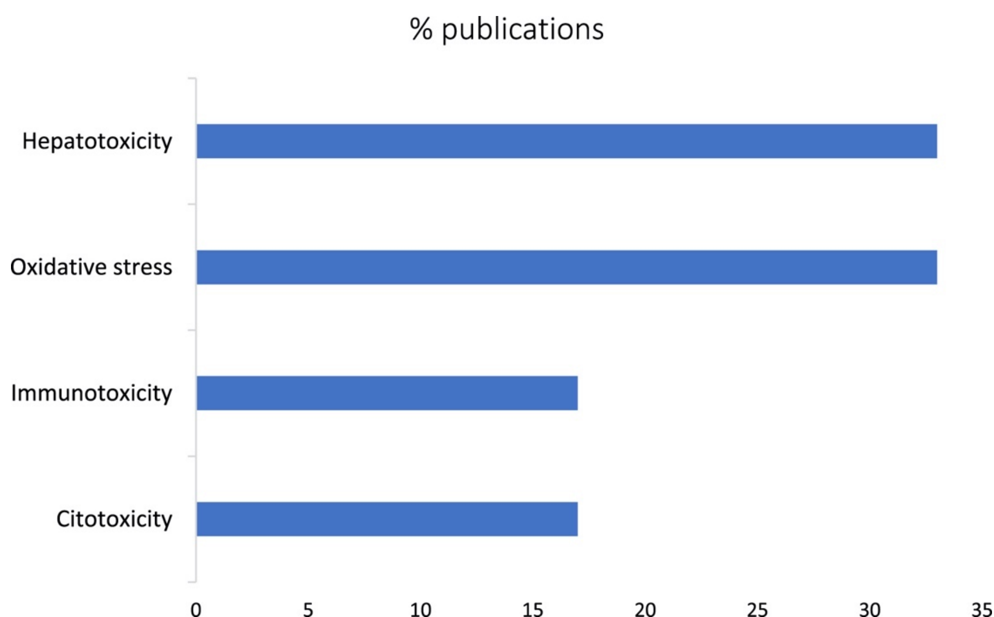

Fig. 6. Percentage of publications according to the main toxicological effect of Fumonisins.

3.5.1. Mice

Mice studies had both acute and subchronic duration, using low concentrations of mycotoxin in the acute one (0,1–10 mg/kg) and the highest in the subchronic one (150 mg/kg). During the 72h exposure, the genotoxic potential of FB1 was assessed by induction of MN formation in bone marrow polychromatic erythrocytes. Femora's slides were examined microscopically by analysing the ratio of polychromatic erythrocytes and normochromatic erythrocytes (PCE/NCE). The reduced ratio PCE/NCE suggested the cytotoxic but non-genotoxic nature of FB1 (Karuna and Rao, 2013). Similar responses were obtained in p53+/− heterozygous and p53+/+ homozygous

mice, in which FB1 resulted hepatocarcinogenic but non-genotoxic in a subchronic exposure. Hepatic adenomas and cholangiomas were observed in both transgenic mice exposed to 150 mg/kg (Bondy et al., 2012).

3.5.2. Chicken

Similar duration of exposure to FBs was employed in studies using chicken as experimental model with concentrations ranging from 18.6 to 100 mg/kg. A concentration of 20 mg/kg of FBs affected the intestinal microbial homeostasis and the gut microbiota changes stimulating C. perfringens colonization and predisposing the model to necrotic enteritis. PCR analysis was used to generate DNA fragments from broiler chicks' small intestine, previously separated by denaturing gradient gel electrophoresis. qPCR was employed for the quantification of total C. perfringens in ileal contents and revealed an increased level in FBs contaminated diet fed chickens (Antonissen et al., 2015). In the other subacute treatment, FB1 had a significant negative effect on oxidative stress parameters SOD, CAT and glutathione-S- transferase (GST) in the liver of broiler chicks concomitantly with Sa/So ratio accumulation in blood (Poersch et al., 2014).

3.5.3. Pigs

Transcriptomics and enzymatic studies have been analysed in pigs. Doses ranged between 1 and 10 mg/kg for FB1, while from 2 to 15 mg/kg in a combined FB1 and FB2 assay by employing both pigs and turkeys. FCM, PCR,

qPCR, MLR, APC, microarray, ELISA and HPLC-MS/MS were the main techniques used across the reported assays.

FB1 mediated reduction of intestinal antigen presenting cells (APC) maturation. A reduced intestinal expression of IL-12p40 and impaired function of APC, with decreased upregulation of Major Histocompatibility Complex Class II molecule (MHC-II) and reduced T cell stimulatory capacity upon stimulation were observed. Treated animals showed a longer shedding of F4+ enterotoxigenic Escherichia coli (ETEC) following infection and a lower induction of the antigen-specific immune response following oral immunization (Devriendt et al., 2009).

Transcriptome and kinome of liver and jejunum, two organs targeted by FB1, were analysed by qPCR analysis, finding that the most significantly altered genes were in jejunum and related to cell structure, cell energy, immunity, metallothioneins, calcium homeostasis and cyclin modulation, while in liver only a number of genes related to cell structure, cellular energy, defence response, cation homeostasis and metallothioneins were identified (Régnier et al., 2017).

Dietary FBs concentrations below the recommended guideline levels caused disruption of the sphingolipid metabolism in turkeys and pigs in a 14 days-study, as reflected by significantly increased Sa/So ratios. Feed supplementation with the commercial application of FumD prevented alterations of the Sa/So ratio in both species (Masching et al., 2016).

3.6. Deoxynivalenol

DON belongs to trichothecenes group which are small sesquiterpenoids produced by fungi of Fusarium and Stachybotrys species, all having in common epoxide groups at position 12–13 critical for their toxicity. It is also known as vomitoxin, due to his strong emetic effects after consumption and occurs in several food and feed, especially in cereals (EFSA, 2017). The toxicity of DON in humans and animals has been widely demonstrated in numerous studies. But, however, is classified by IARC as Group 3 (Not classifiable relating to its carcinogenicity to humans). Exposure can cause reduced growth in farm animals, with pigs being one of the most sensitive species. Moreover, it can affect the intestinal barrier function and cause reproductive disorders, but its major damage is related to immune alterations (Pinton and Oswald, 2014; Maresca, 2013; EFSA, 2017). It has been shown that it is cytotoxic at molecular level, interfering with ribosomes by activating mitogen-activated kinases (MAPKs), and inducing trans-activation of a number of pro-inflammatory cytokines, increasing the stability of their mRNA (Pierron et al., 2016).

As shown in Table 6, the main species studied for DON toxicity was pig, followed by mice, chicken and fish. Not very high doses were used (from 0.002 to 10 mg/kg) and in the majority of the studies the administration route was oral by gavage or in contaminated diet. The main target organ was mostly the intestine (stomach, duodenum, jejunum, ileum, cecum), but also spleen, thymus, liver, blood and lung. The main toxicological effects reported in the bibliography were immunotoxicity, oxidative stress, morphometric changes, cytotoxicity, hepatotoxicity, genotoxicity, embryotoxicity and neurotoxicity (Fig. 7). qPCR, ELISA, and microscopy were the principal techniques employed.

Table 6. DON toxicity studies: dose and administration route, exposure time, animal species, sex and age, biological sample, technique, damage and reference.

DON							
Dose/ administration route	**Exposure time**	**Specie**	**Sex/age**	**Biological sample**	**Technique**	**Damage**	**Reference**
2.5 mg/kg / oral gavage	2,6h	Mice	Male	Spleen	qPCR	mRNA upregulation of cytokines associated with acute trichothecene toxicity.	Wu et al., 2014
5, 10, 25 mg/kg / oral gavage	3, 6, 24h		Male	Thymus	qPCR	Induction of T-cell activation response, rapidly followed by apoptosis and depletion of thymocytes.	van Kol et al., 2011
5.0, 2.5, 1.0 mg/kg / oral gavage	18 days		Female	Placenta	Enzymatic assays, qPCR, IHC	Embryotoxicity caused by DON-induced oxidative stress mediated by Mrf2/HO-1 pathway.	Yu et al., 2017
0.002,0.2 mg/kg / contaminated diet	51 days		Male/Fema le	Serum, liver, thymus, spleen, kidney, stomach, duodenum	HPLC, HP, microscopy	Decrease of body weight, blood indices, ratios of liver	Wang et al., 2017
0.27, 1.68, 12.21 mg/kg /oral gavage	36 days	Rats	Male	Brain	ELISA, HPLC-FD, qPCR	Alteration of neurotransmitter levels, lipid peroxidation, changes of calcium homeostasis.	Wang et al., 2018

7.5±2.20 mg/kg / oral administration + illite-ambrosite clay 1.5 kg/ton feed	3 weeks		Male	Liver, intestine (duodenum, jejunum, ileum)	qPCR	Increase of intestinal barrier permeability after chronic exposure and modification of gut wall morphology in duodenum and jejunum; Absorbing agent: lead to higher concentrations of DON in the more distal parts of the small intestine.	Osselaere et al., 2013
10 mg/kg / oral administration	5 weeks		Male	Plasma, heart, kidney, duodenum and jejunum	CA, enzymatic assays	Cytotoxicity, genotoxicity.	Awad et al., 2014
2.5,5,10 mg/kg / oral administration	5 weeks		Male	Cecum	PCR, MiSeq	Altered cecal bacterial diversity and composition	Lucke et al., 2018
4.6 mg /kg + FBs (25.4 mg/kg) / oral administration	15 days		Male	Intestine, duodenum	qPCR, LC-MS/MS	Changes in the intestinal mucus layer and intestinal epithelial antioxidative mechanisms.	Antonissen et al., 2015b
0.05 mg/kg + activated carbon (0.001 mg/Kg) / gavage	12h	Pigs	Male	Blood	LC-MS/MS	Plasma toxicity; activated carbon prevent the absorption of DON	Devreese et al., 2014

1, 3 mg/kg + chito-oligosaccharides (6.48 mg/mL) / intravenous injections)	3 days	Male	Jejunum	Microscopy	Induction of a significant decrease in the histological score and a significant decrease in morphometric parameters.	Gerez et al., 2018
2.5, 3.5 mg/kg / oral administration + PRRSV vaccination	7 days	Male	Blood	qPCR, ELISA	DON decrease PRRSV viremia, inhibit vaccination efficiency of PRRSV by severely impairing in viral replication.	Savard et al., 2015
2.5, 3.5 mg/kg + PRRSV / contaminated diet	21 days	Male	Blood, lung	ELISA, qPCR, microscopy	Lung lesions, mortality, Increase of PRRSV replication.	Savard et al., 2014
0.5 mg/kg-1mg/kg / gavage administration	21,14 days	Male	Blood, small intestine, intestinal tissues (jejunum, ileum with and without Peyer's patches), fragments of	ELISA, qPCR	Impairments of the morphology of jejunum and ileum, reduction of villi height, decreasing of E-cadherin expression and modification of the intestinal expression of cytokines, hepatotoxicity.	Pierron et al., 2018

			lung, liver, lymph nodes, spleen			
10 µM + *S. cerevisiae* / oral administration	22 days	Male	Duodenum, jejunum	Microscopy, qPCR	Increase of s-IgA, number of goblet cells and growth parameters.	Garcia et al., 2019
4.59 mg/kg / oral administration	29 days	Male	Blood	AHA	Decrease of glucose uptake and glucose transporter activity.	Bannert et al., 2015
2.3 mg/kg / oral administration	4 weeks	Male	Jejunum	WB	Intestinal lesions and activation of MAPK.	Lucioli et al., 2013
DON, ZEN, T-2/HT-2 0.56 mg/kg) + Boron-fructose additive (1650 mg/kg) / oral administration	24 days	Male	Blood, liver	ELISA, FCM	Alteration of growth rate, immune functions (increase of plasma IgG and IgM concentrations, PBMC, respiratory burst of porcine granulocytes, % of peripheral T); CFrB reduce cellular immune responses but not counteract the effect of mycotoxins on biochemistry parameters.	Taranu et al., 2011
DON + ZEA (10 mg/kg) + Hydrogen-rich water + lactulose	25 day	Female	Serum, liver	ELISA	Oxidative stress (increased serum peptide and cholecystokinin levels); HRW and LAC lead attenuated mycotoxin-induced oxidative stress, protect against growth reduction and oxidative damage.	Zheng et al., 2018

(500 mg/kg) / oral 3.50mg /kg , DON+NIV(2.89-0.72 mg/kg) / feed	28 days			Blood, jejunum	IHC	Disruption of intestinal balance proliferation/apoptosis	Cheat et al., 2016
2 mg/kg / oral administration	23, 32 days	Fish	NR	Blood, liver, caudal kidney, gill	qPCR	Oxidative stress (altered enzymatic activity).	Šišperová et al., 2015
0.352, 0.619, 0.954 mg/kg / oral administration	6 weeks		NR	Blood	ELISA	Increase of superoxide dismutase and catalase activities, cytotoxicity on immune cells.	Pietsch et al., 2014

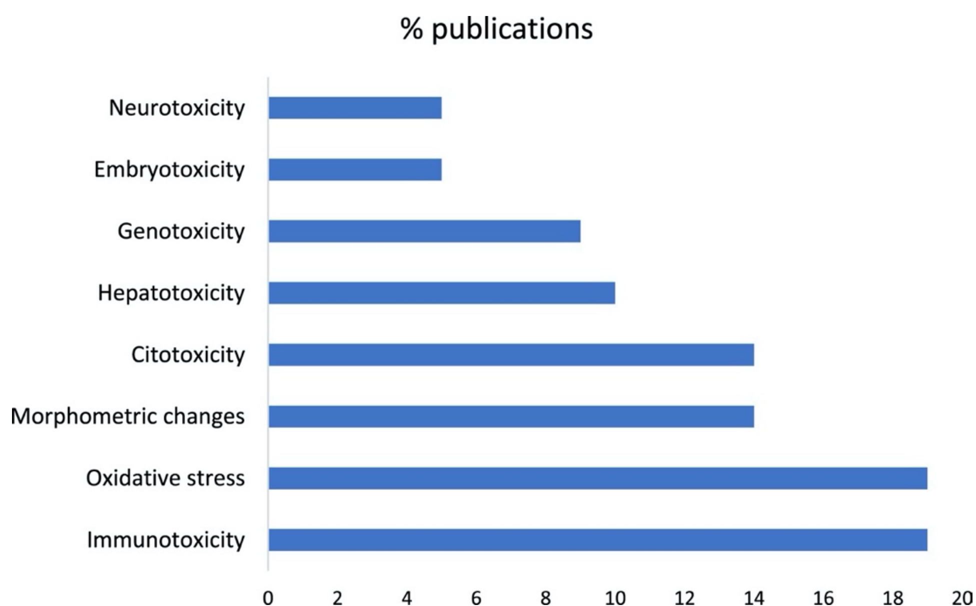

Fig. 7. Percentage of revised publications according to the main toxicological effect of DON.

3.6.1. Mice

Acute and subchronic DON exposure was examined by employing doses from 0.002 to 2.5 mg/kg. Various biological samples were analysed, such as spleen, thymus, placenta, liver, kidney and intestine to investigate immunotoxicity, oxidative stress and alterations in body parameters. The main techniques used were qPCR, IHC, enzymatic assays, HPLC, HP and microscopy.

The acute effects of oral DON exposure on splenic expression of representative cytokine mRNAs induced upregulation of proinflammatory

genes TNF-α IL-1β, IL-6, CXCL-2, CCL-2 and CCL-7 expression associated with acute and chronic trichothecene toxicity (Wu et al., 2014). In another immunotoxicity acute study, a T cell activation response after exposure to 5, 10 and 25 mg/kg DON was observed, rapidly followed by apoptosis and depletion of thymocytes. More especially, this effect is reversible at lower exposure levels (5 and 10 mg/kg), while it is irreversible at 24h after exposure to 25 mg/kg (van Kol et al., 2011).

In a subchronic toxicity study, mice fed on DON-contaminated wheats (DCWs) had a significantly decreased in pro-inflammatory cytokines and blood indices, while showing an increase in alanine aminotransferase, aspartate aminotransferase, blood creatinine and blood urea nitrogen levels. Moreover, it has been shown that ozone mitigates the adverse effects of DON (Wang et al., 2017).

DON treatment in mice during pregnancy led to ROS accumulation in the placenta, which resulted in embryotoxicity, blocked by up-regulation of Nrf2/HO-1 pathway to protect placenta cells from oxidative damage (Yu et al., 2018).

3.6.2. Chicken

Testing the subchronic toxicity of DON was the main goal on these models, and time exposure varied from 36 days to 15 days, alone or in combination with FBs. The main biological samples used were brain, liver,

intestine, heart, kidney, blood and plasma with medium-high doses of DON (0.27–12.21 mg/kg).

In a 36 days DON exposure study at three different concentrations, the cerebral lipid peroxidation, neurotransmitters secretion and the balance of calcium homeostasis were affected. Moreover, the activities of SOD, glutathione peroxidase, CAT activities and nitric oxide contents were significantly decreased (Wang et al., 2018).

The toxicity of DON on the gut wall morphology, intestinal barrier function and inflammation has been studied in broiler chicks three weeks fed with contaminated diet DON 10 mg/kg. qPCR analysis revealed that DON acts in a specific way on the intestinal barrier, since only an up-regulation in the expression of claudin 5 in jejunum was observed. Addition of a mycotoxin adsorbing agent illite-ambrosite clay resulted detrimental in more distal parts of the small intestine (Osselaere et al., 2013). In a more recent study on the intestinal barrier, it has been shown that DON affect the expression of CLDN1, TRL2 and IL6 in duodenal mucosa and of CLDN1, TRL2 and TGFB1 in the jejunal mucosa. Moreover, tended to increase duodenal TJP1 and MUC1 expression (Lucke et al., 2018). Furthermore, it has been demonstrated that a dietary exposure of DON during 5 weeks is cytotoxic and genotoxic to the chicken intestinal and immune cells by causing the increase of DNA damage in blood lymphocytes, might be induced by lipid peroxidation (Awad et al., 2014).

The toxicity of DON on intestinal morphology and microbial homeostasis was evaluated also in combination with FBs at concentrations close

to the UE maximum guidance (5 mg DON and 20 mg FB1+FB2/Kg) levels during 15 days. Both mycotoxins affected the duodenal mucus layer by suppressing the intestinal mucin (MUC) 2 gene expression and altering the mucin monosaccharide composition. Furthermore, they decreased gene expression of the intestinal zinc transporter (ZnT)-1, and regulated intracellular methionine homeostasis.

3.6.3. Pigs

In vivo studies using pigs had acute and subacute duration, from 12h to 29 days. Single DON doses (from 0.05 to 4.59) or in combination with other mycotoxins were given to the experimental animals. Furthermore, the addition of natural compounds like activated carbon, chito-oligosaccharides, boron-fructose additive, hydrogen rich water, lactulose and biological compounds like S. cerevisiae and PRRSV has been demonstrated to be effective in counteract the toxicity of DON. ELISA, qPCR and microscopy were the main techniques employed.

When pigs received a single oral bolus of DON, significant plasma amounts of the mycotoxin were detected by LC-MS/MS and kinetic parameters were calculated using a one compartmental model proving that activated carbon completely prevented the absorption of DON, no plasma amount was detected after its administration (Devreese et al., 2014).

The toxicity of DON and the effect of low doses of chito-oligosaccharides to reduce its induced intestinal histological changes was

evaluated. Microscopic analysis showed that DON induced a significant decrease in the histological score, as a consequence of moderate to severe histological changes and a significant decrease in morphometric parameters. Low levels of chito-oligosaccharides did not counteract DON-induced intestinal lesions (Gerez et al., 2018).

An immunoenzymatic ELISA assay was conducted to assess the impact of DON on the immune response generated following vaccination with PRRSV attenuated vaccine of pigs exposed to 0, 2.5 and 3.5 DON/Kg. DON naturally contaminated feed, significantly decreases porcine reproductive and respiratory syndrome virus (Savard et al., 2015). DON contamination significantly increased viremia and lung viral load in pigs inoculated with PCV2, but had no clinical manifestation of PCV2 associated disease in PCV2b infected animals (Savard et al., 2014). The immune response of pigs against toxicity of DON and its diepoxy-metabolite (DOM-1), was analysed in intestinal tissues (jejunum and ileum) by ELISA and qPCR. DOM-1 was not toxic in the animals but retained some immune-modulatory properties of DON, in particular its ability to stimulate a specific antibody response during a vaccination protocol (Pierron et al., 2018).

Oral administration of S. cerevisiae in pigs against DON pro-inflammatory effects increased s-IgA, the number of goblet cells in small intestine and growth parameters. Also, changes in expression of mRNA encoding for cytokines CCL20, interleukin (IL)-1β, IL-8 and IL-22 were determined by qPCR in small intestine of jejunal explants (Garcia et al., 2019).

Chronic oral DON exposure modulated Escherichia coli lipopolysaccharide (LPS)- induced systemic inflammation in pigs. To demonstrate it, an AHA (Celltac alpha MEK-6450) and an automated blood gas and electrolyte analyzer (GEM Premier 4000) were used for a red hemogram assessment, blood gases, electrolytes, pH, glucose and lactate (Bannert et al., 2015). Low doses of DON induced histological changes in the intestine score and activated the MAPK ERK ½, p38 and JNK in piglets' jejunum. These effects impaired the homeostasis of intestinal tissue in the aspects of barrier function and immune protection (Lucioli et al., 2013).

Changes in concentration of Ig subsets and the percentage of T-lymphocyte were determined by ELISA and FCM in cross-bred piglets exposed to a mix of DON, ZEA, T-2 and HT-2. Boron-fructose additive reduced the exacerbated cellular immune responses induced by Fusarium toxins, but its consumption did not counteract the effect of mycotoxins on biochemistry parameters and increased plasma IgM and IgG (Taranu et al., 2011). Zheng et al. (2018) reported that hydrogen-rich water and lactulose, protected against the adverse effects of Fusarium mycotoxins in female piglets by mitigating growth reduction and oxidative damaged caused by DON and ZEA.

DON an NIV, individually and combined, caused the decrease of total cell proliferation at the villus tips in intestinal pig mucosa. Apoptosis assessments have also shown that in loops model, apoptotic enterocytes at villus tips increased dose-dependently after DON, NIVtreatment, alone or associated (Cheat et al., 2015).

3.6.4. Fish

The two studies described here focused on oxidative stress response in carps and trout during a subchronic exposure. Doses ranged from 0.352 to 2 mg/kg. Investigation of the altered activity of oxidative stress parameters was developed in blood, liver, kidney and gill.

Immunosuppressive effects of DON at three different concentrations were demonstrated by an increase of SOD and CAT activity on carps' immune cells (Pietsch et al., 2014). Oxidative stress markers and detoxifying enzymes expressions were determined also by using a higher dose of DON (2 mg/kg) during 6 weeks in trout. Significant differences were observed for CAT, GR, GPx and GST activities, while no significant differences were found in SOD gene expression, lipid peroxidation or ferric reducing ability of plasma (FRAP) (Šišperová et al., 2015).

3.7. Zearalenone

ZEA mycotoxin can be conjugated with polar molecules by plants and fungi. The main producers of ZEA belong to fungi species of Fusarium genera, such as F. graminearum, F. culmorum, F. crookwellense, F. semitectum and F. equiseti (Rai et al., 2019). Its occurrence has widely been studied in foods, feeds and environmental samples and according to present data it has been proved to be hepatotoxic, hematotoxic, cytotoxic, genotoxic and can cause reproductive and developmental toxicity in vivo (Gao et al., 2017). ZEA also possesses an estrogenic effect, which manifests itself clinically in hyperestrogenism, by

transactivating estrogen receptors (ERs) and promoting the expression of estradiol (E2)- responsive genes (Lecomte et al., 2019). However, carcinogenicity of ZEA is still questionable as it has been listed by IARC as Group 3 (Rai et al., 2019). As well as in DON, in ZEA the most studied specie was pig, followed by mice, rats and fish. The administration doses ranged from 5×10^{-4} to 40 mg/kg and in most cases the administration route was oral, occasionally in gelatine capsules. Immunotoxicity, reproductive damage, oxidative stress, genotoxicity, teratogenicity, neurotoxicity, hepatotoxicity and cardiotoxicity were the main purposes reported in the bibliography analysed (Fig. 8). The most used technique was qPCR, followed by WB, microscopy and ELISA. Moreover, it has been shown that certain compounds such as crocin and Vitamin C mitigate the toxicity of ZEA. Results are summarized in Table 7, in order of exposure time.

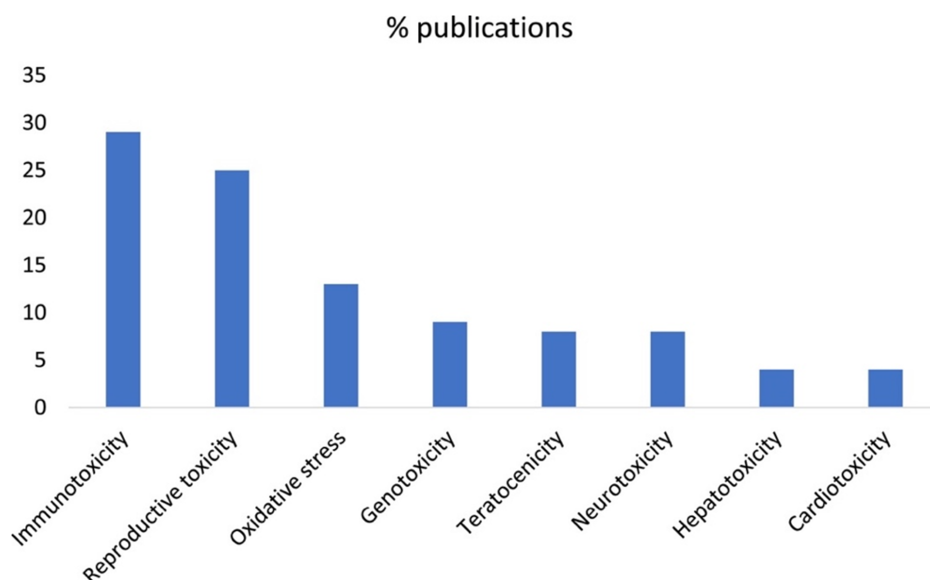

Fig. 8. Percentage of revised publications according to the main toxicological effect of ZEA.

Table 7. ZEA toxicity studies: dose and administration route, exposure time, animal species, sex and age, biological sample, technique, damage and reference.

ZEA							
Dose/ administration route	**Exposure time**	**Specie**	**Sex/age**	**Biological sample**	**Technique**	**Damage**	**Reference**
40 mg/kg / intraperitoneal injections + CRO (100-250 mg/kg single dose or mycotoxin addiction)	24h	Mice	Male	Heart	Enzymatic assays, WB	Oxidative stress, apoptosis in cardiac tissue; CRO: significant reduction of ZEA-induced toxicity for all tested markers in a dose dependent manner.	Salem et al., 2016
4.5 mg/kg daily gavage	1 week		NR	Colon	semiqPCR, WB, ELISA	Toxic effect on the epithelial barrier and accumulation of ROS. Induction of caspase-1 activation via the NLRP3 inflammasome complex, cleaving pro-IL-1β and pro-IL-18 into their biologically active forms, which initiates an intestinal inflammatory cascade.	Fan et al., 2018
40 mg/kg / intraperitoneal injections	10 days		Male	Blood, serum, testes	qPCR, WB	Testis oxidative damage, downregulation of gene	Long et al., 2017

						expressions and protein of Bcl-2, upregulation of gene expression and protein of Bax and caspase-3, upregulation of GRP78, CHOP and XBP-1.	
40 mg/kg + LAB (2 mg/kg) / gavage	15 days		Male	Blood, spleen, thymus	FCM, AHA	Toxicity in immunologic and hematologic parameters; LAB reduce immunotoxic effects.	Abbès et al., 2013
0.15, 0.150 mg/kg / oral administration	70 days		Male	Testes, epididymis, seminal vesicles, prostate	qPCR	Changes in the expression of genes important for spermatogenesis, impairment of sperm quality (morphology and increase of apoptotic spermatozoa).	Zatecka et al., 2014
25 mg/kg b.w./ intraperitoneal administration	3 days	Rats	Male	Liver	qPCR	Changes in mRNA expression of the proteins involved in detoxification and biotransformation of xenobiotics.	Duca et al., 2012
1 mg/kg / subcutaneous injections	Gestation day 7-20 (every 24h)		Female pregnant	Maternal organs (duodenum, placenta, mammary gland,	HPLC, qPCR, WB	Modulation of mRNA levels of ABC transporters in fetal liver and maternal tissues, modulation of	Koraichi et al., 2012

				ovary, uterus, liver), fetal liver (0.2-4g)		protein levels in maternal uterus and fetal liver.	
0, 5, 10 and 20 mg/kg	21 days		Male	Blood, brain, testis	qPCR, ELISA, WB, HPLC-fluo	Disruption of the system regulating the reproductive hormones and testis development through hormone related genes, which may result in a reproductive dysfunction in adult male offspring.	Gao et al., 2018
1.0 mg/kg / oral-intravenous administration	48h		Male	Serum	LC-EC/MS/MS	Changes in ZEA metabolism.	Fleck et al., 2017
0.1 mg/kg / oral administration in gelatin capsules	14, 28, 42 days	Pigs	Male	Peyer's patch (ileum), liver	ELISA	Disruption of the immune response by influencing the secretion of cytokines from Th1 and Th2 cells in GALT.	Obremski, 2014
0.25 mg/kg / oral administration	18 days		Male	Liver	ELISA, qPCR	Hepatoimmunotoxicity, disruption of endocrine oestrogens.	Pistol et al., 2014
1.0 mg/kg + vit C oral administration	28 days		Male	Serum, kidneys, spleen, thymus, pancreas,	ELISA, IHC	Induction of reproductive toxicity, increasing of reproductive organs' index and the length, width and	Su et al., 2018

				reproductive organs		area of the vulva, disrupting serum hormone levels.	
0.10 mg/kg / gelatin capsules	42 days		Male	Ileum	Microscopy, IHC	Changes in immunoreactivity of nervous structures in the ileal wall.	Gonkowski et al., 2015
0.04 mg/kg, DON (0.012 mg/kg), ZEA+DON (0.04 + 0.012 mg/kg) , oral administration	7, 14, 21, 28, 35, 42 days.		Male	Liver, colon	qPCR	Disorder of pathways involved in cell proliferation and survival.	Brzuzan et al., 2015
0.6 mg/kg + T-2 (0.12 mg/kg) / gelatine capsules	42 days		Female, 8 weeks old	Colon	Microscopy, IHC	Changes in neurochemical profile of enteric neurons.	Makowska et al., 2017
0.40 mg/kg + DON (0.12 mg/kg) / contaminated diet	6 weeks		Male	Jejunum	Microscopy	Immunotoxicity.	Przybylska-Gornowicz et al., 2015
10 mg/kg / intraperitoneal injections	24, 72, 168h	Fish	Female	Liver, ovarios	qPCR	Disturbance of blood coagulation, iron storage and different cellular components (cytoskeleton).	Woźny et al., 2012

0.0005, 0.001, 0.005, 0.010 mg/L / oral administration	21 days	NR	Gonads, liver, brain	qPCR, microscopy	Alteration of reproductive functions.	Muthulakshmi et al., 2018
0.332, 0.621, 0.797 mg/kg / oral administration	6 weeks	NR	Liver	qPCR	Alteration of the expression of immune, antioxidative and lysosomal genes.	Pietsch, 2017

3.7.1. Mice

Mice studies using ZEA had different duration from 24h to 70 days. Doses ranged from 0.15 to 40 mg/kg and the administration route was intraperitoneal or by gavage. The researches principally focused on changes in oxidative stress parameters and gene expression using qPCR, WB, ELISA, enzymatic assays, FCM and AHA.

ZEA caused the increase of creatine phosphokinase, lactate dehydrogenase, aspartate transaminase, alanine transaminase, total cholesterol and triglyceride levels and induced oxidative stress and apoptosis in cardiac tissues of mice. The natural carotenoid CRO was effective to prevent ZEA-induced cardiotoxicity (Salem et al., 2016).

In a study conducted by Zatecka et al. (2014), toxicological effect of ZEA on reproductive parameters and expression of testicular genes were studied by qPCR in male mice. ZEA caused decrease in sperm concentration, increase of morphologically abnormal spermatozoa, increased binding of apoptotic marker annexin V and changes in the expression of key genes for proper spermatogenesis, were observed. Long et al. (2017) explored the testicular oxidative damage of ZEA in male mice and the preventive role of proanthocyanidin (PC). ZEA exposure measured by qPCR, significantly reduced SOD and GSH-Px activities, upregulated the expression of Bax, caspase-3 and endoplasmatic reticulum (ER) stress-related genes (GRP78, CHOP, XBP-1). PC could ameliorate ZEA-induced testicular reproductive toxicity in mice by decreasing ER stress and testicular cell apoptosis.

ZEA administration caused changes in mice spleen and thymus weights, as well as in organ cellularity after 2 week-exposure. It also induced toxicity in immunologic and hematologic parameters by changing lymphocyte cell numbers and splenocyte cellularity. LAB addition strongly reduced the adverse effect of ZEA on weight loss and immunotoxic effects (Abbès et al., 2013). In addition, an increased mRNA expression of NLRP3 inflammasome, pro-interleukin-1β, and pro-interleukin-18 was observed in intestinal tissues of ZEA-treated mice and histological analysis reported an obvious inflammatory cell infiltration and tissue damage in the colon (Fan et al., 2018).

3.7.2. Rats

Reproductive toxicity and changes in the expression of proteins involved in detoxification and biotransformation were the main goal of the research conducted in this in vivo model. Administrated doses were 1, 5, 10, 20 and 25 mg/kg. Liver, blood, brain, maternal organs and testes were analysed in order to evaluate changes in gene and protein expression by using qPCR, WB, ELISA, HPLC and HPLC-fluo.

ZEA effects on the expression of proteins involved in the detoxification of rat xenobiotics were studied by Duca et al. (2012). Significant changes of mRNA expression were observed for the efflux transporter, P-glycoprotein, mono-oxygenases (CYP2C7, CYP2E1, CYP3A1, CYP3A2, and aromatase), steroid dehydrogenases, and Uridine diphospho-glucuronyl transferases (UGTs) after ZEA exposure.

ZEA exposure modulated the expression of ABC transporters and nuclear receptors in tissues of female rats repeatedly exposed during pregnancy, and fetal liver. ZEA concentration in maternal organs was determined by HPLC. qPCR and WB were used respectively to measure mRNA expression and protein expression in pregnant rat tissues and fetal liver (Koraichi et al., 2012). In addition, prenatal exposure to ZEA delayed the male foetal development and induced long-term toxicity on male offspring, including disrupted circulating concentrations of sexual hormones (E2, LH and T) and impaired testis development. These impairments concurred with an altered gene and protein expression of GnRHr and Esr1 in foetal brain and the down-regulation of 3β-HSD and StAR expressions in both mRNA and protein levels in developing testis (Gao et al., 2018).

3.7.3. Pigs

Different doses of ZEA (from 0.012 to 1 mg/kg), alone or in combination with other mycotoxins were administered to pigs in different ways such as intravenous injections or in contaminated diet (feed or gelatine capsules). The exposure time ranged between 48h and 6 weeks. ELISA, microscopy and IHC were the main techniques used in order to investigate the immunotoxicity or changes in the structure of the tissues.

ZEA contaminated diet altered several important parameters of the hepatic cellular immune response in pigs. It leaded to a significant decrease in the levels of pro- and anti-inflammatory cytokines at both gene expression and protein levels, correlated with a decrease in the levels of other inflammatory

mediators, MMP and TIMP. It was also observed that dietary ZEA induced a reduction in the expression of NF-κB1 and TAK1/p38α MAPK genes in the liver, suggesting that the toxic action of the toxin begins in the upstream of the MAPK signalling pathway by the inhibition of TAK1, a MAPK/NF-κB activator (Pistol et al., 2014). Changes in cytokine levels produced by Th1 and Th2 were also observed by ELISA in ileal Payer's patches in pigs exposed to ZEA (0,1 mg/kg). ZEA and its metabolites disrupted the immune response by influencing the secretion of cytokines in gut-associated lymphoid tissue, the gastrointestinal system protection against external pathogens (Obremski, 2014).

ZEA can change immunoreactivity of pigs by affecting the expression of active substances in nerve fibers in the ileal wall, even at low doses. IF technique have shown changes in the expression of antibodies against vasoactive intestinal peptide, neuronal form of nitric oxide synthase, cocaine and amphetamine regulatory peptide, pituitary adenylate cyclase-activating peptide-27 and substance P except galanin which expression was less after administration of ZEA (Gonkowski et al., 2015).

Dietary ZEA induced reproductive toxicity, immunotoxicity and haematological toxicity of piglets. In particular, could increase the length, width and area of vulva, the genital organ coefficient, the level of IgA, IgG and IgM, the level of BUN, CRE, AST and TBIL and decrease the level of E2, PROG, LH and FSH. The addition of Vitamin C prevented deformities in the vulva, decrease in immune response capacity, changes in serum biochemical indicators and disorders in hormones level of the piglets (Su et al., 2018). In order to evaluate the metabolism of ZEA via phase I and II enzymes to understand the

relevance of several competing pathways for the overall metabolic fate a tandem MS analysis was used. Clearly, phase II conjugation and keto-reduction to α- and β-ZEL were the predominant reactions. Moreover, reductive metabolism to ?-ZEA (?-ZEL) was extensive with smaller amounts of β-ZEL (Fleck et al., 2017).

Regarding mixtures, low doses of ZEA and T-2 toxin led to an increase in the percentage of calcitonin gene related peptide-like immunoreactive (CGRP-LI) neurons in the enteric nervous system (ENS) of the porcine descending colon in female pigs, determined by using a double immunofluorescence technique. Moreover, both mycotoxins changed the degree of co-localization of CGRP with other neuronal active substances, such as substance P, galanin, nitric oxide synthase, and cocaine and amphetamine-regulated transcript peptide (Makowska et al., 2017).

The role of microRNAs (miRNA) in responses of the porcine digestive system to ZEA and DON was examined by Brzuzan et al. (2015). It was shown that the pathways involved in cell proliferation and survival were disordered. Moreover, changes in miRNA expression in the liver and the descending colon were smaller and were associated more with treatment duration than the exposure to mycotoxins.

The administration of a mixture of ZEA and DON in pigs increased the number of goblet cells and lymphocyte in the villus epithelium of female gilts. Histological analysis revealed an increase in the mucosal thickness and the crypt depth, as well as a decrease in the ratio of the villus height to the crypt depth after six weeks of exposure (Przybylska-Gornowicz et al., 2015).

3.7.4. Fish

A complex network of genes associated with diverse molecular functions were identified by droplet digital PCR (dd-PCR) in rainbow trout treated with ZEA. The mRNA alterations were subsequently confirmed by qPCR. The results of the analysed genes indicate the ability of ZEA to interfere with important biological processes (blood coagulation or iron-storage) and different cellular components (cytoskeleton) (Woźny et al., 2012).

The influence of ZEA on the activity of immune, antioxidant and lysosomal genes has been studied in carp liver tissue by qPCR, reporting a clearly affected the expression of immune (IL-1β, IL-8, IL-10, arg2), antioxidative (mn-sod, cu-sod, cat) and lysosomal (cathep, v-atp-ase) genes. It also influenced estrogen-regulated genes (estrogen receptors er-α, er-β2, vitellogenin vtg transferrin tf) at mycotoxin concentrations that remained far below the maximum allowable level recommended by the European Commission (Pietsch, 2017).

ZEA induced gonadal impairment and altered gene expression in the hypothalamic-pituitary-gonadal axis of adult female zebrafish exposed to ZEA for 21 days. Environmentally, concentrations of ZEA could adversely affect the gonad functions by changing growth indices, histology of the ovary and apoptosis. Furthermore, this toxin alters the HPG axis by varying the expression of steroidogenic hormone encoding genes, affecting the reproductive function (Muthulakshmi et al., 2018).

3.8. Alternaria

3.8.1. Alternariol

AOH is a mycotoxin produced by species of fungi of the Alternaria genus. AOH is often found in a multitude of agricultural commodities, especially in fruits and in processed fruit products such as juices, wine and vegetables (Tu et al., 2019). It has been studied that AOH is cytotoxic and induce apoptotic cell death through the mitochondrial pathway (Fernández-Blanco et al., 2016). It has been also demonstrated that is genotoxic, mutagenic and induces reproductive disturbs (Tiessen et al., 2017; Schoevers et al., 2019). However, no indication of carcinogenicity to humans has been listed by IARC. Regarding its mode of action, it forms ROS and interacts with DNA topoisomerase, causing single and double DNA strand breaks (Dellafiora et al., 2015). Moreover, AOH has been reported to undergo aromatic hydroxylation by CYP450 enzymes and enzymes of phase 1 metabolism (Simon et al., 2016) and has been suggested as a potential ligand of the aryl hydrocarbon receptor (AhR), by potentially enhancing its own metabolism inducing the expression of AhR-dependent CYP1A1 (Schreck et al., 2012). Therefore, compounds like cyclodextrins polymers (Fliszár-Nyúl et al., 2019)

The dermal toxicity of AOH was proved in mice during 24h, through a single topic application o 12.5, 25, 50 µg/kg. The techniques used were IHC and WB. The study concerning AOH's toxicity is reported in Table 8.

Table 8. AOH toxicity studies: dose and administration route, exposure time, animal species, sex and age, biological sample, technique, damage and reference.

AOH							
Dose/ administration route	**Exposure time**	**Speci e**	**Sex/age**	**Biological sample**	**Technique**	**Damage**	**Reference**
12.5, 25, 50 µg/animal/ single topical application	24 hours	Mice	Male	Skin	IHC, WB	Induction of dermal manifestation through PGE2/EP2/cAMP/pCREB signalling cascade at low concentrations (50 nM and 100 nM)	Bansal et al., 2019

3.9. Fusarium emerging mycotoxins

Emerging mycotoxins gain a lot of interest due to their frequent contamination of food and feed, although in vivo toxicity data are limited. However, in vitro studies have shown genotoxic and cytotoxic effects of ENs A, A1 and B1, beauvericin and moniliformin (Prosperini et al., 2017; Fraeyman et al., 2018). Moreover, immunotoxicity has been studied for many emerging mycotoxins but reproductive disorders only for beauvericin and enniatin B (Schoevers et al., 2016; Albonico et al., 2017). Recently, BEA and ENs toxicity and genotoxicity have been confirmed in vivo in rodents (Maranghi et al., 2018). An increasing number of in vitro and in vivo studies are developing to understand their complex mechanism of action. Moniliformin may be concomitant mainly with Fusarium mycotoxins. In a recent review has been reported that oral exposure in poultry, in pigs and catfish reduces feed consumption and body weight gain, and may induce cardiotoxic effects and/or alterations in serum biochemical and hematological parameters (Fremy et al., 2019). Furthermore, it has been shown that natural compounds such as dietary carotenoids, dietary fibers (galactomannan, glucomannan, citrus fiber, bamboo fiber, carrot fiber, xilan and cellulose) and trans-resveratrol dietary supplements can mitigate its toxic effect on human health (Alonso-Garrido et al., 2020; Luz et al., 2017; Mallebrera et al., 2017).

As regard emerging mycotoxins, ENs, BEA and MON were studied. The only laboratory animal employed was rat in sub-acute studies, from 2 to 28 days. Doses ranged from 5 to 465 mg/kg of mycotoxin, administrated orally or

through intraperitoneal injections. Urine, faeces and blood were the most analysed biological samples. Carcinogenicity, immunotoxicity and neurotoxicity were the main toxicological effects reported (Fig. 9). The main techniques employed were UPLC-Q-TOF-MS, LC-MS/MS, IHC and FCM in carcinogenetic research. Results are shown in Table 9, in order of exposure time.

Table 9. Emerging mycotoxins toxicity studies: dose and administration route, exposure time, animal species, sex and age, biological sample, technique, damage and reference.

Emerging mycotoxins							
Dose/ administration route	**Exposure time**	**Specie**	**Sex/age**	**Biological sample**	**Technique**	**Damage**	**Reference**
MON (5,10,25,50,50 mg/kg / oral administration)	14 days	Rats	Male	Urine, faeces, liver, spleen, kidney, lung, thymus, heart, stomach, intestine, testes, pancreas, brain	UPLC-Q-TOF-MS	Carcinogenicity, neurotoxicity, immunotoxicity.	Jonsson et al., 2013
ENNA (465 mg/kg of food / oral administration)	28 days	Rats	Male	Blood, urine, faeces	FCM, LC-MS	Changes in peripheral blood lymphocytes.	Juan et al., 2014
ENB, BEA (5 mg/kg) / intraperitoneal injection	2, 3 days	Rats	Male	Urine, blood	LC-MS/MS, IHC	Tissue accumulation, tumour accumulation.	Rodríguez-Carrasco et al., 2016

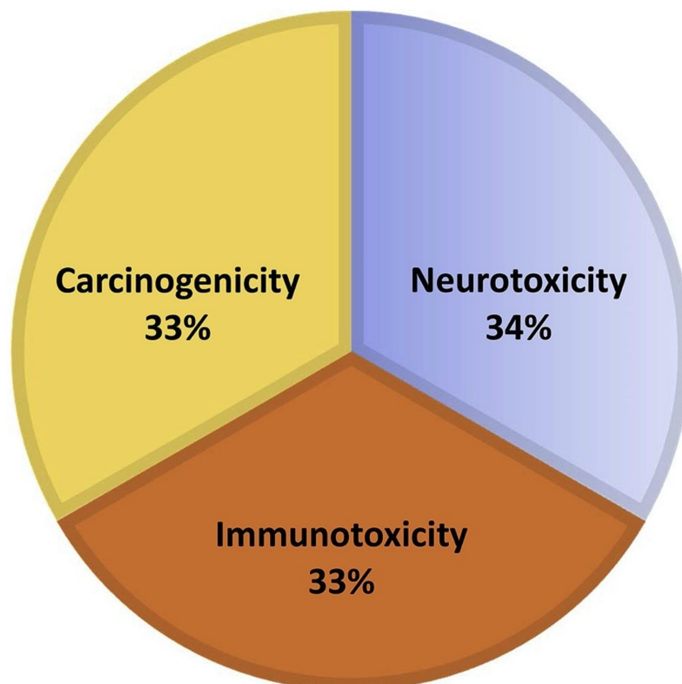

Fig. 9. Pie chart of scientific articles according to the main toxicological effect of emerging mycotoxins.

3.9.1. Moniliformin

3.9.1.1. Rats

UPLC-Q-TOF-MS method was used to investigate acute MON on male rats intoxicated by gavage with a single dose of MON (5, 10, 25, 40 and 50

mg/kg b. w.) and observed during 14 days. At the end of the experiment, tissue samples were collected from the liver, spleen, kidneys, lungs. Adrenal glands, thymus and heart to make a complete necropsy. In the 50 mg/kg dose group were also collected samples of stomach, intestine, testicles, pancreas, skeletal muscle and brain. The highest levels of MON were detected in urine in the first 6h after exposure (Jonsson et al., 2013).

3.9.2. Enniatins

3.9.2.1. Rats

Manyes and collaborators (2014) did not observe adverse effects in any intoxicated female rat with ENA (20.91 mg/kg bw/day) during the 28 day-experiment. ENA quantitation by LC-MS/MS in biological fluids ranged from 1.50 to 9.00 mg/kg, whereas in the gastrointestinal organs the concentration ranged from 2.50 to 23.00 mg/kg. The ENA concentration found in jejunum liquid and tissue indicates an absorption area. Moreover, two ENA degradation products were identified in duodenum, jejunum and colon content, probably produced by gut microflora.

Juan et al. (2014) reported a method for ENA extraction, determination and detection by LC-MS/MS in serum, urine and faeces of Wistar rats to evaluate immunologic effect in peripheral blood lymphocytes (PBL). For immunotoxicity assay, blood samples were analysed by FCM. ENA in urine and faeces was not detected, but its presence in serum might be the cause of changes in lymphocyte phenotyping.

LC-MS/MS was also used to analyse the distribution of ENB and BEA in selected tissue samples and biological fluids in mice. Both toxins were found in all tissues and serum and the highest amounts were measured in liver and fat, demonstrating the molecules tendency to bioaccumulate in lipophilic tissues. ENB three phase I metabolites (deoxygenated-ENB, mono- and di-demethylated-ENB) were found in liver and colon, with deoxygenated-ENB being most prominent (Rodríguez- Carrasco et al., 2014).

3.10. Other mycotoxins

3.10.1. Sporidesmin

Sporidesmin is a mycotoxin produced by the saprophytic fungus Pithomyces chartarum and is the cause of liver and bile duct damage that leads to elevated blood phytoporphyrin concentrations and a secondary photosensitization disease known as facial eczema (FE), which predominantly affects sheep and cattle (Lindsay et al., 2018). The unique study analysed in this review is described below and it is centred on changes in hepatic gene expression in sheep.

3.10.1.1. Sheep

After orally administrating sheep with 0.25 mg/kg during 7 weeks (Table 10), RNA-seq technology and qPCR were used to investigate hepatic gene expression changes (including antioxidant enzymes, detoxification enzymes and Wnt inhibitors). The expression of multiple genes critical for detoxification,

particularly members of the cytochrome P450 (CYP), was higher in animals exhibiting mycotoxin resistance, probably due to the individual ability to detoxify secondary metabolites. Furthermore, in resistant animals a marked suppression of multiple key Wnt inhibitor genes was reported (Zheng et al., 2018).

Table 10. Other mycotoxins toxicity studies: dose and administration route, exposure time, animal species, sex and age, biological sample, technique, damage and reference.

Other mycotoxins							
Dose/administration route	**Exposure time**	**Specie**	**Sex/age**	**Biological sample**	**Technique**	**Damage**	**Reference**
Sporidesmin (0.25 mg/kg / oral administration)	7 weeks	Sheep	Male	Blood, liver	qPCR, RNA-Seq	Changes in hepatic gene expression.	Zhang et al., 2014

4.Discussion

In the articles screened and analyzed in full text, for all the species studied, the most used mycotoxin was AFB1 (22%). Afterwards DON (18%), ZEA (16%), OTA (15%), FBs (7%), PAT (4%), CIT (2%) and a 1% for ENA, MON, AOH and Sporidesmin. There is also a 12% of the studies that used the combination of two or more mycotoxins (Fig. 10).

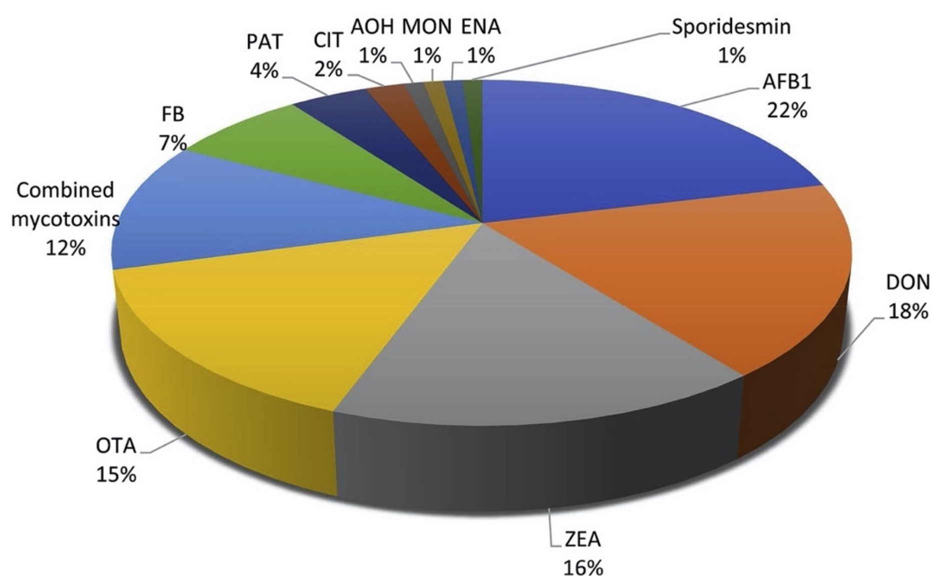

Fig. 10. Pie chart of the percentage of studies found depending on the mycotoxin studied.

Regarding the use of laboratory animals in the studies, the main species used was mouse (29%), followed by pigs (24%) and rats (23%). Other animals used were chicken (14%), fish (7%), alpacas (2%), turkeys (2%) and sheep (1%) (Fig. 11A). The main targeted organ used was the liver (26%), followed by

blood/plasma analysis (18%), kidney (16%), spleen (11%) and intestine (10%). A smaller percentage included the use of thymus (4%), lung (4%), reproductive organs (3%), brain (2%), urine (2%), skin (1%), pancreas (1%), embryo/larvae (1%) and heart (1%) (Fig. 11B).

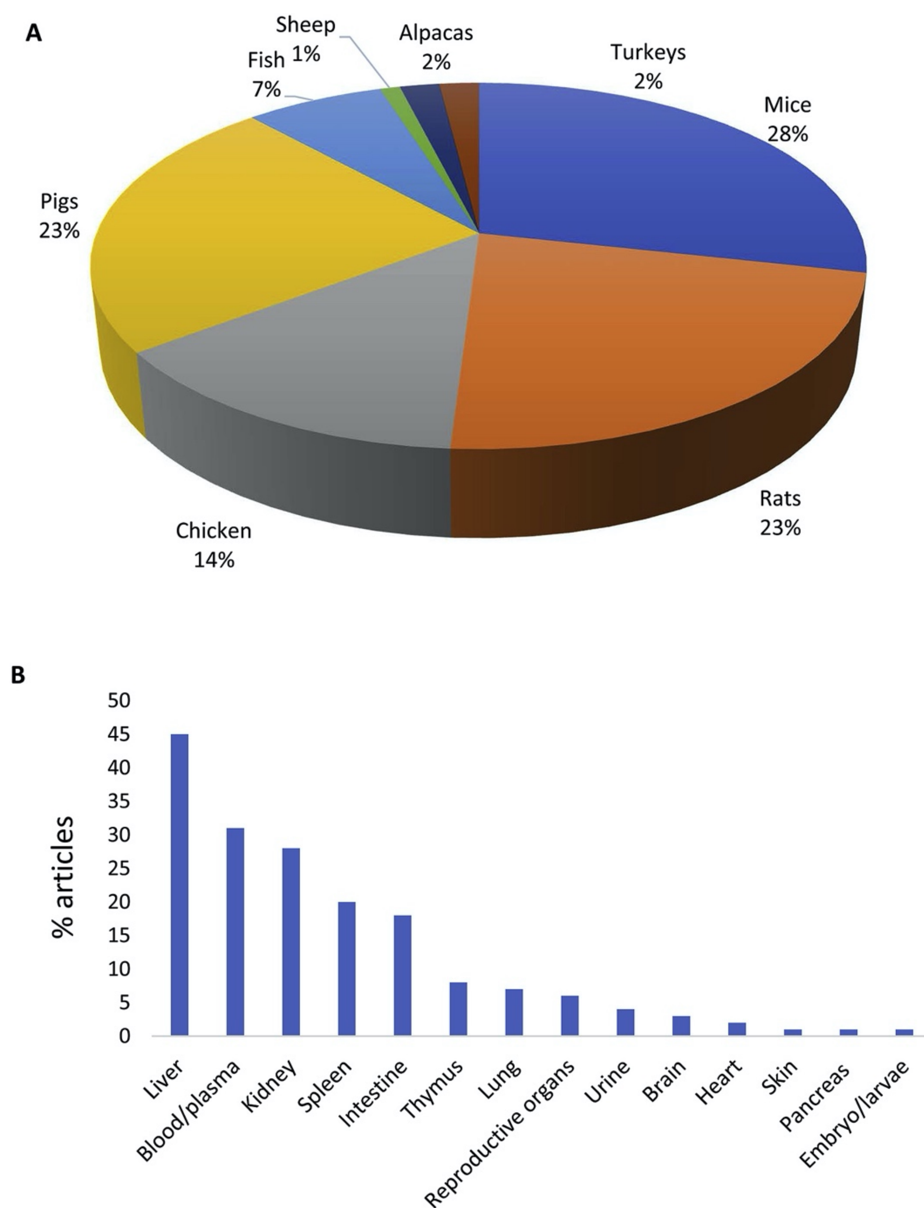

Fig. 11. A. Percentage of the studies according to the animal species. **B.** Percentage of articles according to the organ studied.

As shown in Fig. 12, the most common purpose of the studies was the immunotoxicity (30%). A similar percentage is constituted by genotoxicity (19%), oxidative stress (18%) and hepatotoxicity (16%). Less frequent was the study of cytotoxicity (7%), teratogenicity (5%) and neurotoxicity (5%).

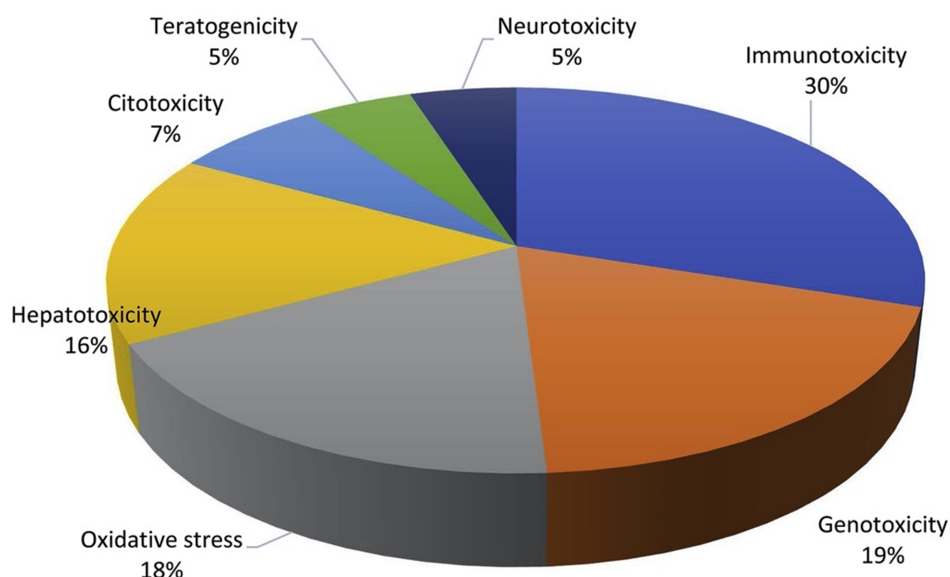

Fig. 12. Pie chart of the percentage of the papers according to the main purpose of the study.

The most common technique used in these studies is the qPCR (27%). Secondly, ELISA (12%) and Immunohistochemistry (IHC) (12%), followed by WB (9%), LC-MS/MS (5%), microscopy (5%), PCR (3%) enzymatic assays (3%), microarray (3%), RNA- Seq (3%), and a 2% of FCM, comet assay (CA), Histopathology (HP), gel electrophoresis (GE), High-performance liquid chromatography with fluorescence detection (HPLC-FLD), HPLC. Lastly, only a 1% of DNA-Seq, Electrochemistry coupled to liquid chromatography/mass

spectrometry (LC-EC/MS/MS), Automated haematology analyzer (AHA), antibody assays, Orbitrap, LC-TOF-MS, Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay, SDS-PAGE, mixed lymphocyte reaction (MLR), MiSeq, semiqPCR, UPLC-Q-TOF/MS (Fig. 13).

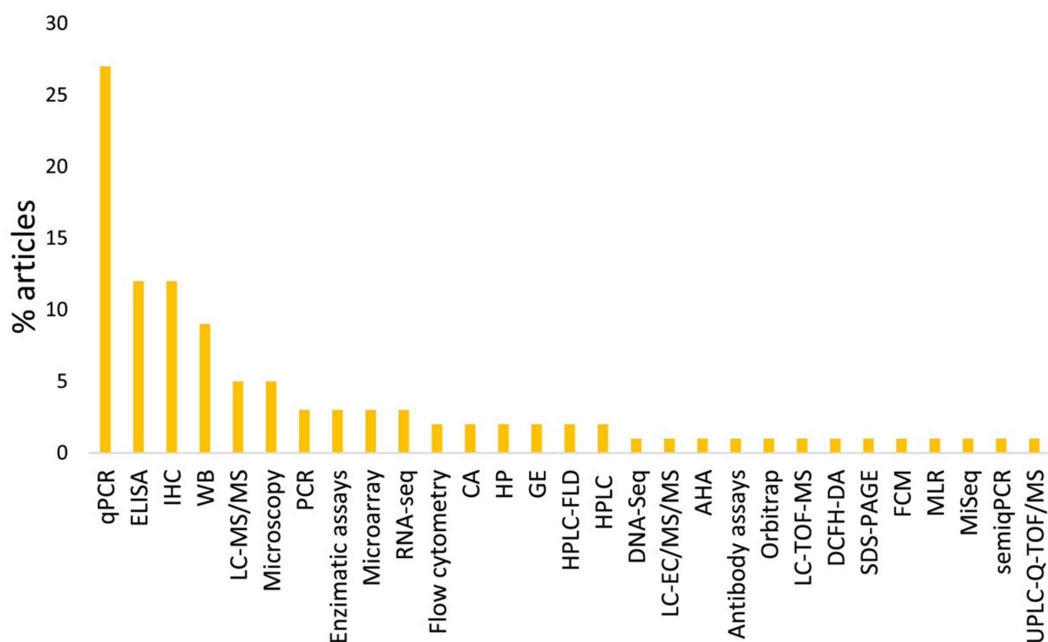

Figura 13. Percentage of scientific articles according to the techniques used.

5.Conclusions

In the last few years, several efforts have been developed in order to minimize the content of mycotoxin in food. Degradation of toxic fungal metabolites may be an ideal approach to remove or neutralize the toxins activity if the process does not alter its nutritional composition. Physical, chemical, and

biological methods may be used to completely inactivate or remove mycotoxins in food, such as: cold atmospheric pressure plasma in demolition of plant pathogens, hydrolysis, ammonization, ozonation, peroxidation, the use of hydrochloric acid, ascorbic acid, sodium bisulfite, hydrogen peroxide, ammonium hydroxide, probiotic bacteria and enzymes (Abbas, 2019; Čolović et al., 2019). Despite all the attempts to reduce its content in food, the research has not succeeded in solving this major problem. Therefore, there is a need to pursue the study of their toxicity.

The literature reviewed shows that the most studied mycotoxins in vivo in the last decade were AFB1, DON, ZEA and OTA while the less studied were FBs, PAT, CIT, AOH and the emerging mycotoxins. Interestingly, combinations of mycotoxins were also frequent, especially with AFB1, DON and ZEA. Furthermore, the effect of several natural and biological compounds was investigated to counteract their toxic mode of action. As expected, the predominant laboratory animals employed were the rodents (mice and rats) and pigs, whereas less studied were chicken, turkeys, fish, alpacas, sheep and fish. The main targeted organ analysed was the liver, followed by blood, kidney, spleen, intestine, thymus, lung, reproductive organs, brain, urine, skin, pancreas, embryo and heart. Regarding the main purpose of the researches, the majority focused on immunotoxicity and a smaller percentage was constituted by genotoxicity, oxidative stress, hepatotoxicity, cytotoxicity, teratogenicity and neurotoxicity studies.

To study the toxicity of mycotoxins, a wide range of different techniques were employed, among which the most used was the qPCR, followed by ELISA,

IHC, WB, LC-MS/MS, microscopy, PCR, enzymatic assays, microarray, RNA-Seq, FCM, CA, HP, GE, HPLC-FLD. Less used were DNA-Seq, LC-EC/MS/MS, AHA, antibody assays, orbitrap, LC-TOF-MS, DCFH-DA, SDS-PAGE, MLR, MiSeq, semiqPCR, UPLC-Q-TOF/MS. Moreover, the study of immunologic oxidative stress mechanisms and transcriptomic analysis, through promising innovative and accurate techniques, possess a very important role in understanding the mechanisms of action of these secondary metabolites. However, despite these new approaches, more in vivo trials are needed to investigate their mode of action on human health.

In conclusion, it can be confirmed that according to its well-known toxicity and IARC classification, AFB1 is the most toxic and studied mycotoxin, while PAT, CIT, AOH, emerging mycotoxins and sporidesmin need more accurate research in vivo to ensure their toxicological effect.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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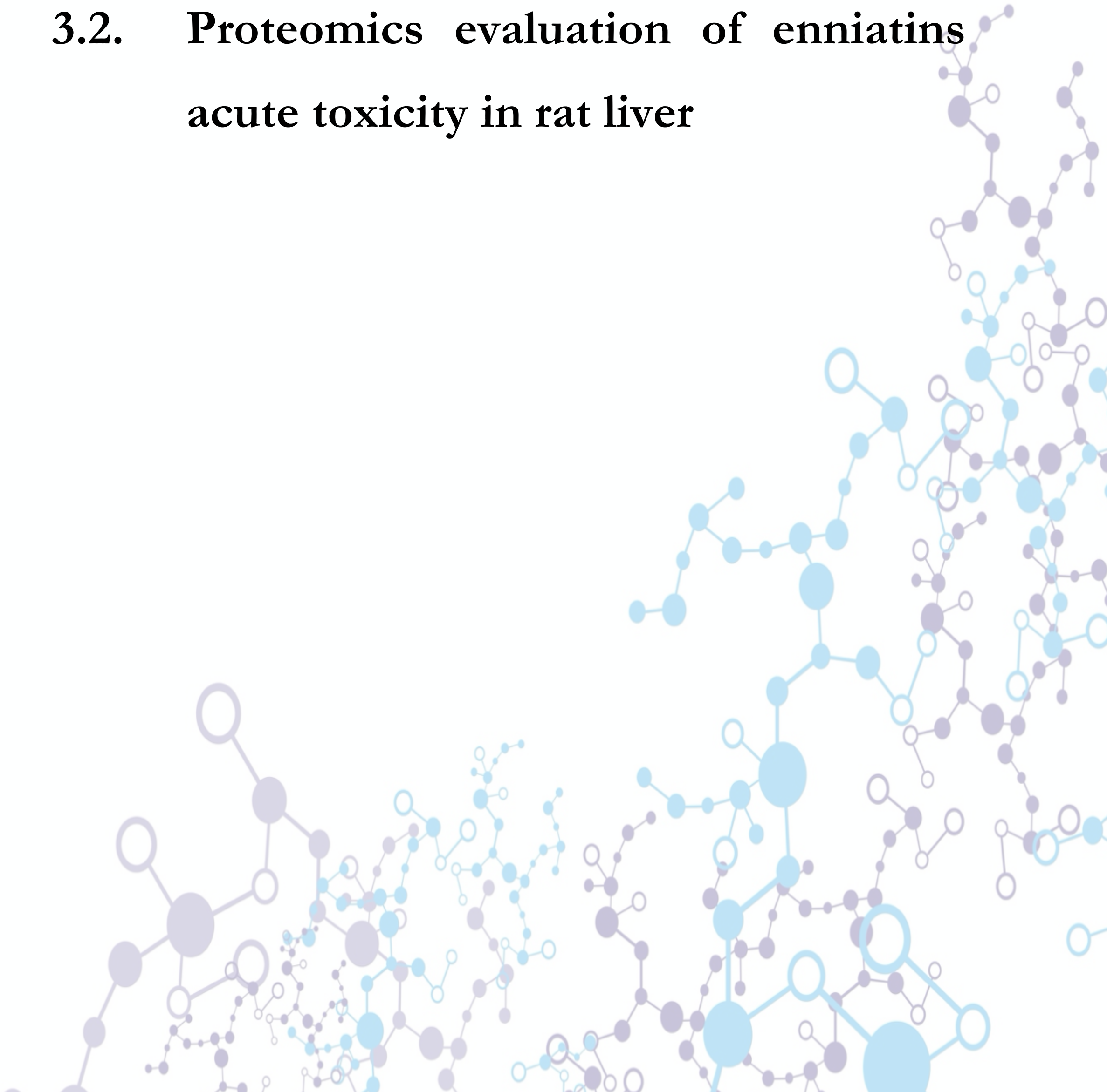
3.2. Proteomics evaluation of enniatins acute toxicity in rat liver



Food and chemical toxicology

Proteomics evaluation of enniatins acute toxicity in rat liver

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Abstract

Enniatins (ENs) are emerging mycotoxins produced by *Fusarium* fungi which are cytotoxic also at low concen- trations due to its ionophoric properties. The aim of this study was to evaluate the hepatic toxicity of ENs exposure at different concentrations in Wistar rats through a proteomic approach. Animals were intoxicated by oral gavage with medium (EN A 256, ENA1 353, ENB 540, ENB1 296 µg/mL) and high concentrations (ENA 513, ENA1 706, ENB 1021, ENB1 593 µg/mL) of an ENs mixture and sacrificed after 8 h. Protein extraction was performed using powdered liver. Peptides were analyzed using a liquid chromatography coupled with a quad- rupole time-of-flight mass spectrometer. Proteins were filtered by abundance using Mass Professional Profiler software (Agilent Technologies) and 57 were differentially expressed when compared to the control. In terms of abundance, the liver biomarker Carboamoyl-phosphate synthase showed the highest levels in all conditions employed while actin-1 had the lowest. Bioinformatic analysis using DAVID platform reported acetylation, nucleotide phosphate-binding region:NAD and catalytic activity as the most represented terms. Furthermore, metabolism was the most significant and enriched pathway in Reactome overrepresentation. In conclusion, ENs acute exposure caused protein expression changes related to major cellular processes in rats, hinting its involvement in liver disturbance.

Keywords: Mycotoxins, oxidative stress, electron transport chain, *in vivo*

1. Introduction

Mycotoxins are toxic chemical substances produced by filamentous fungi. Even after the introduction of good practices in the food pro- duction, storage and distribution chain, they continue to be a concern in food safety. Significant economic losses are associated with the impact of mycotoxins on human health, animal productivity and international trade (Food and Agriculture Organization of the United Nations, 2020; Gil et al., 2016).

Fusarium species are common pathogens of cereal grains, animal feeds, and food commodities worldwide. Under favorable conditions, their secondary metabolism can produce enniatins (ENs), hexadep- sipeptidic mycotoxins comprising enniatin A (ENA), A1 (ENA1), B (ENB) and B1 (ENB1). ENs are commonly found in several grains and their derived products, in fish, dried fruits, nuts, spices, cocoa, coffee prod- ucts. Furthermore, some food processes including cooking, baking, frying or roasting do not affect their chemical structure, so detoxifica- tion strategies to mitigate the risks of ENs presence in foods and feed may be difficult (Prosperini et al., 2017). Lately, in Rumanian wheat-based products detectable values of at least one EN were found in 56% of the samples, where ENB was detected 44% of the samples and ENB1 was present in the 38% (Stanciu et al., 2017).

The lipophilic nature of ENs allows them to be incorporated into lipid bilayers of cell membranes and creates cation selective pores that cause an increase in the permeability for cations, resulting in disturbances of the physiological cation level in the cell. The ionophoric property of ENs promote

the transport of mono- and divalent cations through mem- branes leading to toxic actions by loss of homeostasis. Moreover, they decrease the calcium retention capacity of the mitochondrion matrix leading to the mitochondrial membrane potential collapse via perme- ability transition pore opening (Prosperini et al., 2017).

ENs toxicity in combined mixtures has been tested *in vitro* but not *in vivo* (Cimbalo et al., 2020). There is quantitative evidence that ENs cytotoxicity depend on their concentrations, and also on their combi- nation with other mycotoxins (Juan-García et al., 2013). The individual ENs toxicity results obtained point liver as an important target in mammals. After 28-day repeated dose assay in rats, using enniatin A (ENA) naturally contaminated feed (20.91 mg/kg bw/day), the con- centration found in liver was 23 mg/kg (Manyes et al., 2014) and al- terations reflected in lymphocyte phenotyping was observed (Juan et al., 2014) ENB was selected for *in vivo* toxicity and genotoxicity testing in mice and the results supported a genotoxic effect in bone marrow and liver cells after acute treatment. ENB increased lobular liver inflamma- tion without a clear dose-effect relationship, while the increasing of mitotic cells was dose-related without effect on necrosis or apoptosis (Maranghi et al., 2018).

Other mycotoxins have already been confirmed as hepatotoxic, like aflatoxin B1, citrinin, deoxinivalenol, fumonisins, ochratoxin A, patulin and zearalenone. Among them, the most dangerous is aflatoxin B1, recognized by the International Agency for Research on Cancer (IARC) as a group 1 human carcinogen (Cimbalo et al., 2020).

Following the different molecular initiating events that may take place, biological effects of mycotoxins can be observed at the different omics levels. Thus, mycotoxin toxicology should be investigated from a holistic perspective. The clinic manifestations of mycotoxins exposure result from the disruption of different cell functions, which are often the consequence of the effect of that toxicant on multiple molecular targets, thereby triggering a pleiotropic toxicity, whose study requires the use of global technical approaches such as proteomics (Dellafiora and Dal- l'Asta, 2017).

In the present study a proteomic approach is used to investigate toxic effects of ENA, A1, B and B1 in rat liver after acute exposure. The samples were analyzed by quadrupole time-of-flight mass spectrometers (QTOF-LC/MS) and the mass spectrometric data were identified using a bioinformatics and UniProt/SwissProt database.

2. Material and methods

2.1. Reagents

The standards of standard solution stock (purity: 99%) of ENA (mw: 681.92 g/mol), ENB (mw: 639.82 g/mol), ENA1 (mw: 667.87 g/mol), ENB1 (mw: 653.85 g/mol) and phosphate buffer saline (PBS) were ob- tained from Sigma–Aldrich (Madrid, Spain). All the stock solutions were prepared by dissolving 1 mg of mycotoxin in 1 ml of pure methanol, obtaining a 1 mg/ml solution. These stock solutions were diluted with methanol in order to obtain the appropriate multi-compounds working standard solutions. All the standards were kept at − 20 ◦C.

For proteins extraction and digestion, DL-Dithiothreitol (DTT) ≥99.0%, Trizma® hydrochloride (Tris-HCl), ≥99.0% and Trypsin were purchased from Sigma Aldrich (St. Louis, USA). Iodoacetamide (IAA), 98% was acquired from ACROS OrganicsTM, Thermo Fisher Scientific (New Jersey, USA). For lysis buffer preparation Thiourea (TU), 99% was obtained from Thermo Fisher Scientific (Kandel, Germany) and Urea (U), 99% was acquired from F.E.R.O.S.A (Barcelona, Spain). Deionized water (resistivity < 18 MV cm) was obtained using a Milli-Q water pu- rification system (Millipore, Bedford, MA, USA). Acetonitrile (AcN) LC/ MS-grade OPTIMA® (≥99.9% purity) were supplied by Fisher Chemical (Geel, Belgium). Formic acid (≥98%) was obtained from Sigma Aldrich (St. Louis, USA).

2.2. In vivo experimental design

Fourteen female Wistar rats (243–278 g) were acquired from Phar- macy animal facility (Universitat de Val`encia, Spain). The institution Animal Care and Use Committee of the University of Valencia approved all animal procedures (protocol no A13338818442265). Animals were divided in three groups: four rats in the control group and ten rats in the treated ones, with medium (M) and high (H) exposure. Animals were housed in one cage in a windowless room with a 12-h light-dark cycle. The study rooms were maintained under controlled conditions appro- priate for the species (temperature 22 ◦C, relative humidity 45–65%). The control group was exposed to 1 ml of vehicle (PBS) by esophageal intubation, while five of the treated ones were intoxicated with 1 ml of medium concentrations: single dose of ENA 256, ENA1 353, ENB 540, ENB1 296

μg/mL; and other five with 1 ml of the higher ones: single dose of ENA 513, ENA1 706, ENB 1021, ENB1 593 μg/mL (Table 1). After 8 h exposure, they were sacrificed by isoflurane gas overdose and the organs were removed and stored at − 20 ◦C.

2.3. Protein extraction

Tissue was washed by deionized water to neutral pH, then dried and smashed to powder. Protein extraction was performed using 10 mg of powdered liver tissue resolved in 200 μL of lysis buffer (8 M U/2 M TU/ 50 mM Tris-HCl), subsequently homogenized by vortexing and sonified 3 times using a methanol: H2O mixture until protein solubilization (USC 1200D ultrasonicator, VWR, International bvba, Leuven, Belgium). Af- terwards, the samples were centrifuged for 30 min at 4 ◦C and 13000 g. The supernatant was collected and protein concentration was deter- mined using a NanoDropTM 2000 (Thermo ScientificTM, Spain).

2.4. Protein denaturation, alkylation, enrichment and digestion

Firstly, samples were standardized for protein digestion to 1 mg/mL and subsequently mixed with DTT (200 mM, pH 7.8) for 1 h at 60 ◦C. Therefore, IAA 200 mM (pH 7.8) was added for alkylation of cysteine residues, followed by incubation for 30 min at 37 ◦C. Protein digestion was initiated with addition of trypsin (1:40) and incubated for 16 h. Digestion was stopped with addition of 5% acetic acid down to pH 5. The samples were dried for 2 h in a vacuum concentrator (lyophilizer Freezone 2.5 freeze dryer Benchtop, Labconco) at a −

40 ◦C temperature and vacuum pressure of 0.080 mBar. After the lyophilization, peptides were eluted in 0,1% acetic acid: acetonitrile (98:2 v/v) to a final concentration of 100 μg/μL.

2.5. Q-TOF mass spectrometry and data analysis

The Q-TOF-LC/MS analysis was performed using an Agilent Technologies (Santa Clara, CA, USA) 1200 Infinity Series LC coupled with an Agilent Technologies 6540 UHD Accurate-Mass Q-TOF-LC/MS. This device was equipped with an electrospray ionization Agilent Technol- ogies Dual Jet Stream ion source (Dual AJS ESI).

Peptides were separated by a C18 RP capillary column AdvanceBio peptide mapping column (120 Å, 2.7 μm, 2.1 × 150 mm) (Agilent Technologies Inc., California, USA). The chromatographic gradient was conducted using a mobile phase of H2O (0.1% formic acid) as phase A and AcN (0.1% formic acid) as phase B. The gradient elution was applied at a flow rate of 0.5 mL/min, with the following gradient: 0–1 min, 3% B; 1–21 min, 40% B; 21–23 min, 95% B and maintained during 1min; 24–29 min 3% B; this initial conditions were maintained during 11 min. The total run time was 40 min. The injection volume was 10 μL and the column temperature was maintained at 50 ◦C.

Q-TOF-MS conditions were the following: drying gas flow (N2), 13.0 L/min; nebulizer pressure, 35 psi; gas drying temperature, 325 ◦C; capillary voltage, 4000 V; nozzle voltage 500 V; fragmentor voltage,175 V; skimmer voltage 65 V and octopole RF peak, 750 V.

Dual AJS ESI interface was used in positive ionization mode and positive ions were acquired in the range of 100–3000 m/z for MS scans, and 50–3000 m/z for auto MS/MS scans, at a scan rate of 8 scans/s for MS and 3 scans/s for MS/MS, respectively. Automatic acquisition mode MS/MS were carried out using a ramped collision energy with charge state preference of 2, 3, >3 and slope 3.1, 3.6 and 3.6, respectively. Internal mass correction was enabled using two reference masses at 121.0509 and 922.0098 m/z. Instrument control and data acquisition were performed using Agilent MassHunter Workstation software B.08.00 (Agilent Technologies).

2.6. Bioinformatics and statistical analysis

Data was analyzed using the Spectrum Mill MS Proteomics Work- bench software package Rev B.06.00.201 from Agilent Technologies, which is capable of performing database searches and rapidly identi- fying matched proteins and peptides by using the UniProt catalog. MS/ MS spectra were searched against the SwissProt human database (downloaded on 7/4/2020) and validated at the spectral level using 1.2% FDR as the criteria. Data were searched with trypsin specificity, with the following search parameters maximum missed cleavages 2; fixed modification carbamidomethylation (C); variable modification oxidized methionine (M); minimum matched peak intensity 50%; pre- cursor mass tolerance ±20 ppm; product mass tolerance ±50 ppm; maximum ambiguous precursor charge 3; minimum detected peaks 3 and precursor isolation purity >70%. Validation of peptide identifica- tions was based on a maximum FDR of 1.2% across each LC run, mini- mum peptide length of six amino acids, and precursor charge range of 2–6. These data were also searched with ±20 ppm

precursor and ±50 ppm fragment ion tolerance. Spectrum Mill determines the area for each precursor and, following peptide spectral matching, assigns the peak area to the corresponding peptide. Protein intensity was calculated as the median intensity of the peptides from the protein.

Identified proteins were exported to the Mass Profiler Professional (MPP) 15.0 version software from Agilent Technologies for statistical data analysis. This analysis was carried out based on the total spectra intensity of the proteins which were considered as entities in MPP. The baseline of the spectra was adjusted to the mean across all samples. The entities were then filtered based on their frequency of occurrence across at least all replicates of one group. Contrasts for each rat between the experimental mycotoxin dose front the control were analyzed using unpaired *t*-test with Benjamin-Hochberg adjustment. Results were considered significant with a fold change ≥0.7 and p-values cut-offs < 0.05. The whole list can be found in supplementary material table.

2. Results

The analysis of the livers from exposed rats to two different ENNs concentrations (M and H, Table 1) and their controls was performed by gel-free shotgun proteomic approach. By identifying features across all QTOF-LC/MS runs, analysis in MassHunter software from Agilent allowed identification of 108 unique proteins at 1% false discovery rate (peptide level). Subsequently, analyses were confined to 57 proteins with differentiated expression which could also be

used for the stringent assessment of quantitative difference between control and treated groups (see supplementary material for detailed results).

Table 1. Medium and high doses of ENNs B, B1, A1, A (μg/ml) administered to rats.

	Medium dose (μg/ml)	**High dose (μg/ml)**
ENB	256	513
ENB1	353	706
ENA1	540	1021
ENA	296	593

Moreover, from 57 of these proteins with differentiated abundance with respect to controls, 41 proteins displayed higher and 12 lower levels in treated groups. Among the proteins showing the same trend (increasing or decreasing) with mycotoxins mixtures concentration, the most abundant protein was carbamoyl-phosphate synthase, mitochondrial, meanwhile actin-1 had the lowest level. The remaining 4 proteins have altered levels in-between treated groups depending on mycotoxins concentrations used, but did not follow any pattern relative to control.

In order to assess the gene ontology (GO) analysis using DAVID platform, each protein number was checked. The detailed identification of each Uniprot number assigned and its conversion to the equivalent protein number

assigned for *Rattus norvegicus* led to exclude 11 Uniprot IDs because they were protein duplicates in other mammal species or assigned to bacteria.

DAVID analysis revealed acetylation, nucleotide phosphate-binding region:NAD and catalytic activity as the most represented terms in the bioinformatics analysis (Table 2). Moreover, 13 of these proteins were found in the mitochondrion and 12 were related to oxidoreductase ac- tivity. Regarding Reactome overrepresentation test results, metabolism was both the most significant pathway and the most enriched (Table 3).

Table 2. Functional annotation chart of the 5 most significant terms using DAVID resources of 46 proteins selected in the liver proteomics analysis after enniatins exposure of rats in the categories UniProt Keyword, UniProt Sequence Feature and Gene Ontology Molecular Function.

UniProt Keyword

Term	Count	%	p-value	p-adjusted
Acetylation	33	71.7	1.10E-24	1.40E-22
Phosphoprotein	36	78.3	4.40E-17	2.70E-15
Cytoplasm	23	50	6.70E-10	2.70E-08
Methylation	12	26.1	4.90E-09	1.50E-07
Mitochondrion	13	28.3	1.10E-08	2.60E-07

UniProt Sequence Feature

Term	Count	%	p-value	p-adjusted
nucleotide phosphate-binding region:NAD	6	13	1.10E-04	1.10E-02

transit peptide:Mitochondrion	9	19.6	1.40E-04	1.10E-02
binding site:NAD	5	10.9	5.40E-04	2.70E-02
metal ion-binding site:Iron (heme distal ligand)	3	6.5	1.00E-03	3.20E-02
binding site:Substrate	7	15.2	1.10E-03	3.20E-02
Gene Ontology Molecular Function				
Term	**Count**	**%**	**p-value**	**p-adjusted**
catalytic activity	34	73.9	1.40E-07	4.30E-05
oxidoreductase activity	12	26.1	6.00E-06	8.90E-04
drug binding	6	13	3.30E-05	3.10E-03
NAD binding	5	10.9	4.20E-05	3.10E-03
antioxidant activity	5	10.9	6.00E-05	3.40E-03

Table 3. Reactome's pathways results using 46 proteins selected in the liver proteomics analysis after enniatins exposure of rats by entities p value and false discovery rate (FDR).

Pathway name	Count	Ratio	p-value	FDR
Metabolism	28	0.187593	1.66E-10	1.04E-07
Fructose metabolism	3	6.12E-04	3.20E-06	0.001001
Urea cycle	3	8.75E-04	9.25E-06	0.0019233
Heme degradation	3	0.001312	3.08E-05	0.0048019

Chaperone Mediated Autophagy	3	0.001925	9.53E-05	0.0103414
Metabolism of amino acids and derivatives	8	0.032899	9.94E-05	0.0103414
Fructose catabolism	2	4.37E-04	1.87E-04	0.0150683
Metabolism of porphyrins	3	0.00245	1.93E-04	0.0150683
Gluconeogenesis	3	0.002975	3.40E-04	0.0234761
Erythrocytes take up oxygen and release carbon dioxide	2	7.00E-04	4.75E-04	0.0294771
The citric acid (TCA) cycle and respiratory electron transport	5	0.015399	6.34E-04	0.0354825

3. Discussion

These results delve into the mechanism of action of ENs acute liver poisoning, taking one more step into the *in vivo* ENB acute toxicity in liver described by Maranghi et al. (2018). The liver is an essential metabolic organ where glucose is converted into pyruvate through glycolysis in the cytoplasm, and pyruvate is subsequently oxidized in the mitochondria to generate ATP through the TCA cycle and oxidative phosphorylation. In the fed state, glycolytic products are used to syn- thesize fatty acids through de novo lipogenesis. In the fasted state, the liver secretes glucose through both glycogenolysis and

gluconeogenesis. Aberrant energy metabolism in the liver promotes insulin resistance, diabetes, and nonalcoholic fatty liver diseases (Rui, 2014).

Regarding acetylation, 33 proteins related to that term were found altered in this study (Table 2). Acetylation plays particular important roles in material and energy metabolism by modifying the activity and/ or specificity of certain enzymes and substrates, thereby regulating glucose, lipid, and amino acid metabolism. Human-related studies have indicated that the change in protein acetylation pattern is associated with the occurrence and/or development of metabolic-related diseases such as obesity, cardiovascular disease, diabetes, and tumorigenesis. In particular, in livers, it has been predicted that acetylation modification is involved in most metabolic pathways by regulating glycolipid meta- bolism and urea cycles (Le-Tian et al., 2020). Proteomics studies have demonstrated that many key metabolic enzymes are acetylated, malo- nylated, or succinylated in mitochondria, and that their enzymatic ac- tivities are regulated by these modifications in response to environmental stimuli (Stein and Imai, 2012).

Methylation is a chemical modification which can alter biological functions such as epigenetics and protein complex formation. In a recent study, methyltransferase nicotinamide N-methyltrasferase (NNMT) expression increased in the stroma of peritoneal metastases, ovarian cancer cells, breast and colon cancer stroma, suggesting the association with its expression and multiple cancer types (Eckert et al., 2019). Moreover, the atypical activity of glutamate methylation and histidine methylation site was observed in diet-induced obese mice, hinting a potential role in diabetes and obesity (Zhang et al., 2018).

According to these findings, there were expression changes of 12 proteins involved in methylation processes in rat's liver after ENs acute exposure.

In ENs exposed livers, results point to an alteration of the respiratory transport chain, fructose, gluconeogenesis, amino acids metabolism and urea cycle (Table 3). A proteomics research in fatty liver disease of dairy cows revealed mitochondria-associated pathways that are involved in the regulation of the pathogenesis of fatty liver disease, including the TCA cycle, propionate metabolism, glycolysis/gluconeogenesis, pyru- vate metabolism, oxidative phosphorylation, fatty acid degradation, valine, leucine, and isoleucine degradation, drug metabolism (Le-Tian et al., 2020).

The citric acid cycle and electron transport chain are among the altered pathways and they have been already associated to ENs exposure (Alonso-Garrido et al., 2020). UQCRC1 found in complex III from the respiratory electron transport chain is upregulated in one treated sample while ATP5 alpha and beta subunits form complex V, included in Table 2 in mitochondrion, are upregulated between 0.96 and 2.51 logFC (fold change) in 5 treated samples. Moreover, LDHA is upregulated in 4 treated samples. This enzyme plays a crucial role in the final step of anaerobic glycolysis and in consequence in NADH production. Its overexpression is observed in many human malignancies in association with tumor progression, among them in cholangiocarcinoma where it is correlated with poor prognosis (Thonsri et al., 2017).

Considering the oxidoreductase and the antioxidant activities (Table 2), four proteins participate in both processes and appear upre- gulated in ENs

exposures, superoxide dismutase 1 (SOD1), peroxir- edoxin 4 (PRDX4), hemoglobin beta (HBB) and hemoglobin alpha (HBA1). SOD scavenges superoxide radicals, a primary ROS produced in the body, and hence plays a central role in protection against oxidative stress. Of the three genes that encode SOD in mammals, SOD1 is present largely in the cytoplasm and partly in the intermembrane space of mitochondria (Fridovich, 1995). PRDX4 catalyzes disulfide bond for- mation in proteins via the action of hydrogen peroxide and hence de- creases oxidative stress and supports oxidative protein folding for the secretion of lipoproteins (Matsumoto et al., 1999). Suppression of oxidative stress by hemoglobin has been described as a mechanism to protect hepatocytes from oxidative damage in nonalcoholic steatohe- patitis. Furthermore, treatment with hydrogen peroxide, a known oxidative stress inducer, increased HBA1 and HBB expression in HepG2 and HEK293 cells (Liu et al., 2011).

Finally, it has been described that increased liver CPS-1 levels in parallel with the duration of alcohol consumption are presumed to reflect mitochondrial damage and redox stress (Carter et al., 2015). The role of mitochondrial carbamoyl phosphate synthase-1 (CPS-1) is to catalyse the synthesis of carbamoyl phosphate from ammonia and bi- carbonate. This is the first rate-limiting step in liver of the urea cycle (Table 3), and is key in humans for nitrogen disposal in ureagenesis (Martínez et al., 2010). In the present study CPS-1 is upregulated in 6 treated samples (1.07–3.09 logFC) confirming ENs induced liver damage.

4. Conclusion

ENs are food and feed contaminants non legislated by the authorities which are related with oxidative phosphorylation changes, particularly studied *in vitro*. In this study it is shown that rats ENs acute exposure lead to protein expression changes in liver related to metabolism, the main function of this organ. The oxidative liver damage caused by ENs causes the modification of the mitochondrial activity and it leads to the alter- ation of acetylation processes and in consequence, in metabolic distur- bance. Moreover, liver injury biomarker CPS-1 shows increased expression in exposed rat livers.

CRediT authorship contribution statement

A. Cimbalo: Investigation, Formal analysis, Writing – original draft. **M. Frangiamone:** Investigation, Formal analysis, Writing – original draft. **C. Juan:** Investigation, Formal analysis, Writing – review & editing. **G. Font:** Project administration, Funding acquisition, Writing – review & editing. **M. Lozano:** Software, Formal analysis. **L. Manyes:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2021.112130.

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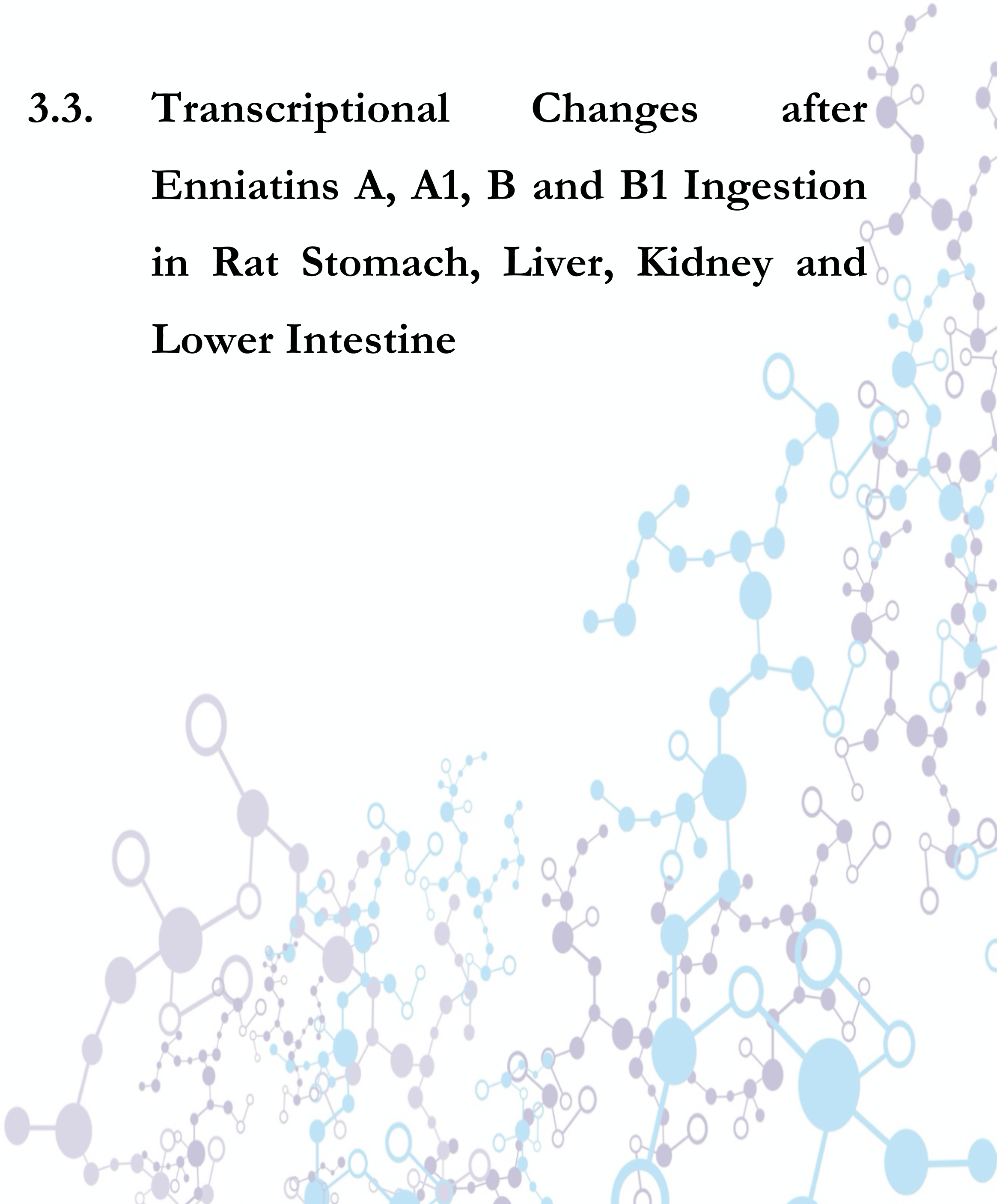
3.3. Transcriptional Changes after Enniatins A, A1, B and B1 Ingestion in Rat Stomach, Liver, Kidney and Lower Intestine



Foods

Transcriptional Changes after Enniatins A, A1, B and B1 Ingestion in Rat Stomach, Liver, Kidney and Lower Intestine

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Abstract

Enniatins (ENs) are depsipeptide mycotoxins produced by *Fusarium* fungi. They are known for their capacity to modulate cell membrane permeability and disruption of ionic gradients, affecting cell homeostasis and initiating oxidative stress mechanisms. The effect of the acute toxicity of ENs A, A1, B and B1 at two different concentrations after 8 h of exposure was analysed in Wistar rats by a transcriptional approach. The following key mitochondrial and nuclear codified genes related to the electron transport chain were considered for gene expression analysis in stomach, liver, kidney and lower intestine by quantitative Real-Time PCR: mitochondrially encoded NADH dehydrogenase 1 (MT-ND1), mitochondrially encoded cytochrome c oxidase 1 (MT-COX1), succinate dehydrogenase flavoprotein subunit A and ATP synthase F1 subunit alpha, respectively. Moreover, the expression of markers involved in oxidative stresssuperoxide dismutase 1 (SOD1), glutathione peroxidase 1 (Gpx1), heme oxygenase 1, apoptosis B-cell lymphoma 2, Bcl2 Associated protein X (Bax), tumor suppressor protein (p53), inhibition of apoptosis nuclear factor kappa of activated B cells, immune system interleukin 1β and intestinal tight junction Occludin merely in lower intestine tissues have been investigated. For mitochondrial genes, the main differences were observed for MT-ND1 and MT-COX1, showing its deficiency in all selected organs. With regard to the intestinal barrier's cellular response to oxidative stress, the activity of the antioxidant gene SOD1 was decreased in a dose- dependent manner. Similarly, the catalytic enzyme GPx1 was also downregulated though merely at

medium dose employed. On the contrary, the pro-apoptotic Bax and p53 regulators were activated after ENs exposure, reporting a significant increase in their expression. Furthermore, the alteration of intestinal permeability was assessed by the abnormal activity of the tight junction protein occludin. In summary, ENs may generate mitochondrial disorders and induce oxidative stress in intestinal barrier function.

Keywords: enniatins; oxidative phosphorylation; in vivo; quantitative Real-Time PCR (qPCR)

1. Introduction

In spite of many years of research and the introduction of good practices in the food production, storage and distribution chain, nowadays mycotoxins are a big risk to food safety. A useful tool capable to protect consumers against their toxic effects is the HACCP for production and storage, but currently this system only applies to legislated mycotoxins (Gil et al., 2016). In the past, emerging mycotoxins have been considered less important because of their low probability of acute toxicity. Nevertheless, they have a high prevalence in food products, sometimes even in high concentrations (Escrivá et al., 2015).

Regarding enniatins (ENs) toxicity, limited data is available until now, hence they are currently under occurrence and toxicity evaluation (Stanciu et al., 2017). ENs are emerging mycotoxins produced by filamentous fungi of *Fusarium* genus, mainly by F. *acumuniatum*, F. *avenaceum*, *F. oxysporum, F. Poae, F. sporothrichioides, F. Sambucinum* and *F. Tricinctum* species (Frayeman et al., 2018). They can be found in a wide variety of food and feed, mainly in cereals and their derivatives, dried fruit, spices, cocoa and coffee. ENs chemical structure corresponds to a cyclic depsipeptide and so far, more than 23 belonging to A, B and J types, have been identified (Prosperini et al., 2017). However, the most frequent ENs detected in food and feed are enniatin A (ENA), enniatin A1 (ENA1), enniatin B (ENB), enniatin B1 (ENB1) (Jonsson, 2017).

The toxicity of ENs is based on their ionophoric properties. They facilitate the transport of mono or divalent cations such as K^+ or Ca^{2+} across membranes, thereby disrupting normal physiological concentrations of these

ions Gruber-Dorninger et al., 2017). Moreover, it has been shown that ENs can induce a cytotoxic effect by producing ROS and subsequently causing lipid peroxidation and alteration of the normal cell cycle due to their anti-proliferative effects on several cell types (Frayeman et al., 2017). Furthermore, they can decrease calcium retention capacity of the mitochondrion matrix leading to the collapse of the mitochondrial membrane potential via permeability transition pore opening and they can cause oxidative phosphorylation decoupling (Maranghi et al., 2018).

The European Food Safety Authority reported that ENB induced genotoxic effect *in vivo* after acute oral administration (Cimbalo et al., 2020). Recently, *in vivo* toxicity of ENs was reviewed, reporting immunotoxicity in peripheral blood lymphocytes in Wistar rats. ENB was found in high concentration in rats' liver and fat, demonstrating the molecules tendency to bioaccumulate in lipophilic tissues. Jejunum, duodenum and colon were also identified as a possible absorption area for ENA in female rats, during a sub-chronic exposure (Jonsson et al., 2016).

In vitro studies have confirmed ENs toxicity in several cell lines. ENB alone or in mixture with other ENs, induced lysosomal disruption and necrosis in Caco-2 cells, lysosomal damage in RAW 267.4 macrophages and apoptosis in H4IIE hepatocyte cell line (Juan-García et al., 2013). Likewise, ENs induced nuclear fragmentation and apoptotic body formation in A549, GLC-4, KB-3-1 and HL-60 cells (Alonso-Garrido et al., 2018). Moreover, the linkage between ENs toxicity and the alteration of mitochondrial related pathways has been recently investigated *in vitro*. Transcriptomics helped to observe how

mitochondria were the main affected organelles in Jurkat cells after ENB exposure, finding the highest expression alterations in oxidative phosphorylation related genes (Alonso-Garrido et al., 2020a). ENs toxicity in ECV304 cells was also confirmed in the expression of several genes belonged to complex I (CI), IV and V, although ATP synthase resulted the most affected (Alonso-Garrido et al., 2020b). Furthermore, after ENB and BEA mixture exposure, proteomics analysis revealed changes in the protein levels in the inner and outer membrane in Jurkat cells in a concentration dependent-manner (Bustin et al., 2009).

In order to better understand ENs toxicity, Wistar rats were chosen as an *in vivo* model to analyze changes in the expression of selected genes involved in ETC, oxidative stress, apoptosis, inflammation and intestinal tight junction in rat stomach, liver, kidney and lower intestine. More specifically, mitochondrially encoded NADH dehydrogenase 1 (MT-ND1) belonging to CI, succinate dehydrogenase flavoprotein subunit A (Sdha) of complex II (CII), mitochondrially encoded cytochrome c oxidase 1 (MT-COX1) of CIV and ATP synthase F1 subunit alpha (ATP5) of CV activity was assessed in all the organs considered. Furthermore, oxidative stress markers superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (GPx1), heme oxygenase 1 (Hmox1), apoptosis regulators B-cell lymphoma 2 (Bcl2), Bcl2 Associated protein X (Bax), tumor suppressor protein p53, nuclear factor kappa of activated B cells (Nf-κB) inflammation cytokine interleukin 1β (Il-1βand intestinal tight junction protein Occludin were analyzed in colon tissues.

2. Materials and Methods

2.1 Reagents

The standards of standard solution stock (purity: 99%) of ENA (mw: 681.92 g/mol), ENB (mw: 639.82 g/mol), ENA1 (mw: 667.87 g/mol), ENB1 (mw: 653.85 g/mol) and phosphate buffer saline (PBS) were obtained from Sigma–Aldrich (Madrid, Spain). All the stock solutions were prepared by dissolving 1 mg of mycotoxin in 1 ml of pure methanol, obtaining a 1 mg/ml (1000 mg/l) solution. These stock solutions were diluted with methanol in order to obtain the appropriate multi-compounds working standard solutions. All the standards were kept at −20 °C.

For RNA extraction, TRIzol™ reagent was purchased from Invitrogen™ (USA) whereas for its purification was employed ReliaPrep™ RNA Miniprep System kit from Promega (USA). Deionized water (resistivity < 18 MV cm) was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA). TaqMan™ Reverse Transcription kit and PowerUp™ SYBR™ Green for qPCR analysis were purchased from Applied Biosystems (Carlsbad, CA, USA).

2.2 In vivo study design

Fourteen female Wistar rats (243-278 g) were acquired from Pharmacy animal facility (Universitat de València, Spain). The institution Animal Care and Use Committee of the University of Valencia approved all animal procedures (protocol nº A13338818442265). Animals were divided in three groups: four rats

in the control group and five rats in each treated one, with medium and high exposure. Each group was housed in one cage in a windowless room with a 12-h light-dark cycle. The study rooms were maintained under controlled conditions appropriate for the species (temperature 22 °C, relative humidity 45-65%). The control group was exposed to the vehicle (PBS), while five of the treated ones were intoxicated with medium concentrations: single dose of EN A 256, ENA1 353, ENB 540, ENB1 296 μg/mL; and other five with the higher ones: single dose of ENA 513, ENA1 706, ENB 1021, ENB1 593 μg/mL. Mycotoxins were administered dissolved by sonication in 1 mL of PBS by oropharyngeal administration using a metal cannula. After 8 h exposure, with water but no feed, they were sacrificed by isoflurane gas asphyxiation and the organs (liver, stomach, kidneys, lower intestine) were removed and stored at -20 °C.

2.3 RNA extraction

Total RNA of the control and exposed rats was isolated using from approximately 50 mg of frozen tissue according to TRIzol™ manufacturer's protocol (Invitrogen™, USA). Samples were homogenized in TRIzol™ (50 mg/mL) with a T25 Ultra-turrax Digital High-Speed Homogenizer (IKA®, Germany). Extracted RNA was purified according to ReliaPrep™ RNA Miniprep System kit (Promega, USA). The purity and quantity of RNA were evaluated spectrophotometrically using a NanoDrop™ 2000 (Thermo Scientific™, Spain), showing concentrations between 370 and 2359 ng/μl and appropriate 260/280 nm and 260/230 nm ratios both around 2 (Table 1). RNA

samples were stored until their dilution to 100 ng/μl with pure Milli-Q H2O until their reverse transcription to cDNA.

2.4. Gene Selection and Primer Design

Gene-specific primers were designed using Primer-BLAST by using default criterion of the software with amplified products ranging from 83 to 122 bp and Tm at 58 °C. Primer sequences were used in qPCR analyses. Standard curve by qPCR was performed for all primer's pairs and a single amplification product for each gene was obtained by the melting curve assay StepOne Plus Real-time PCR instrument (Applied Biosystems, Foster city, CA, USA). Primer amplification efficiency was determined from standard curve generated by serial dilution of cDNA (5-fold each) for each gene. Correlation coefficient (R2 values) and amplification efficiencies (E) for each primer pairs were calculated from slope of regression line by plotting mean Cq values against the log cDNA dilution factor in StepOne software (Table 2).

Table 2. Target organ, samples: control (C), medium dose

	Sample	**[RNA](ng/μL)**	**260/280**	**260/230**
Stomach	C1	738.4	1.93	2.07
	C2	370.8	1.90	2.26
	C3	246.7	1.85	1.95
	C4	1051.2	2.01	2.15
	M1	546.4	1.89	1.76
	M2	647.1	1.96	2.07

	M3	923.2	1.95	2.10
	M4	1003.8	1.98	2.21
	M5	1312.1	1.96	2.07
	H1	607.8	1.92	2.08
	H2	1177.1	1.99	2.21
	H3	1388.9	2.02	2.01
	H4	652.0	1.92	2.08
	H5	1259.3	1.99	2.21
Liver	C1	664.1	2.10	2.24
	C2	695.8	2.10	2.20
	C3	493.9	2.07	2.10
	C4	638.0	2.11	2.28
	M1	914.3	2.16	2.24
	M2	2199.2	2.12	2.05
	M3	1613.9	2.17	2.22
	M4	1592.0	2.15	2.14
	M5	1340.0	2.18	2.14
	H1	1875.7	2.13	2.17
	H2	1017.6	2.15	2.32
	H3	1199.6	2.16	2.35
	H4	1502.6	2.16	2.31
	H5	1334.5	2.15	2.31
Kidneys	C1	1197.6	2.07	2.27
	C2	2359.5	2.01	2.16
	C3	1550.3	2.12	2.14
	C4	1615.1	2.1	2.25

	M1	1995.0	2.10	2.18
	M2	1585.0	2.07	2.26
	M3	1606.0	2.07	2.22
	M4	1211.6	2.11	2.16
	M5	600.8	2.06	2.26
	H1	329.6	2.06	2.25
	H2	247.4	2.11	2.30
	H3	715.7	2.10	2.15
	H4	615.0	2.10	2.15
	H5	2407.3	2.12	2.25
Colon	C1	1728.0	2.16	2.20
	C2	1581.0	2.18	2.20
	C3	759.0	2.17	2.22
	C4	2306	2.10	2.14
	M1	1838.0	2.15	2.20
	M2	1891.0	2.14	2.17
	M3	1407.0	2.16	2.23
	M4	1360.0	2.18	2.23
	M5	1619.0	2.17	2.19
	H1	1961.0	2.17	2.19
	H2	1736.0	2.16	2.19
	H3	838.0	2.17	2.21
	H4	1084.0	2.17	2.21
	H5	859.0	2.16	2.20

Table 3. Gene symbol, forward (F) and reverse (R) primers, efficiency and linearity of the selected genes plus reference genes β-actin and 18S rRNA.

Gene	Sequence	Efficiency	Linearity
MT-ND1	F: CGAGCTCCCTTCGACTTAAC R: GAATAGGGCGAATGGTCCTG	101.806	0.991
Sdha	F: GACGATCTCTGCGGTATGAC R: TCGGTGTATGGACCCATCTT	107.658	0.975
MT-COX1	F: GCTGGAGCATCCGTAGATTT R: ATTGGGTTATAGCAGGGGGT	106.425	0.985
ATP5	F: GTGATGTGTCCGCCTACATT R: ACAAGCCCACATTAATGGCA	107.423	0.978
SOD1	F: ACACAAGGCTGTACCACTGC R: CCACATTGCCCAGGTCTCC	124.573	0.991
GPx1	F: GTCCACCGTGTATGCCTTCTCC R:TCTCCTGATGTCCGAACTGATTGC	105.269	0.990
Hmox1	F: CACGCATATACCCGCTACCT R: AAGGCGGTCTTAGCCTCTTC	146.284	0.981
Bcl2	F: ACTGAGTACCTGAACCGGCATC R: GGAGAAATCAAACAGAGGTCGC	148.237	0.990
Bax	F: AAGAAGCTGAGCGAGTGTCT R: CAAAGATGGTCACTGTCTGC	117.003	0.981
p53	F: GTTCCGAGAGCTGAATGAGG R: TTTTATGGCGGGACGTAGAC	111.153	0.990
Nf-κB	F: CTTCTCGGAGTCCCTCACTG R: CCAATAGCAGCTGGAAAAGC	102.480	0.996
Il-1β	F: CTTGTCGAGAATGGGCAGTCT R: TGTGCCACGGTTTTCTTATGG	105.269	0.990
Occludin	F: AGTACATGGCTGCTGCTGATG	143.830	0.990

	R: CCCACCATCCTCTTGATGTGT		
β-actin	F: AACCTTCTTGCAGCTCCTCCG R: CCATACCCACCATCACACCCT	94.242	0.96
18S rRNA	F: GAGCGTGTGATCACCATCAT R: TCCTTCACGTCCTTCTGTCT	105.487	0.979

2.5. Reverse transcription and qPCR

Real-time amplification reactions were performed in 96 well plates using SYBR Green detection chemistry and were run in triplicate on 96-wells plates with the StepOne Plus Real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). Reactions were prepared as follows: 100 ng template, 500 μM of each primer, the required amount of 2x Fast SYBR Green and completed to 20 μL with RNAse free water (Applied Biosystems, Foster City, CA, USA). The cycling conditions were set as default: initial denaturation step of 95 ◦C for 5 min to activate the Taq DNA polymerase, followed by 40 cycles of denaturation at 95 ◦C for 15 s, annealing at 58 ◦C for 15 s and elongation at 72 ◦C for 30 s. The melting curve was generated by heating the amplicon from 60 to 90 ◦C. Therefore, threshold cycles (Ct) were automatically determined using the StepOne Plus Software version 2.3 (Applied Biosystems, Foster, CA, USA). Three technical replicates were performed for each condition. Experiments were performed according to MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines [16].

2.5. Statistical analysis

Normalized Cp were calculated per sample as ΔCt (experimental Ct—housekeeping Ct mean) by using Ct values obtained by qPCR. A t-Student test was applied to evaluate differences between each mycotoxin exposed sample group and the control considering $p \leq 0.05$ as statistically significant. Statistical analysis was assessed by SPSS 24.0 (IBM Corp., Armonk, NY, USA). For gene expression analysis, three technical replicates of each sample were analyzed for control group (C1, C2, C3, C4), medium dose treated group (M1, M1, M2, M3, M4, M5) and high dose treated group (H1, H2, H3, H4, H5). Log2RQ median of all genes was calculated for each condition, considering C1 as Log2RQ = 0.

3. Results

3.1. Activity of mitochondrial and nuclear encoded genes in stomach

Transcriptional analysis of selected mitochondrial genes was performed by qPCR technique. In the first organ studied, a remarkable downregulation of mitochondrial encoded MT-ND1 at highest dose was observed when compared to the control. Likewise, nuclear encoded genes Sdha and Atp5 resulted downregulated but solely after medium treatment (Log2RQ=-3.5) (Figure 1).

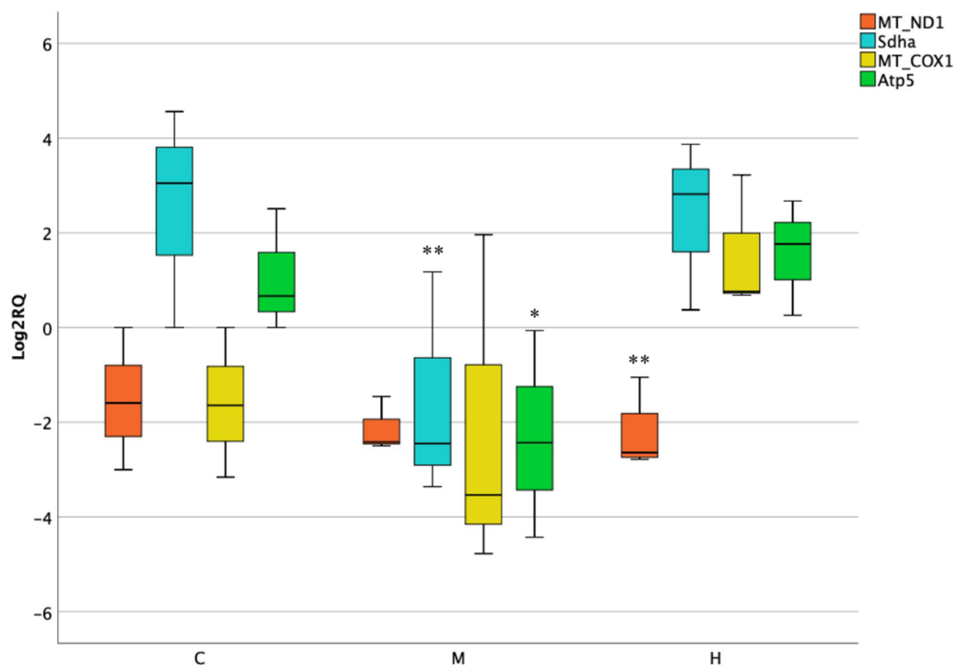


Figure 1. Box plot showing relative expression of mitochondrial and nuclear encoded genes in stomach when compared to control C1 (Log2RQ=0) to medium treatment (M) and high treatment (H) by qPCR. RQ, relative quantification. The box plots show the median value (horizontal line), the top and bottom edges of the boxes (75% and 25% percentiles) and whiskers which are the furthest values away from the boxes that are not considered outliers. *p < 0.05; **p < 0.01 significantly different from the control.

3.2. Activity of Mitochondrial and Nuclear Encoded Genes in Liver

The hepatic investigation after acute exposure to ENs reported significant changes of all genes analyzed when treated with a medium dose. Once administering higher dose, merely MT-ND1 and MT-COX1 revealed a considerable decrease of roughly twofold compared to the control ($p = 0.0002$). Nevertheless, a moderate interindividual variability was observed in the control groups of the latter genes (Figure 2).

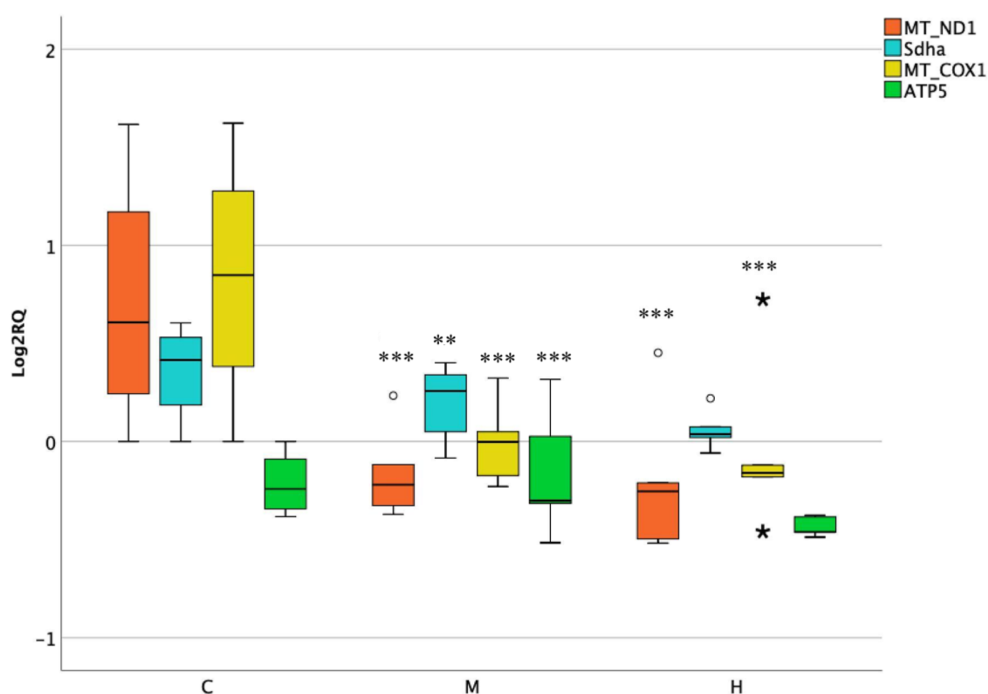


Figure 2. Box plot showing relative expression of mitochondrial and nuclear encoded genes in liver when compared to control C1 (Log2RQ=0) to medium treatment (M) and high treatment (H) by qPCR. RQ, relative quantification. The box plots show the median value (horizontal line), the top

and bottom edges of the boxes (75% and 25% percentiles), (★) extreme cases, (o) outliers (atypical values) and whiskers which are the furthest values away from the boxes that are not considered outliers. **p < 0.01; ***p < 0.001 significantly different from the control.

3.1. Activity of mitochondrial and nuclear encoded genes in kidneys

Regarding kidneys, the expression of MT-ND1 and MT-COX1 was strongly affected at medium dose. A significant down-regulation of both genes was observed (p=0,0001), although showing a noticeable interindividual variability among samples. Similarly, Sdha followed the same trend but in this case, finding a down-regulation of nearly 4-fold in a dose-dependent manner (Figure 3).

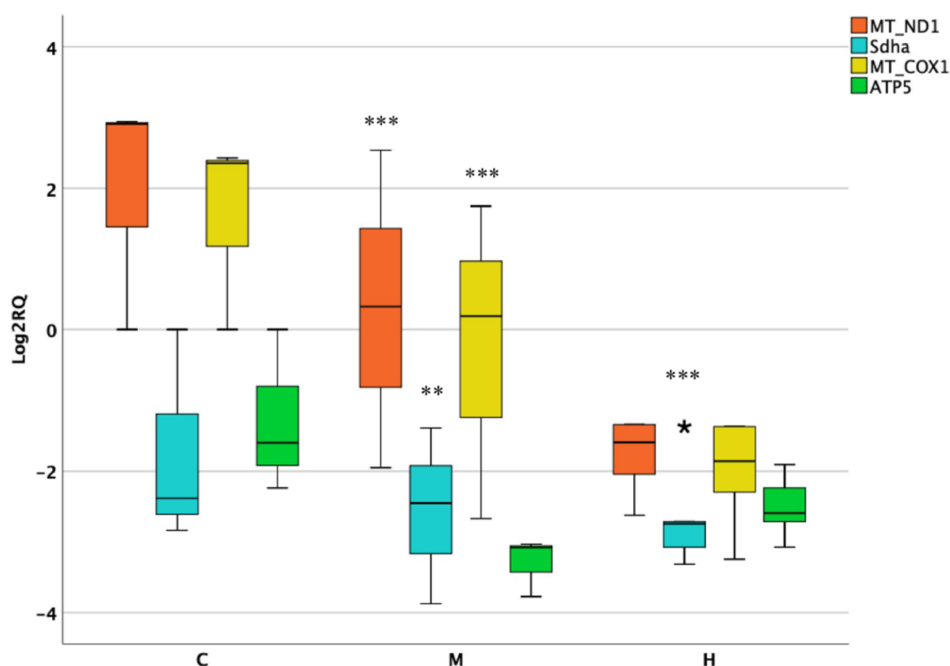


Figure 3. Box plot showing relative expression of mitochondrial and nuclear encoded genes in kidney when compared to control C1 (Log2RQ=0) to medium treatment (M) and high treatment (H) by qPCR. RQ, relative quantification. The box plots show the median value (horizontal line), the top and bottom edges of the boxes (75% and 25% percentiles), (★) extreme cases and whiskers which are the furthest values away from the boxes that are not considered outliers. **$p < 0.01$; ***$p < 0.001$ significantly different from the control.

3.1. Activity of mitochondrial and nuclear encoded genes in lower intestine

When studying rat's colon tissues, MT-ND1 and MT-COX1 were significantly downregulated at medium dose, but not at the higher one. On the contrary, nonsignificant changes were observed for nuclear encoded genes. (Figure 4).

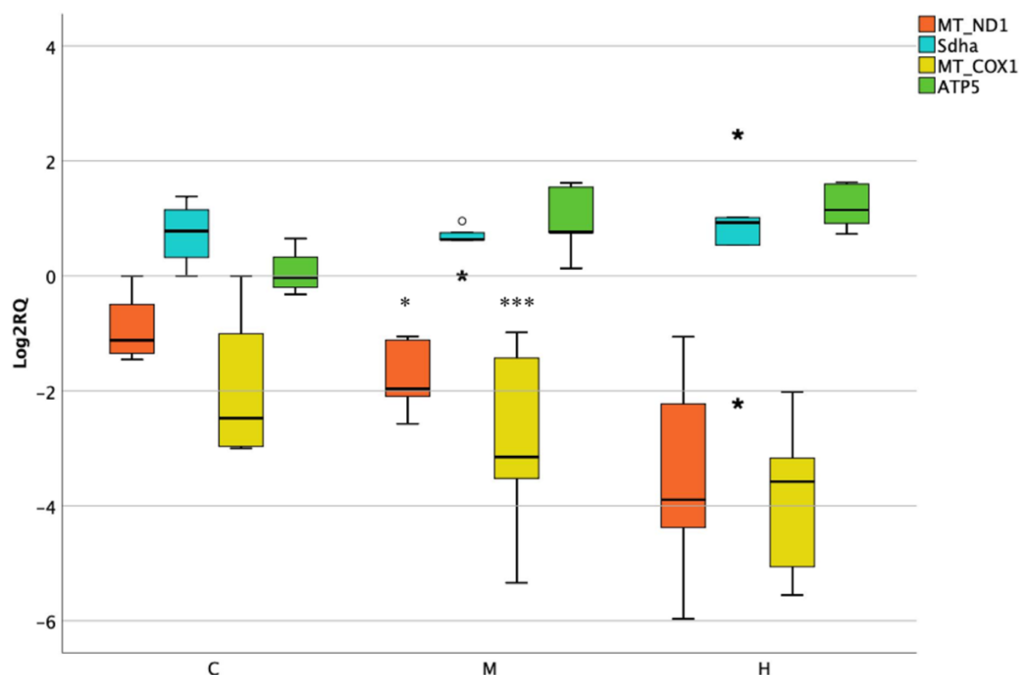
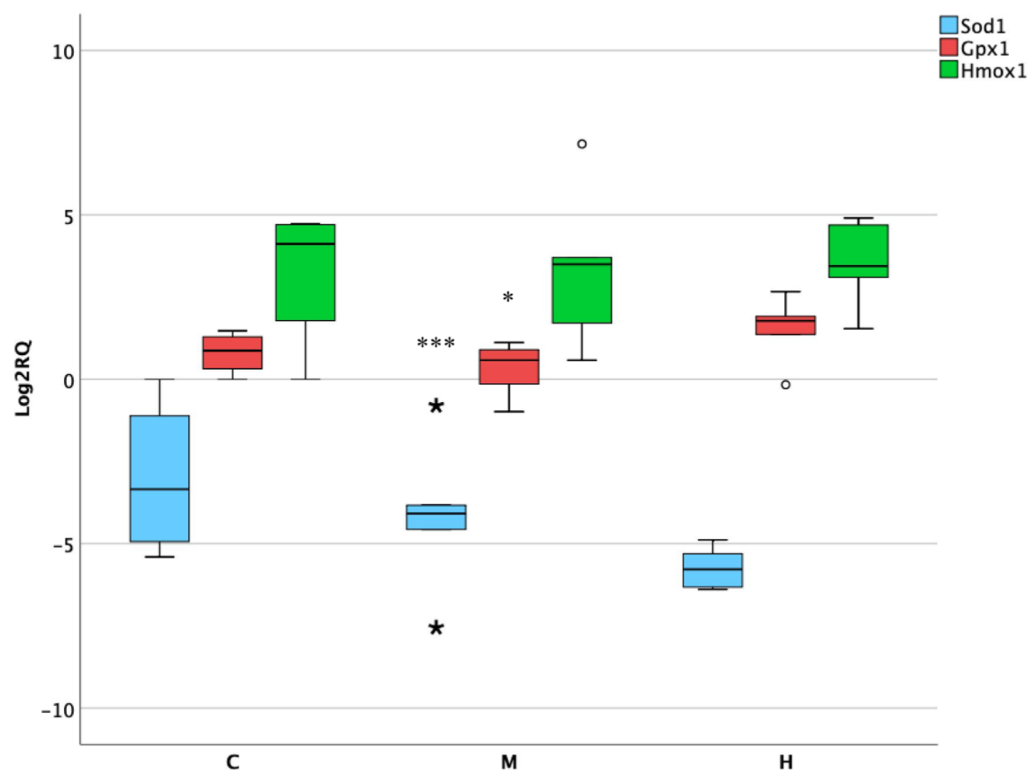


Figure 4. Box plot showing relative expression of mitochondrial and nuclear encoded genes in lower intestine when compared to control C1 (Log2RQ=0) to medium treatment (M) and high treatment (H) by qPCR. RQ, relative quantification. The box plots show the median value (horizontal line), the top and bottom edges of the boxes (75% and 25% percentiles), (★) extreme case and whiskers which are the furthest values away from the boxes that are not considered outliers. *$p < 0.05$; ***$p < 0.001$ significantly different from the control.

3.1. Activity of oxidative stress genes in lower intestine

The intestinal tract is frequently exposed to ROS production and the associated activation antioxidant enzyme defense systems. For this purpose, genes involved in oxidative stress processes were selected. After the treatments, the most altered was SOD1, showing a loss of its expression which was significant at medium dose (p=0,0002). Besides, the antioxidant enzyme GPx1



was slightly down-regulated but had a weaker response to the medium treatment (p=0,05) (Figure 5).

Figure 5. Box plot showing relative expression of oxidative stress genes in lower intestine when compared to control C1 (Log2RQ=0) to medium treatment (M) and high treatment (H) by qPCR. RQ, relative quantification. The box plots show the median value (horizontal line), the top and bottom edges of

the boxes (75% and 25% percentiles), (★) extreme cases, (o) outliers (atypical values) and whiskers which are the furthest values away from the boxes that are not considered outliers. **p < 0.01; ***p < 0.001 significantly different from the control.

3.1. Activity of apoptotic genes in lower intestine

Apoptosis is an important process which maintains the function of intestinal barrier at normal state by regulating its homeostasis. In this study the activity of genes implicated in apoptotic pathways was evaluated. The expression of pro-apoptotic genes Bax and p53 showed an overexpression of nearly 4-fold changes at both doses. On the contrary, nuclear factor Nf-κB and anti-apoptotic gene Bcl2 did not report significant changes when compared to the control (Figure 6).

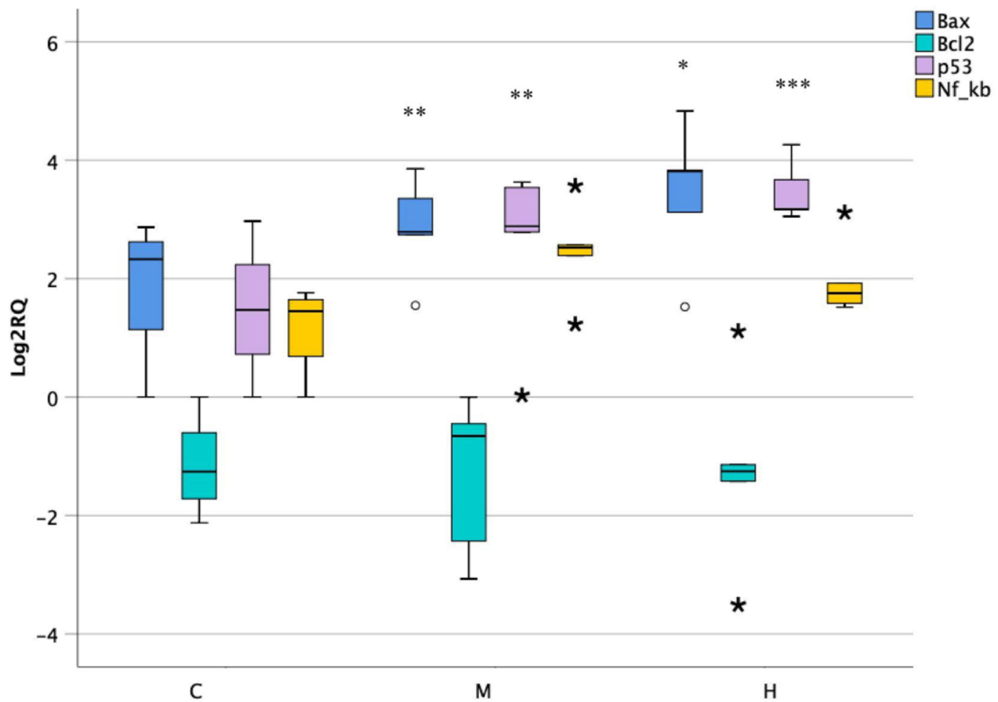


Figure 6. Box plot showing relative expression of apoptotic genes in lower intestine when compared to control C1 (Log2RQ=0) to medium treatment (M) and high treatment (H) by qPCR. RQ, relative quantification. The box plots show the median value (horizontal line), the top and bottom edges of the boxes (75% and 25% percentiles), (★) extreme cases, (o) outliers (atypical values) and whiskers which are the furthest values away from the boxes that are not considered outliers. *p < 0.05; **p < 0.01; ***p < 0.001 significantly different from the control.

3.1. Inflammatory response and permeability in lower intestine

The activation of oxidative stress and apoptotic processes was checked by the activity of pro-inflammatory cytokine Il-1β, which surprisingly did not report significant alterations after ENs exposure. Moreover, the function of intestinal permeability was evaluated by assessing the expression of occludin, an integral membrane protein localized at intestinal epithelial tight junction barrier. In this case, results have shown a significant increase in its expression in both medium and high treatment, reaching up to 10-fold (Figure 7).

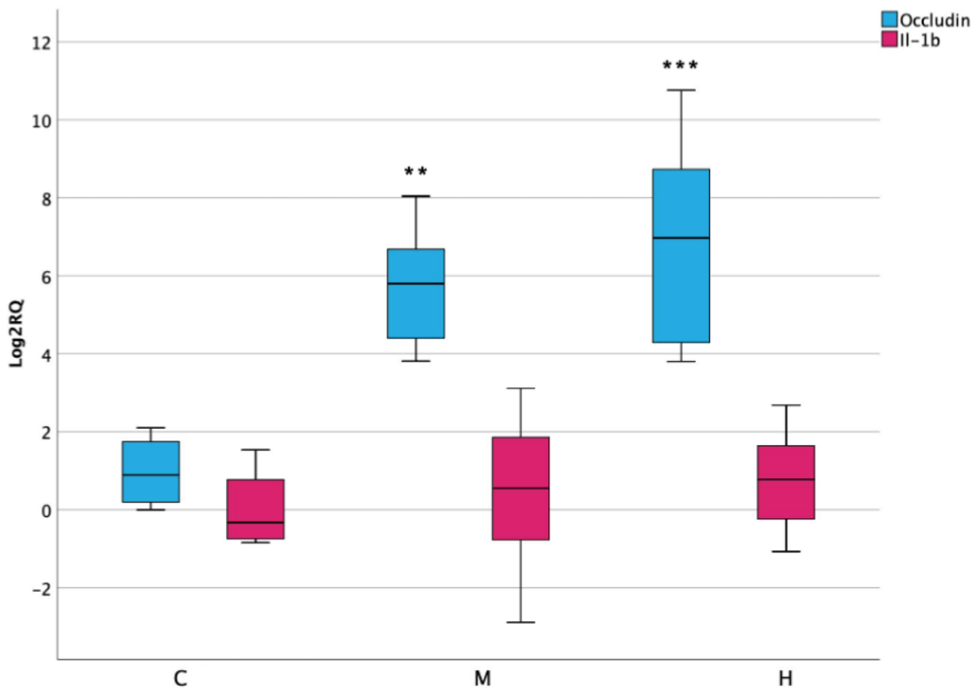


Figure 7. Box plot showing relative expression of Il-1β in lower intestine when compared to control C1 (Log2RQ=0) to medium treatment (M) and high treatment (H) by qPCR. RQ, relative quantification. The box plots show the median value (horizontal line), the top and bottom edges of the boxes (75% and 25% percentiles) and whiskers which are the furthest values away from the boxes that are not considered outliers. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ significantly different from the control.

3. Discussion

A recent proteomics approach confirmed the toxic capacity of ENs after acute exposure in rat's liver and pointed to diverse biological processes as a target (Cimbalo et al., 2021). Proteomics findings showed that 13 of the significantly altered proteins were involved in the mitochondria, among which are ATP5 alpha and beta. ATP5 alpha protein expression was enhanced at both doses (log2FC medium dose: 4.1144; high dose: 2.2354) as well as antioxidant activity, showing the upregulation of four proteins including the SOD1 enzyme, but solely at the highest exposure (log2FC high dose:9.6712). In this study, using those liver samples and adding the stomach, kidney and lower intestine from the same animals, changes in the expression of selected genes related to mitochondria, oxidative stress, apoptosis, inflammatory response and permeability processes were evaluated. These findings have shown that acute exposure to ENs caused changes in the expression of all analyzed genes depending on the type of tissue.

Regarding the gene expression in the ETC, the first complex, mitochondrial CI defi- ciency is the most prevalent defect in the respiratory chain causing mitochondrial disorders. The activity of CI subunit 1 MT-ND1 is involved in the formation of the fourth proton pumping site by promoting proton translocation across the membrane (Iommarini et al., 2018). After ENs administration, it has been observed a leakage of its expression in all organs analyzed, which could be related to an impairment of membrane homeostasis. According to these findings, lower activity of this gene was implicated in non-alcoholic fatty acid liver disease in rats and chronic kidney disease in mice (García-Ruiz et al., 2010; Guo et al., 2017). However, on the contrary, elevated levels of MT-ND1 expression indicated mitochondrial dysfunction in the villous adenoma of human tissue, resulting with the accumulation of mutations in mtDNA (Wallace et al., 2016).

Secondly, succinate-coenzyme Q reductase is a mitochondrial enzyme complex con- sisting of four protein subunits (Sdha, Sdhb, Sdhc and Sdhd), which is involved in the tricarboxylic acid cycle (TCA) and the ETC (Huang et al., 2013). The loss of the normal TCA cycle promotes tumorigenesis due to metabolic alterations with enforced dependence on glycolysis for energy production (Miettinen et al., 2014). CII Subunit -a was altered by ENs mixture, being downregulated in rats' stomach, liver and kidneys, hinting at an impairment caused by these mycotoxins. According to previous findings, the lack of this gene is associated with mitochondrial dysfunction, which led to the development of wild type gastrointestinal stromal tumors and hereditary renal cell carcinoma (Boikos et al., 2016; Kamai et al., 2019).

Thirdly, cytochrome *c* oxidase, localized in the inner mitochondrial membrane, is the final electron acceptor in the ETC and its deficiency is a prevalent cause of oxidative stress status in mitochondria (Abdulhag et al., 2015). MT-COX1 gene is the main subunit of the complex, and it is responsible for the homeostatic synthesis of prostanoids (Jimenez et al., 2010). The expression of MT-COX1 was significantly decreased in the liver at both doses of ENs employed, along with renal and intestinal tissues, although solely at medium dose. Its downregulation is related to the progression of esophageal adenocarcinoma, colon carcinogenesis, reduced proliferation and increased macro autophagy (Cathcart et al., 2012; Martín-Sanz et al., 2010). Moreover, MT-COX1 deficiency exacerbates hepatic diseases in mice (Xiao et al., 2015) as well as in the present research, suggesting that ENs' toxicity could play a role in hepatic function. Contrasting with this evidence, MT-COX1 resulted as overexpressed in mammalian and zebrafish kidneys under oxidative stress conditions (Harris, 2013; Sarkar et al., 2017).

The last complex studied was mitochondrial ATP synthase (CV), which is formed of two functional domains: F_1, situated in the mitochondrial matrix and F_O, located in the inner mitochondrial membrane. It produces most of the cell ATP by rotary catalysis, and its deficiency plays a crucial role in severe human disorders such as neuropathy, ataxia, encephalopathy (Leigh syndrome) and hypertrophic cardiomyopathy (Jonckheere et al., 2012; Aiyar et al., 2014). ATP5, the subunit α of CV F_1 domain, was downregulated in gastric and hepatic tissues, but solely after medium treatment. According with these findings, reduced levels of its expression led to the decrease of oxidative phosphorylation in chicks, prostate and lung cancer, decrease in tissue metabolism, reduction in

protein synthetic capacity and impairment of ATP-biosynthetic functions in rat liver (Zhang et al., 2018; Feichtinger et al., 2018). In contrast, Xu and collaborators (2016) reported highly overexpressed ATP5 in glioblastoma tumor cells and endothelial cells of microvascular proliferation.

Beyond the mitochondrial dysregulation, several studies in vitro have demonstrated ENs intestinal effects in specific cell lines, reporting its cytotoxicity and the activation of oxidative stress processes, even at low molecular concentrations (Bertero et al., 2020). The activity of three essential markers implicated in cellular response to oxidative stress was evaluated in vivo after ENs acute exposure. SOD1 is an antioxidant enzyme which regulates the superoxide levels from cytosol and mitochondrial intermembrane (Eleutherio et al., 2020). It has been observed that its deletion is related to ROS production and the reduction of antioxidant enzymes activities in colon tissues, agreeing with the downregulation observed in the present research (Gonzalez-Menendez et al., 2018). Similarly, it has been observed that the higher activity of the key catalytic enzyme GPx1 promote the progression of distinct types of cancer, including colon cancer, which is in line with the overexpression observed after the highest dose of ENs mixture employed in female rats (Gan et al., 2014; Wei et al., 2020). As regard the intestinal inflammation modulator Hmox1, it has been shown to inhibit the activation of apoptotic pathways in different cell lines but in this case, did not show significant changes in its expression (Ribeiro et al., 2017).

The homeostasis of the intestinal barrier is also disturbed by an imbalance that occurs between pro- and anti-apoptotic genes. In particular, it has been demonstrated that the p53 gene is involved in the initiation of the

apoptosis mechanisms of the cellular cycle and its overexpression has been associated with different types of intestinal cancer (Ryter et al., 2021; Kim et al., 2021). Moreover, tumor suppressor p53 regulates the anti-apoptotic (Bcl2) and pro-apoptotic (Bax) members of the Bcl-2 proteins family, which control apoptosis by monitoring mitochondrial outer membrane permeabilization (Dashzeveg et al., 2015). In this study, p53 and Bax genes were upregulated after ENs treatment, whereas on the contrary, anti-apoptotic Bcl-2 did not show significant changes, suggesting apoptosis activation.

As a consequence of the development of oxidative-stress-induced cellular damage and apoptosis, the onset of inflammation and structural disfunction can successively occur (Netea et al., 2017). The activity of the structural protein occludin, for instance, is related to intestinal epithelial disorders and abnormal secretory function (Zhao et al., 2021). Its upregulation in intestinal cells was implicated in the decrease of gut permeability in accordance with this result, hinting the activation of defense mechanisms (Zhou et al.,2019).

5. Conclusions

ENs generated toxic responses in rat tissues at the mitochondrial level at the medium and high concentrations employed, more evident for MT-ND1 and MT-COX1 in all organs analyzed. Furthermore, the activation of oxidative stress and pro-apoptotic genes was shown in lower intestine tissues but not confirmed by inflammatory cytokine activity Il-1β. At the structural level, changes in the epithelial barrier have been observed, suggesting the decrease of barrier permeability. In conclusion, the results obtained in this study suggest that

ENs could play a role in mitochondrial disorders and intestinal acute toxicity. However, there is a need of further investigation in ENs long-term exposure in order to survey their possible chronic effect on animals.

Author Contributions

Conceptualization, L.M.; methodology, L.M.; software, A.C.; formal analysis, A.C., M.A.-G. and M.F.; investigation, A.C., M.A.-G. and M.F.; writing—original draft preparation, A.C. and M.A.-G.; writing—review and editing, G.F. and L.M.; supervision, G.F. and L.M.; project administration and funding acquisition, G.F. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the institution Animal Care and Use Committee of the University of Valencia (protocol no. A13338818442265).

Conflicts of Interest

The authors declare no conflict of interest.

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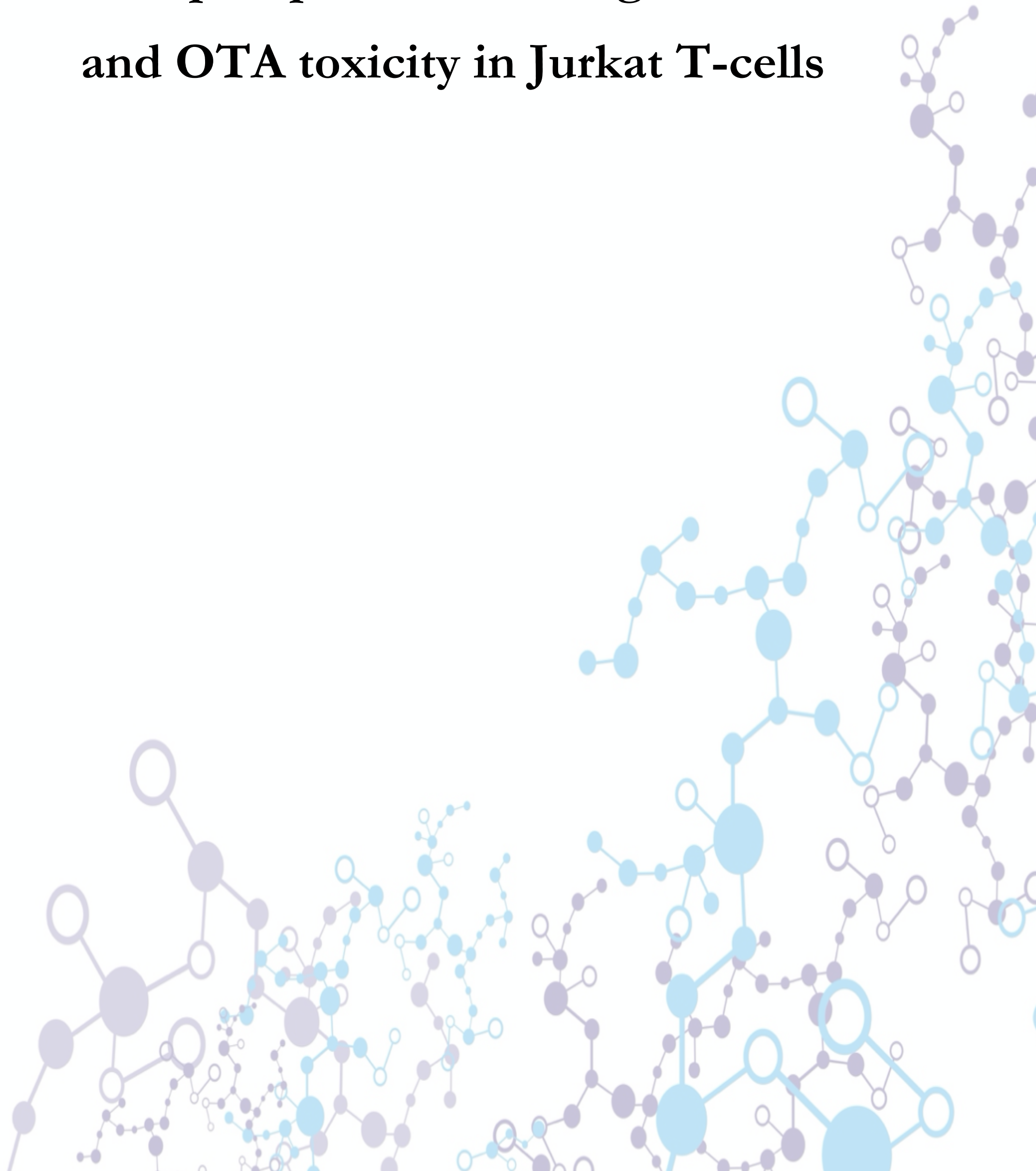
3.4. Protective role of fermented whey and pumpkin extract against AFB_1 and OTA toxicity in Jurkat T-cells



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Protective role of fermented whey and pumpkin extract against AFB_1 and OTA toxicity in Jurkat T-cells

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Abstract

The beneficial effect of fermented whey and pumpkin extract rich in carotenoids was evaluated in Jurkat cells against aflatoxin B_1 (AFB_1) and ochratoxin A (OTA) cytotoxicity through a proteomic approach. The functional ingredients were added into mycotoxin contaminated bread formulation, which digested *in vitro* in order to simulate human intestinal absorption. Cell cultures were exposed during 7 days to these mycotoxins dissolved in: a) 0.1% organic solvent (DMSO), b) in intestinal digest of bread with pumpkin individually (PID) and c) in intestinal digest of bread with pumpkin mixed with whey fermented (PID+WF). Extracted proteins were subjected to reduction and alkylation and subsequently a tryptic digestion in order to be analyzed by liquid chromatography coupled with quadrupole time of flight (Q-TOF). A total of 496 unique proteins at 1% false discovery rate was detected for DMSO conditions, 352 for PID and 316 for PID+WF using Spectrum Mill software (Agilent). Later, differentially expressed proteins were statistically evaluated through Mass Professional Profiler software (Agilent). Results obtained show the identification of proteins involved in several metabolic pathways, mainly in gluconeogenesis, antioxidant activity and nucleosome assembly. Furthermore, histones' expression implicated in the metabolic pathway of nucleosome assembly (H2A, H2B, H2C, H3 and H4) was increased when exposing cells to functional ingredients, as well as repression of cyclin A2, a profile associated to limiting the growth of carcinogenic cells.

Keywords: aflatoxin B1, ochratoxin A, proteomics, Jurkat cells, LC/Q-TOF

1. Introduction

Aflatoxin B_1 (AFB_1) is the most potent natural carcinogenic toxin which contaminate crops and a wide variety of food materials, such as cereals, nuts, milk, spices, vegetables, fruits, oils and meat (Kowalska *et al.*, 2017). They are polyketide compounds synthesized by secondary metabolic pathway in *Aspergillus* and they have been demonstrated to be carcinogenic, teratogenic and mutagenic in nature (Smith, 2020). AFB_1 is absorbed in the small intestine and metabolized firstly in the liver to release reactive intermediate metabolites but its carcinogenicity has been associated also to remote organs such as kidney, pancreas, bladder, bone, viscera and central nervous system (Benkerroum, 2020). Another widespread mycotoxin, ochratoxin A (OTA), contaminates a great variety of foodstuffs, such as grapes, coffee, cocoa, and infant food. It is a pentaketide derived from dihydrocoumarins family produced by fungi of *Penicillium* and *Aspergillus* genera and has been shown to be nephrotoxic, hepatotoxic, teratogenic and immunotoxic to humans and animals (Tao *et al.*, 2018). As regard its mechanism of action, it is rapidly absorbed in the proximal tubule of the kidney but slowly eliminated and excreted leading to potential accumulation in the body (Zhu *et al.*, 2017) Due to their high toxicity, the Panel on Contaminants in the Food Chain has reported a scientific opinion on the human health risks related to the presence of AFs and OTA in food and estimated its dietary exposure of the European Union population (EFSA, 2020).

Cereal and cereal-based products are classified as one of the leading sources of energy and minerals as well as vitamins for human and animal (Khanegan *et al.*, 2019). However, these products can be contaminated by several

mycotoxin species in the field after harvest and storage, or as a consequence of grain infection by fungi. Food processing and innovative technologies can prevent mycotoxins formation and promote its degradation but nonetheless, they cannot eliminate the risk (Gavahian *et al.*, 2020). In order to reduce mycotoxin's absorption and toxicity in humans and animals, the use of functional ingredients has been studied. For instance, the protective effect of dietary antioxidant with high content of carotenoids against mycotoxins induced oxidative stress has been observed at mitochondrial transcriptional level after AFs and OTA exposure in ECV304 cells (Alonso-Garrido *et al.*, 2021; Alonso-Garrido *et al.*, 2020). Moreover, it has been shown that they can prevent the onset of cardiovascular diseases by blocking the formation of low-density lipoproteins, improve the communication of GAP-type intercellular junctions, protect cells from mycotoxin-induced toxicity (Juan-Garcia *et al.*, 2019) and limit abnormal cell growth, reducing the risk of certain types of cancers.

Several bioactive compounds, such as probiotic bacteria, are involved in different functions contributing to human health by acting as antioxidant, anti-inflammatory, and immune modulating mediators (Chugh & Kamal-Eldin, 2020). Among them, milk fermented whey (WF) is a functional food by-product formed in milk coagulation step of cheese manufacturing process, which is able to inhibit mycotoxigenic strains growth in food products (Izzo *et al.*, 2020a, Izzo *et al.*, 2020b, Dopazo *et al.*, 2021). Recently, Escrivá *et al.*, (2021) evaluated the bioaccessibility and bioavailability of bioactive compounds from milk whey and yellow mustard flour- both fermented with *Lactobacillus plantarum* and non-fermented and evidenced these ingredients may have promising applications in food industry as natural preservatives. Moreover, it has been observed that the

addition of milk whey ingredient in contaminated bread formulation, increases its shelf life and antioxidant activity (Luz *et al.*, 2021).

For this purpose, the aim of the present work was to evaluate the *in vitro* effect of gastrointestinal digest of bread prepared with fermented whey and pumpkin extract on AFB_1- and OTA- induced cytotoxicity in Jurkat cells through a proteomic approach.

2. Material and methods

2.1 Reagents

The standards of Standard solution stock (purity: 99%) of AFB_1 and OTA and phosphate buffer saline (PBS) were obtained from Sigma–Aldrich (St. Louis, MO, USA). All the stock solutions (1000 mg/l) were prepared by dissolving 1 mg of mycotoxin in 1 ml of pure methanol. These stock solutions were diluted with methanol in order to obtain the appropriate multi-compounds working standard solutions. All the standards were kept at −20 °C.

DL-Dithiothreitol (DTT), ≥ 99.0%, Trizma® hydrochloride (Tris-HCl) for proteins extraction, ≥99.0% and Trypsin for digestion, were purchased from Sigma Aldrich (St. Louis, MO, USA). Iodoacetamide (IAA), 98% was acquired from ACROS Organics™, Thermo Fisher Scientific (New Jersey, USA). Thiourea (TU), 99% for lysis buffer preparation was obtained from Thermo Fisher Scientific (Kandel, Germany) and Urea (U), 99% was acquired from F.E.R.O.S.A (Barcelona, Spain). Deionized water (resistivity < 18 MV cm) was

obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2 Cell culture and treatments exposure

Jurkat T cells were grown in a humidified incubator with 5% CO_2 and 95% air atmosphere at constant humidity (37°C), using RPMI-glutamax medium supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin and 10% inactivated FBS. Cells were seeded at a density of $5x10^5$ cells/mL in 6-well plates and subsequently exposed to the different treatments. Firstly, cells were treated with AFB_1 and OTA 100 nM in 0.1% DMSO and the solvent concentration as control during 7 days. Secondly, cells were exposed to intestinal digests of two types of bread prepared with lyophilized pumpkin and milk whey fermented (1:10), single or in combination with mycotoxins during 7 days. Carotenoid's content and profile of intestinal digests are reported in Table 1. All the experimental conditions performed were: a) AFB_1 and OTA, individually or in combination, at 100 nM in 0.1% DMSO for 7 days; b) intestinal digest of pumpkin bread (PID), AFB_1 and OTA, individually or in combination; c) PID mixed with fermented whey (WF), AFB_1 and OTA, individually or in combination (Table 2)

Table 1. Concentration (μg/mL) and profile of carotenoids identified from gastrointestinal digests of pumpkin (PID) and PID with fermented whey (WF).

	Carotenoids concentration (μg/mL)					
Bread	β-Carotene	Lutein	Anteraxanthin	Violaxantin	Zeaxanthin	β-Cryptoxantin
PID	0.7364	0.0181	n.d.	1.3309	0.0264	1.0241
PID+AFB_1	0.7091	0.0050	0.0001	0.9779	0.0067	0.6988
PID+OTA	0.5394	0.0036	n.d.	0.4978	0.0053	0.3627
PID+AFB_1+OTA	0.2742	0.0036	0.0002	0.6654	0.0019	0.5470
PID+WF	1.8636	0.0180	n.d.	3.3676	n.d.	2.9880
PID+WF+AFB_1	2.5152	0.0122	0.0054	3.0368	0.0054	2.3976
PID+WF+OTA	4.000	0.0120	n.d.	3.3971	n.d.	2.3614
PID+WF+AFB_1+ OTA	2.2879	0.0086	0.0580	2.7426	0.0580	1.8554

Table 5. AFB_1 and OTA Concentrations of each condition employed: A) mycotoxin standard in organic solvent (DMSO), B) intestinal digest of bread with pumpkin (PID), C) intestinal digest of bread with pumpkin and whey fermented (PID+WF)

	Treatment	**Mycotoxin concentration [nM]**	
		AFB_1	**OTA**
	DMSO 0.1 %	-	-
A	DMSO 0.1 %+AFB_1	-	100
	DMSO 0.1 %+OTA		-
	DMSO 0.1 %+OTA+AFB_1	100	100
	Pumpkin bread intestinal digest (PID)		
	PID	-	-
B	PID +AFB_1	137	-
	PID+OTA	-	777
	PID+AFB_1+OTA	191	704
	Fermented whey-Pumpkin bread intestinal digest (PID)		
	PID+WF	**-**	**-**
C	PID+WF+AFB_1		1968
	PID+WF+OTA	-	1037
	PID+WF+AFB_1+OTA	187	837

2.3 Protein extraction

Cell's pellet was washed by deionized water to neutral pH, then dried and resolved in lysis buffer (8 M U/2 M TU/50 mM Tris-HCl). Afterwards, was homogenized by vortexing and sonified 3 times using a methanol: H_2O mixture until protein solubilization (USC 1200D ultrasonicator, VWR, International bvba, Leuven, Belgium). Samples were centrifuged for 30 min at 4 °C and 13000 g and the supernatant was collected in order to quantify protein concentration using a NeoDot UV/Vis Nano Spectrophotometer (Quimigen, Madrid, Spain).

2.4 Protein denaturation, alkylation, enrichment and digestion

Firstly, samples were standardized to 1 mg/mL and subsequently reduced with DTT (200mM, pH 7.8) for 1 hour at 60 ºC. Therefore, IAA 200 mM (pH 7.8) was added for alkylation of cysteine residues and incubated for 30 minutes at 37 ºC. Protein digestion was initiated with addition of trypsin (1:40) and incubated once again for 16 hours. Digestion was stopped with addition of 5% acetic acid down to pH 5. Samples were dried for 2 hours in a vacuum concentrator (lyophilizer Freezone 2.5 freeze dryer Benchtop, Labconco) at a -40 ºC temperature and vacuum pressure of 0.080 mBar. After the lyophilization, peptides were eluted in 0.1 % acetic acid: acetonitrile (98:2 v/v) to a final concentration of 100 µg/µL.

2.5 Q-TOF mass spectrometry and data analysis

Samples were analyzed using a LC system coupled with quadrupole time of flight (Q-TOF) in duplicate per biological sample. A C18 bioZen™ 2.6 µm, 120 Å, 50 x 2.1 mm (Phenomenex, California, USA) was used to achieve chromatographic separate peptides separation. The injection volume was 10 µL and the column temperature was maintained at 45ºC. Total run time was 40 min, at a flow rate of 0.4 mL/min. The eluent consisted of two different phases: A (H_2O containing 0.1% formic acid) and B (ACN containing 0.1% formic acid). The gradient elution was applied with an initial 3% of phase B for 1 min, which went up to 20% in 21 min. Afterwards, the gradient increased to 95% over 3 min and subsequently decreased at 3% in the last 10 min.

As regard Q-TOF-MS conditions, drying gas flow (N2) was 13.0 L/min; nebulizer pressure, 35 psi; gas drying temperature 325 ºC; capillary voltage 4000 V; nozzle voltage 500 V; fragmentor voltage,175 V; skimmer voltage 65 V and octopole RF peak, 750 V. Positive ionization mode was employed by using Dual AJS ESI interface and positive ions were acquired in the range of 100–3000 m/z for MS scans, and 50–3000 m/z for auto MS/MS scans, at a scan rate of 8 scans/s for MS and 3 scans/s for MS/MS, respectively. MS/MS automatic acquisition mode was carried out using a ramped collision energy with charge state preference of 2, 3, >3 and slope 3.1, 3.6 and 3.6, respectively. Two reference masses of 121.0509 and 922.0098 m/z were used as internal mass correction. Agilent MassHunter Workstation software B.08.00 (Agilent Technologies) was used as instrument controller and data acquisition.

2.6 Bioinformatics and statistical analysis

Spectra were processed using the Spectrum Mill MS Proteomics Workbench Package Rev B.06.00.201 software (Agilent Technologies), which is capable of analyzing data from high quality spectra reducing false positive number and using the Uniprot catalog to identify matched proteins and peptides. MS/MS parameters of spectra were searched and validated according to Cimbalo *et al.*, (2021). Identified proteins were exported to the Mass Profiler Professional (MPP) 15.0 version software (Agilent Technologies) to perform statistical data analysis. This analysis was carried out based on the total spectra intensity of the proteins which were considered as entities in MPP. The baseline of the spectra was adjusted to the mean across all samples. The entities were then filtered based on their frequency of occurrence across at least all replicates of one group. Contrasts for each condition between the experimental mycotoxin dose front the control were analyzed using unpaired t-test with Benjamin-Hochberg adjustment. Results were considered significant with a fold change $\geq$ 0.7 and p-values cut-offs < 0.05. Finally, biological processes, molecular functions and metabolic pathways related to these proteins were identified in a meaningful way by using a database for integrated protein annotation, visualization and discovery (DAVID) (Huang *et al.*, 2009a; Huang *et al.*, 2009b).

3. Results

3.1 Identification and quantification of proteins

The analysis of Jurkat cells exposed to different conditions of AFB_1 and OTA, individually and mixed, and their controls (DMSO 0.1%, PID, PID+WF) was performed by gel-free shotgun proteomic approach. A total of 496 unique proteins at 1% false discovery rate was detected for 0.1% DMSO treatment, 352

for the PID treatment and 316 for PID+WF. Subsequently, differentially expressed proteins were identified by a comparison of any treatment and the control groups (fold change ≥ 0.7 and a p-value ≤ 0.05), as determined by a t-test (See supplementary material for detailed results). From 154 of differentially expressed proteins in DMSO treatment, 74 have shown higher and 80 lower expression levels when compared to the control (Table S1). After PID treatment, 64 were found upregulated and 18 downregulated compared to the control (Table S2). Finally, from 85 proteins identified after PID + WF treatment, 58 displayed higher and 27 lower expressions with respect to the control (Table S3).

3.2 Differentially expressed proteins in Jurkat cells after AFB_1 exposure

After 0.1% DMSO + AFB_1 (100 nM) exposure, gluconeogenesis was as the biological process and metabolic pathway showing more changes in protein expression, identifying glycerol-3-phosphate dehydrogenase (GPDH), glucose-6-phosphate isomerase (GPI), phosphoglycerate kinase (PGK) and phosphoglycerate mutase (PGM) as major proteins, in all cases with lower expression levels compared to the control. Moreover, polyadenylated RNA binding resulted the main molecular function, in which 17 proteins were altered. Among them, ATP-dependent RNA helicase and histone H4 were slightly upregulated (1.17 - 4 LogFC) whereas RNA polymerase II, peroxiredoxin significantly repressed (LogFC= -4) (Table S4).

When cells were exposed to PID + AFB_1, among the overexpressed proteins, there were 4 proteins involved in nucleosome assembly, 2 of mucosal

innate immune response and 2 of natural killer (NK) cell-mediated cytotoxicity. The most significant protein involved in these processes is the antioxidant protein peroxiredoxin 1 (PRDX1). Furthermore, analysing the molecular functions, 7 proteins involved in heterodimerization and protein kinase binding were altered, particularly ATP-dependent RNA helicase, PRDX1, histone H2A, H2B, H4 were significantly overexpressed. As regard the metabolic pathways, 6 proteins involved in telomere stress-induced senescence and senescence-associated phenotype were found. The main proteins involved in this metabolic pathway are histones of the H2A, H2B, H3, H4, I, C family, all with higher expression levels compared to the control and cyclin A2, which was down-regulated.

Finally, upon exposure of cells with PID + WF + AFB_1, the stimulation of biological processes such as mitochondrial tRNA methylation, gene silencing by RNA and nucleosome assembly was observed, in addition to the activation of certain molecular functions such as polyadenylated RNA binding and protein kinase binding. Senescence-associated phenotype, transcriptional regulation by small RNA and DNA methylation were the main metabolic pathways activated after treatment.

3.3 Differentially expressed proteins in Jurkat cells after OTA exposure

Proteins expressed after exposure to OTA (100 nM) in presence of DMSO (Table 6) exert their biological processes as translational regulators (whose main proteins are the eukaryotic translation initiation factor 4A1 and the ribosomal proteins S28, S30 and SA), cotranslational proteins, or as part of

gluconeogenesis. In terms of their molecular functions, the binding to polyadenylated RNA and the contribution to ribosome structure stand out. The major proteins were mitochondrial ATP synthase, eukaryotic translation elongation factor 2 and 4A, and nucleolin. When PID was added together with OTA, the expressed proteins turn out to be significantly involved in the combined processes of nucleosome assembly, cell division and regulation of serine/threonine kinase activity, and in the molecular functions of protein heterodimerization or histone binding. Finally, in the PID + WF + OTA condition, proteins involved in nucleosome assembly, superoxide radical scavenging and hydrogen peroxide catalysis are expressed. The molecular functions are related to antioxidant capacity and thioredoxin peroxidase activity (Table S5)

3.4 Differentially expressed proteins in Jurkat cells after AFB_1 + OTA exposure

Biological processes, molecular functions and metabolic pathways in which differentially expressed proteins were involved after exposure with 0.1% DMSO, PID and PID + WF in combination with AFB_1 and OTA are shown below in Table S6.

When cells were exposed to AFB_1 and OTA standards at 100 nM (0.1% DMSO), activation of biological processes related to gluconeogenesis or translational regulation was observed. In addition, 15 proteins in polyadenylated RNA binding and 13 in ATP were stimulated. The addition of pumpkin highlights 4 proteins involved in biological processes related to nucleosome assembly, in the defense response to Gram-positive bacteria 2 in the regulation

of ubiquitination. The most characteristic molecular functions were histone binding and protein heterodimerization activity. Finally, when pumpkin and WF were added in combination, 3 proteins of nucleosome assembly and 2 of innate immune defense of the mucosa were altered in its expression. On the other hand, the most significant molecular functions were heterodimerization activity of 4 proteins, binding to DNA of 5 proteins and pyridoxal phosphate binding of 2 proteins.

As for metabolic pathways, the most significant one observed after exposure with 0.1% DMSO turned out to be glycolysis and gluconeogenesis, while adding PID with or without WF, 5 proteins related to secretory phenotype associated with senescence and 4 to DNA methylation were identified.

5. Discussion

Bioactive compounds are substances which naturally occur in human diet and have been identified as having a preventive role against specific diseases caused by food contaminants. This study combined the beneficial effect of pumpkin and fermented whey contained in intestinal digest of bread against AFB_1 and OTA cytotoxicity in Jurkat cells, by focusing on its proteomics profile.

Among the biological processes in which identified differentially expressed proteins participate, gluconeogenesis stands out. This process converts non-carbohydrate substrates (such as lactate, amino acids and glycerol) into glucose under fasting conditions and is modulated by external factors such as nutrients, energy, exercise and stress reactions (Zhang *et al.*, 2019). In this

work, 5 of the enzymes implicated in gluconeogenesis process, such as GPDH, GPI, PGK and PGM, were commonly downregulated after DMSO treatment along with mycotoxins. In particular, deficiency of GPDH and GPI strongly downregulated after mycotoxins exposure (LogFC = -4), was implicated in hepatic steatosis and nonalcoholic fatty liver (Zheng *et al.*, 2019; Lin *et al.*, 2020). Moreover, the loss of PGK observed also in the present study after AFB_1 (LogFC=-0.1672) and OTA (LogFC=-0.4104) exposure alone, but not in combination, is associated with early prognosis in cancer, since is the first enzyme to produce ATP in the glycolytic pathway (Fu and Yu, 2020). As regard PGM, which catalyzes the reversible transformation of 3-phosphoglycerate to 2-phosphoglycerate, was downregulated after single mycotoxin exposure (LogFC=4) but upregulated with mix (LogFC=0.4309). Based on these latter results, it has been reported that PGM overexpression induced necroptosis in primary cardiomyocytes whereas on the contrary, its deficiency increased mitochondrial DNA and transcripts levels, normalized mitochondrial respiration and repressed mitochondrial ROS production (Zhu *et al.*, 2021).

Regarding the antioxidant activity, peroxiredoxins and thioredoxins expression changed depending on treatment employed in Jurkat cells. These proteins protect cells from oxidative aggressions produced by mycotoxins and help to maintain their natural homeostasis. In particular, peroxiredoxins catalyze the formation of peroxides species**,** in order to balance H_2O_2 content at cellular level, since they are essential to regulate signaling and oxidative stress mechanisms (Ye *et al.*, 2019). When cells were exposed to AFB_1 and OTA alone or in combination in 0.1% DMSO, PRDX1 decreased its expression down to **4-fold** when compared to the control. On the contrary, when functional

ingredients with antioxidant capacity were added, PRDX 1 and PRXD2 were overexpressed up to 4-fold, along with TRX-1 which was probably activated by the synergistic action of the two mycotoxins. According to that, in *vitro* and *in vivo* studies reported higher expression of peroxiredoxin and thioredoxin as a consequence of the increased cells' natural defenses after different mycotoxins exposure, alone or in combination (Brennan *et al*., 2017). Moreover, higher expression of peroxiredoxins was observed in this work in biological processes related to the mucosal innate immune response, superoxide radical scavenging and H_2O_2 catabolic process.

Nucleosome assembly is a process by which DNA is packaged into nucleosomes by assisting chromatin replication, in order to maintain genome stability. Each nucleosome is composed of an octamer of four core histones which are H2A, H2B, H3 and H4. The expression of histones, which are highly basic proteins that help the assembly of new nucleosomes, facilitate genome replication and conservation of hereditary epigenetic information (Liu *et al*., 2017; Zhao and Shilatifard, 2019). It has been demonstrated that the dysregulation of certain chromatin-associated proteins act as drivers in certain type of cancer. Low expression of histone variants, for instance, was associated with a higher amount of DNA damage in cervical cancer cell lines, suggesting a correlation with DNA repair mechanism (Li *et al*., 2017). In this study, when exposing cells to functional ingredients and mycotoxins, the overexpression of 6 histones species, (H1, H2A, H2B, H2C, H3, H4) was observed in all conditions employed. Moreover, histone mutations which are partly incorporated into chromatin such as H1.3 and H3.3, have been identified in cancer cell growth by changing the structural properties of nucleosome (Arimura

et al., 2018). Also in this case, the histone variants H1.3 and H3.3 levels were strongly increased (LogFC=4) after exposing cells to OTA and mix. Hence, pumpkin and WF could promote the development of new nucleosomes contributing to genome conservation.

Considering metabolic pathways, senescence-associated secretory phenotype related was the most frequent found in cells treated with intestinal digest. This metabolic pathway sensitizes cells and renders them senescent to prevent genomic stress processes such as DNA damage, telomere shortening and chromatin alteration from inducing the loss of vital cell functions. In addition, it has the ability to stimulate the expression of immune-related proteins and paralyze cell proliferation in the process of cancer formation, including promoting tissue repair and development (Özcan *et al.*, 2016). In all conditions where this metabolic pathway was altered, the expression of cyclin A2 was commonly decreased when compared to the control. This protein, has an essential role in progression of cell cycle phase transitions and its up-regulation has been confirmed in certain types of cancer (Pei *et al.*, 2019). On the contrary, Tu *et al.* (2019) demonstrated that down-regulation of cyclin A2 and cyclin-dependent kinases studied in colon cancer cell lines attenuated cell proliferation, as well as in the present study. Based on these findings, functional ingredients can act as protectors against genomic stress caused by AFB_1 and OTA also in this case, preventing the loss of vital cell functions and paralyzing the growth of carcinogenic cells.

6. Conclusions

Digested intestinal extracts of bread enriched with pumpkin and whey fermented contributed positively to the normal functioning of biological processes involved in cells activity against AFB_1 and OTA toxicity, especially gluconeogenesis, antioxidant activity and nucleosome assembly. Moreover, the overexpression of proteins involved in the metabolic pathway of the secretory phenotype associated with senescence (histones of the H2A, H2B, H3 and H4 families) and repression of cyclin A2 confirms the preventive effect of these functional ingredients.

Conflicts of interest

All authors declare have no conflict of interest (financial or non-financial) in the subject matter or materials discussed in this manuscript.

Author's contribution

A.Cimbalo: visualization, conceptualization, experimental part and writing original draft. **M. Frangiamone**: experimental part and writing original draft. **M. Lozano:** methodology, data curation, statistical analysis. **L. Escrivá:** review & editing**. Vila-Donat:** writing final draft, review & editing. **L. Manyes:** conceptualization, funding acquisition, project administration and supervision, review & editing**.**

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4. GENERAL DISCUSSION

4. Discusión general

4. Discusión general

Para alcanzar los objetivos propuestos en la presente Tesis Doctoral, se ha realizado en primer lugar una revisión bibliográfica sobre los efectos toxicológicos que las micotoxinas pueden inducir en la salud humana, centrándose en las diferentes técnicas analíticas utilizadas. Por otra parte, se ha evaluado mediante cromatografía liquida de alta resolución acoplada a espectrometría de masas de tiempo de vuelo (LC-MS-QTOF) la toxicidad aguda de las ENs en hígado de ratas Wistar, analizando su perfil proteómico. Asimismo, se han evaluado cambios de expresión génica en hígado, estómago, riñón y colon de las mismas ratas mediante el uso de la PCR cuantitativa en tiempo real (qPCR). Finalmente, se ha estudiado el efecto beneficioso del suero de leche fermentado y de los carotenoides de la calabaza sobre la toxicidad de AFB1 y OTA en la línea celular Jurkat de linfocitos T humanos mediante un enfoque proteómico.

4.1 Toxicidad de las micotoxinas en vertebrados

En la actualidad, los estudios *in vivo* en Europa son limitados debido a su complejidad y, sobre todo, a las nuevas normas de cuidado y uso de animales vivos con fines científicos, que se rigen por los principios de sustitución, reducción y perfeccionamiento establecidos internacionalmente. Además, las consideraciones éticas y de bienestar animal exigen que se limite al máximo el uso de animales. En este sentido, la Directiva 2010/63/UE sobre la protección de los animales utilizados con fines científicos se incorporó a la legislación nacional de cada Estado miembro de la UE en 2013 y apoya la aplicación del

principio de las tres erres (3R) a la hora de considerar la elección de los métodos a utilizar. No obstante, el uso de animales representa la única forma de corroborar la toxicidad en humanos (Clark, 2018). La Organización Mundial de Sanidad Animal (OIE), en el Código Sanitario para los Animales Terrestres, reconoce el papel vital que desempeña el uso de animales vivos en la investigación y la educación (OIE, 2019). De hecho, los animales de laboratorio, en particular las ratas y los ratones, que son las especies más utilizadas, se han empleado con éxito para la detección de varias enfermedades. Sin embargo, en realidad se consideran modelos deficientes para la mayoría de las enfermedades humanas (Baumans, 2016).

En los últimos años, se han desarrollado varios esfuerzos para minimizar el contenido de micotoxinas en los alimentos. La degradación de los metabolitos fúngicos tóxicos puede ser un enfoque ideal para eliminar o neutralizar la actividad de las toxinas si el proceso no altera su composición nutricional. Se pueden utilizar métodos físicos, químicos y biológicos para inactivar o eliminar completamente las micotoxinas en los alimentos, tales como: plasma a presión atmosférica fría en la demolición de los patógenos de las plantas, hidrólisis, ammonización, ozonización, peroxidación, el uso de ácido clorhídrico, ácido ascórbico, bisulfito de sodio, peróxido de hidrógeno, hidróxido de amonio, bacterias probióticas y enzimas (Abbas, 2019; Čolović et al., 2019). A pesar de todos los intentos por reducir su contenido en los alimentos, la investigación no ha logrado resolver este importante problema. Por lo tanto, es necesario perseguir el estudio de su toxicidad. La literatura revisada muestra que las micotoxinas más estudiadas *in vivo* en la última década fueron AFB1(22%), DON (18%), ZEA (16%) y OTA (15%), mientras que las menos estudiadas fueron

FBs (7%), PAT (4%), CIT (2%), AOH y las micotoxinas emergentes (1%). Curiosamente, las combinaciones de micotoxinas también fueron frecuentes, especialmente con AFB1, DON y ZEA. Además, se investigó el efecto de varios compuestos naturales y biológicos para contrarrestar su modo de acción tóxico. Como era de esperar, los animales de laboratorio empleados predominantemente fueron los roedores (ratones y ratas) y los cerdos, mientras que los menos estudiados fueron los pollos, los pavos, los peces, las alpacas y las ovejas. El principal órgano analizado fue el hígado, seguido de la sangre, el riñón, el bazo, el intestino, el timo, el pulmón, los órganos reproductores, el cerebro, la orina, la piel, el páncreas, el embrión y el corazón. En cuanto al objetivo principal de las búsquedas, la mayoría se centró en la inmunotoxicidad y un porcentaje menor lo constituyeron los estudios de genotoxicidad, estrés oxidativo, hepatotoxicidad, citotoxicidad, teratogenicidad y neurotoxicidad.

Para estudiar la toxicidad de las micotoxinas, se empleó una amplia gama de técnicas diferentes, entre las cuales la más utilizada fue la qPCR, seguida de ELISA, IHC, WB, LC-MS/MS, microscopía, PCR, ensayos enzimáticos, microarray, RNA-Seq, FCM, CA, HP, GE, HPLC-FLD. Menos utilizados fueron DNA-Seq, LC-EC/MS/MS, AHA, ensayos de anticuerpos, orbitografía, LC- TOF-MS, DCFH-DA, SDS-PAGE, MLR, MiSeq, semiqPCR, UPLC-Q-TOF/ MS. Además, el estudio de los mecanismos de estrés oxidativo inmunológico y el análisis transcriptómico, a través de prometedoras técnicas innovadoras y precisas, poseen un papel muy importante en la comprensión de los mecanismos de acción de estos metabolitos secundarios.

4.2 Estudio proteómico de las ENs *in vivo*

Estudios *in vitro* han demostrado la toxicidad de las ENs, que es dependiente de sus propiedades ionofóricas y lipofílicas. Estos metabolitos secundarios pueden incorporarse a las bicapas lipídicas de las membranas celulares y crear poros selectivos que provocan un aumento de la permeabilidad para los cationes mono y divalentes como el K^+ o el Ca^{2+}, lo que da lugar a alteraciones en la homeostasis celular.
Además, se ha observado que las ENs pueden producir especies reactivas de oxígeno (ROS) y, posteriormente, provocar la alteración del ciclo celular debido a sus efectos antiproliferativos. Sin embargo, a pesar de los estudios presentes, los datos disponibles de micotoxinas emergentes son a día de hoy limitados, por lo que actualmente se sigue evaluando su toxicidad.

El presente estudio profundiza el mecanismo de acción de las ENs a diferentes concentraciones en ratas Wistar a través de un enfoque proteómico. El análisis de los hígados de ratas expuestas a dos concentraciones diferentes de ENs permitió la identificación de 108 proteínas únicas y 57 diferencialmente expresadas entre los grupos tratados y el control. Además, de 57 de estas proteínas con abundancia diferenciada respecto a los controles, 41 proteínas mostraron niveles más altos y 12 más bajos en los grupos tratados. Entre las proteínas que mostraron la misma tendencia (aumento o disminución) con la concentración de las mezclas de micotoxinas, la proteína más abundante fue la carbamoil-fosfato sintasa, mitocondrial, mientras que la actina-1 tuvo el nivel más bajo. El análisis bioinformático reveló que la acetilación, la región de unión a fosfato de nucleótidos:NAD y la actividad catalítica eran los términos más

representados. Además, 13 de estas proteínas se encontraban en la mitocondria y 12 estaban relacionadas con la actividad oxidorreductasa. En cuanto a la sobrerrepresentación de Reactome, el metabolismo fue tanto la vía más significativa como la más enriquecida.

El hígado es un órgano metabólico esencial donde la glucosa se convierte en piruvato a través de la glucólisis en el citoplasma, y el piruvato se oxida posteriormente en las mitocondrias para generar ATP a través del ciclo TCA y la fosforilación oxidativa. En el estado de alimentación, los productos glucolíticos se utilizan para sintetizar los ácidos grasos mediante la lipogénesis de novo. En el estado de ayuno, el hígado segrega glucosa a través de la glucogenólisis y la gluconeogénesis. Un metabolismo energético aberrante en el hígado promueve la resistencia a la insulina, la diabetes y las enfermedades del hígado graso no alcohólico (Rui, 2014).

En cuanto a la acetilación, en este estudio se encontraron alteradas 33 proteínas relacionadas con ese término. La acetilación desempeña funciones especialmente importantes en el metabolismo material y energético al modificar la actividad y/o la especificidad de ciertas enzimas y sustratos, regulando así el metabolismo de la glucosa, los lípidos y los aminoácidos. Los estudios relacionados con los seres humanos han indicado que el cambio en el patrón de acetilación de las proteínas está asociado con la aparición y/o el desarrollo de enfermedades relacionadas con el metabolismo, como la obesidad, las enfermedades cardiovasculares, la diabetes y la tumorigénesis. En particular, en el hígado, se ha observado que la modificación de la acetilación está implicada en la mayoría de las vías metabólicas mediante la regulación del metabolismo de

los glicolípidos y los ciclos de la urea (Le-Tian et al., 2020). Los estudios proteómicos han demostrado que muchas enzimas metabólicas clave están acetiladas, maloniladas o succiniladas en las mitocondrias, y que sus actividades enzimáticas están reguladas por estas modificaciones en respuesta a los estímulos ambientales (Stein & Imai, 2012).

La metilación es una modificación química que puede alterar funciones biológicas como la epigenética y la formación de complejos proteicos. En un estudio reciente, la expresión de la metiltransferasa nicotinamida N-metiltrasferasa (NNMT) aumentó en el estroma de las metástasis peritoneales, las células de cáncer de ovario y el estroma del cáncer de mama y de colon, lo que sugiere la asociación con su expresión y múltiples tipos de cáncer (Eckert et al., 2019). Además, se observó la actividad atípica del sitio de metilación del glutamato y de la histidina en ratones obesos inducidos por la dieta, insinuando un papel potencial en la diabetes y la obesidad (Zhang et al., 2018). De acuerdo con estos hallazgos, hubo cambios de expresión de 12 proteínas involucradas en los procesos de metilación en el hígado de rata después de la exposición aguda a ENs.

En los hígados expuestos a ENs, los resultados apuntan a una alteración de la cadena de transporte respiratorio, la fructosa, la gluconeogénesis, el metabolismo de los aminoácidos y el ciclo de la urea. Una investigación proteómica en la enfermedad del hígado graso de vacas lecheras reveló vías asociadas a la mitocondria que están implicadas en la regulación de la patogénesis de la enfermedad del hígado graso, incluyendo el ciclo TCA, el metabolismo del propionato, la glucólisis/gluconeogénesis, el metabolismo del piruvato, la

fosforilación oxidativa, la degradación de los ácidos grasos, la degradación de la valina, la leucina y la isoleucina, y el metabolismo de los fármacos (Le-Tian et al., 2020).

El ciclo del ácido cítrico y la cadena de transporte de electrones se encuentran entre las vías alteradas y ya se han asociado a la exposición a ENs (Alonso-Garrido et al., 2020). UQCRC1, que se encuentra en el complejo III de la cadena respiratoria de transporte de electrones, estaba sobreexpresado solo en una de las muestras tratadas, mientras que las subunidades ATP5 alfa y beta del complejo V de la mitocondria, están sobreexpresadas entre un 0,96 y 2,51 logFC en 5 de las muestras tratadas. Además, la LDHA está aumentaba su expresión en 4 de las muestras tratadas. Esta enzima desempeña un papel crucial en el último paso de la glucólisis anaeróbica y, en consecuencia, en la producción de NADH. Su sobreexpresión se observa en muchas neoplasias humanas en asociación con la progresión del tumor, entre ellas en el colangiocarcinoma donde se correlaciona con un mal pronóstico (Thonsri et al., 2017).

Teniendo en cuenta la actividad de oxidorreductasa y la antioxidante, cuatro de las proteínas identificadas participan en ambos procesos y aparecían sobreexpresadas tras la exposición a ENs: la superóxido dismutasa 1 (SOD1), la peroxiredoxina 4 (PRDX4), la hemoglobina beta (HBB) y la hemoglobina alfa (HBA1). La SOD elimina los radicales superóxidos, uno de las principales especies reactivas del oxigeno (ROS) producidos en el organismo, y por ello desempeña un papel fundamental en la protección contra el estrés oxidativo. De los tres genes que codifican la SOD en los mamíferos, el SOD1 está presente en gran parte en el citoplasma y en parte en el espacio intermembrana de las

mitocondrias (Fridovich, 1995). PRDX4 cataliza la formación de enlaces disulfuro en las proteínas a través de la acción del peróxido de hidrógeno y, por lo tanto, disminuye el estrés oxidativo y apoya el plegamiento oxidativo de las proteínas para la secreción de lipoproteínas (Matsumoto et al., 1999). La supresión del estrés oxidativo por parte de la hemoglobina se ha descrito como un mecanismo para proteger a los hepatocitos del daño oxidativo en la esteatohepatitis no alcohólica. Además, el tratamiento con peróxido de hidrógeno, un conocido inductor del estrés oxidativo, aumentó la expresión de HBA1 y HBB en las células HepG2 y HEK293 (Liu et al., 2011).

Por último, se ha descrito que el aumento de los niveles de CPS-1 en el hígado en paralelo a la duración del consumo de alcohol se presume que refleja el daño mitocondrial y el estrés redox (Carter et al., 2015). La función de la carbamoil fosfato sintasa-1 (CPS-1) mitocondrial es catalizar la síntesis de carbamoil fosfato a partir de amoníaco y bicarbonato. Este es el primer paso limitante en el hígado del ciclo de la urea, y es clave en humanos para la eliminación de nitrógeno en la ureagénesis (Martínez et al., 2010). En el presente estudio, el CPS-1 resultó sobreexpresada en 6 muestras tratadas, lo que confirma el daño hepático inducido por las ENs.

4.3 Estudio transcripcional de las ENs *in vivo*

En el pasado, las micotoxinas emergentes se han considerado menos importantes debido a su baja probabilidad de toxicidad aguda. Sin embargo, tienen una alta prevalencia en los productos alimenticios, a veces incluso en altas concentraciones. Hasta la fecha, se han identificado más de 23 especies de ENs

pertenecientes a los tipos A, B y J, pero las más frecuentes detectadas en alimentos y piensos por la Autoridad Europea de Seguridad Alimentaria (EFSA) son la eniatina A (ENA), la eniatina A1 (ENA1), la eniatina B (ENB) y la eniatina B1 (ENB1). Recientemente, se ha revisado la toxicidad *in vivo* de las ENs, reportando su inmunotoxicidad en linfocitos de sangre periférica en ratas Wistar. También, la ENB se encontró en altas concentraciones en el hígado de ratas, lo que demuestra la tendencia de la molécula a bioacumularse en los tejidos lipofílicos. El yeyuno, el duodeno y el colon también se identificaron como una posible zona de absorción de ENA en ratas hembras durante una exposición subcrónica.

En la presente investigación, se utilizó un modelo *in vivo* de ratas Wistar para analizar los cambios en la expresión de genes seleccionados implicados en la cadena de transporte de electrones (ETC), en el estrés oxidativo, apoptosis, inflamación y unión estrecha intestinal en estómago, hígado, riñón y colon de las ratas. Más concretamente, se evaluó la actividad del NADH deshidrogenasa 1 (MT-ND1) codificada por la mitocondria y perteneciente al complejo I (CI), la flavoproteína subunidad A de la succinato deshidrogenasa (Sdha) del complejo II (CII), la citocromo c oxidasa 1 (MT-COX1) codificada por la mitocondria y perteneciente al complejo IV (CIV) y la subunidad alfa de la ATP sintasa F1 (ATP5) del complejo V (CV). Además, los marcadores de estrés oxidativo superóxido dismutasa 1 (SOD1), glutatión peroxidasa 1 (GPx1), hemo oxigenasa 1 (Hmox1), reguladores de la apoptosis linfoma de células B 2 (Bcl2), proteína X asociada a Bcl2 (Bax) la proteína supresora de tumores p53, el factor nuclear kappa de las células B activadas (Nf-κB), la citoquina de inflamación

interleucina 1β (Il-1β) y la proteína de la unión estrecha intestinal Occludin se analizaron en los tejidos del colon.

En este estudio, utilizando las muestras de hígado analizadas en el estudio proteómico y añadiendo las de estómago, riñón y colon de los mismos animales, se ha demostrado que la exposición aguda a ENs causó cambios en la expresión de todos los genes analizados dependiendo del tipo de tejido. Con respecto a la expresión de genes en la cadena de transporte de electrones, el primer complejo, la CI mitocondrial defectuosa es el defecto más prevalente en la cadena respiratoria que causa trastornos mitocondriales. La actividad de la subunidad 1 del CI MT-ND1 está implicada en la formación del cuarto sitio de bombeo de protones al promover la translocación de protones a través de la membrana (Iommarini et al., 2018). Tras la administración de ENs, se ha observado una pérdida de su expresión en todos los órganos analizados, lo que podría estar relacionado con una alteración de la homeostasis de la membrana. De acuerdo con estos hallazgos, la menor actividad de este gen se implicó en la enfermedad hepática por ácido graso no alcohólico en ratas y en la enfermedad renal crónica en ratones (Garcia-Ruiz et al., 2010, Guo et al., 2017**)**. Sin embargo, por el contrario, los niveles elevados de expresión de MT-ND1 indicaban una disfunción mitocondrial en el adenoma velloso del tejido humano, lo que resultaba en la acumulación de mutaciones en el ADNmt (Wallace et al., 2016).

En segundo lugar, la succinato-coenzima Q reductasa es un complejo enzimático mitocondrial compuesto por cuatro subunidades proteicas (Sdha, Sdhb, Sdhc y Sdhd), que participa en el ciclo del ácido tricarboxílico (TCA) y en el ETC (Huang et., 2013). La pérdida del ciclo TCA normal promueve la

tumorigénesis debido a las alteraciones metabólicas con una dependencia forzada de la glucólisis para la producción de energía (Miettinen et al., 2014). La subunidad -a de la CII fue alterada por la mezcla de ENs, siendo reprimida en el estómago, hígado y riñones de las ratas, lo que conduce a un deterioro causado por estas micotoxinas. En estudios previos, la falta de este gen estaba asociada a la disfunción mitocondrial, que condujo al desarrollo de tumores del estroma gastrointestinal de tipo salvaje y al carcinoma hereditario de células renales (Boikos et al., 2016; Kamai et al., 2019).

En tercer lugar, la citocromo c oxidasa, localizada en la membrana mitocondrial interna, es el aceptor final de electrones en el ETC y su deficiencia es una causa prevalente del estado de estrés oxidativo en las mitocondrias (Abdulhag et al., 2015). El gen MT-COX1 es la subunidad principal del complejo, y es responsable de la síntesis homeostática de prostanoides (Jimenez et al., 2010). La expresión de MT-COX1 disminuyó significativamente en el hígado en ambas dosis de ENs empleadas, junto con los tejidos renal e intestinal, aunque sólo en la dosis media. Su represión está relacionada con la progresión del adenocarcinoma de esófago, la carcinogénesis de colon, la reducción de la proliferación y el aumento de la macroautofagia (Cathcart et al., 2012; Martín-Sanz et al., 2010). Además, la deficiencia de MT-COX1 exacerba las enfermedades hepáticas en ratones (Xiao et al., 2015), al igual que en la presente investigación, lo que sugiere que la toxicidad de las ENs podría desempeñar un papel en la función hepática. En contraste con esta evidencia, MT-COX1 resultó estar sobreexpresado en riñones de mamíferos y de pez cebra en condiciones de estrés oxidativo (Harris et al., 2013; Sarkar et al., 2017).

El último complejo estudiado fue la ATP sintasa mitocondrial (CV), que está formada por dos dominios funcionales: F1, situado en la matriz mitocondrial y Fo, localizado en la membrana mitocondrial interna. Produce la mayor parte del ATP celular por catálisis rotativa, y su deficiencia desempeña un papel crucial en trastornos humanos graves como la neuropatía, la ataxia, la encefalopatía (síndrome de Leigh) y la cardiomiopatía hipertrófica (Aiyar et al., 2014). ATP5, la subunidad α del dominio F1 de la CV, fue reprimida en los tejidos gástricos y hepáticos, pero únicamente tras el tratamiento con el medio. De acuerdo con estos resultados, los niveles reducidos de su expresión condujeron a la disminución de la fosforilación oxidativa en pollos, cáncer de próstata y pulmón, disminución del metabolismo tisular, reducción de la capacidad de síntesis de proteínas y deterioro de la ATP-biosintética funciones en el hígado de rata (Zhang et al., 2018, Feichtinger et al., 2018). Por el contrario, Xu y colaboradores (2016) observaron una alta sobreexpresión de ATP5 en células tumorales de glioblastoma y en células endoteliales de proliferación microvascular.

Más allá de la desregulación mitocondrial, varios estudios *in vitro* han demostrado los efectos intestinales de las ENs en líneas celulares específicas, reportando su citotoxicidad y la activación de procesos de estrés oxidativo, incluso a bajas concentraciones moleculares (Bertero et al., 2020). La actividad de tres marcadores esenciales implicados en la respuesta celular al estrés oxidativo fue evaluada *in vivo* tras la exposición aguda a ENs. SOD1 es una enzima antioxidante que regula los niveles de superóxido del citosol y de la intermembrana mitocondrial (Eleutherio et al., 2020). Se ha observado que su supresión está relacionada con la producción de ROS y la reducción de las

actividades de las enzimas antioxidantes en los tejidos del colon, lo que coincide con la regulación a la baja observada en la presente investigación (Gonzalez-Menendez et al., 2018). De la misma manera, se ha observado que la mayor actividad de la enzima catalítica clave GPx1 promueve la progresión de distintos tipos de cáncer, incluido el de colon, lo que coincide con la sobreexpresión observada tras la dosis más alta de la mezcla de ENs empleada en ratas hembras (Gan et al., 2014). En cuanto al modulador de la inflamación intestinal Hmox1, se ha demostrado que inhibe la activación de las vías apoptóticas en distintas líneas celulares, pero, en este caso, no mostró cambios significativos en su expresión (Ribeiro et al., 2017).

La homeostasis de la barrera intestinal también se ve alterada por el desequilibrio que se produce entre los genes pro- y anti-apoptóticos. En particular, se ha demostrado que el gen p53 está implicado en el inicio de los mecanismos de apoptosis del ciclo celular y su sobreexpresión se ha asociado a diferentes tipos de cáncer intestinal (Ryter et al., 2021; Kim et al., 2021). Además, el supresor tumoral p53 regula los miembros antiapoptóticos (Bcl2) y proapoptóticos (Bax) de la familia de proteínas Bcl-2, que controlan la apoptosis mediante el control de la permeabilización de la membrana externa mitocondrial (Dashzeveg et al., 2015). En este estudio, los genes p53 y Bax fueron regulados al alza tras el tratamiento con ENs, mientras que, por el contrario, el antiapoptótico Bcl-2 no mostró cambios significativos, lo que sugiere la activación de la apoptosis.

Como consecuencia del desarrollo del daño celular inducido por el estrés oxidativo y la apoptosis, puede producirse sucesivamente la aparición de

inflamación y disfunción estructural (Netea et al., 2017). La actividad de la proteína estructural ocludina, por ejemplo, está relacionada con trastornos epiteliales intestinales y con una función secretora anormal (Zhao et al., 2021). Su sobreexpresión en las células intestinales se implicó en la disminución de la permeabilidad intestinal de acuerdo con este resultado, insinuando la activación de mecanismos de defensa (Zhou et al., 2019).

4.4 Evaluación del efecto beneficioso del suero fermentado y del extracto de calabaza frente a la toxicidad de la AFB1 y la OTA en linfocitos T Jurkat.

AFB1 y OTA son las toxinas carcinógenas naturales más potentes que contaminan los cultivos y una gran variedad de productos alimentarios. Debido a su elevada toxicidad, la Comisión Técnica de Contaminantes de la Cadena Alimentaria ha emitido una opinión científica sobre los riesgos para la salud humana relacionados con la presencia de AFs y OTA en los alimentos y ha estimado su exposición dietética de la población de la Unión Europea (EFSA, 2020).

Varios compuestos bioactivos, como las bacterias probióticas, se encuentran de forma natural en la dieta y participan en diferentes funciones que contribuyen a la salud humana actuando como mediadores antioxidantes, antiinflamatorios e inmunomoduladores. El presente estudio combinó el efecto beneficioso de la calabaza y del suero de leche fermentado contenidos en un digerido intestinal del pan para contrarrestar la toxicidad de la AFB1 y la OTA en células Jurkat, centrándose en su perfil proteómico.

El análisis de las células Jurkat expuestas a diferentes condiciones de AFB1 y OTA, individualmente o en combinación, y sus controles se realizó mediante un enfoque proteómico de EM sin gel. Se detectó un total de 496 proteínas únicas para el tratamiento con DMSO al 0,1%, 352 para el tratamiento con digerido intestinal de pan de calabaza (PID) y 316 para digerido intestinal de pan de calabaza y suero de leche (PID+WF). De 154 de las proteínas expresadas diferencialmente en el tratamiento con DMSO, 74 han mostrado niveles de expresión más altos y 80 más bajos en comparación con el control. Tras el tratamiento con PID, 64 fueron reprimidas y 18 sobreexpresadas en comparación con el control. Por último, de las 85 proteínas identificadas tras el tratamiento con PID + WF, 58 mostraron una expresión mayor y 27 menor con respecto al control. La ontología genética de la clasificación de las funciones de los genes mostró los parámetros más significativos identificados al analizar los procesos biológicos, las funciones moleculares y las vías metabólicas de las proteínas implicadas tras la exposición de las células Jurkat con DMSO al 0,1%, PID y PID + WF, en presencia de AFB1 y OTA singularmente o en combinación.

Entre los procesos biológicos en los que participan las proteínas identificadas con expresión diferencial, destaca la gluconeogénesis. Este proceso convierte sustratos no carbohidratos (como el lactato, los aminoácidos y el glicerol) en glucosa en condiciones de ayuno y está modulado por factores externos como los nutrientes, la energía, el ejercicio y las reacciones de estrés (Zhang et al., 2019). En este trabajo, 5 de las enzimas implicadas en el proceso de gluconeogénesis, como GPDH, GPI, PGK y PGM, fueron comúnmente

reprimidas después del tratamiento con DMSO junto con las micotoxinas. En particular, la deficiencia de GPDH y GPI después de la exposición a micotoxinas, fue implicada en la esteatosis hepática y el hígado graso no alcohólico (Zheng et al., 2019; Lin et al., 2020).

Además, la pérdida de PGK observada también en el presente estudio tras la exposición a AFB1 solo, pero no en combinación, esta asociada a la detección temprana del cáncer, ya que es la primera enzima que produce ATP en la vía glucolítica (Fu & Yu., 2020). En cuanto a la PGM, que cataliza la transformación reversible de 3-fosfoglicerato a 2-fosfoglicerato, fue reprimida tras la exposición a una sola micotoxina, pero sobreexpresada con la mezcla. Sobre la base de estos últimos resultados, se ha observado de que la sobreexpresión de PGM indujo la necroptosis en los cardiomiocitos primarios, mientras que, por el contrario, su deficiencia aumentó los niveles de ADN y transcritos mitocondriales, normalizó la respiración mitocondrial y reprimió la producción de ROS mitocondriales (Zhu et al., 2021).

En cuanto a la actividad antioxidante, la expresión de peroxiredoxinas y tiorredoxinas cambió en función del tratamiento empleado en las células Jurkat. Estas proteínas protegen a las células de las agresiones oxidativas producidas por las micotoxinas y ayudan a mantener su homeostasis natural. En particular, las peroxiredoxinas catalizan la formación de especies de peróxidos, para equilibrar el contenido de H_2O_2 a nivel celular, ya que son esenciales para regular los mecanismos de señalización y estrés oxidativo (Ye et al., 2019). Cuando las células fueron expuestas a AFB1 y OTA solas o en combinación en DMSO al 0,1%, PRDX1 disminuyó su expresión hasta 4 veces en comparación con el

control. Por el contrario, cuando se añadieron ingredientes funcionales con capacidad antioxidante, PRDX 1 y PRXD2 se sobreexpresaron hasta 4 veces, junto con TRX-1 que probablemente se activó por la acción sinérgica de las dos micotoxinas. De acuerdo con esto, los estudios *in vitro* e *in vivo* informaron de una mayor expresión de peroxiredoxina y tiorredoxina como consecuencia del aumento de las defensas naturales de las células tras la exposición a diferentes micotoxinas, solas o en combinación (Brennan et al., 2017). Además, en este trabajo se observó una mayor expresión de peroxiredoxinas en procesos biológicos relacionados con la respuesta inmune innata de la mucosa, la eliminación de radicales superóxidos y el proceso catabólico del H_2O_2.

El ensamblaje de nucleosomas es un proceso por el cual el ADN se empaqueta en nucleosomas ayudando a la replicación de la cromatina, con el fin de mantener la estabilidad del genoma. Cada nucleosoma está compuesto por un octámero de cuatro histonas centrales que son H2A, H2B, H3 y H4. La expresión de las histonas, que son proteínas altamente básicas que ayudan al ensamblaje de nuevos nucleosomas, facilitan la replicación del genoma y la conservación de la información epigenética hereditaria (Liu et al., 2017; Zhao & Shilatifard, 2019). Se ha demostrado que la desregulación de ciertas proteínas asociadas a la cromatina actúa como impulsores en cierto tipo de cáncer. La baja expresión de variantes de histonas, por ejemplo, se asoció con una mayor cantidad de daños en el ADN en líneas celulares de cáncer de cuello uterino, lo que sugiere una correlación con el mecanismo de reparación del ADN (Li et al., 2017). En este estudio, al exponer las células a ingredientes funcionales y micotoxinas, se observó la sobreexpresión de 6 especies de histonas, (H1, H2A, H2B, H2C, H3, H4) en todas las condiciones empleadas. Además, se han

identificado mutaciones de histonas que se incorporan parcialmente a la cromatina, como H1.3 y H3.3, en el crecimiento de las células cancerosas al cambiar las propiedades estructurales del nucleosoma (Arimura et al., 2018). También en este caso, los niveles de las variantes histónicas H1.3 y H3.3 aumentaron fuertemente después de exponer las células a la OTA y la mezcla. Por lo tanto, la calabaza y el WF podrían promover el desarrollo de nuevos nucleosomas que contribuyen a la conservación del genoma.

Considerando las vías metabólicas, la relacionada con el fenotipo secretor asociado a la senescencia fue la más frecuente encontrada en las células tratadas con digestión intestinal. Esta vía metabólica sensibiliza a las células y las hace senescentes para evitar que los procesos de estrés genómico, como el daño al ADN, el acortamiento de los telómeros y la alteración de la cromatina, induzcan la pérdida de funciones celulares vitales. Además, tiene la capacidad de estimular la expresión de proteínas relacionadas con el sistema inmunitario y de paralizar la proliferación celular en el proceso de formación del cáncer, incluso de promover la reparación y el desarrollo de los tejidos (Özcan et al., 2016). En todas las condiciones en las que se alteró esta vía metabólica, la expresión de ciclina A2 disminuyó comúnmente en comparación con el control. Esta proteína, tiene un papel esencial en la progresión de las transiciones de fase del ciclo celular y su sobreexpresión se ha confirmado en ciertos tipos de cáncer (Pei et al., 2019). Por el contrario, Tu et al. (2019) demostraron que la represión de la ciclina A2 y de las quinasas dependientes de ciclina estudiadas en líneas celulares de cáncer de colon atenuaba la proliferación celular, al igual que en el presente estudio. En base a estos resultados, los ingredientes funcionales pueden actuar como protectores contra el estrés genómico causado por la AFB1 y la

OTA también en este caso, impidiendo la pérdida de funciones celulares vitales y paralizando el crecimiento de las células cancerígenas.

4. CONCLUSIONS

4. Conclusiones

5. CONCLUSIONES

1. La literatura revisada muestra que las micotoxinas más estudiadas *in vivo* en la última década fueron AFB1, DON, ZEA y OTA, y combinaciones de micotoxinas, especialmente con AFB1, DON y ZEA. Los animales de laboratorio predominantes empleados fueron los roedores (ratones y ratas) y cerdos siendo el órgano más estudiado el hígado. Se emplearon diversas técnicas, entre las cuales la más utilizada fue la qPCR. Se estudió principalmente inmunotoxicidad seguida de genotoxicidad, hepatotoxicidad, teratogenicidad y neurotoxicidad.

2. Las eniatinas generan un descenso en la transcripción génica a nivel mitocondrial en todos los tejidos analizados, siendo el riñón el más afectado. En el colon se observa la activación de genes relacionados con estrés oxidativo y apoptosis. A nivel estructural, se destaca en colon el incremento de la expresión de la proteína de unión estrecha ocludina, de manera dependiente a la cantidad de eniatinas ingerida por las ratas, apuntando a una muy probable alteración de la permeabilidad intestinal.

3. La ingesta aguda de eniatinas en ratas provoca cambios de expresión en el perfil proteómico del hígado. Estos cambios afectan a proteínas relacionadas con actividad mitocondrial, procesos de acetilación y metabolismo, siendo esta última la principal función del hígado.

4. Los ingredientes funcionales empleados en la preparación del pan, el suero fermentado de leche de cabra y la calabaza liofilizada, contribuyeron al normal funcionamiento celular *in vitro* en presencia de

las micotoxinas aflatoxina B1 y ocratoxina A incrementando la expresión de proteínas relacionadas con la gluconeogénesis, actividad antioxidante y estabilidad cromosómica.

5. La exposición *in vitro* de digeridos gastrointestinales con ingredientes funcionales junto con micotoxinas muestra un perfil proteómico de prevención de procesos de estrés genómico y paralización de la proliferación celular a la vez que promueve la reparación de tejidos dañados.

6. Se debe seguir avanzando en los estudios toxicológicos sobre los efectos de las micotoxinas, que han cambiado el paradigma del estudio con la utilización de las tecnologías omicas, poniendo de manifiesto cambios moleculares de forma amplia, lo que supondrá un gran avance en la evaluación del riesgo en seguridad alimentaria

5. CONSCLUSIONS

1. The literature reviewed shows that the most studied mycotoxins *in vivo* in the last decade were AFB1, DON, ZEA and OTA, and combinations of mycotoxins, especially AFB1, DON and ZEA. The predominant laboratory animals used were rodents (mice and rats) and pigs, being the most studied organ the liver. Several techniques were employed, among which the most widely used was qPCR. Immunotoxicity was mainly studied, followed by genotoxicity, hepatotoxicity, teratogenicity and neurotoxicity.

2. Enniatins generate a decrease in gene transcription at mitochondrial level in all tissues analyzed, being the kidney the most affected. In intestinal tissues, the activation of genes related to oxidative stress and apoptosis is observed. At structural level, the increase in the expression of the tight junction protein occludin is highlighted in colon, depending on the amount of enniatins ingested by the rats, pointing to a probable alteration of intestinal permeability.

3. Acute ingestion of enniatins in rats causes expression changes in proteomic profile of the liver. These changes affect proteins related to mitochondrial activity, acetylation processes and metabolism, being the latter the main function of the liver.

4. The functional ingredients used in the preparation of bread, fermented goat milk whey and dried pumpkin, contributed to normal cellular functioning *in vitro* in presence of mycotoxins aflatoxin B1 and

ochratoxin A, by increasing the expression of proteins related to gluconeogenesis, antioxidant activity and chromosomal stability.

5. *In vitro* exposure of gastrointestinal digests with functional ingredients combined with mycotoxins shows a proteomic profile of prevention of genomic stress processes and paralysis of cell proliferation, promoting the repair of damaged tissues at once.

6. Further studies are needed on toxicological effects of mycotoxins, which have changed the paradigm of the study of effects with the use of -omics technologies, revealing molecular changes in a broad way, which will be a breakthrough in the evaluation of food safety risk.

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