VNIVERSITAT DE VALÈNCIA

Facultat de Farmàcia

*Departament de Medicina Preventiva i Salut Pública, Ciències de l'Alimentació, Toxicologia i Medicina Legal*

**Programa de Doctorat amb Menció cap a l'Excel·lència en Ciències de l'Alimentació**

# EVALUATION THROUGH OMICS AND MOLECULAR TECHNIQUES OF AFB1 AND OTA TOXICITY PREVENTION BY FUNCTIONAL INGREDIENTS

# Evaluación por técnicas ómicas y moleculares de la prevención de la toxicidad de AFB1 y OTA por ingredientes funcionales

**Tesi Doctoral Internacional**

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**Presentada per:**

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**Professora Lara Manyes i Font**

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**CERTIFIQUEN QUE:**

**Don Massimo Frangiamone** ha realitzat sota la seua direcció el treball **"Evaluación por técnicas ómicas y moleculares de la prevención de la toxicidad de AFB1 y OTA por ingredientes funcionales"**, i autoritzen la seua presentació per a optar al títol de Doctor per la Universitat de València.

I, perquè així conste, expedeixen i signen el present certificat.

Burjassot (València), Juny, 2023.

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**Aquest treball ha sigut plasmat en 5 articles publicats o que es publicaran en les següents revistes:**

1. *In vitro* and *in vivo* evaluation of AFB1 and OTA-toxicity through immunofluorescence and flow cytometry techniques: A systematic review (2022). *Food and chemical Toxicology*. 160, 112798. *Impact factor: 5.572*
2. Pumpkin extract and fermented whey individually and in combination alleviated AFB1- and OTA-induced alterations on neuronal differentiation *in vitro* (2022). *Food and chemical Toxicology*. 164, 113011. *Impact factor: 5.572*
3. AFB1 and OTA promote Immune toxicity in Human Lymphoblastic T cells at Transcriptomic Level (2023). *Foods*. 12(2), 259. *Impact factor: 5.561*
4. The protective effect of pumpkin and fermented whey mixture against AFB1 and OTA immune toxicity *in vitro*. A transcriptomic approach (2023). *Under revision*. *Molecular Nutrition and Food Research. Impact factor: 6.575*
5. Fermented Whey modulated AFB1 and OTA-induced hepatotoxicity and nephrotoxicity *in vivo*. A relative and absolute quantification about sex differences. *Toxicology mechanism and methods*, 1-12. *Impact factor: 4.019*

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“Ad maiora semper”

Alla mia famiglia e agli amici più cari.

# RESUMEN

Las micotoxinas son metabolitos secundarios tóxicos producidos por ciertas especies de hongos que pueden contaminar una amplia gama de alimentos y materias primas en condiciones ambientales específicas. La aflatoxina B1 (AFB1) y la ocratoxina A (OTA) figuran entre las micotoxinas más tóxicas y estudiadas.

Con el fin de contextualizar los avances en los últimos años sobre los efectos tóxicos de AFB1 y OTA en modelos celulares y animales, se realizó una revisión bibliográfica centrada en técnicas como la inmunofluorescencia y la citometría de flujo. Se puso de manifiesto que la citometría de flujo se empleó principalmente en la evaluación de la apoptosis y el estrés oxidativo mientras que la inmunofluorescencia en la detección de alteraciones genéticas, epigenéticas y metabólicas. En los estudios *in vitro* la línea celular más utilizada fue HepG2, seguida por HEK-293T y PK-15. En cuanto a los estudios *in vivo*, los animales de laboratorio con mayor número de estudios fueron los pollos y ratas mientras que los principales órganos diana analizados fueron el timo, la bolsa de Fabricio y el bazo. Los objetivos principales de las investigaciones se centraron en la inmunotoxicidad, nefrotoxicidad, y hepatotoxicidad.

Varios compuestos funcionales contenidos en la calabaza (CL) y en el suero de leche fermentado (FW) han demostrado su potencial aplicación

en la mitigación de toxicidad inducida por micotoxinas debido a sus propiedades antioxidantes y antinflamatorias. Para conocer el posible papel neuroprotector e inmunoprotector de CL y FW, se llevaron a cabo varios ensayos moleculares y *ómicos* con el fin de investigar los posibles efectos de estos ingredientes funcionales frente a AFB1 y OTA en células neuronales y linfocitos T humanos.

El estudio sobre la diferenciación neuronal *in vitro* evidenció que el tratamiento con OTA fue el más perjudicial no sólo por reprimir la expresión de biomarcadores neuronales, sino también por detener el ciclo celular en la fase $G_0/G_1$. Resultados menos pronunciados se observaron tras la exposición a AFB1 y a la mezcla de micotoxinas, sugiriendo un posible efecto antagónico. Sin embargo, la adición de CL y FW restableció los niveles de los biomarcadores de forma similar al control y moduló tanto las alteraciones del ciclo celular como los cambios de expresión génica promovidos por las micotoxinas.

Los resultados de la secuenciación del ARN realizada en una línea celular de linfocitos T humanos revelaron que AFB1 alteró principalmente la vía de señalización de la ataxia telangiectasia mutada, indicando posibles daños en el ADN y alteraciones en sus mecanismos de reparación. La exposición a OTA activó principalmente la ruta de la glucólisis, sugiriendo la posible reprogramación del metabolismo energético hacia la anaerobiosis. En combinación, AFB1 y OTA activaron la ferroptosis, una

forma de necrosis programada con peroxidación lipídica y represión de la enzima antioxidante glutatión peroxidasa 4.

El análisis transcriptómico en células inmunitarias Jurkat expuestas a un digerido gastrointestinal de pan preparado con micotoxinas y la mezcla de CL y FW puso de manifesto la promoción tras la exposición a AFB1 y OTA de una clara respuesta proinflamatoria mediante la activación de la vía de señalización de los eicosanoides y del interferón gamma. No obstante, la presencia de los ingredientes funcionales no solo atenuó la sobreexpresión de genes proinflamatorios sino también la inducción de factores y rutas metabólicas estrictamente inflamatorias.

Para estudiar los efectos del FW frente la hepatotoxicidad y nefrotoxicidad de AFB1 y OTA *in vivo* en ratas Wistar, se analizó la expresión génica de biomarcadores biológicos como la carbamoil fosfato sintetasa 1 (CPS1) y la molécula de lesión renal 1 (KIM-1) mediante PCR cuantitativa en tiempo real y PCR digital en gotas (ddPCR).

Los resultados de la cuantificación relativa y absoluta de CPS1 demostraron que en el hígado de ratas macho, la exposición a AFB1 fue la más tóxica al reprimir la expresión génica de CPS1, lo que se revirtió totalmente en presencia del FW. En las hembras, el daño inducido por las micotoxinas así como el efecto hepatoprotector del FW fueron menos pronunciados. En el riñón de ratas macho y hembra, la exposición a OTA

fue la más dañina aumentando en mayor medida la expresión génica de KIM-1. Sin embargo, el análisis por ddPCR demostró que el FW aliviaba los cambios de expresión génica y el daño renal en ambos sexos.

Es necesario seguir investigando los mecanismos de acción de AFB1 y OTA, así como los posibles efectos beneficiosos de los compuestos funcionales, que representan una eficiente estrategia alimentaria en la protección de la salud humana frente a contaminantes alimentarios de carácter global como las micotoxinas.

# SUMMARY

Mycotoxins are toxic metabolites produced by certain fungal species, which can contaminate a wide range of food and raw materials under specific environmental conditions. Among mycotoxins, aflatoxin B1 (AFB1) and ochratoxin A (OTA) are some of the most toxic and studied.

In order to contextualize the knowledge generated in recent years on AFB1 and OTA toxicity in cell and animal models, a literature review was performed focusing on techniques such as immunofluorescence and flow cytometry. It was revealed that flow cytometry was mainly used in the evaluation of apoptosis and oxidative stress while immunofluorescence in the detection of genetic, epigenetic and metabolic alterations. The most commonly used cell line for *in vitro* assays was HepG2, followed by HEK-293T and PK-15. As for *in vivo* studies, laboratory animals with the highest number of assays were chickens and rats, while the main target organs analyzed were thymus, bursa of Fabricius and spleen. The main focus of the research was on immune toxicity, nephrotoxicity, and hepatotoxicity.

Several bioactive compounds contained in pumpkin (P) and fermented milk whey (FW) have shown a potential application in the mitigation of mycotoxin-induced toxicity due to their antioxidant and anti-inflammatory properties. To gain insight into the possible neuroprotective and immune protective role of P and FW, several molecular and *omics* assays were carried out to investigate the possible effects of these functional

ingredients against AFB1 and OTA in human neuronal cells and T lymphocytes.

The analysis of neuronal differentiation *in vitro* showed that OTA treatment was the most detrimental not only by repressing neuronal biomarkers expression but also by arresting the cell cycle at $G_0/G_1$ phase. It has been also observed less marked results after exposure to AFB1 and the mixture of mycotoxins, suggesting their possible antagonistic effect. However, the addition of P and FW restored biomarker levels similar to the control and modulated both cell cycle alterations and mycotoxin-promoted gene expression changes.

The results of RNA sequencing performed on a human T lymphocyte cell line revealed that AFB1 mainly altered the ataxia telangiectasia mutated signaling pathway, indicating possible DNA damage and alterations in its repair mechanisms. Conversely, OTA exposure activated glycolysis pathway, suggesting a possible reprogramming of energy metabolism toward anaerobiosis. In combination, AFB1 and OTA activated ferroptosis, a type of programmed necrosis with lipid peroxidation and repression of glutathione peroxidase 4 antioxidant enzyme activity.

Transcriptomic analysis in Jurkat immune cells exposed to a digested bread extract prepared with mycotoxins and P-FW mixture showed that AFB1 and OTA promoted a clear proinflammatory response *in vitro* by

activating eicosanoid and interferon gamma signaling pathways. Nevertheless, the presence of functional ingredients not only attenuated the overexpression of proinflammatory genes but also the induction of factors and metabolic pathways, strictly related to inflammation.

To assess the effects of FW against AFB1 and OTA hepatotoxicity and nephrotoxicity *in vivo* using Wistar rats, the gene expression of biological biomarkers such as carbamoyl phosphate synthetase 1 (CPS1) and kidney injury molecule 1 (KIM-1) was analyzed by quantitative real-time PCR and droplet digital PCR.

The results of CPS1 relative and absolute quantification showed that in the liver of male rats, AFB1 was the most toxic exposure by repressing CPS1 gene expression, which was totally reversed upon FW administration. In females, mycotoxin-induced damage as well as the hepatoprotective effect of FW were less evident. In the kidney of male and female rats, OTA exposure was the most damaging by increasing KIM-1 gene expression to a greater extent than other conditions. However, the absolute KIM-1 quantification showed that FW alleviated gene expression changes and kidney damage in both sexes.

Further research is needed to investigate AFB1 and OTA mechanisms of action, as well as the potential beneficial effects of functional ingredients,

which represent an efficient dietary strategy in protecting human health from global food contaminants such as mycotoxins.

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# List of abbreviations

AFB1: Aflatoxin B1

ALT: Alanine aminotransferase

AST: Aspartate aminotransferase

BW: Body weight

C6 : Rat glioma cells

CAT : Catalase

CHME5: Human microglial cells

COX-2: Cyclooxygenase 2

CPS1: Carbamoyl phosphate synthetase 1

DNA: Deoxyribonucleic acid

ECV304: Human endothelial cells

FCM: Flow cytometry

FW: Fermented milk whey

GPX: Glutathione peroxidase

GSH: Glutathione

HepG2: Human hepatocellular carcinoma cells

HGMC: Human glomerular mesangial cells

HHL-5: Human liver cells

HK-2: Human renal epithelial cells

IF: Immunofluorescence

IHC: Immunohistochemistry

Jurkat: Human lymphoblastic T cells

KIM-1: Kidney injury molecule 1

LDH: Lactate dehydrogenase

LLC-PK1: Epithelial-like pig kidney cells

LMH: Chicken hepatocarcinoma cells

MDA: Malondialdehyde

MDCK: Madin-darby canine kidney cells

MoA: Mechanism of action

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADPH: Nicotinamide adenine dinucleotide phosphate

NO: Nitric oxide

OTA: Ochratoxin A

P: Pumpkin

PAM: Porcine alveolar macrophage cells

PBCEC: Primary porcine brain capillary endothelial cells

PCV2: Porcine circovirus type 2

PK-15: Porcine kidney cells

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RT-qPCR: Real-time polymerase chain reaction

SH-SY5Y: Human neuroblastoma cells

SOD: Superoxide dismutase

WB: Western blo

# Lista de abreviaturas

ADN: Ácido desoxirribonucleico

AFB1: Aflatoxina B1

AFs: Aflatoxinas

ARN: Ácido ribonucleico

AST: Astaxantina

ATM: Ataxia telangiectasia mutada

BAL: Bacterias ácidos lácticas

BEA: Beauvericina

BHE: Barrera hematoencefálica

CL: Calabaza

CONTAM: Panel de Contaminantes de la Cadena Alimentaria

CPS1: Carbamoil fosfato sintetasa 1

ddPCR: PCR digital en gotas

DEG: Genes diferencialmente expresados

DON: Deoxinivalenol

EA: Enfermedad de Alzheimer

EFSA: Agencia Europea de Seguridad Alimentaria

ENNs: Enniatinas

EP: Enfermedad de Parkinson

FB1: Fumonisina B1

FCM: Citometría de flujo

FW: Suero de leche fermentado

GPX4: Glutatión peroxidasa 4

IARC: Agencia Internacional para la Investigación sobre el Cáncer

IF: Inmunofluorescencia

IFN: Interferón

KIM-1: Molécula de lesión renal 1

MoA: Mecanismo de acción

MTT: bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio

OTA: Ocratoxina A

ROS: Especies reactivas de oxígeno

RT-qPCR: PCR cuantitiativa en tiempo real

TUBB3: Tubulina beta III

ZEA: Zearalenona

# 1. INTRODUCTION

# 1. Introducción

# 1. INTRODUCCIÓN

## *1.1. Aspectos generales sobre las micotoxinas*

El término "micotoxina" define compuestos químicos tóxicos producidos como metabolitos secundarios por ciertas especies de hongos filamentosos que pertenecen principalmente a los géneros: *Aspergillus, Penicillium, Fusarium, Alternaria y Claviceps* (Wang et al., 2022). Estos tóxicos pueden contaminar alimentos básicos de la dieta humana en distintas etapas de la cadena de producción, especialmente durante la pre y postcosecha (Conte et al., 2020). La contaminación de alimentos por micotoxinas está relacionada con factores ambientales como la composición del aire, la temperatura, la actividad del agua, la humedad relativa, la sequía, el pH, la concentración de oxígeno y la zona geográfica. Asimismo, factores genéticos y nutricionales pueden también contribuir a la colonización fúngica (Peter Mshelia et al., 2020).

Aunque se hayan considerado varias estrategias de descontaminación tanto en el cultivo como en el almacenamiento, las micotoxinas constituyen un grave problema para la seguridad agroalimentaria con graves repercusiones sociales y económicas a escala global. En consecuencia, la contaminación de alimentos por micotoxinas es continuamente monitoreada por la Agencia Europea de Seguridad Alimentaria (EFSA). En el 2021, el 67-73% de alimentos y piensos a nivel mundial estaban contaminados con múltiples micotoxinas. El 81% de las

muestras analizadas presentaban valores de aflatoxinas (AFs) que superaban hasta 30 veces los límites permitidos, con un elevado riesgo de aflatoxicosis en ciertas regiones asiáticas. Asimismo, la Comisión Europea ha reportado más de 400 alertas de contaminación fúngica en alimentos y piensos, siendo AFs y ocratoxina A (OTA) las toxinas más notificadas, con el 89% y 10% de notificaciones, respectivamente (Annual Report RASFF, 2020; Dey et al., 2022; Johns et al., 2022).

Una vez ingeridas, las micotoxinas pueden causar efectos adversos para la salud humana y animal favoreciendo el desarrollo de enfermedades agudas y crónicas. Los episodios agudos se caracterizan por un inicio rápido con una respuesta tóxica que incluye dolor abdominal, coma, convulsiones, edema pulmonar y en última instancia la muerte. Por otra parte, las exposiciones crónicas pueden conllevar graves consecuencias sistémicas como: disfunción hepática, insuficiencia renal, trastornos inmunológicos y alteraciones neurológicas. Cabe señalar que no todas las micotoxinas tienen el mismo grado de toxicidad, ya que sus efectos tóxicos están relacionados con su metabolización. Asimismo, la susceptibilidad de los seres humanos y animales varía con respecto a la especie, sexo, edad, dieta e historial clínico (Magnoli et al., 2019).

Considerando el grave impacto para la salud pública y la economía agroalimentaria, la Agencia Internacional para la Investigación sobre el Cáncer (IARC) inició hace años un programa de Monografías, en el que

recopila una serie de pruebas científicas sobre la exposición a micotoxinas con el fin de evaluar el riesgo carcinogénico en los seres humanos. El programa condujo a la clasificación de las micotoxinas en cinco grupos (1, 2ª, 2B, 3, 4). Entre ellas, AFs fueron clasificadas en el grupo 1 como carcinógenos humanos, siendo a su vez potentes agentes mutagénicos y hepatotóxicos. Seguidas de OTA, que junto a fumonisina B1 (FB1) fueron clasificada en el grupo 2B como agentes cancerígenos para los animales (sobre todo roedores) y posiblemente carcinógenos para los humanos. Además, las recientes pruebas científicas sobre la genotoxicidad de OTA y la presunta formación de aductos con el ácido desoxirribonucleico (ADN) podrían llevar a clasificar la toxina en el grupo 2A (es decir, probable carcinógeno humano) (IARC, 2012; Ostry et al., 2017). Teniendo en cuenta que AFs y OTA son los metabolitos más dañinos Para el organismo humano, a continuación se tratarán con más detalle estos dos grupos de micotoxinas.

### *1.1.1. Aflatoxinas*

Las AFs son compuestos policétidos producidos principalmente por dos especies del género *Aspergillus*, *A. flavus* y *A. parasiticus.* La contaminación por AFs se produce en una amplia variedad de productos alimenticios como: cereales (maíz, arroz, cebada y trigo), aceite, especias y frutos secos (Shen & Singh, 2021). Aunque se hayan identificado más de 20 especies de AFs, las más comunes son: aflatoxina B1 (AFB1), AFB2, AFG1,

AFG2. Cabe mencionar también AFM1, un metabolito de AFB1, que se encuentra en la leche y productos lácteos (Liu et al., 2020) (Fig.1).

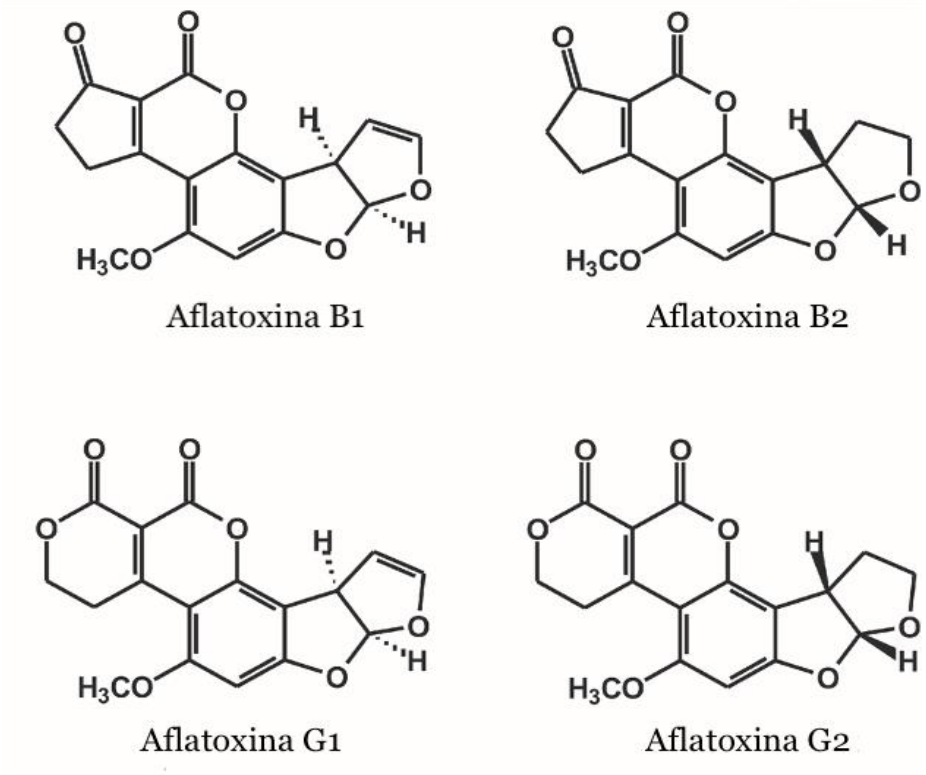


**Fig. 1.** Estructura química de AFB1, AFB2, AFG1, AFG2.

Considerando las repercusiones tóxicas sobre la salud humana y animal, AFB1 es el metabolito más nocivo debido a sus propiedades mutagénicas y carcinogénicas (Guo Y. et al., 2021). El mecanismo de acción (MoA) de esta toxina implica su metabolización en un epóxido de aflatoxina-8,9 por el citocromo P450. Este derivado tiene la capacidad de reaccionar con las macromoléculas y, en particular, con el ADN, formando aductos tóxicos

como el 8,9-dihidro-8(N7-gua-nil)-9-hidroxi-AFB1, que se considera como el principal responsable de sus efectos genotóxicos y tumorigénicos (Dai et al., 2017; Benkerroum, 2020; EFSA, 2020a).

Por ello, la exposición a AFB1 puede conllevar graves consecuencias para el sistema inmunitario, el hígado y el riñón (Manyes & Font, 2022). Asimismo, la alta solubilidad lipídica de AFB1 y su presencia en el tejido cerebral post mortem sugieren su capacidad de atravesar y alterar la barrera hematoencefálica (BHE) (Bahey et al., 2015; Qureshi et al., 2015). En cuanto al sistema nervioso central, AFB1 puede alterar las funciones de las células neuronales induciendo: estrés oxidativo, apoptosis, disfunciones mitocondriales e inhibición de la síntesis de macromoléculas (Alsayyah et al., 2019).

### *1.1.2. Ocratoxina A*

Como se muestra en la Figura 2, OTA es un metabolito pentacetácido producido por muchas especies fúngicas que pertenecen a los géneros *Aspergillus* y *Penicillium*, en particular *A. circumdati, P. nordicum* y *P. verrucosum.* Los alimentos típicamente contaminados por esta toxina son: cereales (maíz, trigo, arroz, sorgo, cebada, avena), y productos derivados (pan, harina y pasta), uvas, cacao, frutos secos, especias, legumbres, café, vino y cerveza (Chen et al., 2018; EFSA, 2020b).

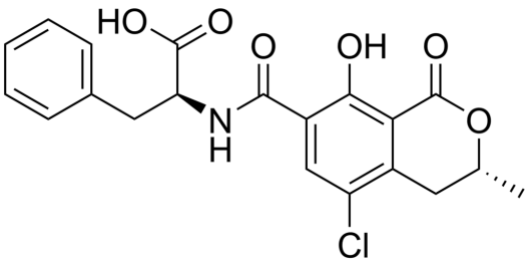


**Fig. 2.** Estructura química de OTA.

Esta toxina se absorbe rápidamente por el túbulo renal, no obstante su lenta excreción del organismo puede causar graves alteraciones sistémicas. Por lo tanto, en varios modelos animales, OTA es la principal causa de enfermedades renales crónicas, así como cáncer (Liu et al., 2022; Miljkovic & Mantle, 2022). Su MoA implica un aumento de la actividad de las enzimas NADPH oxidasa y del citocromo P450, activación de la vía de señalización de las caspasas y promoción de la muerte celular. Además, OTA favorece la producción de especies reactivas de oxígeno (ROS) tanto en las mitocondrias como en el retículo endoplásmico, lo que conlleva alteraciones en el metabolismo energético y lipídico (EFSA, 2020b; Zhu et al., 2017; Tao et al., 2018).

Consecuentemente, la exposición a OTA se asocia a varios efectos toxicológicos como: nefrotoxicidad, hepatotoxicidad e inmunotoxicidad (Frangiamone et al., 2022a). De forma similar, estudios recientes en ratas sugieren que OTA es capaz de atravesar la BHE y de acumularse en distintas zonas del cerebro como: cerebelo, mesencéfalo ventral, estriado y corteza cerebral (Niaz et al., 2020). Otra de las zonas en las que se han hallado altas

dosis de OTA es el hipocampo, principal lugar que se ve afectado por la neurodegeneración en la enfermedad de Alzheimer (EA) (Bhat et al., 2016). En el cerebro, OTA desencadena la generación de estrés oxidativo, alteración del potencial de la membrana mitocondrial, citotoxicidad y muerte neuronal (Park et al., 2019; Babayan et al., 2020).

### *1.2. Mitigación de micotoxinas por compuestos bioactivos*

Considerando que las micotoxinas constituyen un problema global para la seguridad alimentaria, la Organización Mundial de la Salud ha alertado sobre la importancia de controlar y prevenir la contaminación fúngica en las distintas fases de la cadena de producción. Con este fin, se han considerado varias intervenciones físicas, químicas y biológicas. Por un lado, los métodos que implican el uso de compuestos químicos (amoníaco, hidróxido de sodio, ácido clorhídrico y otros) o de técnicas físicas (luz ultravioleta, luz pulsada, plasma frio, irradiación y empleo de adsorbentes) han demostrado ser eficientes herramientas para la descontaminación de micotoxinas. Todavía, los posibles riesgos para la salud pública, junto con la pérdida de nutrientes y de las propiedades organolépticas así como los elevados costes, impiden su aplicación en la industria alimentaria. Por otro lado, los métodos biológicos para la detoxificación microbiana, en particular los que emplean compuestos funcionales y nutracéuticos, han revelado ser especialmente eficaces no sólo para desintoxicar y biodegradar las micotoxinas, sino también para limitar su absorción y toxicidad en el

organismo (Matumba et al., 2021; Makhuvele et al., 2020). Estos compuestos bioactivos pueden ser de origen vegetal, como polifenoles y carotenoides o de origen bacteriano, como los probióticos. Teniendo en cuenta la seguridad alimentaria de estos ingredientes funcionales y su bajo coste de producción, las perspectivas futuras de las intervenciones biológicas son prometedoras (Zhou et al., 2022; Tian et al., 2022). A continuación, se trataran dos de los compuestos bioactivos de mayor interés a nivel industrial: la calabaza (CL) y el suero de leche.

### *1.2.1. Calabaza*

La CL, del género *Cucurbita*, es una planta que se encuentra en differentes regiones del mundo. Pertenece a la familia de las *cucurbitáceas* y se distinguen esencialmente tres especies: *Cucurbita moschata, Cucurbita máxima* y *Cucurbita pepo* (Pereira et al., 2020). La CL se utiliza ampliamente por su alto contenido en micronutrientes y fitoquímicos, que le confieren propiedades antiinflamatorias, antioxidantes, antiangiogénicas, antilipogénicas y antihiperglucémicas. Por ejemplo, el alto contenido en antioxidantes en forma de carotenoides como β-caroteno, luteína, licopeno, criptoxantina y zeaxantina previene el riesgo de enfermedades cardiovasculares ya que son capaces de reaccionar con los radicales libres y suprimirlos. Asimismo, el alto contenido en grasas poliinsaturadas, presentes en las semillas, favorece la eliminación del colesterol en la sangre

de humanos y animales, otorgándoles propiedades hepatoprotectoras y antiaterogénicas (Bergantin et al., 2018; Hussain et al., 2022; Kaur et al., 2020).

Cabe mencionar que la corteza, las semillas y la pulpa de la CL han demostrado un efecto antimicrobiano contra: *Pseudomonas aeruginosa, Candida albicans, Acinetobacter baumanii, Enterococcus faecalis, Klebsiella pneumonia, Escherichia coli y Staphylococcus aureus* (Ahmad & Khan, 2019). Además, se demostró que la administración *in vivo* de un extracto acuoso de CL reducía los efectos tóxicos de la exposición a AFB1 y *Aspergillus Flavius* mejorando la histología y la función pulmonar de ratas. Se ha observado también que los carotenoides presentes en la CL modulan los efectos tóxicos de AFB1 y OTA en modelos celulares así como en el riñón, hígado, corazón y cerebro de ratas (Alonso-Garrido et al., 2021a; Cimbalo et al., 2022a; Frangiamone et al., 2021; Anlar & Bacanli, 2020; Saddiq & Awedh, 2019).

Teniendo en cuenta que la CL es capaz de mejorar las propiedades organolépticas de los alimentos y de aportar enormes beneficios para la salud del consumidor, la industria agroalimentaria la ha utilizado como materia prima para la producción de alimentos funcionales novedosos como el ogiri (Kaur et al., 2020).

### *1.2.2. Suero de leche fermentado*

El suero de leche es un residuo alimentario típico de la producción quesera con propiedades nutricionales de gran relevancia, ya que contiene aproximadamente el 55% de los nutrientes de la leche. Por lo tanto, la industria agroalimentaria ha sugerido varios métodos para valorizar y reutilizar este producto, como la incorporación de bacterias ácido lácticas (BAL). Las BAL son un grupo de organismos gram positivos, clasificados como "Generalmente Reconocidos como Seguros" por la EFSA y la Organización de las Naciones Unidas para la Agricultura y la Alimentación. En este sentido, las BALs se pueden utilizar como probióticos en los alimentos, con el fin de producir, mediante la fermentación, ingredientes funcionales capaces de atenuar la exposición oral a los contaminantes químicos (Chiocchetti et al. 2019; Luz et al., 2020).

La fermentación del suero de leche produce varios metabolitos bioactivos, entre ellos enzimas proteolíticas que hidrolizan grandes proteínas lácteas generando pequeños péptidos y aminoácidos libres con diversas propiedades beneficiosas para la salud humana. Dependiendo de la secuencia de aminoácidos, los compuestos derivados pueden ejercer actividades antioxidantes, cardioprotectoras, inmunomoduladoras y anticancerígenas (Tomazou et al., 2019).

Se ha demostrado que el suero de leche de cabra, fermentado con diferentes cepas de *Lactobacillus,* posee actividades antimicrobianas capaces de inhibir la actividad patógena de *Escherichia coli, Salmonella, Micrococcus luteus y Proteus mirabilis* (Biadała et al., 2020). Análogamente, este producto ha demostrado la capacidad de formar aductos con las micotoxinas, favoreciendo su biodegradación. Además, el suero de leche fermentado (FW) de forma individual o combinado con la CL, ha reducido en manera significativa la absorción intestinal, la immunotoxicidad, la neurotoxicidad, la hepatotoxicidad y la nefrotoxicidad de AFB1 y OTA *in vitro* e *in vivo* (Cimbalo et al., 2022a; Escrivá et al., 2022; Frangiamone et al., 2022b; 2023; Nasrollahzadeh et al., 2022).

Considerando los efectos beneficiosos para la salud humana y el medio ambiente así como los bajos costes de producción, el FW puede considerarse un excelente ingrediente funcional con aplicaciones prometedoras en la industria alimentaria. Puede emplearse como fuente de compuestos bioactivos o conservante natural que se incorpora en los productos alimenticios, prolongando su vida útil y enriqueciendo potencialmente su valor nutritivo (Escrivá et al., 2021).

### *1.3. In vitro and in vivo assessment of AFB1 and OTA toxicity and the beneficial role of bioactive compounds.*

The toxicity related to aflatoxin B1 (AFB1) and ochratoxin A (OTA) exposure was assessed by analyzing systematically all studies (n=60) found in literature over the past 5 years (2018-2023). The majority of articles focused on hepatotoxicity, nephrotoxicity, immunotoxicity, and neurotoxicity. Very few studies on intestinal and reproductive toxicities were found (Fig.3). In addition, 40% of studies (n=32) used cellular models, whose phenotype changed according to the toxicity investigated, while 60% of papers (n=48) used animal models (Fig.4). In detail, *in vitro* studies showed mycotoxins concentration ranging from 0.001 to 100 μM with exposure times between 1 h and 7 days, while *in vivo* doses varied between 25 μg/kg/ per body weight (bw) and 320 mg/kg/bw administered during 1 days to 90 days.

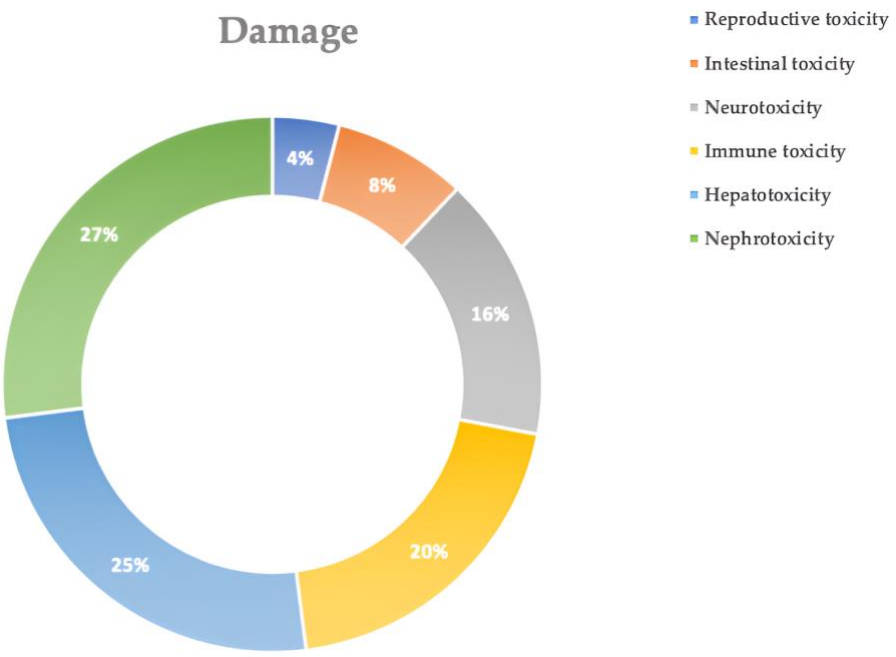


**Fig 3.** Ring plots showing the percentage of studies according to the main toxicity investigated (n=60).

Regarding biological compounds, they were employed in numerous studies (n=34) in order to modulate AFB1 and OTA toxicity *in vitro* and *in vivo*. In the present study, the functional compounds used were: lemon peel, *Erythrina latissima* bark extract, purple rice husk, gallic acid, apigeninidin-rich extracts of *Sorghum bicolor*, grape seed meal, *Mentha piperita*, *Bacillus* extract, probiotics, selenomethionine, curcumin, *Allium sativum, Withania somnifera,* lycopene, hydroxytyrosol, ursolic acid, silymarin, astragalus polysaccharide, quercetin, *Chelidonium majus* ethanolic extract, *Artichoke leaf* extract, L-dopa, pumpkin (P) and fermented milk whey (FW). In addition,

29% of studies (n=13) tested such bioactive compounds in cell models using doses ranging from 0.5 to 2500 μM during 2 h-7 days, while 71% of papers (n=32) employed animal models with doses ranging from 0.2 to 800 mg/kg/bw during 24 h-90 days (Fig.4).

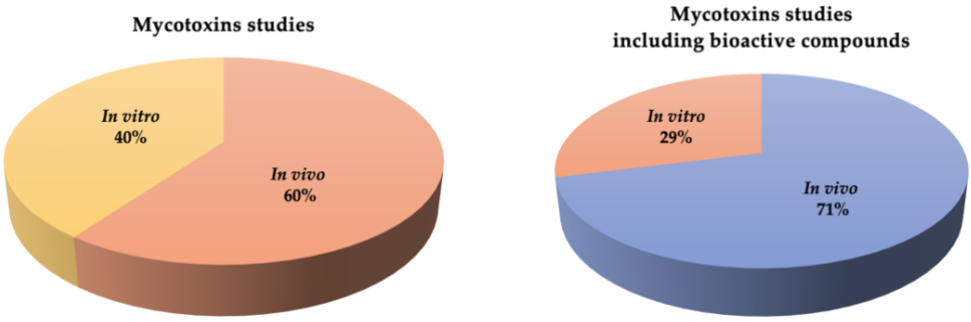


**Fig. 4.** Pie charts of the % articles based on *in vitro* and *in vivo* assays.

In relation to experimental techniques, the most used were: flow cytometry (FCM), immunofluorescence (IF), real-time quantitative polymerase chain reaction (RT-qPCR), western blot (WB), ELISA, immunohistochemistry (IHC), microscopy, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and enzymatic assays. Less used were: ribonucleic acid (RNA) sequencing, comet assay, lipid analysis, as well as deoxyribonucleic acid (DNA) fragmentation analysis, gelatin zymography test and permeability barrier measurement. Naturally, the techniques used to evaluate mycotoxin toxicity were the same ones employed to assess the

protective role of bioactive compounds. Fig.5 shows the percentage of studies according to the experimental assay performed.

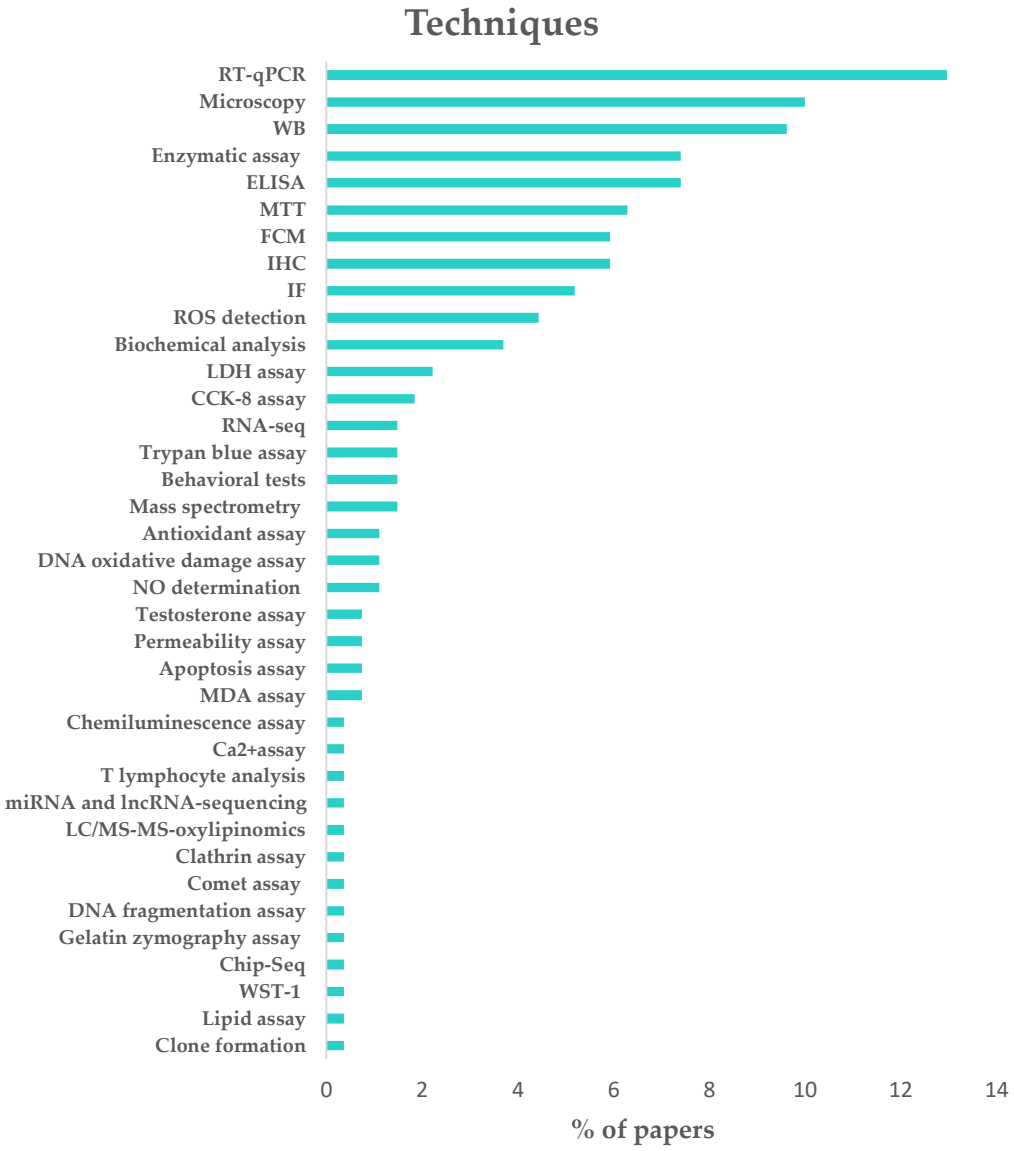


**Fig. 5.** Bar plots of the percentage of articles depending on the experimental techniques used.

Finally, all selected studies were organized according to the toxicity investigated. Then, for each toxic effect, articles were classified by mycotoxin and exposure time. The same disposition was also maintained for studies testing bioactive compounds.

### *1.3.1. Neurotoxicity*

Concerning neurotoxicity, human neuroblastoma cells (SH-SY5Y), human microglial cells (CHME5), human endothelial cells (ECV304), rat glioma cells (C6) and primary porcine brain capillary endothelial cells (PBCEC) were used for *in vitro* experiments while the animal models used were only mice and rats (Table 1). The functional compounds used to attenuate AFB1 and OTA neurotoxic effects were: P, FW, probiotics, quercetin, L-dopa, *Chelidonium majus* ethanolic extract, *Artichoke leaf* extract, *Allium sativum* and *Withania somnifera* (Table 2).

#### *1.3.1.1. Exposure to mycotoxins*

As shown in Table 1, acute exposure to naturally occurring levels of AFB1 can induce neuroimmune dysfunctions. Indeed, in human microglia cells, AFB1 upregulated the gene expression of several proinflammatory factors such as TLRs, MyD88, NF-kB and CxCr4, causing also intracellular ATP depletion and increased caspase-3/7 activity in a time dependent manner. Therefore, the potential damaging effects of environmental AFB1

levels on human brain homeostasis were demonstrated (Mehrzad et al., 2018). In SH-SY5Y cells, Frangiamone et al. (2022b) tested the putative effect of digested bread extracts containing low AFB1 dose on neuronal differentiation. Results showed that AFB1 slightly disrupted the fluorescent expression of dopamine and βIII Tubulin neuronal markers. In addition, a mild alteration in cell cycle phases as well as in mRNA expression of several cell cycle and neuronal differentiation related genes was observed.

In male rats brain, very low AFB1 doses prompted histopathological damages characterized by impairments of pyramidal cells structure and congestion of blood vessel in the cerebral cortex. In addition, AFB1 not only triggered anxiety and depression-like behavior but also increased oxidative stress and inflammation parameters in the brain (Sahin et al., 2022). In the same animal models, 28 days AFB1-intoxication significantly decreased acetylcholine, dopamine and serotonin levels in the cortex and hippocampus. In addition, AFB1 increased nitric oxide (NO) and malonaldehyde (MDA) levels with a strong inhibition of antioxidant enzymes activity. A clear induction of inflammation and apoptosis was also detected. Due to its oxidative proprieties, AFB1 was associated with the onset of neurodegenerative diseases (Kasem et al., 2022).

In rat hippocampus, 42 days exposure to toxin caused not only tissue histopathological changes and inflammation but also a strong increase in HDL, insulin and glycemia concentration. The promotion of oxidative and nitrosative stress with impairment of antioxidant enzymes activity was also

found (Ibrahim et al., 2022). In the longest exposure, oral AFB1 administration altered mice locomotor activity, spatial learning and memory as well as triggered an anxiety-like behavior. Intoxicated mice also showed high levels of inflammation and oxidative stress with decreased levels of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) (Gugliandolo et al., 2020).

As regards OTA, a short exposure (2 h) to low toxin doses (100 nM) disrupted the integrity of blood brain barrier (formed using ECV304 and C6 cells) and altered the metabolic cell profile by triggering the expression of proinflammatory eicosanoids (Alonso-Garrido et al., 2021b). Similar results were obtained by Behrens et al. (2021), wherein 48 h exposure to OTA caused porcine astrocytes death and altered the integrity of neuronal barrier. Thus, OTA-ability to reach the brain and induce brain homeostasis alteration was demonstrated. In SH-SY5Y cells, exposure to OTA provoked neuronal dysfunction and apoptosis with alterations in several genes related to apoptosis (p53, Bax, MAPT, BDNF and TPPP) (Sharma et al., 2023). In the same cell line, digested bread extracts containing low OTA doses altered neuronal differentiation and cell cycle. A clear alteration in several cell cycle (p21, p53, cyclin B and D) and neuronal differentiation (GAP43, Wnt5a and TUBB3) genes was also found (Frangiamone et al., 2022b).

In the most aggressive exposure, OTA administration in mice brain induced several tissue lesions such as meningeal, cerebral cortex and

subependymal hemorrhages with granular stratification in the cerebellum region. In addition, toxin altered neurotransmitter levels (dopamine, serotonin and norephedrine) and antioxidant enzymes (SOD, CAT, GPX) activity. Interestingly, the results obtained by behavioral experiments, such as: spontaneous activity, gait analysis, pole and cylinder test demonstrated that OTA provoked the onset of parkinsonian physiognomies (Bhat et al., 2018). In rat brains, OTA-administration during 21, 42 and 63 days triggered oxidative stress and lipid peroxidation with a significant decrease of total and reduced glutathione (GSH) levels. OTA-intoxicated rats also showed histological brain injuries accompanied by neuronal degeneration, increased perineuronal spaces and neuropil oedema (Jamuna et al., 2020). Izco et al. (2021) evaluated how *in vivo* OTA exposure can lead to biochemical and pathological changes typically reported in Parkinson disease. In fact, mice exposed for 28 days to low OTA doses and then fed for six months with a normal diet showed a loss of striatal dopaminergic innervation and dysfunctions in dopaminergic cells, which are responsible for motor disturbances typically related to the neurogenerative disease. In addition, elevated levels of alpha-synuclein, a typical marker of Parkinson syndrome, were detected. The authors associated OTA neurotoxicity with decreased LAMP-2A protein expression, altered chaperone-mediated autophagy, and reduced alpha-synuclein turnover.

In the only *in vitro* study evaluating the combined AFB1 and OTA effects on neuronal differentiation, Frangiamone et al. (2022b) demonstrated

not only a clear alteration in the fluorescent expression of neuronal markers and cell cycle phases but also changes in gene expression of cell cycle and neuronal differentiation markers. However, a possible antagonism between mycotoxins was found as the results were less altered compared to the control condition than after OTA single exposure (Table 1).

**Table 1.** Neurotoxicity studies: mycotoxin, dose, exposure time, experimental model, techniques, effects and references.

| Mycotoxin | Dose | Exposure time | Experimental model | Techniques | Effects | References |
|---|---|---|---|---|---|---|
| **AFB1** | 0.06 μM | 24-48 h | CHME5 cells | MTT, chemiluminescence assay, RT-qPCR, FCM | Inflammatory response, ATP depletion and apoptosis | Mehrzad et al., 2018 |
| | 0.06 μM | 7 days | SH-SY5Y cells | MTT, FCM, IF, RT-qPCR | Disruption of neuronal differentiation with cell cycle arrest | Frangiamone et al., 2022b |
| | 25 μg/kg bw/ gastric gavage administration | 7 days | Rats brain | Behavioral, recognition, forced test, Biochemical analysis, IHC | Alterations of brain tissue with oxidative stress and inflammation | Sahin et al., 2022 |
| | 80 μg/kg bw/ oral gavage administration | 28 days | Rats brain | Enzymatic assay, biochemical analysis, ELISA | Promotion of apoptosis, inflammation and oxidative stress | Kasem et al., 2022 |
| | 72 μg/kg bw/ oral gavage administration | 42 days | Rats brain | Microscopy, enzymatic assay, biochemical analysis, ELISA | Alteration of lipid, glucose, insulin profiles, oxidative stress | Ibrahim et al., 2022 |
| | 75 μg/kg bw/ oral gavage administration | 45 days | Mice brain | Behavioral tests, ELISA, enzymatic assay | Oxidative stress, behavioral alterations and impairment of antioxidant defense | Gugliandolo et al., 2020 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 0.1 μM | 2 h | ECV304-C6 cells | LC-ES-QTOF-MS | Disruption of blood brain barrier and inflammation | Alonso-Garrido et al., 2021b |
| | 10 μM | 48 h | PBCEC cells | HPLC-MS/MS, CCK-8 assay, permeability assay | Apoptosis and blood brain barrier disruption | Behrens et al., 2021 |
| | 0.001-1 μM | 48 h | SH-SY5Y cells | RT-qPCR, Trypan blue | Neuronal death | Sharma et al., 2023 |
| | 0.24 μM | 7 days | SH-SY5Y cells | MTT, FCM, IF, RT-qPCR | Disruption of neuronal differentiation with cell cycle arrest | Frangiamone et al., 2022b |
| **OTA** | 3.5 mg/kg bw/ intraperitoneal administration | 14 days | Mice brain | Behavioral tests, WB, biochemical analysis | Alterations of neurotransmitters with oxidative stress and Parkinson onset | Bhat et al., 2018 |
| | 2 mg/kg/contaminated feed | 21,42,63 days | Rats brain | Microscopy, enzymatic and antioxidant assay | Brain injury with oxidative stress and impairment of antioxidant defense | Jamuna et al., 2020 |
| | 0.21, 0.5 mg/kg bw/ oral gavage administration | 28 days | Mice brain | Behavioral tests, WB, IHC, RT-qPCR | Parkinson onset | Izco et al., 2021 |
| **AFB1 – OTA** | 0.06 μM (AFB1) 0.24 μM (OTA) | 7 days | SH-SY5Y cells | MTT, FCM, IF, RT-qPCR | Disruption of neuronal differentiation with cell cycle arrest | Frangiamone et al., 2022b |

*1.3.1.2. Exposure to mycotoxins and bioactive compounds*

The addition of P and FW, individually and combined, in digested bread extracts limited the toxic effects of AFB1, OTA and their combination in SH-SY5Y cells, by enhancing the fluorescent expression of dopamine and βIII Tubulin markers and modulating cell cycle alterations. An overall transcriptomic improvement of several genes related to cell cycle and neuronal differentiation was also obtained (Frangiamone et al., 2022b).

*In vivo* probiotics administration, especially *Bifidobacterium* and *Lactobacillus*, also exhibited the ability to modulate AFB1-neurotoxicity, due to their antioxidant and anti-inflammatory activities. In effect, compared to AFB1 group, the supplementation of probiotics increased SOD and GPX activities and decreased MDA, TNF-a, and IL-6 levels. In addition, probiotics ameliorated anxiety and depression-like behavior and preserved the normal structure of cerebral cortex and neuronal blood vessels (Sahin et al., 2022). Kasem et al. (2022) also showed the strong neuroprotective *in vivo* effect of *Chelidonium majus* ethanolic extract, a plant compound containing alkaloids, acid phenolics and flavonoids with pharmacological activities. The oral compound supplementation downregulated the expression of inflammatory, oxidative and apoptotic markers as well as restored the normal values of neurochemical markers (acetylcholine, dopamine and serotonin) that were deteriorated by AFB1 intake. The protective *Chelidonium majus* ethanolic extract effect in rats brain could be mediated by its

antioxidant and free radical scavenging activities (Table 2). Likewise, *Artichoke leaf* extract, a Mediterranean plant rich in flavonoids, showed a strong neuroprotection activity against AFB1-toxic effects in adult male rats. High *Artichoke leaf* extract doses relieved inflammation and oxidative stress promoted by AFB1-poisoning, by increasing the activity of anti-inflammatory and antioxidant mediators. A clear improvement in brain histological changes as well as in glycemia and insulin levels was also found. In view of this, the possible *Artichoke leaf* extract application as food conservative to safeguard animal and human cerebral health can be considered (Ibrahim et al., 2022). A similar application in food industry can be proposed for the natural carotenoid quercetin. Indeed, 45 days *in vivo* exposure to quercetin increased GSH, SOD and CAT activities and reduced oxidative stress, lipid peroxidation and inflammation in AFB1-exposed mice. As a result, improved cognitive and spatial memory capacity as well as a better profile in anxiety and lethargy disorders was achieved (Gugliandolo et al., 2020).

The metabolic analysis performed in a neuronal brain barrier model, exposure with low doses of pumpkin pulp, rich in flavonoids, has been showed to play a neuroprotective *in vitro* effect by reducing the production of proinflammatory eicosanoids prompted by OTA exposure (Alonso-Garrido et al., 2021b). The supplementation of L-Dopa, a dopamine precursor, also showed the ability to alleviate OTA induced Parkinsonian physiognomies in mice brain. In effect, L-Dopa administration ameliorated

locomotor and memory capacities of OTA intoxicated mice. In addition, L-Dopa enhanced neurotransmitter levels and general brain parameters related to antioxidant and anti-inflammatory status, which were impaired by fungal compound (Bhat et al., 2018). Jamuna et al. (2020) also demonstrated how high doses of *Allium sativum* and *Withania somnifera* increased the activities of enzymatic and non-enzymatic antioxidants as well as reduced histopathological brain changes caused by oral OTA intake. These functional ingredients can modulate OTA-neurotoxicity due to their ability in preventing lipid peroxidation and replenishing GSH content. Therefore, a possible implementation of *Allium sativum* and *Withania somnifera* in food industry to treat and prevent the onset of neurotoxic processes can be raised.

**Table 2.** Neurotoxicity studies: mycotoxin, dose, bioactive compound, exposure time, experimental model, techniques, effects and references.

| Mycotoxin | Dose | Bioactive compound | Exposure time | Experimental model | Techniques | Effects | References |
|---|---|---|---|---|---|---|---|
| **AFB1** | 0.06 μM | P, FW (10% in medium) | 7 days | SH-SY5Y cells | MTT, FCM, IF, RT-qPCR | Mitigation of neurotoxicity with improvement in neuronal differentiation | Frangiamone et al., 2022b |
| | 25 μg/kg bw/ gastric gavage administration | Probiotics ($2.5 \times 10^{10}$ CFU/ml) | 7 days | Rats brain | Behavioral, recognition, forced test, Biochemical analysis, IHC | Modulation of oxidative stress and neuroinflammation | Sahin et al., 2022 |
| | 80 μg/kg bw/ oral gavage administration | *Chelidonium majus* extract (300 mg/kg bw) | 28 days | Rats brain | Enzymatic assay, biochemical analysis, ELISA | Neuroprotection | Kasem et al., 2022 |
| | 72 μg/kg bw/ oral gavage administration | *Artichoke leaf* extract (100 mg/kg bw) | 42 days | Rats brain | Microscopy, enzymatic assay, biochemical analysis, ELISA | Modulation of neurotoxicity | Ibrahim et al., 2022 |
| | 75 μg/kg bw/ oral gavage administration | Quercetin (30 mg/kg bw) | 45 days | Mice brain | Behavioral tests, ELISA, enzymatic assay | Protection against oxidative stress, behavioral alterations and impairment of antioxidant defense | Gugliandolo et al., 2020 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 26.5 μM | Pumpkin pulp (0.5 μM) | 2 h | ECV304-C6 cells | LC-ES-QTOF-MS | Modulation of neuroinflammation | Alonso-Garrido et al., 2021b |
| | 0.240 μM | P, FW (10% in medium) | 7 days | SH-SY5Y cells | MTT, FCM, IF, RT-qPCR | Mitigation of neurotoxicity with improvement in neuronal differentiation | Frangiamone et al., 2022b |
| **OTA** | 3.5 mg/kg bw/ intraperitoneal administration | L-dopa (20 mg/kg bw) | 14 days | Mice brain | Behavioral tests, WB, biochemical analysis | Alterations of neurotransmitters with oxidative stress and Parkinson onset | Bhat et al., 2018 |
| | 2 mg/kg/contaminated feed | *Allium sativum* (20 mg/kg bw) and *Withania somnifera* (100 mg/kg bw) | 21, 42, 63 days | Rats brain | Microscopy, enzymatic and antioxidant assay | Brain protection against oxidative stress and histological damage | Jamuna et al., 2020 |
| **AFB1-OTA** | 0.06 μM (AFB1) 0.24 μM (OTA) | P, FW (10% both in medium) | 7 days | SH-SY5Y cells | MTT, FCM, IF, RT-qPCR | Mitigation of neurotoxicity with improvement in neuronal differentiation | Frangiamone et al., 2022b |

### *1.3.2. Immune toxicity*

Regarding immune toxicity, the cell lines used for *in vitro* experiments were: human lymphoblastic T cells (Jurkat) and porcine alveolar macrophage cells (PAM) while the animal models employed were: mice, pigs, and chickens (Table 3). The functional ingredients utilized to alleviate AFB1 and OTA immune toxicity were: P, FW, quercetin, curcumin, astragalus polysaccharide, grape seed meal and lycopene (Table 4).

#### *1.3.2.1. Exposure to mycotoxins*

The shortest exposure to AFB1 in chickens elicited the release of heterophils extracellular traps, a network of DNA-based structures consisting of histone 3 and elastase. Extracellular traps formation was mediated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity along with glycolysis and p38 signaling pathways activation. A direct consequence of extracellar traps release was the increase of liver and kidney biochemical indexes in chicken serum. Additionally, AFB1 exposure not only decreased SOD and GSH-Px levels, but also increased the mRNA expression of inflammatory and apoptotic markers (Gao X. et al., 2022). In Jurkat cells, the proteomic analysis conducted after 7 days exposure to low AFB1 doses (100 nM) revealed the alteration of several proteins related to antioxidant, metabolic and genomic pathways (Cimbalo et al., 2022a).

In mice, 21 days oral AFB1 administration altered intestinal immune function by suppressing the activity of M2 macrophages and Paneth cells. Using an oxylipinomics approach, authors associated toxin immune toxicity with an increase of soluble epoxide hydrolase levels, which in turn enhanced eicosanoid signaling pathway and proinflammatory cytokines production (Wang et al., 2023).

AFB1 most aggressive exposure led to severe histological damages in chicken spleen, thymus and bursa of Fabricius, with a decrease in the number and ratio of plasma cells. RT-qPCR analysis also showed the promotion of inflammation with a marked decrease of IL-2 and IFN-γ gene expression (Li et al., 2022). In pig mesenteric lymph nodes, 28 days exposure to AFB1 resulted in oxidative stress and lipid peroxidation induction by decreasing the activity of CAT, SOD and GPX enzymes. It has been also observed a clear inflammatory response with the increased gene expression of MAPK, NF-kB and proinflammatory mediators (Marin et al., 2020). In the less aggressive exposure, intragastric AFB1 administration caused splenic damage and inflammation in mice with a marked reduction of T-lymphocytes subsets. In addition, AFB1 not only promoted reactive oxygen species (ROS) generation with alteration of total antioxidant status but also provoked impairment of mitochondrial membrane potential and apoptosis via cytochrome c release and caspase 3-9 activity (Xu et al., 2019). Interestingly, Xu et al. (2022) investigated AFB1-immune toxicity by integrating RNA and small-RNA sequencing data of chicken immune organs

in order to elucidate both differentially expressed transcriptional profiles and related pathways. RNA-seq analysis revealed the alteration of immune and metabolic pathways while differentially expressed miRNAs were mainly associated with cancer progression and inflammation. A potential mRNA–miRNA pathway crosstalk was also found.

In macrophages, exposure to low doses of OTA impaired cell viability, proliferation and phagocytic capacity. In addition, the fungal compound not only favored the polarization of macrophages towards a proinflammatory M1 phenotype but also suppressed the expression of several anti-inflammatory mediators and promoted lactate dehydrogenase (LDH) release. The repression of autophagy, a protective biological process, was also observed (Su et al., 2018). Likewise, Gan et al. (2022) demonstrated the ability of porcine circovirus type 2 (PCV-2) to boost OTA-immunosuppressive effects in porcine macrophages and pigs spleen. *In vitro* results showed that PCV-2 infection aggravated OTA-promoted nuclear apoptosis, γ-H2AX foci formation and NF-kB pathway activation. *In vivo* results also indicated as the viral infection exacerbated OTA-induced NF-κB p65 phosphorylation and inflammation with increased TLR4 and MyD88 protein expression. In porcine macrophages, 48 h exposure to toxin decreased cell viability and triggered cell death with apoptotic nuclei formation. A significant proinflammatory cytokines production was also found. These outcomes were confirmed in mice spleen, in which OTA administration caused tissue inflammation and histopathological damages

(Liu et al., 2018). In Jurkat cells, the proteomic analysis carried out after 7 days exposure to low OTA doses (100 nM) showed the altered expression of several DNA-linked histones with impairment of cell antioxidant capacity and thioredoxin peroxidase activity (Cimbalo et al., 2022a).

Notably, Gao et al. (2023) exposed for 28 days mice jejunum to OTA in order to screen for key intestinal immunotoxicity-related pathway, by analyzing differentially expressed mRNAs, microRNAs and long non-coding RNAs. Results demonstrated a possible crosstalk between mRNA-microRNA and long non-coding RNA in which NF-kB signaling was considered the main responsible for OTA-immunosuppression *in vivo*. Tissue damages with proinflammatory cytokines production were also detected. Analogously, in chicken immune organs, 42 days exposure to toxin induced pathological tissue lesions with a significant decrease in the antibody response to Newcastle Disease, Infectious Bronchitis and Avian Influenza vaccination. OTA also fostered oxidative stress and lipid peroxidation by decreasing CAT and GSH levels. A marked apoptosis promotion with gene expression changes in PTEN, Bax and Caspase-3 were also found (Abdelrahman et al., 2022).

As shown in Table 3, only one study evaluated the combined effect between AFB1 and OTA. In this case, QTOF-MS proteomic analysis revealed not only the alteration of several proteins related to immune mucosa defense but also the possible antagonism between mycotoxins (Cimbalo et al., 2022a).

**Table 3.** Immune toxicity studies: mycotoxin, dose, exposure time, experimental model, techniques, effects and references.

| Mycotoxin | Dose | Exposure time | Experimental model | Techniques | Effects | References |
|---|---|---|---|---|---|---|
| AFB1 | 1,2,4,8 μM | 1.5 h | Chicken heterophils | IF, ROS measurement | Promotion of immune toxicity with extracellular traps release and glycolysis activation | Gao X. et al., 2022 |
| | 0.1, 0.14, 0.2 μM | 7 days | Jurkat cells | Trypan blue assay, QTOF-MS | Alteration in proteins expression leading to immune toxicity | Cimbalo et al., 2022a |
| | 0.06, 0.18, 0.6 mg/kg bw/oral gavage administration | 21 days | Mice macrophages and Paneth cells | FCM, RT-qPCR and LC/MS-MS-oxylipinomics | Disruption of intestinal immunity with high epoxide hydrolase levels | Wang et al., 2023 |
| | 5 mg/kg/contaminated diet | 28 days | Chicken immune organs | Microscopy, RT-qPCR | Induction of immune organs damage and inflammation | Li et al., 2022 |
| | 320 μg/kg/contaminated feed | 30 days | Pigs mesenteric lymph nodes | Enzymatic assay, ELISA, RT-qPCR, WB | Promotion of inflammation and oxidative stress | Marin et al., 2020 |
| | 75 μg /kg bw oral gavage administration | 30 days | Mice spleen | T lymphocyte analysis, ELISA, RT-qPCR, WB, microscopy, FCM, apoptosis and oxidative stress assay | Immunosuppression with inflammation, oxidative stress, apoptosis and mitochondrial dysfunction | Xu et al., 2019 |
| | 0.3, 0.6,1.2 mg/kg/contaminated diet | 35 days | Chicken bursa of Fabricius and spleen | RNA-seq, RT-qPCR | Inflammation and immune toxicity | Xu et al., 2022 |

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| | 0.4 μM | 24-48-72h | PAM cells | MTT, LDH assay, RT-qPCR, FCM, IF, | Immunosuppression with proinflammatory biomarkers expression, macrophage polarization and autophagy inhibition | Su et al., 2019 |
| | 1, 3, 5, 7, 9 μM | 48h | PAM cells | MTT, FCM, RT-qPCR, IF,WB, ELISA | Reduction of cell viability, DNA damage, apoptosis and inflammatory biomarkers expression | Gan et al., 2022 |
| **OTA** | 0.6 μM | 48h | PAM cells | MTT, LDH assay, RT-qPCR, FCM, WB | Decrease of cell viability and induction of apoptosis and inflammatory biomarkers expression | Liu et al., 2018 |
| | 0.1, 0.7, 1 μM | 7 days | Jurkat cells | Trypan blue assay, QTOF-MS | Alteration in proteins expression leading to immune toxicity | Cimbalo et al., 2022a |
| | 75 μg/kg bw intraperitoneal administration | 21 days | Mice spleen | Microscopy | Immune stress and damage | Liu et al., 2018 |
| | 3 mg/kg bw oral gavage administration | 28 days | Mice jejunum | Microscopy, RT-qPCR, RNA, miRNA, lncRNA-sequencing | Intestinal dysfunction with immunosuppression by NF-kB signaling activation | Gao et al., 2023 |
| | 400 μg/kg contaminated feed | 42 days | Pigs spleen | Microscopy, IHC | Immune toxicity with NF-kB-p65 phosphorylation and TLR4 and MyD88 expression | Gan et al., 2022 |
| | 0.5 mg/kg contaminated feed | 42 days | Chicken immune organs | RT-qPCR, microscopy, ELISA, antioxidant assay | Promotion of immune stress, apoptosis and histopathological damage | Abdelrahman et al., 2022 |

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|---|---|---|---|---|---|---|
| **AFB1-OTA** | 0.1, 0.18, 0.20 μM (AFB1)<br>0.1, 0.7, 0.8 μM (OTA) | 7 days | Jurkat cells | Trypan blue assay, QTOF-MS | Alteration in protein expression leading to immune toxicity | Cimbalo et al., 2022a |

*1.3.2.2. Exposure to mycotoxins and bioactive compounds*

As shown in Table 4, P and FW, two bioactive compounds widely used as food conservatives for their beneficial properties on human health, have been shown to reduce the toxic effects of AFB1, OTA and their combination in Jurkat cells, by enanching the expression of several proteins related to antioxidant and immune protective pathways (Cimbalo et al., 2022a).

Li et al. (2022) also provided an experimental scientific basis for the application of curcumin as a therapeutic compound in livestock production practice. Indeed, curcumin alleviated AFB1-induced chicken immune organs damage and plasma cell alterations in a dose-dependent manner. A strong immunomodulatory effect with the production of anti-inflammatory cytokines was also observed. In a similar way, grape seed meal, a waste food product rich in phenolic acids, resveratrol, proanthocyanins, and flavonoids, also demonstrated the ability to mitigated tissue damages inflammation induced by AFB1 oral administration in pigs mesenteric lymph nodes. The addition of grape seed in contaminated diet also showed a strong antioxidant effect by improving the activity of CAT, SOD and GPX enzymes (Marin et al., 2020). Likewise, the supplementation of lycopene in mice spleen during 30 days alleviated AFB1-induced immunosuppression by relieving splenic damages and by increasing tissue weight, T lymphocyte subsets, and the levels of IL-2, IFN-$\gamma$, and TNF-$\alpha$ in serum and spleen.

Furthermore, this natural carotenoid inhibited oxidative stress by decreasing ROS levels and enhancing the activities of antioxidant enzymes. A marked reduction of cell death via mitochondria pathways was also observed (Xu et al., 2019).

The oral supplementation of astragalus polysaccharide, a common plant compound used in Chinese medicine and known for its multiple biological activities, modulated OTA-immune stress *in vitro* and *in vivo.* In PAM cells, astragalus polysaccharide could alleviate cell cytotoxicity, apoptosis and proinflammatory cytokines production. In mice spleen, this compound relieved OTA-promoted inflammation, apoptosis and tissue injuries. The protective mechanism of action (MoA) by which astragalus polysaccharide suppressed toxin immune toxicity was related to NF-kB repression and AMPK/SIRT-1 signaling pathway activation (Liu et al., 2018). In chicken immune organs, 42 days exposure to quercetin, a common flavonoid produced as a plant-secondary metabolite, has been shown to reverse OTA-induced tissue damages and improve the antibody response to several viral infections. In addition, the supplementation of this flavonoid limited oxidative stress and ROS generation by increasing the activity of antioxidant enzymes activity. The immunomodulatory quercetin effect was associated with PI3K/AKT signaling pathway activation (Abdelrahman et al., 2022) (Table 4).

**Table 4.** Immune toxicity studies: mycotoxin, dose, bioactive compound, exposure time, experimental model, techniques, effects and references.

| Mycotoxin | Dose | Bioactive compound | Exposure time | Experimental model | Techniques | Effects | References |
|---|---|---|---|---|---|---|---|
| **AFB1** | 0.1, 0.14, 0.2 μM | P, FW (both 10% in medium) | 7 days | Jurkat cells | Trypan blue assay, QTOF-MS | Modulation effect on immune toxicity | Cimbalo et al., 2022a |
| | 5 mg/kg/contaminated diet | Curcumin (150, 300, 450 mg/kg bw) | 28 days | Chicken immune organs | Microscopy, RT-qPCR | Reduction of immune damage and inflammation | Li et al., 2022 |
| | 320 μg/kg/contaminated feed | Grape seed (8% in diet) | 30 days | Pigs mesenteric lymph nodes | Enzymatic assay, ELISA, RT-qPCR, WB | Reduction of immune toxicity, inflammation and improvement of antioxidant enzymes activity | Marin et al., 2020 |
| | 75 μg /kg bw oral gavage administration | Lycopene (5 mg/kg bw) | 30 days | Mice spleen | T lymphocyte analysis, ELISA, RT-qPCR, WB, microscopy, FCM, apoptosis and oxidative stress assay | Prevention of inflammation, oxidative stress, apoptosis and mitochondrial dysfunction | Xu et al., 2019 |

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| **OTA** | 0.6 μM | Astragalus polysaccharide (20 μg/ml) | 48h | PAM cells | MTT, LDH assay, RT-qPCR, FCM, WB | Alleviation of immune toxicity and inflammation | Liu et al., 2018 |
| | 0.1, 0.7, 1 μM | P, FW (10% medium) | 7 days | Jurkat cells | Trypan blue assay, QTOF-MS | Modulation effect on immune toxicity | Cimbalo et al., 2022a |
| | 75 μg/kg bw intraperitoneal administration | Astragalus polysaccharide (200 mg/kg bw) | 20 days | Mice spleen | Microscopy | Ameliorative effect on immune stress and damage | Liu et al., 2018 |
| | 0.5 mg/kg contaminated feed | Quercetin (5 g/kg bw) | 42 days | Chicken immune organs | RT-qPCR, microscopy, ELISA, antioxidant assay | Antiapoptotic, antioxidant and immunomodulatory activities | Abdelrahman et al., 2022 |
| **AFB1-OTA** | 0.1, 0.18, 0.20 μM (AFB1) 0.1, 0.7, 0.8 μM (OTA) | P, FW (both 10% in medium) | 7 days | Jurkat cells | Trypan blue assay, QTOF-MS | Modulation effect on immune toxicity | Cimbalo et al., 2022a |

### *1.3.3. Hepatotoxicity*

In relation to hepatotoxicity, the cell lines used were: human liver cells (HHL-5), human hepatocellular carcinoma cells (HepG2), and chicken hepatocarcinoma cells (LMH) while mice, rats, chickens, rabbits and quails were used for *in vivo* experiments (Table 5). The bioactive compounds used to mitigate AFB1 and OTA hepatotoxic effects were: lemon peel, *Erythrina latissima* bark extract, purple rice husk, *Sorghum bicolor*, gallic acid, grape seed meal, *Mentha piperita, Bacillus* extract, probiotics, selenomethionine, curcumin, *Allium sativum* and *Withania somnifera* (Table 6).

#### *1.3.3.1. Exposure to mycotoxins*

Acute exposure to high doses of AFB1 inhibited HHL-5 cell proliferation, arrested the cell cycle in the $G_0/G_1$ phase and induced oxidative stress with DNA damage. The toxicity of AFB1 was mainly associated with the increased activity of NADPH oxidase (Cao et al., 2022). In HepG2 cells and mice liver, exposure to AFB1 provoked liver damage steatosis with increased levels of alanine (ALT) and aspartate (AST) aminotransferase as well as an aberrant accumulation of cholesterol, triglycerides and lipid droplets. Authors concluded that AFB1-toxicity was related to cyclooxygenase (COX-2) and PTEN signaling pathways activation (Ren et al., 2020).

In chicken livers, a daily toxin administration promoted histopathological damage, inhibited the activity of antioxidant enzymes such as: SOD and GPX and increased the gene expression of inflammatory (TNF-$\alpha$, IL-6 and IL-1$\beta$, iNOS, COX-2) and apoptotic (caspase 1-3-11) markers (Gao X. et al., 2022). Interestingly, the intraperitoneal toxin administration after 48 h in chimeric mice with humanized livers caused cytochrome CYP3A4 bioactivation, which in turn led to hepatic vacuolation and inflammatory cells infiltration. Thus, the modulation of cytochrome activity has proven to be crucial in protecting human liver health against AFB1 toxic effects (Ishida et al., 2020). Similarly, the presence of hepatic necrosis and fibrosis with a clear dilation of the portal vein and bile ducts was observed by exposing rats to low doses of AFB1 for only 72 h (Zarev et al., 2020).

In the same animal model, AFB1 exposure showed genotoxic effects with micronuclei formation and a clear alteration of several detoxifying enzymes activity (CYP1A1, 1A2, 3A2) (Chariyakornkul et al., 2019). The formation of AFB1-DNA adducts was also observed by Zhang J. et al. (2022) after 12 days exposure to toxin. In addition, body weight loss and histopathological lesions with hepatocellular edema and inflammatory cell infiltrates were clearly found. Likewise, rat livers exposed during 28 days with AFB1 showed clear histopathological alterations characterized by Kupffer cell vacuolization. A significant increase in TNF-$\alpha$ and IL-6 expression was also detected (Sabry et al., 2022). In the less aggressive

studies, 28 days oral exposure to AFB1 (50 and 75 μg/kg/bw) in rats caused body weight loss and pathological liver damages characterized by apoptotic bodies and microvesicular steatosis. Furthermore, AFB1 suppressed the activity of antioxidant enzymes by inducing oxidative and nitrosative stress. The increased protein expression of proinflammatory cytokines (IL-1β, IL-10, TNF-$\alpha$) and apoptosis markers (caspase 3 and 9) was also observed (Owuni et al., 2022; 2020a).

In the most aggressive study, pigs treated with very high doses of AFB1 (320 mg/kg in diet) showed histological liver changes and portal vein fibrosis as well as genetic alterations with DNA fragmentation. Additionally, the expression of 34 proinflammatory markers was significantly increased. AFB1-MoA was associated with MAPK and NF-kB signaling pathways activation (Taranu et al., 2020).

Longest exposures tested were 35 days in quails and 42 days in rats. In quails, toxin administration impaired liver bird structure and promoted oxidative stress by altering SOD and GPX activity (Masouri et al., 2022). In rats, AFB1 fostered oxidative stress with disruption of antioxidant enzymes activity (GPX and SOD) and upregulated the gene expression of pro-apoptotic marker (p53 and Bax). Significant chromosomal aberrations with DNA fragmentation were also found (Abdel-Wahhab et al., 2020) (Table 5).

As regard OTA studies, the shortest exposure to toxin showed genotoxic effects by promoting DNA damage and fragmentation in LMH liver cells (Markowiak et al., 2019). In HepG2 cells, OTA promoted cell death and oxidative stress in a dose and time dependent manner. Moreover, toxin depleted intracellular GSH reserves and suppressed CAT gene expression (García-Pérez et al., 2021).

In rabbits, 7 days exposure to toxin caused fibrosis and hepatic vacuolation with a significant reduction in glycogen levels. Furthermore, OTA promoted oxidative stress by suppressing the activity of several antioxidant enzymes. RT-qPCR and WB assays also showed the clear overexpression of proinflammatory cytokines such as: IL-1β, IL-6 and TNF-$\alpha$ (Zhang Z. et al., 2022). In rats, 14 days OTA exposure induced histopathological liver lesions with a marked macrophagic infiltration. In addition, NO levels accompanied by SOD, CAT and GPX enzymes activity suppression were observed. ELISA assay also showed not only a marked expression of proinflammatory cytokines (TNF-$\alpha$, IL-1β, IL-6) but also the presence of oxidative DNA damage (Damiano et al., 2021; Longobardi et al., 2021). In rats, the longest exposure to OTA promoted oxidative stress, lipid peroxidation and histopathological alterations (hepatocytomegaly, karyomegaly and binucleations) in a time-dependent manner. In addition, OTA decreased the enzymatic activity of SOD, CAT and GPX (Jamuna et al., 2018).

**Table 5.** Hepatotoxicity studies: mycotoxin, dose, exposure time, experimental model, techniques, effects and references.

| Mycotoxin | Dose | Exposure time | Experimental model | Techniques | Effects | References |
|---|---|---|---|---|---|---|
| **AFB1** | 1.25-80 μM | 1-72h | HHL-5 cells | MTT, ROS detection, clone formation, FCM, IF, RT-qPCR, WB | Inhibition of cell proliferation and viability. Promotion of $G_0/G_1$ cell cycle arrest, oxidative stress and DNA damage by NADPH oxidase | Cao et al., 2022 |
| | 5 μM | 24-48h | HepG2 cells | RT-qPCR, IF, WB, Lipid assay | Promotion of liver damage with alteration in mitochondrial lipid metabolism by COX-2 expression | Ren et al., 2020 |
| | 5 mg/kg bw oral gavage administration | 24h | Chickens liver | Enzymatic assay, microscopy, RT-qPCR | Histopathological damage, oxidative stress and up-regulation of apoptotic and inflammation biomarkers | Gao X. et al., 2022 |
| | 3 mg/kg bw/ intraperitoneal administration | 48h | Mice liver | IHC, RT-qPCR, WST-1 | Induction of hepatocyte vacuolization and inflammation by CYP3A4 bioactivation | Ishida et al., 2020 |
| | 1 mg/kg bw/ oral gavage administration | 72h | Rats liver | Microscopy | Alterations in liver tissue structure | Zarev et al., 2020 |
| | 200 μg/kg bw/ intraperitoneal administration | 8 days | Rats liver | Enzymatic assay | Hepatotoxicity by alterations in antioxidant and detoxification enzymes | Chariyakornkul et al., 2019 |
| | 5 mg/kg bw oral gavage administration | 12 days | Rats liver | IHC | Liver damage | Zhang J. et al., 2022 |

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| | 80 µg/kg bw/ oral gavage administration | 28 days | Rats liver | Biochemical analysis, ELISA, IHC | Liver damage and inflammation | Sabry et al., 2022 |
| | 50 µg/kg bw/ oral gavage administration | 28 days | Rats liver | enzymatic assay, ELISA, Microscopy | Promotion of oxidative stress, inflammation, apoptosis with histopathological tissue alteration | Owumi et al., 2022 |
| | 75 µg/kg bw/ oral gavage administration | 28 days | Rats liver | enzymatic assay, ROS measurement, ELISA, Microscopy | Promotion of inflammation, oxidative stress and reduction of antioxidant defenses | Owumi et al., 2020a |
| | 0.6 mg/kg bw/ intraperitoneal administration | 28 days | Mice liver | IHC, enzymatic assay | Promotion of liver injury and steatosis | Ren et al., 2020 |
| | 320 mg/kg/contaminated feed | 28 days | Pigs liver | Microscopy, IHC, WB, RT-qPCR, DNA/RNA oxidative damage assay | Liver histological damage, oxidative stress, inflammation by MAPK and NF-kB signaling | Taranu et al., 2020 |
| | 2.5 mg/kg/contaminated feed | 35 days | Quails liver | Enzymatic assay | Liver damage with oxidative stress | Masouri et al., 2022 |
| | 80 µg/kg bw/ oral gavage administration | 42 days | Rats liver | RT-qPCR, DNA fragmentation assay, microscopy | Liver damage with oxidative stress, inflammation and DNA damage | Abdel-Wahhab et al., 2020 |
| **OTA** | 10 µM | 24h | LMH cells | Comet assay MTT, | Hepatotoxicity with DNA damage | Markowiak et al., 2019 |
| | 0.01-100 µM | 24-72h | HepG2 cells | enzymatic assay, ROS measurement, RT-qPCR | Impairment of cell viability, induction of oxidative stress with CAT and GSH depletion | García-Pérez et al., 2021 |

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|---|---|---|---|---|---|
| 0.2 mg/kg/contaminated feed | 7 days | Rabbits liver | ELISA, RT-qPCR, microscopy, ROS measurement biochemical and enzymatic assay | Liver injury with ROS production and impairment of antioxidant enzymes | Zhang Z. et al., 2022 |
| 0.5 mg/kg bw/ oral gavage administration | 14 days | Rats liver | IHC, ELISA, oxidative DNA assay, NO determination | Liver injury with nitrosative stress, inflammation and DNA damage | Longobardi et al., 2021 |
| 0.5 mg/kg bw/ oral gavage administration | 14 days | Rats liver | Microscopy, biochemical analysis, enzymatic assay | Liver damage with oxidative stress | Damiano et al., 2021 |
| 2 mg/kg/contaminated feed | 21, 42, 63 days | Rats liver | Microscopy, enzymatic assay | Liver injury with oxidative stress and impairment of antioxidant enzymes | Jamuna et al., 2018 |

### 1.3.3.2. *Exposure to mycotoxins and bioactive compounds*

The oral administration of *Erythrina latissima* bark extract, a leguminous plant rich in polyphenols, showed not only a high anti-genotoxic activity but also a clear protective effect against histopathological liver damages promoted by 72 h exposure to AFB1 in rats (Zarev et al., 2020). The purple rice husk extract, a natural compound containing high quantity of phenolic acids and vitamin E, also demonstrated a strong chemopreventive and antigenotoxic effect against AFB1. Moreover, the bioactive compound enhanced the activity of various detoxifying enzymes, including glutathione S-transferase and heme oxygenase, by inducing toxin degradation (Chariyakornkul et al., 2019). Furthermore, the addition in contaminated feed of lemon peel extract, rich in flavonoids and alkaloids, attenuated AFB1-induced liver damage in rats by reducing the expression of pro-inflammatory markers (TNF-$\alpha$, IL-6) and increasing the activity of antioxidant enzymes (CAT, SOD). A significant amelioration in liver tissue histology was also found (Sabry et al., 2022). Owumi et al. (2020a) and (2022) demonstrated the marked ameliorative effect of *Sorghum bicolor*, a polyphenol-rich plant, and gallic acid against AFB1-induced hepatic derangements in rats. These functional ingredients also exhibited clear antioxidant and anti-inflammatory effects, by reducing oxidative and nitrosative stress as well as the production of proinflammatory cytokines.

In pigs, the administration of grape seed meal, known for its high polyphenols content, attenuated AFB1-prompted histological liver injuries

and oxidative stress. The bioactive compound showed the ability to act at transcriptomic level, modulating not only MAPK and NF-kB signaling pathway activation but also the overexpression of 34 genes related to inflammation and immune response (Taranu et al., 2020). In quails, high doses of *Mentha piperita*, a low-calorie plant containing vitamins A and C, iron, potassium and fibers, revealed a clear protective effect on liver damage and oxidative stress caused by AFB1. In addition, *Mentha piperita* supplementation improved meat quality, bone strength and performance in AFB1-treated animals (Masouri et al., 2022). The addition of *Bacillus* MERNA97 extract, rich in phenolic acids, in contaminated feed during 42 days also exhibited therapeutic benefits against AFB1 exposure. Indeed, the functional compound showed not only a protective role on rat livers damage but also significant antioxidant, anti-inflammatory and antigenotoxic properties in a dose-dependent manner (Abdel-Wahhab et al., 2020) (Table 6).

The administration during 24 h of probiotics (*Saccharomyces cerevisiae* and various *Lactobacillus* strains) in LMH cells has been shown to modulate OTA-genotoxicity, by reducing DNA damage and fragmentation (Markowiak et al., 2019). In rabbit livers, selenomethionine supplementation in contaminated feed significantly modulated pathological liver damage and ameliorated the alteration of several physiological blood indices promoted by OTA poisoning. Selenomethionine also increased SOD and GPX activities and decreased IL-1$\beta$, IL-6 and TNF-$\alpha$ expression, thus revealing antioxidant

and anti-inflammatory properties (Zhang Z. et al., 2022). Moreover, curcumin, a natural polyphenolic compound extracted from the rhizomes of Turmeric plant, has been shown to counteract the harmful effects of chronic OTA exposure in rat livers. The bioactive compound reduced the multifocal lymphoplasmacellular hepatitis, periportal fibrosis and necrosis observed in OTA-treated animals. A significative reduction in NO levels and oxidative DNA damage was also found (Damiano et al., 2021; Longobardi et al., 2021). Jamuna et al. (2018) also demonstrated how the long-term administration of *Allium sativum* and *Withania somnifera*, two traditional medicinal plants rich in flavonoids, modulated OTA chronic effects in rat livers. These compounds not only improved the histopathological tissue injuries promoted by mycotoxin but also enhanced the antioxidant activity of SOD, CAT and GPX enzymes (Table 6).

**Table 6.** Hepatotoxicity studies: mycotoxin, dose, bioactive compound, exposure time, experimental model, techniques, effects and references.

| Mycotoxin | Dose | Bioactive compound | Exposure time | Experimental model | Techniques | Effects | References |
|---|---|---|---|---|---|---|---|
| **AFB1** | 1 mg/kg bw/ oral gavage administration | *Erythrina latissima* extract (20-50-100 mg/kg bw**)** | 72 h | Rats liver | Microscopy | Mitigation of AFB1-induced hepatotoxicity | Zarev et al., 2020 |
| | 200 μg/kg bw/ intraperitoneal administration | Purple husk rice extract (50-500 mg/kg bw) | 8 days | Rats liver | Enzymatic assay | Modulation of AFB1 liver toxicity increasing the activity of antioxidant and detoxification enzymes | Chariyakornkul et al., 2019 |
| | 80 μg/kg bw/ oral gavage administration | Lemon peel extract **(**100 mg/kg bw**)** | 28 days | Rats liver | Biochemical analysis, ELISA, IHC | Reduction of liver damage | Sabry et al., 2022 |
| | 75 μg/kg bw/ oral gavage administration | Gallic acid (20-40 mg/kg bw) | 28 days | Rats liver | Enzymatic assay, ROS measurement, ELISA, Microscopy | Reduction of inflammation and oxidative stress promoted by mycotoxin. | Owumi et al., 2020a |
| | 50 μg/kg bw/oral gavage administration | Apigeninidin-rich extracts of *Sorghum bicolor* (5-10 mg/kg bw) | 28 days | Rats liver | Enzymatic assay, ELISA, Microscopy | Inhibition of oxidative and nitrosative stress, inflammation, apoptosis. | Owumi et al., 2022 |

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|---|---|---|---|---|---|---|---|
| | 320 mg/kg/contaminated feed | Grape seed (8% of contaminated feed) | 28 days | Pigs liver | Microscopy, IHC, WB, RT-qPCR, DNA/RNA oxidative damage assay | Liver histological damage, oxidative stress, inflammation by MAPK and NF-kB signaling | Taranu et al., 2020 |
| | 2.5 mg/kg/ contaminated feed | *Mentha piperita* (400-800 mg/kg bw) | 35 days | Quails liver | Enzymatic assay | Modulation of liver injury | Masouri et al., 2022 |
| | 80 μg/kg bw/ oral gavage administration | *Bacillus* extract (2,4 mg/kg bw) | 42 days | Rats liver | RT-qPCR, DNA fragmentation assay, microscopy | Liver damage with oxidative stress, inflammation and DNA damage | Abdel-Wahhab et al., 2020 |
| **OTA** | 10 μM | Probiotics 2× $10^9$ CFU/g | 24 h | LMH cells | Comet assay | Protection of liver cells vitality | Markowiak et al., 2019 |
| | 0.2 mg/kg/contaminated feed | Selenomethionine (0.2,0.4,0.6 mg/kg bw) for 21 days | 7 days | Rabbits liver | ELISA, RT-qPCR, microscopy, ROS measurement, biochemical and enzymatic assay | Preservation of liver health with improvement of antioxidant enzymes activity | Zhang Z. et al., 2022 |
| | 0.5 mg/kg bw/ oral gavage administration | Curcumin (100 mg/kg bw) | 14 days | Rats liver | Microscopy, biochemical analysis, enzymatic assay | Mitigation of liver injury and oxidative stress | Damiano et al., 2021 |
| | 0.5 mg/kg bw/ oral gavage administration | Curcumin (100 mg/kg bw) | 14 days | Rats liver | IHC, ELISA, oxidative DNA assay, NO determination | Protection against inflammation, DNA damage and nitrosative stress | Longobardi et al., 2021 |

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|---|---|---|---|---|---|---|---|
| | 2 mg/kg/contaminated feed | *Allium sativum* (20 mg/kg bw) and *Withania somnifera* (100 mg/kg bw ) | 21, 42, 63 days | Rats liver | Microscopy, enzymatic assay | Reduction of liver injury and oxidative stress | Jamuna et al., 2018 |

### *1.3.4. Nephrotoxicity*

As regards nephrotoxicity, the cellular models used were: porcine kidney cells (PK-15), madin-darby canine kidney cells (MDCK), human renal epithelial cells (HK-2), epithelial-like pig kidney cells (LLC-PK1) and human glomerular mesangial cells (HGMC). Only mice and rats were the species used for *in vivo* experiments (Table 7). The biological compounds used to relieve the nephrotoxic effects of AFB1 and OTA were: lemon peel, lycopene, *Sorghum bicolor*, gallic acid, hydroxytyrosol, selenomethionine, curcumin, silymarin and ursolic acid (Table 8**).**

#### *1.3.4.1. Exposure to mycotoxins*

In the shortest *in vitro* exposure, AFB1 increased cell death and intracellular ROS generation in a dose-dependent manner. RNA-seq and WB assays revealed not only the disruption of antioxidant cell defenses but also the close correlation between AFB1 toxic effects and BACH1 increased activity (Zhang J. et al., 2022).

In the most aggressive exposure, the oral toxin administration caused severe intertubular hemorrhage and tubular dilatation in rats kidney tissue. AFB1 also promoted oxidative stress, by decreasing the activity of antioxidant enzymes (SOD, CAT and GPX) (Yilmaz et al., 2018). Whilst, in the less aggressive studies, 28 days exposure to AFB1 (50 and 75 μg/kg/bw)

fostered histopathological injury characterized by inflammatory cell infiltration and disseminated tubular necrosis. AFB1 also suppressed the activity of antioxidant enzymes (SOD, CAT, GPX) by inducing oxidative and nitrosative stress. The increased protein expression of proinflammatory cytokines (IL-1β, IL-10, TNF-α) and apoptosis markers (caspase 3 and 9) was also found (Owuni et al., 2022; 2020a). Similarly, 28 days exposure to AFB1 induced a marked vacuolization of the renal tubular epithelium, nuclei pyknosis, distension of Bowman's space and congestion of intertubular blood vessels. In addition, a strong kidney inflammation with high levels of TNF-α and IL-6 was detected (Sabry et al., 2022). As shown in Table 7, in the longest exposure, the fungal compound not only increased the serum concentration of urea nitrogen and creatinine but also caused pathological lesions in renal structure with glomerular atrophy. The gene expression of antioxidant enzymes such as: SOD, CAT and GSH was also repressed. Authors associated AFB1-MoA with alterations in Nrf2 signaling pathway (Yu et al., 2018).

In relation to OTA studies, 24 h exposure to mycotoxin induced MDCK cell death and upregulation of several genes related to renal fibrosis (α-SMA, Vimentin, TGF-β) and pyroptosis (GSDMD, IL-1β, IL-18, caspase-1) in a dose-dependent manner. Renal tissue fibrosis was also observed in mice, in which the intraperitoneal toxin administration (2 mg/kg for 14 days) not only increased histological injuries and the expression of various renal fibrosis molecules but also promoted pyroptosis, by activating NLRP3

signaling pathway. However, the use of small interfering RNA to knockdown NLRP3 and caspase-1, inhibited pyroptosis and alleviated OTA-toxic effects (Li et al., 2021; Mao et al., 2022). In HEK-293T cells, a short treatment with OTA disrupted cell viability and increased ROS production through the inhibition of Lonp1, Aco2 and Hsp75 protein expression (Li et al., 2019). Likewise, in canine, pig and rabbit renal cell lines, OTA exposure dose-dependently promoted cell death, oxidative and nitrosative stress. These outcomes were confirmed in rat kidneys after 90 days exposure to toxin, which prompted ROS generation and renal fibrosis with severe degenerations in the inner cortex zone (Crupi et al., 2020).

In human tubular and glomerular cell lines, marked levels of oxidative stress with cell death were detected after 24 h exposure to OTA. OTA administration provoked more severe lesions in the glomerular area compared to the tubular one owing to a differential autophagy regulation. Similarly, a significant renal fibrosis with extensive tissue degeneration was also confirmed *in vivo* using mice kidney (Le et al., 2022). In HK-2 cells, an epigenetic explication of OTA tumorigenesis was given. Indeed, exposure to mycotoxin promoted the inhibition of histone acetyltransferases activity as well as the loss of histone acetylation, which in turn lead to a global genetic repression with possible disruptions in mitosis phase and DNA repair mechanisms (Limbeck et al., 2018).

In PK-15 cells, the higher toxicity of OTA compared to fumonisin B1 and deoxynivalenol was demonstrated. Indeed, exposure to toxin caused ROS production and cell death to a greater extent compared with other

mycotoxins (Ledur & Santurio, 2020). Pyo et al. (2020) also demonstrated OTA-nephrotoxicity by exposing HK-2 cells during 48 h with different concentrations of OTA (50, 100 and 200 nM) and mice kidney with low toxin doses (200 and 1000 μg/kg bw) for 12 weeks. It has been observed a clear renal fibrosis with high levels of urea and creatinine in the serum of OTA-treated mice. In addition, α-SMA, fibronectin and E-cadherin gene expression was strongly upregulated by OTA *in vitro* and *in vivo*. Therefore, OTA-MoA was associated with the expression of TGF-β, smad2/3 and β-catenin proteins while the epithelial-mesenchymal transition was considered the driving event in OTA-promoted carcinogenesis.

In rat kidneys, 14 days OTA exposure provoked histopathological injuries with macrophagic infiltrations, severe fibrosis and proteinaceous casts formation. High NO levels accompanied by SOD, CAT and GPX enzymes activity suppression were also obtained. In addition, ELISA assay showed not only the expression of proinflammatory cytokines (TNF-α, IL-1β, IL-6) but also the presence of oxidative DNA damage (Damiano et al., 2020; Longobardi et al., 2021). IF and WB assays also demonstrated the harmful effects of sub-chronic exposure to low OTA doses, which selectively inhibited the activity of renal organic cation transport 1, by disrupting the right functionality of membrane potential as well as kidney homeostasis in rats (Karaica et al., 2022) (Table 7).

**Table 7.** Nephrotoxicity studies: mycotoxin, dose, exposure time, experimental model, techniques, effects and references.

| Mycotoxin | Dose | Exposure time | Experimental model | Techniques | Effects | References |
|---|---|---|---|---|---|---|
| **AFB1** | 0.04, 0.8 μM | 36 h | PK-15 cells | CCK-8 assay, FCM, RNA-seq, RT-qPCR, WB, IF | Oxidative stress with suppression of antioxidant genes expression | Zhang J. et al., 2022 |
| | 0.5, 1.5 mg/kg bw/ oral gavage administration | 3-7 days | Rats kidney | Biochemical assay, microscopy | Kidney injury with reduction of antioxidant enzymes activity | Yilmaz et al., 2018 |
| | 50 μg/kg bw/ oral gavage administration | 28 days | Rats kidney | Enzymatic assay, ELISA, Microscopy | Promotion of oxidative stress, inflammation, apoptosis with histopathological tissue alteration | Owumi et al., 2022 |
| | 75 μg/kg bw/ oral gavage administration | 28 days | Rats kidney | Enzymatic assay, ROS measurement, ELISA, Microscopy | Promotion of inflammation, oxidative stress and reduction of antioxidant defenses | Owumi et al., 2020a |
| | 80 μg/kg bw/ oral gavage administration | 28 days | Rats kidney | Biochemical analysis, ELISA, IHC | Kidney damage and inflammation | Sabry et al., 2022 |
| | 75 μg/kg bw/ oral gavage administration | 30 days | Mice kidney | IHC, RT-qPCR, WB, enzymatic assay | Renal damage with and oxidative stress and reduction of antioxidant enzymes activity | Yu et al., 2018 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 0.8 μM | 24 h | MDCK cells | MTT, LDH assay, FCM, Microscopy, RT-qPCR, ELISA,WB | Renal fibrosis with increased expression of inflammation and pyroptosis related genes | Li et al., 2021 |
| | 8 μM | 24 h | HEK-293T cells | CCK-8 assay, ROS measurement, WB | Reduction of cell viability with induction of oxidative stress by inhibition of Lonp1/Aco2/Hsp75 expression | Li et al., 2019 |
| | 1-10 μM | 24 h | MDCK, LLC-PK1, RK-13 cells | MTT, ROS measurement, LDH and MDA assay, NO detection | Reduction of cell proliferation and oxidative stress promotion | Crupi et al., 2020 |
| **OTA** | 1.25-40 μM | 24 h | HK-2 cells | IF, WB, RT-qPCR, apoptosis, ROS measurement, MTT, LDH assay | Inhibition of cell viability and promotion of apoptosis, oxidative stress and aberrant autophagy | Le et al., 2022 |
| | 10, 25, 50 μM | 24 h | HK-2 cells | MTT, WB, LC-MS/MS, Chip-Seq, Chip-PCR, RT-qPCR | Nephrotoxicity by histone acetyltransferases repression | Limbeck et al., 2018 |
| | 0.8 μM | 24 h | MDCK cells | MTT, RT-qPCR, ROS measurement, WB, LDH assay | Renal fibrosis accompanied by oxidative stress, inflammation and pyroptosis | Mao et al., 2022 |
| | 5 μM | 48 h | PK-15 cells | MTT, FCM, ROS measurement | Reduction of cell viability with ROS and apoptosis induction | Ledur & Santurio, 2020 |

| | | | | | |
|---|---|---|---|---|---|
| 0.05, 0.1, 0.2 μM | 48 h | HK-2 cells | MTT, RT-qPCR, WB, Gelatin zymography assay | Induction of epithelial-mesenchymal transition and renal fibrosis via Smad2/3 and β-catenin signaling pathways | Pyo et al., 2020 |
| 2 mg/kg/bw intraperitoneal administration | 14 days | Mice kidney | Microscopy | Kidney injury | Li et al., 2021 |
| 0.5 mg/kg bw/ oral gavage administration | 14 days | Rats kidney | IHC, ELISA, oxidative DNA assay, NO determination | Kidney injury with nitrosative stress, inflammation and DNA damage | Longobardi et al., 2021 |
| 0.5 mg/kg bw/ oral gavage administration | 14 days | Rats kidney | Microscopy, antioxidant assay | Promotion of kidney injury oxidative stress, inflammation and fibrosis | Damiano et al., 2020 |
| 0.5, 1, 2 mg/kg bw intraperitoneal administration | 21 days | Mice kidney | Biochemical analysis, IHC | Kidney damage and necrosis | Le et al., 2022 |
| 0.125, 0.250 mg/kg/bw oral gavage administration | 21 days | Rats kidney | IHC, IF, WB | Kidney damage by organic cation transporters alteration | Karaica et al., 2022 |
| 200, 1000 μg/kg/ contaminated feed | 84 days | Mice kidney | IHC | Induction of epithelial-mesenchymal transition and renal fibrosis via Smad2/3 and β-catenin signaling pathways | Pyo et al., 2020 |
| 250 μg/kg bw oral gavage administration | 90 days | Rats kidney | Microscopy, RT-qPCR, WB, enzymatic assay | Kidney injury with oxidative stress and fibrosis | Crupi et al., 2020 |

*1.3.4.2. Exposure to mycotoxins and bioactive compounds*

Several functional ingredients have been tested against mycotoxins effects until date. The supplementation of lycopene, a natural carotenoid found in fruits and vegetables, has been shown to be an excellent bioactive compound in reducing AFB1-induced aflatoxicosis *in vivo* (Table 8). In rodent kidneys, the oral lycopene administration reduced renal tissue fibrosis and inflammation promoted by toxin exposure. The carotenoid also improved the activity of several antioxidant enzymes and reduced the oxidative and nitrosative stress by enhancing Nrf2 antioxidant signaling pathway (Yilmaz et al., 2018; Yu et al., 2018). Owumi et al. (2020a) and (2022) also demonstrated the significant ameliorative effect of *Sorghum bicolor* and gallic acid against AFB1-promoted renal lesions in rats. These functional compounds exhibited clear antioxidant and anti-inflammatory proprieties, by reducing oxidative stress and ROS generation as well as the production of proinflammatory cytokines prompted by fungal compound. These outcomes suggested the possibility of using these functional compounds for cancer therapy and like chemopreventive agents to prevent AFB1-related health problems. In a similar way, the supplementation of lemon peel extract (100 mg/kg in the diet) totally reversed the histological damage promoted by AFB1 in rat kidney tissue. Furthermore, the functional compound reduced elevated urea and creatinine levels in the blood and relieved the protein expression of several proinflammatory cytokines. Considering its

antimicrobial activity, lemon peel was proposed as novel feed additive to reduce AFB1-contamination *in vivo* (Sabry et al., 2022).

The administration of hydroxytyrosol, a natural component of extra virgin olive oil, in canine, pig and rabbit renal cell lines during 24 h and in rats for 90 days has been shown to be an important nutritional strategy to treat and prevent OTA kidney dysfunction. *In vitro* hydroxytyrosol exposure prevented cell cytotoxicity and limited intracellular ROS generation with reduction of MDA, LDH and nitrosative stress levels. *In vivo,* the bioactive compound reduced oxidative stress and collagen accumulation in renal tissue and decreased the high levels of AST, ALT and creatinine fostered by OTA-poisoning (Crupi et al., 2020). Similarly, selenomethionine, an essential trace element with multiple biological properties, modulated the cytotoxicity and oxidative stress promoted by OTA exposure in MDCK cells. Furthermore, this compound down-regulated several genes related to renal fibrosis ($\alpha$-SMA, Vimentin, and TGF-$\beta$) and pyroptosis (Caspase-1 and GSDMD) as well as suppressed NLRP3 inflammasome activation. Thus, it was considered an effective strategy to preserve animal health against mycotoxicosis (Mao et al., 2022). In human renal cell line, ursolic acid exposure, a water-insoluble compound found in various cuticular waxes of edible fruits, food materials and medicinal plants, has been shown to revert OTA cytotoxicity, oxidative stress and cell death by modulating the expression of Lonp1, Aco2 and Hsp75, key proteins involved in renoprotection (Li et al., 2019).

Ledur & Santurio, (2020) also demonstrated the strong cytoprotection offered by curcumin and silymarin, two plant compounds largely used in oriental medicine owing to their antioxidant proprieties. Accordingly, these bioactive compounds modulated cell death and oxidative stress prompted by 48 h exposure to OTA *in vitro*. The renoprotective effect of curcumin was also tested in rat kidneys. This bioactive compound improved OTA-chronic effects by reducing glomeruli atrophy and inflammatory infiltrates in kidney tissue. Curcumin also attenuated the production of proinflammatory cytokines (TNF-$\alpha$, IL-1$\beta$, and IL-6) and oxidative DNA damage. A significant reduction in ROS production, NO levels, lipid peroxidation with increased SOD, CAT and GPX activity was also found (Damiano et al., 2020; Longobardi et al., 2021) (Table 8).

**Table 8.** Nephrotoxicity studies: mycotoxin, dose, bioactive compound, exposure time, experimental model, techniques, effects and references.

| Mycotoxin | Dose | Bioactive compound | Exposure time | Experimental model | Techniques | Effects | References |
|---|---|---|---|---|---|---|---|
| AFB1 | 0.5, 1.5 mg/kg bw/ oral gavage administration | Lycopene (5 mg/kg bw) for 15 days | 3-7 days | Rats kidney | Biochemical assay, microscopy | Prevention of kidney damage and ameliorative effect on antioxidant enzymes activity | Yilmaz et al., 2018 |
| | 75 µg/kg bw/ oral gavage administration | Gallic acid (20-40 mg/kg bw) | 28 days | Rats kidney | Enzymatic assay, ROS measurement, ELISA, Microscopy | Reduction of inflammation and oxidative stress promoted by mycotoxin. | Owumi et al., 2020a |
| | 50 µg/kg bw oral gavage administration | Apigeninidin-rich extracts of *Sorghum bicolor* (10 mg/kg bw) | 28 days | Rats kidney | Enzymatic assay, ELISA, Microscopy | Inhibition of oxidative and nitrosative stress, inflammation, apoptosis and tissue damage | Owumi et al., 2022 |
| | 80 µg/kg bw/ oral gavage administration | Lemon peel extract (100 mg/kg in diet) | 28 days | Rats kidney | Biochemical analysis, ELISA, IHC | Modulation of kidney damage and inflammation | Sabry et al., 2022 |
| | 75 µg/kg bw/ oral gavage administration | Lycopene (5 mg/kg bw) | 30 days | Mice kidney | IHC, RT-qPCR, WB, enzymatic assay | Preservation of kidney structure and improvement of antioxidant defense by Nrf2 signaling | Yu et al., 2018 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1-10 μM | Hydroxytyrosol (10-2500 μM) | 24 h | MDCK, LLC-PK1, RK-13 cells | MTT, ROS measurement, LDH and MDA assay, NO detection | Prevention of renal cytotoxicity and oxidative stress | Crupi et al., 2020 |
| | 0.8 μM | Selenomethionine (2, 4, 8 μM) | 24 h | MDCK cells | MTT, RT-qPCR, ROS measurement, WB, LDH assay | Mitigation of oxidative stress, inflammation and pyroptosis | Mao et al., 2022 |
| | 8 μM | Pre- or post-treatment for 2h with ursolic acid (1 μM) | 24 h | HEK-293T cells | CCK-8 assay, ROS measurement, WB | Reduction of cytotoxicity and ROS production by Lonp1/Aco2/Hsp75 increased activity | Li et al., 2019 |
| | 5 μM | Curcumin (0.5, 1, 2 μM) and Silymarin (1, 2.5, 5 μM) | 48 h | PK-15 cells | MTT, FCM, ROS measurement | Improvement of cytotoxicity | Ledur & Santurio, 2020 |
| **OTA** | 0.5 mg/kg bw/ oral gavage administration | Curcumin (100 mg/kg bw) | 14 days | Rats kidney | Microscopy, antioxidant assay | Mitigation of kidney injury and oxidative stress, inflammation and fibrosis | Damiano et al., 2020 |
| | 0.5 mg/kg bw/ oral gavage administration | Curcumin (100 mg/kg bw) | 14 days | Rats kidney | IHC, ELISA, oxidative DNA assay, NO determination | Protection against inflammation, DNA damage and nitrosative stress | Longobardi et al., 2021 |
| | 250 μg/kg bw oral gavage administration | Hydroxytyrosol (20 mg/kg bw) | 90 days | Rats kidney | Microscopy, RT-qPCR, WB, enzymatic assay | Preservation of liver health, reducing fibrosis and oxidative stress | Crupi et al., 2020 |

# 2. OBJECTIVES

# 2. Objetivos

## 2. OBJETIVOS

El objetivo de la presente Tesis Doctoral es profundizar en el conocimiento tanto de los mecanismos de toxicidad y los efectos toxicológicos de AFB1 y OTA como el posible efecto protector de la calabaza y el suero de leche fermentado *in vitro* e *in vivo* mediante ensayos y técnicas *ómicas* y moleculares. Para llevar a cabo este trabajo se han propuesto los siguientes objetivos específicos:

1. Recopilación de los hallazgos presentes en la literatura sobre los efectos adversos que AFB1 y OTA pueden causar en la salud humana y animal, mediante el examen de estudios *in vitro* e *in vivo* que emplean técnicas como la inmunofluorescencia (IF) y la citometría de flujo (FCM).

2. Evaluación de los posibles efectos beneficiosos de la calabaza y el suero de leche fermentado contenidos en digeridos gastrointestinales de pan frente a los efectos tóxicos que AFB1 y OTA provocan en la diferenciación de células neuronales humanas.

3. Análisis transcriptómico del efecto toxicológico que AFB1 y OTA, individualmente y en combinación, inducen sobre el perfil de expresión génica de linfocitos T humanos.

4. Evaluación del efecto *in vitro* de un digerido gastrointestinal de pan preparado con calabaza y suero de leche fermentado frente la inmunotoxicidad inducida por AFB1 y OTA mediante el análisis del transcriptoma de linfocitos T humanos.

5. Estudio *in vivo* del posible efecto protector del suero de leche fermentado contra la hepatotoxicidad y nefrotoxicidad promovidas por AFB1 y OTA en ratas macho y hembra a través la cuantificación relativa y absoluta de biomarcadores biológicos típicos como la carbamoil fosfato sintetasa 1 (CPS1) y la molécula de lesión renal 1 (KIM-1).

## 2. OBJECTIVES

The goal of this Doctoral thesis is to investigate not only AFB1 and OTA mechanisms of toxicity and toxicological effects but also the possible protective role of pumpkin and fermented milk whey *in vitro* and *in vivo* by using *omics* and molecular techniques. In order to achieve this objective, the following specific aims are proposed:

1. To review the bibliographic research about the toxic effects that AFB1 and OTA may induce on human and animal health, by analyzing *in vitro* and *in vivo* studies, which employed novel techniques such as immunofluorescence and flow cytometry.

2. Evaluation of the potential beneficial effect of pumpkin and fermented milk whey contained in digested bread extracts against AFB1 and OTA induced alterations in human neuronal cell differentiation.

3. Transcriptomic analysis by RNA sequencing to assess AFB1 and OTA toxicological effects, individually and in combination, on the gene expression profile of human T lymphocytes.

4. *In vitro* evaluation on the effect of digested bread extracts prepared with pumpkin and fermented milk whey against AFB1 and OTA immunotoxicity, by human T lymphocyte transcriptome analysis.

5. *In vivo* study of the potential therapeutic role of fermented milk whey against AFB1 and OTA promoted hepatotoxicity and nephrotoxicity in male and female rats through the relative and absolute quantification of typical biological biomarkers such as carbamoyl phosphate synthetase 1 (CPS1) and kidney injury molecule 1 (KIM-1).

# 3. RESULTS

# 3. Resultados

## 3.1. *In vitro* and *in vivo* evaluation of AFB1 and OTA-toxicity through immunofluorescence and flow cytometry techniques: A systematic review

Food and Chemical Toxicology

# *In vitro* and *in vivo* evaluation of AFB1 and OTA-toxicity through immunofluorescence and flow cytometry techniques: A systematic review

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**Abstract**

Due to the globalization, mycotoxins have been considered a major risk to human health being the main contaminants of foodstuffs. Among them, AFB1 and OTA are the most toxic and studied. Therefore, the goal of this review is to deepen the knowledge about the toxicological effects that AFB1 and OTA can induce on human health by using flow cytometry and immunofluorescence techniques *in vitro* and *in vivo* models. The examination of the selected reports shows that the majority of them are focused on immunotoxicity while the rest are concerned about nephrotoxicity, hepatotoxicity, gastrointestinal toxicity, neurotoxicity, embryotoxicity, reproductive system, breast, esophageal and lung toxicity. In relation to immunofluorescence analysis, biological processes related to AFB1- and OTA-toxicity were evaluated such as inflammation, neuronal differentiation, DNA damage, oxidative stress and cell death. In flow cytometry analysis, a wide range of assays have been performed across the reviewed studies being apoptosis assay, cell cycle analysis and intracellular ROS measurement the most employed. Although, the toxic effects of AFB1 and OTA have been reported, further research is needed to clarify AFB1 and OTA-mechanism of action on human health.

**Keywords:** Mycotoxins; toxicological mechanism; cell line; animal model; organ.

Abbreviations

α-SMA: Alpha Smooth Muscle Actin

3D4/21: Continuous porcine cell lines from alveolar macrophages

8OhdG: 8-hydroxiguanosine

8-oxyguanine: 8-oxoG

A459: Adeno-carcinomic human alveolar basal epithelial cell line

AKT: Protein-kinase B

Arg-1: Arginase 1

ATP: Adenosine triphosphate

ATR: ataxia telangiectasia and Rad3-related

$a_w$: water activity

BRCA1: Breast cancer 1 susceptibility protein

BRL: Buffalo rat liver cell line

Caco-2: Human colorectal adenocarcinoma cell line

CD20: Cluster differentiation 20

CD45: Cluster differentiation 45

CMT93-II: Mouse rectum carcinoma cell line

CRM1: Chromosomal Maintenance 1

Dcx: Doublecortin

DNA: Deoxyribonucleic acid

DNMT1: DNA Methyltransferase 1

EBNA-2: The Epstein-Barr virus nuclear antigen 2

eDNA: Extracellular DNA

ER : Endoplasmic Reticulum

ET : Extracellular Traps

ERK1/2: Extracellular signal-regulated protein kinases 1 and 2

F2R: Coagulation factor II thrombin receptor

GC-2: Germ cell line

Ges-1: Human gastric epithelial cell line

GFAP: Glial Fibrillary acidic protein

GPX: Glutathione peroxidase

GSDMD: Gasdermin D

H3K9ac: Recombinant Histone H3 acetyl Lys9

H3K9me3: Recombinant Histone H3 trimethyl Lys9

H9-T: Human cutaneous $CD4^+$ T lymphoma cell line

HCT-116: Human colon carcinoma cell line

HCT-8: Human ileocecal adenocarcinoma cell line

HEK293T: Human embryonic kidney 293 cell line

HepaRG: Differentiated hepatic cell line

HepG2: Human liver cancer cell line

HET-1A: Human esophageal epithelial cell line

HIF-1α: Hypoxia inducible Factor 1

HK-2: Human renal proximal tubule epithelial cell line

HKC cells: Human proximal tubular epithelial cell line

HO-1: Heme oxygenase

Iba1:Ionized calcium binding adaptor molecule 1

iNOS: Inducible Nitric Oxide Synthase

IPECJ2: Intestinal Porcine Epithelial cell line-J2

ITGB1: Integrin beta 1

Kim-1: Kidney injury molecule-1

L02: Human fetal hepatocyte

LX-2: Human hepatic stellate cell line

MAP2-AP18/M13: Microtubule-Associated Protein 2 clone AP18 and M13

MDCCs: monocyte-derived dendritic cells

MDCK cells: Madin-Darby Canine Kidney cell line

mGalc: Galactocerebroside

MPO: Myeloperoxidase

NAC: N-acetylcysteine

NADP: Nicotinamide adenine dinucleotide phosphate

NeuN: Neuronal nuclei

NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NLRP3: NLR family pyrin domain containing protein 3

NP: Nucleoprotein

NRF2: The nuclear factor erythroid 2-related factor 2

NRK-52E: Normal rat kidney cells clone 52E

p65: Nuclear factor NF-kappa-B p65 subunit

PCNA: Proliferating cell nuclear antigen

PI: propidium iodide

PI3K: Phosphatidyl-Inositol 3-Kinase

PK15: Porcine kidney 15 cell line

PTEN: Phosphatase and tensin homolog

Rad51: DNA repair protein RAD51 homolog 1

RAW 264.7: Mouse leukemic monocyte-macrophage cell line

RIP3: Receptor-interacting serine/threonine-protein kinase 3

RNA: Ribonucleic acid

SOD: Superoxide dismutase

TM3-TM4 cell line: Mouse Leydig and Sertoli cell line

TNF: Tumor Necrosis Factor

t-RNA: RNA transfer

TOM20: Mitochondrial import receptor subunit TOM20 homolog

Zo1: Tight junction protein-1

γH2A: Trypanosomal histone γH2AX

**1. Introduction**

Mycotoxins are toxic secondary compounds synthesized under specific conditions by certain fungal species capable of growing in a wide variety of foodstuffs (Luo et al., 2018). Although more than 400 fungal metabolites with toxigenic potential have been reported, the most common and studied mycotoxins are aflatoxins (Afs), among them aflatoxin B1 (AFB1); ochratoxin A (OTA); citrinin; patulin; trichothecenes: deoxynivalenol (DON), T2 and HT2 toxin; fumonisins; zearalenone (ZEA) and emerging mycotoxins: fusaproliferin, moniliformin, beauvericin and enniatins (Khaneghah et al., 2019).These metabolites are mainly produced by fungi of the genera Aspergillus, Fusarium, Penicillium and Alternaria (Anfossi et al., 2016; Topi et al., 2021).

The growth of toxigenic fungi and mycotoxin production are widely dependent on environmental factors such as microbial competition, water activity (aw), pH and oxygen concentration, temperature and relative humidity, which represent the driving force of fungal colonization (Mshelia et al., 2020). The Food and Agriculture Organization (FAO) estimates that approximately 25% of cereals produced in the world are contaminated by mycotoxins (FAO, 2013). However, other authors reported that 72-79% of feed samples from different part of the world contained mycotoxins, confirming a worldwide spreading (Streit et al., 2013; Kovalsky et al., 2016; Eskola et al., 2020). These toxic chemical compounds can be commonly found

in processed and unprocessed foods: cereals, fruits, spices, coffee, milk, cheese, beer, wine, bread, chocolate and baby foods (Ayofemi Olalekan Adeyeye, 2020; Sarmast et al., 2020).

Due to their toxicological effects, mycotoxins can induce adverse effects on human and animal health even at low concentrations (Bryła et al., 2018). Once ingested, mycotoxins may cause acute or chronic disease: a) acute episodes are characterized by a rapid onset with an obvious toxic response including abdominal pain, coma, convulsions, pulmonary and cerebral edema and death; b) low-dose chronic exposures to mycotoxins over a long period of time reported toxic responses, including cancer, hepatic diseases, immune and neurological disorders (Alshannaq & Yu, 2017) . It should be emphasized that not all mycotoxins exhibit the same toxicity as their detrimental effects depend on the characteristics (age, nutrition, metabolism and other factors as well) of humans and animals affected (Bertero et al., 2018; Cimbalo et al., 2020).

Consequently, the International Agency for Research on Cancer (IARC) has classified mycotoxins into five groups (Group 1, 2A, 2B, 3 and 4) according to their carcinogenicity (IARC, 2012). Nevertheless, due to their widespread occurrence and well-known toxicity, AFB1 and OTA are to be considered with a major caution (Kutsanedzie et al., 2020). Table 1 shows their fungal producing species, maximum tolerable levels, contaminated foods, affected organs, toxicological mechanisms and damages.

**Table 1.** AFB1 and OTA fungi producers, maximum tolerable levels, foodstuffs, organ affected, toxicological mechanism, damage and references.

| | Fungi producers | Maximum Levels | Foodstuffs | Organ target | Damage | References |
|---|---|---|---|---|---|---|
| Aflatoxin B1 | *Aspergillus (Flavius and parasiticus).* | Dried fruits 2-8 μg/kg, cereals 2-5 μg/kg, spices 5 μg/kg, baby foods 0.1 μg/kg. | Cereals, oil seeds, spices, tree nuts, milk, meat and dried fruits. | Liver, Immune system | Mutagenicity, hepatotoxicity, carcinogenicity, neurotoxicity. | Ráduly et al., 2020 Rushing & Selim, 2019 Niaz et al., 2020 EC Commission, 2006 |
| Ochratoxin A | *Aspergillus and Penicillium species* | Soluble coffee 10 μg/kg, unprocessed cereals 5 μg/kg, fruit juice and wine 2 μg/kg, spices 15 μg/kg, dietary and baby foods 0.5 μg/kg. | Cereals, wine, tea, coffee, milk, cheese, meat, fish, eggs, fruits, dried fruits, spices and vegetables. | Liver, kidney. | Nephrotoxicity, hepatotoxicity, immunotoxicity, neurotoxicity. | Zhao et al., 2017 Chen et al., 2018 Ráduly et al., 2020 EC Commission, 2006 |

*1.1 Aflatoxins*

Afs are the most toxic mycotoxins for human health (Singh et al., 2021). They are polypeptide compounds produced by many species of Aspergillus, mainly by Aspergillus flavus and Aspergillus parasiticus (Kumar et al., 2017). The most important factors in fungi colonization and toxin production are temperature and aw. In particular, optimum temperature and aw values are ranged between 16-31 °C and 0.82-0.99 aw (Bernáldez et al., 2017; Sarma et al., 2017). Afs production occurs in a wide range of food commodities: cereals, spices, tree nuts, milk and dried fruits (Umesha et al., 2017).

Several studies have shown that Afs contamination induce mutagenicity, hepatotoxicity, immunotoxicity and carcinogenicity (Ostry et al., 2017; Rushing & Selim, 2019). Therefore, they are classified by IARC in group 1 as carcinogenic to humans (IARC, 2012). In order to protect human health, it is essential to keep these food contaminants at toxicologically acceptable levels. The European Union (EU) sets the maximum tolerable limits of AFB1 at 2-5 μg/kg in cereals, 2-8 μg/kg in dried fruits, 5 μg/kg in spices, 6 μg/kg in dietary foods for medical purpose and 0.1 μg/kg in foods for infants and young children (EC Commission, 2006).

Mechanistically, AFB1-exo isomer 8,9-epoxide (AFBO), formed in the first step of AFB1 metabolism, has been considered as the major responsible

for their genotoxicity. AFBO reacts with DNA, RNA and proteins forming the toxic adduct 8,9-dihydro-8(N7-guanyl)-9-hydroxy-AFB1 (AFB1–N7-Gua), which confers mutagenic properties (Dai et al., 2017; Benkerroum, 2020).

In view of this, AFB1-exposure may induce severe implications on the immune system, liver, epididymis, kidney and heart (Xu et al., 2020). Likewise, the high lipid solubility of AFB1 and its existence in the post mortem brain tissue (Bahey et al., 2015) suggest its ability to cross the blood brain barrier (BBB), thus affecting its integrity (Qureshi et al., 2015). In the central nervous system (CNS), AFB1 may disturb neural cell functions by forming DNA and protein adducts and by inducing oxidative stress, apoptosis and the inhibition of macromolecules synthesis (Alsayyah et al., 2019).

### *1.2 Ochratoxins*

Ochratoxins are the most common mycotoxins detected in foodstuffs (Malir et al., 2016). There are 3 groups: Ochratoxin A (OTA), B (OTB), C (OTC), which are produced by different species of Aspergillus and Penicillium (Heussner & Bingle, 2015). Among these, OTA, an organic toxin with low molecular weight, is the most abundant and detrimental (Kőszegi & Poór, 2016). Optimum temperature and aw values for mycotoxin production are ranged between 15-25°C and 0.90-0.99 aw (Sánchez-Montero

et al., 2019). Typical contaminated foods are cereals (maize, wheat, rice, sorghum, barley, oats) cereal-based products (bread, flour, and pasta), wine, tea, coffee, milk, cheese, dried fruits, spices and vegetables (Chen et al., 2018).

OTA has been classified by IARC in group 2B as possible carcinogen for human (IARC, 2012). The EU sets the maximum permissible levels of OTA in unprocessed cereals at 5 μg/kg, 8 μg/kg in wheat gluten, in roasted coffee beans at 5 μg/kg and 10 μg/kg in soluble coffee, 15 μg/kg in spices, 2 μg/kg in grape juice and wine, in liquorice between 20 and 80 μg/kg, in baby foods and dietary foods for medical purpose at 0.5 μg/kg (EC Commission, 2006).

Mechanistically, OTA exerts its toxicity by increasing NADPH and P450 enzyme activity, or by promoting caspase signaling pathway and apoptosis. In addition, OTA may elicit oxidative stress in mitochondria and alter cell cycle, lipid and nucleotide metabolism (Zhu et al., 2017; Tao et al., 2018).

Consequently, OTA-exposure determinate various toxicological effects, including the disruption of gut microbiota homeostasis, hepatotoxicity, genotoxicity, immunotoxicity, embryotoxicity, neurotoxicity, testicular toxicity and nephrotoxicity (Luz et al., 2018; Leitão, 2019). Despite limited evidences, the association between OTA-exposure

with Balkan Endemic Nephropathy, Chronic Interstitial Nephropathy and other kidney diseases has been demonstrated (Zhao et al., 2017). Furthermore, in vivo studies suggest that OTA crosses the BBB and accumulates in cerebellum, ventral mesencephalon, striatum, pons and cerebral cortex (Niaz et al., 2020). High doses of OTA have been also found in hippocampus, the primary site of neurodegeneration in Alzheimer disease (Bhat et al., 2016). In brain, OTA triggers reactive oxygen species (ROS) generation, alterations in cell cycle and proteome profiles, caspase activation and disruption of mitochondrial membrane potential (MMP) (Park et al., 2019; Babayan et al., 2020).

Nowadays, several molecular techniques can be employed to determinate the toxicological mode of action (MOA) of mycotoxins in organisms. Naturally, the main tool used are -omics techniques, which allow to analyze both in vivo and in vitro systemic changes induced by harmful compounds (Soler & Oswald, 2018; Cimbalo et al., 2020). In addition, flow cytometry (FCM) and microscopy can be excellent tools for studying mycotoxin toxicity. FCM is an automated and high throughput method which allows a very rapid analysis of multiple chemical and physical characteristics of single cells. It is an extremely useful technology which has been used for decades with filamentous fungi (Bleichrodt & Read, 2019). Furthermore, FCM provides an excellent interpretation of cellular processes such as redox balance, proliferation and apoptosis (Zhou et al., 2020; Broemsen et al., 2021). Whilst, microscopy, which is a rapid, cheap and

simple technic, allows to investigate and examine histological changes on tissues induced by mycotoxins and through specific antibodies and fluorescence kits it enables to analyze their specific MOA (Nguyen et al., 2019; Gémes et al., 2021).

Therefore, the goal of this review is to deepen the knowledge about AFB1 and OTA toxicity in vitro and in vivo models by using FCM and immunofluorescence (IF) techniques.

## 2.Material and Methods

The extensive bibliographic research was conducted using four different scientific databases: PubMed, Web of science, Scopus and Google scholar by selecting articles in the last six years (2015-2021). The research was carried out by using the following keywords: AFB1, OTA, microscopy and flow cytometry. To facilitate the screening in google scholar, which covers the same importance of other scientific databases in bibliographic research but includes a less quality publications from some websites (Kousha & Thelwall, 2008; Martín-Martín et al., 2021), the research was refined by using the terms: immunofluorescence, *in vivo*, *in vitro*, mycotoxins, mechanism, toxicity, biological effects and toxicological damage. A number of 733 articles, which met the criteria to be included into the study, were analyzed. Reports obtained were assessed by screening abstract to discard unnecessary, incomplete or irrelevant literature. Finally, a total of 98 articles

were analyzed in full text and classified based on: type of mycotoxin, technique used, analysis model (*in vitro* or *in vivo*), dose administration, exposure time, antibodies implemented or FCM assays and toxicological mechanism (Fig.1)

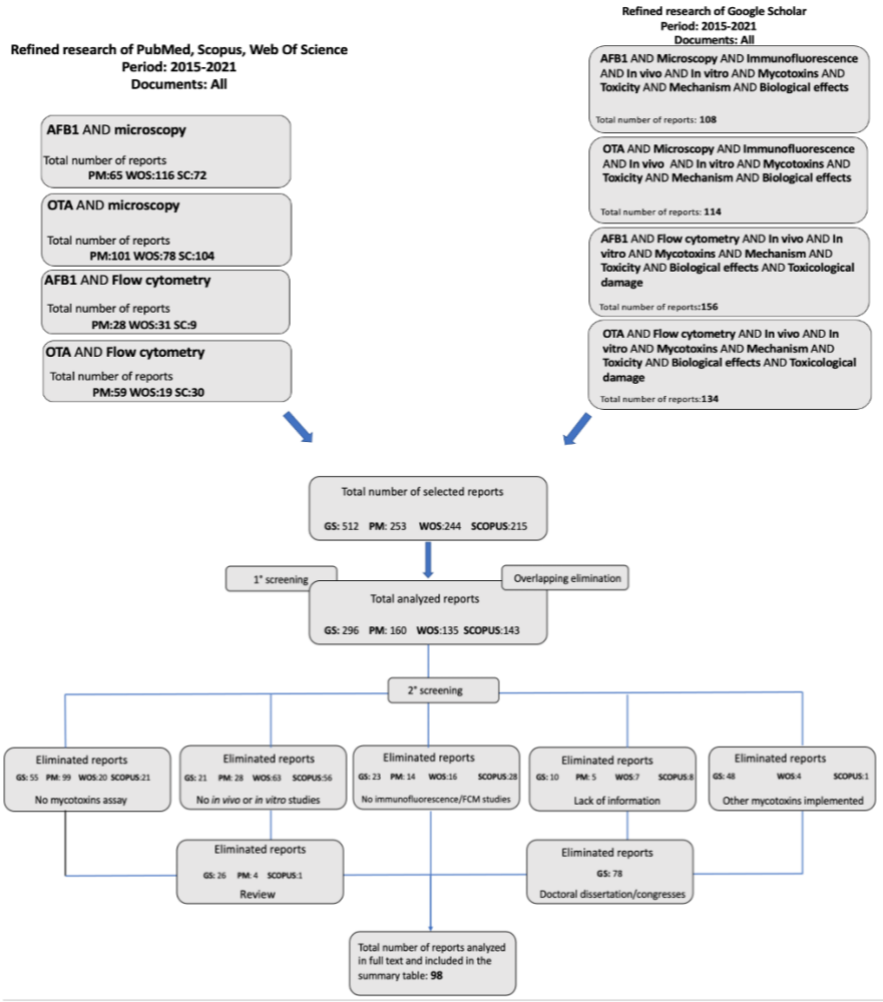


**Figure 1.** Graphic representation of total number of articles screened throughout the bibliographic research

## 3.Results

### *3.1. Aflatoxin B1*

AFB1 is the best known and most studied mycotoxin. In this review, its toxicity was elucidated through IF and FCM techniques. Regarding IF analysis, antibodies varied depending on the specific mechanism or metabolic route searched by authors. FCM was mainly used to detect alterations in cell cycle, mitochondrial balance, ROS generation and apoptosis/necrosis induction. Interestingly, FCM was also employed to quantify and identify particular markers, apoptotic DNA and cell subsets. Furthermore, several natural compounds, food components and non-pathogenic bacteria have been employed to ameliorate AFB1-toxicity such as: β-1,3-glucan, zinc sulfate ($ZnSO_4$) gluconate (GZn) and bentonite (Zn-Bent1), L-proline, selenium (Se), palm kernel cake (PKC), gallic acid, N-acetylcysteine (NAC), *Lactobacillus paracasei* (LP) BEJ01, montmorillonite clay (MT), egg yolk immunoglobulin (IgY) and lactic acid bacteria (LAB).

For each technique, the selected AFB1-studies were organized according to the model used (*in vitro* and *in vivo)*, showing that the main AFB1-toxicological effects *in vitro* are hepatotoxicity, immunotoxicity, gastrointestinal toxicity, nephrotoxicity, neurotoxicity, embryotoxicity and breast toxicity (Fig.2). Whilst, immunotoxicity, gastrointestinal toxicity, hepatotoxicity, nephrotoxicity and neurotoxicity were mainly observed *in vivo* (Fig.3). The summary of the studies found for AFB1 are shown below.

Tables were divided according to the technique, model used and type of toxicity, and were ordered by the exposure time, from the shortest to the longest.

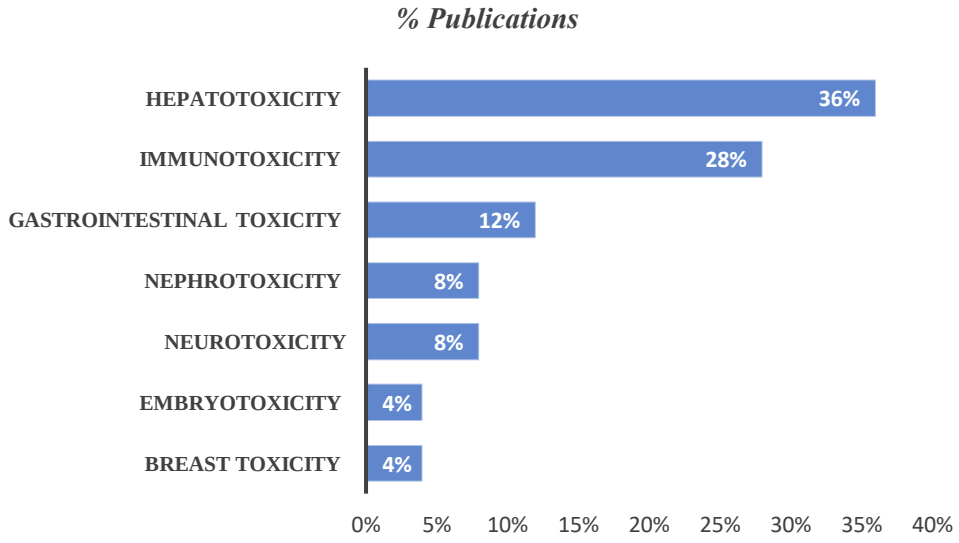


**Fig.2.** Percentage of total publications (n=25) according to the main toxicological effects of AFB1 *in vitro*.

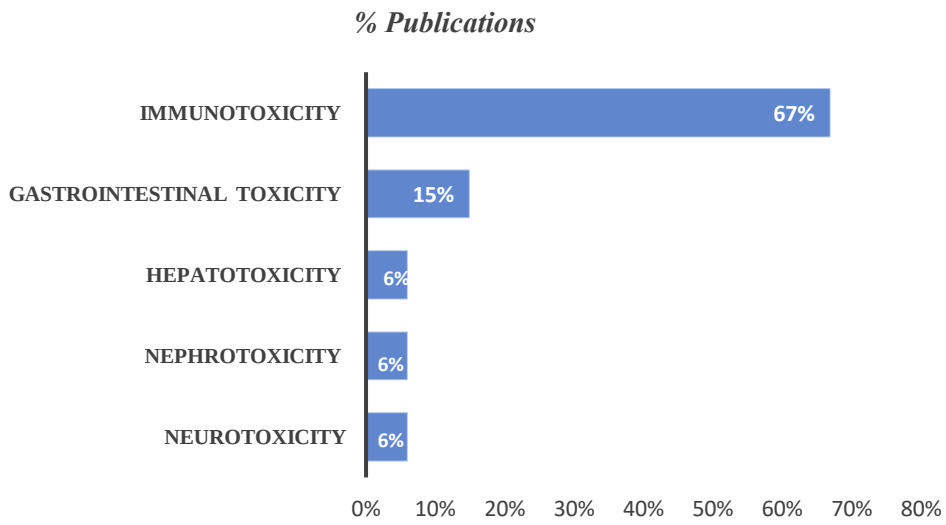


**Fig.3.** Percentage of total publications (n=20) according to the main toxicological effects of AFB1 *in vivo.*

*3.1.1. Immunofluorescence*

*3.1.1.1. In vitro toxicity*

In vitro AFB1-IF studies show exposure times between 2 h and 7 days and concentrations of $3.12 \times 10^{-4}$ - 10 μg AFB1/ml. As shown in Fig. 4, the most used cell line was 3D4/21. The natural compounds implemented to reduce AFB1-damages were zinc, sulfate and gluconate, and NAC.

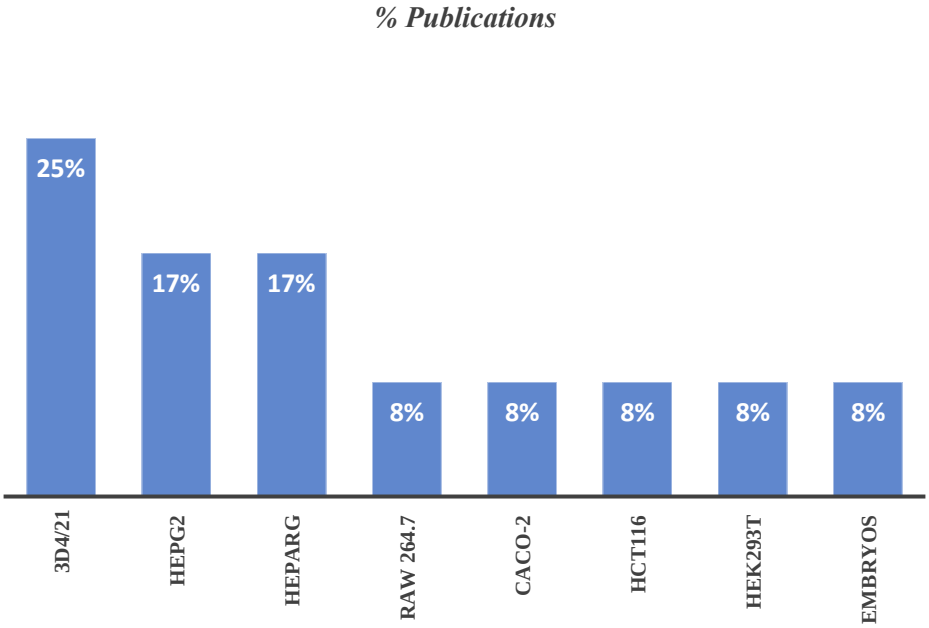


**Fig.4.** Percentage of publications (n=10) according to cell lines used in AFB1-IF *in vitro* studies.

*3.1.1.1.1. Immunotoxicity*

In the shortest exposure, AFB1 triggered an immune and pro-inflammatory response in macrophages through ROS-mediated autophagy and extracellular traps (Ets) production. The major components of Ets, elastase, myeloperoxidase, and histone H3, were disrupted by toxin exposure. In addition, by the analysis of typical markers of macrophage polarization, it has been observed a M1 polarization of macrophages, which acquired pro-inflammatory properties (An et al., 2017).

Furthermore, acute exposure to AFB1 exacerbated swine influenza virus (SIV) infection, inflammation and lung damage by activating TLR4-NFκB signaling pathway. In contrast, TAK242 and TLR4 knockout, TLR4-specific antagonist, alleviated the AFB1-promoted SIV replication, inflammation and lung damage (Sun et al., 2018). In a similar duration study, AFB1 induced oxidative stress, cell death and the production of pro-inflammatory cytokines. It also caused genomic damages, identified by γH2AX fluorescent analysis, a specific histone wrapping around DNA. Authors associated AFB1-immunotoxicity with JAK2/STAT3 pathway activation and the alteration of gene and protein expression of DNA methyl transferases 1 and 3a (Zhou et al., 2019).

In the 48h study, AFB1 alone or in combination with OTA could aggravate immunotoxicity through the degradation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBa), the phosphorylation of nuclear factor kappa B, and its translocation into nuclei. Owing to AFB1-exposure, the expression of pro-inflammatory cytokines and ROS production were significantly increased. However, high doses of NAC and BAY11-7082, a specific inhibitor of NF-κB, suppressed oxidative stress, inflammation and immunotoxicity of both mycotoxins (Hou et al., 2018).

*3.1.1.1.2. Hepatoxicity*

As regards hepatotoxicity, AFB1 promoted nuclear changes with chromatin fracture-induced DNA double strand breaks (DSB) and oxidative DNA damage. However, ZnSO4 and GZn reversed AFB1-promoted epigenetic changes and ameliorated cell survival, DNA integrity and the genome wide methylation (Yang et al., 2016). Zhang et al. (2019) demonstrated that exposure to toxin promoted the pyroptosis of hepatocytes via COX-2, inducing both NLRP3 and caspase 1 activation with IL-1β release. The fluorescent quantification of Gasdermin D, a novel regulator of caspase 1-mediated pyroptosis, confirmed its involvement in liver inflammation.

In the longest exposure, AFB1 enhanced Hepatitis B virus (HBx) infection in mutated liver cells. The combination of HBx and AFB1 exacerbated hepatic steatosis via COX-2 up-regulation and increased the expression of RIP3, a component of TNF receptor and strong inducer of apoptosis and inflammation. Additionally, AFB1 promoted mitochondrial disorders by altering TOMM20 expression, a central component in the recognition and translocation of mitochondrial proteins (Chen et al., 2019).

*3.1.1.1.3. Gastrointestinal toxicity*

As shown in Table 2, only one study about gastrointestinal toxicity was reported. In Caco-2 and HCT116 cells, AFB1 triggered cell death and DNA damage through ATR-Chk1 specific axis. The IF-analysis performed on γH2AX and p53, showed that ATR-axis was activated by replicative stress, a molecular and early response to AFB1, which subsequently led to DNA-DSB formation. In addition, an aberrant intracellular ROS generation (2-fold higher in comparison to the control) has been observed (Gauthier et al., 2020).

*3.1.1.1.4. Nephrotoxicity*

In kidney, the immunostaining of exportin 1, also known as CRM-1, showed that AFB1 increased significantly the nuclear volume inducing abnormal changes in DNA, such as polyploidy. CRM-1 mediates the nuclear proteins export playing a crucial role in cell cycle regulation, centrosome duplication and spindle assembly. Consequently, alterations in CRM-1 expression were closely related to cell cycle arrest and DNA damage (Huang et al., 2019).

*3.1.1.1.5. Embryotoxicity*

Toxicological damages were also detected in porcine embryos. In the longest exposure, 1 week, the lowest concentration of AFB1 reported ($3.12 \times 10^{-4}$ μg/ml) compromised embryonic development by the induction of oxidative stress and DNA damage, revealed by γH2AX fluorescence analysis. Moreover, AFB1 disrupted DNA damage mechanism repair through the dysregulation of p53 binding protein 1 and triggered autophagy, as shown by an over-expression of its typical markers, Beclin-1 and LC3. At the same time, the presence of AFB1 significantly impaired cell proliferation, endpoint for blastocyst quality and growth (Shin et al., 2018).

In summary, 24 h and anti-γH2AX were the most common exposure time and antibody used. The induction of oxidative stress and apoptosis were the main toxicological effects related to AFB1-contamination.

*3.1.1.2. In vivo toxicity*

In vivo AFB1-IF studies were performed exposing rats and mice between 8 and 10 weeks to concentrations ranging 0.016–0.07 mg AFB1/kg administered intraperitoneally and orally. No natural compounds against AFB1-toxicity have been tested.

*3.1.1.2.1. Neurotoxicity*

As shown in Table 2, the only neurotoxicity study was sub chronic (8 weeks) and performed through a microscopic analysis, assessing the effect of AFB1 on different brain sections. The fluorescent quantification of neuronal and astrocyte biomarker, NeuN and GFAP proteins, demonstrated that a low concentration of AFB1 decreased the vitality of neuronal cells in rat hippocampus. Moreover, AFB1 altered the distribution of astrocytes in brain, causing astrogliosis with inflammation and neuronal cell death. Nevertheless, AFB1-withdrawal reversed changes in cerebral cortex and hippocampus (Bahey et al., 2015).

*3.1.1.2.2. Immunotoxicity*

In the longest exposure (8–10 weeks), AFB1 promoted the diffusion of Epstein–Barr virus (EBV), which markedly increased the incidence of Burkitt lymphoma. In addition, AFB1 induced EBV-driven cellular transformation in humanized animal model. Owing to the activation of EBV lytic cycle, the expression of EB-nuclear antigen and EBV replicative load was significantly increased. In view of this, AFB1 can be considered a co-factor of EBV mediated-carcinogenesis and immunotoxicity (Accardi et al., 2015).

To sum up, a similar exposure time (8 weeks) in both analyzed studies was observed.

**Table 2**. AFB1-IF studies: *In vitro* and *in vivo* model, dose and exposure time, antibodies implemented, mechanism and references.

| ***In vitro* / *in vivo* model** | **Dose administration** | **Exposure time** | **Antibodies used** | **Mechanism** | **References** |
|---|---|---|---|---|---|
| ***In vitro*** | | | | | |
| ***Immunotoxicity*** | | | | | |
| RAW264.7 cells | 0.62 μg/ml | 2 hours | Anti-iNOS, anti-Arg-1, anti-MPO, anti-elastase, anti-eDNA, anti-histone H3 | Induction of ROS-Mediated Autophagy and Extracellular Trap Formation. | (An et al., 2017) |
| 3D4/21 cells | $1x10^{-2}$, $2x10^{-2}$, $4x10^{-2}$ μg/ml | 24 hours | Anti-NP | Promotion of Swine influenza virus. | (Sun et al., 2018) |
| 3D4/21 cells | $2x10^{-2}$, $4x10^{-2}$, $8x10^{-2}$, 0.16 μg/ml | 48 hours | Anti-γH2AX | Apoptosis, ROS generation and DNA damage with epigenetic modifications. | (Zhou et al., 2019) |
| 3D4/21 cells | 0.16 μg/mL + NAC (652 μg/ml) | 48 hours | Anti-p65, anti-NF-kB | Activation of NF-κB pathway and promotion of apoptosis, inflammation and oxidative stress. | (Hou et al., 2018) |

| | | | | | |
|---|---|---|---|---|---|
| ***Hepatotoxicity*** | | | | | |
| HepG2 cells | 10 μg/mL + $ZnSO_4$ (8.1 μg/ml) or $ZnSO_4$ (4 μg/ml) + GZn (11.4 μg/mL ) | 24 hours | Anti-AFB1 | Promotion of ROS generation, cell cycle arrest and apoptotic bodies formation. Induction of DNA strand breaks and epigenetic changes. | (Yang et al., 2016) |
| HepaRG cells | 0.31 μg /ml | 24 hours | Anti-GSDMD | Promotion of hepatocytes pyroptosis and activation of Kupffer cells with liver inflammatory. | (Zhang et al., 2019) |
| HepG2 cells<br>HepaRG cells | 0.31 μg//ml | 48-72 hours | Anti-TOM20, anti-RIP3 | Induction of Hepatitis B virus infection, hepatic steatosis and mitochondrial disorders. | (Chen et al., 2019) |
| ***Gastrointestinal toxicity*** | | | | | |
| HCT116 cells<br>Caco-2 cells | 3.12 μg//ml | 8 hours | Anti-yH2AX, anti-p53BP1 | Enhancement of cell death and DNA damage-mediated cell cycle arrest through the ATR-Chk1 axis. | (Gauthier et al., 2020) |
| ***Nephrotoxicity*** | | | | | |
| HEK293T cells | 1, 2, 6 μg/ml | 24 hours | Anti-CRM1 | Cell cycle arrest. | (Huang et al., 2019) |

| | | | | | |
|---|---|---|---|---|---|
| ***Embryotoxicity*** | | | | | |
| Porcine embryos | $3.12x10^{-4}$ μg/ml | 1 week | Anti-LC3, anti-cytochrome C, anti-53BP1, anti-γH2AX, anti-BECLIN1 | Disturbance of embryonic development, production of ROS and DNA damage. Induction of apoptosis and autophagy with a reduction of cell proliferation. | (Shin et al., 2018) |
| ***In vivo*** | | | | | |
| ***Neurotoxicity*** | | | | | |
| Rats brain | 0.016 mg/kg/orally | 8 weeks | Anti-NeuN, anti-GFAP | Structural alterations in rat brain with an increase of astrocyte distribution and a reduction of neuronal cells. | (Bahey et al., 2015) |
| ***Immunotoxicity*** | | | | | |
| Mice spleen | 0.05-0.07 mg/kg/intraperitoneally | 8-10 weeks | Anti-EBNA2, anti-CD20 | Promotion of EBV carcinogenesis. | (Accardi et al., 2015) |

*3.1.2. Flow cytometry*

*3.1.2.1. In vitro toxicity*

In vitro AFB1-FCM studies exposed cells between 2 h and 72 h to doses of 3.12 × $10^{-3}$-100 μg/ml (Table 3). As shown in Fig. 5, HepG2 was the most used cell line. The protective role of different compounds (GZn, ZnSO4, Zn-Bent1, PKC, acid gallic, IgY, L-Proline) was also investigated.

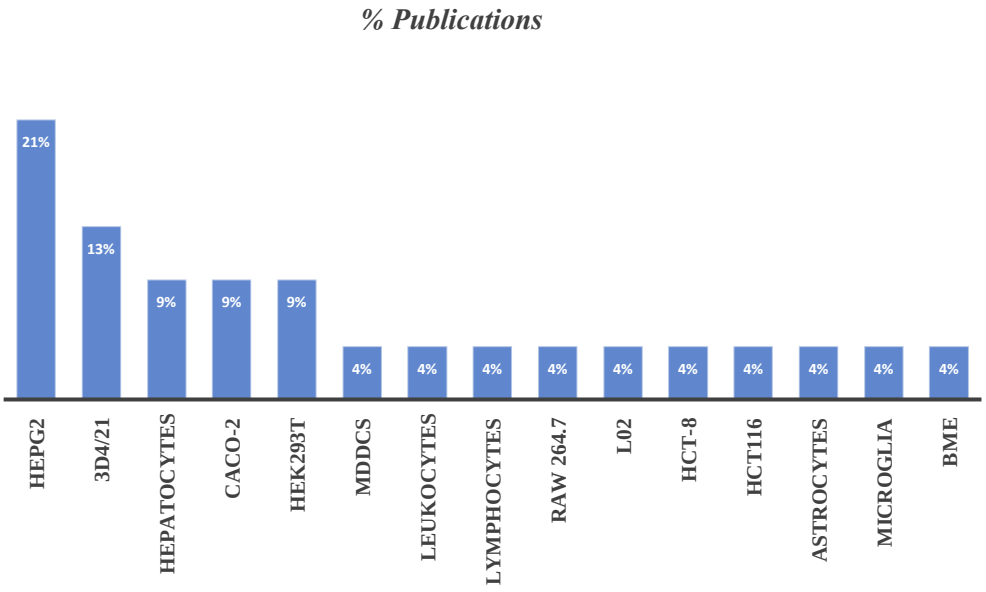


**Fig.5.** Percentage of publications (n=23) according to the cell lines used in AFB1-FCM *in vitro* studies.

*3.1.2.1.1. Immunotoxicity*

AFB1-exposure induced a complete autophagic process in macrophages with Ets generation in a dose-dependent manner (Table 3). FCM analysis revealed AFB1-capacity to exacerbate immunotoxicity by switching macrophages polarization to pro-inflammatory M1 phenotype (An et al., 2017).

Mehrzad et al. (2018) evaluated the effect of AFB1 in MDCCs cells. AFB1 impaired the phagocytosis capacity of MDDCs, promoted cell death and dysregulated the function of key cytokines. Similar results were obtained by the same authors by treating human, canine and bovine leukocytes with low doses of AFB1. As expected, an aberrant activation of caspase 3/7 and a significant decrease of ATP content was observed. FCM-analysis showed a slightly increase of necrotic neutrophils, lymphocytes, and monocytes in all studied models (Mehrzad et al., 2020).

In human lymphocytes, the induction of oxidative stress, lipid peroxidation, with the suppression of superoxide dismutase (SOD) activity were obtained upon AFB1-exposure. Interestingly, FCM was used to detect the 8-oxyguanine (8-oxoG), a typical marker of DNA/RNA oxidative damage. The presence of 8-oxoG may promote mismatches during DNA replication and miscoding in transcriptomic process, thus inducing genetic

mutations. The content of 8-oxoG in samples increased in an AFB1-dose-dependent manner (Su et al., 2017).

Sun et al. (2018) confirmed that exposure to toxin promoted the switch of macrophages and SIV-infection, as demonstrated by the increase of viral titers and NP expression. Moreover, the promotion of inflammation and cell death in a concentration-dependent manner was observed.

In the same cell line, exposure to the same AFB1-doses elicited cell death in a dose-dependent manner via mitochondrial pathway. In comparison to the control, nuclei changes with chromatin condensation, marginalization and fragmentation were observed in AFB1-treated cells. Another important immune index for macrophages is the phagocytic activity, which was significantly decreased. The promotion of oxidative stress with ROS generation and a decreased glutathione (GSH) activity was also detected (Zhou et al., 2019). Similar results were obtained by Hou et al. (2018) who showed that the combined action of AFB1 and OTA exacerbated immunotoxicity due to the marginalization, condensation and fragmentation of chromatin with apoptotic bodies formation. Moreover, the expression of pro-inflammatory cytokines (TNF-$\alpha$ and IL- 6) significantly increased while the release of lactate dehydrogenase and phagocytic index decreased. Mycotoxin-exposure also stimulated the production of free radicals with a significant decrease of GSH activity.

*3.1.2.1.2. Hepatotoxicity*

After a short exposure, AFB1 sparked mitochondrial-ROS generation, decreased MMP and induced apoptosis via Nrf2-signal pathway activation. Moreover, the altered expression of caspase 3/9, NADPH quinine oxidoreductase 1, SOD and HO-1 has been observed (Liu and Wang, 2016). Similarly, 24 h exposure to toxin caused apoptotic bodies formation and arrested the cell cycle at phase S with marked genomic alterations. AFB1 inhibited the global DNA hypomethylation and induced intracellular ROS generation with mitochondrial dysfunctions (Yang et al., 2016). Interestingly, AFB1-exposure could enhance the migration of hepatocellular carcinoma cells to other tissues. A decreased cell ratio at G0/G1 phase and an increased cell distribution at G2/M stage confirmed the aberrant growth and the subsequent extra-tissue expansion of cancer cells (K. Chen et al., 2016).

Furthermore, AFB1 alone or in combination with Fumonisin B1 (FB1) increased both the production of ROS and the percentage of apoptotic cells (10–30% more in comparison to the control) with a significant decrease of MMP in a dose-dependent manner. A significant increase of oxidative stress might contribute to cell apoptosis by disrupting the mitochondria, leading to the release of cytochrome c (cyt c), and activating caspase cascade. Additionally, the cell cycle arrest at phase S was associated with the reduced DNA content in AFB1-treated cells (Du et al., 2017).

Oskoueian et al. (2015) demonstrated that PKC and acid gallic played an important cytoprotective effect on chicken hepatocytes by inhibiting the lipid peroxidation and cell death induced by AFB1. Phenolics compounds (PEF) improved the antioxidant cell system, through the production of SOD, catalase (CAT) and glutathione reductase (GR). As an excessive amount of ROS promoted the NF-kB up-regulation and NRF2 down-regulation and induced the expression of several pro-inflammatory mediators (IL-6, TNF-$\alpha$), PEF may attenuate cell inflammation due to the direct inhibition of oxidative stress.

In HepG2 cells, the identification and quantification by FCM analysis of three different and typical markers of cancer stem cells, CD133, CD44, and aldehyde dehydrogenase1 upon AFB1-exposure demonstrated its capacity to promote hepato-cellular carcinoma development (Ju et al., 2016).

A similar duration study revealed that high concentrations of AFB1 triggered apoptotic bodies formation and DNA damage with cell cycle arrest at G2/M phase. The aberrant ROS production (1.5-fold higher than control) with MMP disturbance has been also observed. Nevertheless, low doses of IgY showed protective effects against oxidative stress, apoptosis and cell cycle arrest associated with AFB1-treatment (Qiu et al., 2018).

*3.1.2.1.3. Gastrointestinal toxicity*

In relation to gastrointestinal toxicity, the molecular explanation on the risks of cross-talk between AFB1 and OTA was provided in intestinal cancer cells by Kim et al. (2016). DNA fluorescence analysis performed by fluorescence-activated cell sorting (FACS) technique revealed that AFB1 was capable to trigger genomic damages, apoptosis and cell cycle arrest at phase S via p53 dysregulation. These mutagenic effects were strongly antagonized by OTA-treatment which may contribute to cancer cells survival by increasing the risk of carcinogenesis.

Nones et al. (2017) demonstrated the cytoprotective effect of Zn-Bent1 against fungal compounds. Low doses of Zn-Bent1 reversed the elevated percentage of apoptotic nuclei and necrotic cells promoted by AFB1 (15% more than control). Likewise, in Caco-2, AFB1 triggered a steady state level of permeabilized PI positive cells indicating necrosis/late apoptosis, whereas in HCT116 cells, AFB1 caused apoptosis in a dose-dependent manner with a significant arrest of cell cycle at G2/M phase (Gauthier et al., 2020).

*3.1.2.1.4. Nephrotoxicity*

One of the main organs affected by AFB1-toxicity is undoubtedly the kidney. Regarding the most severe exposure reported, AFB1 (100 μg/ml)

activated oxidative stress-related pathways and caused kidney injury with a high rate of apoptotic cells (70% more in comparison to the control). However, L-proline preserved kidney integrity alleviating oxidative damage and decreasing downstream apoptosis by regulating proline dehydrogenase levels and pro-apoptotic proteins expression (Li et al., 2019). Furthermore, acute exposure to AFB1 has promoted the alteration of p21 and its specific regulators, PLK1, PLD1 and MYC, thus causing a significant arrest of cell cycle at phase S in a dose-dependent manner. Since p21 is the most important regulator of G1/S and S/G2 cell cycle transition, DNA replication and genomic stability, the alteration of cell cycle has been considered as the main cytotoxic effect of AFB1 in kidney cells (Huang et al., 2019).

*3.1.2.1.5. Neurotoxicity*

Although different studies have suggested AFB1-ability to cross the BBB and disrupt its integrity, the effects on brain are not well reported. In mouse astrocytes, AFB1 led to an aberrant cell death (16.55% higher than control) in a time-dependent manner with ATP depletion and caspases 3/7 activation. Significant cyt c release confirmed the association between apoptosis and mitochondrial impairment (Vahidi-Ferdowsi et al., 2018). In a similar way, AFB1 may provoke microglial cell death due to the intracellular ATP depletion and caspase 3/7 activation. Additionally, toxin could trigger an inflammatory reaction in neuronal cells by the dysregulation of many pro-inflammatory cytokines such as TRLs, NF-κB and myeloid

differentiation primary response 88 (MyD88). In view of this, AFB1 could increase the susceptibility to neurodegenerative diseases, inducing neuroinflammation and altering the homeostasis of human CNS (Mehrzad et al., 2018a, Mehrzad et al., 2018b).

*3.1.2.1.6. Breast toxicity*

Finally, the harmful effects of AFB1 have also been studied in BME cells. Wu et al. (2021) observed the cell cycle arrest, inhibition of cell proliferation and apoptosis upon AFB1-exposure. The cytotoxicity was related to epigenetic modifications; in particular, a marked decrease of RNA methylation was detected.

In summary, 24 h and 48 h were the most common exposure times in FCM assay while the apoptosis assay was the main analysis performed. The enhancement of apoptosis and cell cycle arrest were the main toxicological effects observed owing to AFB1-treatment.

**Table 3.** AFB1-FCM studies: *In vitro* model, dose and exposure time, FCM assay, mechanism and references.

| *In vitro* model | Dose administration | Exposure time | FCM assay | Mechanism | References |
|---|---|---|---|---|---|
| ***Immunotoxicity*** | | | | | |
| RAW264.7 cells | $7.7x10^{-2}$ – 0.62 μg/ml | 1.5 hours | Macrophages subset analysis | Induction of ROS-Mediated Autophagy and Extracellular Trap Formation. | (An et al., 2017) |
| MDDCs cells | $1x10^{-2}$ μg /ml | 2-12 hours | Apoptosis assay Phagocytosis assay | Impairment of cell phagocytosis activity and function. Increase of cell death. | (Mehrzad et al., 2018) |
| Human, dog and cattle leukocytes | $1x10^{-2}$ μg /ml | 24 hours | Apoptosis assay | Depletion of intracellular ATP and apoptosis. | (Mehrzad et al., 2020) |
| Human lymphocytes | 0.2, 2, 20 μg/ml | 24 hours | DNA damage analysis | Promotion of oxidative stress by inducing lipid peroxidation. | (Su et al., 2017) |
| 3D4/21 cells | $2x10^{-2}$, $4x10^{-2}$, $8x10^{-2}$, 0.16 μg/ml | 48 hours | Analysis of immune cells subset | Promotion of Swine influenza virus. | (Sun et al., 2018) |

| | | | | | |
|---|---|---|---|---|---|
| | | | Apoptosis assay | | |
| 3D4/21 cells | $2x10^{-2}$, $4x10^{-2}$, $8x10^{-2}$, 0.16 μg/ml | 48 hours | Apoptosis assay | Apoptosis, ROS generation and DNA damage with epigenetic modifications. | (Zhou et al., 2019) |
| 3D4/21 cells | 0.16 μg/ml | 48 hours | Apoptosis assay<br>Intracellular ROS measurement | Activation of NF-κB pathway and promotion of apoptosis, inflammation and oxidative stress. | (Hou et al., 2018) |
| ***Hepatotoxicity*** | | | | | |
| Broiler hepatocytes | 0.15, 0.31, 0.77, 1.56 μg/ml | 6 hours | Apoptosis assay<br>Measurement of mitochondrial membrane potential | Impairment of mitochondrial functions, ROS generation and induction of apoptosis. | (Liu & Wang, 2016) |
| HepG2 cells | 10 μg/mL + $ZnSO_4$ (8.1 μg/ml) or $ZnSO_4$ (4 μg/ml) + GZn (11.4 μg/ml ) | 24 hours | Cell cycle analysis<br>AFB1-DNA adduct formation | Promotion of ROS generation, cell cycle arrest and apoptotic bodies formation. Induction of DNA damages and epigenetic changes. | (Yang et al., 2016) |

| | | | | | |
|---|---|---|---|---|---|
| HepG2 cells | 0.62 μg/ml | 24 hours | Cell cycle analysis | Pathological impairment and cell cycle alteration. | (K. Chen et al., 2016) |
| HepG2 cells | $3.12x10^{-3}$, 1.88, 3.12 μg /ml | 24, 48 hours | Cell cycle analysis<br>Apoptosis assay<br>Measurement of mitochondrial membrane potential | Disturbance in cell cycle, promotion of apoptosis and ROS through mitochondrial impairments. | (Du et al., 2017) |
| Chicken hepatocytes | 1.56 μg/ml + Palm kernel cake (5, 10, 20, 40 μg/ml) + Acid gallic (1.7 μg/ml) | 48 hours | Apoptosis assay | Induction of lipid peroxidation and apoptosis. | (Oskouean et al., 2015) |
| HepG2 cells | 1.56, 3.12, 6.24 μg/ml | 48 hours | Quantitative determination of hepatocellular cancer stem cells | Promotion of hepatocellular carcinoma. | (Ju et al., 2016) |
| L02 cells | 31.2 μg/ml + IgY ($9x10^{-2}$, $4.7x10^{-2}$, $2.3x10^{-2}$ μg/ml ) | 48 hours | Cell cycle analysis<br>Apoptosis assay<br>AFB1-DNA adduct formation | Apoptotic bodies formation and DNA strand breaks, with $G_2$/M phase cell cycle arrest. High ROS production with MPP disturbance. | (Qiu et al., 2018) |

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|---|---|---|---|---|---|
| ***Gastrointestinal toxicity*** | | | | | |
| HCT-8 cells | 3.12 μg/ml | 24 hours | DNA apoptotic analysis | Promotion of DNA damage and cell cycle alteration via p53 activation. Possible cross-talk between AFB1 and OTA. | (Kim et al., 2016) |
| Caco-2 cells | 9.3 μg/mL + Zn-Bent1 (600 μg/ml) | 48 hours | Apoptosis assay | Promotion of necrosis. | (Nones et al., 2017) |
| HCT116 cells<br>Caco-2 cells | $7.8x10^{-2}$, 0.31,1.56, 3.12, 6.24 μg/ml | 48 hours | Apoptosis assay<br>Cell cycle analysis<br>Intracellular ROS measurement | Enhancement of cell death and DNA damage-mediated cell cycle arrest through ATR-Chk1 axis. | (Gauthier et al., 2020 |
| ***Nephrotoxicity*** | | | | | |
| HEK293T cells | 100 μg/ml + L-proline (1000 μg/ml) | 24 hours | Apoptosis assay | Disturbance in cell vitality. | (Li et al., 2019) |
| HEK293T cells | 1, 2, 6 μg/ml | 24,48 hours | Cell cycle analysis | Cell cycle arrest. | (Huang et al., 2019) |

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| ***Neurotoxicity*** | | | | | |
| Mouse astrocytes | $1x10^{-1}$ µg /ml | 24,48,72 hours | Apoptosis assay | Apoptosis in a time dependent manner with intracellular ATP depletion and caspases 3-7 activation. | (Vahidi-Ferdowsi et al., 2018) |
| Human microglia cells | $2x10^{-2}$ µg/ml | 48 hours | Apoptosis assay | Apoptosis with intracellular ATP depletion and caspases 3-7 activation. | (Mehrzad et al., 2018) |
| ***Breast toxicity*** | | | | | |
| BME cells | 2 µg/ml | 24 hours | Cell cycle analysis<br>Apoptosis assay<br>Intracellular ROS measurement | Promotion of epigenetic modification in RNA-methylation. Impairment in cell cycle, proliferation and apoptosis. | (Wu et al., 2021) |

*3.1.2.2. In vivo toxicity*

*In vivo* AFB1 FCM-studies, the exposure time ranged between 1 and 6 weeks with concentrations of 0.016–1.25 mg/kg administered orally, by gavage or in contaminated feed in broilers, chickens, mice and rats (Fig. 6). The activity of non-pathogenic bacteria (LAB, LP) and natural compounds (MT, Se, β-1,3-glucan) was investigated.

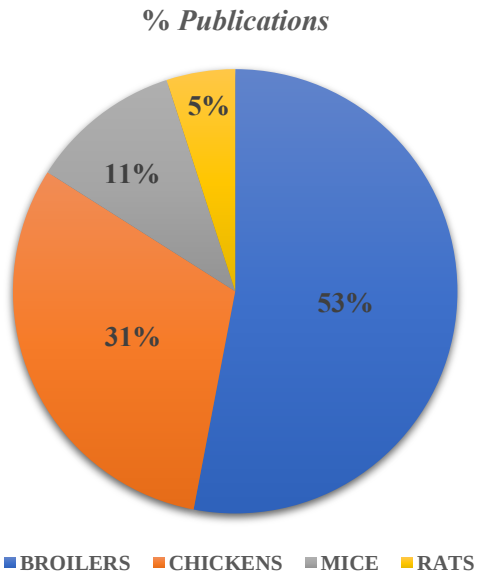


**Fig.6.** Percentage of publications (n=18) according to animal models used in AFB1-FCM *in vivo* studies.

*3.1.2.2.1. Immunotoxicity*

In a sub-acute immunotoxicity study, AFB1 markedly decreased the percentages of lymphocytes subsets, Foxp3+ T cells, and reduced IL-2, TNF-$\alpha$, IL-17 and IFN-$\gamma$ production. Also, it has been demonstrated that AFB1 led to a general immune suppression by the down-regulation of Th1,Th2,Th17 and Treg genes related to immune responses. However, $\beta$-1,3-Glucan improved the responses of lymphocyte subsets, including cytokines production, thus counteracting AFB1-immunotoxicity in spleen (Bakheet et al., 2016). Similarly, AFB1, alone or in combination with FB1, promoted DNA-damage and oxidative stress by increasing caspase-3 activity and IL-4, IL-10 expression. The activity of antioxidant enzymes (Gpx and SOD) was clearly reduced. However, LAB-administration induced protective effects against oxidative stress and immunotoxicity of both mycotoxins (Abbès et al., 2016). Likewise, the pre-treatment with LP BEJ01 and MT prevented the effects of AFB1 on immune response in rats. The protective effects of these compounds could be explained by their ability to bind the toxin in the gastrointestinal tract, thereby reducing its bioavailability. Otherwise, exposure to AFB1 caused inflammation and oxidative stress with a marked reduction of immunoglobulins, B and T-lymphocytes, and natural killer cells (Ben Salah-Abbes et al., 2016).

Noteworthy in Table 4, the presence of several studies with similar experimental conditions, in which AFB1-toxicity and the possible protective

role of selenium on chickens and broilers immune organs were investigated. Histopathology and FCM analysis demonstrated the negative effect of AFB1-dietary exposure (0.6 mg/kg) in chickens bursa of Fabricius (BF). AFB1 arrested the cell cycle in G2/M phase at 7 days and in phase G0/G1 at 14 and 21 days. The arrest of cell cycle was related to alterations in Cyclin D1 signaling pathway (Hou et al., 2018a). These toxicological damages were restored by the dietary supplementation of Se (0.4 mg/kg), which also alleviated histological lesions and oxidative damage induced by AFB1-administration (Hu et al., 2018b). Chickens treated with AFB1 for 21 days have shown a clear splenocytes death (10–15% higher than control group) due to the impairment of death receptors (FAS, FASL, TNF-$\alpha$, TNF-R1) and ER molecules expression (Grp78 and Grp94) (Zhu et al., 2017). Fang et al. (2019) demonstrated that these molecular alterations could be reversed by Se-supplementation (0.4 mg/kg) in contaminated feed.

In broilers thymocytes, Se could alleviate AFB1-induced cell death and histological lesions and may reversed the G0/G1 cell cycle arrest by modulating p21/p27-CyclinE/Cdk2 and p15-CyclinD1/Cdk6 signaling pathways (Guan et al., 2019). In a similar duration exposure (3 weeks), AFB1 (0.15, 0.3, 0.6 mg/kg) induced apoptosis by altering caspase-3, Bax, Bcl-2 expression in broilers thymus and BF (Peng et al., 2016).

Furthermore, consumption of AFB1-contamined feed was associated with immunosuppression in broilers spleen. In particular, ROS-induced

apoptosis, DNA damage and mitochondrial impairment have been observed (J. Chen et al., 2016). In a 21 days study, AFB1 compromised the rate of cell-mediated immunity in broilers cecal tonsil. Authors obtained a clear reduction in cytokines (IL-2, IL-4, IL-6, IL-10, IL-17, IFN-$\gamma$, TNF-$\alpha$) expression and a reduced percentage of T-cells subset (CD3+,CD3+CD4+,CD3+CD8+) (Liu et al., 2016). Whilst, in broilers spleen, thymus and BF the ingestion of AFB1-contaminated feed during 42 days caused histopathological lesions with lymphocytes depletion. In this case, AFB1-MOA was associated with alterations in mitochondrial and ER compartments (Peng et al., 2015).

Finally, the different sensitivity of broilers BF and thymus to mycotoxin-exposure in contaminated feed during 42 days was evaluated. Histopathological lesions and pathological impairment were evident to a greater extent in BF than thymus with higher lymphocyte depletion. At 21 days, both thymocytes and BF cells showed a cell cycle arrest at phase G2/M. Whilst, at 42 days, cell cycle perturbations at phase G0/G1 in thymocytes and at G2/M phase in BF cells was observed. However, a similar apoptotic rate was obtained (Peng et al., 2017).

*3.1.2.2.2. Hepatotoxicity*

Table 4 shows that the ingestion of AFB1-contamined feed (0.6 mg/ml) during 21 days caused degenerative histological lesions in broilers

liver with high percentages of apoptotic cells (2-5-fold higher than control group) and intracellular ROS production. Se-treatment (0.4 mg/ml) could mitigate oxidative stress and relieve AFB1-induced apoptosis through the molecular regulation of death receptors (Wu et al., 2019).

*3.1.2.2.3. Gastrointestinal toxicity*

To evaluate the effects of AFB1 on the gastrointestinal tract, chickens and broilers were exposed to a sub-acute contamination. It has been observed marked morphological changes in chickens jejunum with a reduction of villus height and area. In this case, the apoptosis of jejunal cells was associated with the altered expression of death receptors and ER molecules (Zheng et al., 2017). Fang et al. (2018) observed the protective role of Se against AFB1-toxicological damages by modulating the ataxia telangiectasia mutated (ATM) pathway and by reversing G2/M cell cycle arrest.

In broilers jejunal cells, Yin et al. (2016) demonstrated that ATM kinase and its downstream molecules, the checkpoint kinase Chk2 and the increased expression of p53 and p21, likely contribute to the AFB1-induced G2/M cell cycle arrest.

*3.1.2.2.4. Nephrotoxicity*

In the only study found on nephrotoxicity, Yu et al. (2015) demonstrated that the ingestion of AFB1 (0.3 mg/kg) in contaminated feed during 21 days resulted in apoptosis with altered caspase-3 and Bax expression. A significant reduction of cell proliferation with cell cycle arrest at phase G0/G1 was also revealed. However, Se supplied in diet mitigated apoptosis, cell proliferation and kidney damages.

In summary, 3 weeks was the most used exposure time while the apoptosis assay was the main analysis performed. The most used concentrations were 0.6 mg/kg for AFB1 and 0.4 mg/kg for Se in contaminated feed. The induction of apoptosis, cell cycle arrest and immunosuppression were the main damages related to AFB1-administration.

**Table 4.** AFB1-FCM studies: *In vivo* model, dose and exposure time, FCM assay, mechanism and references

| *In vivo* model | Dose administration | Exposure time | FCM assay | Mechanism | References |
|---|---|---|---|---|---|
| ***Immunotoxicity*** | | | | | |
| Mice spleen | 1.25 mg/kg + β-1,3-Glucan (150 mg/kg ) /orally | 1 week | Measurement of IL-2, TNF-α, IL-17, IFN-γ Assessment of intracellular Foxp3 | Reduction of T-cells subset and cytokines production. | (Bakheet et al., 2016) |
| Mice thymus | 0.08 mg/kg + LAB (2 mg/kg)/ orally | 2 weeks | Apoptotic DNA analysis | Increase of intracellular ROS production, high level of apoptotic DNA and cell death. | (Abbes et al., 2016) |
| Rats plasma | 0.08 mg/kg + *Lactobacillus paracasei* BEJ01 (2 mg/kg) + montmorillonite clay (0.5 mg/kg)/ gavage administration | 2 weeks | Measurement of total white and red blood cells and lymphocyte subtypes | Enhancement of oxidative stress with a reduction of B-lymphocytes, T-lymphocytes, NK cells subset. | (Ben-Salah Abbes et al., 2016) |
| Chickens bursa of Fabricius | 0.6 mg/kg/ contaminated diet | 3 weeks | Cell cycle analysis | Cell cycle arrest. | (Hu et al., 2018a) |

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|---|---|---|---|---|---|
| Chickens bursa of Fabricius | 0.6 mg/kg + Se (0.4 mg/kg)/ contaminated diet | 3 weeks | Cell cycle analysis | Tissue damage and cell cycle arrest. | (Hu et al., 2018b) |
| Chickens spleen | 0.6 mg/kg/ contaminated diet | 3 weeks | Apoptosis assay | Induction of apoptosis with the expression of death receptor and endoplasmic reticulum molecules. | (Zhu et al., 2017) |
| Chickens spleen | 0.6 mg/kg + Se (0.4 mg/kg)/ contaminated diet | 3 weeks | Apoptosis assay | Aberrant apoptotic rate. | (Fang et al., 2019) |
| Broilers thymus | 0.6 mg/kg + Se (0.4 mg/kg)/ contaminated diet | 3 weeks | Cell cycle analysis | Alterations in cell cycle. | (Guan et al., 2018) |
| Broilers thymus and bursa of Fabricius | 0.15, 0.30, 0.60 mg/kg/ contaminated diet | 3 weeks | Apoptosis assay | Promotion of apoptosis. | (Peng et al., 2016) |
| Broilers spleen | 0.15, 0.30, 0.60 mg/kg/contaminated diet | 3 weeks | Apoptosis assay | Induction of oxidative stress, splenocytes apoptosis and immunodepression. | (J.Chen et al., 2016) |
| Broilers cecal tonsil | 0.6 mg/kg/ contaminated diet | 3 weeks | T cells identification | Reduction of T cell subsets and cytokines production. | (Liu et al., 2016) |

| | | | | | |
|---|---|---|---|---|---|
| Broilers immune organs | 0.082, 0.134 mg /kg / contaminated diet | 6 weeks | Apoptosis assay | Promotion of apoptosis, histopathological lesions with lymphocytic and splenic plasmacytes depletion. | (Peng et al., 2015) |
| Broilers thymus and bursa of Fabricius | 0.016, 0.134 mg/kg /contaminated diet | 6 weeks | Cell cycle analysis Apoptosis assay | Cell cycle arrest and pathological impairment. | (Peng et al., 2017) |
| ***Hepatotoxicity*** | | | | | |
| Broilers liver | 0.6 mg/kg + Se (0.4 mg/kg)/contaminated diet | 3 weeks | Apoptosis assay | Induction of apoptosis and ROS generation. | (Wu et al., 2019) |
| ***Gastrointestinal toxicity*** | | | | | |
| Chickens jejunum | 0.6 mg/kg/ contaminated diet | 3 weeks | Apoptosis assay | Induction of apoptosis with the altered expression of death | (Zheng et al., 2017) |

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|---|---|---|---|---|---|
| Chickens jejunum | 0.6 mg/kg + Se (0.4 mg/kg)/ contaminated diet | 3 weeks | Cell cycle analysis | Pathological impairments and cell cycle alteration. | (Fang et al., 2018) |
| Broilers jejunum | 0.6 mg/kg/ contaminated diet | 3 weeks | Cell cycle analysis | Cell cycle arrest. | (Yin et al., 2016) |
| ***Nephrotoxicity*** | | | | | |
| Broilers kidney | 0.3 mg/kg + Se (0.4 mg/kg)/ contaminated diet | 3 weeks | Apoptosis assay<br>Cell cycle analysis | Reduction of cell proliferation and arrest of cell cycle in $G_0/G_1$ phase. | (Yu et al., 2015) |

### *3.2. Ochratoxin A*

The toxicity of OTA, the other mycotoxin analyzed in this review, was also investigated using the techniques mentioned above. In relation to IF analysis, antibodies used varied according to the specific pathway studied by authors while the FCM was mainly performed to detect apoptosis, ROS generation, alterations in cell cycle, mitochondrial and redox balance. Furthermore, several natural compounds have been used to mitigate OTA-toxicity, such as luteolin (LUT), selenomethionine (SeMet), taurine (TAU), melatonin, NAC, resveratrol (RSV), zinc, N-acetyl-tryptophan (NAT), glycyrrhizin (CAG), gluconolactone (GA), L-arginine (L-Arg), silymarin (Sil) and astragalus polysaccharide (APS).

For each technique, the selected OTA-studies were organized according to the model used showing that the main toxicological effects in vitro are nephrotoxicity, gastrointestinal toxicity, hepatotoxicity, immunotoxicity, embryotoxicity, reproductive toxicity, neurotoxicity, lung and esophageal toxicity (Fig. 7). Whilst, only three in vivo toxicity studies have been selected, which confirmed OTA-nephrotoxicity and neurotoxicity (Fig. 8). The summary of the articles found for OTA are shown below. Tables were divided depending on the technique, model used and type of toxicity, and were ordered by the exposure time, from the shortest to the longest.

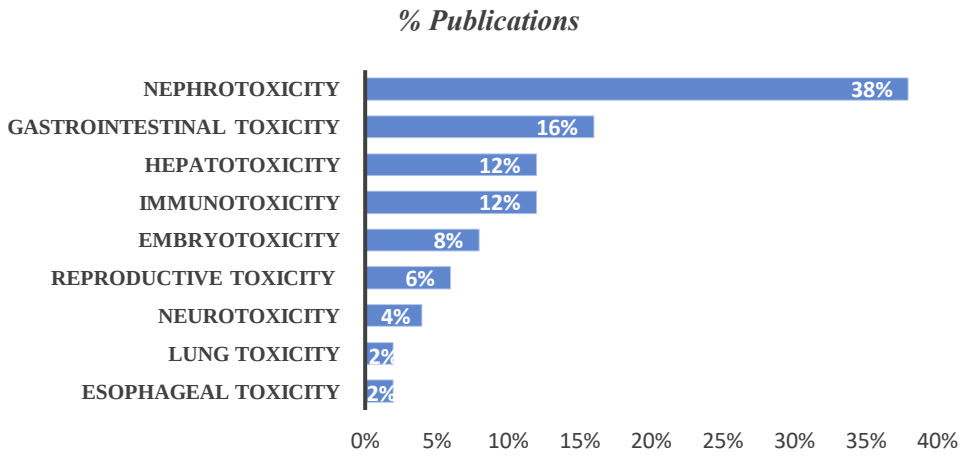



**Fig.7.** Percentage of publications (n=50) according to the main toxicological effects of OTA *in vitro*.

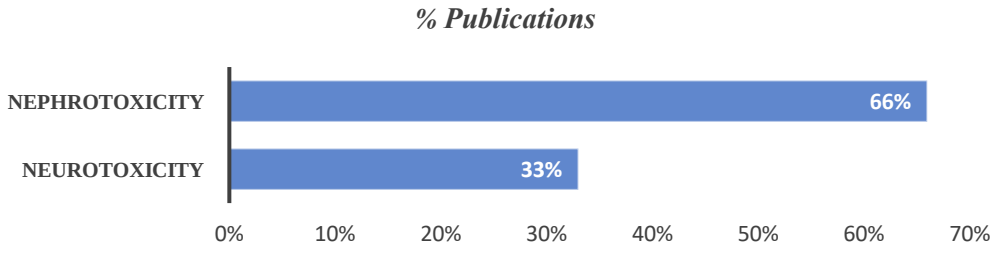



**Fig.8.** Percentage of publications (n=3) according to the main toxicological effects of OTA *in vivo*.

*3.2.1. Immunofluorescence*

*3.2.1.1. In vitro toxicity*

Exposure times chosen for in vitro OTA-IF studies ranged between 2 h and 40 weeks with concentrations of 1 × $10^{-2}$- 40 μg/ml (Table 5). Fig. 9 shows that PK-15 was the most used cell line. LUT, SeMet, NAC, TAU and melatonin were employed to alleviate OTA-toxicity.

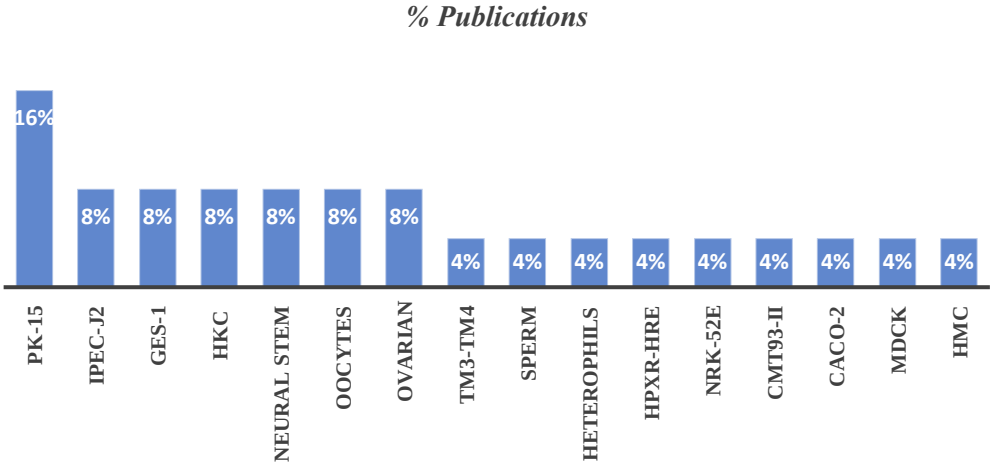



**Fig.9.** Percentage of publications (n=25) according to cell lines used in OTA-IF *in vitro* studies.

*3.2.1.1.1. Immunotoxicity*

In the only study reported, OTA impaired the phagocytosis function of heterophils and enhanced the release of their Ets. The IF analysis of Ets main constituents, histone H3 and elastase, proved their dose-dependent release. In addition, its formation was associated with the activation of NADPH oxidase, ERK1/2, and p38/MAPK signaling pathways, which led to intracellular ROS generation (Han et al., 2019).

*3.2.1.1.2. Hepatotoxicity*

As shown in Table 5, the only study reviewed was conducted by Shen et al. (2020). It has been proved that OTA-dichlorination (OTA Cl/M) and demethylation (OTA M) triggered the downregulation of pregnane X receptor (PXR), whose excessive activation is related to lipid retention, metabolic diseases and toxicological bioactivation of drugs. The fluorescence staining of FLAG, which is widely used as an epitope tag for selective protein detection, showed that OTA and its derivatives interacted with different residues of PXR, providing the molecular basis for a selectivity. In view of this, OTA-derivatives (C1/M and M) may be considered novel PXR-antagonists, reducing liver cytotoxicity.

*3.2.1.1.3. Gastrointestinal toxicity*

The gastrointestinal tract is one of the main sites affected by OTA-toxicity (Ricci et al., 2021). After a short exposure, this mycotoxin not only promoted cytotoxicity but also the phosphorylation and nuclear translocation of ERK1/2, which in turn mediated the autophagy, reduced mitochondrial ROS production and caspase-3 activation and mitigated OTA-detrimental effects. Therefore, OTA may have a bivalent effect on IPEC-J2 cells whereas ERK1/2-mediated autophagy could be considered as one of the main protective mechanisms in maintaining the integrity of intestinal barrier (Wang et al., 2018).

In GES-1 cells, Li et al. (2019) demonstrated that an over-activation of autophagy may produce opposite results. Indeed, OTA-exposure promoted the conversion of LC3B-I to LC3B-II, a typical autophagy marker, indicating that autophagic-cell death was triggered. Moreover, it has been detected an excessive production of autophagic vacuoles, which engulfed the damaged mitochondria and induced the occurrence of mitophagy. In this case, mitophagy was positively related to cell death. Authors concluded that OTA-effects on mitochondria in GES-1 cells are complex as it not only promoted apoptosis via mitochondrial pathway but also enhanced mitochondria biogenesis via AMPK/PGC-1$\alpha$/TFAM pathway, increasing cell survival.

In similar duration exposure, OTA alone or in combination with DON, compromised the intestinal barrier integrity, with a decreased transepithelial electrical resistance (TEER) value (50% less than control cells) and an increased paracellular permeability (2-fold higher in comparison to the control). In addition, the fluorescent analysis of claudin-3 and 4 tight junction and p65 factor demonstrated that OTA was capable to exacerbate DON-induced intestinal barrier dysfunction, inflammation and pro-inflammatory cytokines production (Ying et al., 2019). Interestingly, the analysis conducted by Nakayama et al. (2018) on claudin-2, a specific tight junction localized and expressed only in the deep crypt of the intestine. In CMT93-II cells, which exhibit typical features of deep crypt cells, OTA reduced claudin-2 expression and altered the TEER, by disrupting the intestinal barrier integrity. A reduced expression of claudin 4 and 6 has been detected as well. Similarly, in differentiated Caco-2 cells, the epithelial integrity was disordered due to an increased paracellular permeability and a decreased TEER value. Microscopic analysis, performed on claudin 3 and 4, occludin and ZO-1, revealed their altered expression and structure. A partial involvement of p44/42 mitogen-activated protein kinase (MAPK) in OTA-mediated intestinal barrier disruption has been observed (Gao et al., 2018).

Finally, chronic exposure to OTA (3 days of mycotoxin-exposure each week during 40 weeks) increased the proliferation, migration, and invasion capacity of GES-1 cells. Authors demonstrated that malignant

transformations of GES-1 cells can be attributed to Wnt/β-catenin signaling pathway. Indeed, aberrant de-regulation of Wnt/β-catenin pathway has been closely related to various types of tumors, including gastric cancer. In addition, inoculation of OTA-treated GES-1 cells in mice triggered the formation of tumor xenografts, confirming OTA-carcinogenicity in vivo. The pre-treatment with a high concentration of NAC could mitigate the long-term OTA-toxicity via Wnt/β-catenin pathway inhibition (Jia et al., 2016).

*3.2.1.1.4. Nephrotoxicity*

According to the main target organ related to OTA-toxicity, which is the kidney, the majority of the reviewed studies were focused on nephrotoxicity (Table 5). In NRK-52 E cells, the cytoprotective role of LUT was demonstrated. This compound alleviated OTA-induced oxidative stress by reducing ROS generation and improving the efficiency of antioxidant enzymes (SOD and GSH). Microscopic analysis showed that LUT increased cell antioxidant defenses by activating Nrf2 signaling pathway. At the same time, by modulating HIF-1$\alpha$ pathway, LUT mediated repair and regeneration process, and promoted angiogenesis and endothelial survival (Liu et al., 2020).

In HKC cells, 24 h exposure to toxin suppressed Notch and Ras/MAPK/CREB pathways and impaired cell growth and proliferation. Fluorescence analysis of DNMT1 showed alterations in global DNA

methylation, thus correlating OTA-nephrotoxicity with epigenetic changes. The genome-wide hypermethylation may contribute to OTA-mediated the inhibition of cell cycle progression (Zhang et al., 2020).

Interestingly, the analysis carried out by Yang et al. (2019) on 75 kD glucose-regulated protein (Grp75) and mitochondrial factors, involved in cell protection against OTA-activity. Grp75 is a Heat Shock Protein, which regulates the transfer of calcium from ER stores into the mitochondrial matrix. The IF analysis showed a slightly reduction of Grp75 expression and activity, probably related to OTA-contamination, which mediated the inhibition of mitochondrial metabolism. Exposure to toxin was also linked to Lonp1 suppression, a typical marker involved in reducing oxidative stress and in maintaining mitochondrial DNA stability. Fluorescence quantification of 8-OhdG and Kim-1 showed the tendency of OTA to promote DNA oxidative damage and kidney injury in the examined in vitro model.

In the 24 h study, OTA may induce kidney fibrosis by altering $\alpha$-SMA, vimentin and TNF-$\beta$ expression in a concentration-dependent manner. At the same time, this mycotoxin enhanced the activation of NLRP3 inflammasome, as detected by the fluorescent quantification of caspase 1-dependent pyroptosis. Also, OTA may promote an inflammatory status by modulating the expression of pro-inflammatory mediators (IL-6, TNF-$\alpha$) and pyroptosis-related genes (GSDMD, IL-1$\beta$, IL-18). These harmful effects were

significantly abrogated by MCC950 pre-treatment, a specific NLRP3 inhibitor, and by caspase-1 knockdown (Li et al., 2021).

In a similar study, the fluorescent quantification of vimentin revealed that OTA-administration caused morphological cell changes via ERK1/2 pathway activation. In addition, fluorescence staining of p65 showed its translocation into the nucleus upon IκB-$\alpha$ degradation with the subsequent activation of NF-kB signaling pathway. The pre-treatment with FR180240 and BAY 11–7082, specific inhibitors of ERK1/2 and NF-kB pathways, could withdraw OTA-nephrotoxicity (Le et al., 2020).

As shown in Table 5, several studies with similar experimental conditions, in which OTA-induced autophagy, apoptosis and porcine circovirus 2 (PCV2) replication, were reported. The possible protective role of Se, NAC and taurine was also examined. Qian et al. (2017) observed the increased expression of cap protein and the aberrant viral DNA synthesis upon OTA-administration. However, OTA-induced PCV2-replication was antagonized by NAC-administration. Similar results were obtained by Gan et al. (2015a). In this study, the promotion of PCV-2 replication was associated with p38/ERK1/2 MAPK signaling pathway activation. The cytoprotective role of NAC was also confirmed. Likewise, the pre-treatment with SeMet attenuated OTA-promoted ROS-dependent autophagy and PCV-2 replication by inhibiting AMPK/mTOR signaling pathway (Qian et al., 2018). The same biochemical pathway was modulated by high

concentrations of taurine, which could be considered an effective strategy against autophagy, oxidative stress and PCV2 infection (Zhai et al., 2018).

*3.2.1.1.5. Neurotoxicity*

As shown in Table 5, only two neurotoxicity studies were reviewed. In rat neural stem cells (rNSC), 5 days-exposure to OTA induced morphologic changes, affected cell viability in a concentration-dependent manner and inhibited cell proliferation and neutrospheres formation. Also, the immunostaining of GFAP and Dcx, astrocytes and neurons markers, showed that low doses of toxin significantly decreased neurons maturation and astrocytes density. These results confirmed OTA-negative effects on neural differentiation (Paradells et al., 2015). In vitro differentiation of rat NSC into astrocytes, neurons, and oligodendrocytes was also monitored by Gill and Kumara (2019). Using cell-specific IF staining for undifferentiated rNSC (nestin), neutrospheres (nestin and A2B5), neurons (MAP2 clone M13, MAP2 clone AP18, and Dcx), astrocytes (GFAP), and oligodendrocytes (A2B5 and mGalc), OTA-neurotoxicity was confirmed. Indeed, a non-cytotoxic concentration of OTA (0.08 μg/ml) markedly reduced the differentiation degree of rNSC into mature astrocytes, neurons and oligodendrocytes, thereby affecting the normal brain development.

*3.2.1.1.6. Embryotoxicity*

After 12 h exposure, OTA worsened oocyte quality by impairing meiotic maturation and by disrupting spindle formation and chromosome alignment. Additionally, OTA elicited intracellular ROS production and reduced Gpx activity. The fluorescent quantification of H3K9ac, H3K9me3 revealed the presence of epigenetic alterations in oocytes genome (Jia et al., 2020).

Similarly, microscopic analysis conducted in granulosa cells (GCs) confirmed OTA-ability to activate surface receptors and to promote oxidative stress by decreasing the expression of ITGB 1, a heterodimeric cell-surface receptor involved in different cell functions, and F2R receptor, a G-protein coupled receptor involved in the regulation of GC response. At the same time, fluorescence analysis of Bax and Bcl-2 indicated the positive correlation between OTA-treatment and cell death (Zhang et al., 2019). In the same in vitro model, OTA may exert deleterious effects by inducing DNA damage, disrupting DNA repair-related processes and arresting the cell cycle at phase G2/M. DNA damage was detected by the over-expression of DSB gene, γ-H2AX, and DNA repair-related genes, BRCA1 and RAD51 (Zhang et al., 2020).

In porcine oocytes, Lan et al. (2020) observed disturbances in oocytes maturation owing to the delay of Cdc2-mediated cell cycle progression. The

disruption of meiotic spindle formation was also related to fungal exposure. Moreover, IF analysis showed that OTA triggered aberrant mitochondria dysfunctions in oxidative phosphorylation with the promotion of oxidative stress, apoptosis and autophagy. However, the pre-incubation with elevated concentrations of melatonin significantly ameliorated oxidative stress, apoptosis and prevented the cell cycle arrest.

*3.2.1.1.7. Reproductive toxicity*

In this review, OTA-toxicity was also observed in the reproductive system. In testicular cells, TM3 and TM4, a marked decrease in cell proliferation and growth was detected. The down-regulation of PCNA, a typical cell proliferation marker, may explain the significant inhibition of cell cycle progression. In addition, authors obtained the loss of normal physiological functions, failure of spermatogenesis and male infertility due to the marked reduction of calcium concentration in cytosol and mitochondria (Park et al., 2020).

Zhang et al. (2018) investigated the correlation between OTA-exposure and PI3K/PTEN/AMPK signaling pathway activation. Microscopic analysis showed that high doses of OTA significantly increased the expression of PTEN/PI3K, thereby inducing apoptosis and oxidative stress. Whereas, a reduced AKT fluorescent expression can be associated with a reduced motility of spermatozoa in vitro.

In summary, anti-PCV2 and 24 h were the most common antibody and exposure time used in IF analysis. The induction of oxidative stress with ROS generation was the main effect related to OTA-administration.

*3.2.1.2. In vivo toxicity*

Only two in vivo OTA-IF studies were found and reviewed. The exposure time ranged between 3 and 6 weeks while doses of 2.50 and 3.50 mg/kg were administered in mice intraperitoneally. No microorganism or natural compounds were tested.

*3.2.1.2.1. Nephrotoxicity*

With regard to nephrotoxicity in vivo, Loboda et al. (2017) demonstrated through the immunostaining of $\alpha$-SMA, vimentin and collagen I, OTA pro-fibrotic effects while by the fluorescent analysis of caspase 3 and p53, authors confirmed OTA pro-apoptotic activity. Moreover, the immunofluorescent detection of CD45-positive cells indicated higher infiltration of leukocytes and inflammation in mice kidney. Whilst, the reduced fluorescent quantification of HO-1 activity, a cytoprotective enzyme, could promote an additional enhancement in OTA-induced kidney damage. Accordingly, the reduced Nrf2-expression and pro-inflammatory cytokines production, along with HO-1 inhibition, might be considered potential mechanisms involved in OTA-nephrotoxicity.

*3.2.1.2.2. Neurotoxicity*

As shown in Table 5, fluorescent quantification of astrocytes (GFAP), microglia (Iba) and immature neurons (βIII-Tubulin, Dcx, calretinin) typical markers, revealed both the lack of neuronal differentiation and the reduced presence of astrocytes, microglia and young neurons in the subventricular zone of mice brain. In view of this, OTA-exposure during 6 weeks could affect brain development and neurogenesis, by inducing its neurodegeneration in *in vivo* models (Paradells et al., 2015).

In both in vivo studies, a similar dose administration, intraperitoneally, was evidenced.

**Table 5.** OTA-IF studies: *In vitro* and *in vivo* model, dose and exposure time, antibodies implemented, mechanism and references.

| *In vitro/in vivo* model | Dose administration | Exposure time | Antibodies used | Mechanism | References |
|---|---|---|---|---|---|
| ***In vitro*** | | | | | |
| ***Immunotoxicity*** | | | | | |
| Chicken heterophils | 2,4,8 μg /ml | 2 hours | Anti-NE, anti-histone H3 | Disruption of Ets formation and intracellular ROS generation. | (Han et al., 2019) |
| ***Hepatotoxicity*** | | | | | |
| hPXR-HRE cells | 0.40 μg/ml | 24 hours | Anti-FLAG | Cytotoxicity. | (Shen et al., 2020) |
| ***Gastrointestinal toxicity*** | | | | | |
| IPEC-J2 cells | 0.80 μg /ml | 12 hours | Anti-ERK1/2 | Induction of autophagy, apoptosis and ROS generation. Disruption of intestinal barrier. | (Wang et al., 2018) |
| GES-1 cells | 4 μg /ml | 24 hours | Anti- LC3B, anti α-tubulin | Mitochondrial dysfunction with autophagy, cell death and oxidative stress promotion. | (Li et al., 2019) |

| | | | | | |
|---|---|---|---|---|---|
| IPEC-J2 | 1.6 μg /ml | 24 hours | Anti-p65, anti-claudin-3, anti-claudin-4 | Intestinal barrier dysfunction and inflammatory response. | (Ying et al., 2019) |
| CMT93-II cells | 16 μg /ml | 36 hours | Anti-claudin-2, anti-claudin-4, anti-claudin-6 | Disruption of intestinal barrier. | (Nakayama et al., 2018) |
| Caco-2 cells | $8x10^{-2}$, 8 μg/ml | 48 hours | Anti-claudin-3, anti-claudin-4, anti-occludin, anti-ZO1 | Disruption of epithelial barrier permeability. | (Gao et al., 2017) |
| GES-1 cells | 1 μg /ml | 72 hours for (40 weeks) | Anti-β-catenin | Promotion of cell malignant transformations via intracellular ROS generation. | (Jia et al., 2016) |
| ***Nephrotoxicity*** | | | | | |
| NRK-52E cells | 20 μg /ml + LUT (28.6 μg /ml) | 24 hours | Anti-Nrf2, anti-HIF- 1α | Promotion of oxidative stress, lipid peroxidation and mitochondrial dysfunction. | (Liu et al., 2020) |
| HKC cells | 2,4,8,16 μg /ml | 24 hours | Anti-DNMT1 | Cell cycle alteration and epigenetic modifications. | (Zhang et al., 2020) |

| | | | | | |
|---|---|---|---|---|---|
| HKC cells | 4 μg /ml | 24 hours | Anti-Grp75, Anti-α SMA, anti 8-hydroxyguanosine | DNA damage, oxidative stress and disruption of mitochondrial metabolism. | (Yang et al., 2019) |
| MDCK cells | 0.5, 1.0, 2.0 μg/ml | 24 hours | Anti-NLRP3 | Promotion of cell death, inflammation and kidney fibrosis. | (Li et al., 2021) |
| HMC cells | 0.80, 1.60, 3.20 μg /ml | 48 hours | Anti-vimentin, anti-p65 | Induction of cell morphologic changes, fibrosis, DNA damage and ROS production. | (Le et al., 2020) |
| PK-15 cells | $4x10^{-2}$ μg /ml + NAC (815 μg/ml) | 48 hours | Anti-PCV2 | Enhancement of autophagy and porcine circovirus type 2 replication. | (Qian et al., 2017) |
| PK-15 cells | 0.01, 0.05, 0.1, 0.5,1 μg/ml + NAC (652 μg/ml) | 48 hours | Anti-PCV2 | Promotion of porcine circovirus type 2 replication and oxidative stress. | (Gan et al., 2015a) |
| PK-15 cells | $4x10^{-2}$ μg/ml + SeMet (0.156, 0.312, 0.468 μg/ml) | 48 hours | Anti-PCV2 | Induction of autophagy of porcine circovirus type 2 replication. | (Qian et al., 2018) |

| | | | | | |
|---|---|---|---|---|---|
| PK-15 cells | $4x10^{-2}$ μg /ml + TAU (1250, 2500, 5000 μg/ml) | 60 hours | Anti-PCV2 | Promotion of autophagy and porcine circovirus type 2 replication. | (Zhai et al., 2018) |
| ***Neurotoxicity*** | | | | | |
| Neural stem cells | $1x10^{-2}$, 0.1, 1, 10, 100 μg/ml | 5 days | Anti-GFAP, anti-DCX | Reduction of cell proliferation and differentiation rates with a significant decrease in neuroblasts and glial cells. | (Paradells et al., 2015) |
| Neural stem cells | $2x10^{-2}$, $8x10^{-2}$, 0.40, 2 μg /ml | 7 days | Anti-MAP2 clone M13, Anti-MAP2 clone AP18, Anti-Doublecortin, Anti-GFAP, Anti-Nestin, Anti-A2B5, Anti-mGalc | Impairment of neural differentiation . | (Gill & Kumara, 2019) |
| ***Embryotoxicity*** | | | | | |
| Mouse oocytes | 3 μg /ml | 16 hours | Anti-Gpx, anti-α-tubulin, anti- | Induction of oxidative stress, cell apoptosis, epigenetic | (Jia et al., 2020) |

| | | | | | |
|---|---|---|---|---|---|
| | | | H3K9ac, anti-H3K9me3 | modifications and meiotic failure. | |
| Porcine ovarian granulosa cells | 8,16 μg /ml | 24 hours | Anti-BAX, anti-BCL-2, Anti-ITGB1, anti-F2R | High ROS levels and apoptosis. | (Zhang et al., 2019) |
| Porcine ovarian granulosa cells | 8,16 μg /ml | 24 hours | Anti-ATR, anti-γ-H2AX, anti-Rad51, anti-Brca1 | DNA damage and alterations in cell proliferation. | (Zhang et al., 2020) |
| Porcine oocytes | 3.20 μg /ml + Melatonin ($2.30x10^{-4}$, $2.30x10^{-2}$, 2.30, 230 μg /ml ) | 27-52 hours | Anti-α-tubulin, anti-LC3A | Mitochondrial impairment, promotion of oxidative stress, early apoptosis and autophagy. | (Lan et al., 2020) |
| ***Reproductive toxicity*** | | | | | |
| TM3-TM4 cells | 0.80, 2 μg/ml | 24 hours | Anti-PCNA | Suppression of Sertoli and Leydig cell proliferation. Failure of spermatogenesis. | (Park et al., 2020) |
| Sperm cells | 4, 40 μg/ml | 24 hours | Anti-PTEN, Anti-PI3K, Anti-AKT, Anti-p-AKT | ROS production and cell death with a reduction of sperm motility. | (Zhang et al., 2018) |

| | | | | | |
|---|---|---|---|---|---|
| ***In vivo*** | | | | | |
| ***Nephrotoxicity*** | | | | | |
| Mice kidney | 2.50 mg/kg/ intraperitoneally | 3 weeks | Anti-vimentin, anti-collagen I, anti-p53, anti-caspase 3, anti-CD45, anti-HO-1 | Promotion of inflammation, apoptosis, oxidative stress. | (Loboda et al., 2017) |
| ***Neurotoxicity*** | | | | | |
| Mice brain | 3.50 mg/kg/ intraperitoneally | 6 weeks | Anti-GFAP, anti-Iba1, anti-beta III Tubulin, anti-Dcx, anti-calretinin. | Impairment of neurogenesis and brain development. | (Paradells et al., 2015) |

*3.2.2. Flow cytometry*

*3.2.2.1. In vitro toxicity*

In vitro OTA FCM-studies were performed using exposure times between 30 min and 40 weeks and doses of $5 \times 10^{-4}$ – 40 μg/ml. As shown in Fig. 10, the most used cell line was HEK293T. Table 6 points out that various compounds (LUT, SeMet, NAC, TAU, RSV, Zn, CAG, GA, L-Arg, Sil and APS) were implemented to alleviate OTA-toxicity.

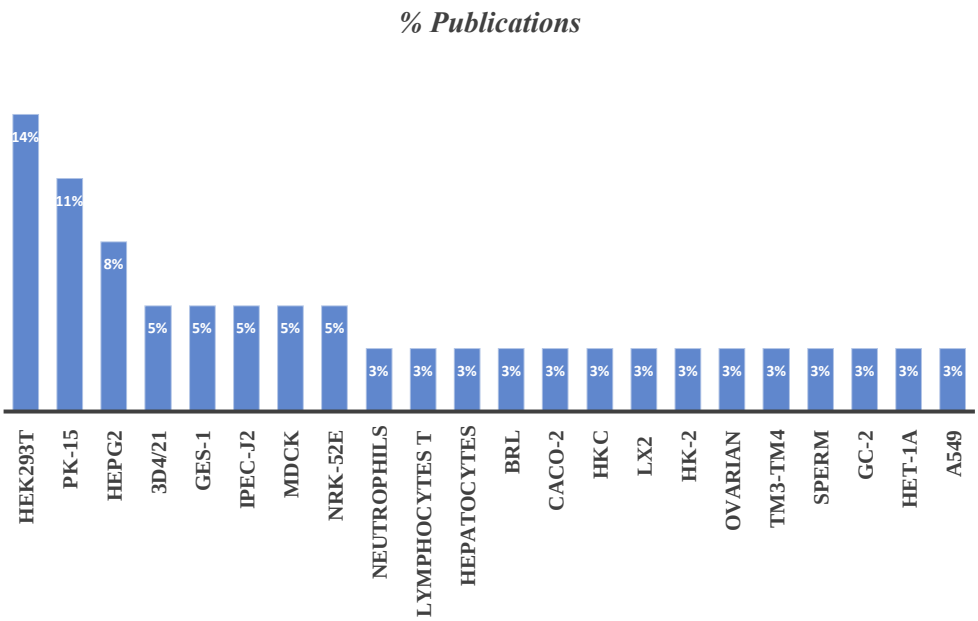



**Fig.10.** Percentage of publications (n=36) according to the cell lines used in OTA-FCM *in vitro* studies.

*3.2.2.1.1. Immunotoxicity*

In a time-dependent manner, OTA promoted apoptosis by mitochondria dependent and independent pathways. FCM analysis demonstrated the impairment of cell viability, high percentage of apoptotic necrotic cells (2–20% higher than control cells) and caspase-3 activation. Marked changes in cell morphology and DNA fragmentation were also observed. In addition, OTA triggered the significant modulation of IL-2 and enhanced the autocrine pro-apoptotic effect of TNF-$\alpha$ (Darif et al., 2016).

In human neutrophils, OTA mediated the release of calcium from internal stores, causing oxidative burst, ATP depletion and MMP disturbance. As mitochondria play a key role in cell vitality, their disruption led to apoptosis in a dose and time depended manner. At the same time, the biodegradation of OTA into its metabolite, OTA-$\alpha$, by gastrointestinal microorganisms, could be a promising strategy to alleviate OTA-immunotoxicity (Kupski et al., 2016). In alveolar macrophages, OTA-administration promoted both nuclear condensation and apoptotic bodies formation with alterations in caspase 3/9 expression and cytokines production. In contrast, high doses of APS could attenuate the immune stress induced by OTA via AMPK/SIRT-1 signaling pathway (Liu et al., 2018). In the same in vitro model, 48 h exposure to toxin significantly caused cell death and intracellular ROS generation in a dose-dependent manner. Also, OTA may enhance the phosphorylation of ERK1/2, p38, and NF-κB p65

pathways, thereby increasing the production of pro-inflammatory cytokines. However, high doses of NAC reversed OTA-induced apoptosis, oxidative stress and inflammation (Xu et al., 2017).

Regarding long lasting exposure, the association between OTA and autoimmune diseases such as rheumatoid arthritis (RA) was analyzed. The release of pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α, in macrophages was markedly observed after low levels of OTA-exposure. In addition, this mycotoxin increased the susceptibility to develop RA via Th1/Th17 cell differentiation and STAT signaling pathway activation (Jahreis et al., 2017).

*3.2.2.1.2. Hepatotoxicity*

In HepG2 cells, exposure to different concentrations of OTA reduced both protein synthesis and intracellular ATP content in a dose-dependent manner. Moreover, it induced cell death with a marked disorganization of intracellular structures, especially in actin filaments of cytoskeleton (Sali et al., 2016).

As shown in Table 6, in chicken hepatocytes, the promotion of oxidative stress, lipid peroxidation with suppressed SOD and GSH activities were detected upon 24 h OTA-contamination. It also increased cell death and caspase-3 activity, suggesting the association between apoptosis and

mitochondrial dysfunctions. The pre-treatment with CAG, L-Arg, Sil and GA could protect the liver by alleviating OTA-induced oxidative stress and apoptosis (Yu et al., 2018).

Zhu et al. (2016) suggested that cell death is the primary MOA in OTA-induced hepatotoxicity while miR-122 is the primary effector of apoptosis via CCNG1/p53 and Bcl/caspase-3 pathways activation. As confirmation of this hypothesis, the treatment with has-miR-122 inhibitor significatively reversed OTA-toxic effects. In rat liver cells, FCM analysis demonstrated the positive association between cell death and OTA-exposure. In this case, the expression of typical apoptotic proteins was significantly altered (Wang et al., 2020). In HepG2 cells, Juan-García et al. (2019) observed by FCM analysis the G0/G1 cell cycle arrest with DNA damage upon 48 h OTA-contamination.

*3.2.2.1.3. Gastrointestinal toxicity*

In IPEC-J2 cells, OTA-exposure caused typical apoptotic changes in the nucleus and promoted cell death in a time-dependent manner. Also, it triggered mitochondrial dysfunctions with ROS production, cyt-c release and caspase-3 activation. However, ERK1/2 pathway activation alleviated OTA-damage, by protecting the integrity of intestinal barrier (Wang et al., 2018). Similarly, in the same cell line, mitochondrial ROS generation, cyt-c release and caspase-3 activation were observed upon 12 h toxin-exposure.

Nevertheless, Mito-TEMPO, a mitochondria-targeted ROS-scavenger, played a protective role against oxidative stress and mitochondrial dysfunctions (Wang et al., 2017).

Li et al. (2019), in GES-1 cells, analyzed the involvement of mitochondria in OTA-MOA. Authors detected the promotion of oxidative stress, apoptosis and mitochondrial disturbances with MMP loss and ATP depletion. At the same time, OTA enhanced AMPK activation and up-regulated PGC-1$\alpha$, NRF-1 and TFAM factors, by stimulating the biogenesis of new mitochondria, which might be a cellular response to damaging agents.

Interestingly, Yang et al. (2019) suggested that the impairment of small intestine, the main site of OTA-absorption, might be partly attributed to apoptosis, which was associated in turn with the loop interaction between MDM2 and p53 signaling pathway. OTA suppressed MDM2 expression and led to cell death through CASP3 activation and its downstream pattern.

In gastric cells, chronic exposure to OTA (3 days of toxin-exposure each week during 40 weeks) caused malignant transformations via Wnt/β-catenin pathway activation. FCM analysis confirmed the increased rate of cells in the proliferative stage (phases G2/M and S) and the intracellular ROS generation. High concentration of NAC could mitigate the long-term OTA-toxicity confirming its excellent antioxidant capacity (Jia et al., 2016).

*3.2.2.1.4. Nephrotoxicity*

FCM-analysis conducted by Agarwal et al. (2020) revealed that OTA triggered intracellular ROS generation and cell cycle arrest at phase S and G0 in a time linked spiral response. In addition, the promotion of cell death and mitochondrial impairment with MMP loss was significantly observed. In contrast, NAT largely ameliorated OTA-mediated oxidative stress, cell cycle dysregulation, MMP destabilization and cell death. It has been demonstrated NAT-capacity to compete with OTA binding-pocket on phenylalanyl t-RNA synthetase, by preventing eventual alterations in protein synthesis.

In NRK-52 E cells, exposure to toxin increased intracellular ROS production, lipid peroxidation with a reduced activity of antioxidant enzymes (SOD and GSH). Pre-treatment with LUT effectively neutralized ROS production and oxidative stress owing to its ROS-scavenging capacities (Liu et al., 2020).

Furthermore, OTA-nephrotoxicity might be promoted by apoptosis signal-regulating kinase 1 (ASK1) activation. ASK-1, a mitogen-activated protein kinase kinase kinase (MAPKKK, MAP3K), is activated by OTA and promoted ROS generation, apoptosis and mitochondrial dysfunctions. Nevertheless, these toxicological effects could be reversed by ASK-1

knockdown, indicating its clear involvement in OTA-induced kidney injury (Liang et al., 2015).

As shown in Table 6, OTA may induce a global alteration of DNA methylation, leading to a hypermethylation of essential genes associated with G1/S phase transition, G1/DNA damage checkpoints signal transduction and anaphase-promoting complex/cyclosome protein. Consequently, OTA-triggered G0/G1 cell cycle arrest was observed (Zhang et al., 2020).

Exposure to OTA, alone or in combination with ZEA, promoted cell death by altering Bax, Bcl-2, and caspase-3 expression. However, a novel mode to suppress toxins activity has been proposed. A recombinant fusion enzyme (ZHDCP), by combining two single genes, zearalenone hydrolase and carboxypeptidase, suppressed OTA and ZEA-cytotoxicity, degrading them with 100% efficiency (Azam et al., 2019).

In the 24 h study, OTA impaired the expression of organic cation transport 1, 2, 3 (OCT1, OCT2 and OCT3) and induced cell death via mitochondrial pathway. Additionally, ROS generation and DNA damage were detected. However, OCT-2 knockout and TEA-treatment, a specific OCT-2 inhibitor, significantly improved OTA-mediated cell death, oxidative stress and DNA damage in vitro (Qi et al., 2018). Another toxicological mechanism whereby OTA induced kidney toxicity was the induction of

pyroptosis, a novel mechanism of programmed cell death. Indeed, OTA promoted the activation of NLRP3 inflammasome complex, which in turn activated caspase 1. This enzyme cleaved the precursors of inflammatory cytokines, IL-18 and IL-1β, by inducing the inflammatory response in neighboring cells (Li et al., 2021).

In MDCK cells, OTA induced apoptosis and oxidative stress by altering the expression of metallothionein-1 (MT-1) and metallothionein-2 (MT-2). Nevertheless, MTs, a group of small cysteine-rich proteins known for its strong free scavenging and antioxidant capacity, were up-regulated by zinc supplementation, thereby reversing intracellular ROS production and cell death (Li et al., 2019). Similarly, RSV confirmed its potential anti-oxidant and chemo-preventive proprieties by suppressing oxidative stress and DNA damage induced by OTA-administration (Raghubeer et al., 2015).

Gong et al. (2019) showed the involvement of microRNAs (miRNAs) in OTA-promoted nephrotoxicity. Indeed, has-miR-1-3p and has-miR-122–5p played an important role in the induction of apoptosis and cell cycle arrest by regulating the expression of their target genes, programmed cell death 10 and cyclin G1, respectively.

In HK-2 cell line, the cell cycle arrest was related to cyclin-dependent kinase 2 (CDK2). This kinase was downregulated by OTA exposure while its overexpression partially blocked OTA-mediated G1 cell cycle arrest. In view

of this, CDK2 can be considered one of the key regulators of G1 cell cycle arrest promoted by low concentrations of OTA (Dubourg et al., 2020).

In relation to PK15 cell line, 48 h exposure to OTA significantly promoted cell death and oxidative stress with a reduction of GSH content (20% less than control cells) and an increase ROS and oxidants production. NAC-treatment significantly abrogated ROS production and oxidative stress by improving cell antioxidant defenses (Gan et al., 2015a; Qian et al., 2017). Furthermore, Liu et al. (2020) associated the high apoptotic rate with the altered expression of Bax, caspase-3 and Bcl-2. In this study, taurine-supplementation alleviated cell death by restoring the expression of apoptosis-related proteins to the control level. In a similar way, SeMet could alleviate OTA-induced kidney damage, apoptosis and oxidative stress by improving selenoenzyme expression and by restoring Gpx activity (Gan et al., 2015b).

*3.2.2.1.5. Embryotoxicity*

As reported in Table 6, only two studies were reviewed. Zhang et al. (2019) observed that OTA-exposure compromised ovarian GCs viability in a concentration-dependent manner, by inducing late apoptosis/necrosis with oxidative stress and ROS generation. In addition, a positive correlation between cell death and PI3K/AKT signaling pathway was observed. By reducing the ratio of p-AKT/AKT, OTA led to a dysregulation of apoptosis-

related genes, Bax and caspase 9, and consequently a high percentage of apoptotic cells.

Zhang et al. (2020) observed the clear arrest of cell cycle at G2/M stage with the reduction of DNA content in a dose-dependent manner. These findings were closely associated with DNA damage and dysregulation of DNA-repair processes promoted by OTA-administration.

*3.2.2.1.6. Reproductive toxicity*

As mentioned above, Park et al. (2020) revealed that OTA was capable to promote a significant decrease in the proliferative rate of TM3 and TM4 cell lines. Also, toxin reduced the concentration of calcium ions in the cytosol and mitochondria, by disrupting both cellular homeostasis and physiological functions. FCM analysis showed a high percentage of cells in the sub-G1 stage, indicating a cell cycle arrest at phase G0/G1. The antiproliferative effects of OTA were related to PI3K and MAPK signaling pathways.

As shown in Table 6, high doses of OTA significantly decreased sperm motility in vitro via PTEN/AMPK pathway activation. As expected, OTA increased intracellular ROS generation and triggered cell death by altering p53 and Bax expression. Nevertheless, neither the vitality nor mitochondrial functions were altered by OTA-exposure (Zhang et al., 2018).

Finally, the role of miRNAs in OTA-reproductive toxicity was examined. In germ cells, it has been demonstrated that several pathways involved in cell proliferation and differentiation were dysregulated. Moreover, the high percentage of apoptotic cells, associated with p53 and caspase-3 pathways, was partly mediated by miR-122 activation (Chen et al., 2015).

*3.2.2.1.7. Other toxicity*

Finally, OTA-detrimental effects have been also studied in esophageal and lung cells. In a 24 h study, exposure to toxin caused DNA damage and chromosome aberrations in Het-1A cells. DNA damage was followed by G2 cell cycle arrest, with the down-regulation of Cdc2 and cyclin B1 expression. In this study, the cell death was related to mitochondrial dysfunctions (Liu et al., 2015). Whereas, in A549 cells, it has been confirmed that apoptosis is one of the main OTA-MOA to induce lung toxicity (Csepregi et al., 2018).

To sum up, 24 h and apoptosis assay were the most common exposure time and assay used. The promotion of apoptosis and ROS generation were the main toxicological effects obtained upon OTA-administration.

*3.2.2.2. In vivo toxicity*

As regards in vivo toxicity, only one study was reported. It was performed during 26 weeks on mice with concentrations ranged between 0.5 and 40 mg/kg administered in contaminated diet. No natural compounds were tested to counteract OTA-effects (Table 6).

*3.2.2.2.1. Nephrotoxicity*

In kidney, the transcriptional regulator p53 triggers DNA damage repair processes and reverses cell cycle arrest due to the activation of cell-cycle checkpoints. Chronic exposure to OTA in p53 heterozygous (p53+/−) and homozygous (p53+/+) mice, provoked kidney injury with alterations in cell proliferation, apoptosis and tubular degeneration in a dose-dependent manner (Bondy et al., 2015).

**Table 6.** OTA-FCM studies: *In vitro and in vivo* model, dose and exposure time, FCM assay, mechanism and references.

| *In vitro/ In vivo* model | Dose administration | Exposure time | FCM assay | Mechanism | References |
|---|---|---|---|---|---|
| ***In vitro*** | | | | | |
| ***Immunotoxicity*** | | | | | |
| H9 T cells | 0.40, 2, 4, 8 μg/ml | 3, 6, 12, 18, 24 hours | Cell viability<br>Apoptosis assay<br>Detection of caspase-3 | Marked changes in cell morphology and DNA fragmentation with high apoptotic rate. | (Darif et al., 2016) |
| Human neutrophils | 0-120 μg/ml | 4-20 hours | Apoptosis assay<br>Mitochondrial membrane potential measurement<br>Intracellular $Ca^{2+}$ measurement | Promotion of neutrophils oxidative burst, apoptosis, ATP depletion and MMP disruption. | (Kupski et al., 2016) |
| 3D4/21 cells | 1.5 μg/ml + APS (10, 20, 30 μg/ml) | 48 hours | Apoptosis assays | Induction of pro-inflammatory cytokines expression and cell death. | (Liu et al., 2018) |

| | | | | | |
|---|---|---|---|---|---|
| 3D4/21 cells | 0.1, 0.5, 1, 1.5, 2 μg/ml + NAC (652 μg/ml) | 48 hours | Apoptosis assay Intracellular ROS measurement | Enhancement of ROS generation, apoptosis and inflammation. | (Xu et al., 2017) |
| Lymphocytes T | $1x10^{-3}$– $5x10^{-2}$ μg/ml | 72 hours | Analysis Th1/Th17 cell differentiation | Promotion of a pro-inflammatory status and RA development. | (Jaheis et al., 2017) |
| ***Hepatotoxicity*** | | | | | |
| HepG2 cells | 2, 5, 8, 20 μg/ml | 24 hours | Apoptosis assays | Promotion of apoptosis, ATP depletion and intracellular structure disruption. | (Sali et al., 2016) |
| Chicken hepatocytes | 1 μg/ml + CAG, L-Arg, Sil, GA (1 μg/ml) | 24 hours | Apoptosis assay | Induction of ROS generation, lipid peroxidation and reduction of antioxidant enzymes activity. | (Yu et al., 2018) |
| HepG2 cells | 4,8,12 μg/ml | 24 hours | Apoptosis assay | Induction of cell death. | (Zhu et al., 2016) |
| BRL cells | 6.8, 7.6, 8.4 μg/ml | 48 hours | Apoptosis assay | Induction of cell death. | (Wang et al., 2020) |
| HepG2 cells | 1.25, 2.5, 5, 10 μg/ml | 48 hours | Cell cycle analysis | Arrest of cell cycle with DNA damage. | (Juan-Garcia et al., 2019) |

| | | | | | |
|---|---|---|---|---|---|
| ***Gastrointestinal toxicity*** | | | | | |
| IPEC-J2 cells | 0.80 μg/ml | 6,12,24 hours | Apoptosis assay | Induction of autophagy, apoptosis and ROS generation. Disruption of intestinal barrier. | (Wang et al., 2018) |
| IPECJ-J2 cells | 0.80, 1.60, 3.20 μg/ml | 12 hours | Apoptosis assay | Promotion of apoptosis and ROS generation with impairment in mitochondrial permeability. | (Wang et al., 2017) |
| GES-1 cells | 2,4,8 μg /ml | 24 hours | Apoptosis assay Intracellular ROS measurement Mitochondrial membrane potential measurement Mitochondrial mass determination | Mitochondrial dysfunction with autophagy, cell death and oxidative stress promotion. | (Li et al., 2019) |
| Caco-2 cells | $5x10^{-4}$, $5x10^{-3}$, 4 μg/ml | 48 hours | Apoptosis assay | Impairment of intestinal function and apoptosis. | (Yang et al., 2019) |

| | | | | | |
|---|---|---|---|---|---|
| GES-1 cells | 1 μg/mL + NAC (652 μg/ml) | 72 hours for 40 weeks | Cell cycle analysis<br>Intracellular ROS measurement | Promotion of cell malignant transformation via intracellular ROS generation. | (Jia et al., 2016) |
| ***Nephrotoxicity*** | | | | | |
| HEK293T cells | 1-10 μg/ml + NAT (2.5 μg/ml) | 0.5,1, 4, 24, 48 hours | Intracellular ROS measurement<br>Apoptosis assay<br>Cell cycle analysis | Induction of mitochondrial membrane de-stabilization and oxidative stress. | (Agarwal et al., 2020) |
| NRK-52E cells | 20 μg /ml + LUT (28.6 μg /ml) | 1,3,6,12,24 hours | Intracellular ROS measurement | Promotion of oxidative stress, lipid peroxidation and mitochondrial dysfunction. | (Liu et al., 2020) |
| HEK293T cells | 8 μg/ml | 1, 24 hours | Intracellular ROS measurement | Enhancement of ROS generation, cell apoptosis and mitochondrial impairment. | (Liang et al., 2015) |
| HKC cells | 4,8,12,16 μg/ml | 24 hours | Cell cycle analysis | Cell cycle alteration and epigenetic modifications. | (Zhang et al., 2020) |
| LX2 and HEK293T cells | 20 μg/ml | 24 hours | Apoptosis assay | Induction of apoptosis. | (Azam et al., 2019) |

| | | | | | |
|---|---|---|---|---|---|
| NRK-52E cells | 8, 20 μg/ml | 24 hours | Intracellular ROS measurement<br>Apoptosis assay | Alteration in OCT1,2,3 expression with oxidative stress and DNA damage. | (Qi et al., 2018) |
| MDCK cells | 0.5, 1.0, 2.0 μg/ml | 24 hours | Determination of Caspase-1 | Promotion of cell death, inflammation and kidney fibrosis. | (Li et al., 2021) |
| MDCK cells | 1 μg/ml + Zn (6.5 μg/ml) | 24 hours | Apoptosis assay | ROS generation and apoptosis. | (Li et al., 2019) |
| HEK293T cells | 0.60, 3.76 μg/ml + RSV (5.7 μg/ml) | 24, 48 hours | Intracellular ROS measurement | Promotion of oxidative stress and ROS generation. | (Raghubeer et al., 2015) |
| HEK293T cells | 6.4 μg/ml | 48 hours | Cell cycle analysis | Promotion of cell apoptosis, ROS generation and DNA damage. | (Gong et al., 2019) |
| HK-2 cells | $4x10^{-3}$, $4x10^{-2}$ μg /ml. | 48 hours | Cell cycle analysis | Oxidative stress induction and cell cycle alteration. | (Dubourg et al., 2020) |
| PK-15 cells | 0.01, 0.05, 0.1, 0.5 μg/ml + NAC (652 μg/ml) | 48 hours | Intracellular oxidants assay<br>Intracellular ROS measurement | Promotion of porcine circovirus type 2 replication and oxidative stress. | (Gan et al., 2015a) |

| | | | | | |
|---|---|---|---|---|---|
| PK-15 cells | $4x10^{-2}$ μg /ml + NAC (815 μg/ml) | 48 hours | Intracellular ROS measurement | Enhancement of autophagy and porcine circovirus type 2 replication. | (Qian et al., 2017) |
| PK-15 cells | 0.40, 0.80, 1.60, 2.40 μg /ml + TAU (625 μg/ml) | 48 hours | Apoptosis assay | Induction of apoptosis. | (Liu et al., 2020) |
| PK15 cells | 2.5 μg/ml + SeMet ($3.9x10^{-2}$, $7.8x10^{-2}$, 0.156, 0.312 μg/ml) | 48 hours | Apoptosis assay Intracellular ROS measurement | Cell death and oxidative stress promotion. | (Gan et al., 2015b) |
| ***Embryotoxicity*** | | | | | |
| Porcine ovarian granulosa cells | 8,16 μg/ml | 24 hours | Apoptosis assay | High ROS levels and apoptosis. | (Zhang et al., 2019) |
| Porcine ovarian granulosa cells | 8,16 μg /ml | 24 hours | Cell cycle analysis | DNA damage and alterations in cell proliferation. | (Zhang et al., 2020) |
| ***Reproductive toxicity*** | | | | | |
| TM3-TM4 cells | 0.20, 0.40, 1 μg/ml | 24 hours | Cell cycle analysis Intracellular ROS measurement | Suppression of Sertoli and Leydig cell proliferation. Failure of spermatogenesis. | (Park et al., 2020) |

| | | | | | |
|---|---|---|---|---|---|
| | | | Mitochondrial $Ca^{2+}$ concentration | | |
| Sperm cells | 4,40 μg/ml | 24 hours | Apoptosis assay | ROS production and cell death with a reduction of sperm motility. | (Zhang et al., 2018) |
| GC-2 cells | 2, 4, 6, 8, 12 μg/ml | 24 hours | Apoptosis assay | Alteration in miRNAs synthesis and processing. | (Chen et al., 2015) |
| ***Other toxicity*** | | | | | |
| Het-1A cells | 1, 2, 4, 8 μg/ml | 24 hours | Apoptosis assay<br>Cell cycle analysis | Induction of DNA strand breaks and chromosome aberrations.<br>Cell cycle arrest and apoptosis. | (Liu et al., 2015) |
| A549 cells | 2, 5, 8 μg/ml | 24 hours | Apoptosis assay | Induction of apoptosis. | (Csepregi et al., 2018) |
| ***In vivo*** | | | | | |
| ***Nephrotoxicity*** | | | | | |
| Mice kidney | 0.5, 1, 2, 10, 15, 40 mg/kg/ contaminated diet | 26 weeks | Lymphocyte subset analyses | Induction of apoptosis, karyomegaly and tubular degeneration in a dose-dependent manner. | (Bondy et al., 2015) |

## 4. Discussion

Analyzing all the studies reviewed in this work, it emerges that the most used cell lines were HepG2 (10%), HEK-293T (9%), PK15 (8%), 3D4/21 (6%), Caco-2 (5%), hepatocytes and IPEC-J2 cell line (4%). A similar percentage is constituted by HepaRG, human lymphocytes, GES-1, MDCK, NRK-52E, HKC, neural stem and porcine ovarian granulosa cells (3%). The less frequent were astrocytes, microglia, porcine embryos, BME cells, heterophils, neutrophils, H9-T, GC-2, hPXR-HRE, BRL, CMT93-II, HMC, HK-2,LX2, A549, Het-1A, sperm and TM3-TM4 cells, porcine and mouse oocytes, L02, HCT-8, HCT116, RAW264.7, MDDCs cells and leukocytes (1%) (Fig. 11).

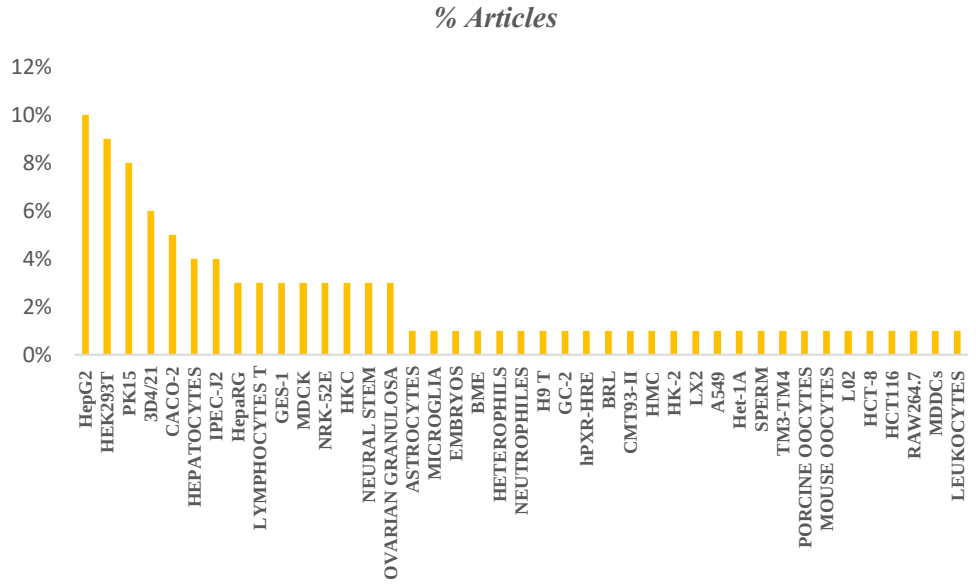



**Fig.11.** Percentage of studies according to the main cell lines employed.

Regarding the use of laboratory animals in the studies, the main specie used was broilers (42%), followed by mice (25%), chickens (25%) and rats (8%) (Fig. 12A). The main targeted organs used were thymus, BF and spleen (20%), followed by jejunum and kidney (12%). A smaller percentage included the use of brain (7%), tonsil, liver and blood/plasma analysis (3%) (Fig. 12B).

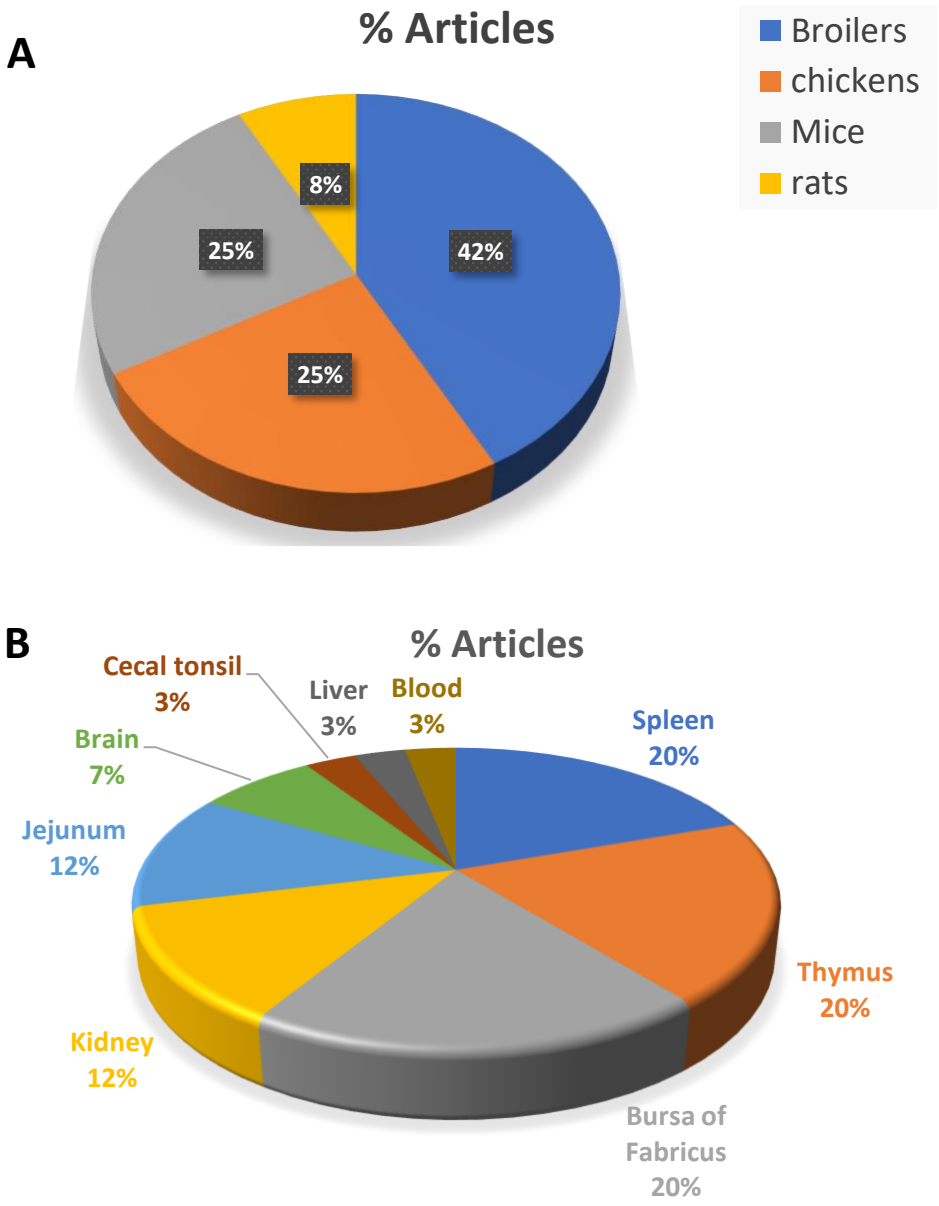


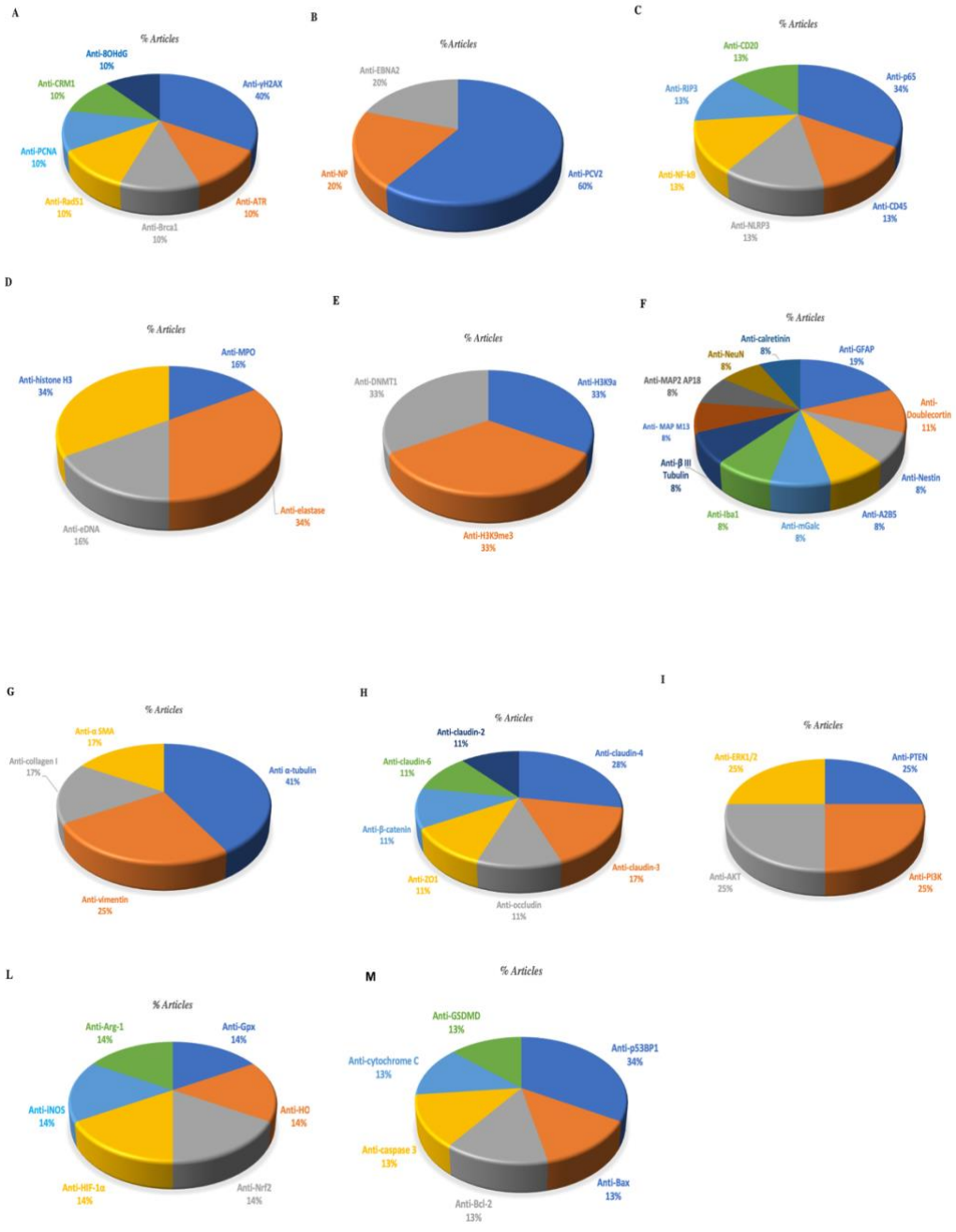

**Fig. 12 A.** Percentage of studies according to the animal species. **B.** Percentage of studies according to the organ studied.

As shown in Fig. 13, the most common purpose of the studies was immunotoxicity (29%), followed by nephrotoxicity (24%), hepatotoxicity (16%), gastrointestinal toxicity (14%). Less frequent were the studies of neurotoxicity (6%), embryotoxicity (5%), reproductive system (2%), breast, esophageal and lung toxicity (1%).


**Fig.13.** Percentage of articles (n=98) according to the main purpose of the study.

In relation to IF staining, the antibodies were selected depending on the specific route or biological processes searched by authors, in order to clarify AFB1 and OTA-MOA. Therefore, anti-γH2AX (40%), anti-8OHdG, anti-ATR, anti-CRM1, anti-PCNA, anti-Rad51 and anti-Brca1 (10%) were implemented to reveal DNA damage (Fig. 14A). To assess AFB1 and OTA ability to promote viral replication, anti-PCV2 (60%), anti-EBNA-2 and anti-NP (20%) were employed (Fig. 14B). Whilst, inflammation was detected

through anti-p65 (34%), anti-CD45 (14%), anti-CD20, anti-RIP3, anti-NF-kB and anti-NLRP3 (13%) (Fig. 14C). Moreover, anti-elastase and anti-histone H3 (34%) anti-eDNA and anti-MPO (16%) were used to identified ETs formation (Fig. 14D) while anti-DNMT1, anti-H3K9ac and anti-H3K9me3 (33%) to confirm epigenetic modifications (Fig. 14E). Several antibodies were also utilized to evaluate neural differentiation: anti-GFAP (19%), anti-Dcx (11%), anti-mGalc, anti-calretinin, anti-Iba1, anti-NeuN, anti-MAP2-AP18/M13, anti-βIII- Tubulin, anti-Nestin and anti-A2B5 (8%) (Fig. 14F). Furthermore, anti-$\alpha$ tubulin (41%), anti-vimentin (25%) anti-collagen I and anti-$\alpha$ SMA (17%) were used to assess fibrosis and cell integrity (Fig. 14G) whereas anti-claudin-4 (28%), anti-claudin-3 (17%), anti-occludin, anti-ZO1, anti-β-catenin, anti-claudin-6, anti-claudin-2 (11%) to investigate intestinal barrier integrity (Fig. 14H). Metabolic routes involved in AFB1 and OTA-MOA were detected by anti-ERK1/2, anti-AKT, anti-PTEN and anti-PI3K (25%) (Fig. 14I). Regarding the oxidative stress and hypoxia, anti-Arg1, anti-iNOS, anti-HIF-1$\alpha$, anti-NRF2, anti-HO-1 and anti-Gpx (14%) were employed (Fig. 14L). Whilst, anti-p53BP1 (34%), anti-Bax, anti-Bcl-2, anti-caspase-3, anti-cyt C and anti-GSDMD (13%) were used to reveal cell death (Fig. 14M). Moreover, Beclin-1 (33%) and LC3 (67%) were implemented to detect autophagy while Kim-1 and Grp75 (50%) to identify kidney injury. Finally, embryogenesis (anti-ITGB1), thrombosis (anti-F2R), specific epitope identification (anti-FLAG), AFB1-DNA adduct formation (anti-AFB1) and mitochondrial import process (anti-TOM20) were also investigated.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Anti-p53BP1 34%
Anti-cytochrome C 13%
Anti-caspase 3 13%
Anti-Bax 13%
Anti-Bcl-2 13%

**Fig.14.** Pie charts of antibodies used in the reviewed studies.

**A.** Pie chart of antibodies related to DNA damage in the revised papers. **B.** Pie chart of antibodies related to viral replication in the revised articles. **C**. Pie chart of antibodies related to inflammation in the revised publications. **D.** Pie chart of antibodies related to extracellular traps in the revised papers. **E.** Pie chart of antibodies related to epigenetic modifications in the revised articles. **F**. Pie chart of antibodies related to neural differentiation in the revised publications. **G.** Pie chart of antibodies related to cell integrity in the revised papers. **H.** Pie chart of antibodies related to intestinal barrier integrity in the revised articles**. I.** Pie chart of antibodies used to detect metabolic route in the revised publications. **L.** Pie chart of antibodies related.

As regard FCM technique, the most common analysis was apoptosis assay (41%), followed by cell cycle analysis (21%) and ROS measurement (15%). A smaller percentage was constituted by immune cell subset analysis and MMP measurement (4%) and DNA damage analysis (3%), T cells identification, intracellular/mitochondrial calcium determination and AFB1-DNA adduct formation (2%). The less used were TH1/TH17 analysis, Caspase 1/3 detection, intracellular oxidants assay, cell viability assay, immune markers and cytokine evaluation, FOXP3 determination, cancer cells quantification, phagocytes assay and mitochondrial mass determination (1%) (Fig.15)

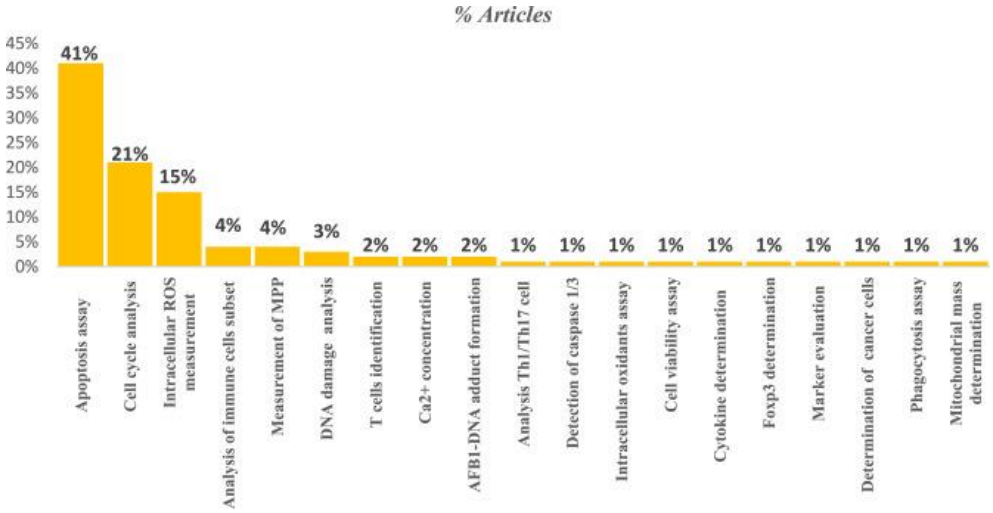


**Fig. 15.** Percentage of FCM assays employed in the reviewed studies.

With regard to AFB1, *in vitro* IF-studies showed that 24 h and anti-γH2AX were the most common exposure time and antibody used. The induction of oxidative stress with ROS generation and apoptosis were the main toxicological effects detected. For *in vivo* studies, a similar exposure time (8 weeks) was observed. As regards *in vitro* AFB1-FCM studies, 24 h and 48 h were the most common exposure times while the apoptosis assay was the main analysis performed. The promotion of apoptosis and cell cycle arrest were the main toxicological damages observed. *In vivo* studies, 3 weeks was the main exposure time while the apoptosis assay was mostly performed. The enhancement of apoptosis, cell cycle arrest and immunosuppression were mainly detected. In relation to OTA *in vitro* IF-studies evidenced that anti-PCV2 and 24 h were the most common antibody and exposure time used. The induction of oxidative stress was the main

effect related to OTA-administration. *In vivo* studies showed a similar dose administration (intraperitoneally). As regards *in vitro* OTA-FCM studies, 24 h and apoptosis assay were the most common exposure time and assay employed. As expected, the promotion of apoptosis and ROS generation were the main toxicological damages detected. Whilst, *in vivo*, only one study was screened. It has been performed during 26 weeks in mice with high doses of OTA, which were supplied in contaminated feed.

## 5. Conclusions

In the last six years, in the study of AFB1- and OTA-toxicity by IF and FCM techniques, the most used cell line was HepG2, followed by HEK-293T, PK15, 3D4/21, Caco-2, hepatocytes, IPEC-J2, HepaRG, human lymphocytes, GES-1, MDCK, NRK-52E, HKC, neural stem and porcine ovarian granulosa cells. In *in vivo* studies, the predominant laboratory animals employed were broilers followed by mice, chickens and rats while the main targeted organs used were thymus, BF, and spleen. The main purpose of the researches was immunotoxicity, followed by nephrotoxicity, hepatotoxicity, gastrointestinal toxicity, neurotoxicity, embryotoxicity, reproductive system, breast, esophageal and lung toxicity. The effect of several compounds to counteract AFB1- and OTA-toxicological damages was also investigated.

Regarding the IF analysis, different antibodies were employed to elucidate alterations in various biological process related to AFB1- and OTA-

toxicity such as: DNA, cell and intestinal integrity, inflammation, neural differentiation, oxidative stress and cell death. Whilst, in FCM analysis, several assays were performed. The predominant were the apoptosis assay, cell cycle analysis and intracellular ROS measurement.

In conclusions, the present work confirms AFB1 and OTA-toxicity and their potential harmful effects on in vitro and in vivo models. Nevertheless, the high number of in vitro studies and the scarceness of in vivo researches can be clearly discerned. In relation to OTA, only two studies were reviewed, indicating the lack of information obtained by FCM and IF in the last six years. Regarding AFB1, very scarce sources were found for neurotoxicity, nephrotoxicity and hepatotoxicity.

In view of this, more in vivo trials are needed to provide a more extensive and precise overview on AFB1- and OTA-toxicity and their MOA on human health.

**Conflicts of interest**

Authors declare no have conflicts of interest.

**CRediT authorship contribution statement**

Massimo Frangiamone: Visualization, Writing – original draft. Alessandra Cimbalo: Visualization, Writing – original draft. Manuel Alonso-Garrido: Writing – original draft, Writing – review & editing. Pilar Vila-Donat: Writing – review & editing. Lara Manyes: Funding acquisition, Project administration, 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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## *3.2.* Pumpkin extract and fermented whey individually and in combination alleviated AFB1- and OTA-induced alterations on neuronal differentiation *in vitro*

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# Pumpkin extract and fermented whey individually and in combination alleviated AFB1- and OTA-induced alterations on neuronal differentiation *in vitro*

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**Abstract**

Food and feed are daily exposed to mycotoxin contamination which effects may be counteracted by functional compounds like carotenoids and fermented whey. Among mycotoxins, the most toxic and studied are aflatoxin B1 (AFB1) and ochratoxin A (OTA), which neurotoxicity is not well reported. Therefore, SH-SY5Y human neuroblastoma cells ongoing differentiation were exposed during 7 days to digested bread extracts contained pumpkin and fermented whey, individually and in combination, along with AFB1 and OTA and their combination, in order to evaluate their presumed effects on neuronal differentiation. The immunofluorescence analysis of βIII-tubulin and dopamine markers pointed to OTA as the most damaging treatment for cell differentiation. Cell cycle analysis reported the highest significant differences for OTA-contained bread compared to the control in phase $G_0/G_1$. Lastly, RNA extraction was performed and gene expression was analyzed by qPCR. The selected genes were related to neuronal differentiation and cell cycle. The addition of functional ingredients in breads not only enhancing the expression of neuronal markers, but also induced an overall improvement of gene expression compromised by mycotoxins activity. These data confirm that *in vitro* neuronal differentiation may be impaired by AFB1 and OTA-exposure, which could be modulated by bioactive compounds naturally found in diet.

**Keywords:** Mycotoxins; Bioactive compounds; Neurotoxicity; Immunofluorescence; qPCR.

**Abbreviations**

AD: Alzheimer Disease

AFB1: Aflatoxin B1

C6: Rat glioma cell line

DNA: Deoxyribonucleic acid

EU: European Union

GAP43: Growth Associated Protein 43

HT22: Mouse Hippocampal cell line

IARC: International Agency for Research on Cancer

LAB: Lactic acid bacteria

MPP: 1-methyl-4-phenylpyridinium

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide

OTA: Ochratoxin A

P: Pumpkin

p21: cyclin-dependent kinase inhibitor 1

p53: cellular tumor antigen p53

PCR: Polymerase Chain Reaction

PD: Parkinson Disease

RNA: Ribonucleic acid

SH-SY5Y: Human Neuroblastoma cell line WF: Whey Fermented

Wnt5a: Human protein Wnt5a

## 1.Introduction

Mycotoxins, secondary metabolites synthesized under specific conditions by certain fungal species, are considered a major risk factor in food and feed to human health (Janik et al., 2020). Due to their toxicological effects, the presence of mycotoxins in foods has severe implications on human and animal health even at very low concentrations (Cimbalo et al., 2020). In particular, AFB1 and OTA are some of the most toxic and studied fungal compounds (Kutsanedzie et al., 2020).

AFB1 is a polypeptide compound produced by *Aspergillus flavus* and *parasiticus*, which occurrence includes a wide range of food commodities: cereals, oil seeds, spices, tree nuts, milk, meat and dried fruits (Umesha et al., 2017). Several studies have shown that AFB1-contamination induces mutagenicity, hepatotoxicity, immunotoxicity, nephrotoxicity and neurotoxicity (Ostry et al., 2017; Rushing & Selim, 2019). Thus, AFB1 is classified by the International Agency for Research on Cancer (IARC) in group 1 as carcinogenic to humans (IARC, 2012). In order to protect human health, it is essential to keep these food contaminants at toxicologically acceptable levels. The European Union (EU) sets the maximum tolerable limits of AFB1 at 2-5 μg/kg in cereals, 2-8 μg/kg in dried fruits, 5 μg/kg in spices, 6 μg/kg in dietary foods for medical purpose and 0.1 μg/kg in foods for infants and young children (EC Commission, 2006).

OTA, a phenylalanyl derivative of a substituted isocoumarin, is mainly produced by *Penicillium verrucosum* and by *Aspergillus ochraceus* and *niger*. Typically contaminated foods by this mycotoxin are cereals (maize, wheat, rice, sorghum, barley, oats), cereal-based products (bread, flour, and pasta), wine, tea, coffee, milk, cheese, dried fruits, spices and vegetables (Chen et al., 2018). The ubiquitous presence of OTA in food commodities has been associated with nephrotoxicity, hepatotoxicity, genotoxicity, immunotoxicity, embryotoxicity and neurotoxicity (Leitao, 2019; Frangiamone et al., 2022). In view of this, OTA has been classified by IARC in group 2B as possible carcinogen for humans (IARC, 2012). The EU sets the maximum permissible levels of OTA in unprocessed cereals at 5 μg/kg, 8 μg/kg in wheat gluten, in roasted coffee beans at 5 μg/kg and 10 μg/kg in soluble coffee, 15 μg/kg in spices, 2 μg/kg in grape juice and wine, in liquorice between 20 and 80 μg/kg, in baby foods and dietary foods for medical purpose at 0.5 μg/kg (EC Commission, 2006).

However, several bioactive compounds have been shown to mitigate mycotoxins activity (Taroncher et al., 2021). Pumpkin, which belongs to the *Cucurbitaceae* family, is rich in carotenoids that play an important role in protecting cells from oxidative stress and damage, thus preventing the incidence of several human diseases (Bergantin et al., 2018). Milk whey is a by-product of the dairy industry with high nutritional values and it represents an important source of functional compounds, such as antifungal peptides. Milk whey is also an optimum substrate for lactic acid bacteria

(LAB) fermentation (Gupta & Prakash, 2017). Therefore, pumpkin and milk whey have recently been proposed as excellent bio-preservative candidates in food production (Sharma et al., 2020; Escrivá et al., 2021). The administration of these bioactive ingredients in bread preparation improved its shelf life, reduced the growth of mycotoxigenic fungi as well as AFB1- and OTA-production (Sadeghi et al., 2019; Luz et al., 2020; Llorens et al., 2022).

In relation to human health risk assessment, the most important factors are bio-accessibility and bioavailability parameters (Trujillo-Rodríguez et al., 2020). Bio-accessibility represents the fraction of metabolite released from the food matrix in the gastrointestinal tract, whereas bioavailability is the proportion of the compound, ingested through food, that reaches the bloodstream and may exert toxic effects (Rebellato et al., 2021). In light of this, several *in vitro* models have been used to reproduce a simulated human gastrointestinal system in order to evaluate mycotoxins metabolism (Manyes et al., 2014; De Boevre et al., 2015).

Gonzalez-Arias et al. (2013) postulated that AFB1 is the mycotoxin with the highest bio-accessibility, regardless of the food matrix. In nuts and corn, bio-accessibility values ranged between 94-98% (Simla et al., 2009), 88-96% in peanut and infant foods (Kabak et al., 2009), 92-94% in spices and wheat (Kabak and Ozbey, 2012). It has been also demonstrated that AFB1 presents a bio- accessibility 3-fold higher than OTA, which should be

considered in risk assessment and toxicological studies (Kabak et al., 2009). Related to OTA, very scarce information is available. Very low bio-accessibility values, ranging between 45-86% in wheat and 22-32% in infant foods and corn, have been reported (Gonzalez-Arias et al., 2013). On the contrary, Escrivá et al. (2022) demonstrated as the addition of pumpkin extract (P) in contaminated AFB1 and OTA breads, significatively reduced bio-accessibility up to 74% and 34%, respectively. Similarly, fermented milk whey (WF), and the combination of P-WF showed intestinal bio-accessibility reductions between 57–68% for AFB1, and between 11–20% for OTA. A large reduction in bio-accessibility parameter has been also observed for both toxins after LAB-administration in contaminated digested breads (Saladino et al., 2018; Luz et al., 2018).

Regarding bioavailability, Sobral et al. (2019) observed that AFB1 and OTA passively cross the gastrointestinal barrier albeit the absorbed fraction (FA) was quite different. AFB1 showed a higher FA percentage (>96%) both in the isolated and combined transport, while OTA reported an FA of 11% when absorbed individually and 66% in combination with other fungal compounds. Once absorbed, AFB1 and OTA can reach the peripheral organs, such as the brain, by crossing the blood-brain barrier (BBB). Low doses of AFB1 (3-32 nM) were sufficient to cross the BBB and induce cytotoxic effects in microvascular endothelial cells, causing damage to brain tissue (Qureshi et al., 2015). Furthermore, AFB1 (60 nM) not only crossed the BBB but also induced neuroinflammation in microglial cells with oxidative stress and

apoptosis (Mehrzad et al. 2018). At the same concentrations, AFB1-exposure was related to BBB disruption and neurotoxicity, leading to a brain-wide decompensation with astrocytes death (Vahidi-Ferdowsi et al., 2018). Similarly, OTA (250 nM) crossed the BBB inducing cell death in a neuronal model, reproduced using SH-SY5Y cells (Zhang et al., 2009). Exposure to OTA (200 nM) also triggered BBB alterations with a reduced differentiation degree of astrocytes, neurons and oligodendrocytes, thereby disrupting the normal brain development (Gill and Kumara, 2019). Chronic low exposure to OTA enhanced negative effects on striatal dopamine metabolism, promoting neuroinflammation and brain degeneration (Sava et al., 2006a; Petrulli et al., 2017). In ECV304 cells, a typical BBB model, the protective role of pumpkin (rich in carotenoids and flavonoids) against AFB1 and OTA-induced neurotoxicity, was also assessed (Alonso-Garrido et al., 2020; Alonso-Garrido et al., 2021, Frangiamone et al., 2021).

In view of the above, the goal of this study is to evaluate the effects of AFB1 and OTA on neuronal differentiation and the presumed role of pumpkin extract and fermented whey to modulate their toxicological effects in SH-SY5Y human neuroblastoma cell line using digested bread extracts.

## 2.Material and methods

### *2.1. Reagents*

The reagent grade chemicals and cell culture compounds used were DMEM Ham's-F12, penicillin, trypsin/EDTA solutions, fetal bovine serum (FBS), phosphate buffer saline (PBS), trizma base (Tris), *t*-octylphenoxypolyethoxyethanol (Triton-X 100) and paraformaldehyde (PFA) from Sigma Chemical Co (St. Louis, MO); Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Geel, Belgium); Methanol (MeOH), sodium chloride (NaCl) and hydrochloric acid (HCl) w ere obtained from VWR International (LLC, Monroeville, PA); Deionized water (<18, MΩcm resistivity) was obtained using Milli-QSP ® Reagent Water System (Millipore, Bedford, MA, USA); Cycletest™ Plus DNA Reagent Kit for the cell cycle assay by flow cytometry was purchased from BD Biosciences (San Diego, CA); Rabbit polyclonal primary antibody anti-dopamine was obtained from Antibodies (Cambridge, UK); Mouse monoclonal primary antibody anti-βIII-Tubulin (TUBB3), the secondary antibodies, goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) and goat Anti-Mouse IgG H&L (Alexa Fluor® 555), and the nuclear dyes, Hoechst 33258 and 33342 were purchased from Invitrogen (Thermo Fisher Scientific, Carlsbad, CA); Standard mycotoxins AFB1 (MW: 312.28 g/mol) and OTA (MW: 403.81 g/mol) were obtained from Sigma-Aldrich (St. Louis, MO) and stock solutions were dissolved in methanol at 1000 ppm and maintained at -20 °C.

*2.2. Cell culture and treatments exposure*

Human neuroblastoma SH-SY5Y cells were cultured in DMEM Ham's-F12 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. The incubation conditions were pH 7.4, 5% $CO_2$ at 37 °C and 95% air atmosphere at constant humidity. The medium was changed every 2-3 days. In this work, the different experiments were performed in SH-SY5Y cells ongoing differentiation, promoted by cell exposure for 7 days to the differentiation factor All-Trans-Retinoic-Acid (ATRA) (10µM) added at 0.1% (v/v) to the differentiation medium (DMEM-F12 with 100 U/mL penicillin, 100 mg/mL streptomycin and 1% FBS). To achieve the goal of the study, cells ongoing differentiation were treated during 7 days with bread extracts obtained after a simulated *in vitro* human digestion, as described Escrivá et al. (2022). These digested bread extracts, containing P and WF (both at 1% w/w), individually and in combination, along with mycotoxins (AFB1, OTA and their combination), were added to the differentiation medium using a final dilution of 1:50. Except for the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay, the concentrations of AFB1 and OTA contained in the different digested bread extracts were normalized at 60 nM and 240 nM, respectively, in order to work with the same doses in all cases. These concentrations were achieved by adding standard mycotoxin to the digested bread extracts with a final methanol (MeOH) concentration of 0.1% (v/v) (Table 1). This solvent dose was also added in control loaves.

**Table 1.** AFB1 and OTA concentrations for each condition employed: a) control digested breads; b) WF digested breads; c) P digested breads; d) P+WF digested breads.

| | **Control digested breads** | **WF digested breads** | **P digested breads** | **P+WF digested breads** |
|---|---|---|---|---|
| **CONTROL** | 0.1% MeOH | 0.1% MeOH | 0.1% MeOH | 0.1% MeOH |
| **AFB1** | 60 nM | 60 nM | 60 nM | 60 nM |
| **OTA** | 240 nM | 240 nM | 240 nM | 240 nM |
| **AFB1+ OTA** | 60+240 nM | 60+240 nM | 60+240 nM | 60+240 nM |

### *2.3. In vitro cytotoxicity*

The MTT was used to assess differentiated SH-SY5Y viability following the exposure to contaminated bread extracts. The assay was performed according to the protocol described by Manyes et al. (2018) with some arrangements. SH-SY5Y cells were plated in 24-well microplates at a density of $5 \times 10^4$ cells/well. After 7 days, the required differentiation time, culture medium was replaced with fresh medium containing five serial dilutions (dilution factor = 5) of contaminated bread extracts (Table 2).

**Table 2.** AFB1 and OTA concentrations used in MTT assay. These doses were obtained through dilutions 1:5 (1/2 to 1/1250 in medium) from the mother extract.

| Dilution (1:5) | **Control digested breads** | **WF digested breads** | **P digested breads** | **P+WF digested breads** |
|---|---|---|---|---|
| **AFB1** | 14.75-0.02 nM | 6.50-0.01 nM | 6.05-9x$10^{-3}$ nM | 10.43-0.01 nM |
| **OTA** | 130-0.20 nM | 113.12-0.18 nM | 103.70-0.16 nM | 134.31-0.21nM |

| | | | | |
|---|---|---|---|---|
| **AFB1** | 2.85-4x10$^{-3}$ nM | 14.25-0.02 nM | 7.05-0.01 nM | 7.55-0.01 nM |
| **+** | | | | |
| **OTA** | 93.12-0.15 nM | 87.50-0.14 nM | 95.75-0.15 nM | 94.24- 0.15 nM |

After incubation for 24, 48h, and 72h, the contaminated medium was replaced with 250 µL of fresh medium containing 50 µL of MTT solution (0.5 mg/ml). After 4 h of incubation (37 °C in darkness), 400 µL of DMSO were added and cytotoxicity was determined. The absorbance was measured at 620 nm using a Wallace Victor2, model 1420 multi-label counter (Perkin Elmer Life Sciences, Waltham, MA, USA). Cell viability was expressed in percentages compared to the control (cells and medium). Four independent experiments with eight replicates were performed.

*2.4. Immunofluorescence staining*

To assess the neuronal differentiation, the fluorescent expression of two typical markers, dopamine and TUBB3 was determined. SH-SY5Y cells ($2 \times 10^4$ cells/well) were seeded in 48-well culture plates (Thermo Fisher, USA) and exposed to different treatments for 7 days. Afterwards, SH-SY5Y cells were fixed with 4% of PFA for 20 min, washed three time with 0.1 M phosphate buffer PB (prepared by mixing 0.2 M $Na_2HPO_4$ and 0.2 M $NaH_2PO_4$) and incubated in blocking solution (0.1 M PB, 0.2% Triton X-100, and 5% FBS) for 1 h at room temperature. Subsequently, cells were incubated with primary rabbit polyclonal antibody anti-Dopamine (1:500 in blocking solution) and primary mouse monoclonal antibody anti-TUBB3 (1:800

dilution in blocking solution) at 4 °C overnight. Cells were then washed three times with 0.1 M PB and incubated with Alexa Fluor® 488 affinity purified anti-rabbit IgG secondary antibody and Alexa Fluor® 555 affinity purified anti-mouse IgG secondary antibody (both at 1:800 in blocking solution) for 1 h at room temperature. Nuclei were stained with Hoechst 33258 (for Dopamine) and Hoechst 33342 (for TUBB3) (both at 1:2000 dilution in PBS) for 10 min. The images were captured and analyzed using IN Cell Analyzer 2000 (GE Healthcare, Chicago, IL). For quantification, 60 fields for well were observed at a magnification of 20×. Three independent experiments were performed and at least 300 cells for field were counted. The results were analyzed with IN Cell Analyzer 2000 Workstation software (GE, healthcare) and expressed as fluorescence intensity of cells positive for the antibody studied.

*2.5. Cell-cycle analysis by flow cytometry*

After 7 days-exposure to different treatments, SH-SY5Y cells were detached by trypsin, centrifuged at 1500 rpm for 10 min and washed thrice with cold PBS. Then, cells were pelleted and analyzed using CycleTest™ plus DNA reagent kit according to manufacturer instructions. The pellets were washed thrice with a buffer solution (DMSO in sucrose-sodium citrate), suspended in Solution A (trypsin buffer) and incubated at room temperature for 10 min. Solution B (trypsin inhibitor and RNase buffer) was added and cells were incubated at room temperature for 10 min. Then, solution C (Propidium Iodide solution) was added and cells were incubated at 4 °C for

10 min. Once filtered, samples were analyzed. Three independent experiments were performed using a BD LSRFortessa flow cytometry (BD Biosciences, Franklin Lakes, NJ) with at least 10.000 events for each sample. Results obtained were analyzed using Modfit LT 3.0 program (San Jose, CA).

*2.6. RNA extraction*

Total RNA of the control and SH-SY5Y cells exposed to different treatments was isolated using ReliaPrep™ RNA Cell Miniprep System Kit (Promega, USA) and purified to remove genomic DNA contamination. Firstly, the RNA extracted from each sample was checked for quantity and quality using a NanoDrop™ 2000 (Thermo Scientific™, Madrid, Spain), showing appropriate 260/280 nm and 260/230 nm ratios both around 2. RNA samples were stored at −20 °C until their dilution to 100 ng/μl with sterilized milli-Q $H_20$. Then, cDNA was synthesized, using TaqMan Reverse Transcription Kit (Applied Biosystems) for qPCR analysis.

*2.7. Gene selection and primer design*

Gene-specific primers were designed through Primer-BLAST by using default criterion of the software with amplified products ranging from 65 to 150 bp and Tm at 60 °C. Primer sequences were used in qPCR analyses. Standard curve by qPCR was performed for all primers 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 obtained from standard curve generated by serial dilution of cDNA (5-fold each) for each gene. Correlation coefficient ($R^2$ values) and amplification efficiencies (E) for each primer were calculated from slope of regression line by plotting mean Cq values against the log cDNA dilution factor in StepOne software. The gene-specific primers used in the present study are listed in Table 3.

**Table 3.** Gene symbol, forward and reverse primers, efficiency and linearity of the selected genes plus the reference gene GAPDH.

| Gene | Primer forward sequence | Primer reverse sequence | Efficiency (%) | Linearity |
|---|---|---|---|---|
| **Housekeeping** | | | | |
| GAPDH | GAGAAGGCTGGGGCTCATTT | AGTGATGGCATGGACTGTGG | 110 | 0.990 |
| **Neuronal development** | | | | |
| Wnt5a | AGCAGACGTTTCGGCTACAG | TGCCCCCAGTTCATTCACAC | 98 | 0.992 |
| TUBB3 | CCGAAGCCAGCAGTGTCTAA | AGGCCTGGAGCTGCAATAAG | 118 | 0.991 |
| GAP43 | AGGGAGAAGGCACCACTACT | GGAGGACGGCGAGTTATCAG | 121 | 0.983 |
| **Cell cycle** | | | | |
| Cyclin B | TCGAAAGTGTCGCATCAAACT | CACAGAAGATGTGAGAGCAGG | 97 | 0.987 |
| Cyclin D | CCCTCGGTGTCCTACTTCAA | AGGAAGCGGTCCAGGTAGTT | 94 | 0.991 |
| p21 | GATGAGTTGGGAGGAGGCAG | GAGCGAGGCACAAGGGTACA | 108 | 0.992 |
| p53 | CCACCATCCACTACAACTACAT | CACAAACACGCACCTCAAA | 109 | 0.984 |

*2.8. 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 for cell-cycle related genes were set as default: initial denaturation step of 95 °C for 5 min to activate Taq DNA polymerase, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s. For neuronal differentiation related genes, the cycling conditions were: initial denaturation step of 95 °C for 10 min to activate Taq DNA polymerase, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. The melting curve was generated by heating the amplicon from 60 to 90 °C. Threshold cycles (Ct) were determined using the StepOne Plus Software version 2.3 and data was analyzed with Quant Studio 5 software (Applied Biosystems, Foster, CA, USA). Three technical replicates were performed for each condition. Experiments were carried out according to MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Bustin et al., 2009).

*2.9. Statistical analysis*

In order to assess the statistical analysis, ΔCt (experimental Ct – housekeeping Ct mean) obtained by qPCR was used. Levene's test was applied to evaluate the equality of group variances and T-student test was used to evaluate differences between groups. For statistically significant differences, a $p \leq 0.05$ was considered.

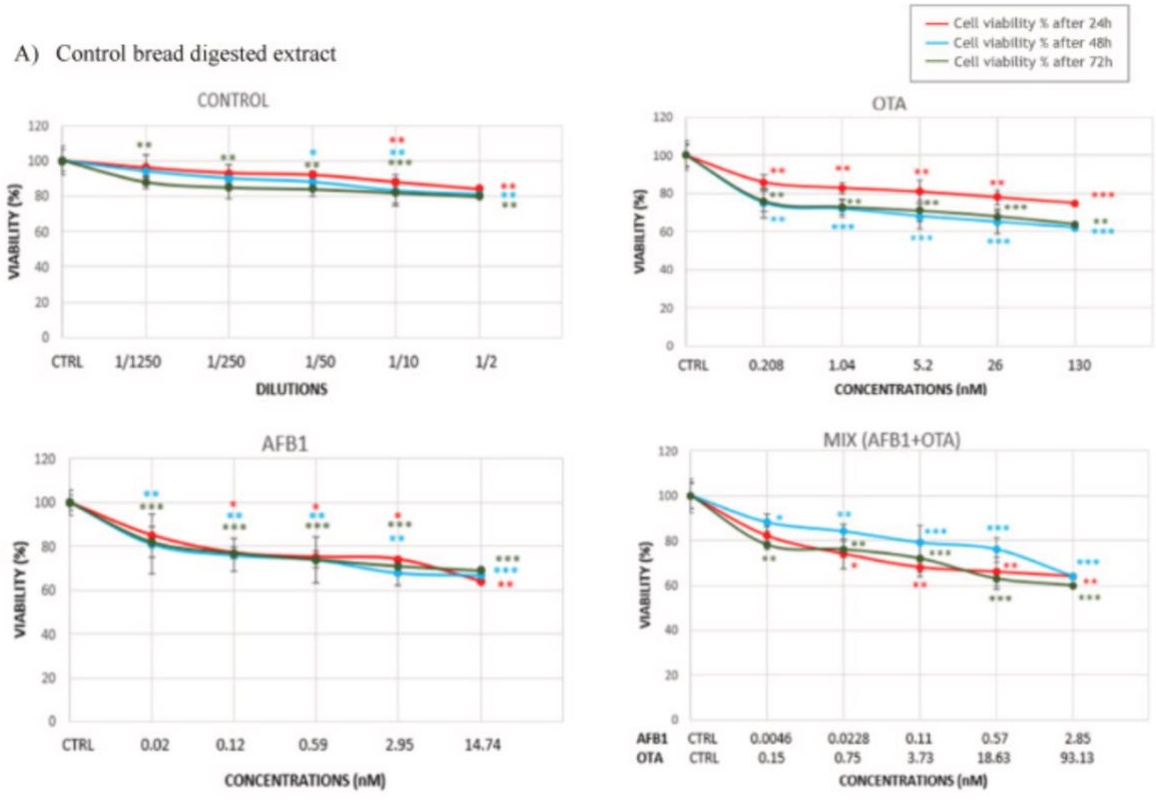
**3. Results**

*3.1. In vitro cytotoxicity*

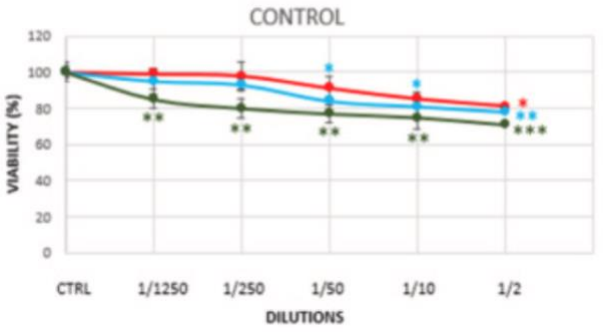
In order to evaluate the neuronal cell viability, the MTT assay was performed. In control breads, cell viability decreased in a dose dependent manner for all exposure times tested. No remarkable differences among treatments were found (Fig.1a). About WF enriched breads, the enhancement of cell viability was mainly observed after 24h of exposure in control, AFB1 and mixed conditions. Interestingly, in AFB1-exposure, cell viability statistically decreased in a time and concentration dependent manner (Fig.1b). In P enriched breads, a comparable decrease in cell viability was obtained in all conditions after 24h and 48h of exposure. Notably, the treatment up to 72h showed higher viability values compared with those obtained in shorter exposure times (Fig.1c). Lastly, the presence of P+WF increased cell viability after 72h of exposure in all tested conditions. The

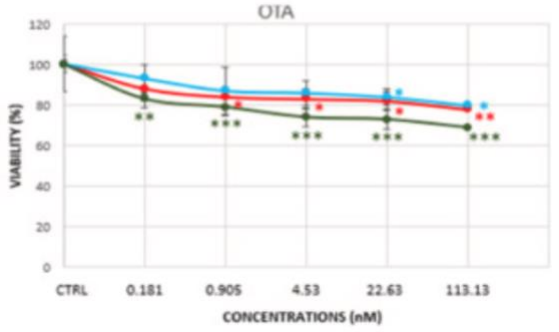
toxic damages caused by the binary combination of OTA and AFB1 were lower than the ones detected in the other bread extracts (Fig.1d). Overall, cell viability was always greater than 50%

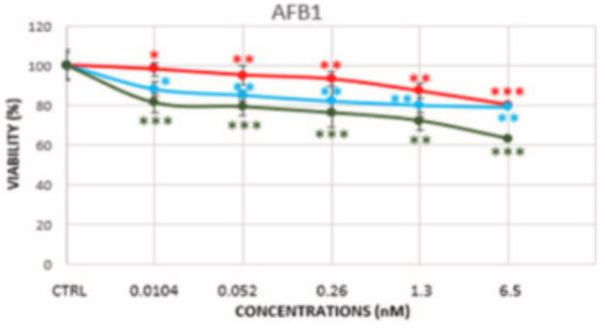
A) Control bread digested extract

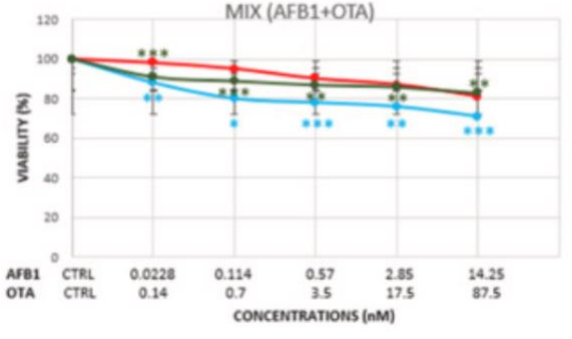


B) WF bread digested extract

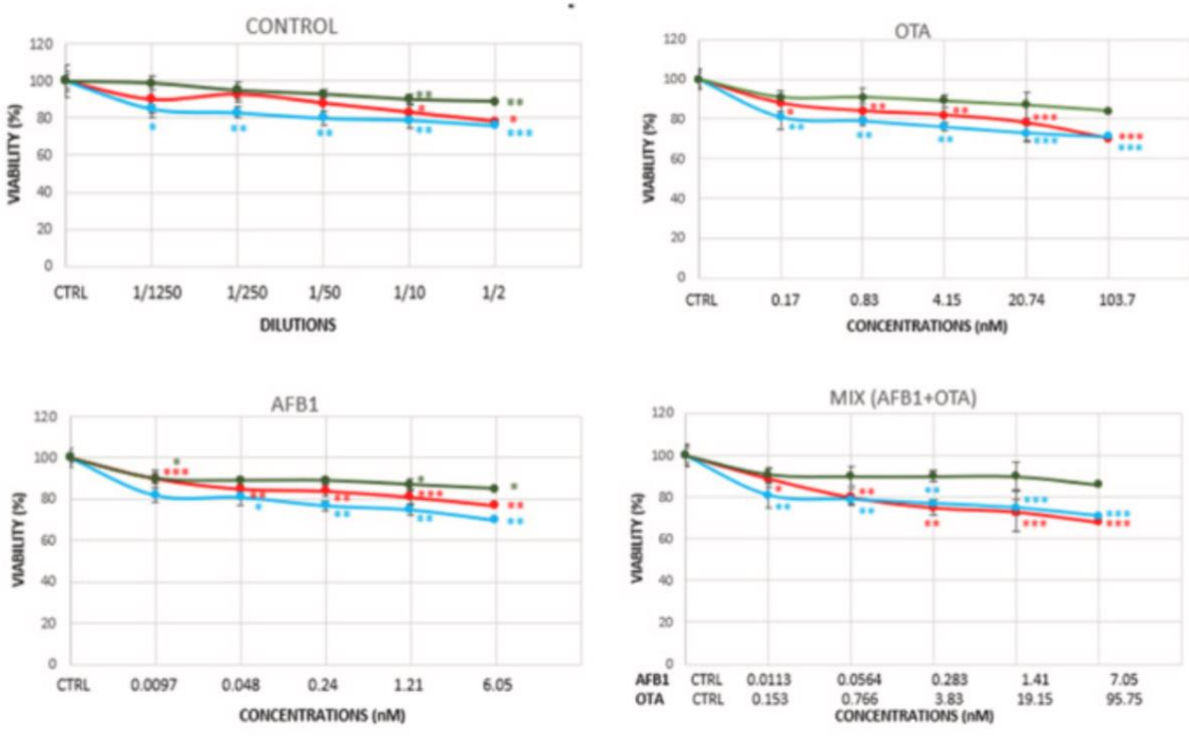


C) P bread digested extract



D) P+WF bread digested extract

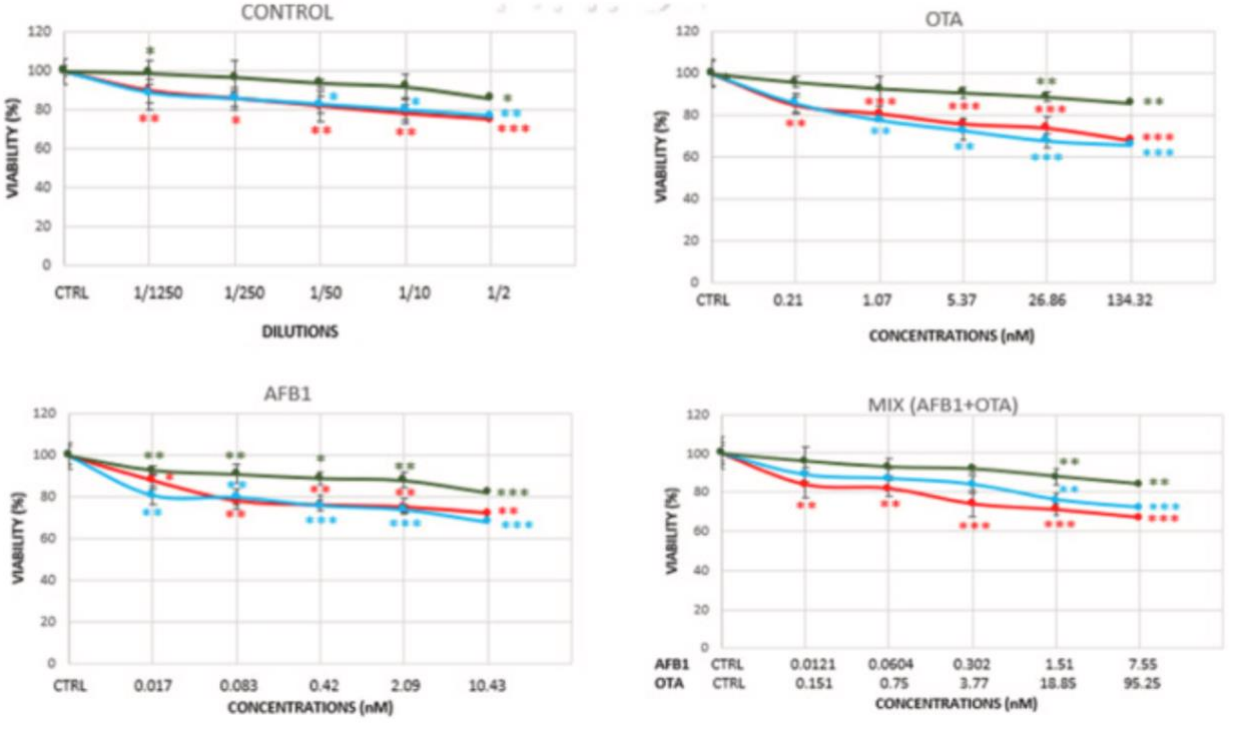
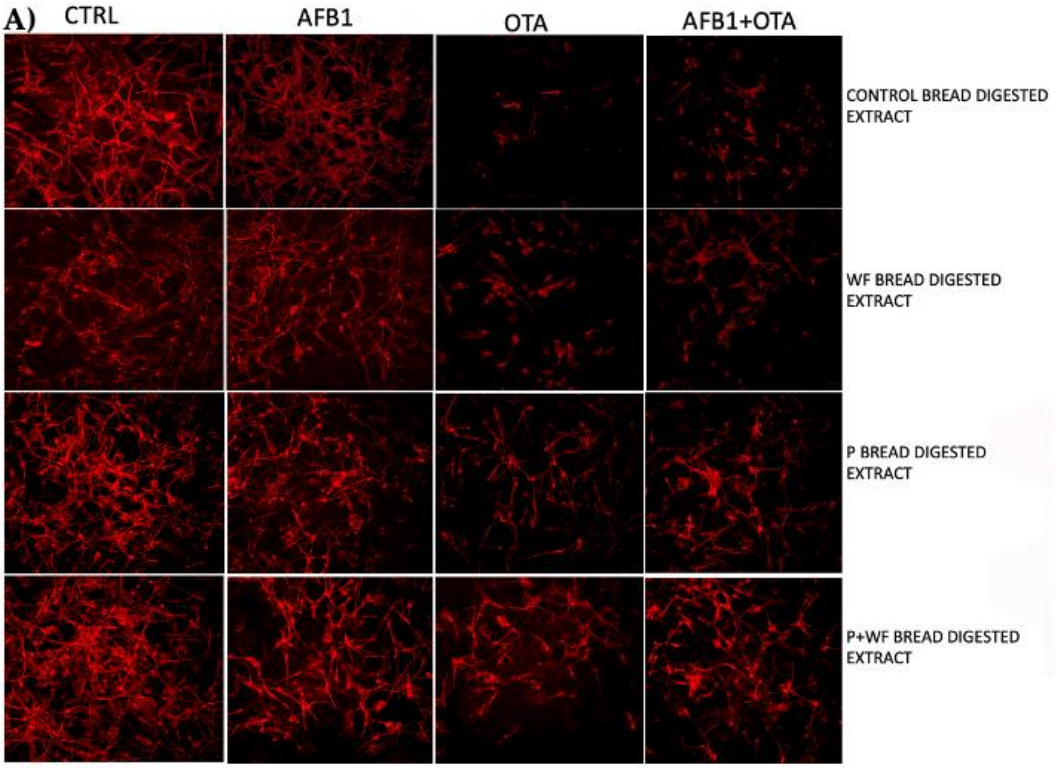


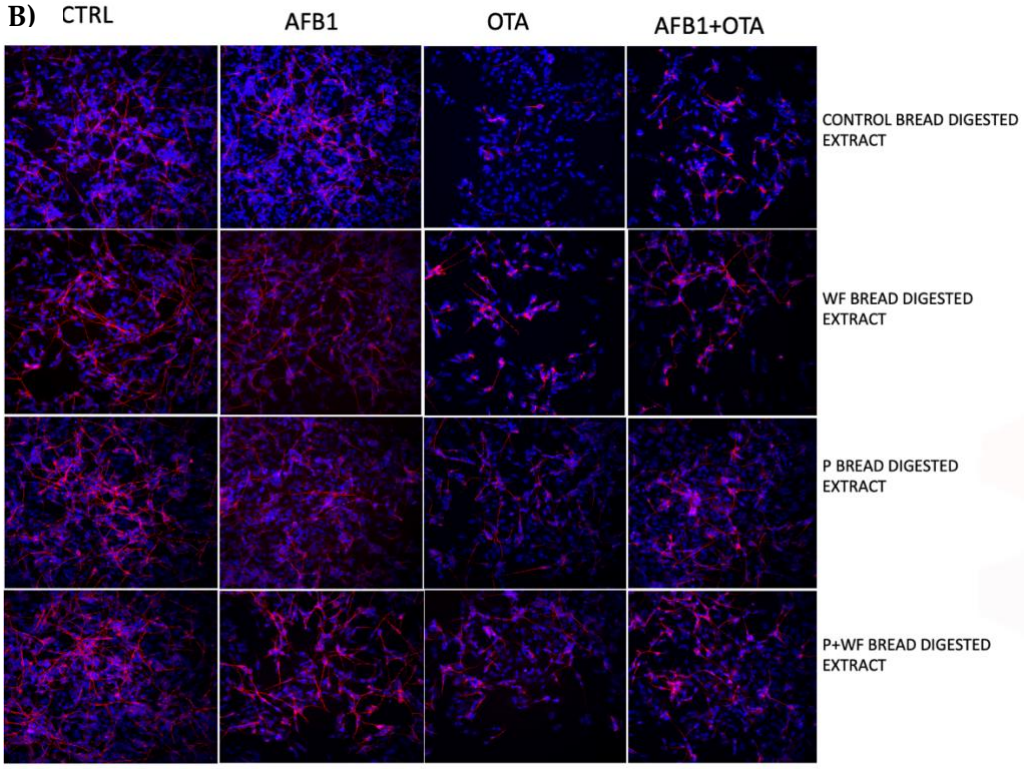
**Figure 1.** Cell viability (%) results obtained in differentiated SH-SY5Y cells after exposure to: a) control bread digested extract, b) WF bread digested extract, c) P bread digested extract, d) P+WF bread digested extract. $p \leq 0.05$ (*) $p \leq 0.01$ (**), $p \leq 0.001$ (***). P: Pumpkin; WF: Whey Fermented.

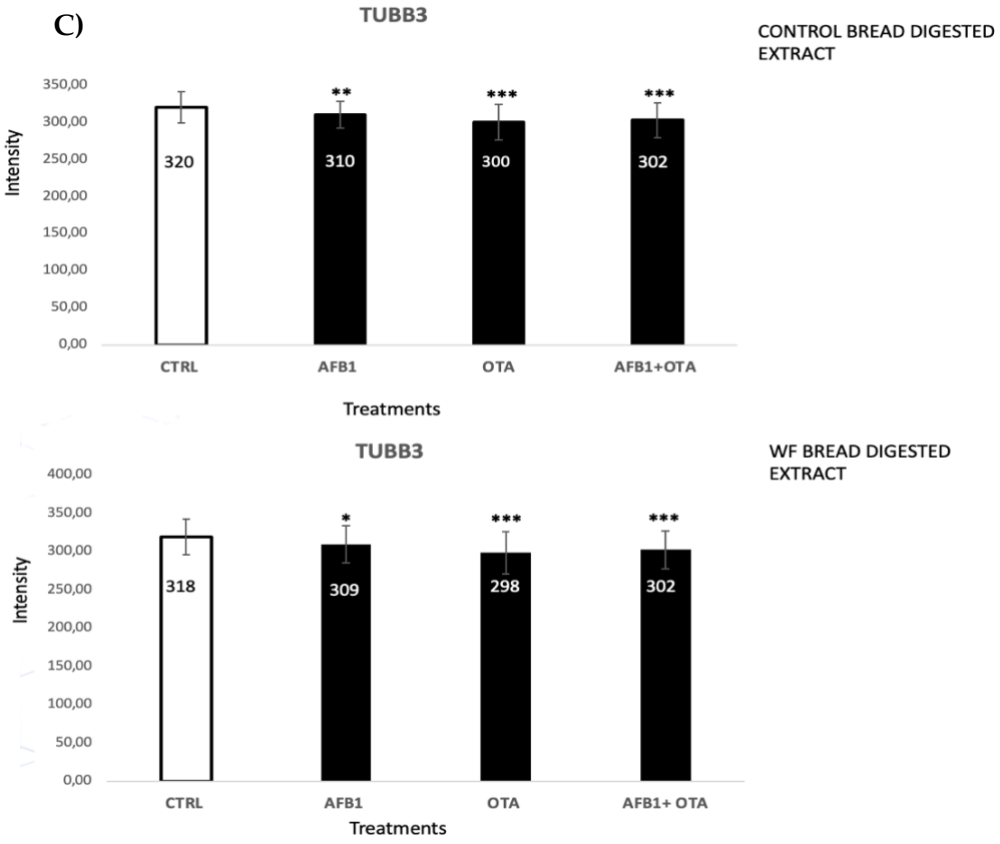
### *3.2. Effect of AFB1 and OTA on neuronal differentiation*

To evaluate the effect of AFB1 and OTA, individually and in combination, the fluorescent expression of dopamine and TUBB3, two typical neuronal markers, was investigated. In relation to control breads, OTA was the treatment that most affected the neuronal differentiation. Fig. 2a and 3a show a significative reduction of TUBB3 and dopamine expression in OTA-contained bread. A slight but significant difference compared to the control was also observed in bread with AFB1. The binary combination of toxins impaired the neuronal differentiation to a lesser extent than OTA individually and to a greater extent in comparison to AFB1 singly. The bar graphs of staining intensity of cells positive for the antibodies under study are shown in Figure 2c and 3c. Regarding WF enriched breads, OTA confirmed to be the most damaging treatment for SH-SY5Y cells ongoing differentiation, followed to a lesser extent by the combined exposure. AFB1 slightly reduced the differentiation degree of SH-SY5Y as confirmed by the fluorescent expression of TUBB3, which was quite similar to the control (Fig.2b). The same results were obtained in dopamine staining (Fig.3b) and further confirmed by statistical analysis in both experiments (Fig 2c and 3c).

In relation to P enriched breads, an overall enhancement of TUBB3 and dopamine expression were observed (Fig 2a and 3a). The quantitative analysis showed that OTA remained the most harmful treatment compared to AFB1 and mixed conditions (Fig 2c and 3c). In P+WF breads, the positive effect of functional ingredients in promoting neuronal differentiation was confirmed (Fig 2b and 3b). Indeed, neuronal markers were clearly expressed in all treatments performed. In this case, as with all other bread extracts, OTA induced a statistically significant reduction in SH-SY5Y differentiation compared to the control, followed by the combined and AFB1 exposures, respectively (Fig.2c and 3c).


A)
CTRL
AFB1
OTA
AFB1+OTA
CONTROL BREAD DIGESTED EXTRACT
WF BREAD DIGESTED EXTRACT
P BREAD DIGESTED EXTRACT
P+WF BREAD DIGESTED EXTRACT


B)
CTRL
AFB1
OTA
AFB1+OTA
CONTROL BREAD DIGESTED EXTRACT
WF BREAD DIGESTED EXTRACT
P BREAD DIGESTED EXTRACT
P+WF BREAD DIGESTED EXTRACT


C)
TUBB3
CONTROL BREAD DIGESTED EXTRACT
Intensity
350,00
300,00
250,00
200,00
150,00
100,00
50,00
0,00
320
310
300
302
**
***
***
CTRL
AFB1
OTA
AFB1+OTA
Treatments
TUBB3
WF BREAD DIGESTED EXTRACT
Intensity
400,00
350,00
300,00
250,00
200,00
150,00
100,00
50,00
0,00
318
309
298
302
*
***
***
CTRL
AFB1
OTA
AFB1+ OTA
Treatments

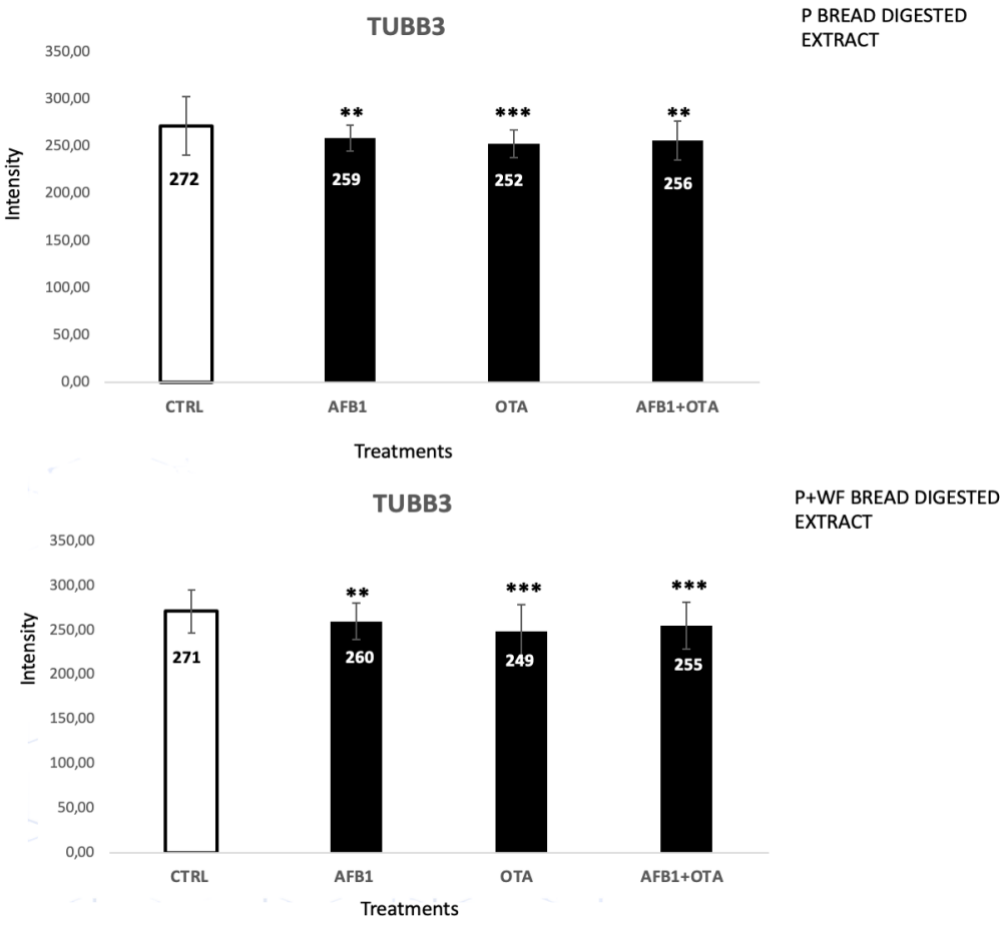
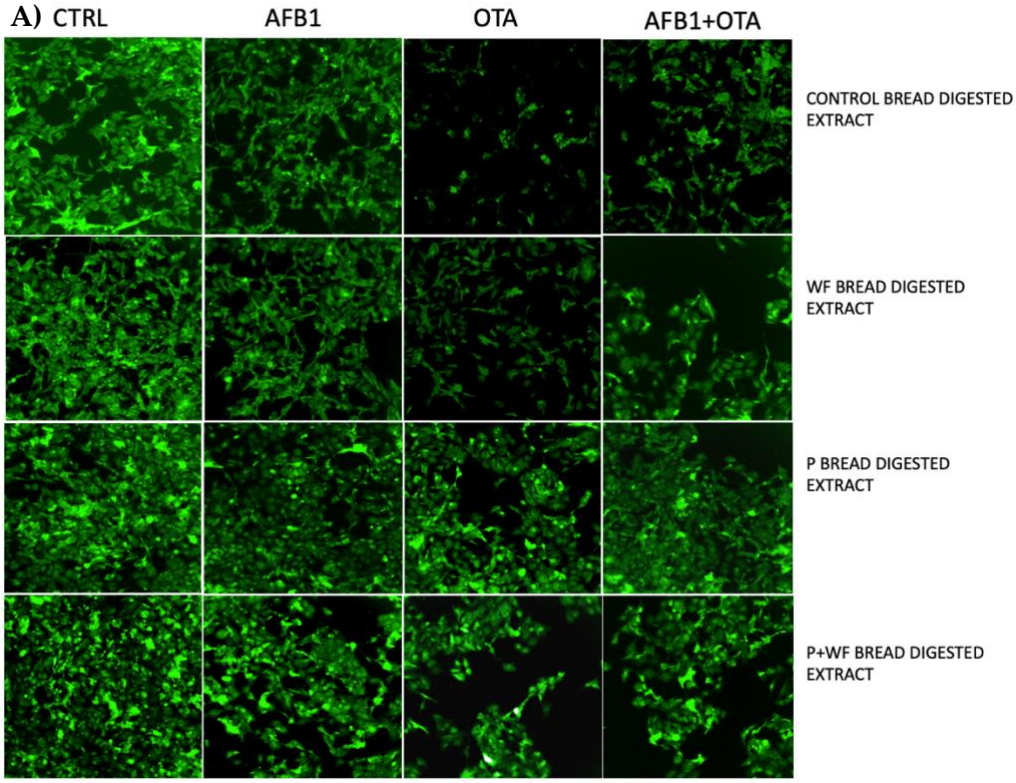
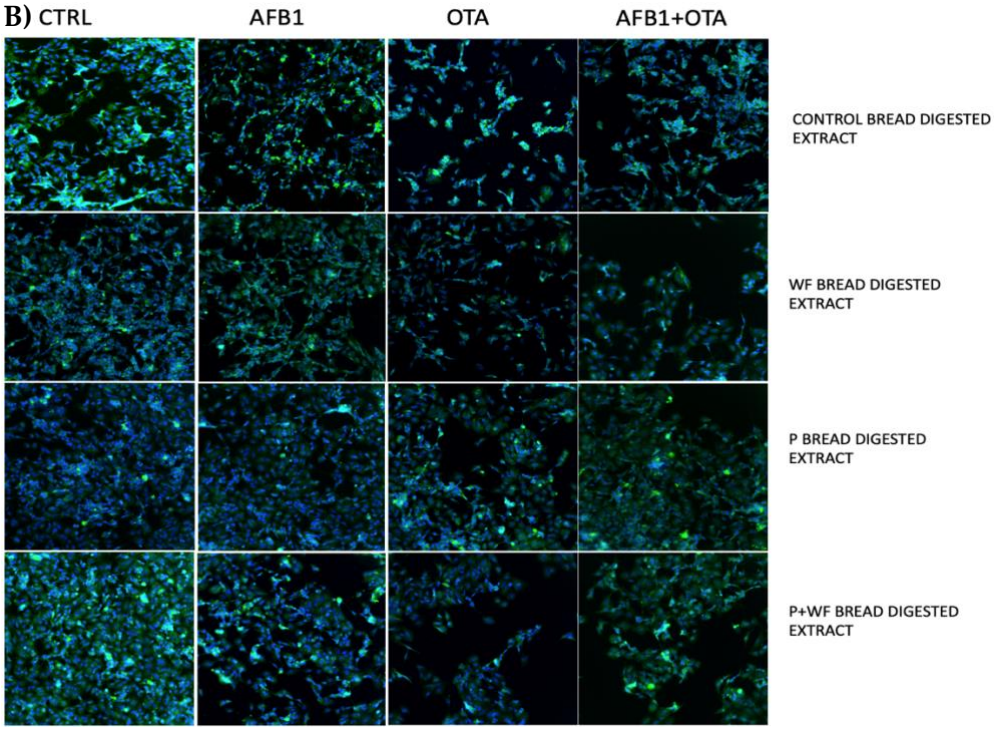


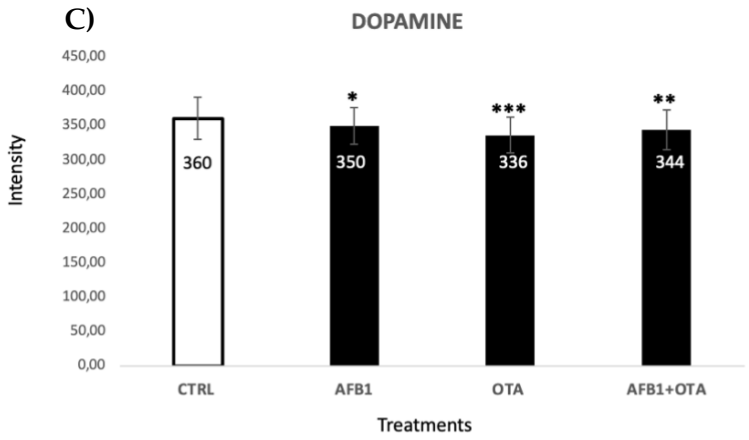

**Figure 2.** Effect of digested bread extracts (in all cases: AFB1:60nM; OTA:240nM; AFB1+OTA:60nM+240nM) on TUBB3 expression. (a) SH-SY5Y cells were exposed during 7 days to control bread digested extract; WF bread digested extract; P bread digested extract; and P+WF bread digested extract and then fixed and stained with Cy3 conjugated secondary antibody (red) for TUBB3 detection. (b) Overlay images were obtained staining cell nuclei with HOECHST (blue) and with Cy3 conjugated secondary antibody (red) for TUBB3 detection. All figures were obtained by using

the automated microscope IN Cell Analyzer 2000 (20× magnification). The data shown are representative of three independent experiments. (c) Quantification of TUBB3 fluorescence intensity. Data are expressed as mean ± SEM ($n = 3$). $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***). TUBB3: βIII-Tubulin; Cy3: Cyanine dye 3; P: Pumpkin; WF: Whey Fermented.




B) CTRL
AFB1
OTA
AFB1+OTA
CONTROL BREAD DIGESTED EXTRACT
WF BREAD DIGESTED EXTRACT
P BREAD DIGESTED EXTRACT
P+WF BREAD DIGESTED EXTRACT

C)


DOPAMINE
CONTROL BREAD DIGESTED EXTRACT
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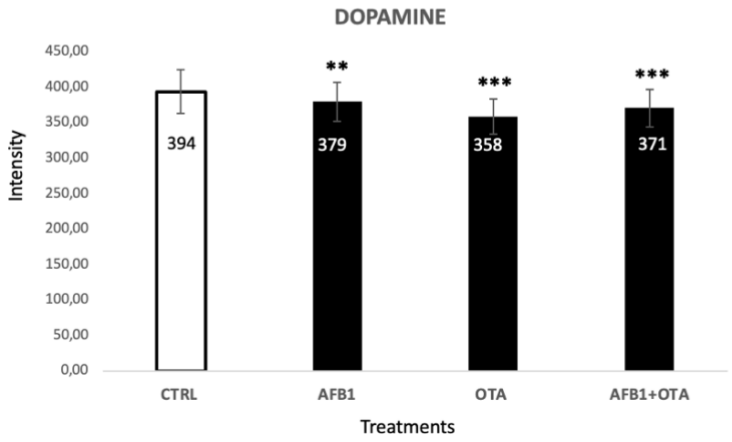
350,00
300,00
250,00
200,00
150,00
100,00
50,00
0,00
360
350
336
344
*
***
**
CTRL
AFB1
OTA
AFB1+OTA
Treatments


DOPAMINE
WF BREAD DIGESTED EXTRACT
Intensity
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400,00
350,00
300,00
250,00
200,00
150,00
100,00
50,00
0,00
394
379
358
371
**
***
***
CTRL
AFB1
OTA
AFB1+OTA
Treatments

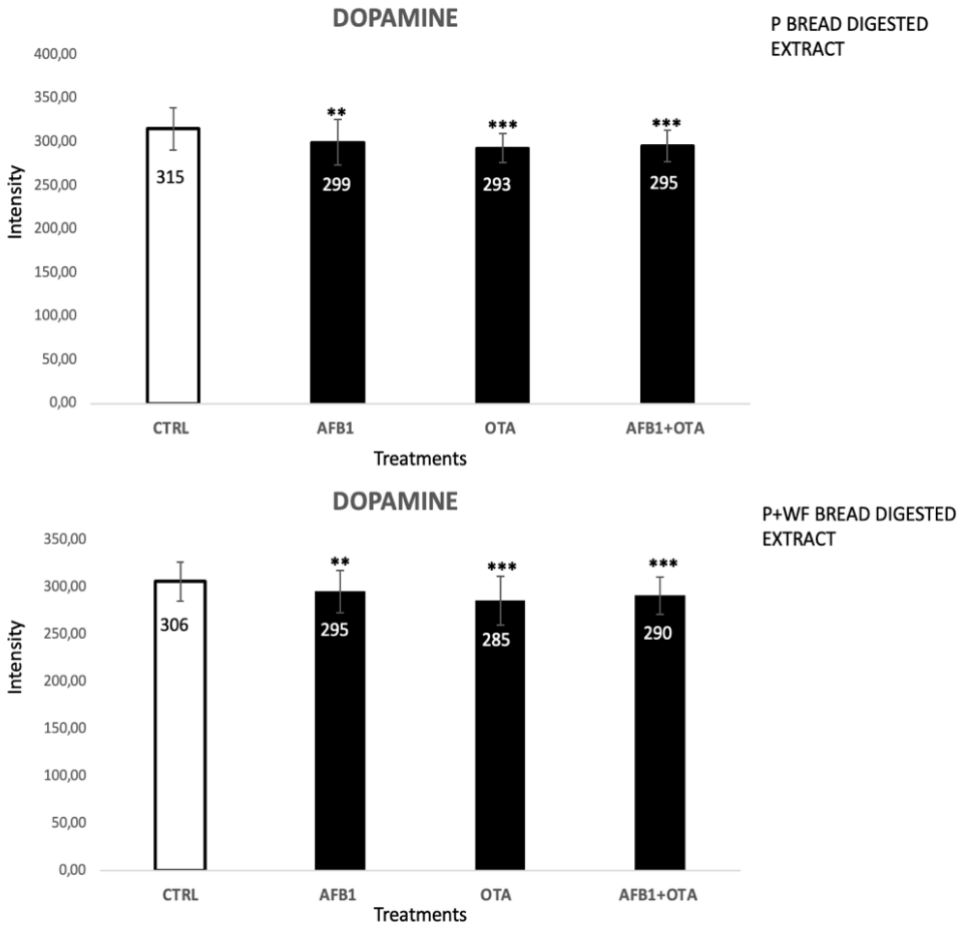


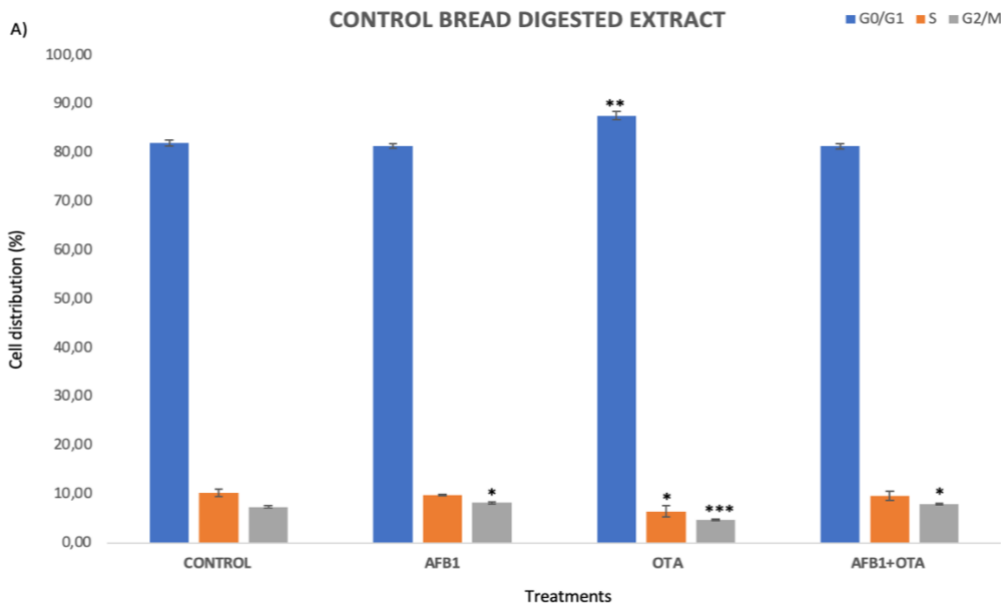

**Figure 3.** The effect of digested bread extracts (in all cases: AFB1:60nM; OTA:240nM; AFB1+OTA:60nM+240nM) on dopamine expression. (a) The SH-SY5Y cells were exposed during 7 days to control bread digested extract; WF bread digested extract; P bread digested extract; P+WF bread digested extract and then stained with FITC conjugated secondary antibody (green) for dopamine detection. (b) Overlay images obtained staining cell nuclei with HOECHST (blue) and with FITC conjugated secondary antibody (green) for dopamine detection. All figures were obtained by using the automated microscope IN Cell Analyzer 2000 (20× magnification). The

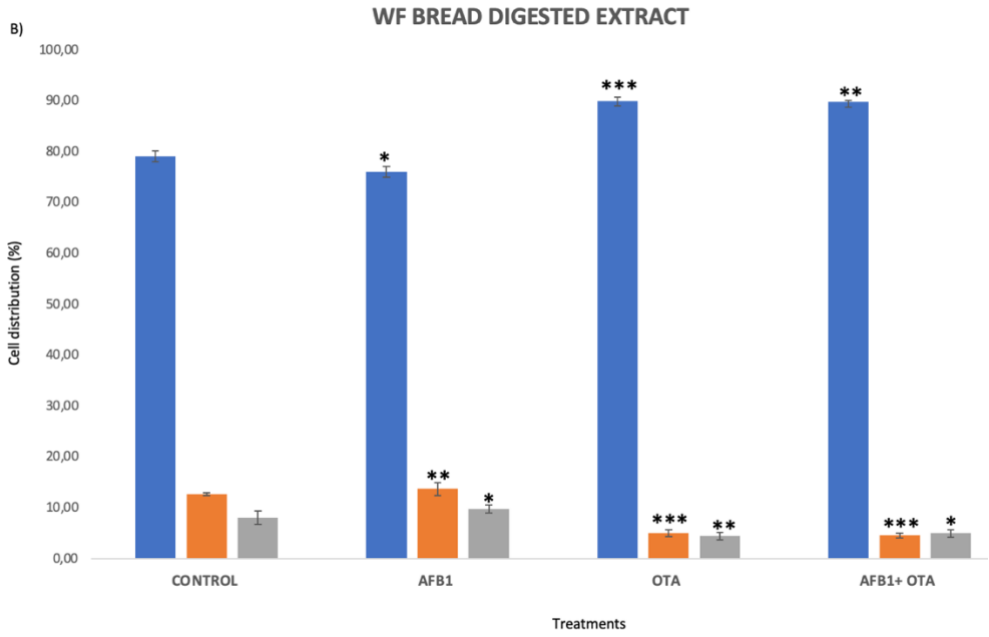
data shown are representative of three independent experiments. (c) Quantification of dopamine fluorescence intensity. Data are expressed as mean ± SEM ($n = 3$). $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***). FITC: Fluorescein; P: Pumpkin; WF: Whey Fermented.

### *3.3. Effects of bread extracts on cell cycle-progression*

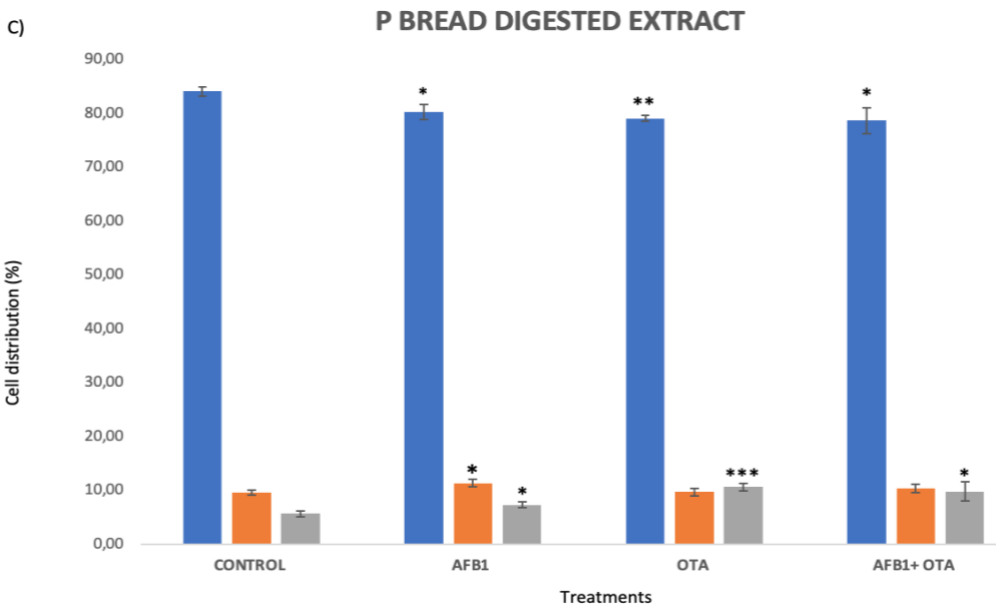
Possible cell cycle alterations in SH-SY5Y ongoing differentiation were analyzed through flow cytometry analysis. In relation to control breads, OTA was the most damaging treatment compared to the control. Indeed, it has been observed an increased percentage of SH-SY5Y cells at $G_0/G_1$ phase of cell cycle (88.63% vs 82% of control) and a lower cell percentage at S (5.50% vs 10.26%) and $G_2$/M phases (4.71% vs 7.41%). AFB1 and the combined exposure induced no remarkable differences in cell cycle, when compared to the control (Fig.4a). The addition of WF triggered no clear alterations in cell cycle. Except for AFB1, in which SH-SY5Y distribution remained similar to the control, a high percentage of cells was found at $G_0/G_1$ phase after OTA (90.03% vs 79.13% of control) and combined exposure (89.80% vs 79.13%) (Fig.4b). In the same treatments, a clear and marked reduction of SH-SY5Y percentage at S and $G_2$/M phases was also observed. In P enriched breads, statistically significant differences between control and treatments were observed. Despite that, the presence of P preserved the distribution of cell cycle similar to control levels in all conditions tested (Fig.4c). The binary combination of functional compounds induced clear

alterations for every treatment in all cell cycle phases. It has been observed a high percentage of cells at $G_0/G_1$ phase (AFB1:84.02%, OTA:86.53%, AFB1+OTA: 85.97% vs 72.10% of control) and a marked decrease of cell distribution in the synthesis and mitotic phase of cell cycle (Fig.4d). Interestingly, in P+WF control, a lower percentage of cells at $G_0/G_1$ phase was observed and this event might correlate with a lower dopamine and TUBB3 fluorescent intensity, compared to the other controls (Fig.2c and 3c). These results could suggest a possible interaction between P and WF in SH-SY5Y cells ongoing differentiation.




B)
WF BREAD DIGESTED EXTRACT
100,00
90,00
80,00
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
Cell distribution (%)
CONTROL
AFB1
OTA
AFB1+ OTA
Treatments

C)


P BREAD DIGESTED EXTRACT
Cell distribution (%)
90,00
80,00
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
CONTROL
AFB1
OTA
AFB1+ OTA
Treatments

D)

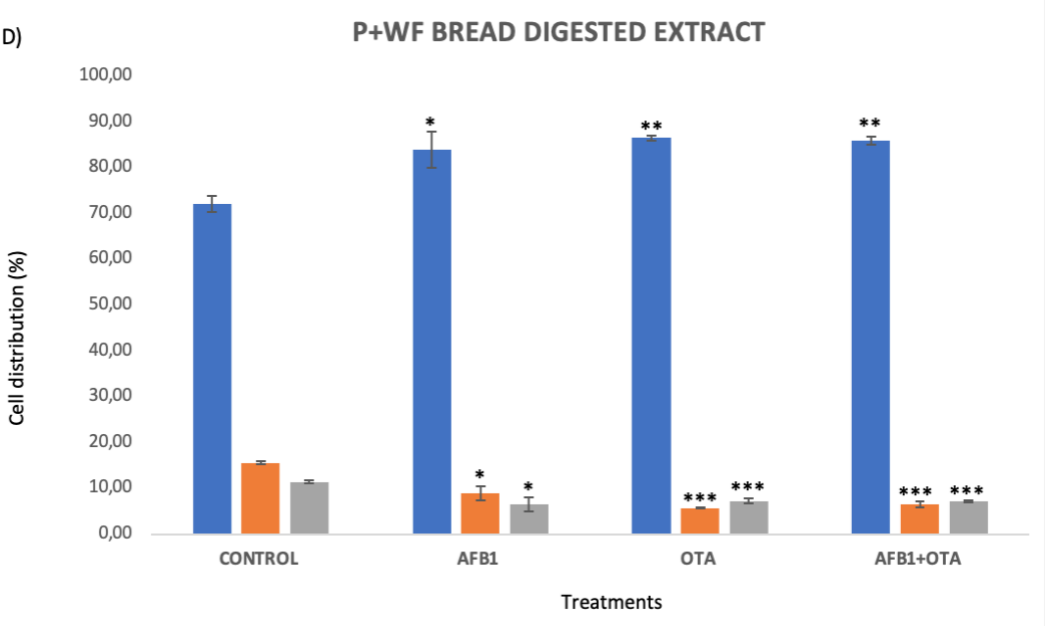

P+WF BREAD DIGESTED EXTRACT
Cell distribution (%)
100,00
90,00
80,00
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
CONTROL
AFB1
OTA
AFB1+OTA
Treatments

**Figure 4.** Flow cytometry analysis of cell cycle distribution in SH-SY5Y cells exposed during 7 days to: a) control bread digested extract, b) WF bread digested extract, c) P bread digested extract, d) P+WF bread digested extract. In all digested bread extracts (AFB1:60nM; OTA:240nM; AFB1+OTA:60nM+240nM). Data are expressed as mean ± SEM (n = 3). $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) compared to control. Acronyms used for cell cycle phases are: G for growth, S for DNA synthesis and M for mitosis phase. P: Pumpkin; WF: Whey Fermented.

### *3.4 Effects of bread extracts on cell cycle and neuronal differentiation related genes*

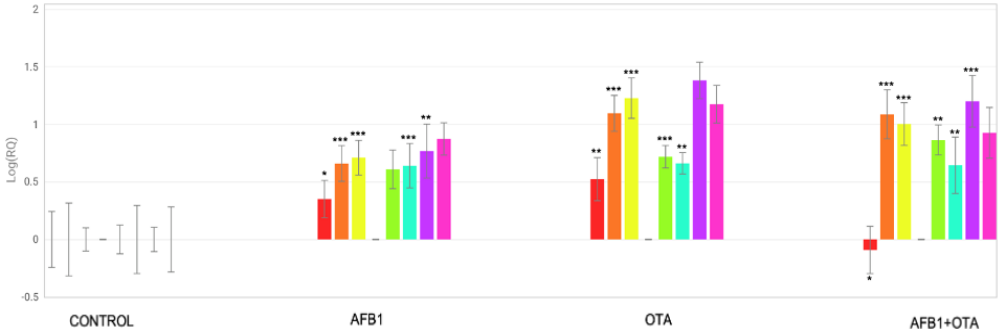
The transcriptional analysis of selected genes was performed by qPCR technique. In breads with no bioactive compounds, the clear down-regulation of cyclin D, cyclin B and Wnt5a ($p<0.01$) was observed for every treatment performed, when compared to the control (Fig.5a). For the other analyzed genes, although the relative gene expression is quite similar to control levels (Log2RQ<1), statistically significant differences were obtained. In this neuronal model, the administration of WF induced a significant upregulation of all studied genes (Fig.5b). The relative expression of Wnt5a, cyclin B and D was completely reversed compared to the one observed in control digested extracts. In P enriched breads, the relative expression of analyzed genes was similar to control levels (Log2RQ<1) for each condition performed (Fig.5c). In OTA and mixed conditions, the mild overexpression of TUBB3, GAP43, cyclin B and p21 was observed. Moreover, it has been obtained the slight downregulation of Wnt5a in all treatments. When

combined, P and WF preserved the expression of analyzed genes to control levels (Log2RQ<1). Fig.5d shows the significant overexpression of GAP43 and p21 and the down-regulation of Wnt5a for each treatment. The relative expression of TUBB3, cyclin B, D and p53 changes significantly according to the condition performed.

**A)** **CONTROL BREAD DIGESTED EXTRACT**

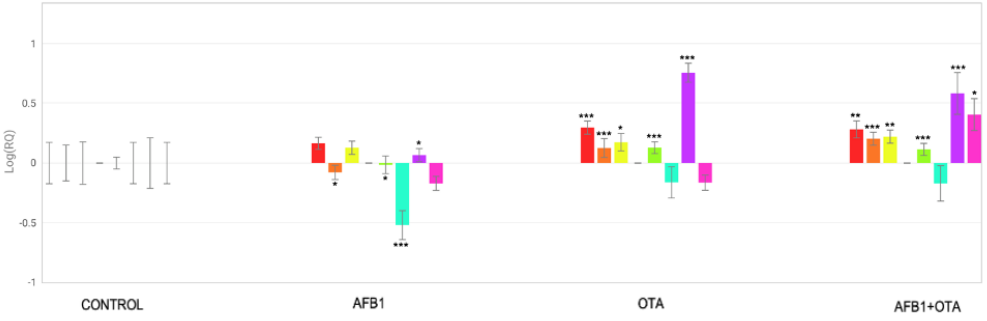
**B)**

**WF BREAD DIGESTED EXTRACT**




**C)**

**P BREAD DIGESTED EXTRACT**



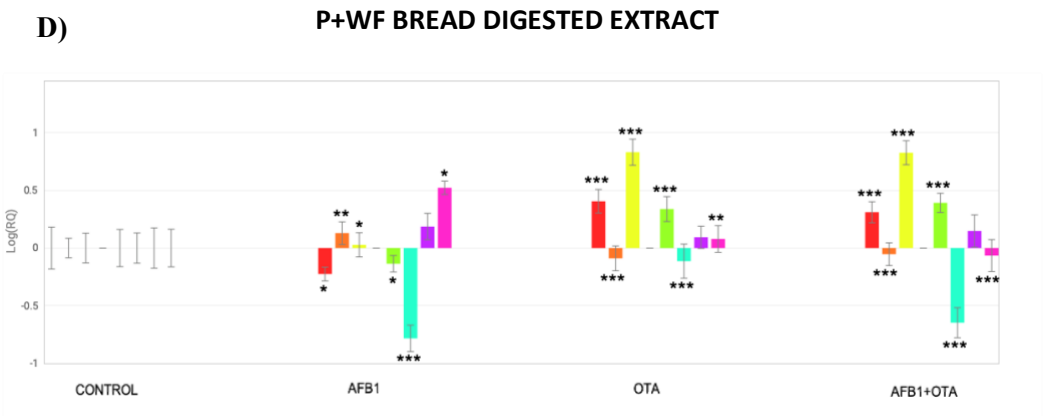



**Figure 5.** Bar plot showing the relative expression of cell cycle and neuronal differentiation genes when compared to control (log2RQ = 0) in SH-SY5Y cells after 7 days-exposure to a) control bread digested extract, b) WF bread digested extract, c) P bread digested extract, d) P+WF bread digested extract by qPCR analysis. In all digested bread extracts (AFB1:60nM; OTA:240nM; AFB1+OTA:60nM+240nM). $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***). RQ: relative quantification; P: Pumpkin; WF: Whey Fermented.

## 4. Discussion

In the present study, the effects of AFB1 and OTA on SH-SY5Y cells ongoing differentiation and the possible protective role of P and WF was evaluated. To reproduce a real scenario, bread extracts from a simulated *in vitro* human digestion were used, in which AFB1 and OTA doses were normalized to mimic the amounts reaching and crossing the BBB.

Firstly, the cytotoxic effect of bread extracts was measured by the reduction of formazan by mitochondrial dehydrogenases of living cells in the MTT assay. The results obtained in this work demonstrate the slight toxicity of diluted digested bread extracts at neuronal level since the cell viability never falls below 60% for all conditions tested. These results are in agreement with Escrivá et al. (2021), whose work testing the same digested bread extracts in a gastrointestinal barrier *in vitro* model, obtained a cell viability between 60-100% indicating the absence of any damaging effects. However, as shown in Fig.1, a non-toxic but significant reduction of cell viability was observed in a dose dependent manner in all digested breads. A similar trend has been obtained by Paradells et al. (2015) testing OTA-cytotoxicity in rat neural cells and by Vahidi-Ferdowsi et al. (2018) treating mouse astrocytes with different doses of AFB1. Interestingly, the presence of P in digested breads significantly increased neuronal viability after 72h treatment compared to shorter exposure times (Fig.1). Similarly, Montesano et al. (2020) testing carotenoids-enriched extracts in SH-SY5Y cell line obtained an increase of cell viability in a time dependent-manner. A possible explanation for these results was provided by Zhang et al. (2014), suggesting that a multiple and prolonged use of carotenoids is possibly required for a persistent protection against brain injuries in *in vivo* models. Also, Ochiai et al. (2007) demonstrated that the incorporation of carotenoids in PC12 neuronal cells occurred in a time-dependent manner and the ability to penetrate the cell membrane was determined by their lipophilic proprieties such as the number of glucose esters.

SH-SY5Y cell line represent a well-established model of neuronal differentiation *in vitro*. Once differentiated, SH-SY5Y cells exhibit a neuron-like phenotype characterized by prominent morphological alterations, including the neurite outgrowth, and by the synthesis of the typical dopamine neurotransmitter (Chinta et al., 2005; Lv et al., 2012; Avola et al., 2018). TUBB3 is one of the earliest neuronal cytoskeletal proteins expressed in brain development. TUBB3 has been also strictly associated with neurite outgrowth and is largely used as neuronal marker in developmental studies (Katsetos et al., 2003). Dopamine is one of the most widely studied neurotransmitters due to its involvement in several brain functions and neurological diseases (Chinta et al., 2005). In this work, the expression of TUBB3 and dopamine was analyzed following the exposure to digested bread extracts by immunofluorescence technique.

In control breads, a clear reduction in the differentiation degree was observed in OTA-treatment, showing to be more toxic compared to AFB1 and their combination at neuronal level (Fig. 2 and 3). In relation to TUBB3, similar results were obtained by Tanaka et al. (2015), who showed no remarkable change in TUBB3-expression after AFB1-exposure *in vivo*, suggesting that TUBB3 cannot be a possible effector of AFB1-induced neurotoxicity. Whilst, Hong et al. (2002) demonstrated the positive correlation between the inhibition of neurite outgrowth and OTA-administration (0.1-0.3 μM) in rat midbrain cells. *In vitro* differentiation of

rat neural stem cells into astrocytes, neurons, and oligodendrocytes was also monitored by Gill and Kumara (2019). Using cell-specific immunofluorescence staining, authors concluded that a non-cytotoxic concentration of OTA (0.2 μM) significantly reduced the differentiation degree of stem cells, thereby affecting the normal brain development. Paradells et al. (2015), through the fluorescent quantification of typical neuronal markers, among them TUBB3, revealed both the lack of neuronal differentiation and the reduced presence of astrocytes, microglia and young neurons in mice brain after six weeks-exposure to OTA. In view of this, OTA may affect neurogenesis, by inducing brain degeneration.

Regarding the dopaminergic system, very few studies on AFB1-toxicity have been reported as its harmful effects have been well-studied and confirmed on the cholinergic system (Richards et al., 2020). The only study found was conducted by Coulombe and Sharma (1985), showing the reduction of dopamine levels in specific regions of rats brain after AFB1-admnistration. Likewise, the brain tyrosine hydroxylase (TH) activity, the main enzyme involved in dopamine synthesis, was altered by AFB1-exposure *in vivo* (Weekley et al., 1984). In mice, OTA-exposure was positively correlated with striatal dopamine depletion and decreased TH activity, which in turn promoted Parkinson disease (PD), motor abnormalities and brain degeneration (Sava et al., 2006a; Sava et al., 2006b; Izco et al., 2021). The inhibitory effect of OTA on neuronal differentiation with altered dopamine levels was also detected *in vitro* (Hong et al., 2000).

For the first time, the presumed effect of P and WF against AFB1 and OTA on neurite growth and dopamine levels have been evaluated in the present study In agreement with our results, Goda et al. (2016) showed that astaxanthin (AST), a well-known carotenoid, ameliorated and reversed the alterations on the dopaminergic system induced in rat brain homogenates by Penitrem A, a potent neurotoxin produced by several terrestrial and marine *Penicillium* species. Accordingly, the dopamine levels were restored to control ones. Similarly, Lee et al. (2011) demonstrated the positive role of AST in preventing both the depletion of dopamine content and the impairment of TH activity promoted by the neurotoxin $MPP^+$ in the substantia nigra of rats brain. Therefore, AST-treatment has been considered a valuable therapeutic strategy in the modulation of PD progression. Similarly, the neuroprotective role of lycopene against neurotoxic agents has been reported in several *in vitro* and *in vivo* models. This compound prevented the alteration of dopaminergic system and PD progression (Hedayati et al., 2019). In relation to WF, Ano et al. (2018) demonstrated its beneficial role in mice brain by increasing dopamine levels, which in turn improved memory function and cognitive decline. In the same experimental models, WF may prevent the progression of neurodegenerative diseases such as dementia and Alzheimer disease (AD) (Katayama and Nakamura, 2019).

Cell cycle arrest is a transient condition induced by several factors and generally cells spontaneously revert this condition restoring their

proliferation rate or becoming apoptotic/necrotic (Juan-Garcia et., 2013). Although, AFB1 may disrupt the normal cell cycle progression in several cell lines (hepatic, renal, immune and epithelial) (Ricordy et al., 2002; Yu et al., 2015; Li et al., 2019), in our study no relevant differences have been detected in the digested loaves with no functional ingredients when compared to the control (Fig.4a). Different findings were obtained by Huang et al. (2020), treating IRM-32 neuronal cells with AFB1 (0.5-2 μM) during 24-48 h. Mycotoxin exposure resulted in a significant cell cycle arrest at phase S with marked DNA damage. Likewise, AFB1 (5-50 μM for 48 h) decreased the proliferation rate and arrested the cell cycle progression to the sub-$G_0/G_1$ stage with mitochondrial dysfunction and apoptosis in human astrocytes (Park et al., 2020). The discrepancy of results may be correlated with different experimental conditions used by other authors. In relation to OTA-exposure, it has been observed a significant accumulation of SH-SY5Y cells at $G_0/G_1$ phase of cell cycle (Fig.4a). In line with these results, OTA-promoted $G_0/G_1$ cell cycle arrest has been reported in astrocytes, renal, hepatic and immune cells (Liu et al., 2012; Juan-García et al., 2019; Park et al., 2019; Zhang et al., 2020). Conversely, Babayan et al. (2020) observed no significant difference in all phases of cell cycle treating SH-SY5Y and HT22 cell lines with high doses of OTA (2.5-30 μM) during 24 h. The discordance of results can be related not only to experimental conditions (mainly referred to the doses) but also to undifferentiated models employed by authors.

Chronic neuronal impairment induced by cell cycle dysregulation could obstruct various motor, behavioural, cognitive and regulatory functions of the brain. Therefore, the preservation of cell cycle machinery represents an essential aspect of neuroprotection (Joseph et al., 2020). In agreement with our results, Sathasivam and Ki (2018) demonstrated as the administration of carotenoids avoids cell cycle alterations *in vitro* and could be proposed as a novel strategy in neuroprotection. Whilst, the addition of WF showed unclear effects on cell cycle (Fig.4b and 4d). The high percentage of SH-SY5Y cells found at $G_0/G_1$ phase can be correlated with WF composition. The presence of several bioactive compounds in WF, such as phenolic acids, may interfere and potentiate ATRA effects (Xu et al., 2013; Chairez-Cantu et al., 2021). Indeed, ATRA promotes neuronal differentiation by prolonging the $G_0/G_1$ phase and by inducing $G_0$ cell cycle exit (Zhang et al., 2009; Qiao et al., 2012). However, further research is needed to confirm this hypothesis.

Finally, results obtained through immunofluorescence and flow cytometry techniques were validated at transcriptional level by qPCR analysis. To achieve this goal, the expression of several neuronal differentiation genes (Wnt5a, TUBB3 and GAP43) and cell cycle related genes (cyclins B and D, p21, p53) were analysed.

Wnt proteins are involved in several cellular functions including neuronal migration, polarisation, development and maturation, axon

guidance, dendrite development and synapse formation (Inestrosa & Arenas, 2010; Rosso & Inestrosa, 2013). The Wnt proteins play an important role not only in neuronal connectivity, but also in synaptic modulation, neurodevelopment and neurodegenerative diseases (De Ferrari & Moon, 2006; Salinas & Zou, 2008). According to previous findings, the aberrant down-regulation of Wnt5a, obtained in bread with no functional ingredients (Fig.5a), is involved in β-amyloid induced cortical neuron apoptosis and AD progression (Zhou et al., 2017). Similarly, the repression of Wnt5a could induce developmental neurotoxicity with deficits at cognitive and behavioral level in *in vivo* models (Coullery et al., 2020). In contrast, Blakely et al. (2011) demonstrated as the increased expression of Wnt5a, mainly observed in WF-enriched breads (Fig.5c), promotes the elongation and maturation of dopaminergic axons, driving them to forebrain targets in mice ventral midbrain.

GAP43, also known as neuromodulin, is a neurite outgrowth-related protein involved in presynaptic membrane changes, leading to the phenomena of neurotransmitter release, endocytosis and synaptic vesicle recycling, long-term potentiation, spatial memory formation, and learning (Denny, 2006). In the present work, AFB1 and OTA exposure induced a slight downregulation of GAP43 (Fig.5a), while the addition of P and WF, individually and in combination, promoted GAP43 over-expression for each condition performed (Fig.5b,c,d). Although GAP43 down-regulation might contribute to the disruption of axon outgrowth (Martinez et al., 2020), its

over-expression in C6 glioma cells and IRM-32 neuronal cells has been positively related to neurite formation and outgrowth (Konar et al., 2011) and to neuronal regeneration and plasticity in spiral ganglion neurons (Lei and Tang, 2017).

In line with immunofluorescence results, the mild down-regulation of TUBB3 in control breads (Fig 5a) and its significant over-expression in P and WF enriched breads was observed. According to these findings, the downregulation of TUBB3 expression was linked to the reduced differentiation of dopaminergic neurons *in vitro* (Lupu et al., 2018), while TUBB3 upregulation promoted the neuronal differentiation of both C6 and mouse embryonic stem cells (Chao et al., 2015; Pereckova et al., 2021).

In relation to cell cycle, cyclins are a family of proteins that control the cell cycle progression by activating cyclin-dependent kinase (CDK) enzymes (Galderisi et al., 2003). Cyclin B1, a key component in promoting cell cycle progression from $G_2$ to M phase, is implicated in the early events of mitosis, such as chromosome condensation and spindle pole assembly (Tu et al., 2013). Cyclin D1 is well known as a key protein in cell cycle evolution from $G_1$ to S phase in response to growth factors stimulation (Long et al., 2008). In this study, Cyclin B1 and D1 showed a similar pattern, being downregulated in control loaves and significantly overexpressed in P and WF-enriched breads (Fig.5). According with previous findings, in which non-canonical functions of cyclins have been investigated, the

downregulation of cyclin B1 was positively related to homocysteine-induced neurotoxic effects in SH-SY5Y cells (Curro et al., 2014). Likewise, a reduced cyclin D1 gene expression, typically observed in OTA-induced $G_0/G_1$ cell cycle arrest (Liu et al., 2012; Celik et al., 2020), was observed in bisphenol A-disrupted neurogenesis and neuronal differentiation in rats brain (Tiwari et al., 2015).

p21 is the main regulator of cell cycle arrest by inhibiting the activity of cyclin-CDK complexes in response to DNA damage. Moreover, p21 regulates $G_1$/S checkpoint and its activation plays a key role in the differentiation and maturation of neural precursors (Galderisi et al., 2003; Cazzalini et al., 2010; Zhen et al., 2012). Therefore, the overall upregulation of p21 observed in our work (Fig. 5), which was more prominent in functional ingredients enriched breads, might be considered an intrinsic event associated with ATRA-promoted neuronal differentiation (Qiao et al., 2012; Fu et al., 2020).

Another function of p21 is controlling the cell cycle through the negative regulation of p53 dependent or independent apoptosis pathways (Benson et al., 2014). p53 involvement in the cell cycle regulation is mainly correlated with post-translational modifications, acetylation and phosphorylation, and with its subcellular localization (Tedeschi and Di Giovanni, 2009; Solá et al., 2011). AFB1 (1 μM) has been reported to induce apoptosis in astrocytes and oligodendrocytes in brain of zebrafish embryos

by upregulating p53 and caspase cascade (Park et al., 2020). OTA-exposure has also proved p53 increase and therefore apoptosis induction *in vivo* and *in vitro* (Çelik et al., 2020; Kuroda et al., 2015; Hibi et al., 2013; Yoon et al., 2009). In this case, p53 gene expression reported an unclear trend, being slightly downregulated or overexpressed depending on the treatment (Fig.5).

Overall, our results confirm the negative effects of AFB1 and OTA on neuronal differentiation *in vitro* and the beneficial role of P and WF, individually and in combination, against the tested mycotoxins even at low doses. This is the first approach on the potential preventive effects of these bioactive compounds against AFB1- and OTA-neurotoxic effects *in vitro*.
ors.

## 5. Conclusion

These findings contribute to a better understanding of the underlying molecular mechanisms triggered by AFB1, OTA, dietary carotenoids and whey fermented on the neuronal differentiation *in vitro.* Compared to AFB1 and mixed exposure, OTA revealed to be the most damaging condition by affecting to a greater extent the expression of neuronal markers, TUBB3 and dopamine. Also, OTA induced $G_0/G_1$ cell cycle arrest in SH-SY5Y cells ongoing differentiation. However, the addition of pumpkin extract and whey fermented, individually and in combination , reported a beneficial

effect on neuronal development when added to the mixture. In qPCR analysis, the downregulation of neuronal differentiation markers Wnt5, TUBB3 and GAP43 by AFB1 and OTA was reverted by pumpkin extract and whey fermented. Cyclins were also positively regulated by the bioactive compounds when added to the mixture of mycotoxins. Further investigations are needed to better explore the positive role of pumpkin extract and whey fermented as neuroprotective agents against AFB1 and OTA-induced neurotoxicity, as well as to verify their activity in *in vivo* models, but results are promising.

**Authorship statement**

Manuscript title: Pumpkin extract and fermented whey alleviated AFB1 and OTA-induced alterations on neuronal differentiation in vitro. All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the Hong Kong Journal of Occupational Therapy.

**CRediT authorship contribution statement**

Massimo Frangiamone:. Conception and design of study: M. Frangiamone, M.Alonso-Garrido, L.Manyes; Acquisition of data: M.Frangiamone, M. Alonso- Garrido; Analysis and/or interpretation of data: M.Frangiamone; Drafting the manuscript: M. Frangiamone, M. Alonso-Garrido, A. Cimbalo; Revising the manuscript critically for important intellectual content: L. Manyes, G. Font

**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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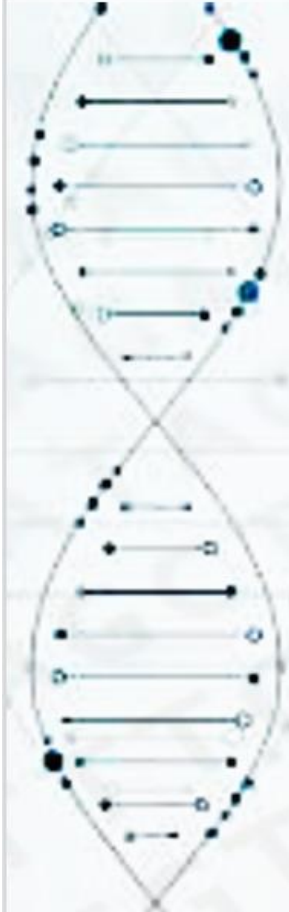

## 3.3. AFB1 and OTA promote Immune toxicity in Human Lymphoblastic T cells at Transcriptomic Level

Foods

# AFB1 and OTA promote Immune toxicity in Human Lymphoblastic T cells at Transcriptomic Level

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**Abstract**

Aflatoxin B1 (AFB1) and ochratoxin A (OTA) are typical contaminants of food and feed, which have serious implications for human and animal health, even at low concentrations. Therefore, a transcriptomic study was carried out to analyze gene expression changes triggered by low doses of AFB1 and OTA (100 nM; 7 days), individually and combined, in human lymphoblastic T cells. RNA-sequencing analysis showed that AFB1-exposure resulted in 99 differential gene expressions (DEGs), while 77 DEGs were obtained in OTA-exposure and 3236 DEGs in the combined one. Overall, 16% of human genome expression was altered. Gene ontology analysis revealed, for all studied conditions, biological processes and molecular functions typically associated with the immune system. PathVisio analysis pointed to ataxia telangiectasia mutated signaling as the most significantly altered pathway in AFB1-exposure, glycolysis in OTA-exposure, and ferroptosis in the mixed condition (Z-score > 1.96; adjusted p-value ≤ 0.05). Thus, the results demonstrated the potential DNA damage caused by AFB1, the possible metabolic reprogramming promoted by OTA, and the plausible cell death with oxidative stress prompted by the mixed exposure. They may be considered viable mechanisms of action to promote immune toxicity in vitro.

**Keywords**: mycotoxins; Jurkat cells; RNA-sequencing; mechanism of action; immune toxicity

## 1.Introduction

AFB1 is the most dangerous compound produced by several species of *Aspergillus,* which contamination occurs in a wide range of food commodities (Umesha et al., 2017). AFB1 is absorbed in the small intestine and metabolized in the liver releasing reactive intermediate metabolites, which are considered the causative agents of growth suppression, malnutrition, immune system alterations and onset of hepatocellular carcinoma (Monson et al., 2015). AFB1-toxicity has been also reported in pancreas, bladder, kidney and central nervous system (Frangiamone et al., 2022b; Rushing and Selim, 2019). In view of this, AFB1 has been classified by the International Agency for Research on Cancer (IARC) in group 1 as carcinogenic to humans (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012).

Another widespread mycotoxin, OTA, is mainly produced by *Penicillium verrucosum, Aspergillus ochraceus and Aspergillus niger.* Typical OTA-contaminated foods are cereals (maize, wheat, rice, sorghum, barley, oats), cereal-based products (bread, flour, and pasta), wine, tea, coffee, cheese, meat, fruits, dried fruits, spices and vegetables (Chen et al., 2018b). OTA is absorbed in the proximal tubule of kidney and its slow excretion led to a potential accumulation in the body (Zhu et al., 2017). Several studies have showed as OTA-exposure can led to hepatotoxicity, nephrotoxicity and neurotoxicity (Cimbalo et al., 2022b; Tao et al., 2018). According to these

evidences, OTA has been classified in group 2B as possible human carcinogen (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012).

AFB1 and OTA are also well-known to be immunotoxic agents *in vivo* and *in vitro* (Cimbalo et al., 2022a; Frangiamone et al., 2022a). Exposure to AFB1 may induce an immune and pro-inflammatory response in macrophages with reactive oxygen species (ROS) generation, autophagy and extracellular traps formation (An et al., 2017). In this cell line, AFB1 exacerbated swine influenza virus infection, inflammation and lung damage by activating TLR4-NFκB signaling pathway (Sun et al., 2018). The suppression of immune response has been also observed in rats and broilers (Bakheet et al., 2016; Ben Salah-Abbes et al., 2016; Guan et al., 2019). Similarly, OTA may disrupt the phagocytosis function of heterophils with intracellular ROS production (Han et al., 2019). In macrophages, OTA-exposure was also correlated with rheumatoid arthritis progression (Jahreis et al., 2017) while *in vivo* OTA-administration enhanced apoptosis, immune stress, inflammation and spleen damage (Liu et al., 2018). Furthermore, Hou et al. (2018) observed that AFB1 and OTA in combination can promote inflammation, oxidative stress and apoptosis in 3D4/21 cell line via NF-κB signaling pathway. However, very scarce information on AFB1 and OTA-induced toxicity in human T lymphocytes has been reported as well as the underlying mechanism has been rarely investigated.

In toxicological studies for human health risk assessment, the use of low experimental doses is necessarily required to reproduce a real scenario (Hernández & Tsatsakis, 2017). In human blood, AFB1 and OTA concentrations varied according to the studied population (Arce-Lopez et al., 2020). For AFB1, doses ranging from 0.6 to 237 nM were observed in the serum of Gambian children, while low values between 0.5 and 4 nM were detected in serum samples from healthy Iraqi patients (Kareem et al., 2021; Leong et al., 2012). As regards OTA, values of 22-25 nM were observed in plasma samples from Chinese and Bolivian patients whereas only 2 nM in the serum of Swedish adolescents (Fan et al., 2019; Ferrufino-Guardia et al., 2019; Warensjo Lemming et al., 2020). In relation to AFB1-metabolites, their detection is quite difficult (Ali et al., 2017). For the main AFB1-metabolite, i.e. AFM1, extremely low values ranging from 0.01 to 0.03 nM were observed in urinary samples from Brazilian, Guinean and Bangladeshi patients (Ali et al., 2020; De Cassia Romero et al., 2010; Leong et al., 2012). Regarding OTA-metabolism, the European Food Safety Authority (ESFA) reported that *in vivo* OTA-biotransformation, especially in humans and animals, is very low (EFSA, 2020). For OT$\alpha$, the main OTA-metabolite, values of 0.5 nM were detected in the plasma of Bangladeshi adolescents as well as in urinary samples from Spanish and German patients (Ali et al., 2018; Coronel et al., 2011; Munoz et al., 2010). Nevertheless, OT$\alpha$ has been described as a non-toxic compound and its formation has been considered an important OTA-detoxification pathway (EFSA, 2020; Wu et al., 2011).

In view of the above, a transcriptomic analysis was designed to investigate changes in gene expression profile triggered by low doses of AFB1 and OTA (both at 100 nM), individually and in combination, after 7 days of exposure in Jurkat cells.

## 2. Material and Methods

### *2.1. Chemicals*

The reagents and compounds used for cell culture, Roswell Park Memorial Institute (RPMI)-glutamax medium, fetal bovine serum (FBS), penicillin/streptomycin, and phosphate buffer saline were purchased by Sigma Chemical Co. (St. Louis, MO, USA); Dimethyl sulfoxide (DMSO) and methanol were obtained from Fisher Scientific (Madrid, Spain); Deionized water (<18, MΩcm resistivity) was obtained using Milli-QSP® Reagent Water System (Millipore, Bedford, MA, USA); AFB1 (MW: 312.28 g/mol) and OTA (MW: 403.81 g/mol) standards were acquired from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions were prepared in methanol solvent at a concentration of 1000 mg/L and kept at −20 °C.

### *2.2. Cell Culture and Exposure Conditions*

Jurkat cells (ATCC-TIB152) were maintained in RPMI-glutamax medium complemented with 100 U/mL penicillin, 100 mg/mL streptomycin

and 10% FBS. Cells were incubated at pH 7.4, 5% CO2 at 37 °C and air atmosphere at a constant humidity of 95%. The medium was changed every 2–3 days. To achieve the goal of the study, Jurkat cells were plated at a density of $2.5 \times 10^5$ cells/mL in 6-well tissue culture plates and exposed for 7 days to DMSO solvent at 0.1% as control condition in maintenance medium (n = 3) as well as AFB1 and OTA at 100 nM, individually and in combination, in 0.1% DMSO (n = 3).

### *2.3. RNA Extraction and Next Generation Sequencing (NGS)*

Firstly, RNA was extracted from Jurkat cells and purified from DNA contamination by using a ReliaPrepTM RNA Cell Miniprep System Kit (Promega, WI, USA). Quantity and quality of obtained RNA were assessed using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific™, Madrid, Spain), showing 260/280 nm and 260/230 nm ratios both around 2.

Secondly, Illumina NextSeq 500, supplied by the Genomics section of the Central Service for Experimental Research (SCSIE, University of Valencia), was employed for sequencing high-quality RNA samples, being the Integrity Numbers above 8. The standard protocol of Illumina was also carried out to create RNA-seq libraries, using TruSeq-stranded mRNA. Subsequently, results were generated as one archive for each sample, 12 in total.

*2.4. Data Processing*

FastQC software v0.11.8 (Babraham bioinformatics, Cambridge, UK) was used to calculate the percentage of mapped reads and to ensure quality control (QC) of reads (Andrews, 2010). The trimming was carried out employing FASTX-Toolkit v0.13 (Hannon, 2010), eliminating bases from 5′- and 3′-extremes. Then, reads with low quality and identical sequences were reduced into a single sequence but maintained counts. The trimmed reads alignment was performed by Spliced Transcripts Alignment to a Reference (STAR) software v2.7 (Cold Spring Harbor Laboratory, New York, NY, USA) (Dobin et al., 2013), using the Genome Reference Consortium Human Build 38 version as a reference. SAM tools software v1.10 (GitHub, San Francisco, CA, USA) (Li et al., 2009) allowed to transform Sequence Alignment Map (SAM) files into their binary version (BAM). BAM files were used with STAR to generate an expression matrix in R software (R core Team, 2018). Annotation, normalization and statistical analysis were performed according to the user guide of edgeR package (Robinson et al., 2010) to contrast differential expression among mycotoxin exposures and control, using gene-wise negative binomial generalized linear models with quasi-likelihood tests. Differentially expressed genes (DEGs) with p-value ≤ 0.05 were considered significant. Venn diagrams and heat maps were built by using Vennerable and pheatmap packages (Kolde, 2019; Swinton, 2013), to assess coincident DEGs between conditions (Alonso-Garrido et al., 2018; Escrivá et al., 2018).

*2.5. DEGs Analysis*

DEGs were submitted to gene ontology (GO) analysis by ConsensusPathDB (Kamburov & Herwig, 2022). Pathway assignments were carried out through PathVisio software (University of Maastricht, Maastricht, The Netherlands) by using WikiPathways as biological pathways database (Kutmon et al., 2015; Martens et al., 2021). Z-score > 1.96 and adjusted $p \leq 0.05$ were used as thresholds to identify significant pathways.

*2.6. Gene Selection and Primer Design*

Specific primers for each gene were designed by Primer-BLAST establishing the default software settings with PCR products of amplification ranging from 97 to 145 bp and melting temperature of 60 °C. qPCR analysis was performed by StepOne Plus Real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). The reliability of primer amplification was determined from standard curves of each gene, by measuring linearity ($R^2$ values) and efficiency of Ct mean values against the log cDNA dilution factor. Table 1 reports primers employed in the present study.

**Table 1.** Gene names, forward and reverse primer sequences, PCR efficiency and linearity value for the target genes and the reference gene GAPDH.

| Gene | Primer forward sequence | Primer reverse sequence | Efficiency (%) | Linearity ($R^2$) |
|---|---|---|---|---|
| GAPDH | GGAGTCCACTGGCGTCTT | GAGTCCTTCCACGATACCAAA | 110 | 0.990 |
| CSTA | AAACCCGCCACTCCAGAAAT | GCACAGCTTCCAATTTTCCGT | 107 | 0.991 |
| DNTT | GCCTCGTCAAAGAGTGGACA | GTCTCTCTCAAACCGGGAGC | 109 | 0.991 |

*2.7. Reverse Transcription and qPCR*

Amplification solutions were prepared in 96 well plates using SYBR Green as fluorescent dye. Reactions mixes consisted of 100 ng template, 500 nM of each primer, and the required amount of 2× Fast SYBR Green in a reaction volume of 10 μL. The PCR temperature cycling conditions for cystatin A (CSTA) and DNA nucleotidylexotransferase (DNTT) were as follows: initial denaturation step at 95 °C for 10 min to activate Taq DNA polymerase, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 30 s, and elongation at 72 °C for 15 s. The melting curve was generated by heating the amplicon from 60 to 90 °C. Threshold cycles (Ct) were generated by StepOne Plus Software version 2.3 and relative gene expressions were assessed using the $2^{-\Delta\Delta CT}$ method (Litvak & Schmittgen, 2001). The relative quantification values were transformed to log2 (Log2RQ) for normalization. Samples were run in triplicate according to Minimum

Information for Publication of Quantitative Real-Time PCR Experiments guidelines (Bustin et al., 2009).

*2.8. Statistical Analysis*

Statistical analysis was carried out by ΔCt values (experimental Ct and housekeeping Ct mean) obtained using the StepOne Plus Software version 2.3 (Applied Biosystems, Foster City, CA, USA). Variances among groups were evaluated by Levene's test and all the group variances were equal. T-Student defined differences between controls and treated groups. A p value ≤ 0.05 was considered for statistically significant differences.

**3. Results**

*3.1. Gene expression profile*

The overall gene expression of lymphocytes T treated for 7 days with AFB1 and OTA (100 nM), individually or in combination, significantly differs from the expression of untreated cells. Considering a p-value ≤ 0.05 and log2FC |0.5|, AFB1-exposure resulted in 99 DEGs with 61 up- and 38 down-regulated. 77 DEGs (23 up- and 54 down-regulated) were obtained in OTA-exposure and 3236 DEGs (2255 up- and 981 down-regulated) in the combined AFB1-OTA exposure. Overall, the total number of DEGs was 3412 with 68.5% up-regulation and 31.5% down-regulation, by compromising the

usual expression levels up to 16% of human genome, which contains 21000 protein coding genes (HGNC database). Interestingly, 42 DEGs (1.3%) overlapped in AFB1 and combined exposure, 8 DEGs (0.2%) among AFB1 and OTA individually, 32 DEGs (1%) between OTA and mixed treatment. Solely 3 DEGs (0.1%): DNTT, adhesion G protein-coupled receptor E1 (ADGRE1) and voltage-dependent T-type calcium channel subunit alpha-1I (CACNA1l) were found for each of the three studied conditions (Fig.1). Although DNTT was up-regulated in all conditions performed, ADGRE1 and CACNA1l were down-regulated in AFB1 and OTA individually and over-expressed in the combined exposure.

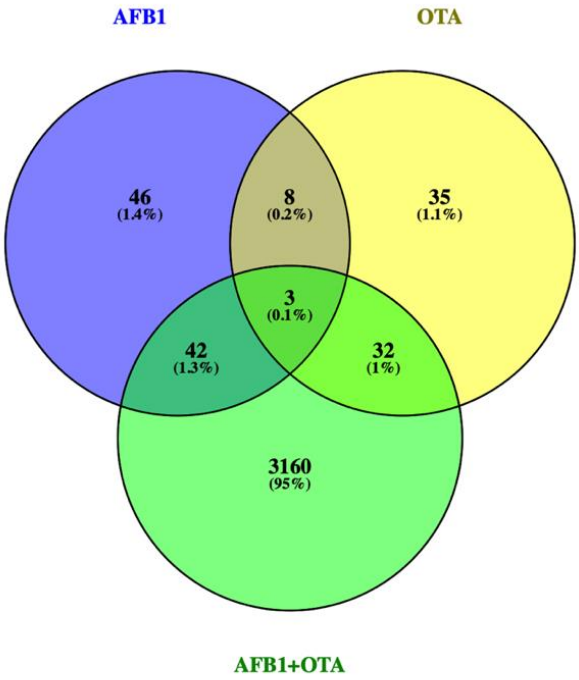

**Figure 1.** Venn diagram for the DEGs found by RNA-seq in Jurkat cells treated with AFB1, OTA, AFB1+OTA at 100 nM in 0.1% DMSO compared to 0.1% DMSO control exposure. DEGs p-value ≤ 0.05 and log2FC |0.5|.

### *3.2. Gene ontology and pathway analysis*

One of the most important steps in a transcriptomics study is the characterization of biological processes (BP) and molecular functions (MF) in which DEGs are involved by comparing the DEGs list with the rest of the genome for over-represented functions and gene set enrichment analysis (Alexeyenko et al., 2012; Conesa et al., 2016). Table 2 shows relevant GOs with their relative categories obtained by the over-representation analysis in ConsensusPathDB.

**Table 2.** Relevant GOs resulting from the over-representation analysis of the selected DEGs obtained by AFB1, OTA, AFB1+OTA treatment (100 nM in all cases) in ConsensusPathDB.

| Gene ontology term | | categories | set size | genes candidates | p-value |
|---|---|---|---|---|---|
| **AFB1 exposure** | | | | | |
| GO:0098609 | Cell-cell adhesion | BP 3 | 871 | 7 (0.8%) | <0.05 |
| GO:0048731 | System development | BP 3 | 4796 | 25 (0.5%) | <0.01 |
| GO:0002684 | Regulation of immune system process | BP 4 | 1238 | 9 (0.7%) | <0.05 |
| GO:0004896 | Cytokine receptor activity | MF 4 | 97 | 3 (3.1%) | <0.01 |
| GO:0043169 | Cation binding | MF 3 | 4346 | 22 (0.5%) | <0.05 |
| GO:0051020 | GTPase binding | MF 4 | 388 | 4 (1.0%) | <0.05 |
| GO:0000981 | DNA and RNA transcription factor activity | MF 3 | 1401 | 9 (0.6%) | <0.05 |
| GO:0005496 | Steroid binding | MF 3 | 100 | 2 (2.0%) | <0.05 |
| GO:0035579 | Plasma membrane | CC 4 | 91 | 2 (2.2%) | <0.05 |
| **OTA exposure** | | | | | |
| GO:0046903 | Secretion | BP 4 | 1469 | 12 (0.8%) | <0.001 |
| GO:0007596 | Blood coagulation | BP 3 | 344 | 5 (1.5%) | <0.01 |
| GO:0007599 | Hemostasis | BP 4 | 348 | 5 (1.4%) | <0.01 |
| GO:0006954 | Inflammatory response | BP 4 | 842 | 7 (0.8%) | <0.05 |
| GO:0002683 | Regulation of immune system process | BP 4 | 468 | 5 (1.1%) | <0.05 |
| GO:0001666 | Response to hypoxia | BP 3 | 338 | 4 (1.2%) | <0.05 |
| GO:0051213 | Dioxygenase activity | MF 3 | 90 | 2 (2.2%) | <0.05 |
| GO:0004842 | Ubiquitin-protein transferase activity | MF 4 | 431 | 4 (0.9%) | <0.05 |
| GO:0016323 | Basolateral plasma membrane | CC 3 | 242 | 3 (1.2%) | <0.05 |
| GO:0016021 | Integral component of membrane | CC 3 | 5401 | 22 (0.4%) | <0.05 |
| **AFB1-OTA exposure** | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| GO:0007155 | Cell adhesion | BP 2 | 1457 | 48 (3.3%) | <0.001 |
| GO:0001816 | Cytokine production | BP 2 | 746 | 20 (2.7%) | <0.01 |
| GO:0030098 | Lymphocyte differentiation | BP 4 | 355 | 12 (3.4%) | <0.01 |
| GO:0002684 | Regulation of immune system process | BP 4 | 1238 | 30 (2.4%) | <0.01 |
| GO:0001913 | T cell cytotoxicity | BP 3 | 50 | 4 (8.0%) | <0.01 |
| GO:0008528 | G protein-coupled peptide receptor activity | MF 4 | 150 | 8 (5.3%) | <0.01 |
| GO:0004896 | Cytokine receptor activity | MF 4 | 97 | 9 (9.3%) | <0.001 |
| GO:0019957 | C-C chemokine binding | MF 5 | 24 | 3 (12.5%) | <0.01 |
| GO:0009925 | Plasma membrane | CC 3 | 49 | 4 (8.2%) | <0.01 |
| GO:0042612 | MHC class I protein complex | CC 5 | 10 | 2 (20%) | <0.01 |
| GO:0072562 | Blood microparticle | CC 2 | 148 | 7 (4.7%) | <0.01 |
| GO:0005938 | Cell cortex | CC 5 | 304 | 11 (3.2%) | <0.01 |
| GO:0000785 | Chromatin | CC 4 | 1231 | 30 (2.4%) | <0.01 |

*3.2.1 AFB1-exposure*

Regarding AFB1-condition, the over-representation analysis by ConsesusPathDB provided a list of GO terms in which several BP linked to system development, cell adhesion, and regulation of immune system process were some of the most over-represented. MF associated with cation, steroid and GTPase binding, DNA and RNA polymerase transcription factor activity and cytokine receptor function were statistically significant in the DEGs set. Cellular components (CC) such as the plasma membrane was significantly represented in the individual AFB1-exposure (Table 2).

PathVisio analysis showed a total of 3691 data points (N), among them 753 met criterion (R). (N) denotes the total number of genes measured in the dataset whereas (R) indicates the filter analysis criterion (Kutmon et al., 2015). Several pathways were statistically significant after AFB1-treatment (Z-score >1.96; *adj.* p-value ≤ 0.05). According to the number of genes affected, the most significant pathways were: ApoE and miRNA-146 in inflammation and atherosclerosis (80%), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) actives NLRP3 inflammasome (67%), miRNA-124 interaction with cell cycle and differentiation (67%) and SARS-CoV-2 antagonizes innate immune activation (62%). The analysis also showed a significant alteration in the mitochondrial immune response to SARS-CoV-2 and ataxia telangiectasia mutated (ATM) signaling in

development and disease, with 45% of genes affected in each pathway (Table 3).

**Table 3.** Pathways overlapped in AFB1-exposure (100 nM) by PathVisio.

| Pathway | Positive (r) | Measured (n) | Total | % | Z Score | *adj.* p-value |
|---|---|---|---|---|---|---|
| ATM signaling in development and disease | 20 | 44 | 49 | 45 | 4.15 | <0.01 |
| ApoE and miR-146 in inflammation and atherosclerosis | 4 | 5 | 13 | 80 | 3.31 | <0.01 |
| SARS-CoV-2 antagonizes innate immune activation | 5 | 8 | 15 | 62 | 2.96 | <0.01 |
| Mir-124 predicts cell cycle and differentiation | 4 | 6 | 8 | 67 | 2.81 | <0.01 |
| Mitochondrial immune response to SARS-CoV-2 | 8 | 18 | 62 | 44 | 2.54 | <0.05 |
| Activation of NLRP3 inflammasome by SARS-CoV-2 | 2 | 3 | 10 | 67 | 1.99 | <0.05 |

The genes involved in ATM signaling pathway (Z-score: 4.15) for *Homo sapiens* are shown in figure 2, indicating in red the over-expressed genes and in green the down-regulated genes ones. A slight alteration in ATM-related gene expression was obtained.

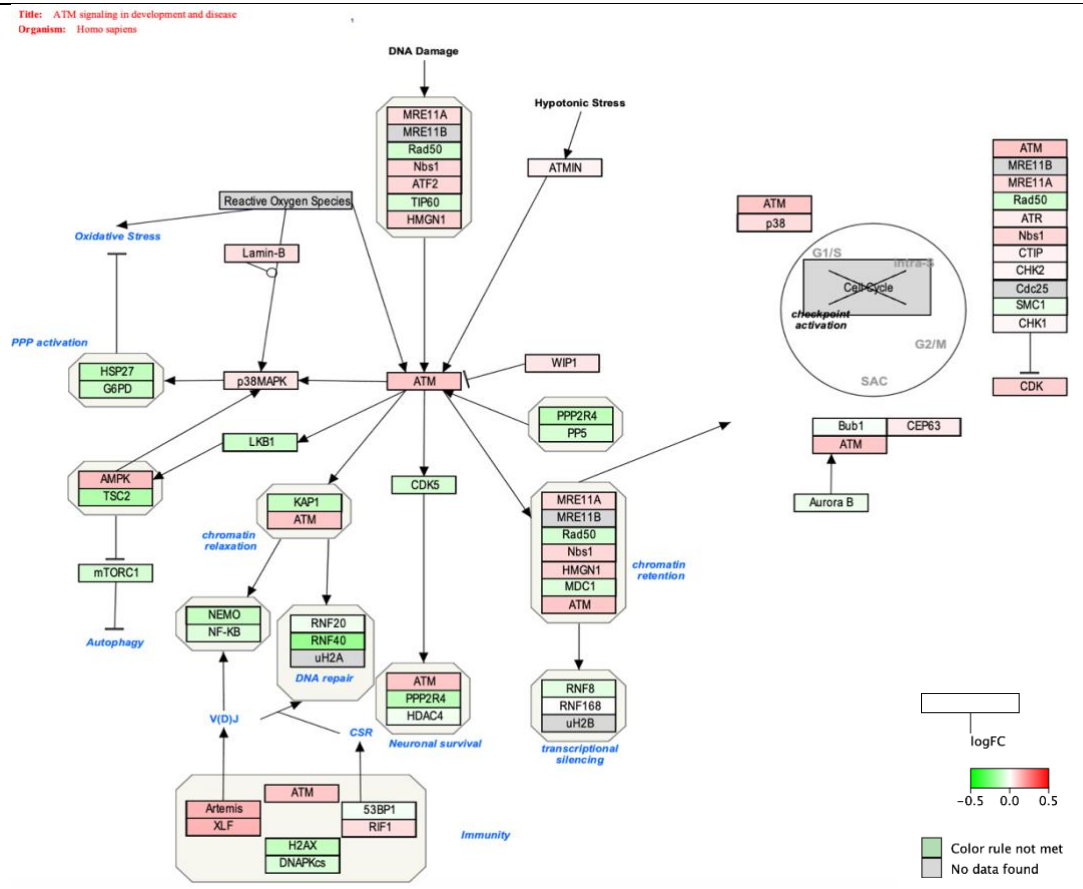
Title: ATM signaling in development and disease
Organism: Homo sapiens
DNA Damage
MRE11A
MRE11B
Rad50
Nbs1
ATF2
TIP60
HMGN1
Hypotonic Stress
ATMIN
Reactive Oxygen Species
Oxidative Stress
Lamin-B
PPP activation
HSP27
G6PD
p38MAPK
ATM
WIP1
PPP2R4
PP5
LKB1
AMPK
TSC2
mTORC1
Autophagy
KAP1
ATM
chromatin relaxation
CDK5
MRE11A
MRE11B
Rad50
Nbs1
HMGN1
MDC1
ATM
chromatin retention
NEMO
NF-KB
RNF20
RNF40
uH2A
DNA repair
ATM
PPP2R4
HDAC4
Neuronal survival
RNF8
RNF168
uH2B
transcriptional silencing
V(D)J
CSR
Artemis
XLF
ATM
53BP1
RIF1
H2AX
DNAPKcs
Immunity
ATM
p38
G1/S
Cell Cycle
checkpoint activation
G2/M
SAC
ATM
MRE11B
MRE11A
Rad50
ATR
Nbs1
CTIP
CHK2
Cdc25
SMC1
CHK1
CDK
Bub1
ATM
CEP63
Aurora B
logFC
−0.5
0.0
0.5
Color rule not met
No data found

**Figure 2.** Genes involved in ATM signaling for *Homo sapiens* are showed: in red the up-regulated genes and in green the down-regulated after AFB1-exposure (100 nM).

*3.2.2. OTA exposure*

ConsensusPathDB analysis pointed to blood coagulation, secretion, hypoxia, hemostasis and regulation of the immune and inflammatory response as some of the most over-represented BP upon OTA-exposure. The activity of dioxygenase and ubiquitin proteins was the most significant MF in the DEGs set at level 3 and 4 whereas CC such as basolateral plasma membrane and its integral components were significantly affected (Table 2). PathVisio analysis reported a total number of 3662 data points, of which only 193 met the criterion. Several pathways were statistically significant upon OTA-treatment (Z-score>1.96; *adjusted* p-value≤0.05). Based on the number of genes affected, the most significant pathways were: glycolysis (43%) and Cori cycle (34%). The analysis indicated a significant alteration in HIF1α and PPAR-γ regulated glycolysis (29%) and innate immune response to SARS-CoV-2 with 25% of genes affected (Table 4).

**Table 4.** Pathways overlapped in OTA-exposure (100 nM) by PathVisio.

| Pathway | Positive (r) | Measured (n) | Total | % | Z Score | *adj.* p-value |
|---|---|---|---|---|---|---|
| Glycolysis in senescence | 3 | 7 | 26 | 43 | 4.45 | <0.01 |
| Cori cycle | 4 | 12 | 53 | 33 | 4.36 | <0.01 |
| Metabolic reprogramming | 7 | 40 | 80 | 17 | 3.48 | <0.01 |

| | | | | | | |
|---|---|---|---|---|---|---|
| HIF1α and PPAR-γ regulation of glycolysis | 2 | 7 | 19 | 29 | 2.76 | <0.05 |
| SARS-CoV-2 antagonizes innate immune activation | 2 | 8 | 15 | 25 | 2.5 | <0.05 |

Figure 3. displays the genes involved in glycolysis signaling pathway (Z-score: 4.45) for *Homo sapiens*. Also in this case, low values of fold changes were observed.

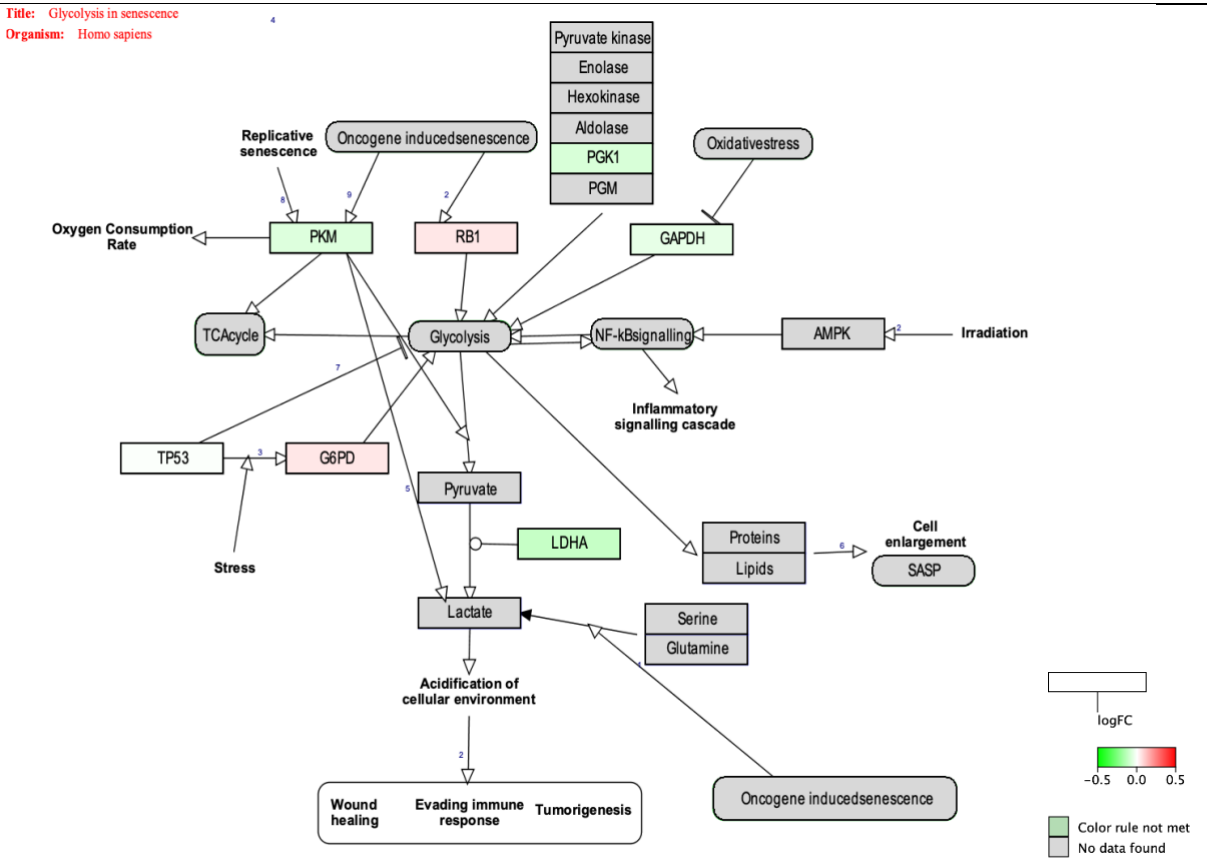
Title: Glycolysis in senescence
Organism: Homo sapiens
Pyruvate kinase
Enolase
Hexokinase
Aldolase
PGK1
PGM
Replicative senescence
Oncogene inducedsenescence
Oxidativestress
Oxygen Consumption Rate
PKM
RB1
GAPDH
TCAcycle
Glycolysis
NF-kBsignalling
AMPK
Irradiation
Inflammatory signalling cascade
TP53
G6PD
Pyruvate
LDHA
Stress
Proteins
Lipids
Cell enlargement
SASP
Lactate
Serine
Glutamine
Acidification of cellular environment
Wound healing
Evading immune response
Tumorigenesis
Oncogene inducedsenescence
logFC
−0.5 0.0 0.5
Color rule not met
No data found

**Figure 3.** Genes implicated in glycolysis senescence pathway for *Homo sapiens* are showed: in red the up-regulated genes and in green the down-regulated upon OTA-treatment (100 nM).

### *3.2.3. AFB1-OTA combined exposure*

The analysis conducted by ConsensusPathDB showed that in AFB1-OTA mixed exposure the most over-represented BP were: regulation of immune system process, cytokine production, cell adhesion, lymphocyte differentiation and T cell cytotoxicity, among others. MF linked to G protein, cytokine receptor activity and C-C chemokine binding were statistically significant in the DEGs set at level 4 and 5. CC such as cell cortex and membrane, chromatin, blood microparticle and MHC class I protein complex were significantly affected to a greater extent (Table 2). PathVisio analysis showed 3744 data points, of which a large number (2489) matched the criterion. Solely 9 of the pathways were statistically relevant following the combined exposure (Z-score>1.96; *adjusted* p-value ≤ 0.05). In this case, all pathways found showed more than 84% of genes affected. The 100% of genes involved in tricarboxylic acid cycle (TCA), dual hijack model in HIV infection and hyperlipidemia were statistically altered (Table 5).

**Table 5.** Pathways overlapped in AFB1-OTA-exposure (100 nM) by PathVisio.

| Pathway | Positive (r) | Measured (n) | Total | % | Z Score | *adj.* p-value |
|---|---|---|---|---|---|---|
| Ferroptosis | 33 | 39 | 88 | 84 | 2.41 | <0.05 |
| PPAR-α pathway | 14 | 15 | 28 | 93 | 2.21 | <0.05 |
| TCA cycle in senescence | 9 | 9 | 30 | 100 | 2.13 | <0.05 |
| Dual hijack model of HIV infection | 8 | 8 | 10 | 100 | 2.01 | <0.01 |
| Familial hyperlipidemia type 1 | 8 | 8 | 35 | 100 | 2.01 | <0.05 |

The genes involved in ferroptosis signaling pathway (Z-score: 2.41) for *Homo sapiens* are shown in figure 4 and the majority of them were down-regulated.

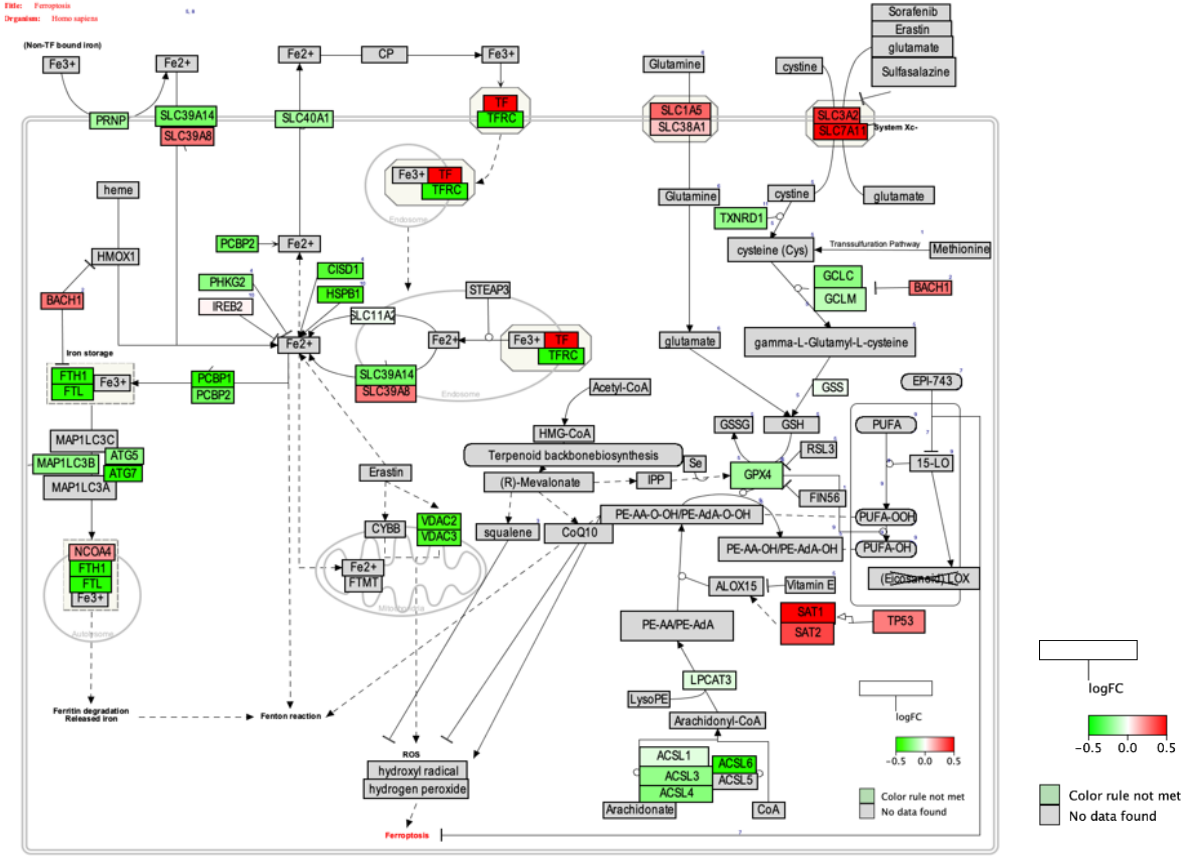
Title: Ferroptosis
Organism: Homo sapiens
(Non-TF bound iron)
Fe3+
Fe2+
PRNP
SLC39A14
SLC39A8
SLC40A1
CP
TF
TFRC
Glutamine
cystine
Sorafenib
Erastin
glutamate
Sulfasalazine
SLC1A5
SLC38A1
SLC3A2
SLC7A11
System Xc-
heme
HMOX1
BACH1
PCBP2
PHKG2
IREB2
CISD1
HSPB1
SLC11A2
STEAP3
Endosome
Iron storage
FTH1
FTL
PCBP1
TXNRD1
cysteine (Cys)
Transsulfuration Pathway
Methionine
GCLC
GCLM
gamma-L-Glutamyl-L-cysteine
GSS
EPI-743
Acetyl-CoA
HMG-CoA
Terpenoid backbonebiosynthesis
GSSG
GSH
PUFA
RSL3
15-LO
Se
GPX4
FIN56
(R)-Mevalonate
IPP
Erastin
VDAC2
VDAC3
CYBB
squalene
CoQ10
PE-AA-O-OH/PE-AdA-O-OH
PUFA-OOH
PE-AA-OH/PE-AdA-OH
PUFA-OH
MAP1LC3C
MAP1LC3B
MAP1LC3A
ATG5
ATG7
NCOA4
Fe2+
FTMT
Mitochondria
ALOX15
Vitamin E
(Eicosanoid) LOX
SAT1
SAT2
TP53
PE-AA/PE-AdA
LPCAT3
LysoPE
Arachidonyl-CoA
Autophagy
Ferritin degradation
Released iron
Fenton reaction
ROS
hydroxyl radical
hydrogen peroxide
ACSL1
ACSL3
ACSL4
ACSL6
ACSL5
Arachidonate
CoA
Ferroptosis
logFC
-0.5 0.0 0.5
Color rule not met
No data found

**Figure 4.** Genes involved in ferroptosis pathway for *Homo sapiens* are showed: in red the up-regulated genes and in green the down-regulated following AFB1-OTA exposure (100 nM for both toxins).

### 3.3. Validation of NGS results by qPCR

The expression of CSTA, a cysteine protease inhibitor gene, was assessed after exposure to AFB1 whereas DNTT expression, an independent DNA polymerase gene, was evaluated upon exposure to AFB1, OTA and mycotoxins mixture. The experimental conditions used in qPCR analysis were the same as those used for the NGS assay. Also, CSTA and DNTT were chosen being ones of most affected genes in the sequencing analysis. qPCR assay confirmed NGS results with the strong up-regulation of CSTA and DNTT when compared to the control (Fig. 5a and 5b). Ribosomal protein 18S was employed as endogenous gene control.

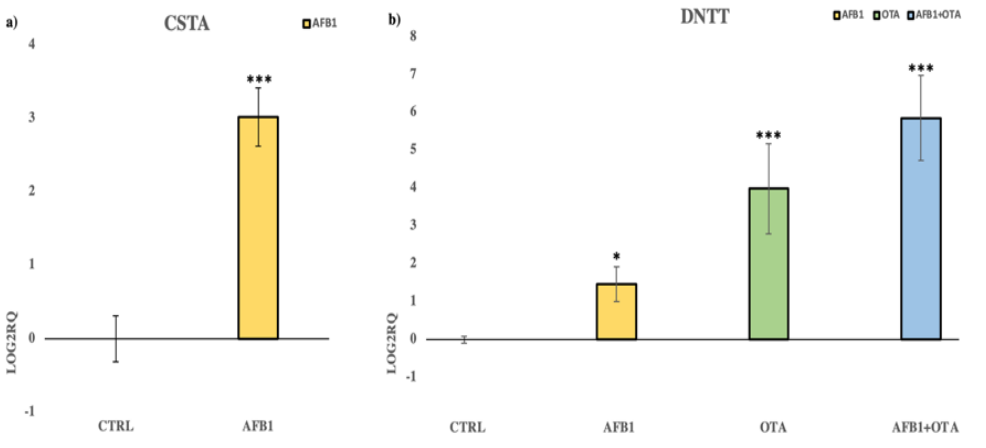

**Figure 5.** Bar plots showing the relative expression of: a) CSTA and b) DNTT when compared to the control (Log2RQ = 0) after 7 days of exposure in Jurkat cells to AFB1 (100 nM) for CSTA and to AFB1, OTA, AFB1+OTA (100 nM in all cases) for DNTT by qPCR. RQ: relative quantification; CTRL: control; CSTA: cystatin A; DNTT: DNA nucleotidylexotransferase. $p \leq 0.05$ (*), $p \leq 0.001$ (***).

## 4. Discussion

In the present study, the possible mechanism of action (MoA) by which low doses of AFB1 and OTA, comparable with those found in human blood, promoted immune toxicity was investigated after 7 days *in vitro* exposure. The choice of this exposure time reflects the peak of the immune response *in vivo*, which precisely occurs after day 7 of infection. Thereafter, the number of T lymphocytes undergoes a programmed contraction as well as the immune system gradually tends to deactivate (Badovinac et al., 2002; Lieberman et al., 2020). Reproducing a realistic scenario, it is interesting to observe as the number of DEGs was significantly increased in the combined exposure (3236) when compared to the individual ones (AFB1 DEGs=99 and OTA DEGs=77). This finding is relevant in human health risk assessment as the human population is constantly exposed to multiple mycotoxins contaminating food and our findings may suggest that AFB1 and OTA have an additive *in vitro* effect (Arce-Lopez et al., 2020). Similar results were obtained by Corcuera et al. (2011), exposing HepG2 liver cells with high concentrations of AFB1 (1.5-150 μM; 3 and 24h) and OTA (50-800 μM; 3 and

24h). It has been observed more prominent toxic effects in the combined exposure compared to individual treatment, suggesting a synergism between mycotoxins. In Vero kidney cells, AFB1 and OTA (1-50 μM; 24h) not only showed an additive cytotoxic effect but also synergism to promote genotoxicity with increased DNA fragmentation (Golli-Bennour et al., 2010). Sedmikova et al. (2001) also showed that OTA significantly increased AFB1 mutagenicity with a higher percentage of mutations than AFB1 alone, indicating the potential risk of mycotoxins co-occurrence. On the contrary, different findings were obtained by Choi et al. (2020) treating chicken LMH liver cells with AFB1 (0-3 μM; 48h) and OTA (0-20 μM; 48h). The cytotoxicity assay and transcriptome analysis revealed the antagonist effect between mycotoxins to induce chicken liver toxicity. The discrepancy of results was probably associated with the use of experimental conditions (doses, exposure time and non-human cell line) less realistic than those employed in the present study.

*4.1 AFB1 exposure*

ATM signaling was the most affected pathway after AFB1-exposure (Table 3). It is well-known that ATM plays a major role in the cellular response to DNA damage, by involving ATM protein kinase as the main downstream signal effector (Zhang et al., 2012). Several studies showed as *in vitro* and *in vivo* exposure to AFB1 induced DNA double-strand breaks (DSBs), activation of ATM signaling and the up-regulation of all ATM

kinases related to chromatin relaxation, cell cycle regulation and immunity (Sancar et al., 2004; Stracker et al., 2013; Yin et al., 2016). Yang et al. (2013) also demonstrated that low AFB1 doses (5-80 nM; 24h) were sufficient to promote DNA damage and ATM up-regulation in BEAS-2B cells with possible genome alterations in human respiratory system.

ATM signaling is also a core component of DNA repair system by activating the ubiquitin ligase RNF40, which in turn covers a key role in chromatin reorganization and timely DNA-DSBs repair (Jin & Oh, 2019; Moyal et al., 2011). The reduced expression of RNF40 was associated with replicative stress, chromosomal instability, cell cycle checkpoint inactivation and inhibition of DNA repair mechanism *in vitro* (Chernikova et al., 2012; Kari et al., 2011; Zhang et al., 2014). In turkey embryos, exposure to AFB1 (1µg/for injection; 24h) has been shown to downregulate the activity of ubiquitin ligase and impair the cellular response to genotoxic damage (Engin & Engin, 2019; Monsom et al., 2016). Therefore, the activation of ATM signaling with up-regulation of ATM kinases and RNF40 down-regulation pointed to the induction of DNA damage and the disruption of repair mechanism as possible MoA by which AFB1 induce immune toxicity *in vitro* (Fig. 2).

The central position of ATM signaling to maintain the genomic stability is demonstrated by its involvement in $G_2$/M cell cycle transition (Lavin & Kozlov, 2007). Yang et al. (2013) observed as AFB1-treatment

prompted the up-regulation of ATM kinase, checkpoint kinase 2 (chk2) and Ataxia telangiectasia and Rad3 related protein (ATR), which in turn caused $G_2$/M cell cycle arrest in bronchial epithelial cells. Similar findings were also observed in chicken bursa of Fabricius, spleen and jejunum, by confirming the correlation between *in vivo* AFB1-administration and the disruption of cell cycle machinery at $G_2$/M stage by ATM signaling activation (Hu et al., 2018; Li et al., 2019; Yin et al., 2016). In line with these findings, 7 days exposure to AFB1 can induce a potential alteration in cell cycle distribution (Fig. 2). However, gene expression changes (i.e ATR, chk2) are very mild and further research is needed to confirm this hypothesis.

Moreover, ATM signaling is involved in T cell development by controlling V(D)J recombination process, which is indispensable to constitute the variable domain of T cell receptor (Helmink & Sleckman, 2012; Nussenzweig and Nussenzweig, 2010). Alterations in ATM signaling and its main effectors can disrupt V(D)J recombination process and predispose human T cells to leukemia and lymphoma (Matei et al., 2006). In this context, Artemis protein, a well-known ATM-substrate, has a critical role in V(D)J recombination (Jeggo & Lobrich, 2005; Rivera-Munoz et al., 2016). It has been reported the association between *in vitro* deleterious effects such as immunodeficiency, cell cycle arrest and DNA damage and Artemis over-expression (Multhaup et al., 2010; Sridharan et al., 2012; Ulus-Senguloglu et al., 2012). Thus, ATM signaling activation with ATM kinase and Artemis

upregulation may suggest the possible interference of AFB1 in T cell recombination and functionality (Fig.2).

*4.2 OTA exposure*

Mycotoxins can induce *in vitro* toxicity by reprogramming cell metabolism (Prusinkiewicz & Mymry, 2019). Wang et al. (2020) showed as OTA (1.25-5 μM; 24h) promoted mitochondrial toxicity by reprogramming energy metabolism from oxidative phosphorylation (OxPhos) to glycolysis in human gastric cells. Similar metabolic disturbances were obtained by Zhao et al. (2021) in human esophageal cells after 24h exposure to OTA (2.5-10 μM; 24h) and by Zhang et al. (2011) analyzing the liver of rats treated with moderate doses of AFB1 in contaminated feed (1.6 mg/kg; 12 days). In line with these evidences, PathVisio analysis revealed the promotion of metabolic reprogramming and pointed to glycolysis as the most affected pathway after 7 days *in vitro* exposure to OTA (Table 4). In detail, the mild over-expression of RB1 and G6PD and the slight down-regulation of PGK1, PKM, GAPDH and LDHA were observed.

Retinoblastoma 1 (RB1) is a tumor suppressor involved in cell cycle regulation, cell differentiation, proliferation and death (Lai et al., 2006). Wu et al. (2020) demonstrated that RB1 over-expression was associated with metabolic changes and glycolysis promotion in human breast cells. Similarly, the up-regulation of glucose-6-phosphate dehydrogenase (G6PD),

a pivotal enzyme in NADPH production and cell redox balance, enhanced glycolysis activation in acute myeloid leukemia cells (Poulain et al., 2017). G6PD over-expression has been also observed in BEA and ENNB-promoted mitochondrial disturbance and reduced ATP production *in vitro* (Soderstrom et al., 2022).

Phosphoglycerate kinase 1 (PGK1) catalyzes the first reaction of anaerobic glycolysis (Fu & Yu, 2020). The microarray analysis performed in HepG2 cells after OTA-exposure (2.5-10 μM; 24h) showed the down-regulation of PGK1 and its involvement in OTA-disrupted liver metabolism (Hundhausen et al., 2008). Likewise, the reduced expression and activity of pyruvate kinase M2 (PKM2), which convert phosphoenolpyruvate to pyruvate with ATP production, has been correlated with OTA-induced metabolic disturbance and glycolysis in human gastric cells (Ferguson & Rathmell, 2008; Wang et al., 2020). Also, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), previously considered a simple housekeeping gene, has been showed to be involved in many cellular processes such as glycolysis and cell metabolism (Tristan et al., 2011). Karaman & Ozden (2019) observed the down-regulation of GAPDH expression with clear alterations in energy metabolism after exposure to ZEA (10 μM; 24h) in human breast MCF10F cells. Lastly, lactate dehydrogenase A (LDHA) catalyzes the last step of anaerobic glycolysis by converting pyruvate to lactate (Xian et al., 2015). Interestingly, the correlation between LDHA expression and Fusaric acid toxicity (4 and 256 μM; 6h). This neglected

foodborne mycotoxin downregulated LDHA gene expression, among others, and induced bioenergetic adaptations by switching energy metabolism from mitochondrial OxPhos to glycolysis in HepG2 cells (Abdul et al., 2020). The metabolic disturbance promoted by OTA-exposure in Jurkat cells is further supported by the alteration of HIF1α, PPAR-γ and Cori cycle signaling pathways (Table 4). HIF1a and PPAR-γ pathways are key regulators of metabolic reprogramming and glycolysis *in vitro* (Feng et al., 2020; Prigione et al., 2014) whereas Cori cycle is a typical metabolic route activated with high lactate concentrations (Suhara et al., 2015).

*4.3 AFB1-OTA combined exposure*

Ferroptosis was the main signaling pathway altered by the combined AFB1-OTA exposure with 85% of genes affected (Table. 5). Ferroptosis is a type of regulated necrosis triggered by the combination of excessive intracellular iron overload, induction of lipid peroxidation with plasma membrane damage and inhibition of glutathione peroxidase 4 (GPX4) activity (Chen et al., 2021).

The intracellular iron accumulation can be mediated by several factors. Firstly, transferrin (TF), a strong inducer of the process, is a carrier protein that bind extracellular ferric iron ($Fe^{3+}$) and transport it into cells through its transporter (TFR) by endocytosis (Shi et al., 2019). Zhao et al. (2021) demonstrated the strong correlation between TF over-expression and

ferroptosis induction in broiler heart upon AFB1-administration in contaminated feed (1 mg/kg; 21 days). It was also observed the down-regulation of Heat Shock Protein Family B1 (HSPB1), a negative regulator of intracellular iron uptake and accumulation, thus confirming the imbalance of iron levels in AFB1-promoted ferroptosis. Conversely, Ding et al. (2021) showed as TFR1 downregulation may lead to iron accumulation, oxidative stress and ROS generation *in vitro*. Indeed, RNA-seq analysis performed on DON-treated IPEC-J2 cells (0.5 μg/ml; 48h) revealed the alteration of several genes related to iron homeostasis, including the down-regulation of TFR1, which was considered a key initial signal of ferroptosis (Lin et al., 2021). Another pivotal driver of ferroptosis is the transcription factor BTB domain and CNC homolog 1 (BACH1), a regulator of iron metabolism. *In vitro* upregulation of BACH1 can induce the repression of several genes involved in iron storage (such as ferritin, a protein complex consisting of heavy, FTH1, and light chains, FTL), thereby causing the release of unstable iron into the cytoplasm and worsening the ferroptotic condition (Nishizawa et al., 2020; Xie et al., 2016).

High concentrations of free iron in the cytoplasm increase the production of endogenous hydrogen peroxide and hydroxyl radical by Fenton reaction, by promoting lipid peroxidation (Au et al., 2008; He et al., 2020). During ferroptosis, lipid oxidation occurs as an intermediate event, in which ASCL4 and ASCL3 are the main regulators (Magtanong et al., 2019; Lee et al., 2021). Wang et coworkers (2021) showed that T-2 toxin (2.5-10 nM

for 6-20h) promoted ferroptosis, lipid peroxidation and intracellular ROS generation through the alteration of several genes expression, including the slight downregulation of ASCL4. Likewise, the downregulation of ASCL3 has been related to ferroptosis, and oxidative stress *in vitro* and *in vivo* (Ubellacker et al., 2020).

GPX4, a selenium-dependent glutathione peroxidase, protects cells from lipid hydroperoxides, formed during oxidative stress, by using reduced glutathione as enzyme co-substrate (Dixon & Stockwell, 2019). In human T cells, the reduced GPX4-activity can lead to lipid peroxidation and ferroptotic cell death, altered cellular homeostasis, and increased susceptibility to acute infections (Chen et al., 2021; Matsushita et al., 2015). It has been reported that exposure to AFB1 and OTA downregulated GPX4 gene expression inducing lipid peroxidation, ROS generation and ferroptosis in several *in vitro* and *in vivo* models (Gan et al., 2015; Ren et al., 2019; Vettorazzi et al., 2019; Zhao et al., 2019). Based on these evidence, AFB1-OTA combined exposure may induce ferroptosis in Jurkat cells by altering the expression of several key genes involved in intracellular iron overload (TF, TFR1, BACH1, FTH1, FTL and NCO4), lipid peroxidation (ASCL4, ASCL3, SAT1) and antioxidant cell defense (GPX4) (Fig.4). Interestingly, AFB1 and OTA also altered PPAR-$\alpha$ signaling pathway, a novel route associated with ferroptosis due to its active function in lipid remodeling and peroxidation (Table 5) (Venkathes et al., 2020).

In the present study, AFB1 and OTA, individually and in combination, resulted in DNTT over-expression. qPCR analysis also confirmed the synergism between mycotoxins as their combined transcriptional effect on DNTT was more pronounced than individual exposure. DNTT plays a key role in T cell recombination and proliferation showing a strong anti-apoptotic function. Consequently, DNTT up-regulation has been reported to confer resistance to tumor cells against chemotherapeutic agents (Wernicke et al., 2012). Since the increased DNTT activity has been also found in peripheral blood cells derived from patients with acute lymphoblastic leukemia, it cannot be excluded that exposure to AFB1 and OTA may worsen the leukemic condition (Martínez-Jiménez et al., 2019; Ulger et al., 2020; Yasmineh et al., 1980).

## 5. Conclusion

These results contribute to a better understanding of the main cellular pathways involved in AFB1 and OTA induced immune toxicity in Jurkat cells. RNA-seq analysis revealed that ATM signaling pathway was the most altered in AFB1-exposure. In detail, potential alterations in $G_2$/M cell cycle checkpoint, T cell recombination and functionality, and induction of DNA damage with impairment of repair mechanisms have been mainly related to AFB1-toxicity. Regarding OTA-treatment, glycolysis signaling pathway was the most affected. Therefore, energy metabolism reprogramming by glycolysis activation may be considered as the main

OTA-MoA in Jurkat cells. In combination, AFB1 and OTA mainly affected the ferroptosis signaling pathway. In this case, the potential accumulation of intracellular iron with lipid peroxidation and oxidative stress accompanied by the disruption of antioxidant cell defense may explain the intrinsic MoA by which the mixture of mycotoxins promoted immune toxicity *in vitro.* Although the transcriptome analysis identified the main pathways altered by low doses of AFB1 and OTA in a human T cell line, further investigations are required to confirm these hypotheses and to better explore the underlying mechanism by which AFB1 and OTA may weaken the immune system, by rendering it more susceptible to infections.

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## 3.4. The protective effect of pumpkin and fermented whey mixture against AFB1 and OTA immune toxicity *in vitro*. A transcriptomic approach

Molecular Nutrition and Food Research (under revision)

# The protective effect of pumpkin and fermented whey mixture against AFB1 and OTA immune toxicity *in vitro*. A transcriptomic approach

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**Abstract**

Aflatoxin B1 (AFB1) and ochratoxin A (OTA) are well-known to be immunotoxic agents *in vitro* and *in vivo*, which can be modulated by functional compounds like pumpkin (P) and fermented whey (FW). Therefore, human lymphoblastic T cells were exposed during 7 days to digested bread extracts containing P-FW mixture along with AFB1 and OTA, individually and combined, in order to assess the potential effect of bioactive compounds mixture against mycotoxins-induced alterations in gene expression profile. RNA-sequencing analysis showed that AFB1 P-FW exposure resulted in 34 differentially expressed genes (DEGs) while 3450 DEGs were found in OTA P-FW exposure and 3264 DEGs in AFB1-OTA P-FW treatment. Gene ontology analysis revealed biological processes and molecular functions related to immune system and inflammatory response. Moreover, PathVisio analysis pointed to eicosanoid signaling via lipoxygenase as the main pathway altered by AFB1 P-FW exposure whereas interferon signaling was the most affected pathway after OTA P-FW and AFB1-OTA P-FW treatments. In all cases, the mitigation of genes and inherent pathways typically associated with the inflammatory response suggested the beneficial and protective role of P-FW mixture against AFB1 and OTA promoted inflammation and immune toxicity *in vitro*.

**Keywords:** Mycotoxins; Bioactive compounds; Jurkat cells; RNA-sequencing; Inflammation.

**Abbreviations**

AFB1: Aflatoxin B1

AFM1: Aflatoxin M1

ALOX12: Arachidonate 12-Lipoxygenase

ALOX15: Arachidonate 15-Lipoxygenase

AST: Astaxanthin

BEA: Beauvericin

BP: Biological process

CC: Cellular component

COX: Cyclooxygenase

DAVID: Database for Annotation, Visualization and Integrated Discovery

DEG: Differentially expressed gene

FC: Fold change

FW: Fermented Whey

GO: Gene ontology

GPR17: G protein-coupled receptor

IFN: Interferon

IRF3: Interferon regulator factor 3

IRF7: Interferon regulator factor 7

LAB: Lactic acid bacteria

LOX: Lipoxygenase

LTA4H: Leukotriene A4 Hydrolase

MF: Molecular function

OTA: Ochratoxin A

P: Pumpkin

RHOB: Ras Homolog Family Member B

RQ: Relative quantification

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2

Seq: Sequencing

TIGD5: Tigger Transposable Element Derived 5

TLR: Toll-like receptors

TNF: Tumor necrosis factor

TRAF3: TNF Receptor Associated Factor 3

TRAF6: TNF Receptor Associated Factor 6

ZEA: Zearalenone

## 1.Introduction

Aflatoxin B1 (AFB1) is a polyketide compound synthesized by *Aspergillus flavus* and *parasiticus*, which toxic effects have been reported in liver, kidney, brain and immune system. Thus, AFB1 has been classified by the International Agency for Research on Cancer (IARC) in the group 1 as human carcinogen (EFSA, 2020a). This mycotoxin is commonly found in cereals, oil seeds, dried fruits, spices, milk and meat. In order to protect human health, the European Union (EU) sets the maximum tolerable limits of AFB1 at 2–5 μg/kg in cereals, 5 μg/kg in spices, 2–8 μg/kg in dried fruits, 6 μg/kg in dietary foods for medical purpose and 0.1 μg/kg in foods for infants and young children (EC European Commission, 2006).

Another widespread mycotoxin, ochratoxin A (OTA), is mainly produced by *Penicillium* and *Aspergillus* species. Several studies have demonstrated that OTA-exposure can elicit nephrotoxicity and immune toxicity *in vitro* and *in vivo*. Hence, IARC has classified OTA in the group 2B as possibly carcinogenic to humans (EFSA, 2020b). Typical foods contaminated by OTA are cereals, wine, spices, meat and dried fruits. The EU established the maximum tolerable levels of OTA at 5 μg/kg in unprocessed cereals, 8 μg/kg in wheat gluten, 10 μg/kg in soluble coffee, 15 μg/kg in spices, 2 μg/kg in grape juice and wine whereas in infants and dietary foods for medical purpose at 0.5 μg/kg (EC European Commission, 2006).

Mycotoxin bioaccessibility is one of the main aspects to take into account for human health risk assessment. For instance, AFB1 and OTA are found in a wide range of food commodities, but nonetheless cereals and cereal-based products are the main foods to be contaminated (Luz et al., 2018b; Sarmast et al., 2021). Thus, AFB1 and OTA bioaccessibility was widely assessed in cereals by using *in vitro* human digestion models. In relation to AFB1, high values of intestinal bioaccessibility were obtained. In detail, values of 87% in wheat, 88-92% in maize, 95% in corn and 114% in bread were detected (Kabak and Ozbey, 2012; Simla et al., 2009; Escrivá et al., 2022; Saladino et al., 2018). Regarding OTA, values of 100% in buckwheat, 99% in cereal-based foods and 38-88% in bread were observed (Versantvoort et al., 2005; Assunção et al., 2016; Luz et al., 2018a; Escrivá et al., 2022). Once absorbed, AFB1 and OTA can reach the bloodstream and induce severe systemic effects, such as the impairment of immune system function (Manyes & Font, 2022).

Food processing and innovative technologies can reduce mycotoxin contamination by promoting its degradation, even though the risk to humans cannot be eliminated (Gavahian et al., 2020). Nowadays, the use of bioactive compounds has been proposed as a new food strategy to mitigate mycotoxins absorption and prevent their systemic effects on human and animal health (Escrivá et al., 2022). Pumpkin, which belongs to *Cucurbitaceae* family, is rich in carotenoids (β-carotene, lutein, lycopene and zeaxanthin)

that play an important role to protect cells from oxidative stress and damage, by preventing the onset of several human diseases (Bergantin et al., 2018). Milk whey, a cheese by-product with high nutritional values, is an excellent source of functional compounds, such as antifungal peptides, and an optimal substrate for lactic acid bacteria (LAB) fermentation (Gupta and Prakash, 2017).

The addition of these functional ingredients in bread formulation improved its shelf life, inhibited the growth of mycotoxigenic fungi, reduced AFB1 and OTA production as well as their intestinal bioaccessibility (Sadeghi et al., 2019; Luz et al., 2020). Escrivá et al. (2022) showed how the introduction of pumpkin (P) in bread preparation significantly reduced bioaccessibility values by up to 74% for AFB1 and 34% for OTA. Fermented whey (FW) and the combination of P-FW showed intestinal bioaccessibility reductions between 57 and 68% for AFB1, and between 11 and 20% for OTA. A large reduction of bioaccessibility value by up to 95% was also observed for both toxins after including specific LAB strains in contaminated breads (Luz et al., 2018a; Saladino et al., 2018). Carotenoids and FW-derived bioactive compounds also reported high *in vitro* bioavailability values (10-60% and 10-22%, respectively), thus showing the ability to reach the bloodstream and potentially exert a systemic effect (Escrivá et al., 2021; Juan et al., 2022). The beneficial role of pumpkin has been also proved at mitochondrial transcriptional level after AFB1 and OTA exposure in a blood-brain barrier *in vitro* model (Alonso-Garrido et al., 2020; 2021a; Frangiamone

et al., 2021). FW-derived bioactive compounds can also inhibit AFB1 and OTA production and toxicity *in vitro* (Illueca et al., 2021). Moreover, it is interesting to emphasize the synergistic and additive effect of P and FW *in vitro* and *in vivo.* In SH-SY5Y cells, the addition of P-FW mixture in AFB1 and OTA contaminated breads not only enhanced the expression of typical neuronal differentiation markers, but also attenuated cell cycle alterations and improved the overall gene expression impaired by mycotoxins exposure (Frangiamone et al., 2022b). In spontaneously hypertensive rats, the administration of pumpkin pectin and milk whey mixture improved blood pressure and gut microbiota dysbiosis (Agarkova et al., 2019).

Therefore, the aim of the present study was to evaluate the potential effect of P-FW mixture against AFB1 and OTA promoted immune toxicity at transcriptional level in human lymphoblastic Jurkat cells by using digested bread extracts.

## 2. Material and methods

### *2.1. Reagents*

The reagent grade chemicals and cell culture compounds used, Roswell Park Memorial Institute (RPMI)-glutamax medium, fetal bovine serum (FBS), penicillin/streptomycin and phosphate buffer saline were purchased by Sigma chemical Co (St. Louis, MO, USA) while methanol was

acquired from Fisher Scientific (Madrid, Spain). Deionized water (<18, MΩcm resistivity) was obtained by Milli-QSP ® Reagent Water System (Millipore, Bedford, MA, USA). Natural ingredients for bread preparation: pumpkin, wheat flour, mineral water, salt (NaCl), and sugar (sucrose) were acquired from a commercial supermarket (Valencia, Spain). Goat milk whey coagulated by commercial rennet (starter culture R-604) was purchased from the ALCLIPOR society, S.A.L. (Benassal, Spain) while LAB used in this study (*Lactobacillus Plantarum CECT 220*) was obtained from CECT (Paterna, Valencia, Spain).

*2.2. Cell culture and treatments exposure*

Jurkat cells derived from human T lymphocytes peripheral blood were maintained in RPMI-glutamax medium supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin and 10% FBS. The incubation conditions were pH 7.4, 5% $CO_2$ at 37 °C and 95% air atmosphere at constant humidity. The medium was changed every 2-3 days. To achieve the goal of the study, Jurkat cells were seeded at a density of $3x10^5$ cells/mL in 6 well plates and treated during 7 days with bread extracts obtained after a simulated *in vitro* human digestion. These extracts, containing the combination of P and FW (both at 1% w/w) along with mycotoxins (AFB1, OTA and their combination), were added to the culture medium using a final dilution of 1:10. Table 1 showed the average concentration and profile of carotenoids identified in digested bread extracts using the method described

by Juan et al. (2022). Table 2 shows AFB1 and OTA concentrations in digested bread extract after the dilution 1:10. Mycotoxins doses were measured as explained by Escrivá et al. (2022).

**Table 1.** Average concentration (μg/mL) and profile of carotenoids identified in digested bread extracts.

| | Carotenoids concentration (μg/mL) | | | | | |
|---|---|---|---|---|---|---|
| **Bread type** | β-carotene | Lutein | Antheraxanthin | Violaxanthin | Zeaxanthin | cryptoxanthin |
| **P-FW** | 1.8636 | 0.0180 | n.d. | 3.3676 | n.d. | 2.9880 |
| **AFB1 P-FW** | 2.5152 | 0.0122 | 0.0054 | 3.0368 | 0.0054 | 2.3976 |
| **OTA P-FW** | 4.0000 | 0.0120 | n.d. | 3.3971 | n.d. | 2.3614 |
| **AFB1-OTA P-FW** | 2.2879 | 0.0086 | 0.0580 | 2.7426 | 0.0580 | 1.8554 |

n.d.: not detected.

**Table 2.** AFB1 and OTA concentrations in digested bread extracts after a dilution of 1:10.

| **Treatment** | **AFB1 (nM)** | **OTA (nM)** |
|---|---|---|
| **P-FW** | - | - |
| **AFB1 P-FW** | 196 | - |
| **OTA P-FW** | - | 1037 |
| **AFB1-OTA P-FW** | 187 | 837 |

*2.3. RNA extraction and next generation sequencing (NGS)*

Total RNA of the control and treated Jurkat cells was isolated through ReliaPrep™ RNA Cell Miniprep System Kit (Promega, USA) and purified to remove genomic DNA contamination. The RNA extracted from each sample was checked for quantity and quality using a NeoDot UV/Vis Nano Spectrophotometer (Quimigen, Madrid, Spain) showing appropriate 260/280 nm and 260/230 nm ratios both around 2. All sequenced samples were generated from high quality RNA samples having an RNA Integrity Number > 8. The standard Illumina protocol was followed to develop RNA-seq libraries, using TruSeq stranded mRNA. Then, Illumina NextSeq 500 platform was employed for sequencing. RNA-seq data acquisition, which consists of several steps from raw reads to read alignment and quantification, was specifically checked at each step. The percentage of mapped reads, which indicates high sequencing accuracy and low presence of contaminating DNA was calculated by FastQC version 0.11.8 (Andrews, 2010). Afterwards, one archive for each sample was obtained. RNA quality control and sequencing were performed by the Genomics section of the Central Service for Experimental Research (SCSIE, University of Valencia).

*2.4. Data processing*

The quality control (QC) of reads was performed by FastQC software v0.11.8 (Andrews, 2010). The trimming was performed with FASTX-Toolkit

v0.13, discarding the low-quality bases from 5'- and 3'- extremes (Hannon, 2010). Then, low quality reads and the identical sequences found in each file were collapsed into a single sequence while reads counts were maintained. The trimmed reads alignment was carried out by Spliced Transcripts Alignment to a Reference (STAR) software v2.7, using as reference the Genome Reference Consortium Human Build 38 version (Dobin et al., 2013). The Sequence Alignment Map (SAM) files were transformed in their binary version (BAM), ordered and indexed by SAM tools software v1.10 (Li et al., 2009). BAM files were used with STAR to generate an expression matrix in *R* software (Team R C., 2018). Annotation, normalization and statistical analysis to contrast differential expression between treatments and control were carried out according to the user guide of *edgeR* package, by using gene wise negative binomial generalized linear models with quasi-likelihood tests (Robinson et al., 2010). Differentially expressed genes (DEGs) with p-value ≤ 0.05 were considered significant. Gene expression compared to the control was expressed as the logarithm of the fold change (log2FC). Venn diagrams and heat maps were plotted by *Vennerable* and *pheatmap* packages assessing coincident DEGs between conditions (Alonso-Garrido et al., 2018; Escrivá et al., 2018; Kolde, 2019; Swinton, 2013).

### *2.5. Gene expression analysis*

The selected DEGs were submitted to over-representation and gene ontology (GO) analysis by DAVID bioinformatics resources in order to

obtain GO annotations and functional DEGs classifications (Huang et al., 2009a, 2009b). Pathway assignments were carried out through PathVisio software using WikiPathways as biological pathways database (Martens et al., 2021; Kutmon et al., 2015). Z-score > 1.96 and *adj.* $p \leq 0.05$ were used as thresholds to identify significant pathways.

### *2.6. Gene selection and primer design*

Gene-specific primers were designed by Primer-BLAST using the default software settings and with amplified PCR products ranging from 203 to 228 bp and Tm at 57-60 °C. Standard curves by qPCR were performed for all primers and a single amplification product for each gene was obtained by the melting curve assay in StepOne Plus Real-time PCR instrument (Applied Biosystems, CA, USA). Primer amplification efficiency was determined from the standard curve generated by serial dilution of cDNA (5-fold each) for each gene. Correlation coefficient ($R^2$ values; i.e linearity) and amplification efficiencies were calculated from the slope of the regression line by plotting Ct mean values against the log cDNA dilution factor. The gene-specific primers used in the present study are listed in Table 3.

**Table 3.** Gene names, forward and reverse primer sequences, PCR efficiency and linearity value for the target genes and the reference gene GAPDH.

| Gene | Primer forward sequence | Primer reverse sequence | Efficiency (%) | Linearity |
|---|---|---|---|---|
| **GAPDH** | GGAGTCCACTGGCGTCTT | GAGTCCTTCCACGATACCAAA | 110 | 0.991 |
| **RHOB** | TCGTGTTCAGTAAGGACGAG | ACTTCTCGGGGATGTTCTC | 108 | 0.996 |
| **TIGD5** | TGGAGCCACCTGACTTACCT | AGAACCAGGGTTTGACAGGC | 94 | 0.990 |

*2.7. Reverse transcription and qPCR*

Also for qPCR analysis, total RNA of the control and treated cells was isolated using ReliaPrep™ RNA Cell Miniprep System Kit (Promega, USA) and purified to remove genomic DNA contamination. The RNA extracted from each sample was checked for quantity and quality using a NeoDot UV/Vis Nano Spectrophotometer (Quimigen, Madrid, Spain) showing concentrations around 500 ng/μl along with appropriate 260/280 nm and 260/230 nm ratios both around 2. RNA samples were stored at −20 °C until their dilution to 100 ng/μl with sterilized Milli-Q $H_2O$. Then, cDNA was synthesized by TaqMan Reverse Transcription Kit (Applied Biosystems). Real-time amplification reactions were performed in 96 well plates using SYBR Green detection chemistry and were run in triplicate by StepOne Plus Real-time PCR instrument (Applied Biosystems, CA, USA). Reactions were prepared as follows: 100 ng of template, 500 nM of each primer, the required

amount of 2x Fast SYBR Green in a reaction volume of 10 μL. The PCR temperature cycling conditions for Ras Homolog Family Member B (RHOB) and Tigger Transposable Element Derived 5 (TIGD5) were as follows: initial denaturation step at 95 °C for 5 min to activate Taq DNA polymerase, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 15 s. The melting curve was generated by heating the amplicon from 60 to 90 °C. Threshold cycles (Ct) and relative quantification (RQ) were automatically generated by StepOne Plus Software version 2.3. Experiments were carried out in triplicate according to MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Bustin et al., 2009).

*2.8. Statistical analysis*

Statistical analysis was performed using the ΔCt (experimental Ct – housekeeping Ct mean) obtained by StepOne Plus Software version 2.3. Group variances were assessed by Levene's test and all group variances were equal. Differences between groups were analyzed by T-student test. A p value ≤ 0.05 was considered for statistically significant differences.

## 3. Results

### *3.1. Gene expression profile*

The overall gene expression of Jurkat cells treated during 7 days with AFB1 P-FW, OTA P-FW and AFB1-OTA P-FW significantly differed from the control. Using a p-value ≤ 0.05 and log2FC |1.0|, AFB1 P-FW exposure resulted in 34 DEGs with 23 up- and 11 downregulated, 3450 DEGs (2135 up- and 1315 downregulated) were found in OTA P-FW exposure and 3264 DEGs (2050 up- and 1214 downregulated) in the combined treatment. Considering all conditions, the total number of DEGs was 6748 with 62.3% upregulation and 37.7% downregulation, compromising the usual expression levels up to 32% of human genome, which contains 21000 protein-coding genes (HGNC database). Venn diagram showed that no gene overlapped between exclusively AFB1 and OTA exposure, only one (UMODL1) in AFB1 and combined exposure, and 2927 genes (78%) between OTA and mixed treatment (Fig.1). From the 30 DEGs (0.8%) overlapping in all studied conditions, 20 were upregulated and 10 downregulated, reaching log2FC values up to 4.00 in overexpression (GPR17 and TIGD5) and approximately -4.00 in repression (RHOB) (Fig.2).

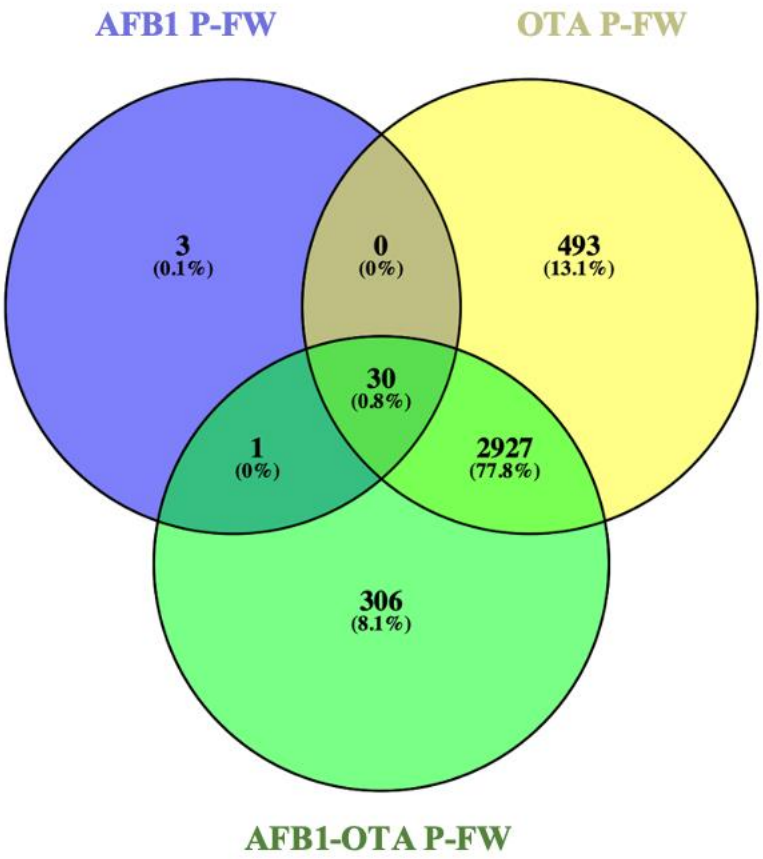


**Figure 1.** Venn diagram for the DEGs obtained in Jurkat cells treated during 7 days with AFB1 P-FW (196 nM), OTA P-FW (1037 nM) and AFB1-OTA P-FW (187-837 nM). DEGs p-value ≤ 0.05 and log2FC |1.0|. DEGs: Differentially expressed genes; P: Pumpkin; FW: Fermented Whey; FC: Fold change.

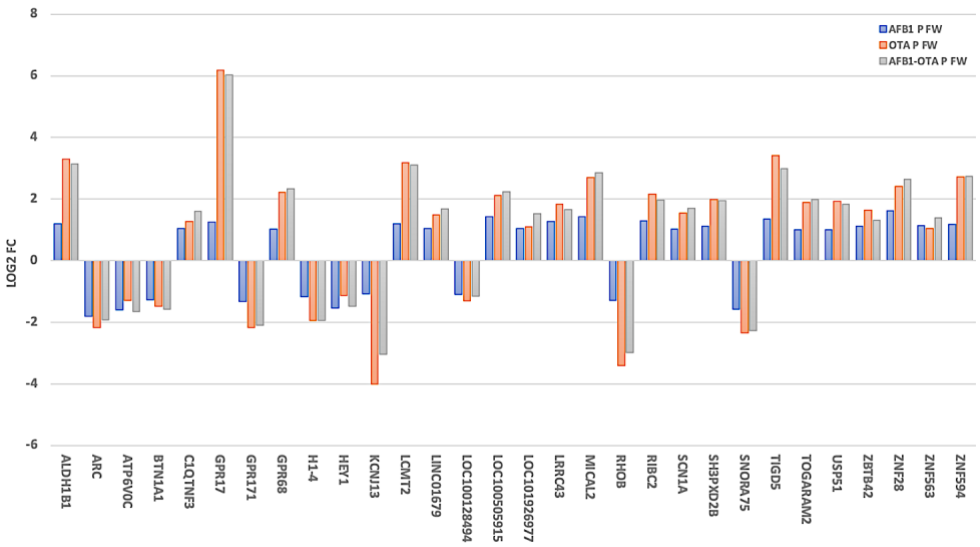


**Figure 2**. Log2FC values for the 30 DEGs overlapped in all studied conditions: AFB1 P-FW (196 nM), OTA P-FW (1037 nM) and AFB1-OTA P-FW (187-837 nM). DEGs p-value ≤ 0.05 and log2FC |1.0|. DEGs: Differentially expressed genes; P: Pumpkin; FW: Fermented Whey; FC: Fold change.

### *3.2. Gene ontology analysis*

In transcriptomics studies, a critical step is the characterization of biological processes (BP), molecular functions (MF) and cellular component (CC) in which DEGs are implicated by comparing the DEGs list with the rest of genome for over-represented functions and gene set enrichment analysis (Conesa et al., 2016).

In relation to AFB1 P-FW exposure, DAVID analysis provided a list of GO terms in which regulation of cell morphogenesis and development were the most over-represented BP. Double-stranded DNA binding was the only MF to be statistically significant in the DEGs list while actin cytoskeleton was the unique CC to be significantly represented after the treatment (Table 4).

In OTA P-FW exposure, some of the most over-represented BP were interferon type I signaling pathway, cellular response to cytokine, T cell differentiation and blood vessel development. MF related to double-stranded DNA binding, chemokine and cytokine receptor activity were statistically significant in the DEGs list. CC such as plasma membrane, ion channel complex, chromatin and MHC protein complex were significantly affected (Table 4).

In the mixed condition, DAVID analysis showed as the regulation of adaptative immune response, immune system development, lymphocyte activation, interferon type I signaling pathway and lymph vessel development were some of the most over-represented BP. MF linked to double-stranded DNA binding, chemokine and cytokine receptor activity were statistically significant in the DEGs set. CC like plasma membrane, ion channel complex, MHC protein complex and chromatin were significantly represented to a greater extent (Table 4).

**Table 4.** Relevant GOs resulted from the over-representation analysis of DEGs obtained by treatments through DAVID bioinformatics resources. DEGs p-value ≤ 0.05 and log2FC |1.0|.

| Treatment | Category | Term | Level | Count | % | P-Value |
|---|---|---|---|---|---|---|
| **AFB1 P-FW** | | | | | | |
| | GO:0022604 | regulation of cell morphogenesis | BP5 | 4 | 11.8 | <0.05 |
| | GO:0060284 | regulation of cell development | BP5 | 5 | 14.7 | <0.05 |
| | GO:0003690 | double-stranded DNA binding | MF5 | 6 | 17.6 | <0.01 |
| | GO:0015629 | actin cytoskeleton | CC5 | 4 | 11.8 | <0.05 |
| **OTA P-FW** | | | | | | |
| | GO:0071345 | cellular response to cytokine stimulus | BP5 | 207 | 6.0 | <0.001 |
| | GO:0030217 | T cell differentiation | BP5 | 56 | 1.6 | <0.001 |
| | GO:0034340 | response to type I interferon | BP5 | 27 | 0.8 | <0.01 |
| | GO:0001568 | blood vessel development | BP5 | 135 | 3.9 | <0.01 |
| | GO:0003690 | double-stranded DNA binding | MF5 | 353 | 10.2 | <0.001 |
| | GO:0004950 | chemokine receptor activity | MF5 | 11 | 0.3 | <0.01 |
| | GO:0004896 | cytokine receptor activity | MF5 | 24 | 0.7 | <0.05 |
| | GO:0044459 | plasma membrane part | CC5 | 513 | 14.9 | <0.001 |
| | GO:0034702 | ion channel complex | CC5 | 66 | 1.9 | <0.001 |
| | GO:0000785 | chromatin | CC5 | 212 | 6.1 | <0.05 |
| | GO:0042611 | MHC protein complex | CC5 | 10 | 0.3 | <0.05 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **AFB1-OTA P-FW** | | | | | | |
| | GO:0060337 | type I interferon signaling pathway | BP5 | 26 | 0.8 | <0.001 |
| | GO:0001945 | lymph vessel development | BP5 | 11 | 0.3 | <0.01 |
| | GO:0002520 | immune system development | BP5 | 170 | 5.2 | <0.01 |
| | GO:0046649 | lymphocyte activation | BP5 | 135 | 4.1 | <0.01 |
| | GO:0002821 | regulation of immune response | BP5 | 26 | 0.8 | <0.05 |
| | GO:0003690 | double-stranded DNA binding | MF5 | 336 | 10.3 | <0.001 |
| | GO:0004950 | chemokine receptor activity | MF5 | 11 | 0.3 | <0.01 |
| | GO:0004896 | cytokine receptor activity | MF5 | 25 | 0.8 | <0.01 |
| | GO:0044459 | plasma membrane part | CC5 | 499 | 15.3 | <0.001 |
| | GO:0034702 | ion channel complex | CC5 | 63 | 1.9 | <0.001 |
| | GO:0000785 | chromatin | CC5 | 199 | 6.1 | <0.05 |
| | GO:0042611 | MHC protein complex | CC5 | 10 | 0.3 | <0.05 |

### *3.3. PathVisio analysis*

#### *3.3.1. AFB1 P-FW condition*

PathVisio analysis revealed a total of 3596 data points (N) and only 108 of them met criterion (R), in which (N) indicates the total number of genes measured in the dataset whereas (R) denotes the filter criterion of the analysis (Kutmon et al., 2015). Numerous pathways were statistically significant after AFB1 P-FW exposure (Z-score >1.96; *adj.* p-value ≤ 0.05). It is interesting to observe as three different pathways of eicosanoid metabolism (via lipoxygenases, cyclooxygenases and cytochrome P450 monooxygenases) were statistically altered with 29%, 21% and 50% of genes affected, respectively. The analysis also showed a significant alteration in SARS coronavirus and innate immunity (17% of genes affected), PI3K/AKT/mTOR (14%) and Wnt/beta catenin (14%) signaling pathways (Table 5).

**Table 5.** Pathways overlapped in AFB1 P-FW exposure (196 nM) by PathVisio.

| Pathway | Positive (r) | Measured (n) | Total | % | Z Score | *adj.* p- value |
|---|---|---|---|---|---|---|
| Eicosanoid metabolism via lipooxygenases | 5 | 17 | 70 | 29 | 6.39 | <0.01 |
| Eicosanoid metabolism via cytochrome P450 monooxygenases | 2 | 4 | 23 | 50 | 5.51 | <0.01 |
| Eicosanoid metabolism via cyclooxygenases | 3 | 14 | 70 | 21 | 4.05 | <0.01 |
| SARS coronavirus and innate immunity | 2 | 12 | 33 | 16 | 2.78 | <0.05 |
| PI3K/AKT/mTOR - VitD3 signaling | 2 | 14 | 34 | 14 | 2.48 | <0.05 |
| Regulation of Wnt / B-catenin signaling | 2 | 14 | 33 | 14 | 2.48 | <0.05 |

According to Z-score value, the most affected pathway was eicosanoid metabolism via lipoxygenases (6.39). Figure 3 showed the genes involved, indicating in red and blue the overexpressed and downregulated, respectively. ALOX12 and ALOX15, key pathway regulators, were the most affected genes.

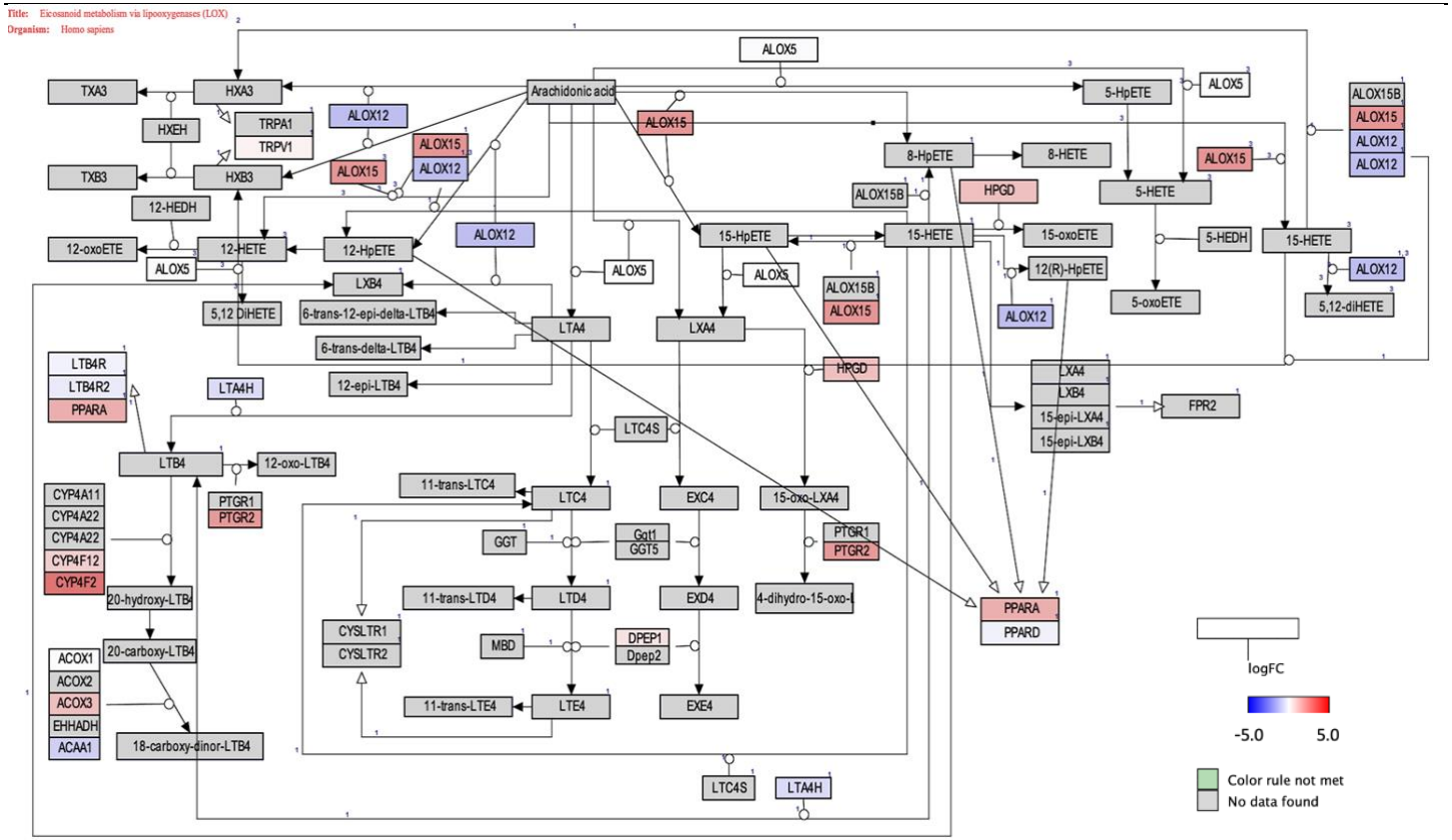
Title: Eicosanoid metabolism via lipooxygenases (LOX)
Organism: Homo sapiens
ALOX5
TXA3
HXA3
Arachidonic acid
5-HpETE
ALOX5
ALOX15B
ALOX15
ALOX12
ALOX12
HXEH
TRPA1
TRPV1
ALOX12
ALOX15
ALOX15
ALOX12
ALOX15
8-HpETE
8-HETE
ALOX15
TXB3
HXB3
ALOX15
ALOX15B
HPGD
5-HETE
12-HEDH
12-oxoETE
12-HETE
12-HpETE
ALOX12
15-HpETE
15-HETE
15-oxoETE
5-HEDH
15-HETE
ALOX5
ALOX5
ALOX5
ALOX15B
ALOX15
12(R)-HpETE
ALOX12
ALOX12
LXB4
5-oxoETE
5,12 DiHETE
6-trans-12-epi-delta-LTB4
LTA4
LXA4
5,12-diHETE
6-trans-delta-LTB4
HPGD
LXA4
LXB4
15-epi-LXA4
15-epi-LXB4
FPR2
LTB4R
LTB4R2
PPARA
LTA4H
12-epi-LTB4
LTC4S
LTB4
12-oxo-LTB4
11-trans-LTC4
LTC4
EXC4
15-oxo-LXA4
CYP4A11
CYP4A22
CYP4A22
CYP4F12
CYP4F2
PTGR1
PTGR2
GGT
Ggt1
GGT5
PTGR1
PTGR2
20-hydroxy-LTB4
11-trans-LTD4
LTD4
EXD4
4-dihydro-15-oxo-L
PPARA
PPARD
CYSLTR1
CYSLTR2
MBD
DPEP1
Dpep2
ACOX1
ACOX2
ACOX3
EHHADH
ACAA1
20-carboxy-LTB4
11-trans-LTE4
LTE4
EXE4
18-carboxy-dinor-LTB4
LTC4S
LTA4H
logFC
-5.0
5.0
Color rule not met
No data found

**Figure 3.** Genes involved in eicosanoid metabolism via lipoxygenases for *Homo sapiens* are shown: in red the upregulated genes and in blue the downregulated upon AFB1 P-FW exposure (196 nM). P: Pumpkin; FW: Fermented Whey.

### *3.3.2. OTA P-FW condition*

The analysis conducted by PathVisio showed a total of 3696 data points, of which a large number (2557) met the criterion. Several pathways related to immune and inflammatory responses were statistically significant upon OTA P-FW treatment (Z-score > 1.96; *adj* p-value ≤ 0.05). Based on the number of genes affected, all pathways showed more than 85% of genes affected. The 100% of genes involved in type I interferon induction, TRL4 signaling, SARS coronavirus and innate immunity, and development of immune cells were significantly altered (Table 6).

**Table 6.** Pathways overlapped in OTA P-FW exposure (1037 nM) by PathVisio.

| Pathway | Positive (r) | Measured (n) | Total | % | Z Score | *adj*.p-value |
|---|---|---|---|---|---|---|
| Host-pathogen interaction of human coronaviruses induced interferon signaling | 30 | 31 | 47 | 96 | 3.34 | <0.01 |
| Type I interferon induction and signaling during SARS-CoV-2 | 22 | 22 | 45 | 100 | 3.14 | <0.01 |
| SARS coronavirus and innate immunity | 13 | 13 | 33 | 100 | 2.41 | <0.01 |
| TLR4 signaling and tolerance | 13 | 13 | 30 | 100 | 2.41 | <0.05 |
| Interleukin-1 structural pathway | 34 | 40 | 52 | 85 | 2.18 | <0.05 |
| Toll-like receptor signaling related to MyD88 | 19 | 21 | 32 | 90 | 2.12 | <0.05 |
| Development of dendritic cells and macrophage subsets | 9 | 9 | 14 | 100 | 2.00 | <0.05 |

The genes associated with host-pathogen interaction of human coronaviruses induced interferon signaling (Z-score: 3.34) were showed in Fig.4. High fold changes values reaching up to 2.90 in overexpression (RIPK1) and -4.56 in repression (FOS) were found

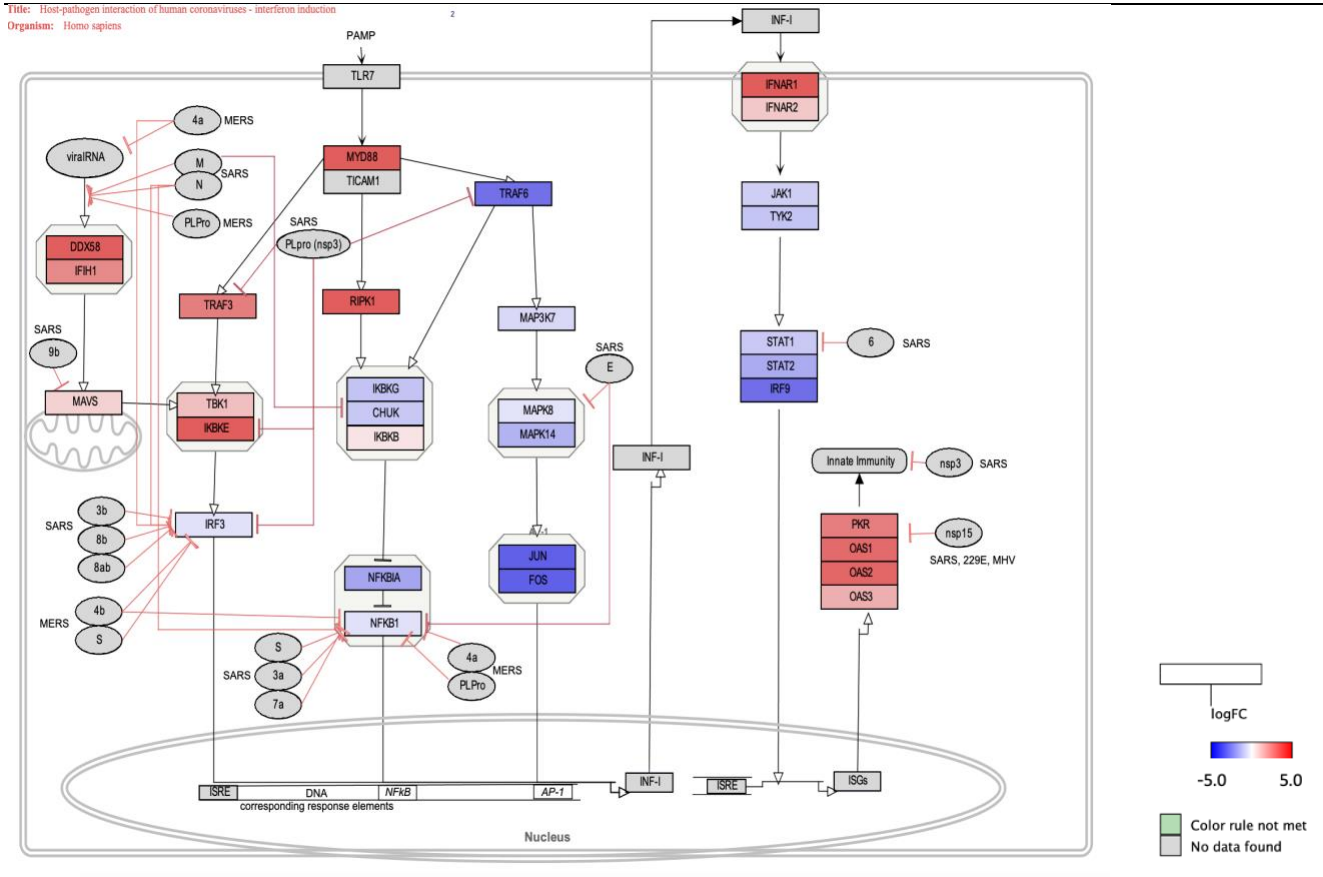
Title: Host-pathogen interaction of human coronaviruses - interferon induction
Organism: Homo sapiens
PAMP
TLR7
4a
MERS
viralRNA
M
N
SARS
PLPro
MERS
MYD88
TICAM1
TRAF6
SARS
PLpro (nsp3)
DDX58
IFIH1
TRAF3
RIPK1
MAP3K7
SARS
9b
MAVS
TBK1
IKBKE
IKBKG
CHUK
IKBKB
MAPK8
MAPK14
SARS
E
INF-I
SARS
3b
8b
8ab
IRF3
NFKBIA
NFKB1
JUN
FOS
MERS
4b
S
S
SARS
3a
7a
4a
MERS
PLPro
ISRE
DNA
NFkB
AP-1
corresponding response elements
INF-I
Nucleus
INF-I
IFNAR1
IFNAR2
JAK1
TYK2
STAT1
STAT2
IRF9
6
SARS
Innate Immunity
nsp3
SARS
PKR
OAS1
OAS2
OAS3
nsp15
SARS, 229E, MHV
ISRE
ISGs
logFC
-5.0
5.0
Color rule not met
No data found

**Figure 4.** Genes involved in host-pathogen interaction of human coronaviruses induced interferon signaling for *Homo sapiens* are shown: in red the upregulated genes and in blue the downregulated after OTA P-FW exposure (1037 nM). P: Pumpkin; FW: Fermented Whey.

### *3.3.3. AFB1-OTA P-FW condition*

PathVisio assessment indicated a total of 3681 data points, of which a higher number (2413) matched the criterion. In this case, only 6 routes were statistically significant following the combined exposure (Z-score > 1.96; *adj* p-value ≤ 0.05). As shown in Table 7, some pathways found in the individual exposures were similarly affected by the mixed condition. Also in this case, all pathways found showed more than 80% of genes affected.

**Table 7.** Pathways overlapped in AFB1-OTA P-FW exposure (187-837 nM) by PathVisio.

| Pathway | Positive (r) | Measured (n) | Total | % | Z Score | adj. p-value |
|---|---|---|---|---|---|---|
| Type I interferon induction and signaling during SARS-CoV-2 infection | 22 | 22 | 45 | 100 | 3.41 | <0.01 |
| Host-pathogen interaction of human coronaviruses - interferon induction | 27 | 31 | 47 | 87 | 2.53 | <0.01 |
| SARS coronavirus and innate immunity | 12 | 12 | 33 | 100 | 2.51 | <0.01 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Regulation of chromatid separation at the metaphase-anaphase transition | 14 | 15 | 16 | 93 | 2.27 | <0.05 |
| Interleukin-1 pathway | 33 | 40 | 52 | 82 | 2.27 | <0.01 |
| Eicosanoid metabolism via cyclooxygenases (COX) | 12 | 13 | 70 | 92 | 2.03 | <0.05 |

Fig. 5 showed the genes involved in type I interferon induction and signaling during SARS-CoV-2 infection (Z-score: 3.41). Interestingly, the alternative NF-kB pathway activation via TRAF proteins was clearly affected.

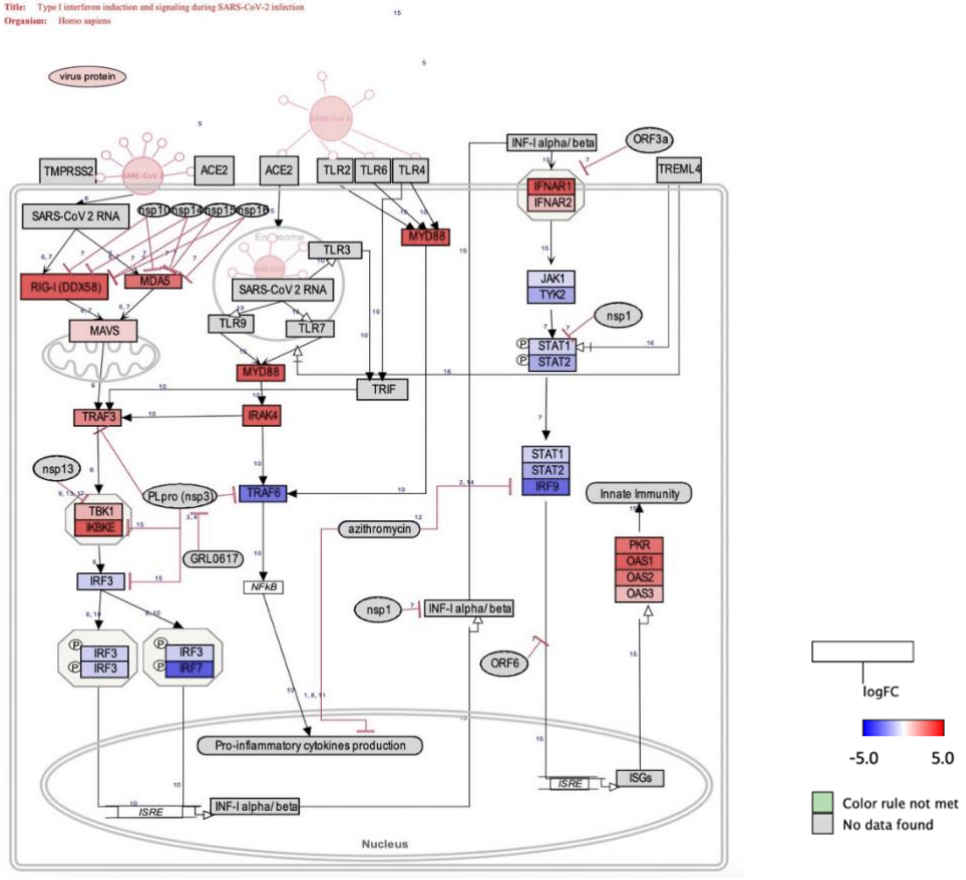
Title: Type I interferon induction and signaling during SARS-CoV-2 infection
Organism: Homo sapiens
virus protein
TMPRSS2
ACE2
ACE2
TLR2
TLR6
TLR4
INF-I alpha/ beta
ORF3a
TREML4
IFNAR1
IFNAR2
SARS-CoV 2 RNA
nsp10
nsp14
nsp15
nsp16
MYD88
TLR3
RIG-I (DDX58)
MDA5
SARS-CoV 2 RNA
JAK1
TYK2
nsp1
TLR9
TLR7
MAVS
STAT1
STAT2
MYD88
TRIF
TRAF3
IRAK4
STAT1
STAT2
IRF9
nsp13
PLpro (nsp3)
TRAF6
Innate Immunity
TBK1
IKBKE
azithromycin
PKR
OAS1
OAS2
OAS3
GRL0617
IRF3
NFkB
nsp1
INF-I alpha/ beta
IRF3
IRF3
IRF3
IRF7
ORF6
Pro-inflammatory cytokines production
ISRE
ISGs
ISRE
INF-I alpha/ beta
Nucleus
logFC
-5.0
5.0
Color rule not met
No data found

**Figure 5**. Genes involved in type I interferon induction and signaling during SARS-CoV2 infection for *Homo sapiens* are shown: in red the upregulated genes and in blue the downregulated after AFB1-OTA P-FW exposure (187-837 nM). P: Pumpkin; FW: Fermented Whey; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

### *3.4. Confirmation of NGS results by qPCR*

The sequencing-based results were validated through qPCR assay. The expression of TIGD5, a DNA-binding gene, and RHOB, a Ras homolog gene, was evaluated in Jurkat cells after exposure to AFB1 P-FW, OTA P-FW and AFB1-OTA P-FW. The experimental conditions used in qPCR analysis were the same as those used for the NGS assay. RHOB and TIGD5 were also chosen being ones of most affected genes in RNA-seq analysis. The results obtained were in line with NGS assay with TIGD5 upregulation and RHOB downregulation when compared to the control (Fig. 6). House-keeping gene GAPDH was used as endogenous gene control.

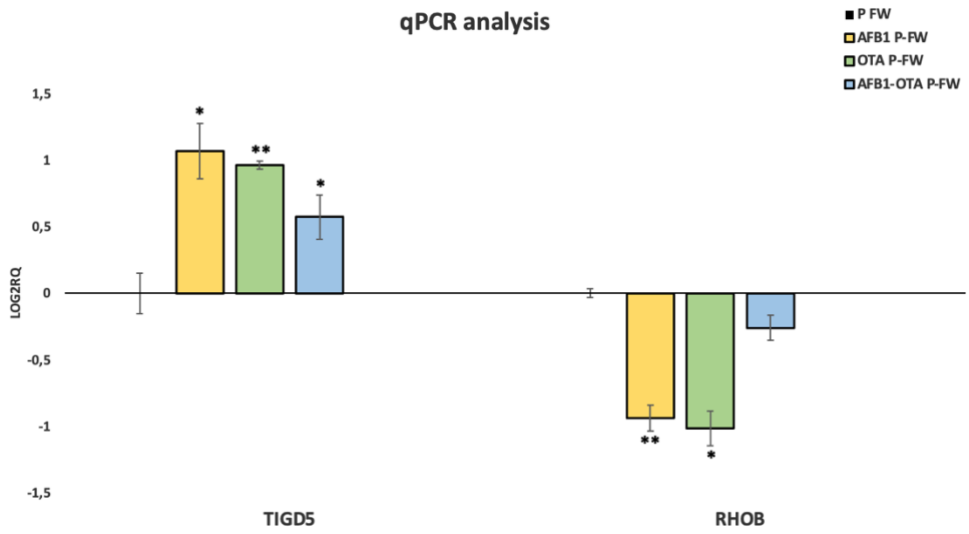


**Figure 6.** Bar plot showing the relative expression of TIGD5 and RHOB when compared to the control (Log2RQ = 0) after 7 days of exposure in Jurkat cells to AFB1 P-FW (196 nM), OTA P-FW (1037 nM) and AFB1-OTA P-FW (196-837 nM) by qPCR. RQ: relative quantification; RHOB: Ras Homolog Family Member B; TIGD5:Tigger Transposable Element Derived 5; P: Pumpkin; FW: Fermented whey. $p \leq 0.05$ (*), $p \leq 0.01$ (**).

## 4. Discussion

In the present study, the possible beneficial effect of P-FW mixture against AFB1 and OTA induced immune toxicity was investigated at transcriptomic level. To reproduce a realistic scenario, human lymphoblastic Jurkat cells were employed, being a robust immune model to reproduce human situations *in vitro* (Shao et al., 2014; Cassioli et al., 2021). The selected exposure time (7 days) reflects the peak of immune response *in vivo*, thereafter the number of T-lymphocytes decreases and the immune system gradually tends to deactivate (Badovinac et al., 2002; Lieberman et al., 2020). Analyzing the number of DEGs following the exposure with mycotoxins and functional ingredients, it should be emphasized as only 34 DEGs were altered by AFB1, 3450 by OTA and 3264 by combined exposure, which could exclude an additive effect between AFB1 and OTA when combined with bioactive compounds (Fig.1). The possible antagonism between mycotoxins was further confirmed by qPCR analysis (Fig.6). Likewise, our research group, using the same experimental conditions (cell line, exposure time and digested bread extracts) for a proteomic analysis, found a lower number of differentially expressed proteins in the combined exposure compared to the individual ones (Cimbalo et al., 2022a). Frangiamone et al. (2022b) also showed an antagonist effect between AFB1 and OTA when combined with P-FW mixture treating SH-SY5Y cells ongoing differentiation during 7 days. Moreover, it is interesting to note as the mixed treatment shared 80% of DEGs with OTA individual exposure (Fig.1). These results can be related to

OTA-concentration, which is almost 5-folds higher than AFB1 in the mixture, and may be explained by the greater effectiveness of bioactive compounds to reduce AFB1 intestinal bioaccessibility (a reduction of 70%) compared to OTA (only 10%) in contaminated breads (Escrivá et al., 2022).

Considering all studied conditions, the overlapping of 30 DEGs was obtained (Fig.2). Among them, the clear overexpression of several genes belonging to the zinc finger family (ZNF28, 563, 594), GPR17 (G protein-coupled receptor) and the downregulation of RHOB was observed. The upregulation of zinc finger proteins has been reported to attenuate NF-kB signaling activation, brain inflammatory injury and proinflammatory cytokines production *in vivo* and *in vitro* (Hong et al., 2016; Zhan et al., 2016; Agnihotri & Gaur, 2022). Likewise, Maekawa et al. (2009) revealed that GPR17 upregulation exerted inhibitory effects on inflammatory response and leukotrienes production in mouse bone marrow-derived macrophages. RHOB downregulation has been also shown to modulate TLR-mediated inflammatory response and cytokines production in macrophages, being a novel target for the treatment of inflammatory diseases (Zhang et al., 2017). In line with DAVID data analysis, in which most DEGs were related to the immune system, these results also demonstrated a clear effect on the inflammatory response *in vitro* after mycotoxins and bioactive compounds exposure (Table 4). In addition, an interesting confirmation of proteomic results obtained by Cimbalo et al. (2022a) was the altered expression of several genes encoding for the histone family, which play a key role in

maintaining genome topology. Herein, the decreased H1.4 expression was observed, which was associated with a strong induction of IFN response *in vitro* (Fig. 2)

*4.1. AFB1 P-FW exposure*

Exposure to AFB1 P-FW promoted the activation of several pathways associated with eicosanoid metabolism (via LOX,COX and cytochrome P450 monooxygenase) (Table 5). Eicosanoids are lipid-based signaling molecules that play a unique role in the inflammatory response, by regulating the production of certain cytokines and chemokines (Sheppe & Eldemann, 2021). Eicosanoids are also actively involved in the immune toxicity mediated by mycotoxins. For instance, AFB1-exposure (25 μM for 48 h) elicited the production of proinflammatory eicosanoids and cytokines in human macrophages, which in turn triggered the necrosis of HepG2 liver cells as well as the growth and development of hepatocellular carcinoma *in vivo* (Fishbein et al., 2018). Similarly, the RNA-seq performed on bovine macrophages revealed the activation of eicosanoid signaling pathway and its active involvement in the immune cell death mediated by OTA-exposure (10 μM for 6 h) (Brennan et al., 2017). In addition, the proteomic analysis conducted on human macrophages showed as the emerging mycotoxin alternariol (1 μM for 3 h) caused immune toxicity, by increasing the production of eicosanoids such as 12-HETE, which enhanced the transcription of proinflammatory mediators (Del Favero et al., 2020). Kostro

et al. (2012) also demonstrated that zearalenone-induced mycotoxicosis in sheep (3.07-14.49 μg/kg for 70 days) was mediated by eicosanoids, which fostered the infiltration of neutrophils in the digestive tract and the onset of systemic inflammation.

Conversely, the use of bioactive compounds such as carotenoids, probiotics and phenolic compounds, typically contained in P-FW mixture, has been reported to be an effective strategy in the modulation of eicosanoid production and inflammatory response (Kamalian et al., 2020). Storniolo et al. (2020) showed as the mixture of carotenoids and phenolic acids relieved oxidative stress and eicosanoids production in human monocytes, by promoting the resolution of inflammation and atherosclerosis disease. Likewise, a mixture of antioxidant and anti-inflammatory compounds (naturally contained in Brazilian propolis) not only modulated cytokines production but also selectively inhibited the synthesis of proinflammatory eicosanoids *in vivo* (Ferreira et al., 2021). Pumpkin pulp also demonstrated a protective role against mycotoxins exposure in ECV304 cells (OTA, BEA, ZEA; 0.1 μM for 9 days), inhibiting the production of inflammatory eicosanoids (prostaglandin D2 and leukotrienes B4 and E4) and protecting the integrity of blood-brain barrier *in vitro* (Alonso-Garrido et al., 2021b).

In this study, the eicosanoid metabolism via LOX was the main pathway to be altered, in which the downregulation of ALOX12 and LTA4H as well as the upregulation of ALOX15 were found (Fig.3). ALOX12 and

ALOX15 are key mediators of eicosanoid production via LOX with different functions. Indeed, the strong downregulation of ALOX12 has been reported to show anti-inflammatory effects by suppressing the production of eicosanoids and pro-inflammatory cytokines in mice, pigs, and monkeys (Zhang et al., 2021). Whilst, ALOX15 upregulation has been widely associated with the resolution of several human pathologies through the synthesis of several anti-inflammatory mediators such as lipoxins (Singh & Rao, 2019; Snodgrass et al., 2020). For instance, RNA-seq performed on T98G neuroglia cells revealed the anti-inflammatory effect of functional compounds (derived from plants extracts) via ALOX12 downregulation, which in turn repressed eicosanoid cascade. The production of anti-inflammatory lipoxins prompted by ALOX15 was also investigated (Panossian et al., 2019). Similarly, LTA4H downregulation was correlated with the anti-inflammatory response and modulation of NF-kB and eicosanoid signaling pathways activation (Wei et al., 2021; Sheppe & Eldemann, 2021). In view of this, the results obtained may confirm the beneficial protective effect of P-FW mixture against eicosanoid activation and the possible proinflammatory cascade induced by AFB1 in Jurkat cells.

*4.2. OTA P-FW exposure*

The main pathway to be affected by OTA P-FW exposure was the interferon signaling (Fig. 4). Interferons (IFNs) play a crucial role in modulating the immune response and inflammation, being produced especially during stress and infection (Barrat et al., 2019; Rojas et al., 2021). Several studies have reported the close connection between interferon induction and mycotoxin toxicity (Frangiamone et al., 2022a). The combination of OTA and deoxynivalenol promoted the release of proinflammatory cytokines such as IL-6, IL-17 and IFN-γ in mice splenocytes. Mycotoxin exposure also caused the activation of IFN signaling pathway, which in turn stimulated the differentiation of naive T-cells into Th1/17 phenotype, increasing the susceptibility to inflammatory diseases (Jahreis et al., 2017). The integrative transcriptomic and proteomic analysis carried out using differentiated Caco-2 cells demonstrated that OTA and AFM1 combined exposure (10 μM for 48 h) triggered the activation of several pathways associated with the immune and inflammatory response. A marked production of proinflammatory cytokines was also observed, leading to intestinal barrier disruption and severe gut inflammation (Gao et al., 2020). In addition, exposure to low doses of OTA *in vivo* (0.5-250 μg/kg for 2-7 weeks) induced IFN signaling pathway activation and cytokines storm (TNF-α , IL-2, IL-4 and IFN- γ) resulting in liver and kidney inflammation along with intestinal dysbiosis ( Prasanna et al., 2007; Wang et al., 2019).

The promotion of IFN signaling pathway by which OTA induced immune toxicity and inflammation *in vitro* and *in vivo* involves the activation of several chain factors, including the overexpression of MyD88 and MAPK proteins, phosphorylation and upregulation of NF-kB and JUN/FOS cascade, which in turn active AP-1 transcriptional complex, a master regulator of inflammatory response (Xu et al., 2017; Kumar et al., 2013; Gan et al., 2017; Wan et al., 2022). In the present study, although MyD88 overexpression indicated IFN pathway activation, the strong downregulation of JUN/FOS, MAPK and NF-kB suggested a clear downstream anti-inflammatory effect of P-FW mixture against OTA in Jurkat cells (Fig.4). Similar results were obtained by Ha et al. (2020) testing a mixture of carotenoids on HaCaT cells previously treated with TNF-$\alpha$ and IFN-$\gamma$. The bioactive compounds showed a marked anti-inflammatory effect by reducing the production of inflammatory cytokines and inhibiting NF-kB and MAPK signaling activation. Furthermore, astaxanthin (AST) and Kefir, a fermented milk product, alleviated OTA-induced cecal injury and inflammation in mice (5 mg/kg for 21 days in both studies) downregulating the gene expression of INF-$\gamma$, NF-kB, TRL-4 and modulating the activation of several immunity-associated pathways (Chen et al., 2021; Du et al., 2022). In the present study, OTA P-FW exposure also led to JAK/STAT downregulation, which has been associated with the suppression of proinflammatory response *in vitro* and *in vivo* (Kowshik et al., 2014; Mohan et al., 2021; Salas et al., 2021).

*4.3. AFB1-OTA P-FW exposure*

AFB1-OTA P-FW exposure triggered a transcriptional effect quite similar to OTA P-FW treatment with IFN signaling pathway activation (Fig.5). The simultaneous administration of AFB1 and OTA has been widely associated with inflammation and immune response. Corcuera et al. (2012) exposing rats to AFB1 and OTA (0.25 and 0.50 mg/kg for 0.8-96 h) obtained inflammatory infiltrates in liver and kidney tissues with cirrhosis and glomerulonephritis outbreak. Similarly, AFB1 and OTA combined exposure (0.5 and 1 μM for 48 h) promoted immune toxicity in macrophages, activating certain pathways associated with the immune response (JUN and MAPK cascade). In this case, the administration of bioactive compounds mitigated mycotoxin toxicity (Hou et al., 2018). The RNA-seq conducted on differentiated Caco-2 cells after AFM1 and OTA combined exposure (50 μM and 4 μM for 48 h) also revealed transcriptional alterations in some pathways related to inflammation and immunity such as NF-kB and IFN activation. Although the number of DEGs excluded the additive effect of mycotoxins, the promotion of gut inflammation by AFM1-OTA treatment was clearly proven (Gao et al., 2022).

Also for AFB1 OTA P-FW exposure, IFN pathway activation occurred via MyD88 overexpression, but nonetheless the involvement of additional drivers such as TRAF3, TRAF6, IRF3, and IRF7 was observed. In detail, TRAF3 was upregulated while the others drivers were

downregulated (Fig.5). TRAFs are specific factors associated with TNF receptor and play a major role in the pathogenesis of inflammatory diseases (Lalani et al., 2018). Among them, TRAF3 is a tumor suppressor and a negative regulator of NF-kB alternative activation. Thus, TRAF3 overexpression has been shown to attenuate the inflammatory response in immune cells (Zhang et al., 2018). Likewise, TRAF6 downregulation can exert an inhibitory effect on NF-kB cascade and cytokine storm in mammary epithelial cells (Wang et al., 2017). Li et al. (2017) associated the anti-inflammatory effect of lycopene supplementation (5 mg/kg for 21 days) in mice with cardiac injury with TRAF6 and NF-kB downregulation. Similarly, a mixture of *Lactobacillus,* typically used for milk fermentation, relieved ZEA-inflammatory effects (25 μM for 1h) in IPEC-J2 cells, through the downregulation of several proinflammatory markers, including TRAF-6 (Taranu et al., 2015). A similar anti-inflammatory response has been reported to be mediated by IRF3 and IRF7 downregulation, two master regulators of IFN induction against pathogens infection. The RNA-seq performed on mice lungs evidenced that AST suppressed lipopolysaccharide-induced inflammation and acute lung injury through the down-regulation of 11 immune markers, including IRF7 (Mao et al., 2020). The downregulation of IRF3 and IRF7 with mitigation of IFN signaling cascade and inflammatory response was also achieved by treating fish hepatocytes and murine macrophages with high doses of AST and LAB strains, respectively (Fang et al., 2021; Cai et al., 2018). In line with these results, our findings may further

support the potential anti-inflammatory *in vitro* effect of P-FW mixture against AFB1-OTA combined exposure (Fig.5).

## 5. Conclusions

This is the first transcriptomic study assessing the possible beneficial effect of P-FW mixture against AFB1 and OTA promoted immune toxicity *in vitro*, by using digested bread extracts. RNA-seq not only revealed as the majority of DEGs were correlated with the immune system and inflammatory response but also the possible antagonistic effect between AFB1 and OTA when combined with bioactive compounds. Furthermore, PathVisio analysis showed that eicosanoid pathway via LOX was the main pathway to be altered by AFB1 P-FW. However, the modulation of several genes associated with the inflammatory response (ALOX12, ALOX15, LTA4H) suggested the protective role of P-FW mixture against eicosanoid activation and the proinflammatory cascade prompted by AFB1 in Jurkat cells. On the other hand, exposure to OTA P-FW mainly affected IFN signaling cascade. The repression of intrinsic pathways such as NF-kB, JUN/FOS and MAPK pointed to the strong downstream anti-inflammatory effect of P-FW mixture *in vitro.* In AFB1-OTA P-FW exposure, IFN signaling was again the most altered, but nonetheless the involvement of additional drivers was observed. Herein, the decreased expression of genes: TRAF6, IRF3, IRF7 and inherent pathways (NF-kB/TRAF3) typically involved in the proinflammatory response further supported the beneficial role of P-FW

against the mixture of mycotoxins. Although the transcriptome analysis showed promising results, further investigations are needed to better explore the use of such food preservatives *in vivo* as immunomodulatory agents against AFB1 and OTA induced proinflammatory response and immune toxicity.

**CRediT authorship contribution statement**

**M. Frangiamone:** Formal analysis, Methodology, Visualization, Writing – original draft, Writing- Review. **M. Lozano:** Formal analysis, Methodology, Writing – original draft. **A. Cimbalo:** Formal analysis, Visualization, Writing- Review. **A. Lazaro**: Formal analysis, Visualization**. G. Font:** Funding, Supervision, Writing- Review, Project administration. **L. Manyes:** Conceptualization, Formal analysis, Methodology, Supervision, Writing- Review, Project administration**.**

**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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## 3.5. Fermented Whey modulated AFB1 and OTA induced hepatotoxicity and nephrotoxicity *in vivo*. A relative and absolute quantification about sex differences

Toxicology Mechanism and Methods

# Fermented Whey modulated AFB1 and OTA-induced hepatotoxicity and nephrotoxicity *in vivo*. A relative and absolute quantification about sex differences

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**Abstract**

Aflatoxin B1 (AFB1) and ochratoxin A (OTA) are well-known to promote hepatotoxicity and nephrotoxicity *in vivo*, which may be counteracted by natural compounds like fermented whey (FW). Carbamoyl phosphate synthetase 1 (CPS1) and kidney injury molecule 1 (KIM-1) are typical biomarkers used to detect liver and kidney damage, respectively. Thus, RT-qPCR and droplet digital PCR (ddPCR) analysis were performed to assess the potential beneficial effect of FW against AFB1 and OTA hepatotoxicity and nephrotoxicity in male and female Wistar rats by analyzing the altered gene expression of hepatic CPS1 and renal KIM-1 after 28 days of oral exposure. In male livers, the most damaging treatment was AFB1 by reducing CPS1 expression, which was totally reversed by FW-administration. This bioactive compound also improved gene expression changes induced by OTA and mycotoxins mixture. In female livers, a significant CPS1 overexpression was observed for each exposure performed, in which FW-supplementation reported no remarkable differences compared with mycotoxins exposure. Conversely, in the kidneys of male and female rats, exposure to mycotoxins promoted renal damage by altering KIM-1 gene expression, being OTA-exposure the most harmful condition. In both sexes, ddPCR analysis demonstrated that FW-addition modulated mycotoxins induced KIM-1 gene expression changes, thus reducing kidney damage. In this organ, sex-related responses were not clearly observed.

Therefore, these findings confirmed that AFB1 and OTA-promoted hepatotoxicity and nephrotoxicity *in vivo*, which could be modulated by dietary FW supplementation.

**Keywords:** Mycotoxins; Fermented whey; RT-qPCR; Droplet digital PCR; *in vivo*.

**Abbreviations**

AFB1: Aflatoxin B1

CI: Confidence interval

CPS1: Carbamoyl phosphate synthetase-1

ddPCR: Droplet digital PCR

DON: Deoxynivalenol

FW: Fermented Whey

FB1: Fumonisin B1

IARC: International Agency for Research on Cancer

KIM-1: Kidney injury molecule 1

LAB: Lactic acid bacteria

Log2RQ: Log2 transformed relative quantification

MoA: Mechanism of action

OTA : Ochratoxin A

RQ: Relative quantification

RT-qPCR: Reverse transcription quantitative PCR

UC: Urea cycle

WHO: World Health Organization

## 1. Introduction

Mycotoxins, secondary metabolites synthesized under specific conditions by certain fungal species, are typical contaminants of food and feed and pose a major risk to human and animal health even at low concentrations. The consumption of contaminated food and feed is associated with neurotoxic, immunosuppressive, and carcinogenic effects *in vivo.* Although more than 400 mycotoxins are identified, the most toxic and studied are aflatoxin B1 (AFB1) and ochratoxin A (OTA), representing a significant economic and public health concern (Cimbalo et al. 2020; Kutsanedzie et al. 2020).

AFB1 is a polypeptide compound produced by *Aspergillus flavus* and *Aspergillus parasiticus,* found in a wide variety of food commodities such as cereals, dried fruits, spices, tree nuts, milk and meat (Umesha et al. 2017). Once ingested, AFB1 is absorbed in the small intestine and metabolized by hepatocytes releasing reactive metabolites, whose carcinogenicity has been reported in pancreas, bladder, bone and central nervous system (Benkerroum 2020). In view of this, AFB1 has been classified by the International Agency for Research on Cancer (IARC) in group 1 as carcinogenic to humans (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2012). On the other hand, OTA is a phenylalanyl derivative of a substituted isocoumarin, mainly produced by *Penicillium verrucosum*, *Aspergillus ochraceus* and *Aspergillus niger*. OTA

typically contaminated a wide variety of foodstuffs such as: cereals, cereal-based products, wine, fruits, and vegetables (Chen et al. 2018). After ingestion, OTA is rapidly absorbed in the renal proximal tubule but it is excreted slowly, leading to a potential accumulation in the body with systemic side effects (Liu et al. 2022). Thus, OTA has been classified by IARC in group 2B as a possible carcinogen for humans (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2012). Considering 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 AFB1 and OTA in food and estimated the European population's dietary exposure (EFSA 2020a, 2020b).

The main organs to be affected by AFB1 and OTA are undoubtedly the liver and kidney, respectively. Indeed, several studies have reported the close connection between AFB1-exposure and the onset of human hepatocarcinogenesis and liver cirrhosis (Mekuria et al. 2020). Similarly, AFB1-exposure may promote acute liver injury in rats and broilers resulting in histopathological damage and abnormal liver function (Deng et al. 2020; Wu et al. 2019). It has been also demonstrated that AFB1 induced hepatoxicity via oxidative stress, apoptosis, cell cycle arrest, mitochondrial damage, and transcriptome alterations *in vitro* and *in vivo* (Frangiamone et al. 2022a).OTA is also involved in liver damage by inducing oxidative stress and inflammation in animal models such as rats, ducks, and pigs (Marin et al. 2018; Zhai et al. 2020; Zhang et al. 2021). Regarding kidney toxicity,

exposure to OTA has been considered as one of the main factors associated with the onset of chronic kidney diseases and tumors in rodents (Damiano et al. 2020; Longobardi et al. 2022; Miljkovic & Mantle 2022). The mechanism of action (MoA) by which OTA elicited nephrotoxicity encompasses lipid peroxidation, apoptosis, inflammation, and DNA damage *in vitro* and *in vivo* (Cimbalo et al. 2022b; Manyes and Font 2022). AFB1 also prompted kidney oxidative stress, genomic damage, and cell cycle arrest in rats and broilers (Wang et al. 2022; Yu et al. 2015).

Considering the high toxicity of AFB1 and OTA, the World Health Organization (WHO) has warned about the importance of controlling and preventing fungal contamination at the different stages of production chain. To this end, several physical, chemical and biological interventions have been tested. On the one hand, methods involving the use of chemical compounds or physical techniques have proven to be efficient tools for mycotoxin decontamination. However, the potential risks to public health along with the high-cost production prevent their application in food industry. On the other hand, biological methods involving the use of functional ingredients has been recently proposed as a new food strategy to reduce mycotoxins absorption and prevent their toxic effects on animal and human health (Zhou et al. 2022). Among them, milk whey is an important source of bioactive compounds with antifungal, antioxidant, and anti-inflammatory properties as well as an excellent substrate for lactic acid bacteria (LAB) fermentation (Gupta & Prakash 2017; Escrivá et al. 2022).

Moreover, several studies have demonstrated that fermented goat milk whey (FW) is an excellent food preservative. It can increase the shelf life and antioxidant activity of bread, inhibit the growth of mycotoxigenic strains in food, reduce AFB1 and OTA intestinal bioaccessibility as well as mitigate AFB1 and OTA induced neurotoxicity and immune toxicity *in vitro* (Cimbalo et al. 2022a; Dopazo et al. 2022; Escrivá et al. 2022; Frangiamone et al. 2022b, 2023; Luz et al. 2020).

For *in vivo* toxicological studies concerning mycotoxins, a main aspect to take into account is the sex-dependent response. For instance, in mice, chickens, and humans, the liver carcinogenicity of AFB1 is greater in males than females. Similarly, OTA prompted kidney tumors much more frequently in male rats than in females (EFSA 2020a, 2020b; Soler & Oswald 2018). The beneficial effects of probiotics have been also reported to show sex-related responses (He et al. 2019; Myles et al. 2020). Furthermore, the findings achieved with animal models can be extrapolated to humans by using biological biomarkers (Kraus 2018). In this sense, carbamoyl phosphate synthetase-1 (CPS1), the most abundant protein in liver mitochondria, represents a robust biomarker to detect hepatic diseases in humans and rats (Weerasinghe et al. 2014). Likewise, kidney injury molecule 1 (KIM-1) has been accepted by the Food and Drug Administration and European Medicines Agency as a highly sensitive and specific biomarker to monitor chemical-induced kidney injury in preclinical studies (Griffin et al. 2019).

Nowadays, innovative technologies have been developed in order to evaluate the presence/absence of specific biomarkers as well as to determine their absolute concentration (Fan et al. 2020). Droplet digital PCR (ddPCR) is a novel technique that enables the absolute gene expression assessment without the need for a standard curve or inter-run calibrator (Košir et al. 2017). This technology is insensitive to PCR inhibitors and its high reproducibility and specificity allow the identification of low abundant targets and obviate the use of technical replicates (Ferracin & Negrini 2018; Hindson et al. 2013; Miotto et al. 2014). ddPCR employs the microfluidic technology to partition the sample into droplets, i.e. multiple and independent micro reactions (20.000), by which the absolute concentration is calculated under the assumption of Poisson distribution. Therefore, more accurate, reproducible, and statistically significant results are provided (Huggett et al. 2013; Taylor et al. 2017).

In light of this, the aim of the present study was to investigate the potential beneficial effect of FW against AFB1 and OTA induced hepatotoxicity and nephrotoxicity in male and female rats by analyzing the altered gene expression of hepatic CPS1 and renal KIM-1 after 28 days of oral exposure. The relative and absolute quantification of these biomarkers have been assessed by RT-qPCR and ddPCR techniques, respectively.

## 2. Material and methods

### *2.1. Reagents*

Natural ingredients for feed preparation: wheat flour, mineral water, salt (NaCl), and sugar (sucrose) were acquired from a commercial supermarket (Valencia, Spain). *Aspergillus steynii* 20510 was obtained from Spanish Type Culture Collection, CECT, Science Park of the University of Valencia (Paterna, Valencia, Spain) while *Aspergillus flavus* ITEM 8111 was acquired from the Agro-Food Microbial Culture Collection of the Institute of Sciences and Food Production (ISPA, Bari, Italy). Goat milk whey coagulated by commercial rennet (starter culture R-604) was purchased from the ALCLIPOR society, S.A.L. (Benassal, Spain) while LAB used in this study (*Lactobacillus Plantarum CECT 220*) was obtained from CECT (Paterna, Valencia, Spain). For RNA extraction, TRIzol™ reagent was purchased from Invitrogen™ (Carlsbad, CA, USA), whereas ReliaPrep™ RNA Miniprep System kit from Promega (Madison, WI, USA) was employed for RNA purification. Deionized water (<18, MΩcm resistivity) was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA). iScript™ Advanced cDNA Synthesis Kit, iTAQ Universal SYBR® Green Supermix, QX200™ Droplet Generation Oil for EvaGreen, QX200™ Buffer Control for EvaGreen, and QX200™ ddPCR™ EvaGreen Supermix were acquired from Bio-Rad (Irvine, CA, USA).

*2.2. Animals*

Animals (male and female Wistar rats of 260-340 g) were acquired from the Pharmacy animal facility (University of Valencia, Spain). At the beginning of the study, Wistar rats were housed in polycarbonate cages in a windowless room with a 12h light-dark cycle. The study room was maintained under controlled conditions appropriate for the species (temperature 22 °C, relative humidity 45–65%). To maintain a sterile condition, nitrile gloves and FFP3 masks were used for all procedures performed, including the manipulation of treated animals or contaminated samples. This project was approved by the institution Animal Care and Use Committee of the University of Valencia (2021/VSC/PEA/0112).

*2.3. Treatments and experimental design*

Wistar rats (n = 80) were randomly distributed into eight groups (10 rats for each, 5 males and 5 females). After 7 days of acclimatization, the control group was supplied with uncontaminated feed while the seven test groups were fed with mycotoxins and FW-containing feed. Feed preparation was carried out by using the basic recipe described by Escriva et al. (2022) with several modifications. Starting with a recipe for 1 kg of feed, the initial amount required for each treatment (4.6 kg) was extrapolated. Due to the moisture loss (25% - 30%), a final weight of 3.5 kg per feed was obtained. The control feed was prepared as follows: 2800.2 g wheat flour, 1726.8 ml mineral

water, 93.4 g sugar (sucrose) and 46.7 g salt (NaCl). Then, some adjustments were made in order to prepare contaminated and enriched feeds. As shown in Table 1, the addition of contaminated flours was performed by replacing an amount of wheat control flour with 1381.43 g of maize flour naturally contaminated with *Aspergillus flavus* ITEM 8111, an AFB1-producing fungal species, and/or 466.7 g of barley flour naturally contaminated with *Aspergillus steynii* 20510, an OTA-producing fungal species. The addition of these quantities of contaminated flours aimed to reach final concentrations of 7.0 (mg/kg) for AFB1 and 11.4 (mg/kg) for OTA in order to create a realistic scenario of mycotoxins ingestion, derived from a biological comparison with Mediterranean diet and humans habits (WHO 2018). Also, differences in the amount of added contaminated flours (1381.43 g for AFB1 and 466.7 g for OTA) are related to natural cereals contamination that is usually higher for OTA compared with AFB1 (Escrivá et al. 2022). Finally, AFB1 and OTA concentrations in each feed were determined by high-performance liquid chromatography with fluorescence detection. To prepare FW-containing feeds, the bioactive compound was added at 1% (w/w). It is interesting to note as feeds showed different mycotoxin concentrations. This phenomenon is related to nutrients concentration and weight changes (mainly related to water loss) occurring during the baking process. According to the initial dough ingredients, the baking process may be different, obtaining feeds with different physicochemical properties. For instance, FW addition influenced the rheological properties of wheat (Escrivá et al. 2022) (Table 1).

**Table 1**. The amount of each ingredient used for feeds preparation.

| Feeds (3.5 kg final weight) | Wheat flour (g) | AFB1-contaminated maize flour (g) | OTA-contaminated barley flour (g) | Fermented milk whey (g) | Mineral water (ml) | Sucrose (g) | Salt (g) |
|---|---|---|---|---|---|---|---|
| CTRL | 2800.2 | | | | | | |
| AFB1 | 1418.8 | 1381.43 | | | | | |
| OTA | 2333.5 | | 466.7 | | | | |
| AFB1+OTA | 952.1 | 1381.43 | 466.7 | | 1726.8 | 93.4 | 46.7 |
| FW | 2765.2 | | | 35 | | | |
| FW-AFB1 | 1383.8 | 1381.43 | | 35 | | | |
| FW-OTA | 2298.5 | | 466.7 | 35 | | | |
| FW AFB1+OTA | 917.2 | 1381.43 | 466.7 | 35 | | | |

Afterwards, rats were orally exposed during 28 days to: 1) control feed; 2) AFB1 (5 ± 0.6 mg/kg); 3) OTA (10.2 ± 1.1 mg/kg); 4) AFB1 and OTA (8.8 ± 1.5 and 10.9 ± 1.5 mg/kg, respectively); 5) FW (1% of feed); 6) FW (1%) with AFB1 (6.1 ± 1.4 mg/kg); 7) FW (1%) with OTA (6.1 ± 0.3 mg/kg); 8) FW (1%) with AFB1 and OTA (8.4 ± 0.4 mg/kg and 8.4 ± 0.3 mg/kg, respectively). The experimental conditions with mycotoxins doses and their respective standard deviations were summarized in Table 2.

**Table 2.** The experimental conditions and mycotoxin doses used for the *in vivo* study design.

| Experimental rats groups | AFB1 (mg/kg) | OTA (mg/kg) |
|---|---|---|
| CTRL | - | - |
| AFB1 | 5.0 ± 0.6 | |
| OTA | | 10.2 ± 1.1 |
| AFB1+OTA | 8.8 ± 1.5 | 10.9 ± 1.2 |
| FW | - | - |
| FW-AFB1 | 6.1 ± 1.4 | |
| FW-OTA | | 6.1 ± 0.3 |
| FW AFB1+OTA | 8.4 ± 0.4 | 8.4 ± 0.3 |

Once the exposure time elapsed, Wistar rats were euthanized with isoflurane by inhalation and organs (liver and kidneys) stored at -80 °C.

*2.5. RNA Extraction*

Total RNA of the control and exposed rats were isolated using approximately 50 mg of frozen tissue according to TRIzol™ manufacturer's protocol. Samples were homogenized in TRIzol™ solution (50 mg/mL) with T25 Ultra-turrax Digital High-Speed Homogenizer (IKA®, Staufen, Germany). Once extracted, RNA was purified according to ReliaPrep™ RNA Miniprep System kit. The purity and quantity of RNA were spectrophotometrically assessed using a NanoPhotometer® N60/N50 (Implen, Westlake Village, USA) showing concentrations between 1250 and

6652 ng/μL along with appropriate 260/280 nm and 260/230 nm ratios both around 2. RNA samples were diluted to 100 ng/μL with an ultrapure Milli-Q $H_2O$ and stored at -20 °C until their reverse transcription to cDNA.

*2.6. Gene Selection and Primer Design*

Gene-specific primers were designed by Primer-BLAST using the default software settings with amplified PCR products ranging from 85 to 185 bp and Tm at 58-61 °C. Standard curves were performed for all primers and a single amplification product for each gene was obtained by the melting curve assay in Bio-Rad CFX96 real-time PCR System (Irvine, CA, USA). Primer amplification efficiency was determined from the standard curve generated by serial dilution of cDNA (5-fold each) for each gene. Correlation coefficient ($R^2$ values, i.e linearity) and amplification efficiencies were calculated from the slope of the regression line by plotting Ct mean values against the log cDNA dilution factor. The gene-specific primers used in the present study are listed in Table 3.

**Table 3.** Gene names, forward and reverse primer sequences, PCR efficiency and linearity value for the target genes and the reference gene β-actin.

| Gene | Primer forward sequence | Primer reverse sequence | Efficiency (%) | Linearity |
|---|---|---|---|---|
| **β-actin** | TCGTGCGTGACATTAAGGAG | AGGAAGGAAGGCTGGAAGAG | 104 | 0.996 |
| **CPS1** | CGGGAAGTAGAGATGGACGC | CCTTGGCTGATGGTCTGTGT | 116 | 0.997 |
| **KIM-1** | TGGCACTGTGACATCCTCAGA | GCAACGGACATGCCAACATA | 107 | 0.995 |

*2.7. Reverse Transcription and RT-qPCR*

Real-time amplification reactions were performed in 96 well plates using SYBR Green detection chemistry and were run in triplicate by using the Bio-Rad CFX96 real-time PCR System (Irvine, CA, USA). Reactions were prepared as follows: 100 ng template, 250 nM of each primer, the required amount of (2x) iTAQ Universal SYBR® Green in a reaction volume of 20 μL RNAse-free PCR grade water (Bio-Rad, CA, USA). The PCR temperature cycling conditions for CPS1 were as follows: initial denaturation step at 95 °C for 5 min to activate Taq DNA polymerase, followed by 40 cycles of

denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s. For KIM-1, the cycling conditions were as follows: initial denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s and annealing at 60 °C for 1 min. The melting curve analysis was generated by heating the amplicon from 65 °C to 95 °C with increments of 0.5 °C for 5 s to determine the specificity of PCR reactions. Threshold cycles (Ct) were automatically generated by Bio-Rad CFX Maestro software and relative gene expressions were evaluated using the $2^{-\Delta\Delta CT}$ method. The relative quantification values were transformed to log2 (Log2RQ) for normalization. Six biological replicates were used for each condition. Experiments were carried out according to MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Bustin et al. 2009).

*2.8. Droplet digital PCR*

The absolute quantification of hepatic CPS1 and renal KIM-1 was performed with QX200™ AutoDG PCR system (Bio-Rad, Irvine, CA, USA). The 22 μL ddPCR mixture was prepared in multiplate™ 96-Well PCR Plates (Bio-Rad, Irvine, CA, USA) as follows: 11 μL of (2x) QX200™ ddPCR™ EvaGreen Supermix, 6.6 μL of primers and 4.4 μL of template. According to the manufacturer's instructions, final primers concentration was 150 nM, while template concentration was < 66 ng. No template controls (NTC) were also included to confirm the method validity. Once prepared, the plate was

shaken for 30 s at 500 rpm (Eppendorf Thermo Mixer C) and then centrifuged at 2000 rpm for 2 min (Eppendorf Centrifuge, 5430 R). The reaction mixture was divided into approximately 20.000 droplets within the AutoDG system, using EvaGreen droplet generator oil and DG32 automated droplet generator cartridge (Bio-Rad, CA, USA). The resulting droplets (≈40 μL) were transferred by a robotic arm to a new sample plate, which was heat-sealed with a pierceable aluminum foil using a PX1 PCR plate sealer (Bio-Rad, Irvine, CA, USA) set at 180 °C for 5s. Then, droplets amplification was performed in C1000 Touch™ Thermal Cycler (Bio-Rad, Irvine, CA, USA) using the following conditions: enzyme activation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing/extension at 57.3 °C for 1 min (after a 55-65 °C thermal gradient optimization) followed by signal stabilization at 4 °C for 5 min & 90 °C for 5 min. Finally, the reaction mixture was held at 4 °C. For each step, a ramp rate of 2 °C/s and lid heated to 105 °C were employed. After thermal cycling, the plate containing amplified droplets was placed in a QX200™ Droplet Reader (Bio-Rad, Irvine, CA, USA) for reading. The software package provided with the ddPCR system was used for data acquisition (QuantaSoft™ 1.6.6.0320; Bio-Rad). Finally, data generated by the software were discarded from subsequent analyses: if fewer than 10.000 droplets were processed or if 99.99 % of droplets were positive (i.e. reaction saturation). Experiments were performed using six biological replicates according to digital MIQE guidelines (Huggett et al. 2013).

*2.9. Statistical analysis*

The qPCR Ct values were generated by Bio-Rad CFX Maestro software while statistical analysis was performed using GraphPad Prism software version 9.4.0. Differences between groups were evaluated with one-way ANOVA followed by Dunnett's post-hoc test for multiple comparisons. For ddPCR results, data analysis was carried out by Quanta Soft™ based on the Poisson distribution with the 95% confidence interval (CI). The fluorescence amplitude thresholds were also applied to discriminate positive and negative droplets. The absolute quantification data of control and treated samples were provided in target copies/μL reaction. Also in ddPCR, to assess statistically significant differences between test groups and control group, one-way ANOVA followed by Dunnett's post-hoc test was performed (GraphPad Prism software 9.4.0). For RT-qPCR and ddPCR, p-value ≤ 0.05 was considered significant.

## . 3. Results

### *3.1. Relative quantification of hepatic CPS1*

The relative quantification of hepatic CPS1 was performed by qPCR analysis. Sex-related differences were also assessed. In male rats, AFB1-exposure induced a significant CPS1 downregulation while OTA individually and in combination with AFB1 slightly upregulated the hepatic biomarker. The administration of FW individually and combined with mycotoxins promoted a clear and significant CPS1 overexpression when compared to the control (Log2RQ >1). Interestingly, in FW-AFB1 group, the presence of the functional compound totally reversed CPS1 downregulation promoted by individual exposure to AFB1. In female rats, all treatments led to a significant CPS1 upregulation (Log2RQ >2). The addition of FW individually or in combination with AFB1 and OTA revealed no remarkable differences compared to individual mycotoxins exposure. In both sexes, statistically significant changes (marked in blue) were also observed when FW group was used as reference. In relation to sex differences, it can be observed the opposite molecular effect of AFB1, which promoted the downregulation of CPS1 in males and its overexpression in females. In this sex, the possible AFB1 and OTA additive effect was observed as well as the greater sensitivity to gene expression changes when compared to males for each treatment performed (Fig.1).

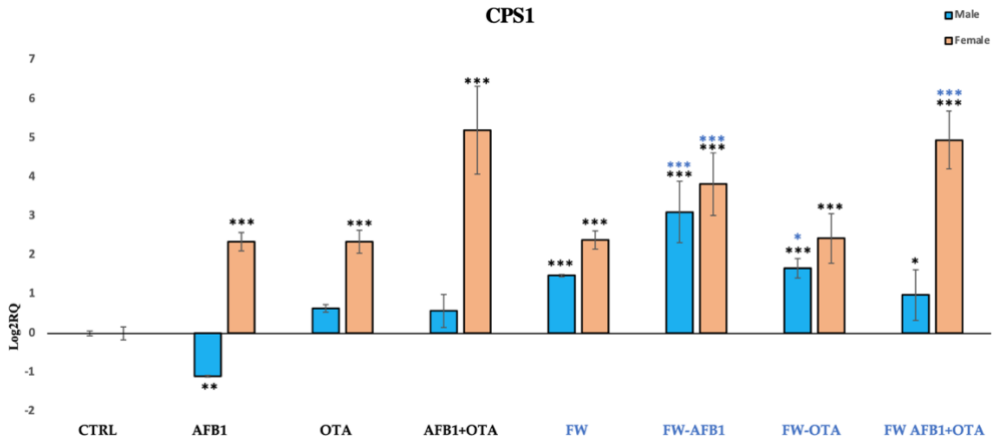


**Fig. 1.** Bar plot showing the relative expression of CPS1 liver biomarker in male (blue) and female rats (orange) when compared to the control group (Log2RQ = 0) after 28 days of exposure to different treatments (mycotoxin doses are reported in material and methods section) by qPCR. In black is shown the statistical analysis compared with the control group while in blue compared to FW group. RQ: relative quantification; CTRL: control; FW: fermented whey; CPS1: Carbamoyl phosphate synthetase I. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

### *3.2. Absolute quantification of hepatic CPS1*

The absolute quantification of CPS1 was carried out by ddPCR approach. Fig. 2a and 2b demonstrated the high accuracy of the method used in both sexes, considering the high number of processed droplets (>10.000) as well as the clear and pronounced separation between positive (with peak frequency at 20.000) and negative droplets (with peak frequency at 5000).

The further confirmation of method validity was the reduced "droplets rain", i.e. droplets that occur between the clusters of negative and positive droplets showing intermediate fluorescence values. Avoiding “droplets rain” is considered one of the critical steps in ddPCR technology (Rowlands et al. 2019). The ddPCR analysis confirmed qPCR results in both sexes with an overall increase of CPS1 quantification (than control group) comparable with the general upregulation observed in the relative quantification analysis. In males, FW-AFB1 totally reversed the transcriptional effect induced by individual AFB1 exposure. In females, the possible synergistic effect of AFB1 and OTA was also confirmed. In relation to sex differences, the opposite effect of AFB1 was again found, by reducing the concentration of CPS1 in males and increasing it in females (Fig. 2c).

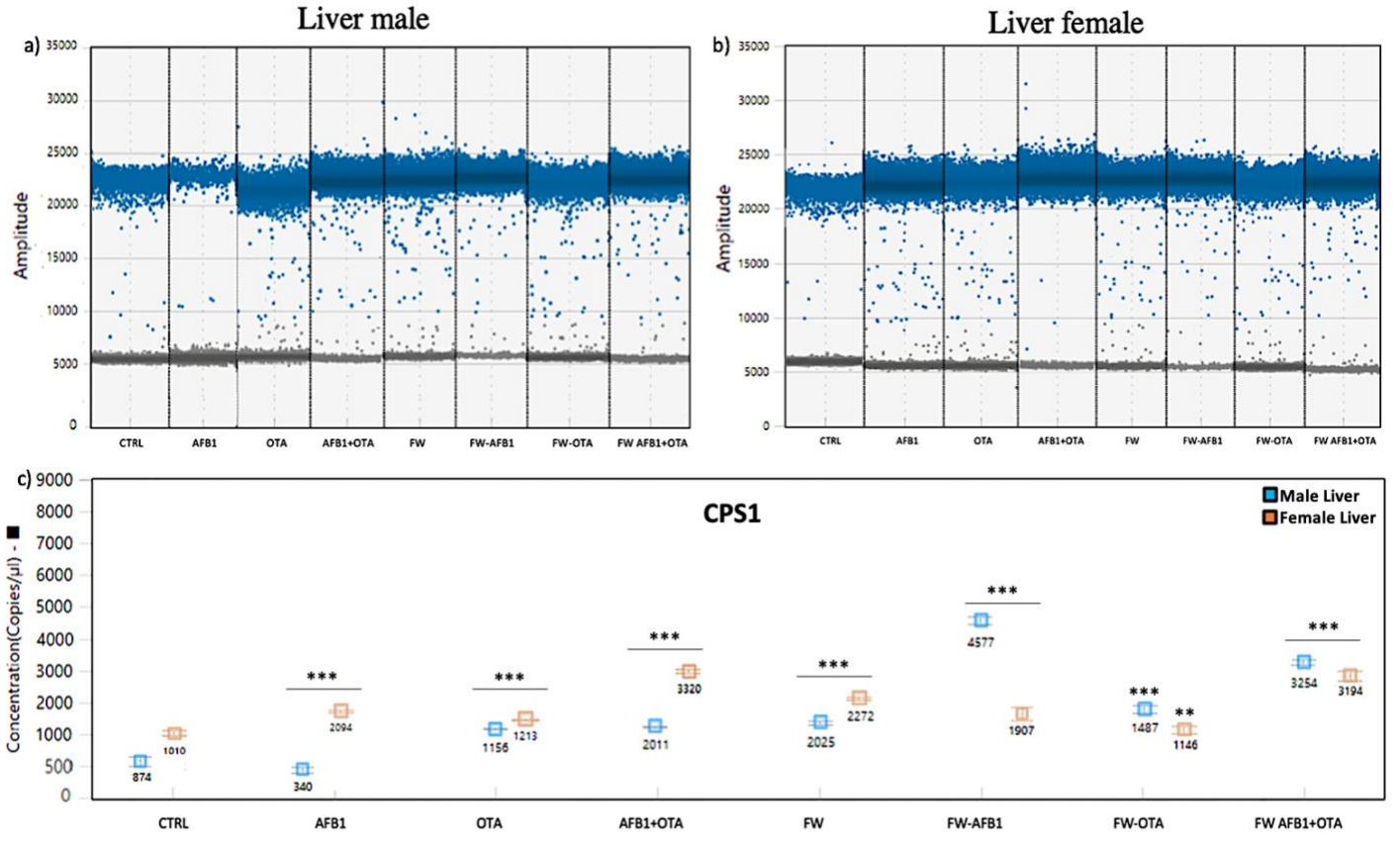
Liver male
a)
Amplitude
35000
30000
25000
20000
15000
10000
5000
0
CTRL
AFB1
OTA
AFB1+OTA
FW
FW-AFB1
FW-OTA
FW AFB1+OTA
Liver female
b)
Amplitude
CPS1
c)
Concentration(Copies/µl) - ■
Male Liver
Female Liver
9000
8000
7000
6000
5000
4000
3000
2000
1000
500
0
***
**
874
1010
340
2094
1156
1213
2011
3320
2025
2272
4577
1907
1487
1146
3254
3194

**Fig. 2.** Fluorescence amplitude of droplet populations obtained for CPS1 in the liver of male (a) and female rats (b) after 28 days of exposure to the different treatments (mycotoxin concentrations are shown in the material and methods section) by ddPCR. The separation between positive (with peak frequency at 20.000) and negative droplets (with peak frequency at 5000) was also shown for both sexes. (c) The absolute quantification of hepatic CPS1, expressed as concentration (copies/μL), in male (blue) and female rats (orange) for each condition performed. The error bars associated with each measurement represent the 95% CI. CTRL: control; FW: fermented whey; CPS1: Carbamoyl phosphate synthetase I; CI: Confidence interval. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

### *3.3. Relative quantification of renal KIM-1*

The possible kidney damage was assessed by analyzing KIM-1 gene expression. In males, AFB1 and OTA individual exposure promoted a clear and significant KIM-1 upregulation (Log2RQ >1) while their combination induced gene expression changes quite similar to the control, suggesting a putative antagonistic effect between AFB1 and OTA in this sex. Likewise, FW administration individually and in combination with mycotoxins significantly upregulated KIM-1 to a greater extent than mycotoxins individual exposure. In females, a different scenario was found. Indeed, AFB1-exposure significatively downregulated KIM-1 whereas OTA and the mixture of mycotoxins overexpressed KIM-1 when compared to the control (Log2RQ >1.5). In FW individual exposure, the relative gene expression was similar to the control while in combination with mycotoxins a significative

KIM-1 overexpression was observed, albeit to a lesser extent than mycotoxins alone. In both sexes, statistically significant changes (marked in blue) were also obtained when FW group was used as reference. Regarding sex differences, AFB1 induced an opposite effect in kidneys, upregulating KIM-1 in males and reducing its expression in females. Also, the combined AFB1-OTA treatment showed an antagonistic effect in males and a possible synergism in females. In this sex, FW administration tended to mitigate alterations in gene expression compared to males that were generally more susceptible to treatments (Fig.3).

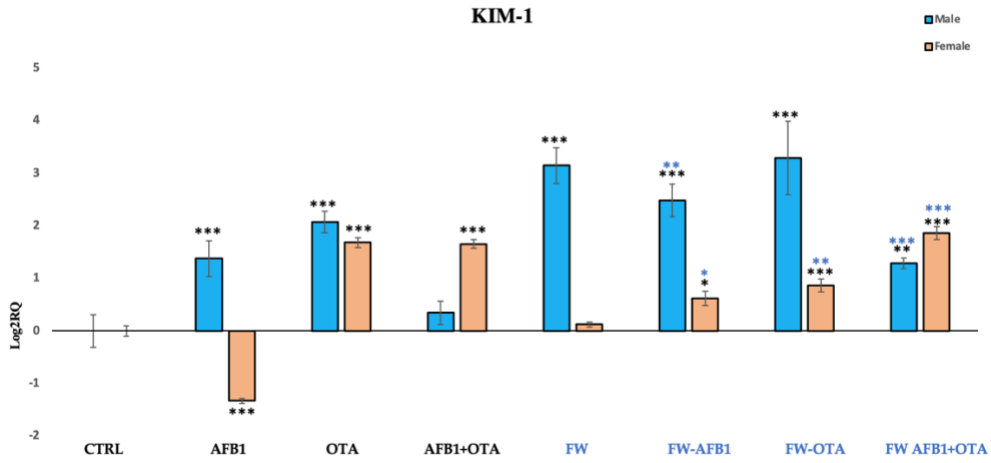


**Fig. 3.** Bar plot showing the relative expression of KIM-1 kidney biomarker in male (blue) and female rats (orange) when compared to the control group (Log2RQ = 0) after 28 days of exposure to different treatments (mycotoxin doses are reported in material and methods section) by qPCR. In black is reported the statistical analysis compared with the control group while in blue compared to FW group. RQ: relative quantification; CTRL: control; FW: fermented whey; KIM-1: Kidney injury molecule 1. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

### *3.4. Absolute quantification of renal KIM-1*

The absolute quantification of KIM-1 confirmed the high ddPCR reliability. As with the liver, a clear separation between positive (with peak frequency at 20.000) and negative droplets (with peak frequency at 7000) as well as the reduced "droplets rain" were observed in both sexes (Fig. 4a and 4b). Herein, ddPCR analysis showed an overall and slight increase of KIM-1

quantification for each condition performed. In males, the antagonistic AFB1 and OTA effect was confirmed, whereas their possible synergism in females was not clearly detected. In contrast to qPCR analysis in which FW alleviated gene expression changes only in female rats, ddPCR assay demonstrated that FW mitigated KIM-1 overexpression in both sexes. Moreover, the opposite sex effect of AFB1 was confirmed while the greater susceptibility of males to treatments was not found (Fig.4c).

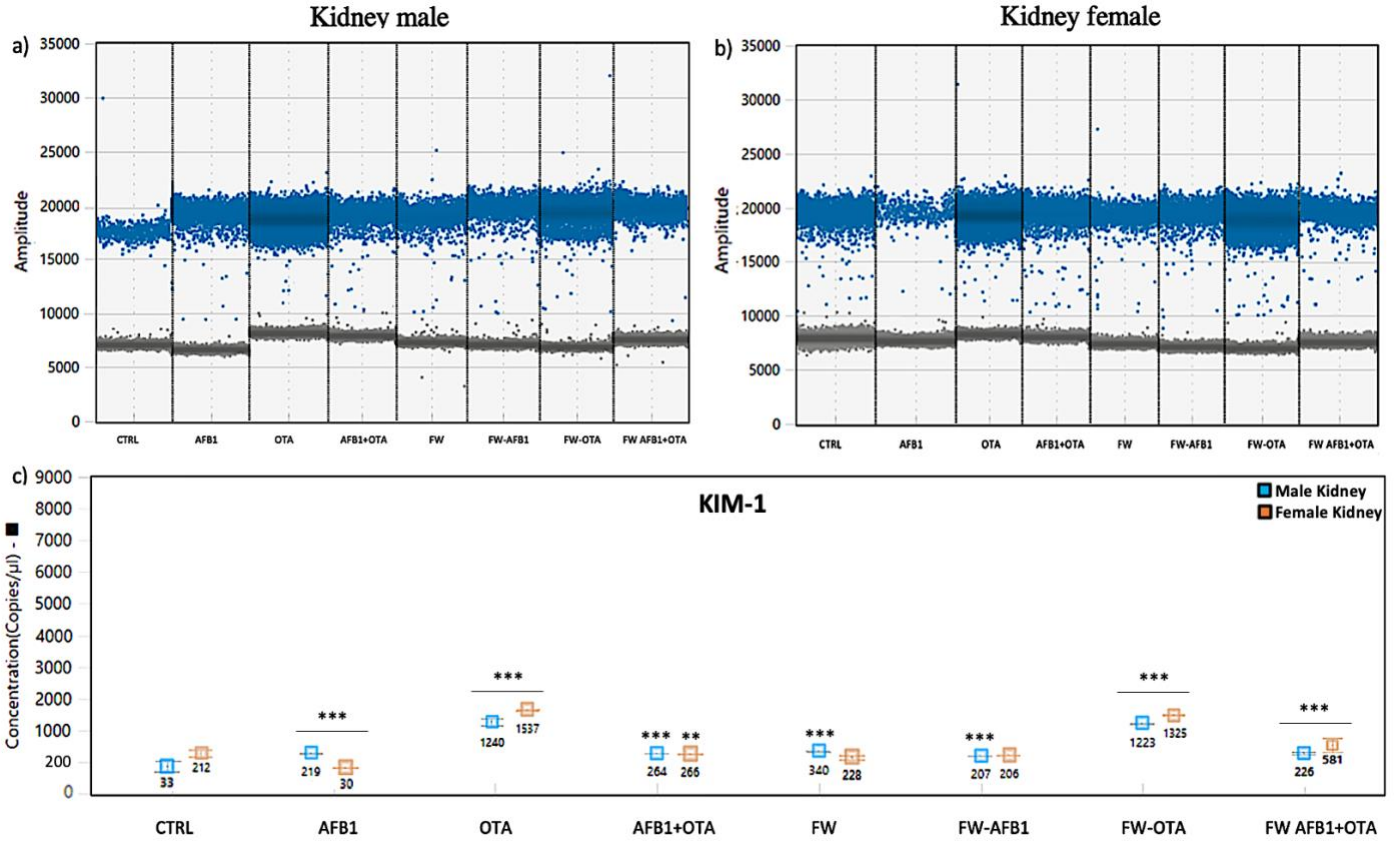
Kidney male
Kidney female
a)
b)
Amplitude
35000
30000
25000
20000
15000
10000
5000
0
CTRL
AFB1
OTA
AFB1+OTA
FW
FW-AFB1
FW-OTA
FW AFB1+OTA
c)
KIM-1
Male Kidney
Female Kidney
Concentration(Copies/µl) - ■
9000
8000
7000
6000
5000
4000
3000
2000
1000
200
0
***
**
33
212
219
30
1240
1537
264
266
340
228
207
206
1223
1325
226
581

**Fig. 4.** Fluorescence amplitude of droplet populations obtained for KIM-1 in the kidney of male (a) and female rats (b) after 28 days of exposure to the different treatments (mycotoxin doses are shown in the material and methods section) by ddPCR. The separation between positive (with peak frequency at 20.000) and negative droplets (with peak frequency at 7000) was also shown for both sexes. (c) The absolute quantification of KIM-1, expressed as concentration (copies/μL), in male (blue) and female rats (orange) for each condition performed. The error bars associated with each measurement represent the 95% CI. CTRL: control; FW: fermented whey; KIM-1: kidney injury molecule 1; CI: Confidence interval. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

## 4. Discussion

The results obtained in the present study delve into the MoA by which AFB1 and OTA, individually and in combination, promoted liver and kidney toxicity at transcriptomic level with a special focus on possible sex-related responses after a sub-chronic *in vivo* exposure. The putative beneficial role of FW against such food contaminants was also assessed. This is the first study in which an innovative technique such as ddPCR was employed to explore mycotoxin toxicity, and the homogeneity with qPCR results showed the consistency of reported findings.

*4.1 Liver*

Liver is the main organ involved in the production of hormones and biochemicals necessary for digestion and growth, organism detoxification, and regulation of energy metabolism. Errors in liver energy metabolism, including urea cycle (UC) disorders, lead to severe hepatic dysfunctions (Ranucci et al. 2019). The UC converts toxic ammonia to urea through the activity of several enzymes, among them CPS1, and has been described as one of the main metabolic processes to be disrupted by mycotoxins activity (Gerdermann et al. 2022). Exposure to AFB1 in rats (65 μg/mL; 10 weeks) and Chang liver cells (5 μg/mL; 12-48h) caused mitochondrial dysfunction with UC impairment and a significative CPS1 downregulation, leading to a massive accumulation of ammonium in the blood (Zhuang et al. 2014). Yang et al. (2016) also demonstrated that exposure to AFB1 in Chang liver cells (40 μg/mL; 24h) triggered CPS1 downregulation, which in turn enhanced tissue proliferation and differentiation, typical processes detected during hepatocarcinogenesis. Thus, the close connection between repression of CPS1 activity and onset of hepatocellular carcinoma was confirmed. Moreover, CPS1 deficiency has been observed during liver cirrhosis and hepatic encephalopathy (Deutsch-Link et al. 2022; Nitzahn and Lipshutz 2020; Yan et al. 2019). On the other hand, Cimbalo et al. (2021) evaluating the acute toxicity of enniatins (ENs) (256-1021 μg/mL; 8h) in rats liver, obtained a marked overexpression of CPS1 with prominent alterations in hepatic energy metabolism. Likewise, exposure to OTA (70-210 μg/kg; 2-23 weeks)

in rats liver imbalanced the energy metabolism and exhausted its repair system with a strong CPS1 upregulation (Qi et al. 2014a). Therefore, it can be affirmed that CPS1 gene expression changed according to the different MoA by which AFB1, ENs and OTA promoted liver damage. In line with these findings, ddPCR and qPCR results showed that in male rats AFB1 downregulated CPS1 while OTA and mixed treatment slightly upregulated the liver biomarker. In females, AFB1 and OTA individually and in combination significatively upregulated CPS1 and to a greater extent than males (Fig.1 and 2). The strong biomarker overexpression found for each condition in female rats may be related to a constitutively higher expression of CPS1, which can offer a greater sex protection from UC dysregulation by external stimuli (Agnelli et al. 2016; Natesan et al. 2016). In this sex, AFB1 and OTA also showed a clearly additive effect compared to males, suggesting that sex can be considered a novel factor to take into account in toxicological risk assessment linked to mycotoxins co-occurrence.

The use of bioactive compounds and food preservatives has been reported to show beneficial effects on liver function, by limiting mycotoxin toxicity. For instance, the administration of probiotics (including several LAB strains) in liver cells modulated AFB1-hepatotoxicity, by relieving inflammation and restoring the normal metabolic activity (Guo et al. 2021). In rats liver, *Lactobacillus casei* not only alleviated AFB1-induced hepatic damage and inflammation but also reduced toxic ammonia production (Nikbakht Nasrabadi et al. 2013; Nurul Adilah et al. 2018). Moreover, the

administration of compounds rich in phytochemicals such as polyphenols and carotenoids upregulated CPS1 gene expression with improved hepatic gluconeogenesis and increased ammonium excretion. Therefore, the effectiveness of these bioactive compounds to reverse hepatic dysfunction was demonstrated (Lomas-Soria et al. 2015; Zhang et al. 2020). Interestingly, CPS1 may also perform a different function from the enzymatic one, acting as a protective anti-inflammatory cytokine. In presence of liver damage, a further increase of CPS1 activity triggered monocyte M2-polarization, augmented the number of hepatic macrophages and enhanced their phagocytic activity, thus accelerating the recovery of normal liver function (Park MJ et al. 2019). Accordingly, FW administration in male rats significatively overexpressed CPS1 for each condition performed and to a greater extent than mycotoxins alone, suggesting a possible beneficial effect on liver function. Noteworthy, FW administration modulated AFB1-toxic effect at molecular level, by reversing CPS1 downregulation and proving to be an effective feeding strategy to counteract AFB1-hepatotoxicity *in vivo* (Fig.1 and 2). In females, FW-supplementation increased the expression of CPS1 than AFB1 alone, whereas for OTA and mixed treatment no remarkable differences were found (Fig.1 and 2). In this sex, the limited effect of FW is possibly related to a hormonal factor that overshadows the nutritional one. Indeed, estrogens are independent regulators of UC enzymes transcription, by maintaining a constant basal level of ureagenesis and ammonium excretion (Patton et al. 2020). However, this is the first *in vivo* study in which the protective effect of FW against mycotoxins was

evaluated, therefore more investigations are needed to confirm this hypothesis.

*4.2 Kidney*

Kidneys are the main organs involved in organism homeostasis, by regulating blood pressure and filtration, acid-base balance and toxin removal. The loss of kidney function is associated with reduced glomerular filtration rate and scattered structural abnormalities, which in turn can lead to bone disease, cardiovascular risk and tumors (Waziri et al. 2019). Exposure to mycotoxins has been also reported to be a major cause of kidney toxicity, in which KIM-1 can be used as prognostic marker for an early damage detection (Ráduly et al. 2021; Webster et al. 2017). For instance, Pyo et al. (2021) observed a clear KIM-1 upregulation, by exposing renal HK-2 cells to low doses of OTA (200 nM; 48h), which promoted hypoxia, cell death and epithelial-mesenchymal transition. In rats kidney, chronic exposure to OTA (70-210 μg/kg; 4-13 weeks) upregulated KIM-1 expression and promoted histopathological abnormalities with cytoplasmic vacuolization in the medulla outer stripe and extensive karyomegaly in tubular epithelium (Qi et al. 2014b). In the same animal model, acute exposure to OTA (1-4 mg/kg; 1 week) resulted in KIM-1 upregulation with phenomena of karyopyknosis, karyolysis, hydropic degeneration, swelling and vacuolization at tubular and glomerular level (Zhu et al. 2016). Li et al. (2021) also demonstrated that a low fumonisin B1 (FB1) dosage exacerbated OTA-

induced nephrocytotoxicity (5 μM; 24 h) by upregulating KIM-1 gene expression and inducing apoptosis and oxidative stress in porcine renal PK-15 cells. In line with these findings, ddPCR and qPCR analysis confirmed that in male rats AFB1 and OTA overexpressed KIM-1 while their mixture induced a transcriptomic effect quite similar to the control, suggesting a possible antagonism between mycotoxins. In females, OTA and mycotoxins mixture upregulated KIM-1 whereas AFB1 individually reduced its expression (Fig.3 and 4). In view of this, a possible hypothesis on site and sex-specificity of AFB1 can be stated. Indeed, the high ability of AFB1 to disrupt the blood renal barrier as well as the high specificity of KIM-1 in detecting proximal tubular injury could suggest a specific glomerular damage in females and a tubular one in males (Abdel-Hamid and Firgany, 2015; Chorley et al. 2021). This finding may confirm the results obtained by other studies, in which the greater susceptibility of males to tubular damage and females to glomerulonephritis was reported (Ashuntantang et al. 2018; Park F et al. 2019). Nevertheless, this is the first study in which the effect of AFB1 on KIM-1 expression is assessed and further research is required to confirm this hypothesis.

*In vivo* administration of probiotics has been proven to be an efficient dietary strategy to prevent kidney damage. In rats, the mixture of *Lactobacillus reuteri* and *Clostridium butyricum* suppressed the expression of KIM-1 and limited inflammation, fibrosis, apoptosis and oxidative stress (Hsiao et al. 2021). Likewise, the supplementation of three LAB strains

(*Lactobacillus casei, reuteri* and *helveticus*) showed a significative renoprotective activity, reducing KIM-1 expression and relieving functional and structural alterations (Ragab et al. 2021). *Lactobacillus plantarum* (the same LAB used in this study) has been also shown to downregulate KIM-1 gene expression and ameliorate histopathological abnormalities and nephrocalcinosis in rats (Paul et al. 2018). Furthermore, probiotics administration revealed beneficial effects against structural alterations, oxidative stress, and mitochondrial damage induced by DON, FB1 and AFB1 in the kidney of piglets, mice and broilers, respectively (Bai et al. 2022; Ezdini et al. 2020; Śliżewska et al. 2019). Other bioactive compounds such as carotenoids, flavonoids and curcumin modulated AFB1 and OTA-nephrotoxicity *in vivo* (Damiano et al. 2020; Soliman et al. 2020; Yu et al. 2018). In the present study, although qPCR analysis showed that in male rats FW addition overexpressed KIM-1 to a greater extent than mycotoxins individual exposure, ddPCR revealed opposite results in which FW mitigated KIM-1 upregulation for each condition (Fig.3 and 4). The discordance of results found in this case can be associated with the different sensibility of employed techniques. Indeed, the greater variability and imprecision of qPCR analysis that can inherently occur during reverse transcription, amplification and reference gene normalization, make ddPCR results more robust, accurate and significative (Campomenosi et al. 2016; Taylor et al. 2017). In females, the relative and absolute quantification assays confirmed that FW administration alleviated KIM-1 overexpression promoted by mycotoxins individual exposure. In view of this, ddPCR

analysis confirmed that FW administration mitigated mycotoxins-induced KIM-1 upregulation in both sexes, thus reducing kidney damage (Fig. 4). Also, except for AFB1, sex-related differences were minimal, in contrast to Garovic and August (2016), whose findings emphasized the inherent renoprotective role of estrogens and the greater males susceptibility to kidney damage.

## 5. Conclusions

These findings contribute to a better understanding of the underlying molecular mechanism promoted by AFB1, OTA and FW in liver and kidney of male and female rats. In males liver, the most damaging treatment was AFB1 by reducing CPS1 expression, which was totally reversed by FW administration. This bioactive compound also improved gene expression changes induced by OTA and mycotoxins mixture. In females, the damage triggered by mycotoxins as well as the protective effect of FW were less pronounced. Therefore, a possible hepatoprotective role of estrogens can be stated. In males and females kidney, exposure to mycotoxins prompted a clear toxic effect on renal tissue by upregulating KIM-1, in which OTA-treatment was the most damaging. A site- and sex-specific effect of AFB1 can be possibly speculated. However, ddPCR analysis confirmed that FW administration mitigated mycotoxins induced KIM-1 upregulation in both sexes, thus reducing kidney damage. Herein, except for AFB1, sex-related responses were minimal. In both organs, the importance of considering sex

as a novel factor in toxicological risk assessment related to mycotoxin co-occurrence was also demonstrated. These data confirmed that AFB1 and OTA promoted hepatotoxicity and nephrotoxicity *in vivo*, which could be mitigated by dietary compounds like FW. Nevertheless, further research is needed to better explore the role of FW *in vivo* as well as to understand the inherent sex-related factors and mechanisms that render individuals of the same species different in response to external stimuli.

**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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# 4. DISCUSSION

# 4. Discusión

## 4. Discusión general

Para alcanzar los objetivos planificados en la presente Tesis Doctoral, en primer lugar, se ha realizado una revisión bibliográfica sobre los efectos toxicológicos que AFB1 y OTA pueden inducir en la salud humana y animal, centrada en dos técnicas novedosas como la IF y la FCM. Posteriormente, mediante el empleo de ensayos como: bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio (MTT), IF, FCM y PCR cuantitativa en tiempo real (RT-qPCR) se evaluaron los efectos dañinos de AFB1 y OTA en la diferenciación neuronal *in vitro* y el posible efecto protector del FW y los carotenoides contenidos en la CL frente a la neurotoxicidad inducida por micotoxinas. Análogamente, se analizaron mediante un enfoque transcripcional los daños inmunológicos inducidos por AFB1 y OTA así como los posibles efectos beneficiosos de la mezcla CL y FW en la línea celular Jurkat de linfocitos T humanos. Finalmente, se investigó en ratas Wistar el papel hepatoprotector y nefroprotector del FW frente la toxicidad de AFB1 y OTA mediante la cuantificación relativa y absoluta de biomarcadores típicos del daño hepático y renal.

### 4.1. Evaluación *in vitro* e *in vivo* de la toxicidad de AFB1 y OTA mediante la inmunofluorescencia y la citometría de flujo.

En los últimos años se han reportado varios casos de intoxicación a gran escala así como problemas económicos internacionales causados por la contaminación fúngica. Es por ello que se han considerado posibles intervenciones, mediante procedimientos físicos, químicos y biológicos, con el fin de reducir la presencia y la actividad tóxica de las micotoxinas. A pesar de todos estos intentos, la investigación no ha logrado resolver este problema global y por lo tanto es necesario seguir estudiando los efectos tóxicos de estos metabolitos fúngicos, en particular de AFB1 y OTA (Matumba et al., 2021; Makhuvele et al., 2020; Zhou et al., 2022; Tian et al., 2022). Por ende, se realizó una revisión bibliográfica sobre la toxicidad de AFB1 y OTA en modelos celulares y animales, centrándose en dos técnicas innovadoras como la IF y la FCM.

La literatura revisada muestra que la línea celular más empleada fue la HepG2, seguida por HEK-293T, PK-15, 3D4/21, Caco-2, IPEC-J2, HepaRG, GES-1, MDCK, NRK-52E, HKC y células de la granulosa ovárica porcina. En cuanto a los estudios *in vivo,* los animales de laboratorio con mayor número de estudios fueron pollos, ratones y ratas en tanto que los principales órganos diana analizados fueron el timo, la bolsa de Fabricio y el bazo. El objetivo principal de la búsqueda se centró en la inmunotoxicidad, nefrotoxicidad, hepatotoxicidad, toxicidad gastrointestinal, neurotoxicidad,

embriotoxicidad junto con la toxicidad inducida en el sistema reproductor y los efectos sobre las glándulas mamarias, esófago y pulmones.

En relación con el análisis de IF, se emplearon diferentes anticuerpos para dilucidar alteraciones en varios procesos biológicos relacionados con los efectos toxicológicos de AFB1 y OTA, tales como: daño del material genético, integridad de la barrera intestinal, inflamación, diferenciación neural, estrés oxidativo y muerte celular. En cuanto al análisis de FCM, se realizaron varios ensayos dirigidos a detectar principalmente alteraciones del ciclo celular y la promoción del estrés oxidativo y apoptosis. Esta revisión demuestra tanto los efectos tóxicos de AFB1 y OTA *in vitro* e *in vivo* como la importancia de la IF y la FCM en la comprensión del MoA de las micotoxinas. Además, dado el gran número de estudios *in vitro*, se necesitan más investigaciones *in vivo* para proporcionar una visión más completa y precisa de la toxicidad de AFB1 y OTA sobre la salud humana.

### 4.2. El efecto protector del extracto de calabaza y el suero de leche fermentado frente las alteraciones inducidas por AFB1 y OTA en la diferenciación neuronal *in vitro*.

AFB1 y OTA son las toxinas cancerígenas naturales más potentes que contaminan una amplia gama de productos alimenticios. Debido a su elevada toxicidad, el Panel de Contaminantes de la Cadena Alimentaria

(CONTAM) publicó un informe científico sobre los riesgos para la salud humana relacionados con la presencia de AFB1 y OTA en los alimentos. Además, la CONTAM estimó la ingesta de AFB1 y OTA en la población europea para monitorizar que sus niveles dietéticos no excediesen los límites máximos recomendados por la Unión Europea y evitar posibles consecuencias para la salud humana (EFSA, 2020a; 2020b).

La toxicidad de AFB1 y OTA también se ha confirmado a nivel neuronal una vez atravesada la BHE. De hecho, AFB1 puede inducir efectos citotóxicos en células endoteliales microvasculares, causando daños en el tejido cerebral (Qureshi et al., 2015). La exposición a AFB1 no solo puede alterar la BHE, sino que también inducir neuroinflamación en células microgliales y descompensación cerebral mediante la promoción del estrés oxidativo y apoptosis (Mehrzad et al., 2018; Vahidi-Ferdowsi et al., 2018). De igual modo, OTA atraviesa la BHE causando apoptosis en células neuronales (Zhang X. et al., 2009). La exposición a OTA también puede provocar efectos negativos sobre el metabolismo de la dopamina, promoviendo neuroinflamación y degeneración cerebral (Sava et al., 2006; Petrulli et al., 2017). No obstante, a pesar de los estudios presentes en literatura, los datos disponibles sobre los efectos tóxicos de AFB1 y OTA en la diferenciación neuronal son bastante limitados.

Por otra parte, varios compuestos bioactivos como la CL y el FW han demostrado efectos beneficiosos sobre la salud humana. La CL es rica en

carotenoides que desempeñan un papel importante en la protección de las células frente al estrés y daño oxidativo, previniendo la incidencia de varias enfermedades humanas. Asimismo, el FW, un subproducto de la industria láctea, representa una excelente fuente de ingredientes funcionales, como los ácidos fenólicos y varios péptidos antifúngicos (Bergantin et al., 2018; Gupta y Prakash, 2017). Por lo tanto, en el presente estudio se evaluó el efecto beneficioso de CL y FW contenidos en digeridos gastrointestinales de pan frente a los efectos tóxicos de AFB1 y OTA sobre la diferenciación neuronal de las células SH-SY5Y, utilizando diferentes ensayos como: MTT, IF, FCM y RT-qPCR.

En detalle, los resultados obtenidos en este trabajo demuestran la moderada toxicidad de los extractos digeridos de pan a nivel neuronal, ya que la viabilidad celular nunca desciende por debajo del 60% para todas las condiciones ensayadas. Estos resultados concuerdan con los de Escrivá et al. (2021), cuyo estudio, experimentando con los mismos digeridos de pan en un modelo de barrera gastrointestinal *in vitro*, obtuvo una viabilidad celular entre el 60 y el 100%. Sin embargo, en el presente estudio, se observó una reducción no tóxica pero significativa de la viabilidad celular en todos los extractos de panes digeridos de forma dependiente a la dosis estudiada. Una tendencia similar fue obtenida por Paradells et al. (2015) probando la citotoxicidad de OTA en células neuronales de ratas y por Vahidi-Ferdowsi et al. (2018) exponiendo astrocitos de ratón a diferentes dosis de AFB1. Curiosamente, la presencia de CL en los digeridos aumentó

significativamente la viabilidad neuronal tras 72 horas de tratamiento en comparación con tiempos de exposición más cortos. De forma similar, Montesano et al. (2020) investigando extractos enriquecidos con carotenoides en la línea celular SH-SY5Y, obtuvieron un aumento de la viabilidad celular en función del tiempo. Zhang et al. (2014) ofrecieron una posible explicación para estos resultados, sugiriendo que el uso múltiple y prolongado de carotenoides podría ser necesario para una protección persistente contra las lesiones cerebrales en modelos animales. Del mismo modo, Ochiai et al. (2007) demostraron que la adición de carotenoides en las células neuronales PC12 incrementaban la viabilidad en función del tiempo de exposición.

La línea celular SH-SY5Y representa un modelo establecido de diferenciación neuronal *in vitro.* Una vez diferenciadas, las células SH-SY5Y muestran un fenotipo neuronal, caracterizado por el crecimiento de neuritas y la síntesis de neurotransmisores dopaminérgicos (Chinta & Andersen, 2005; Lv et al., 2012; Avola et al., 2018). La tubulina beta III (TUBB3) es una proteína del citoesqueleto neuronal que se expresa en el cerebro durante su desarrollo. La TUBB3 se ha asociado estrechamente con el crecimiento de neuritas y se utiliza típicamente como marcador neuronal en los estudios de neurotoxicidad (Katsetos et al., 2003). Por otro lado, la dopamina es uno de los neurotransmisores más estudiados por su implicación en varias funciones cerebrales y enfermedades neurológicas (Chinta y Andersen, 2005). En los extractos de pan control, se observó una clara reducción de la

expresión de TUBB3 y dopamina tras el tratamiento con OTA, la cual resultó ser más tóxica que AFB1 a nivel neuronal. En relación con la TUBB3, resultados similares fueron obtenidos por Tanaka et al. (2015), cuyos resultados no mostraron cambios significativos en la expresión de TUBB3 tras la exposición a AFB1 *in vivo*, sugiriendo que la TUBB3 podría no estar implicada en la neurotoxicidad inducida por AFB1. Por otro lado, Hong et al. (2002) demostraron una correlación positiva entre la inhibición del crecimiento neuronal y la administración de OTA (0,1-0,3 μM) en células del mesencéfalo de ratas. La diferenciación *in vitro* de células madre neurales en astrocitos, neuronas y oligodendrocitos fue también evaluada por Gill y Kumara (2019). Utilizando tinciones fluorescentes específicas, los autores concluyeron que una concentración no citotóxica de OTA (0,2 μM) reducía significativamente el grado de diferenciación de las células madre, afectando negativamente al desarrollo del cerebro. Paradells et al. (2015), mediante el análisis de marcadores neuronales típicos, entre ellos TUBB3, revelaron tanto la ausencia de diferenciación como la menor presencia de astrocitos, microglía y neuronas en el cerebro de ratones tras la exposición crónica a OTA. En vista de ello, OTA podría influir en la neurogénesis induciendo la degeneración cerebral.

Con respecto al sistema dopaminérgico, se han descrito muy pocos estudios sobre la toxicidad de AFB1, ya que sus efectos nocivos han sido bien estudiados y confirmados en el sistema colinérgico (Richard et al., 2020). El único estudio encontrado que muestra una reducción de los niveles de

dopamina en regiones específicas del cerebro de ratas tras la administración de AFB1 es de Coulombe & Sharma (1985). En cambio, la exposición a OTA se correlacionó positivamente con una reducción en los niveles de dopamina en ratones, lo que promovía a su vez la enfermedad de Parkinson (EP), anomalías motoras y degeneración cerebral (Sava et al., 2006, Izco et al., 2021). El efecto inhibidor de OTA sobre la diferenciación neuronal también se ha detectado *in vitro* con niveles alterados de dopamina (Hong et al., 2000).

Por primera vez, en el presente estudio se han observado efectos beneficiosos de la CL y el FW sobre el crecimiento neurítico y los niveles de dopamina. En concordancia con nuestros resultados, Goda et al. (2016) mostraron que la astaxantina (AST), un conocido carotenoide, mejoró y revirtió las alteraciones en el sistema dopaminérgico inducidas en el cerebro de ratas por la Penitrem A, una potente neurotoxina producida por varias especies terrestres y marinas de *Penicillium.* Del mismo modo, Lee et al. (2011) demostraron el papel protector de la AST frente a las alteraciones de los niveles de dopamina promovidas por la neurotoxina $MPP^+$ *in vivo*. Por lo tanto, el tratamiento con AST se ha considerado una valiosa estrategia terapéutica en la modulación de la progresión de la EP. De forma similar, se ha descrito el papel neuroprotector del licopeno frente a agentes neurotóxicos en varios modelos *in vitro* e *in vivo.* Este compuesto modulaba la alteración del sistema dopaminérgico y la progresión de la EP (Hedayati et al., 2019). En relación con el FW, Ano et al. (2018) demostraron su papel beneficioso en el cerebro de ratones al aumentar los niveles de dopamina, lo

que a su vez mejoraba la memoria y prevenía el deterioro cognitivo. En los mismos modelos experimentales, el FW también reducía la progresión de enfermedades neurodegenerativas como la demencia y la EA (Katayama y Nakamura, 2019).

La detención del ciclo celular es una condición transitoria, inducida por varios factores biológicos, que las células pueden revertir espontáneamente restaurando su proliferación o volviéndose apoptóticas (Juan-García et al., 2013). Aunque, AFB1 puede alterar la progresión del ciclo celular en varias líneas celulares (hepática, renal, inmune y epitelial), en el presente estudio no se han detectado diferencias significativas tras la exposición a la toxina (Ricordy et al., 2002; Yu et al., 2015; Li et al., 2019). Hallazgos diferentes fueron obtenidos por Huang et al. (2020), tratando células neuronales IRM-32 con AFB1 (0,5-2 μM) durante 24-48 h. La exposición a AFB1 causó una detención significativa del ciclo celular en fase S con marcado daño en el ADN. Asimismo, AFB1 (5-50 μM durante 48 h) disminuyó la proliferación celular y detuvo la progresión del ciclo celular en fase sub-$G_0/G_1$ con disfunción mitocondrial y apoptosis en astrocitos humanos (Park et al., 2020). La discrepancia de los resultados puede estar relacionada con las diferentes condiciones experimentales utilizadas en estos estudios. Por otro lado, en relación al tratamiento con OTA, se ha observado una acumulación significativa de las células SH-SY5Y en la fase $G_0/G_1$ del ciclo celular. En línea con estos resultados, la exposición a OTA se ha asociado ampliamente con la detención del ciclo celular en la fase $G_0/G_1$ en

astrocitos, células renales, hepáticas e inmunes (Liu et al., 2012; Juan-García et al., 2019; Park et al., 2019). En cambio, Babayan et al. (2020) no observaron diferencias significativas en las fases del ciclo celular tratando las líneas celulares SH-SY5Y y HT22 con altas dosis de OTA (2,5-30 μM) durante 24 h. La discordancia de resultados puede estar relacionada no sólo con las condiciones experimentales (principalmente referidas a las dosis) sino también con los modelos celulares indiferenciados empleados en este estudio.

El deterioro neuronal crónico inducido por la desregulación del ciclo celular puede alterar varias funciones motoras, cognitivas y reguladoras del cerebro. En consecuencia, la preservación del ciclo celular representa un aspecto esencial de la neuroprotección (Joseph et al., 2020). En concordancia con nuestros resultados, Sathasivam & Ki (2018) demostraron como la administración de carotenoides evitó las alteraciones del ciclo celular en varios modelos celulares. En cuanto a la adición del FW, se observaron efectos poco claros sobre el ciclo celular. El alto porcentaje de células SH-SY5Y encontradas en fase $G_0/G_1$ puede correlacionarse con la composición del FW. La presencia de varios ingredientes funcionales en el FW, como los ácidos fenólicos, puede interferir y potenciar los efectos del ácido retinoico (Xu et al., 2013; Chairez-Cantu et al., 2021). El ácido retinoico promueve la diferenciación neuronal prolongando claramente la fase $G_0/G_1$ (Zhang W. et al., 2009; Qiao et al., 2012).

Por último, los resultados obtenidos mediante la IF y la FCM se confirmaron a nivel transcripcional mediante RT-qPCR. Por ello, se analizó la expresión de varios genes relacionados con la diferenciación neuronal (Wnt5a, GAP43 y TUBB3) y el ciclo celular (ciclinas B y D, p21, p53). Las proteínas Wnt están implicadas en varias funciones celulares, como la migración y la polarización neuronal, el desarrollo de dendritas y la formación de sinapsis (Inestrosa y Arenas, 2010; Rosso e Inestrosa, 2013). Concretamente, según hallazgos anteriores, la represión de Wnt5a obtenida en el pan contaminado con micotoxinas, está relacionada con la apoptosis neuronal y la progresión de la EA (Zhou et al., 2017). La represión de Wnt5a podría también inducir neurotoxicidad y déficit cognitivo en modelos animales (Coullery et al., 2020). Asimismo, Blakely et al. (2011) demostraron cómo el aumento de la expresión de Wnt5a, observado principalmente en los panes enriquecidos con FW, podría promover la elongación y maduración de los axones dopaminérgicos, con efectos positivos en el mesencéfalo ventral de ratones. En cuanto a GAP43, también conocida como neuromodulina, es una proteína relacionada con el crecimiento de las neuritas, la memoria espacial y el aprendizaje (Denny, 2006). En el presente estudio, la exposición a AFB1 y OTA indujo una ligera represión de GAP43 mientras que la adición de CL y FW, individualmente y en combinación, promovió la sobreexpresión de GAP43 en cada condición realizada. La sobreexpresión de la neuromodulina en células de glioma C6 y células neuronales IRM-32 se ha relacionado positivamente con la formación y el crecimiento de neuritas, la regeneración neuronal y la plasticidad de las

neuronas ganglionares espirales (Konar et al., 2011; Lei & Tang, 2017). En los panes contaminados con micotoxinas se observó también una ligera represión de TUBB3 y su sobreexpresión en los panes enriquecidos con CL y FW. En línea con estos resultados, la represión de TUBB3 se asoció con una menor diferenciación de las neuronas dopaminérgicas (Lupu et al., 2018), mientras que su sobreexpresión promovió la diferenciación neuronal en células estaminales y en células C6 de ratas (Chao et al., 2015; Pereckova et al., 2021).

En cuanto a la expresión de los genes relacionados con el ciclo celular, las ciclinas son una familia de proteínas que controlan la progresión del ciclo celular mediante la activación de las enzimas quinasas dependientes de ciclinas (Galderisi et al., 2003). La ciclina B1, un componente clave en la progresión del ciclo celular de la fase $G_2$ a la fase M, está implicada en los primeros eventos de la mitosis como la condensación cromosómica (Tu et al., 2013). La ciclina D1 es una proteína clave en la progresión del ciclo celular de la fase $G_1$ a la fase S en respuesta a la estimulación del factor de crecimiento (Long & Wu, 2008). En este estudio, las ciclinas B1 y D1 mostraron una tendencia similar, siendo reprimidas en los panes contaminados con micotoxinas y significativamente sobreexpresadas una vez añadidos los compuestos funcionales. Asimismo, hallazgos anteriores demostraron que la supresión de la ciclina B1 se correlacionaba positivamente con los efectos neurotóxicos inducidos por la homocisteína en células SH-SY5Y (Curro et al., 2014). Del mismo modo, la represión de la

ciclina D1 podría alterar la neurogénesis y la diferenciación neuronal en el cerebro de ratas tras la exposición al bisfenol A (Tiwari et al., 2015).

Por otro lado, p21 regula el punto de control $G_1$/S y su activación desempeña un papel clave en la diferenciación y maduración de los precursores neurales (Galderisi et al., 2003; Cazzalini et al., 2010; Zhen et al., 2012). Por ende, el incremento de la expresión de p21 observada en el presente trabajo podría considerarse un evento intrínseco relacionado con la diferenciación neuronal promovida por el ácido retinoico (Qiao et al., 2012; Fu et al., 2020). Otra función de p21 es el control del ciclo celular mediante la regulación negativa de las vías de apoptosis dependientes o independientes de p53 (Benson et al., 2014). La implicación de p53 en la regulación del ciclo celular está relacionada con modificaciones postraduccionales, es decir acetilación y fosforilación, y con su localización subcelular (Tedeschi y Di Giovanni, 2009; Sola et al., 2011). Se demostró que AFB1 y OTA inducen apoptosis en astrocitos y oligodendrocitos a través de la sobreexpresión de p53 (Kuroda et al., 2015; Hibi et al., 2013; Yoon et al., 2009; Park et al., 2020). En este estudio, la expresión génica de p53 presentó un patrón poco claro, estando ligeramente reprimida o sobreexpresada en función del tratamiento.

En conclusión, cabe destacar que OTA fue la condición más dañina reduciendo en mayor medida la expresión de los marcadores neuronales: TUBB3 y dopamina y deteniendo el ciclo celular en fase $G_0/G_1$. Por otro lado, los efectos de AFB1 sobre la expresión de los biomarcadores neuronales y el ciclo celular fueron menos pronunciados. Sin embargo, la CL y el FW,

individualmente y en combinación, reportaron un efecto beneficioso sobre el desarrollo neuronal y el ciclo celular una vez añadidos a la mezcla de micotoxinas. En cuanto al análisis de RT-qPCR, la represión de marcadores neuronales como: Wnt5, TUBB3 y GAP43 y de las ciclinas por parte de AFB1 y OTA fue revertida por los compuestos funcionales. Por lo tanto, en el presente estudio se demostró tanto la neurotoxicidad de AFB1 y OTA como la función neuroprotectora de CL y FW.

### 4.3. AFB1 y OTA inducen inmunotoxicidad en células linfoblásticas humanas a nivel transcripcional.

La exposición a AFB1 y OTA puede inducir inmunotoxicidad en varios modelos *in vitro* e *in vivo*. La literatura relata que el tratamiento con bajas dosis de AFB1 promovió una respuesta inmune y proinflamatoria en macrófagos con generación de ROS, autofagia y muerte celular (An et al., 2017). En macrófagos, AFB1 también empeoró la infección por el virus de la gripe porcina, la inflamación y el daño del tejido pulmonar mediante la activación de la vía de señalización TLR4/NF-kB (Sun et al., 2018). La supresión de la respuesta inmunitaria también se observó en ratas y pollos (Bakheet et al., 2016; Ben Salah-Abbes et al., 2016; Guan et al., 2019). De forma similar, OTA puede alterar la función fagocitaria de los heterófilos con producción intracelular de ROS (Han et al., 2019). Asimismo, el tratamiento con OTA *in vitro* se relacionó con la progresión de la artritis reumatoide

mientras que la administración *in vivo* de la toxina provocó apoptosis, estrés inmunitario e inflamación en el bazo de pollos (Jahreis et al., 2017; Liu et al., 2018). Además, se observó que la combinación de AFB1 y OTA provocó inflamación, estrés oxidativo y apoptosis *in vitro* a través de la vía de señalización NF-kB (Hou et al., 2018). Sin embargo, la información sobre la toxicidad inducida por AFB1 y OTA en linfocitos T humanos es muy limitada y su MoA han sido raramente investigado. Por ello, en este estudio, se realizó un análisis transcripcional con el fin de evaluar el efecto toxicológico de AFB1 y OTA de forma individual y combinada en el perfil de expresión génica de las células Jurkat.

Por ende, representando un escenario realista, se observó que el número de genes diferencialmente expresados (DEG) aumentaba significativamente en la exposición combinada (3236) con respecto a las individuales (AFB1=99 y OTA=77). Este resultado es relevante en la evaluación de riesgos para la salud humana, ya que la población mundial está constantemente expuesta a múltiples micotoxinas que contaminan los alimentos (Arce-López et al., 2020). Resultados similares fueron obtenidos por Corcuera et al. (2011), exponiendo células hepáticas HepG2 a altas concentraciones de AFB1 (1,5-150 μM; 3 y 24 h) y OTA (50-800 μM; 3 y 24 h). En este sentido, se obtuvieron efectos tóxicos más evidentes en la exposición combinada que en las individuales, sugiriendo un sinergismo entre las micotoxinas. En células renales Vero, AFB1 y OTA (1-50 μM; 24 h) no sólo mostraron un efecto citotóxico aditivo, sino también sinergismo en la

promoción de genotoxicidad con aumento de la fragmentación del ADN (Golli-Bennour et al., 2010). Asimismo, se demostró que OTA aumentaba significativamente la mutagenicidad de AFB1, con un índice de mutación más alto que las exposiciones individuales (Sedmikova et al., 2001). Resultados diferentes fueron obtenidos por Choi et al. (2020) tratando células de hígado LMH de pollos con AFB1 (0-3 μM; 48 h) y OTA (0-20 μM; 48 h). El ensayo de citotoxicidad y el análisis transcripcional revelaron un claro efecto antagonista entre las micotoxinas en la inducción de la hepatotoxicidad. La discrepancia en los resultados se puede asociar con el uso de condiciones experimentales menos realistas (dosis, tiempo de exposición y línea celular no humana) comparadas con aquellas empleadas en el presente estudio.

### *4.3.1 Tratamiento con AFB1*

La vía de señalización de la ataxia telangiectasia mutada (ATM) fue la más afectada tras la exposición a AFB1. Esta ruta biológica desempeña un papel muy importante en la respuesta celular al daño genético mediante la implicación de la proteína quinasa ATM como su principal efector (Zhang et al., 2012). Varios estudios han demostrado que la exposición *in vitro* e *in vivo* a AFB1 promovía roturas en el ADN, activación de la señalización ATM y la sobreexpresión de todas las quinasas ATM implicadas en el estiramiento de la cromatina, regulación del ciclo celular e inmunidad (Sancar et al., 2004; Stracker et al., 2013; Yin et al., 2016). También se demostró que bajas dosis de AFB1 (5-80 nM; 24 h) indujeron daños en el ADN y la activación de la

señalización ATM en células BEAS-2B, lo que causó alteraciones genéticas en el sistema respiratorio humano (Yang et al., 2013).

Asimismo, la señalización ATM es un componente central en el sistema de reparación del ADN mediante la activación de la ubiquitina ligasa RNF40, la cual desempeña un papel clave en la reorganización de la cromatina y la reparación temprana del daño genético (Jin & Oh, 2019; Moyal et al., 2011). La represión de RNF40 se ha asociado con estrés replicativo, inestabilidad cromosómica, alteraciones del ciclo celular e inhibición del mecanismo de reparación del ADN *in vitro* (Chernikova et al., 2012; Kari et al., 2011). En embriones de pavo, se observó que la exposición a AFB1 (1 μg por inyección; 24 h) redujo la actividad de la ubiquitina ligasa y deterioraba la respuesta celular al daño genotóxico (Engin & Engin, 2019; Monson et al., 2015). Por lo tanto, la activación de la señalización ATM mediante el aumento de la expresión de las quinasas ATM y la represión de RNF40 apuntan a la inducción de daños en el ADN y a la alteración de su mecanismo de reparación como posibles MoA mediante los cuales AFB1 promovía toxicidad inmunitaria *in vitro*.

La señalización ATM tiene un papel clave también en la transición $G_2$/M del ciclo celular (Lavin & Kozlov, 2007). Se concluyó que la exposición a AFB1 sobrexpresó la quinasa ATM provocando la detención del ciclo celular en la fase $G_2$/M en células epiteliales bronquiales (Yang et al., 2013). Resultados similares se obtuvieron en la bolsa de Fabricio, bazo y yeyuno de

pollos, lo que confirma la correlación entre la administración *in vivo* de AFB1 y la detención del ciclo celular en la fase $G_2$/M mediante la activación de la vía de señalización ATM (Hu et al., 2018; Li et al., 2019; Yin et al., 2016). En línea con estos resultados, la exposición a AFB1 podría inducir una potencial alteración en el ciclo celular de las células Jurkat.

Además, la señalización ATM está implicada en el desarrollo de las células T mediante el control del proceso de recombinación V(D)J, el cual es indispensable para constituir el dominio variable del receptor TCR de las células linfocitarias (Helmink & Sleckman, 2012; Nussenzweig & Nussenzweig, 2010). Las alteraciones en la señalización ATM puede alterar el proceso V(D)J y predisponer a las células T humanas a leucemia y linfoma (Matei et al., 2006). En este contexto, la proteína Artemis, un conocido sustrato de la ATM, tiene una función clave en la recombinación V(D)J (Jeggo et al., 2005; Riviera-Munoz et al., 2016). Concretamente, se ha descrito la asociación entre varios efectos nocivos *in vitro* como la inmunodeficiencia, detención del ciclo celular y daño en el ADN y la sobreexpresión de la proteína Artemis (Multhaup et al., 2010; Sridharan et al., 2012; Ulus-Senguloglu et al., 2012). Por ende, la activación de la señalización ATM con la sobreexpresión de la proteína Artemis podrían sugerir la posible interferencia de AFB1 en la recombinación y funcionalidad de las células T.

*4.3.2 Tratamiento con OTA*

Las micotoxinas pueden inducir toxicidad *in vitro* mediante varias alteraciones metabólicas (Prusinkiewicz & Mymry, 2019). Concretamente, OTA (1,25-5 μM; 24 h) promovió la toxicidad mitocondrial desplazando el metabolismo energético de la fosforilación oxidativa a la glucólisis en células gástricas humanas (Wang et al., 2020). Alteraciones metabólicas similares fueron identificadas por Zhao M. et al. (2021) en células esofágicas humanas tras la exposición a OTA (2,5-10 μM; 24 h) y por Zhang et al. (2011) analizando el hígado de ratas tratadas con dosis moderadas de AFB1 (1,6 mg/kg; 12 días). En línea con estos estudios, el análisis PathVisio reveló la reprogramación metabólica y señaló la glucólisis como la vía más afectada tras la exposición a OTA en células Jurkat.

Todos los genes relacionados con la vía de la glucólisis se vieron ligeramente afectados por OTA. En particular, RB1 es un supresor tumoral que interviene en la regulación del ciclo celular, la diferenciación, la proliferación y la muerte celular (Lai et al., 2006). Se demostró que la sobreexpresión de RB1 inducía cambios metabólicos con promoción de la glucólisis en células mamarias humanas (Wu et al., 2020). Del mismo modo, el aumento de la actividad de la G6PD, una enzima clave en la producción de NADPH, potenciaba la activación de la glucólisis en células de leucemia mieloide aguda (Poulain et al., 2017). Análogamente, se observó que la sobreexpresión de este enzima tras la exposición a beauvericina (BEA) y

enniatina B promovía la disrupción mitocondrial reduciendo la producción de ATP *in vitro* (Soderstrom et al., 2022). Por otro lado, la enzima PGK1 cataliza la primera reacción de la glucólisis anaeróbica. El análisis del transcriptoma humano realizado en células HepG2 tras la exposición a OTA (2,5-10 μM; 24h) mostró la inhibición de PGK1 y su implicación en la alteración del metabolismo hepático (Hundhausen et al., 2008). De forma similar, la represión de la actividad de PKM2, la enzima que convierte el fosfoenolpiruvato en piruvato para producir ATP, se relaciona con la alteración del metabolismo y la inducción de la glucólisis en células gástricas humanas (Ferguson & Rathmell, 2008; Wang et al., 2020). Se vió también que la enzima GAPDH está implicada en procesos metabólicos como la glucólisis (Tristan et al., 2011). En este sentido, la represión de GAPDH se asoció con alteraciones significativas en el metabolismo energético tras la exposición a zearalenona (ZEA) (10 μM; 24h) en células humanas MCF10F (Karaman & Ozden, 2019). Asimismo, la enzima LDHA cataliza el último paso de la glucólisis anaeróbica convirtiendo el piruvato en lactato (Xian et al., 2015). Abdul et al. (2020) observaron que el ácido fusárico (4 y 256 μM; 6h), una micotoxina de origen alimentario poco conocida, reducía la expresión génica de la LDHA, entre otras, promoviendo adaptaciones bioenergéticas con activación de la glucólisis en células HepG2. La disrupción metabólica promovida por OTA en células Jurkat se confirma por la alteración de las vías de señalización HIF1, PPAR-γ y del ciclo de Cori. Las vías HIF1a y PPAR-γ son reguladoras clave de la reprogramación metabólica y la glucólisis *in vitro* mientras que el ciclo de Cori es una ruta metabólica

típicamente activada por altas concentraciones de lactato (Feng et al., 2020; Prigione et al., 2014; Suhara et al., 2015).

### *4.3.3 Tratamiento combinado de AFB1 y OTA*

La ferroptosis fue la principal vía de señalización alterada tras la exposición combinada a AFB1 y OTA con un 85% de genes expresados de manera diferente a los controles. La ferroptosis es una forma de necrosis inducida por la combinación de: una excesiva acumulación de hierro intracelular, inducción de la peroxidación lipídica con daño oxidativo en la membrana plasmática e inhibición de la actividad de la glutatión peroxidasa 4 (GPX4) (Chen X. et al., 2021).

La acumulación intracelular de hierro se asocia a varios factores biológicos. La transferrina es una proteína que se une al hierro extracelular y lo transporta al interior de las células a través de su transportador por endocitosis (Shi et al., 2019). Zhao L. et al. (2021) demostraron la clara correlación entre la sobreexpresión de la transferrina y la inducción de la ferroptosis en el corazón de pollos tras la administración de AFB1 en piensos contaminados (1 mg/kg; 21 días). Asimismo, la represión de la proteína HSPB1, un regulador negativo de la captación y acumulación de hierro intracelular, también confirmó el desequilibrio en los niveles de hierro durante la ferroptosis promovida por AFB1. De igual modo, la represión del transportador de la transferrina puede causar: acumulación de hierro

intracelular, estrés oxidativo y generación de ROS en varios modelos celulares (Ding et al., 2021). El análisis transcriptomico realizado en células IPEC-J2 tratadas con deoxinivalenol (DON) (0,5 μg/mL; 48 h) reveló la represión de varios genes relacionados con la homeostasis del hierro, incluyendo el receptor de la transferrina, que fue considerado el evento desencadenante del proceso ferroptótico (Lin et al., 2021). Otro inductor fundamental de la ferroptosis es el factor BACH1, un regulador del metabolismo del hierro. La sobreexpresión *in vitro* de esta proteína puede suprimir varios genes implicados en el almacenamiento de hierro (como la ferritina, un complejo proteico formado por cadenas pesada, FTH1, y ligera, FTL), provocando la liberación de hierro inestable en el citoplasma y empeorando así el estado ferroptótico (Nishizawa et al., 2020; Xie et al., 2016).

Altas concentraciones de hierro en el citoplasma pueden aumentar la producción de peróxido de hidrógeno y radical hidroxilo promoviendo la peroxidación lipídica (Au et al., 2008; He et al., 2020). Durante la ferroptosis, la oxidación lipídica se produce como un evento intermedio, en el que las enzimas ASCL4 y ASCL3 son los principales reguladores (Magtanong et al., 2019; Lee et al., 2021). Se ha reportado como la toxina T-2 (2,5-10 nM durante 6-20 h) promovía: ferroptosis, estrés oxidativo y generación intracelular de ROS a través de la alteración de varios genes relacionados con la peroxidación lipídica, incluyendo la ligera represión de ASCL4. Asimismo, la disminución de la expresión de ASCL3 se ha relacionado con la ferroptosis

y el estrés oxidativo en modelos celulares y animales (Ubellacker et al., 2020; Wang et al., 2021).

Por otro lado, la GPX4, una glutatión peroxidasa dependiente del selenio, protege las células frente los peróxidos lipídicos utilizando el glutatión reducido como sustrato (Dixon & Stockwell, 2019). En las células T humanas, una actividad reducida de la GPX4 puede provocar peroxidación lipídica, ferroptosis, alteración de la homeostasis celular y aumento de la susceptibilidad a las infecciones agudas (Matsushita et al., 2015). También se señaló que la exposición a AFB1 y OTA redujo la expresión del gen GPX4 y provocó peroxidación lipídica, generación de ROS y ferroptosis *in vitro* e *in vivo* (Gan et al., 2015; Ren et al., 2019; Vettorazzi et al., 2019).

Considerando estas evidencias, la exposición combinada a AFB1 y OTA podría inducir ferroptosis en las células Jurkat alterando la expresión de varios genes implicados en la acumulación intracelular de hierro (transferrina, BACH1, FTH1, FTL), peroxidación lipídica (ASCL4, ASCL3) y defensa celular antioxidante (GPX4). AFB1 y OTA también alteraron la vía de señalización PPAR-$\alpha$, una nueva vía asociada a la ferroptosis debido a su función activa en la remodelación lipídica (Venkathes et al., 2020).

Estos hallazgos contribuyen a una mejor comprensión de las principales vías celulares implicadas en la toxicidad inmunitaria inducida por parte de AFB1 y OTA en células Jurkat. La secuenciación del ácido

ribonucleico (ARN) reveló que la vía de señalización ATM fue la más alterada tras la exposición a AFB1. En detalle, las posibles alteraciones del ciclo celular en la fase $G_2$/M así como la inducción de daños en el ADN con deterioro de sus mecanismos de reparación fueron los principales MoA relacionados con la toxicidad de AFB1. En cuanto a la exposición a OTA, la ruta de la glucólisis fue la más afectada. Concretamente, la reprogramación del metabolismo energético mediante la activación de la glucólisis puede considerarse el principal MoA mediante el cual OTA causó efectos tóxicos en las células Jurkat. En combinación, AFB1 y OTA alteraron principalmente la vía de la ferroptosis. En este caso, la acumulación de hierro intracelular con peroxidación lipídica, estrés oxidativo y alteración de las defensas celulares antioxidantes pueden explicar como la mezcla de micotoxinas originó la inmunotoxicidad *in vitro*.

### 4.4. El efecto protector de la mezcla de calabaza y suero de leche fermentado contra la toxicidad inmunológica de AFB1 y OTA *in vitro*. Un enfoque transcripcional.

La CL y el FW poseen varios efectos beneficiosos sobre la salud humana. La literatura relata que la adición de estos ingredientes en la elaboración del pan mejoraba su vida útil y capacidad antioxidante, inhibía el crecimiento de hongos micotoxigénicos y reducía tanto la biosíntesis como la bioaccesibilidad de AFB1 y OTA *in vitro* (Escrivá et al., 2022; Sadeghi et al.,

2019; Luz et al., 2020). Los carotenoides de la CL y los compuestos bioactivos contenidos en el FW también muestran altos valores de biodisponibilidad *in vitro* (10-60% y 10-22%, respectivamente), siendo capaces de alcanzar el torrente sanguíneo y ejercer potencialmente un efecto sistémico (Escrivá et al., 2021; Juan et al., 2022). Asimismo, Alonso-Garrido et al. (2021a) demostraron el papel positivo de la CL a nivel transcripcional tras la exposición a AFB1 y OTA en un modelo *in vitro* de la BHE. Además, es interesante destacar el efecto sinérgico y aditivo de CL y FW en modelos celulares y animales. En células neuronales SH-SY5Y, la adición de la mezcla CL-FW en panes contaminados con AFB1 y OTA no sólo aumentó la expresión de marcadores típicos de la diferenciación neuronal, sino que también atenuó las alteraciones genéticas inducidas por la exposición a las micotoxinas (Frangiamone et al., 2022b). En ratas espontáneamente hipertensas, la adición de la mezcla CL-FW mejoró la presión arterial y la disbiosis intestinal (Agarkova et al., 2019).

Sin embargo, los efectos de la mezcla CL-FW frente la toxicidad inmunológica de AFB1 y OTA en escasas ocasiones han sido objeto de estudio. Por ello, en el presente trabajo, se evaluó el posible efecto protector de la mezcla CL-FW frente la inmunotoxicidad promovida por AFB1 y OTA a nivel transcripcional utilizando extractos de pan digeridos. Además, para reproducir un escenario realista, se utilizaron nuevamente las células Jurkat, al ser un sólido modelo inmunitario para representar situaciones humanas *in vitro* (Shao et al., 2014; Cassioli et al., 2021).

Analizando el número de DEGs tras la exposición con micotoxinas e ingredientes funcionales, cabe destacar que sólo 34 DEGs fueron alterados por AFB1, 3450 por OTA y 3264 por la exposición combinada, lo que podría excluir un efecto sinérgico entre AFB1 y OTA cuando se combinan con estos compuestos bioactivos. De forma análoga, Cimbalo et al. (2022a), utilizando las mismas condiciones experimentales del presente estudio (línea celular, tiempo de exposición y extractos de pan digeridos), para realizar un análisis proteómico, hallaron un menor número de proteínas diferencialmente expresadas en la exposición combinada con respecto a las individuales. Frangiamone et al. (2022b), tratando células SH-SY5Y durante 7 días, también observaron un efecto antagónico entre AFB1 y OTA una vez combinadas con la mezcla CL-FW. Es interesante observar cómo el tratamiento combinado de AFB1-OTA compartió el 80% de los DEGs con la exposición individual a OTA. Estos resultados pueden estar relacionados con la concentración de OTA, que es casi 5 veces superior a la dosis de AFB1 en los digeridos gastrointestinales.

Considerando todas las condiciones estudiadas, se obtuvieron 30 DEGs en común. Entre ellos, se observó la clara sobreexpresión de varios genes pertenecientes a la familia dedo de zinc (ZNF28, 563, 594), del gen GPR17 (receptor acoplado a proteína G) y la represión del gen RHOB (una proteína de la familia RAS). Varios estudios han reportado que la sobreexpresión de las proteínas dedos de zinc atenuaba la activación de la señalización NF-kB, las lesiones inflamatorias cerebrales y la producción de

citocinas proinflamatorias *in vitro* e *in vivo* (Hong et al., 2016; Zhan et al., 2016; Agnihotri & Gaur, 2022). Asimismo, Maekawa et al. (2009) revelaron que la sobreexpresión de GPR17 ejercía efectos inhibidores sobre la respuesta inflamatoria y la producción de leucotrienos en macrófagos de ratones. También se ha demostrado que la represión de RHOB modulaba tanto la respuesta inflamatoria mediada por TLR4 como la producción de citoquinas en macrófagos (Zhang et al., 2017). Además, una confirmación interesante de los resultados proteómicos obtenidos por Cimbalo et al. (2022a) fue la expresión alterada de varios genes que codifican para la familia de las histonas, las cuales desempeñan un papel clave en el mantenimiento de la topología del genoma. En este caso, se observó la represión de H1.4, que se ha asociado estrechamente con la producción de interferón (IFN) *in vitro* (Serna-Pujol et al., 2022).

### *4.4.1 Tratamiento AFB1 CL-FW*

El tratamiento con AFB1 CL-FW favoreció la activación de varias rutas biológicas asociadas con el metabolismo de los eicosanoides, los cuales son moléculas lipídicas de señalización que desempeñan una función clave en la respuesta inflamatoria, regulando la producción de citoquinas y quimiocinas (Sheppe y Eldemann, 2021). Se ha descrito que los eicosanoides también participan activamente en la toxicidad inmunitaria mediada por las micotoxinas. De hecho, el tratamiento con AFB1 (25 μM durante 48 h) inició la producción de eicosanoides y citoquinas proinflamatorias en macrófagos

humanos, que a su vez desencadenaron la necrosis de las células hepáticas HepG2, así como el crecimiento y desarrollo del carcinoma hepatocelular *in vivo* (Fishbein et al., 2020). Del mismo modo, la secuenciación del ARN realizada en macrófagos bovinos reveló la activación de la vía de señalización de los eicosanoides y su participación activa en la muerte celular mediada por la exposición a OTA (10 μM durante 6 h) (Brennan et al., 2017). Además, el análisis proteómico realizado en macrófagos humanos mostró como la micotoxina emergente alternariol (1 μM durante 3 h) causaba toxicidad inmunitaria al aumentar la producción de eicosanoides como el 12-HETE, que a su vez potenciaba la síntesis de mediadores proinflamatorios (Del Favero et al., 2020). Kostro et al. (2012) también demostraron que la micotoxicosis inducida por ZEA en ovejas (3,07-14,49 μg/kg durante 70 días) estuvo mediada por los eicosanoides, los cuales fomentaron la infiltración de neutrófilos en el tracto digestivo.

Por otro lado, el uso de compuestos bioactivos como los carotenoides y los compuestos fenólicos, naturalmente presentes en la mezcla CL-FW, se ha considerado una eficiente estrategia nutricional para modular la respuesta inflamatoria y la producción de eicosanoides (Kamalian et al., 2020). Storniolo et al. (2019) demostraron como una mezcla de carotenoides y ácidos fenólicos aliviaba el estrés oxidativo y la producción de eicosanoides en monocitos humanos, promoviendo la resolución de la inflamación y de la aterosclerosis. Asimismo, una mezcla de compuestos antioxidantes y antiinflamatorios (contenidos de forma natural en el propóleo brasileño) no

sólo moduló la producción de citoquinas, sino que también inhibió selectivamente la síntesis de eicosanoides proinflamatorios *in vivo* (Ferreira et al., 2021). De forma similar, la pulpa de la CL mostró un efecto protector frente a la exposición con micotoxinas en células ECV304 (OTA, BEA, ZEA; 0,1 μM durante 9 días), inhibiendo la producción de eicosanoides inflamatorios (prostaglandina D2 y leucotrienos B4 y E4) y protegiendo la integridad de la BHE *in vitro* (Alonso-Garrido et al., 2021b).

En este estudio, el metabolismo de los eicosanoides mediante la lipoxigenasa fue la vía más alterada, en la cual se destaca tanto la represión de ALOX12 y LTA4H como la sobreexpresión de ALOX15. En concreto, ALOX12 y ALOX15 son mediadores clave en el metabolismo de los eicosanoides con funciones diferentes. La literatura relata que la inhibición de ALOX12 mostraba efectos antiinflamatorios, suprimiendo la producción de eicosanoides y citoquinas proinflamatorias en ratones, cerdos y monos (Zhang et al., 2021). De forma similar, la sobreexpresión de ALOX15 se ha asociado ampliamente con la resolución de ciertas enfermedades humanas mediante la síntesis de mediadores antiinflamatorios como las lipoxinas (Singh & Rao, 2019; Snodgrass et al., 2020). Además, la secuenciación del ARN realizada en células neuronales T98G reveló el efecto antiinflamatorio de una mezcla de ingredientes funcionales mediante la represión de ALOX12, lo que a su vez inhibía la síntesis de eicosanoides proinflamatorios (Panossian et al., 2019). Del mismo modo, la represión de LTA4H se ha relacionado con la respuesta antiinflamatoria y la modulación de la vía de

señalización de los eicosanoides *in vitro* (Wei et al., 2021; Sheppe & Eldemann, 2021). En vista de ello, los resultados obtenidos pueden confirmar el efecto beneficioso de la mezcla CL-FW contra la activación de la ruta de los eicosanoides y la posible cascada proinflamatoria inducida por AFB1 en células Jurkat.

### *4.4.2 Tratamiento OTA CL-FW*

La señalización del IFN fue la ruta más perturbada tras la exposición a OTA CL-FW. Los IFNs tienen un rol crucial en la modulación de la respuesta inmune y la inflamación, siendo producidos especialmente durante situaciones oxidativas e infecciosas (Barrat et al., 2019; Rojas et al., 2021). Varios estudios han señalado la estrecha relación entre la inducción de la vía del IFN y la toxicidad de las micotoxinas (Frangiamone et al., 2022a). En este sentido, la combinación de OTA y DON provocó la liberación de citoquinas proinflamatorias como IL-6, IL-17 e IFN-γ en esplenocitos de ratones. La exposición a las micotoxinas también causó la activación de la vía de señalización IFN, que a su vez estimuló la diferenciación de las células T hacia el fenotipo Th1/17, aumentando la susceptibilidad a las enfermedades inflamatorias (Jahreis et al., 2017). De forma análoga, el análisis transcriptómico y proteómico realizado en células Caco-2 demostró que la exposición combinada a OTA y AFM1 (10 μM durante 48h) provocaba la activación de varias rutas asociadas a la respuesta inmune e inflamatoria. Además, se observó una clara producción de citoquinas proinflamatorias, lo

que condujo a una alteración de la barrera intestinal (Gao et al., 2020). Asimismo, la exposición a OTA *in vivo* (0,5-250 μg/kg durante 2-7 semanas) activó la vía de señalización del IFN, causando inflamación sistémica y disbiosis intestinal (Prasanna et al., 2007; Wang et al., 2019).

Varios estudios han demostrado que la activación de la vía de señalización del IFN por OTA implica la inducción de varios factores en cadena, incluida la sobreexpresión de las proteínas MyD88 y MAPK, la fosforilación y activación de las cascadas NF-kB y JUN/FOS (Cimbalo et al., 2022b; Xu et al., 2017; Kumar et al., 2013; Gan et al., 2017; Wan et al., 2022). En el presente estudio, aunque la sobreexpresión de MyD88 indicaba la activación de la vía del IFN, la represión de JUN/FOS, MAPK y NF-kB sugirió un claro efecto antiinflamatorio de la mezcla CL-FW frente OTA en células Jurkat. Resultados similares fueron obtenidos por Ha et al. (2020) investigando una mezcla de carotenoides en células HaCaT previamente expuestas a TNF-$\alpha$ e IFN-$\gamma$. Los compuestos bioactivos mostraron un efecto antiinflamatorio al reducir la producción de mediadores inflamatorios e inhibir la activación de las vías de señalización NF-kB y MAPK. Asimismo, la AST y el Kéfir, un producto lácteo fermentado, aliviaron la inflamación intestinal inducida por OTA en ratones (5 mg/kg durante 21 días en ambos estudios) reprimiendo la expresión génica de INF-$\gamma$, NF-kB, TRL-4 y mitigando la activación de rutas metabólica asociadas a la respuesta inmune (Chen Y. et al., 2021; Du et al., 2022).

*4.4.3 Tratamiento combinado AFB1-OTA CL-FW*

La exposición combinada AFB1-OTA CL-FW desencadenó un efecto transcripcional bastante similar al obtenido con OTA CL-FW mediante la activación de la vía de señalización del IFN. En este contexto, la exposición combinada a AFB1 y OTA se ha asociado ampliamente con la inflamación y la respuesta inmune. Corcuera et al. (2012) intoxicando ratas con AFB1 y OTA (0,25 y 0,50 mg/kg durante 1-96 h) obtuvieron infiltrados inflamatorios en el tejido hepático y renal con aparición de cirrosis y glomerulonefritis. Análogamente, la exposición combinada a AFB1 y OTA (0,5 y 1 μM durante 48 h) fomentó la toxicidad inmunitaria en macrófagos, activando algunas vías metabólicas asociadas a la respuesta inmunitaria (JUN/FOS y MAPK) (Hou et al., 2018). Además, la secuenciación del ARN realizada en células Caco-2 diferenciadas tras la exposición combinada con AFM1 y OTA (50 μM y 4 μM durante 48 h) también reveló alteraciones transcripcionales en ciertas rutas relacionadas con la inflamación y la inmunidad (como la activación de NF-kB e IFN). Aunque el número de DEG excluía el efecto aditivo de las micotoxinas, la inflamación intestinal fue claramente observada (Gao Y. et al., 2022).

También en este caso, la activación de la vía del IFN se produjo mediante la sobreexpresión de MyD88, no obstante, se observó la participación de factores adicionales como: TRAF3, TRAF6, IRF3 e IRF7. En detalle, TRAF3 se vió sobrexpresado mientras que los otros factores,

reprimidos. Los TRAFs son factores específicos asociados con el receptor TNF y desempeñan un rol importante en la patogénesis de las enfermedades inflamatorias (Lalani et al., 2018). Entre ellos, TRAF3 es un supresor tumoral y un regulador negativo de la activación alternativa de NF-kB. Por ende, se demostró que la sobreexpresión de TRAF3 atenuaba la respuesta inflamatoria *in vitro* (Zhang et al., 2018). Del mismo modo, la supresión de TRAF6 puede ejercer un efecto inhibidor sobre la cascada NF-kB y la producción de citoquinas en células epiteliales mamarias (Wang et al., 2017). Además, Li et al. (2017) asociaron el efecto antiinflamatorio del licopeno *in vivo* (5 mg/kg durante 21 días) con la represión de TRAF6 y NF-kB. Asimismo, una mezcla de *Lactobacillus*, típicamente utilizada para la fermentación de la leche, alivió los efectos inflamatorios de ZEA (25 μM durante 1h) en células IPEC-J2, mediante la represión de varios marcadores proinflamatorios, incluso TRAF-6 (Taranu et al., 2015).

La represión de IRF3 e IRF7 también puede promover una clara respuesta antiinflamatoria. En este sentido, la secuenciación del ARN realizada en los pulmones de ratones mostró que la AST (20 mg/mL; 24 h) suprimía tanto la inflamación como las lesiones pulmonares agudas inducidas por lipopolisacáridos mediante la represión de 11 marcadores inmunes, incluyendo IRF7 (Mao et al., 2020). La represión de IRF3 e IRF7 así como la mitigación de la cascada de señalización del IFN también se obtuvo mediante el tratamiento de hepatocitos de peces y macrófagos murinos con altas dosis de AST y BALs, respectivamente (Fang et al., 2021; Cai et al., 2018).

En línea con estos resultados, nuestros hallazgos pueden confirmar el efecto antiinflamatorio *in vitro* de la mezcla CL-FW contra la exposición combinada de AFB1 y OTA.

En resumen, el análisis del transcriptoma de las células Jurkat mostró que la vía de los eicosanoides fue la ruta mayormente alterada por AFB1 CL-FW. No obstante, la modulación de varios genes asociados con la respuesta inflamatoria (ALOX12, ALOX15, LTA4H) indicó el papel inmunoprotector de la mezcla CL-FW contra la síntesis de los eicosanoides y la cascada proinflamatoria desencadenada por AFB1 *in vitro.* Por otro lado, la exposición a OTA CL-FW perturbó principalmente la cascada de señalización del IFN. Sin embargo, la represión de vías intrínsecas como NF-kB, JUN/FOS y MAPK señaló el claro efecto antiinflamatorio de los ingredientes funcionales contra la toxicidad de OTA *in vitro*. Tras la exposición a AFB1-OTA CL-FW, la ruta del IFN fue nuevamente la más afectada, aunque se observó la implicación de otros factores. En este sentido, la represión de TRAF6, IRF3, IRF7 y de la vía NF-kB/TRAF3 típicamente implicada en la respuesta proinflamatoria respaldaron ulteriormente la función beneficiosa de CL-FW frente a la mezcla de micotoxinas *in vitro.*

### 4.5. El papel protector del suero de leche fermentando frente la hepatotoxicidad y la nefrotoxicidad inducida por AFB1 y OTA *in vivo*. Una cuantificación relativa y absoluta de las diferencias entre sexos.

De todos los órganos afectados por AFB1 y OTA, los más importantes son el hígado y el riñón. Es por ello que varios estudios han apuntado a la clara conexión entre la exposición a AFB1 y la aparición de hepatocarcinogénesis y cirrosis hepática en humanos (Mekuria et al., 2020). Del mismo modo, la exposición a AFB1 puede promover lesiones hepáticas agudas en ratas y pollos causando daños histopatológicos y alteraciones en la función hepática (Deng et al., 2020; Wu et al., 2019). Además, se ha demostrado que AFB1 induce hepatotoxicidad a través de estrés oxidativo, apoptosis, detención del ciclo celular, daño mitocondrial y alteraciones del transcriptoma *in vitro* e *in vivo* (Frangiamone et al., 2022a). El tratamiento con AFB1 también puede causar estrés oxidativo renal, daño genómico y detención del ciclo celular en ratas y pollos (Yu et al., 2015).

Por otro lado, OTA también está implicada en el daño hepático al inducir estrés oxidativo e inflamación en modelos animales como ratas, patos y cerdos (Marin et al., 2018; Zhai et al., 2020). En cuanto a la toxicidad renal, la exposición a OTA se ha considerado como uno de los principales factores asociados a la aparición de enfermedades renales crónicas y tumores en roedores (Damiano et al., 2020; Longobardi et al., 2022; Miljkovic & Mantle, 2022). El MoA mediante el cual OTA induce nefrotoxicidad incluye

peroxidación lipídica, apoptosis, inflamación y daño genómico *in vitro* e *in vivo* (Cimbalo et al., 2022b; Manyes & Font, 2022).

En los estudios toxicológicos relativos a las micotoxinas, un aspecto clave es evaluar cómo cambia la respuesta del organismo en función del sexo considerado. Por ejemplo, en ratones, pollos y humanos, la carcinogenicidad hepática de AFB1 es mayor en machos que en hembras. Asimismo, OTA provoca tumores renales con más frecuencia en ratas macho que en las hembras (EFSA, 2020a; 2020b; Soler & Oswald, 2018). También se ha reportado que los efectos beneficiosos de los probióticos cambian en función del sexo considerado (He et al., 2019; Myles et al., 2020). Cabe destacar que los hallazgos conseguidos con modelos animales pueden extrapolarse a humanos mediante el uso de biomarcadores biológicos (Kraus, 2018). En este sentido, CPS1 es la proteína más abundante en las mitocondrias hepáticas y representa un biomarcador típico en la detección de enfermedades hepáticas en humanos y ratas (Weerasinghe et al., 2014). De forma similar, el biomarcador KIM-1 ha sido aceptada por la Food and Drug Administration como un biomarcador altamente sensible y específico para monitorizar el daño renal inducido por sustancias químicas en estudios preclínicos (Griffin et al., 2019). En vista de ello, el objetivo del presente estudio fue investigar el posible efecto protector del FW contra la hepatotoxicidad y la nefrotoxicidad inducidas por AFB1 y OTA en ratas macho y hembra mediante el análisis de la expresión génica del CPS1 hepático y del KIM-1 renal tras 28 días de exposición oral. La cuantificación relativa y absoluta de estos biomarcadores

se llevó a cabo mediante RT-qPCR y PCR digital en gotas (ddPCR), respectivamente.

El hígado es el principal órgano implicado en la producción de hormonas y sustancias bioquímicas necesarias para la digestión y el crecimiento, la desintoxicación del organismo y la regulación del metabolismo energético. Los errores en el metabolismo energético hepático, incluidos los desórdenes del ciclo de la urea, conducen a una disfunción hepática grave (Ranucci et al., 2019). El ciclo de la urea convierte el amoníaco tóxico en urea mediante la actividad de varias enzimas, incluida la CPS1, y se ha descrito como uno de los principales procesos metabólicos que se ven alterados por las micotoxinas (Gerdermann et al., 2022). Concretamente, la exposición a AFB1 en ratas (65 μg/mL; 10 semanas) y en células hepáticas de Chang (5 μg/mL; 12-48 h) causaba disfunción mitocondrial con alteración del ciclo de la urea y una significativa represión de CPS1, lo que condujo a una acumulación masiva de amonio en la sangre (Zhuang et al., 2014). Yang et al. (2016) también demostraron que la exposición a AFB1 en células hepáticas de Chang (40 μg/mL; 24 h) desencadenaba la represión de CPS1, que a su vez potenciaba la proliferación y diferenciación tisular, procesos típicamente detectados durante la hepatocarcinogénesis. De este modo, se confirmó la estrecha relación entre la represión de CPS1 y el desarrollo del carcinoma hepatocelular.

Por otro lado, Cimbalo et al. (2021), evaluando la toxicidad aguda de las enniatinas (ENNs) (256-1021 μg/mL; 8 h) en hígado de ratas, obtuvieron

una marcada sobreexpresión de CPS1 con alteraciones prominentes en el metabolismo energético hepático. Del mismo modo, la exposición a OTA (70-210 μg/kg; 2-23 semanas) en hígado de ratas desequilibró el metabolismo energético y debilitó su sistema de reparación con una fuerte sobreexpresión de CPS1 (Qi et al., 2014a). Por lo tanto, se puede confirmar que la expresión del gen CPS1 cambia según los diferentes MoA mediante los cuales AFB1, ENNs y OTA promueven el daño hepático. En consonancia con estos hallazgos, los resultados obtenidos mediante ddPCR y RT-qPCR mostraron que en las ratas macho AFB1 reprimió la expresión de CPS1, mientras que OTA y el tratamiento combinado incrementaron ligeramente la expresión del biomarcador hepático. En las hembras, AFB1 y OTA aumentaron significativamente la expresión de CPS1. La fuerte sobreexpresión de CPS1 encontrada en las hembras puede estar relacionada con una expresión constitutivamente más elevada de CPS1, lo que podría ofrecer a este sexo una mayor protección frente a la desregulación del ciclo de la urea por estímulos externos (Agnelli et al., 2016; Natesan et al., 2016). En las hembras, AFB1 y OTA también mostraron un claro efecto aditivo en comparación con los machos, sugiriendo que el sexo puede considerarse como un factor novedoso a tener en cuenta en la evaluación del riesgo toxicológico relacionado con la concurrencia de micotoxinas.

Se ha confirmado que el uso de compuestos bioactivos posee efectos positivos sobre la función hepática, al limitar la toxicidad de las micotoxinas. Por ejemplo, el tratamiento con probióticos (incluidas varias cepas de BAL)

en células hepáticas moduló la hepatotoxicidad de AFB1, aliviando la inflamación y restableciendo la normal actividad hepática (Guo H. et al., 2021). Asimismo, en el hígado de ratas, el *Lactobacillus casei* no solo alivió el daño hepático y la inflamación inducida por AFB1, sino que también redujo la producción de amonio tóxico (Nikbakht Nasrabadi et al., 2013; Nurul Adilah et al., 2018). Además, la administración de compuestos ricos en fitoquímicos como polifenoles y carotenoides incrementaron la expresión del gen CPS1 mejorando la gluconeogénesis hepática y aumentando la excreción de amonio *in vitro.* Por lo tanto, se demostró la eficacia de estos compuestos bioactivos en revertir la disfunción hepática (Lomas-Soria et al., 2015; Zhang et al., 2020).

Cabe destacar que la CPS1 también puede actuar como una citoquina antiinflamatoria. Concretamente, en presencia de daño hepático, la sobreexpresión de CPS1 promovió la polarización M2 de los monocitos, aumentó el número de macrófagos hepáticos y potenció su actividad fagocítica, acelerando la recuperación de la función hepática (Park M. et al. 2019). En el presente estudio, la adición del FW en ratas macho incrementó significativamente la expresión de CPS1 y en mayor medida que las exposiciones con micotoxinas solamente, lo que sugiere un posible efecto beneficioso sobre la función hepática. Además, el FW modulaba el efecto tóxico de AFB1 a nivel molecular, revirtiendo totalmente la represión de CPS1, resultando ser una valiosa estrategia alimentaria en la mitigación de la hepatotoxicidad de AFB1 *in vivo*. En las hembras, la suplementación con FW aumentó la expresión de CPS1 en mayor medida respecto a AFB1

individualmente, mientras que para OTA y la exposición combinada no se encontraron diferencias significativas. En este sexo, el efecto limitado del FW está posiblemente relacionado con el factor hormonal. Efectivamente, los estrógenos son reguladores independientes de la transcripción de las enzimas del ciclo de la urea, manteniendo un nivel basal constante de ureagénesis y excreción de amonio a pesar de la dieta (Patton et al., 2020).

Los riñones son los principales órganos implicados en la homeostasis del organismo, al regular la presión arterial y la filtración, el equilibrio ácido-base y la eliminación de toxinas. La pérdida de la función renal se asocia con anomalías estructurales dispersas y reducción de la filtración glomerular, que a su vez pueden provocar enfermedades óseas, riesgo cardiovascular y tumores (Waziri et al., 2019). Además, se ha descubierto que la exposición a las micotoxinas es una de las principales causas de toxicidad renal, en la que KIM-1 puede utilizarse como marcador pronóstico para una detección rápida del daño tisular (Ráduly et al., 2021; Webster et al., 2017). Por lo tanto, Pyo et al. (2021) observaron una clara sobreexpresión de KIM-1, al exponer células renales HK-2 a OTA (200 nM; 48 h), lo que causó hipoxia, muerte celular y la transición epitelio-mesénquima. En el riñón de ratas, la exposición crónica a OTA (70-210 μg/kg; 4-13 semanas) aumentó la expresión génica de KIM-1 induciendo anomalías histopatológicas con vacuolización citoplasmática en la franja externa de la médula y cariomegalia extensa en el epitelio tubular (Qi et al., 2014b). En el mismo modelo experimental, la exposición aguda a OTA (1-4 mg/kg; 1 semana) provocó la

sobreexpresión KIM-1 con fenómenos de cariopicnosis, cariolisis, degeneración hidrópica y vacuolización a nivel tubular y glomerular (Zhu et al., 2016). Li et al. (2021) también demostraron que una baja dosis de FB1 empeoró la nefrocitotoxicidad inducida por OTA (5 μM; 24 h) al incrementar la expresión de KIM-1 y promover estrés oxidativo en células renales PK-15.

En línea con estos hallazgos, los análisis realizados mediante ddPCR y RT-qPCR confirmaron que en ratas macho, AFB1 y OTA indujeron la sobreexpresión de KIM-1 mientras que su mezcla indujo un efecto transcripcional bastante similar al control, sugiriendo un posible antagonismo entre las dos micotoxinas. En las hembras, OTA y la mezcla de micotoxinas aumentaron la expresión de KIM-1 mientras que AFB1 la redujo. En vista de este resultado, se puede afirmar que la toxicidad de AFB1 puede cambiar en función del sexo y del sitio de acción considerado. Por ello, la alta capacidad de AFB1 para alterar la barrera glomerular, así como la alta especificidad de KIM-1 en la detección de la lesión tubular podría indicar un daño glomerular específico en las hembras y uno tubular en los machos (Abdel-Hamid & Firgany, 2015; Chorley et al., 2021). Además, este hallazgo podría confirmar los resultados obtenidos por otros estudios, en los cuales se identificó una mayor susceptibilidad en los machos al daño tubular y en las hembras a la glomerulonefritis (Ashuntantang et al., 2018; Park F. et al., 2019).

La suplementación *in vivo* con probióticos ha demostrado ser una importante estrategia dietética en la prevención del daño renal. En ratas, la mezcla de *Lactobacillus reuteri* y *Clostridium butyricum* suprimió la expresión

génica de varios biomarcadores renales, incluido KIM-1, limitando la inflamación, la fibrosis, la apoptosis y el estrés oxidativo (Hsiao et al., 2021). Asimismo, la suplementación de tres cepas de BAL (*Lactobacillus casei, reuteri y helveticus*) mostró una significativa actividad renoprotectora, reduciendo la expresión de KIM-1 y aliviando las alteraciones funcionales y estructurales *in vivo* (Ragab et al., 2021). También se demostró que el *Lactobacillus plantarum* (la misma cepa utilizada en este estudio) reprimía la expresión génica de KIM-1 y mejoraba las anomalías histopatológicas y la nefrocalcinosis en ratas (Paul et al., 2018). Además, la suministración de probióticos reveló efectos benéficos contra las alteraciones estructurales, el estrés oxidativo y el daño mitocondrial inducidos por DON, FB1 y AFB1 en el riñón de cerdos, ratones y pollos, respectivamente (Bai et al., 2022; Ezdini et al., 2020; Śliżewska et al., 2019). También se ha comprobado que otros compuestos bioactivos como los carotenoides, los flavonoides y la curcumina, modulan la nefrotoxicidad de AFB1 y OTA *in vivo* (Damiano et al., 2020; Soliman et al., 2020; Yu et al., 2018). En el presente estudio, aunque el análisis RT-qPCR mostró que en ratas macho la adición del FW incrementaba la expresión de KIM-1 en mayor medida que las exposiciones individuales con las micotoxinas, la ddPCR reveló resultados opuestos en los cuales el FW mitigaba la sobreexpresión de KIM-1 en cada tratamiento realizado. La discordancia de resultados encontrada en este caso puede estar relacionada con la diferente sensibilidad de las técnicas empleadas. En este sentido, la mayor variabilidad e imprecisión del análisis por RT-qPCR que pueden ocurrir de forma intrínseca durante la transcripción inversa, la

amplificación y la normalización con el gen de referencia, otorgan a los resultados ddPCR mayor robustez, precisión y relevancia (Campomenosi et al., 2016; Taylor et al., 2017). En cuanto a las hembras, los ensayos de cuantificación relativa y absoluta confirmaron que la adición del FW aliviaba la sobreexpresión de KIM-1 promovida por las micotoxinas. En vista de esto, el análisis ddPCR confirmó que el FW mitigaba la sobreexpresión de KIM-1 inducida por AFB1 y OTA en ambos sexos, disminuyendo así el daño renal. Además, excepto para AFB1, las diferencias relacionadas con el sexo fueron mínimas, en contraste con Garovic & August (2016), cuyos hallazgos enfatizaron el papel renoprotector de los estrógenos así como la mayor susceptibilidad de los machos al daño renal.

En conclusión, se destaca que en el hígado de los machos, el tratamiento más perjudicial fue AFB1 al reducir la expresión de CPS1, lo que se revertió totalmente con la administración del FW. Este ingrediente funcional también mejoró los cambios de expresión génica inducidos por OTA y la mezcla de micotoxinas. En las hembras, el daño desencadenado por las micotoxinas así como el efecto protector del FW fueron menos pronunciados. En el riñón de machos y hembras, la exposición a micotoxinas provocó un claro efecto tóxico en el tejido renal mediante la sobreexpresión de KIM-1, siendo el tratamiento con OTA el más dañino. No obstante, el análisis de ddPCR confirmó que la administración del FW mitigaba la sobreexpresión de KIM-1 en ambos sexos, reduciendo así el daño renal. En este caso, excepto para AFB1, las diferencias relacionadas con el sexo fueron

mínimas. En ambos órganos, también se demostró la importancia de considerar el sexo como un factor novedoso en la evaluación del riesgo toxicológico relacionado con la concurrencia de micotoxinas. Por lo tanto, en el presente estudio se confirma la hepatotoxicidad y la nefrotoxicidad de AFB1 y OTA *in vivo*, lo que podría ser mitigado por compuestos dietéticos como el FW.

# 5.CONCLUSIONS

# 5. Conclusiones

## 5. CONCLUSIONES

1. La literatura revisada muestra que en los últimos cinco años la línea celular más empleada fue la HepG2, seguida por las células HEK-293T y PK-15. En cuanto a los estudios *in vivo*, los animales de laboratorio con mayor número de estudios fueron pollos, ratones y ratas en tanto que los principales órganos diana analizados fueron el timo, la bolsa de Fabricio y el bazo. El objetivo principal de la búsqueda se centró en la inmunotoxicidad, nefrotoxicidad, y hepatotoxicidad. La citometría de flujo se empleó principalmente en la evaluación de apoptosis y estrés oxidativo mientras que la inmunofluorescencia en la detección de alteraciones genéticas, epigenéticas y metabólicas.

2. El estudio sobre la diferenciación neuronal *in vitro* muestra que la exposición a OTA fue la condición más dañina al reducir en mayor medida la expresión de los marcadores neuronales y detener el ciclo celular en fase $G_0/G_1$. Por otro lado, los efectos de AFB1 fueron menos pronunciados. Sin embargo, los compuestos bioactivos reportaron un efecto beneficioso sobre el desarrollo neuronal y el ciclo celular una vez añadidos a la mezcla de micotoxinas. En cuanto al análisis de expresión génica, la represión de los marcadores neuronales por parte de AFB1 y OTA fue modulada por los ingredientes funcionales.

3. En correlación con los efectos inmunológicos inducidos por las micotoxinas estudiadas, la secuenciación del ARN reveló que AFB1 provocaba principalmente alteraciones genéticas, OTA inducía alteraciones metabólicas mientras que el tratamiento combinado causaba estrés oxidativo con disfunción de las defensas antioxidantes celulares.

4. El análisis del transcriptoma de las células Jurkat demostró que la exposición a AFB1 y OTA, individualmente y en combinación, provocaba una clara respuesta proinflamatoria a nivel molecular. No obstante, la administración de los ingredientes funcionales promovía la mitigación de genes y vías de señalización relacionadas con la inflamación, lo que demuestra su papel protector y antiinflamatorio en linfocitos T humanos.

5. En relación con el estudio *in vivo*, la cuantificación relativa y absoluta de los biomarcadores estudiados muestra que, en el hígado de ratas macho, AFB1 fue el tratamiento más tóxico al reducir la expresión de CPS1, lo que se revirtió totalmente con la adición del suero de leche fermentado. En las hembras, los resultados obtenidos fueron menos pronunciados. En los riñones de ratas macho y hembra, OTA resultó ser la exposición más tóxica al aumentar claramente la expresión de KIM-1. En cambio, la administración del compuesto bioactivo

limitaba las alteraciones génicas inducidas por las micotoxinas y reducía el daño renal.

6. Se debe seguir investigando los efectos beneficiosos de los ingredientes funcionales, no sólo con tal de preservar la salud humana frente a los efectos adversos causados principalmente por AFB1 y OTA sino también ofrecer a la industria alimentaria una eficiente estrategia dietética contra el problema global de la contaminación por micotoxinas.

# 5. CONCLUSIONS

1. The literature reviewed showed that in the last five years the most commonly used cell line was: HepG2, followed by HEK-293T and PK-15 cells. Regarding *in vivo* studies, the most used animal models were chickens, mice and rats, while the main target organs analyzed were: thymus, bursa of Fabricius and spleen. The research focused on immunotoxicity, nephrotoxicity, and hepatotoxicity. Flow cytometry was mainly used in the evaluation of apoptosis and oxidative stress while immunofluorescence was employed to detect genetic, epigenetic, and metabolic alterations.

2. The evaluation of neuronal differentiation *in vitro* demonstrated that OTA exposure was the most damaging condition, by reducing to a greater extent the expression of neuronal markers and inducing $G_0/G_1$ cell cycle arrest. Conversely, *in vitro* AFB1 neuronal effects were less pronounced. However, functional ingredients showed a beneficial protective effect on neuronal development and cell cycle, once added to mycotoxins mixture. Regarding gene expression analysis, the repression of neuronal markers by AFB1 and OTA was modulated by functional compounds.

3. Regarding the immunological effects induced by mycotoxins, RNA sequencing revealed that AFB1 caused mainly genetic alterations,

OTA promoted metabolic alterations while the combined exposure prompted oxidative stress with dysfunction of cellular antioxidant defenses.

4. The analysis of Jurkat cells transcriptome revealed a clear pro-inflammatory response at molecular level after exposure to AFB1 and OTA, individually and in combination. However, the presence of functional compounds promoted the mitigation of inflammation-related genes and signaling pathways, demonstrating their protective and anti-inflammatory role in human T cells.

5. In relation to *in vivo* study, the relative and absolute quantification of studied biomarkers demonstrated that in male livers AFB1 was the most damaging exposure by reducing the expression of CPS1, which was completely reversed by fermented milk whey administration. In female rats, the results obtained were less pronounced. In the kidneys of male and female rats, OTA proved to be the most toxic exposure by increasing KIM-1 expression. Nevertheless, bioactive compound addition alleviated mycotoxins induced KIM-1 upregulation, by reducing renal damage.

6. The beneficial effects of bioactive compounds should be further investigated, not only to preserve human health from the adverse effects caused by AFB1 and OTA exposure but also to provide the

food industry with an efficient dietary strategy for the worldwide problem of mycotoxin contamination.

# 6. REFERENCES

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