



VNIVERSITAT
ID VALÈNCIA [Q*]

Facultat de Farmàcia i Ciències de l'Alimentació

Departamento de Medicina Preventiva y Salud Pública, Ciencias de la Alimentación,
Toxicología y Medicina Legal

Programa de Doctorado en Ciencias de la Alimentación

**Evaluación de los efectos protectores de ingredientes
bioactivos en la dieta frente a la toxicidad de aflatoxina B1 y
ocratoxina A mediante técnicas ómicas**

València, Mayo 2025

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CERTIFICAN QUE:

Don Álvaro Lázaro Valero ha realizado su tesis doctoral dentro del programa de Ciencias de la Alimentación titulada ***“Evaluación de los efectos protectores de ingredientes bioactivos en la dieta frente a la toxicidad de aflatoxina B1 y ocratoxina A mediante técnicas ómicas”***. Con la aprobación de su trabajo, se autoriza su presentación para optar al título de **Doctor** por la Universitat de València.

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Los resultados de la presente tesis doctoral han sido publicados en diversas revistas científicas internacionales de alto impacto:

1. **Lázaro, Á.**, Frangiamone, M., Maietti, A., Cimbalo, A., Vila-Donat, P., & Manyes, L. (2024). *Allium sativum* L. var. Voghiera Reduces Aflatoxin B1 Bioaccessibility and Cytotoxicity *In Vitro*. *Foods* 13(3), 487; <https://doi.org/10.3390/foods13030487>. Publicado: 2 Febrero de 2024 JCR - Q1 (Food Science and Technology). *Impact factor: 4.7 (2023)*.
2. **Lázaro, Á.**, Vila-Donat, P., & Manyes, L. (2024). Emerging mycotoxins and preventive strategies related to gut microbiota changes: probiotics, prebiotics, and postbiotics - a systematic review. *Food & Function*, 15(18), 8998–9023. <https://doi.org/10.1039/d4fo01705f>. Publicado: 24 septiembre de 2024. JCR - Q1 (Food Science and Technology) *Impact factor: 5.1(2024)*.
3. **Lázaro, Á.**, Gómez-Ramírez, P., Vila-Donat, P., Cimbalo, A., & Manyes, L. (2025). Effects of pumpkin and fermented whey on fecal microbiota profile against AFB1 and OTA exposure in Wistar rats. *Toxicology Mechanisms and Methods*, 1–13. <https://doi.org/10.1080/15376516.2025.2484636>. Publicado: 8 Abril de 2025. JCR – Q2 (Toxicology), *Impact factor: 2.8 (2024)*.

Esta tesis doctoral forma parte del siguiente proyecto:

- Revalorización de residuos de la industria agroalimentaria para el desarrollo de ingredientes antifúngicos y antimicotoxigénicos (PID2022-140722OB-I00), financiado a través del proyecto MCIU/AEI/AEI/10.13039/501100011033/FEDER, UE, del Ministerio de Ciencia, Innovación y Universidades.

El doctorando Álvaro Lázaro Valero agradece:

- Al Ministerio Español de Ciencia, Innovación y Universidades
- A la Universitat de València



*La belleza de los sueños cree en aquellos a los que
pertenece el futuro*

*A mi familia y a mis amigos. En especial, a mi
abuelo Guillermo*

RESUMEN

La presente tesis doctoral se enfoca en la evaluación de los efectos protectores de ingredientes bioactivos en la dieta frente a la toxicidad de la aflatoxina B1 (AFB1) y la ocratoxina A (OTA), dos de las micotoxinas más peligrosas para la salud humana y animal. Las micotoxinas son metabolitos secundarios producidos por hongos de los géneros *Aspergillus*, *Penicillium* y *Fusarium*, y se encuentran en diversos alimentos y piensos, representando un riesgo significativo para la salud pública. Tanto la AFB1 como la OTA son conocidas por sus efectos tóxicos, que incluyen nefrotoxicidad, hepatotoxicidad, carcinogenicidad y neurotoxicidad. La AFB1 ha sido clasificada como carcinógena de Grupo 1 por la Agencia Internacional para la Investigación del Cáncer (IARC), mientras que la OTA se considera potencialmente cancerígena para los seres humanos (Grupo 2B).

Actualmente, existen regulaciones que establecen límites máximos de micotoxinas en alimentos, pero muchas micotoxinas emergentes, como las enniatinas y la beauvericina, aún no están reguladas en la alimentación humana ni animal. En este contexto, los compuestos bioactivos presentes en los alimentos, así como los probióticos, prebióticos, sinbióticos y postbióticos (PPSP), han emergido como estrategias prometedoras para reducir la bioaccesibilidad y biodisponibilidad de las micotoxinas y mitigar sus efectos tóxicos. Estos compuestos también mejoran la salud intestinal, modulan la microbiota y pueden reducir el riesgo de enfermedades crónicas.

En esta tesis se evaluó el efecto de diversos ingredientes bioactivos, como el ajo Voghiera, sobre la bioaccesibilidad y citotoxicidad de la AFB1 en el pan contaminado naturalmente con micotoxina. Los resultados obtenidos demostraron que la inclusión de ajo en el pan redujo de manera significativa la bioaccesibilidad de la micotoxina, lo que indica que el ajo podría disminuir la cantidad de AFB1 disponible para ser absorbida por el organismo. Además, se observó un aumento en la viabilidad celular en las células Caco-2, un modelo utilizado para estudiar la absorción intestinal. La presencia de ajo también contribuyó a mitigar los efectos nocivos de la AFB1, reduciendo la apoptosis y el estrés oxidativo, así como la reactividad celular, que son comúnmente inducidos por la exposición a esta micotoxina. Estos resultados sugieren que el ajo Voghiera puede actuar como un ingrediente funcional capaz de aliviar los efectos tóxicos de AFB1, lo que abre

nuevas perspectivas para su inclusión en la dieta como estrategia preventiva frente a las micotoxinas.

Se llevó a cabo también una revisión sistemática sobre los probióticos más investigados, destacándose géneros como *Lactobacillus*, *Bifidobacterium* y *Saccharomyces*, así como prebióticos como la inulina y el β -glucano. En esta revisión, se demostró claramente la interacción entre la microbiota intestinal y las micotoxinas emergentes, así como el impacto de los PPSP sobre estas micotoxinas. Sin embargo, se identificó una carencia de estudios *in vivo* que profundicen en la evaluación de los efectos de estas interacciones en condiciones biológicas más cercanas a la realidad.

Para abordar esta laguna en la investigación, se llevó a cabo un estudio *in vivo* con ratas Wistar, en el que se investigaron los efectos de la AFB1 y la OTA sobre la microbiota intestinal y los beneficios de ingredientes funcionales como el suero fermentado y la calabaza. Los resultados de este estudio mostraron un efecto sinérgico entre las micotoxinas y los ingredientes funcionales, que contribuyó a mejorar la diversidad α de la microbiota intestinal. Además, se observó que la combinación de estos ingredientes modificaba la abundancia de bacterias intestinales, con un impacto diferencial dependiendo del sexo de los animales, lo que sugiere que los efectos de estos ingredientes pueden variar en función de características biológicas como el dimorfismo sexual. Estos hallazgos abren la puerta a futuras investigaciones que profundicen en los mecanismos de acción de los PPSP y su influencia sobre la microbiota intestinal en relación con las micotoxinas.

Este trabajo resalta la importancia de continuar la investigación sobre las estrategias preventivas frente a las micotoxinas, así como su impacto en la microbiota intestinal y las posibles interacciones con ingredientes funcionales que puedan mitigar sus efectos tóxicos. A medida que se profundiza en la comprensión de estas interacciones, se abren nuevas oportunidades para desarrollar enfoques innovadores y más eficaces en la prevención y reducción de los riesgos asociados a la exposición a micotoxinas, mejorando así la salud pública y animal.

ABSTRACT

This doctoral thesis focuses on evaluating the protective effects of bioactive ingredients in the diet against the toxicity of aflatoxin B1 (AFB1) and ochratoxin A (OTA), two of the most dangerous mycotoxins for human and animal health. Mycotoxins are secondary metabolites produced by fungi of the genera *Aspergillus*, *Penicillium*, and *Fusarium*, and are found in various foods and feed, representing a significant risk to public health. Both AFB1 and OTA are known for their toxic effects, which include nephrotoxicity, hepatotoxicity, carcinogenicity, and neurotoxicity. AFB1 has been classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC), while OTA is considered possibly carcinogenic to humans (Group 2B).

Currently, there are regulations establishing maximum limits for mycotoxins in food, but many emerging mycotoxins, such as enniatins and beauvericin, are not yet regulated in human or animal feed. In this context, bioactive compounds present in foods, as well as probiotics, prebiotics, synbiotics, and postbiotics (PPSP), have emerged as promising strategies to reduce the bioaccessibility of mycotoxins and mitigate their toxic effects. These compounds not only help reduce toxicity but also improve gut health, modulate the microbiota, and may reduce the risk of chronic diseases.

In this thesis, the effect of various bioactive ingredients, such as Voghiera garlic, on the bioaccessibility and cytotoxicity of AFB1 in bread was evaluated. The results showed that the inclusion of garlic in bread naturally contaminated with AFB1 significantly reduced the bioaccessibility of the mycotoxin, suggesting that garlic could decrease the amount of AFB1 available for absorption by the body. Furthermore, an increase in cell viability was observed in Caco-2 cells, a model used to study intestinal absorption. The presence of garlic also helped mitigate the harmful effects of AFB1, reducing apoptosis, oxidative stress, and cellular reactivity, which are commonly induced by exposure to this mycotoxin. These results suggest that Voghiera garlic could act as a functional ingredient capable of alleviating the toxic effects of AFB1, opening new perspectives for its inclusion in the diet as a preventive strategy against mycotoxins.

A systematic review was also conducted on the most studied probiotics, highlighting genera such as *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces*, as well as prebiotics such as inulin and β -glucan. This review clearly demonstrated the interaction between the intestinal microbiota and emerging mycotoxins, as well as the impact of PPSP on these mycotoxins. However, a lack of *in vivo* studies evaluating the effects of these interactions under more biologically relevant conditions was identified

To address this gap in research, an *in vivo* study was conducted with Wistar rats, in which the effects of AFB1 and OTA on the intestinal microbiota were investigated, along with the benefits of functional ingredients such as fermented whey and pumpkin. The results of this study showed a synergistic effect between the mycotoxins and the functional ingredients, which contributed to improving the α -diversity of the intestinal microbiota. Additionally, it was observed that the combination of these ingredients modified the abundance of intestinal bacteria, with a differential impact depending on the sex of the animals, suggesting that the effects of these ingredients may vary based on biological characteristics such as sexual dimorphism. These findings open the door for future research to further explore the mechanisms of action of PPSP and their influence on the intestinal microbiota in relation to mycotoxins.

This work emphasizes the importance of continuing research into preventive strategies against mycotoxins, as well as their impact on the intestinal microbiota and the potential interactions with functional ingredients that could mitigate their toxic effects. As our understanding of these interactions deepens, new opportunities arise to develop innovative and more effective approaches for preventing and reducing the risks associated with mycotoxin exposure, thereby improving public and animal health.

RESUM

Esta tesi doctoral se centra en avaluar els efectes protectors dels ingredients bioactius en la dieta contra la toxicitat de l'aflatoxina B1 (AFB1) i l'ocratoxina A (OTA), dos de les micotoxines més perilloses per a la salut humana i animal. Les micotoxines són metabòlits secundaris produïts per fongs dels gèneres *Aspergillus*, *Penicillium* i *Fusarium*, i es troben en diversos aliments i pinsos, representant un risc significatiu per a la salut pública. Tant l'AFB1 com l'OTA són conegudes pels seus efectes tòxics, que inclouen nefrotoxicitat, hepatotoxicitat, carcinogenicitat i neurotoxicitat. L'AFB1 ha sigut classificada com a carcinogen del Grup 1 per la Agència Internacional per a la Investigació sobre el Càncer (IARC), mentre que l'OTA es considera possiblement carcinogènica per als humans (Grup 2B).

Actualment, hi ha regulacions que estableixen límits màxims per a les micotoxines en els aliments, però moltes micotoxines emergents, com les enniatines i la beauvericina, encara no estan regulades en l'alimentació humana o animal. En aquest context, els compostos bioactius presents en els aliments, així com els probiòtics, prebiòtics, simbiòtics i postbiòtics (PPSP), han emergit com a estratègies prometedores per reduir la bioaccessibilitat de les micotoxines i mitigar els seus efectes tòxics. Aquests compostos no sols ajuden a reduir la toxicitat, sinó que també milloren la salut intestinal, modulen la microbiota i poden reduir el risc de malalties cròniques.

En esta tesi es va avaluar l'efecte de diversos ingredients bioactius, com l'all de Voghiera, sobre la bioaccessibilitat i la citotoxicitat de l'AFB1 en el pa. Els resultats van mostrar que la inclusió d'all en pa contaminat de manera natural amb AFB1 va reduir significativament la bioaccessibilitat de la micotoxina, suggerint que l'all podria disminuir la quantitat d'AFB1 disponible per a l'absorció per part de l'organisme. A més, es va observar un augment en la viabilitat cel·lular en cèl·lules Caco-2, un model utilitzat per estudiar l'absorció intestinal. La presència d'all també va ajudar a mitigar els efectes nocius de l'AFB1, reduint l'apoptosi, l'estrès oxidatiu i la reactivitat cel·lular, que són induïts habitualment per l'exposició a aquesta micotoxina. Aquests resultats suggereixen que l'all de Voghiera podria actuar com a ingredient funcional capaç d'alleujar els efectes

tòxics de l'AFB1, obrint noves perspectives per a la seua inclusió en la dieta com a estratègia preventiva contra les micotoxines.

També es va dur a terme una revisió sistemàtica sobre els probiòtics més estudiats, destacant gèneres com *Lactobacillus*, *Bifidobacterium* i *Saccharomyces*, així com prebiòtics com la inulina i el β -glucà. Esta revisió va demostrar clarament la interacció entre la microbiota intestinal i les micotoxines emergents, així com l'impacte dels PPSP sobre aquestes micotoxines. No obstant això, es va identificar una manca d'estudis *in vivo* que avaluen els efectes d'estes interaccions en condicions biològicament més rellevants.

Per abordar aquesta mancança d'investigació, es va dur a terme un estudi *in vivo* amb rates Wistar, en el qual es van investigar els efectes de l'AFB1 i l'OTA sobre la microbiota intestinal, juntament amb els beneficis d'ingredients funcionals com el sèrum làctic fermentat i la carabassa. Els resultats d'aquest estudi van mostrar un efecte sinèrgic entre les micotoxines i els ingredients funcionals, que van contribuir a millorar l'alfa-diversitat de la microbiota intestinal. A més, es va observar que la combinació d'aquests ingredients modificava l'abundància de bacteris intestinals, amb un impacte diferencial segons el sexe dels animals, suggerint que els efectes d'estos ingredients poden variar segons característiques biològiques com el dimorfisme sexual. Estes troballes obrin la porta a futures investigacions per explorar més a fons els mecanismes d'acció dels PPSP i la seua influència sobre la microbiota intestinal en relació amb les micotoxines.

Este treball posa en relleu la importància de continuar investigant estratègies preventives contra les micotoxines, així com el seu impacte sobre la microbiota intestinal i les possibles interaccions amb ingredients funcionals que podrien mitigar els seus efectes tòxics. A mesura que aprofundim en la comprensió d'estes interaccions, sorgeixen noves oportunitats per desenvolupar enfocaments innovadors i més efectius per prevenir i reduir els riscos associats a l'exposició a micotoxines, millorant així la salut pública i animal.

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LISTA DE ABREVIATURAS

ADN	Ácido desoxirribonucleico
AFG1	Aflatoxina G1
AFG2	Aflatoxina G2
AFB1	Aflatoxina B1
AFB2	Aflatoxina B2
AFM1	Aflatoxina M1
AGCC	Ácidos grasos de cadena corta
AME	Alternariol monometil éter
AOH	Alternariol
AUR	Aurofusarina
BAL	Bacterias ácido lácticas
BEA	Beauvericin
CIT	Citrinina
DAS	Diacetoxiscirpenol
DON	Deoxinivalenol
ENA	Enniatina A
ENA1	Enniatina A1
ENB	Enniatina B
ERC	Enfermedad renal crónica
SNE	Sistema nervioso entérico
Fig.	Figura
F/B	Firmicutes/Bacteroidetes
IARC	Agencia Internacional para la Investigación del Cáncer
LPS	Lipopolisacárido
MON	Moniliformina
OTA	Ocratoxina A
PAT	Patulina
PPC	Polisacárido péctico de calabaza
PPSP	Probióticos, prebióticos, sinbióticos y postbióticos
FW	Suero de leche de cabra fermentado
TeA	Ácido tenuazónico
TEN	Tentoxina

TMAO	N-óxido de trimetilamina
ZEN	Zearalenona



1.INTRODUCCIÓN

1.1 Generalidades sobre las micotoxinas

Las micotoxinas son metabolitos secundarios producidos por diversas especies de hongos, principalmente pertenecientes a los géneros *Aspergillus*, *Penicillium* y *Fusarium*. Se estima que están presentes hasta en el 80% de los alimentos y piensos. La contaminación de los piensos es particularmente preocupante, ya que en los países desarrollados hasta el 70% de la cosecha de cereales - una fuente primaria de micotoxinas - se utiliza en la dieta diaria de los animales (Niaz et al., 2025). Estas toxinas se caracterizan por presentar importantes riesgos para la salud pública a causa de sus propiedades tóxicas tanto en humanos como en animales. Entre otros efectos, estas pueden inducir nefrotoxicidad, hepatotoxicidad, inmunotoxicidad, neurotoxicidad, carcinogenicidad y toxicidad para la reproducción y el desarrollo (Manyes & Font, 2023).

Por otro lado, las micotoxinas también pueden desempeñar un papel como antibióticos al ser capaces de eliminar microorganismos patógenos (Thanh et al., 2020; Wang et al., 2019). El desarrollo del uso de las micotoxinas como antibióticos naturales es muy interesante ya que, lamentablemente, en las últimas décadas, el uso excesivo e indebido de los antibióticos tradicionales, así como factores sociales y económicos, han acelerado la propagación de bacterias resistentes a estos, lo que hace que el tratamiento farmacológico sea ineficaz. Actualmente, al menos 700.000 personas en todo el mundo mueren cada año debido a la resistencia a los antimicrobianos (Mancuso et al., 2021).

1.1.1 Micotoxinas reguladas

El marco legislativo sobre límites máximos de micotoxinas en alimentación humana estaba establecido por el Reglamento de la Comisión (UE) 2023/915 de 25 de abril de 2023. Actualmente, este ha sido enmendado y corregido por (UE) 2024/1756 de 25 junio de 2024 en los que se incluye la regulación para la aflatoxina B1 (AFB1), aflatoxina B2 (AFB2), aflatoxina G1, aflatoxina G2, aflatoxina M1 (AFM1), ocratoxina A (OTA), patulina (PAT), deoxinivalenol (DON), zearalenona (ZEN), fumonisina B1, fumonisina B2 y citrinina (CIT), cuyas estructuras químicas aparecen representadas en la Figura 1 (Fig. 1). Los límites máximos establecidos por la UE para AFB1 y OTA se

indican en la Tabla 1. Además, existen la Recomendación de la Comisión (UE) 2013/165 de 27 de marzo de 2013, la Recomendación de la Comisión (UE) 2022/553 de 5 de abril de 2022 y el Reglamento (UE) 2024/1038 de la Comisión de 9 de abril de 2024, para el alternariol (AOH), el alternariol monometiléter (AME), el ácido tenuazónico (TeA) y los tricotecenos T-2 y HT-2.

Los niveles indicativos de alternariol, éter monometílico de alternariol y ácido tenuazónico son 30 µg/kg, 30 µg/kg y 100 µg/kg para las semillas de sésamo; 30 µg/kg, 30 µg/kg y 1000 µg/kg para las semillas de girasol; y 10 µg/kg, 10 µg/kg y 100 µg/kg para el aceite de girasol, respectivamente. Por su parte, los niveles indicativos para la suma de T-2 y HT-2 a partir/por encima de los cuales deben realizarse investigaciones, sobre todo en caso de resultados repetidos son de 100 µg/kg para el maíz, 200 µg/kg para la avena y 50 µg/kg en el caso de otros cereales. De hecho, en 2021 las micotoxinas ocuparon la tercera categoría de peligro más notificada en todo el mundo (485 notificaciones). Estos se referían principalmente a la detección de aflatoxinas (413) y a la categoría de productos nueces, productos de nueces y semillas (254). El principal origen reportado fue Estados Unidos (53) (Owolabi et al., 2023)

Tabla 1. Límites establecidos por el Reglamento 915/2023 para AFB1 y OTA.

	AFB1		OTA
Maní y otras semillas oleaginosas, avellanas y nueces de Brasil	8 µg/kg	Raíces de malvavisco, diente de león y flores de naranjo (secas) destinadas a infusiones de hierbas o sucedáneos del café	20 µg/kg
Cereales y productos derivados de cereales con algunas excepciones,	5 µg/kg	Cereales en grano sin elaborar	5 mg/kg
Almendras, pistachos y semillas de albaricoque.	5 µg/kg	Productos derivados de cereales sin transformar;	3 mg/kg
		Extracto de regaliz para uso en alimentos, en particular bebidas y dulces	80 µg/kg

Aflatoxina B1 (AFB1). Ocratoxina A (OTA).

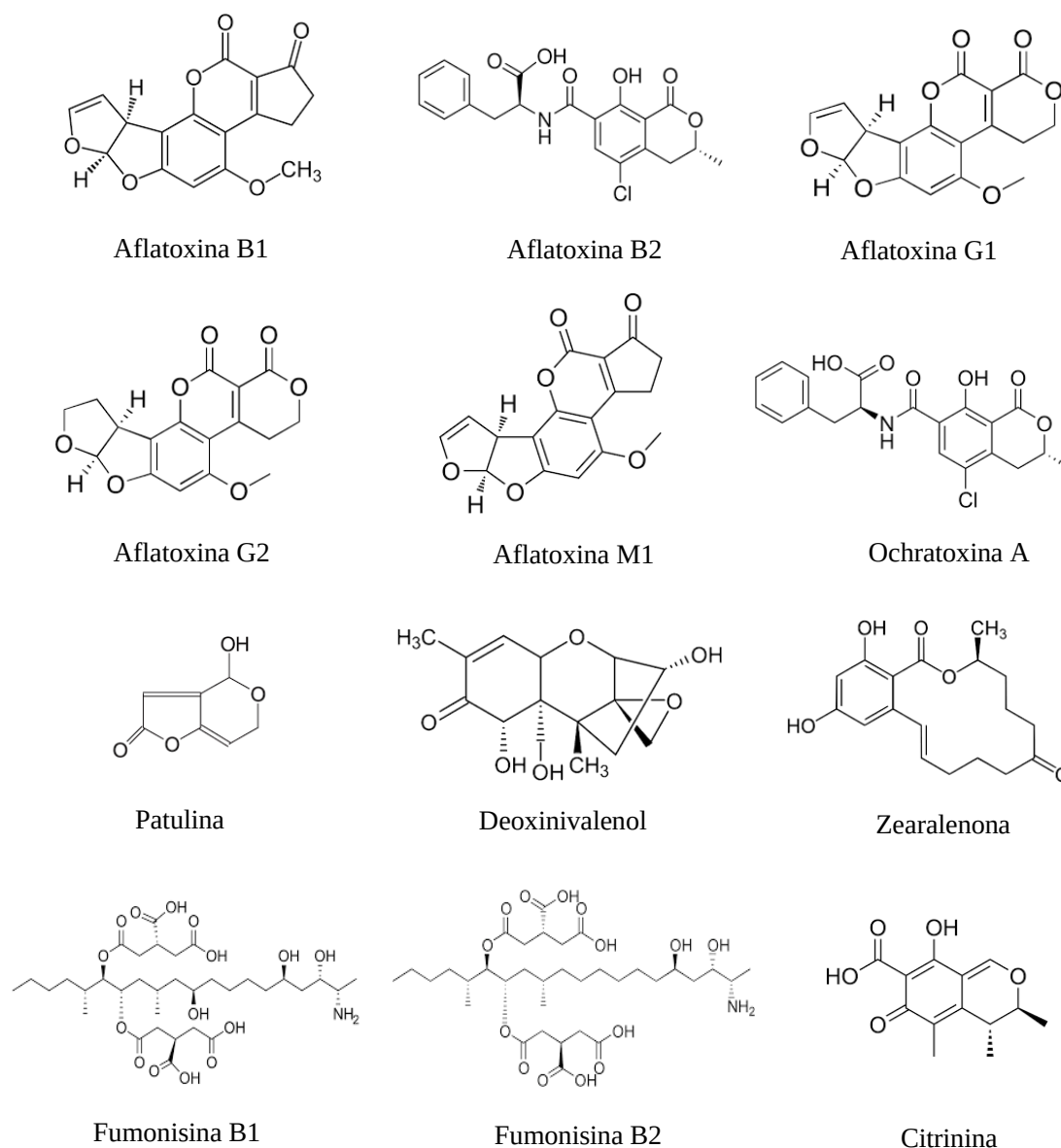


Figura 1. Estructura química de las principales micotoxinas reguladas (Reglamento (UE) 2023/915).

Dentro de las micotoxinas más relevantes, cabe mencionar la AFB1 y la OTA (Escrivà et al., 2023). La Fig. 2 describe los principales efectos que poseen estas dos micotoxinas sobre diferentes órganos. La AFB1 es un metabolito secundario sintetizado principalmente por el género *Aspergillus flavus*, un hongo micotoxigénico que se halla contaminando una amplia diversidad de alimentos como arroz, maíz, carne, leche, nueces, pistachos, especias y semillas (Mahato et al., 2019). Debido a su gran carcinogenicidad tanto en humano como en animales, la AFB1 está muy bien documentada (EFSA, 2020).

De hecho, la Agencia Internacional para la Investigación del Cáncer (IARC) clasificó a la AFB1 como un carcinógeno humano perteneciente al Grupo 1 debido a su participación en la síntesis de estructuras carcinogénicas en el ácido desoxirribonucleico (ADN) (Gramantieri et al., 2022). Estudios *in vitro* e *in vivo* han demostrado su toxicidad tanto aguda como crónica en relación con la inmunosupresión, disfunciones nutricionales, daño hepático y cáncer (Cimbalo et al., 2020; Marchese et al., 2018).

La OTA es otra micotoxina de las más presentes a nivel mundial producida en gran medida por especies de *Aspergillus ochraceus* and *Penicillium verrucosum* (Cabañes et al., 2010). La OTA puede encontrarse contaminando en una gran variedad de alimentos, incluyendo avena, trigo, cebada, cacao, café, cerveza, vino y alimentos de origen animal, especialmente la carne de cerdo (Bayman & Baker, 2006). La contaminación por OTA generalmente ocurre como consecuencia de deficiencias tanto en el almacenamiento como en las prácticas agrícolas durante el secado de los alimentos (Bui-Klimke & Wu, 2015). La literatura científica ha demostrado cómo la exposición a la OTA puede incrementar el estrés oxidativo, la inflamación, el daño al ADN y la muerte celular (Xie et al., 2025). De hecho, la OTA ha sido clasificada por la IARC en el grupo 2B como posiblemente cancerígena para los seres humanos (IARC, 1993).

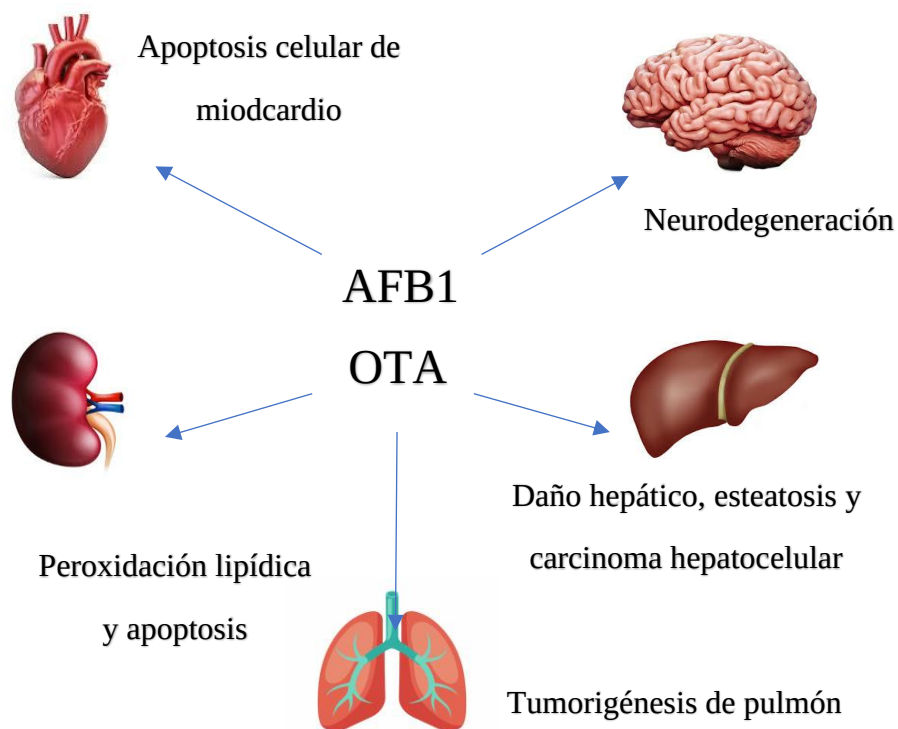


Figura 2. Efectos de la AFB1 y OTA sobre diferentes órganos adaptado de Pożarska et al. (2024).

1.1.2 Micotoxinas emergentes

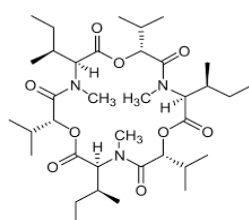
Por otro lado, existen un conjunto de micotoxinas – llamadas micotoxinas emergentes – que aún no han sido reguladas (Kolawole et al., 2024). Algunas de ellas son la enniatina A (ENA), enniatina B (ENB), enniatina A1 (ENA1), beauvericina (BEA), ácido fusárico (FA), moniliformina (MON), fusarenon X, aurofusarina (AUR), fusaproliferina, diacetoxiscirpenol (DAS), altertoxina (ALX), altueno (ALT), tentoxina (TEN), CIT, esterigmatocistina, neosolaniol, citreoviridina, rugulosina y fomopsina (Fig. 3 y Fig. 4).

Es importante destacar que, en muchas encuestas regionales y globales, se ha descubierto que las micotoxinas emergentes coexisten con frecuencia con micotoxinas reguladas (FUM, DON, AFB1, ZEN, T-2/HT-2 y OTA) en cultivos y alimentos para animales. Esto no es sorprendente ya que muchas de estas micotoxinas emergentes también son producidas por varios géneros de hongos (*Aspergillus*, *Alternaria*, *Penicillium* y *Fusarium*) responsables de la producción de micotoxinas reguladas (Kolawole et al., 2024).

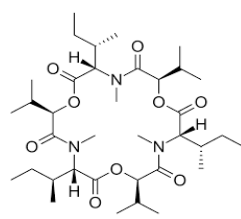
Estas micotoxinas emergentes están involucradas en la apoptosis celular, anomalías cromosómicas, insuficiencia cardíaca aguda, genotoxicidad, hemorragias - en piernas, cráneo y pies - daño hepático, colapso cardiovascular, clorosis, embriotoxicidad, teratogénesis, cáncer, cirrosis hepática, carcinoma hepatocelular, Shoshin - kakke (beriberi cardíaco agudo), degeneración grasa, necrosis de las células hepáticas y muerte celular (Chiminelli et al., 2022; Hasuda & Bracarense, 2024). Por otro lado, como se ha comentado anteriormente, también pueden desempeñar un papel como antibióticos al eliminar los microorganismos patógenos implicados en una gran variedad de enfermedades (Wang et al., 2019; Wu et al., 2018). Por ejemplo, el AOH y AME se han demostrado como inhibidores prometedores de la glucógeno-sintasa-quinasa 3, siendo un objetivo importante en el descubrimiento de fármacos. De hecho, se ha revelado que esta enzima participa en las vías de señalización de la diabetes tipo 2, el cáncer, los trastornos neurológicos y otras enfermedades (Vemuri et al., 2020). Además, la ergotamina, con su modo de acción bloqueador de los receptores adrenérgicos α , también se ha utilizado para el tratamiento de la migraña aguda durante más de 50 años (Gómez Quintero et al., 2022).

De cierto interés es que, se prevé que su presencia aumente en los próximos años como consecuencia del aumento de las temperaturas, lo que favorecerá el desarrollo de hongos en condiciones inéditas en países como Brasil, Argentina y algunas zonas de Asia, entre ellas China e India, que constituyen una proporción considerable de las exportaciones de micotoxinas (Liew & Mohd-Redzwan, 2018). Debido a la ausencia de normativas claras sobre estas micotoxinas emergentes, Europa y la EFSA están realizando estudios para obtener información sobre los niveles de micotoxinas de *Alternaria* — AOH, AME y TeA— en productos alimenticios y piensos (Nagda & Meena, 2024).

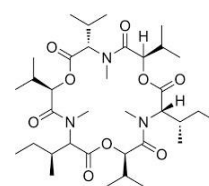
Eusarium



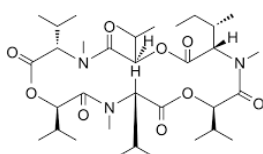
Enniatina A



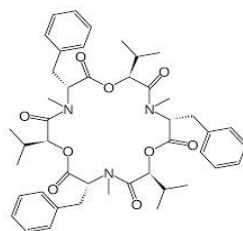
Enniatina B



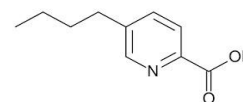
Enniatina A1



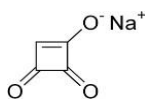
Enniatina B1



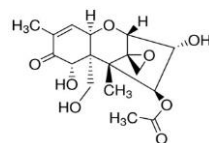
Beauvericina



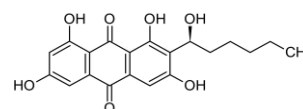
Ácido fusárico



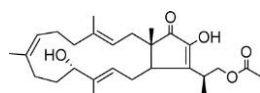
Moniliformina



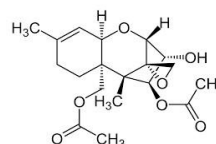
Fusarenon X



Aurofusarina

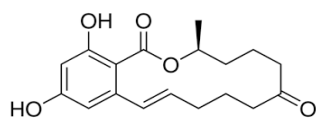


Fusaproliferina

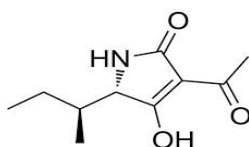


Diacetoxiscirpenol

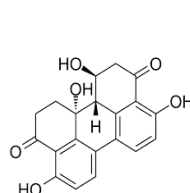
Figura 3. Estructura química de las micotoxinas emergentes producidas por el género *Fusarium*.

Alternaria

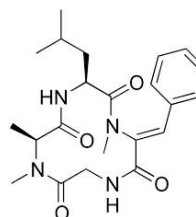
Alternariol monometil éter



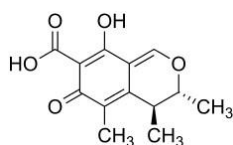
Ácido tenuazónico



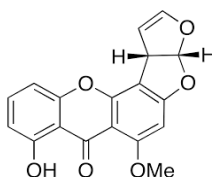
Altertoxina



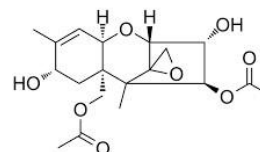
Tentoxina

Monascus

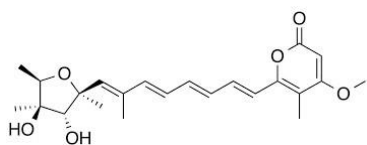
Citrinina



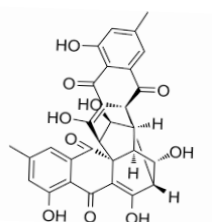
Sterigmatocystina



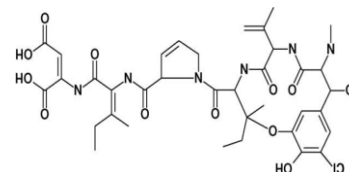
Neosolaniol

Penicillium

Citreoviridina

Diaporthe

Rugulosina



Phomopsina

Figura 4. Estructura química de las micotoxinas emergentes producidas por los géneros *Alternaria*, *Monascus*, *Penicillium* y *Diaporthe*.

1.2 Compuestos bioactivos

En los últimos años, ha habido un creciente interés en los alimentos funcionales, nutraceuticos y suplementos dietéticos de origen vegetal. Esto es debido a su alto contenido en compuestos bioactivos, es decir, sustancias que van más allá de los componentes nutricionales y que presentan beneficios para la salud (Direito et al., 2024). Estos compuestos bioactivos se encuentran en una amplia gama de alimentos de origen vegetal como en verduras, frutas, cereales, especias y sus alimentos y bebidas derivados,

como té, aceite de oliva, zumos de frutas, vino, chocolate y cerveza (Sorrenti et al., 2023; Teodoro et al., 2019). No solo eso, sino que también hay ciertas bacterias como *Saccharomyces boulardii*, *Lactobacillus acidophilus* (*L. acidophilus*), *L. rhamnosus*, *L. casei*, *L. plantarum*, *Bifidobacterium longum* y *Bifidobacterium bifidum* que sintetizan una gran variedad de compuestos bioactivos como bacteriocinas, ácidos grasos de cadena corta (AGCC), vitaminas, aminoácidos, enzimas, oligosacáridos, exopolisacáridos y compuestos inmunomoduladores (Indira et al., 2019).

Los compuestos fenólicos (PC), incluidos los terpenoides (carotenoides y fitoesteroides), polifenoles, alcaloides y otros componentes que contienen nitrógeno (glucosinolatos), representan la mayoría de los llamados compuestos bioactivos (Sorrenti et al., 2023; Teodoro et al., 2019). Según varios estudios epidemiológicos, una dieta rica en compuestos polifenólicos antioxidantes - principalmente flavonoides, ácidos fenólicos, lignanos, estilbenos, taninos y antocianinas - o la ingesta de derivados ricos en polifenoles, parece favorecer un retraso en la aparición de enfermedades degenerativas, incluidas las cardiovasculares, trastornos metabólicos, disfunciones cerebrales, lesiones y cánceres, en las que el estrés oxidativo juega un papel clave (Liu et al., 2023; Tressera-Rimbau et al., 2017). En la Fig. 5, se indica la clasificación de los compuestos bioactivos más importantes.

Compuestos bioactivos

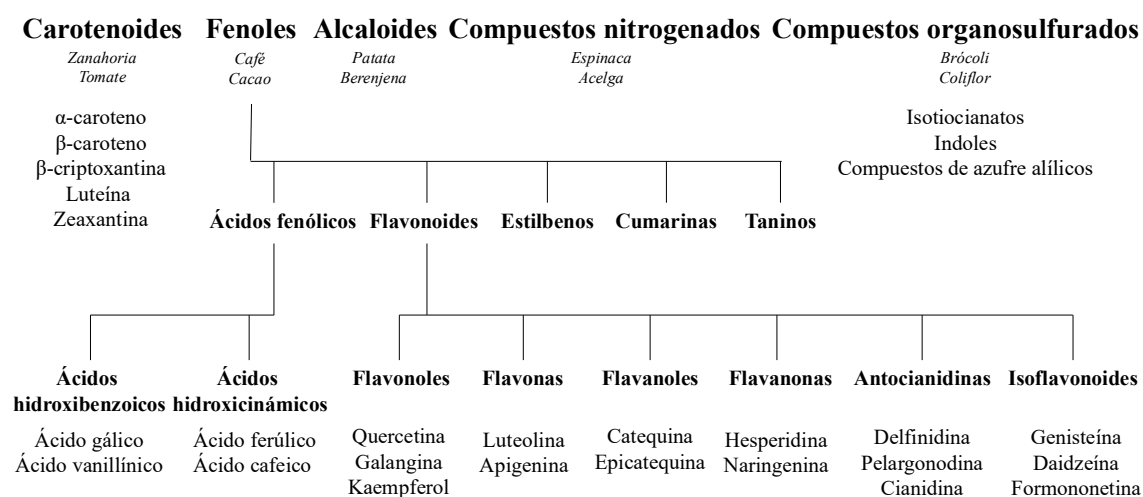


Figura 5. Clasificación de los principales compuestos bioactivos (adaptada de Sindhu et al. 2021).

1.2.1 Probióticos, prebióticos, sinbióticos y postbióticos (PPSP)

Dentro de los ingredientes funcionales/nutracéuticos que presentan una gran gama de compuestos bioactivos son los probióticos, prebióticos, sinbióticos y postbióticos (PPSP). Los probióticos se definen como “microorganismos vivos que, cuando se administran en cantidades adecuadas, confieren un beneficio para la salud del huésped” (Hill et al., 2014). Por su parte, los prebióticos se describen como “un sustrato que es utilizado selectivamente por los microorganismos del huésped, confiriendo un beneficio para la salud” (Hill et al., 2014). Recientemente, hay una tendencia a investigar los sinbióticos y los postbióticos. Los sinbióticos se definen como “una mezcla que comprende microorganismos vivos y sustrato(s) utilizados selectivamente por los microorganismos del huésped que confiere un beneficio para la salud del huésped” (Swanson et al., 2020) mientras que los postbióticos se consideran “una preparación de microorganismos inanimados y/o sus componentes que confiere un beneficio para la salud en el anfitrión” (Guarner et al., 2012). Dentro de algunos ejemplos de PPSP, se hallan el ajo (prebiótico), la calabaza (prebiótico) y el suero de leche (FW) (probiótico, prebiótico y postbiótico).

1.2.2 Ajo

El ajo (*Allium sativum* L.) es una de las especias más consumidas en el mundo (Netzel, 2020). Esta especia ya se utilizaba al comienzo de la historia registrada, encontrándose incluso en las pirámides egipcias y en los antiguos templos griegos. Los textos médicos antiguos de Egipto, Grecia, Roma, China e India prescribían aplicaciones médicas para el ajo (Rivlin et al., 2001). Uno de los tipos de ajo que más está cobrando importancia – tanto por su composición como por sus beneficios - es la variedad de Voghiera. El ajo variedad Voghiera es un ecotipo de ajo típico italiano cultivado en una zona particular al noreste de Voghiera (Ferrara), concretamente, en la región de Emilia-Romaña. Esta variedad de ajo está clasificada como producto con Denominación de Origen Protegida (Tedeschi et al., 2023).

Este alimento tan popular contiene diversos compuestos bioactivos, incluyendo polisacáridos, taninos, saponinas, linalol, geraniol, felandreno, β -felandreno, ajoeno,

aliina y S-alil-mercaptocisteína (El-Saadony et al., 2024). Una gran variedad de estudios ha demostrado que los componentes bioactivos del ajo presentan propiedades antioxidantes, antiinflamatorias, inmunomoduladoras, antibacterianas, antifúngicas, hepatoprotectoras, cardioprotectoras, anticancerígenas, protectoras del sistema digestivo, antidiabéticas, antiobesogénicas y protectoras renales (Bagheri et al., 2011; Kagawa et al., 2019; Shang et al., 2019). De hecho, el análisis fitoquímico del ajo de Voghiera se ha utilizado para el aislamiento de varias saponinas, como voghierósidos (A1, A2, E1, E2), furostanol, diglicósidos de eugenol y espirostanol, que poseen importantes actividades antimicrobianas y antioxidantes. Además, recientemente se ha investigado su papel preventivo en el desarrollo del cáncer (Brugnoli et al., 2021).

El ajo se considera un alimento con actividad prebiótica (Sunu et al., 2019). En un estudio llevado a cabo por Chen *et al.* (2019), los ratones fueron expuestos a diferentes dietas durante un periodo de 12 semanas. La α -diversidad es la diversidad media de especies en un lugar a escala local. Este parámetro, medido a través del índice de Shannon o el valor Chao1, aumentó como consecuencia de la exposición al ajo, especialmente aumentando la abundancia relativa en heces de *Lachnospiraceae*, mientras que reduciendo aquella de *Prevotella*. Un estudio en mujeres obesas reveló que aquellas que recibían el ajo en la dieta, presentaban unas menores abundancias relativas fecales de *Akkermansia*, mientras que en el caso de *Faecalibacterium* y *Bifidobacterium* se veían aumentados (Ettehad-Marvasti et al., 2022). En paciente hipertensos, se vio también como la suplementación con ajo aumentaba las poblaciones de *Lactobacillus* y *Clostridium* en la microbiota fecal después de un período de 3 meses (Ried et al., 2018).

1.2.3 Calabaza

La calabaza es un alimento originario de América - naturalmente distribuida desde los Estados Unidos hasta Argentina - que se difundió a otros continentes después del contacto europeo en 1492 (Gong et al., 2012). Las calabazas fueron dispersadas a otros continentes por viajeros transoceánicos a principios del siglo XVI y se han convertido en un cultivo de hortalizas familiar e importante en muchos países (Paris et al., 2006). Actualmente, los mayores productores de calabaza a nivel mundial son Asia y Europa, y gran parte del resto en América del Norte (FAO, 2006).

La calabaza se caracteriza por contener ácidos grasos poliinsaturados (ácido linoleico, ácido linolénico), tocoferoles (α -tocopherol, γ -tocopherol), ácidos fenólicos (ácido gálico, ácido ferúlico), flavonoles (rutina, isoquercetina), carotenoides (zeaxantina, luteína), delta-7-esteroles, triptófano y muchos otros fitoquímicos (Kulczyński & Gramza-Michałowska, 2019). A nivel general, este alimento multifuncional presenta algunos beneficios como mejora de la espermatogénesis, cicatrización de heridas, propiedades antiinflamatorias, antimicrobianas, antioxidantes, antiulcerosas y tratamiento de la hiperplasia prostática benigna (Batool et al., 2022).

El extracto de los polisacáridos de la calabaza se ha mostrado como un potente antidiabético. Además, estos polisacáridos aumentan los niveles de superóxido dismutasa (SOD), una enzima clave en los mecanismos antioxidantes (Wang et al., 2017b). Por su parte, el aceite de semilla de calabaza también se ha mostrado prometedor en la prevención del cáncer de próstata, ya que redujo el antígeno prostático específico después de 3 meses de ingesta en hombre coreanos con sintomatología de hiperplasia benigna de próstata (Hong et al., 2009). En otro estudio se constató cómo las albúminas 2S, que son proteínas presentes en las semillas de calabaza, presentaban propiedades anticancerígenas, particularmente en el caso del cáncer de mama. La proteína exhibió una fuerte actividad anticancerígena contra líneas celulares de cáncer de mama (MCF-7), teratocarcinoma de ovario (PA-1), cáncer de próstata (PC-3 y DU-145) y carcinoma hepatocelular (HepG2) (Tomar et al., 2014). En otro estudio se reportó que, en ratas inducidas en un estado depresivo, los extractos de semillas de calabaza ejercieron actividades antidepresivas después de 30 días (George & Nazni, 2012).

La actividad prebiótica de extractos de calabaza ha sido previamente estudiada. En un estudio llevado a cabo por Kan *et al.* (2024), un polisacárido péctico de calabaza (PPC) se obtuvo a partir de pulpa de calabaza mediante un método de extracción alcalina. En comparación con la inulina, el PPC promovió *in vitro* de manera más efectiva la proliferación de *L. acidophilus*, *Lactocaseibacillus rhamnosus* GG, *Lactocaseibacillus casei* y *Lactocaseibacillus paracasei* y la producción de ácidos láctico, acético y propiónico. En un estudio llevado a cabo en ratones, se vio como la exposición a polisacáridos de calabaza después de 28 días, aumentaba la abundancia relativa fecal de *Akkermansia*, mientras que aquellas de *Enterorhabdus*, *Colidextribacter*, *Alistipes* y *Blautia* se redujeron significativamente (Wu et al., 2021). En otro estudio, se vio cómo el polisacárido de calabaza cambió la estructura de la microbiota intestinal y tuvo un

enriquecimiento selectivo en especies clave de *Bacteroidess*, *Prevotella*, *Deltaproteobacteria*, *Oscillospira*, *Veillonellaceae*, *Phascolarctobacterium*, *Sutterella* y *Bilophila* (Liu et al., 2018).

1.2.4 Suero de leche fermentado (FW)

El suero es un subproducto de la industria quesera cuyo residuo suponía una alta contaminación ambiental en el pasado (Escrivá et al., 2023; Mateo-Díaz et al., 2023). Recientemente, con la finalidad de reducir la contaminación ambiental, se han realizado esfuerzos considerables encontrando nuevas salidas para la utilización del suero. Hoy en día, tecnologías como el secado por aspersión y la ultrafiltración son económicas y permiten separar diferentes fracciones del suero (lactosa, proteínas del suero como concentrado de proteína de suero, lactoalbúmina, lactoglobulina, etc.) que se comercializan en la industria alimentaria y farmacéutica (Vijayasanthi et al., 2020). El suero y su concentrado proteico se utilizan como ingredientes en la industria alimentaria principalmente debido a sus propiedades emulsionantes y espumantes (Pescuma et al., 2008; Zhu, 2024). Además, las proteínas del suero fortifican los alimentos aumentando la calidad nutricional del queso, postres lácteos, productos de panadería, etc. (Pescuma et al., 2008).

El alto valor nutricional característico del suero de leche viene del hecho de ser una excelente fuente de proteínas, lactosa, pequeñas cantidades de vitaminas y minerales, y un sustrato óptimo para la fermentación de las bacterias del ácido láctico (BAL) como aquellas del género *Lactobacillus* y *Bifidobacterium* (Frangiamone et al., 2022; Gupta y Prakash, 2017). Los efectos beneficiosos del FW están relacionados con una mejora del estrés oxidativo sistémico, la función urinaria, los síntomas del tracto urinario, y también la protección contra la infección bacteriana (Ausmees et al., 2018; Frangiamone et al., 2022; Frangiamone et al., 2023). En un estudio llevado a cabo en adultos coreanos de mediana edad durante 8 semanas, se observó que la suplementación con proteína de suero fermentada mejoró la fuerza muscular, los parámetros musculares y el rendimiento físico (Kim et al., 2023). En otro estudio, se evaluó el efecto protector del FW ante la infección provocada por *Salmonella Typhimurium* en ratones. Después de 28 días, se vio que el FW tenía un mayor efecto protector – incluso que la combinación de este mismo junto con un

probiótico - contra la infección por *Salmonella Typhimurium* en el modelo murino (Cordeiro et al., 2019). Por su parte, Escrivà *et al.* (2021) llevaron a cabo un estudio del efecto del FW sobre la bioaccesibilidad de la AFB1 en el pan. Se vio que tanto el FW como el mix del FW y la P, redujeron la bioaccesibilidad de la AFB1 entre 57-68%. De hecho, el FW se ha utilizado como un conservante natural en pan y queso con resultados prometedores (Dopazo et al., 2022; Izzo et al., 2020).

El FW, debido a presentar una fuente de nutrientes para las BAL, ejerce una función prebiótica al modular la microbiota intestinal. Sin embargo, hay únicamente un estudio que analiza el impacto del FW en la microbiota intestinal (Smith et al., 2020). Además, el FW presenta una actividad postbiótica, ya que la fermentación microbiana llevada a cabo por las BAL da lugar a una serie de metabolitos como bacteriocinas, ácidos orgánicos, aminoácidos, vitaminas y derivados de los exopolisacáridos presentes en el mismo FW (Tang et al., 2023). De hecho, el FW también influye en la proporción de unos de los metabolitos producidos por la microbiota más importantes desde el punto de vista fisiológico: los AGCC (acetato, butirato y propionato). Por ejemplo, un estudio de 6 semanas en muestras fecales de humanos - 5 hombres y 13 mujeres sanos en condiciones de vida libre - demostró que el FW disminuía el acetato, mientras que aumentaba el butirato y el propionato. Sin embargo, debido a la ausencia de un grupo control, estos resultados deben ser verificados en posteriores ensayos controlados (Smith et al., 2020).

1.3 Evaluación del efecto protector de los PPSP frente a micotoxinas en la interacción con el eje microbiano

1.3.1 Microbiota

El tracto gastrointestinal representa una de las interfaces más grandes (250–400 m² en el caso de los humanos) entre el huésped, los factores ambientales y los xenobióticos (Thursby & Juge, 2017). El conjunto de bacterias, virus, hongos y arqueas que colonizan el tracto gastrointestinal se denomina "microbiota intestinal" y ha evolucionado juntamente con el huésped durante miles de años para formar una relación mutuamente beneficiosa (Neish, 2009). El número de células bacterianas presentes en el

intestino de los mamíferos muestra un continuo que va de 10^1 a 10^3 bacterias por gramo de contenido en el estómago y el duodeno, progresando a 10^4 a 10^7 bacterias por gramo en el yeyuno e íleon y culminando en 10^{11} a 10^{12} células por gramo en el colon (O'Hara & Shanahan, 2006). Se ha estimado que hay aproximadamente 10 veces más células bacterianas que el número de células humanas y más de 100 veces la cantidad de contenido genómico (microbioma) que el genoma humano (Gill et al., 2006). No obstante, un estudio reciente ha propuesto que la proporción entre células humanas y bacterianas se sitúa en torno a 1:1 (Sender et al., 2016). Como resultado de la gran cantidad de células bacterianas en el cuerpo, el huésped y los microorganismos que lo habitan a menudo se denominan "superorganismo" (Thursby & Juge, 2017).

La microbiota intestinal es considerada como uno de los elementos clave que contribuyen a la salud del huésped. De hecho, como indica la Fig. 6, se ha visto cómo una pérdida en el equilibrio de la flora intestinal - denominada disbiosis - está implicada en un número considerable de enfermedades como la ansiedad, la depresión, la enfermedad inflamatoria intestinal (EII), la demencia, la enfermedad de Parkinson, la diabetes, la obesidad y el cáncer (Li et al., 2021).

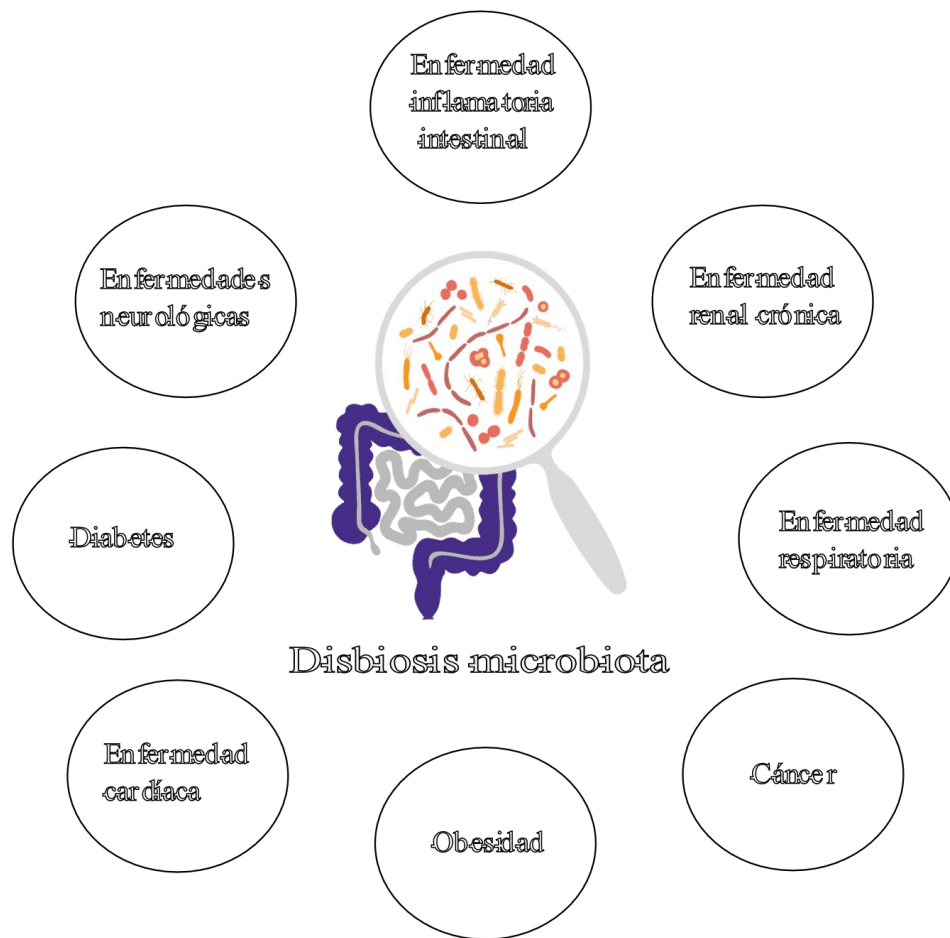


Figura 6. Esquema adaptado de Li *et al.* (2021) donde se representan las enfermedades relacionadas con una disbiosis de la microbiota intestinal.

Hay una serie de factores que modulan la microbiota intestinal: la dieta, el ejercicio, el sueño, los fármacos, la edad, la genética, los factores ambientales, los PPSP y las toxinas (Hou *et al.*, 2022). Los PPSP están relacionados con una protección frente a las enfermedades metabólicas mediante la regulación de la abundancia de bacterias beneficiosas y dañinas (Nambiar *et al.*, 2023). Además, los PPSP se caracterizan también por mejorar indirectamente las enfermedades metabólicas gracias a la producción de AGCC (acetato, propionato, butirato, isovalerato), así como ácido láctico y palmitoiletanolamida; mientras que reducen la producción de lipopolisacárido, N-óxido de trimetilamina (TMAO) y las reservas de ácidos biliares (Li *et al.*, 2021). De hecho, una

óptima regulación de todos estos metabolitos está relacionada con una mejora de la función de la barrera intestinal que, a su vez, contribuye a la atenuación de las enfermedades metabólicas, con una regulación positiva de las expresiones de proteínas que mantienen la integridad del epitelio intestinal como claudina 1, péptido similar al glucagón tipo 1, interleucina 10, ocludina 1 y la proteína de unión estrecha ZO-1 (Li et al., 2021).

La relación existente entre la pérdida del equilibrio de la microbiota intestinal con la gran variedad de enfermedades que se ha descrito en la Fig. 6, se debe en gran medida a la estrecha conexión de la microbiota intestinal con otros órganos. Entre las conexiones más importantes, se hallan aquellas del eje microbiota-hígado, microbiota-cerebro, microbiota-riñón y microbiota-sistema inmune. De hecho, el sistema nervioso entérico (SNE) participa en la regulación metabólica intestinal, y las neuronas entéricas y los neurotransmisores intestinales desempeñan un papel importante en la regulación del SNE. El intestino contiene circuitos reflejos completos del SNE, como neuronas motoras, interneuronas y neuronas sensoriales, y estas neuronas transfieren información entre el SNE y el sistema nervioso central (Wang et al., 2020).

Por su parte, el eje intestino-hígado está sostenido por la vena porta, que permite el transporte de productos derivados del intestino directamente al hígado, y la ruta de retroalimentación hepática de la secreción de bilis y anticuerpos al intestino (Albillos et al., 2020). Respecto al eje microbiota-riñón, se ha podido observar cómo la acumulación patológica de productos del metabolismo microbiano como los sulfatos de p-cresilo e indoxilo en la circulación producen inflamación sistémica en los vasos sanguíneos, resistencia a la insulina, activación del sistema renina-angiotensina-aldosterona y disfunción endotelial, todas ellas características comunes de la hipertensión y la enfermedad renal crónica (Yang et al., 2018).

En relación con el eje microbiota-sistema inmune, la ausencia de microbiota intestinal lleva a un desarrollo deficiente del GALT58 y a una inmunidad sistémica y central anormal. De hecho, los animales que carecen de microbios intestinales tienen una reducción sustancial en los niveles de células B, células plasmáticas, células T colaboradoras 17 e inmunoglobulina A, una función alterada de las células T reguladoras y un desequilibrio de las respuestas TH1 y TH2 (Yang et al., 2018).

Por otra parte, dado que la ingestión es la principal vía de exposición a las micotoxinas en los alimentos y piensos, el tracto gastrointestinal es el primer órgano con el que se encuentran estos compuestos (Bertero et al., 2020). De hecho, se ha visto cómo la relación entre la microbiota intestinal y las micotoxinas también es bidireccional. Las micotoxinas son capaces de cambiar la composición de la microbiota intestinal, mientras que la microbiota también metaboliza las micotoxinas. Dentro de esta metabolización, la microbiota se considera una espada de doble filo, puesto que puede reducir o aumentar la exposición a la forma tóxica de las micotoxinas mediante desintoxicación o desenmascaramiento de una forma enmascarada (Jin et al., 2021). Por un lado, el microbioma intestinal puede transformar las micotoxinas en formas menos tóxicas, lo que puede considerarse como una desintoxicación de las toxinas. Por otro lado, la microbiota intestinal también puede cambiar las formas modificadas menos tóxicas a su forma original, aumentando así la exposición a la toxina original y, por consiguiente, incrementando su toxicidad (Jin et al., 2021). Por ejemplo, la AFB1 ha demostrado alterar varias vías metabólicas importantes de la microbiota intestinal, incluida la síntesis de SCFA, vías relacionadas con el ácido pirúvico, metabolismos de ácidos biliares, aminoácidos y ácidos grasos de cadena larga, afectando al correcto funcionamiento del tracto digestivo y el suministro de energía, producción de neurotransmisores y comunicación cruzada enterohepática e inmunidad intestinal (Zhou et al., 2018). Del mismo modo, bacterias como *L. rhamnosus* y *S. cerevisiae* han mostrado reducir la presencia de AFB1 en un 30.46-31.14% (Zolfaghari et al., 2020). Por su parte, se ha visto cómo una exposición subcrónica a dosis bajas de OTA altera la diversidad y estructura de la comunidad microbiana intestinal (Izco et al., 2021). A su vez, *L. rhamnosus* tiene la capacidad de degradar OTA en un 83.58% (Zheng et al., 2023). Por tanto, estos estudios sugieren que estas alteraciones de vías metabólicas y de la misma microbiota intestinal forman parte de los mecanismos patológicos causados por estas micotoxinas.

1.3.2 Interacción microbiota-hígado

El hígado es un órgano clave que participa en numerosos procesos fisiológicos, incluyendo las vías de señalización, el metabolismo de los macronutrientes, la homeostasis de los lípidos y el colesterol, el apoyo al sistema inmunológico, la regulación del volumen sanguíneo y la descomposición de

compuestos xenobióticos, el control endocrino del crecimiento, así como en muchos fármacos actuales (Trefts et al., 2017). Numerosos estudios han mostrado cómo las alteraciones de la microbiota intestinal están relacionadas con la fisiopatología hepática mediante el eje intestino-hígado (Hsu & Schnabl, 2023; Lang & Schnabl, 2020). La disbiosis se ha asociado con la enfermedad del hígado graso no alcohólico, la esteatohepatitis no alcohólica y la enfermedad hepática alcohólica, así como con cirrosis y complicaciones (Shen et al., 2018). El eje intestino-hígado está sostenido, por una parte, por la vena porta - que permite el transporte de productos derivados del intestino directamente al hígado – y, por otra, por la ruta de retroalimentación hepática de la secreción de bilis y anticuerpos al intestino (Albillos et al., 2020). De hecho, además de recibir drenaje del bazo y la vesícula biliar, la vena porta también drena los intestinos delgado y grueso (Harkins & Ahmad, 2023) – siendo el último donde se encuentra la mayor densidad de microbios en el cuerpo (alrededor de 10^{13} – 10^{14}) (Senghor et al., 2018).

1.3.2.1 Micotoxinas en la interacción microbiota-hígado

Previamente, se ha demostrado que la ingesta dietética de bisfenol A (BPA) induce esteatosis hepática, la cual se encuentra estrechamente relacionada con la disbiosis de la microbiota intestinal, niveles elevados de endotoxinas y un aumento de la inflamación hepática a través de la vía TLR4/NF- κ B (Feng et al., 2020). Por otra parte, se ha visto que la acrilamida, un contaminante alimentario común, induce disbiosis de la microbiota intestinal y trastornos del metabolismo de los ácidos biliares (Yue et al., 2022). Además, la exposición crónica al arsénico se ha relacionado con daño hepático, niveles séricos elevados de marcadores autoinmunes y un mayor riesgo cardiovascular (Das et al., 2012), así como con una reducción de la diversidad alfa en la microbiota intestinal (Chi et al., 2017). En la Tabla 2 se detallan los estudios evaluando las micotoxinas en el eje microbiota-hígado y el efecto preventivo de los PPSP.

Tabla 2. Recopilación de las características más importantes de los estudios encontrados sobre micotoxinas en la interacción de la microbiota y la enfermedad renal. Para cada estudio, se describen la micotoxina, la muestra animal, la dosis de administración de micotoxinas, el tiempo de exposición, el ensayo experimental, los resultados y las referencias.

Introducción

Micotoxina	Animal /sexo	Dosis administrada	Tiempo de exposición	Diseño experimental	Resultados		Referencias
					Microbiota	Hígado	
AFB1	Ratones macho	750 µg/kg peso corporal	2 semanas	Los ratones macho se dividieron en dos tratamientos: control y AFB1. Bioquímica sérica, ELISA, análisis de expresión génica, transferencia Western y análisis de microbiota intestinal mediante secuenciación génica del ARNr 16S	A nivel de filo, se redujo el número de Bacteroidetes y Verrucomicrobia, pero se incrementó el número de Firmicutes en la AFB1. Las abundancias relativas de <i>Prevotella</i> , <i>Blautia</i> , <i>Akkermansia</i> y <i>Bacteroides</i> disminuyeron, pero la abundancia de <i>Clostridium_XIVa</i> , <i>Bifidobacterium</i> , <i>Lactobacillus</i> , <i>Corynebacterium</i> , <i>Ruminococcus</i> , <i>Allobaculum</i> y <i>Coprococcus</i> aumentó en el tratamiento con AFB1.	Los niveles séricos de ALT, AST, ALP y ROS aumentaron en ratones expuestos a AFB1. AFB1 también causó inflamación y necrosis del hígado, hiperplasia difusa grave de los conductos biliares e infiltración inflamatoria.	Liu et al., 2023
AFB1	Ratones macho	25 µg/kg peso corporal	4 semanas	Los ratones se dividieron aleatoriamente en 4 grupos: control, AFB1, ABX y AFB1+ABX. El análisis histológico, bioquímico y de microbiota se realizó mediante el gen bacteriano 16S rRNA amplificado por PCR	El tratamiento con AFB1 redujo Bacteroidetes y Cyanobacteria, pero aumentó Firmicutes y Proteobacteria. AFB1 provocó un aumento de la abundancia relativa de <i>Parabacteroides</i> , <i>Bacteroides</i> , <i>Escherichia-Shigella</i> y <i>Lactobacillus</i> , y una disminución de la abundancia relativa de <i>Prevotella</i> en comparación con el control.	El grupo AFB1 mostró una inducción significativa en el peso del hígado y provocó un infiltrado inflamatorio extenso e hipertrofia de hepatocitos. Además, AFB1 mejoró el LPS, NLRP3, caspasa-1 y ALT, ASP, LDH y MPO en suero y en hígado	Ye et al., 2023
OTA	Patitos macho	235 µg/kg peso corporal	2 semanas	Los patitos se dividieron aleatoriamente en tratamientos de control y OTA. Análisis bioquímico y secuenciación del amplicón 16S rRNA.	La OTA alteró significativamente la microbiota en el ciego y mejoró la abundancia relativa de Bacteroidetes y <i>Bacteroides</i> .	El tratamiento con OTA aumentó las actividades de AST y ALT séricas y los niveles de LPS, IL-1β e IL-6 en el hígado, pero redujo la IL-10.	Wang et al., 2019b
DON	Pollos de engorde macho	5-10 mg/kg de alimento	8 semanas	Los pollos de engorde se dividieron aleatoriamente en control, L-DON y H-DON.	A nivel de filo, DON aumentó la abundancia relativa de Firmicutes y Actinobacteria y disminuyó la abundancia relativa de Bacteroidetes y Proteobacteria. A nivel de género, la abundancia relativa de <i>Bifidobacterium</i> , <i>Escherichia-Shigella</i> y	H-DON aumentó los niveles medios de ALT, TBIL y UA, pero disminuyó los niveles medios de ALB y TG	Jia et al., 2023

				Análisis bioquímico y secuenciación del ARNr 16 S de bacterias intestinales del yeyuno.	<i>Romboutsia</i> aumentó, y <i>Bacteroides</i> disminuyó después de la alimentación con DON.		
DON	Lechones machos y hembras	2524 µg/kg de alimento	2 semanas	Los lechones se dividieron en cuatro tratamientos: control DON, EB y EB+DON. Análisis histológico, bioquímico y metagenómico.	A nivel de filo, DON disminuyó Proteobacteria y Firmicutes, pero mejoró Ascomycota. A nivel de especie, el DON redujo <i>S. cerevisiae</i> y <i>F. prausnitzii</i> , pero el DON aumentó <i>Aureobasidium namibiae</i> , <i>L. mucosae</i> y <i>L. amylovorus</i> en comparación con el control.	DON redujo los niveles séricos de ALP, ALT y AST, pero aumentó ALB, CREA1 y urea en comparación con el control. Además, el DON provocó vacuolización de hepatocitos de moderada a grave y megalocitosis, apoptosis y necrosis focal en comparación con el control	Novak et al., 2021
ZEN	Gallinas ponedoras	2.0 mg/kg peso corporal	3 meses	Gallinas ponedoras se dividieron aleatoriamente en 2 tratamientos: control y ZEN. Análisis serológico bioquímico, examen histopatológico y análisis de secuenciación del microbioma	A nivel de filo, ZEN aumentó Bacteroidetes, mientras que disminuyó Firmicutes y Proteobacteria. A nivel de género, ZEN aumentó <i>Bacteroides</i> y <i>Oscillospira</i> , pero redujo <i>Lactobacillus</i> en comparación al control	ZEN aumentó AST, ALT, MDA, SOD, GSH-Px, GSH, mientras que redujo ALP respecto al control	Wang et al., 2024

Aflatoxina B1 (AFB1). Albúmina (ALB). Alcalina fosfatasa (ALP). Alcalina transaminasa (ALT). Aspartato aminotransferasa (AST). Creatinina 1 (CREA1). Deoxinivalenol (DON). Glutacion (GSH). Glutacion peroxidasa (GSH-Px). Interleucina 1 beta (IL-1β). Interleucina 6 (IL-6). Interleucina 10 (IL-10). Lactato deshidrogenasa (LDH). Lipopolisacárido (LPS). Malondialdehído (MDA). Mieloperoxidasa (MPO). Dominio de pirina de la familia NLR que contiene 3 (NLRP3). Ocratoxina A (OTA). Especies reactivas de oxígeno (ROS). Bilirrubina total (TBIL). Triglicéridos (TG). Ácido úrico (UA). Zearalenona (ZEN).

Tabla 3. Recopilación de las características más importantes de los estudios encontrados sobre el efecto protector de PPSP contra micotoxinas en la interacción de la microbiota y la enfermedad hepática. Para cada estudio, se describen la micotoxina, los PPSP, la muestra animal, la dosis de administración de micotoxinas, el tiempo de exposición, el ensayo experimental, los resultados y las referencias.

Micotoxina	PPSP	Animal /sexo	Dosis administrada	Tiempo de exposición	Diseño experimental	Resultados		Referencia
						Microbiota	Hígado	
AFB1	Melatonina	Ratones macho	0.75 mg AFB1 /kg de peso corporal 20 mg melatonina /kg de peso corporal	2 semanas	Los ratones macho se dividieron aleatoriamente en cuatro grupos: control, AFB1, (AFB1 + melatonina, AFB1 + melatonina + Gly- β -MCA	A nivel de filo, la suplementación con melatonina (AFB1 + melatonina aumentó Firmicutes, Proteobacteria y Actinobacteria, pero disminuyó Bacteroidetes. A nivel de género, dicha suplementación incrementó <i>Akkermansia</i> , y <i>Barnesiella</i> , mientras que redujo <i>Desulfohalobium</i> , <i>Lactobacillus</i> y <i>Clostridium</i> en comparación al grupo AFB1	El pretratamiento con melatonina (AFB1 + melatonina) alivió notablemente la lesión hepática inducida por AFB1, la inflamación de los hepatocitos y la infiltración de células inflamatorias. Además, la melatonina redujo ALP, ALT, ASP en comparación al grupo AFB1	Liu et al., 2022
AFB1	Flavonoides de <i>Penthorum chinense</i> Pursh	Pollos de engorde (no especificado)	3 mg AFB1/kg de alimento 200-60 mg PCPCF/kg de alimento	4 semanas	Los pollos de engorde se dividieron aleatoriamente en 7 grupos: control, AFB1, PCPCF, AFB1 + YCHT, PCPCF alto, PCPCF medio y PCPCF en dosis baja Análisis histopatológico, bioquímica sérica, ELISA y secuenciación génica	A nivel de filo, la exposición a PCPCF aumentó Firmicutes, mientras que redujo Bacteroidetes, Proteobacteria y Actinobacteria. A nivel de género, PCPCF incrementó <i>Lactobacillus</i> y <i>Ruminococcus</i> , pero redujo <i>Bacteroides</i> en comparación al grupo control	La suplementación con PCPCF aumentó ALP, ALT, ASP, GSH, GSH-Px, SOD, mientras que redujo los niveles de MDA respecto al grupo control	Tao et al., 2023
DON	Butirato	Lechones (no especificado)	4 mg DON/kg alimento	4 semanas	Los machos castrados se asignaron aleatoriamente a uno de cuatro tratamientos	A nivel de filo, la exposición a butirato (DON + butirato) aumentó Firmicutes y	La suplementación con butirato (DON + butirato) alivió la necrosis,	Wang et al., 2020b

			0.2 % butirato		control, DON, butirato y DON + butirato Análisis bioquímico e histopatológico y secuenciación génica	Actinobacteria y disminuyó Bacteroidetes. A nivel de género, el butirato aumentó <i>Blautia</i> y disminuyó <i>Lactobacillus</i> respecto al grupo DON	vacuolización y degeneración citoplasmática de hepatocitos, así como trastornos del cordón hepático causado por DON. Además, el butirato redujo ALP, ALT y ASP en comparación al grupo DON	
ZEN	Rutina	Ratones hembra	10 mg de ZEN /kg de alimento 500 mg de rutina/kg de alimento	4 semanas	Los ratones se dividieron en cuatro grupos: control, ZEN, rutina, y ZEN y rutina Análisis bioquímico e histopatológico y secuenciación microbiana	A nivel de filo, la rutina (ZEN + rutina) aumentó Firmicutes y <i>Desulfobacteria</i> , mientras que redujo Actinobacteria. A nivel de género, aumentó <i>Lactobacillus</i> y <i>Bacillus</i> , pero redujo <i>Dubosiella</i> en comparación al grupo ZEN	La suplementación con rutina (ZEN + rutina) redujo ALP, ALT, ASP séricas. Además, dicha suplementación disminuyó la esteatosis, vacuolación citoplasmática e infiltración de células inflamatorias respecto al grupo ZEN	Wang et al., 2024b

Aflatoxina B1 (AFB1). Alcalina fosfatasa (ALP). Alcalina transaminasa (ALT). Aspartato aminotransferasa (AST). Deoxinivalenol (DON). Enzimoimmunoanálisis de adsorción (ELISA). Ácido β -muricólico conjugado con glicina (Gly- β -MCA). Glutation (GSH). Glutation peroxidasa (GSH-Px). Malondialdehído (MDA). Complejo de flavonoides de *Penthorum chinense* Pursh (PCPCF). Superóxido dismutasa (SOD). Extracto de Yin-Chen-Hao-Tang (YCHT). Zearalenona (ZEN)

1.3.3 Interacción microbiota-cerebro

Recientemente, un creciente conjunto de investigaciones ha relacionado la disbiosis de la microbiota intestinal con enfermedades del sistema nervioso central, como las enfermedades del Parkinson y del Alzheimer sugiriendo que dichas patologías pueden tener su origen en una inflamación crónica en el sistema gastrointestinal (Szandruk-Bender et al., 2022). Hay varias rutas por las que microbiota y cerebro se comunican entre sí: el metabolismo del triptófano, el nervio vago, el sistema inmunológico y el sistema nervioso entérico, incluyendo metabolitos microbianos como los aminoácidos de cadena ramificada, los peptidoglicanos y los AGCC (Cryan et al., 2019). De hecho, estudios en ratones han mostrado que aquellos ratones que poseían una microbiota estándar se caracterizaban por tener expresiones aumentadas del factor neurotrófico derivado del cerebro y del subtipo 2A del receptor de ácido N-metil-D-aspartico tanto en la corteza como en el hipocampo, en comparación a aquellos que carecían de microbiota (Wang & Wang, 2016).

1.3.3.1 Micotoxinas en la interacción microbiota-cerebro

En un estudio llevado a cabo por Subramaniam *et al.* (2022) las ratas fueron asignadas aleatoriamente a cuatro grupos: control, grupos tratados con dosis bajas y altas de AFB1 y grupo control positivo. La administración de AFB1 llevó a un recuento fecal de *Lactobacillus spp.* significativamente bajo después de la exposición a AFB1, con un recuento creciente de *Bifidobacterium spp.* en comparación con el control. A su vez, la exposición a AFB1 estuvo relacionada con un comportamiento similar a la anhedonia, tiempo de inmovilidad prolongado que indica desesperación conductual y cambios en el peso relativo del cerebro. Esta disbiosis intestinal provocada por AFB1 puede ser contrarrestada por los PPSP. En otro estudio en ratas por Aytekin Sahin *et al.* (2022), se vio como un probiótico a base de cepas de *Lactobacillus* y *Bifidobacterium* redujo significativamente los efectos neuroconductuales adversos de la exposición a AFB1, caracterizados por ansiedad y comportamientos similares a la depresión. Este efecto se asoció con la prevención del estrés oxidativo cerebral, la gliosis y la pérdida neuronal, incluida la pérdida de células piramidales.

1.3.4. Interacción microbiota-riñón

Estudios recientes se han centrado en el papel que desarrollan los microbios intestinales en la enfermedad renal crónica (ERC) y sus complicaciones asociadas (Yang et al., 2018). La ERC constituye un creciente problema de salud pública asociado con la pérdida de la función renal y la enfermedad cardiovascular, siendo esta última la principal causa de morbilidad y mortalidad en la ERC (Rukavina Mikusic et al., 2020). Actualmente, se proponen una serie de mecanismos que permiten unir la microbiota con la patología renal: alteraciones del microbioma intestinal, activación del receptor de aril hidrocarburo (AHR), aumentos de N-óxido de trimetilamina (TMAO) y toxinas urémicas derivadas de la microbiota, y desregulación de los AGCC y sus receptores. Se ha visto cómo la alteración de la microbiota intestinal contribuye a una producción excesiva de toxinas urémicas como el sulfato de p-cresil, el TMAO y el sulfato de indoxilo, todas ellas implicadas en los procesos variantes del desarrollo de enfermedades renales (Chen et al., 2019). En un estudio llevado a cabo en humanos, se vio cómo aproximadamente 190 unidades taxonómicas operativas microbianas (OTU) fueron significativamente diferentes en abundancia cuando se comparó el microbioma intestinal de pacientes con enfermedad renal terminal con controles sanos (Vaziri et al., 2013).

1.3.4.1 Micotoxinas en la interacción microbiota-riñón

Previamente, se ha demostrado que el bisfenol P causa disbiosis de la microbiota intestinal, lo que induce la alteración de la barrera intestinal y aumenta el estrés oxidativo en células renales de ratones y humanos (Ma et al., 2023; Ma et al., 2024). Además, se ha visto que la acrilamida reduce la producción de AGCC e induce trastornos del metabolismo de los ácidos biliares (Xu et al., 2024), lo que también causa peroxidación lipídica y daño al ADN, a su vez aumentando el estrés oxidativo en el riñón (Quasmi et al., 2025).

Tabla 4. Recopilación de las características más importantes de los estudios encontrados sobre micotoxinas en la interacción de la microbiota y la enfermedad renal. Para cada estudio, se describen la micotoxina, la muestra animal, la dosis de administración de micotoxinas, el tiempo de exposición, el ensayo experimental, los resultados y las referencias.

Micotoxina	Animal /sexo	Dosis administrada	Tiempo de exposición	Diseño experimental	Resultados		Referencia
					Microbiota	Riñón	
AFB1	Carpa gibel (no especificado)	50-100 µg AFB1/kg	28 días	Los peces fueron seleccionados al azar y divididos en tres grupos: control, AFB1 baja y AFB1 alta Determinación de la carga viral y secuenciación microbiana	A nivel de filo, la AFB1 aumentó Bacteroidetes y Proteobacteria, mientras que redujo <i>Fusobacteria</i> . A nivel de género, A nivel de género, la AFB1 aumentó <i>Bacteroides</i> y <i>Aeromonas</i> y disminuyó <i>Cetobacterium</i>	La AFB1 aumentó la carga viral de la carpa gibelio infectada con CyHV-2	Xue et al., 2023
DON	Pollos de engorde machos	5-10 mg DON/kg de alimento	8 semanas	Los pollos de engorde se dividieron aleatoriamente en control, L-DON y H-DON. Análisis bioquímico e histopatológico y secuenciación del ARNr 16 S de bacterias intestinales del yeyuno.	A nivel de filo, DON aumentó la abundancia relativa de Firmicutes y Actinobacteria y disminuyó la abundancia relativa de Bacteroidetes y Proteobacteria. A nivel de género, la abundancia relativa de <i>Bifidobacterium</i> , <i>Escherichia-Shigella</i> y <i>Romboutsia</i> aumentó, y <i>Bacteroides</i> disminuyó después de la alimentación con DON.	DON aumentó la infiltración de células inflamatorias, causando daño en el tejido renal. Además, redujo el peso relativo del riñón	Jia et al., 2023
DON	Cerdos (no especificado)	10 mg DON/kg de alimento	8 semanas	Los cerdos se dividieron aleatoriamente en control, L-DON y H-DON. Análisis bioquímico e histopatológico y secuenciación del ARNr 16 S de bacterias intestinales del yeyuno	A nivel de género, el DON redujo <i>Prevotella</i> y <i>Streptococcus</i> en comparación al grupo control	DON aumentó la infiltración de células inflamatorias, causando daño en el tejido renal. Además, redujo el peso relativo del riñón respecto al grupo control	Jia et al., 2023

Aflatoxina B1 (AFB1). ARN ribosómico 16s (ARNr 16S). Cyprinid herpesvirus 2 (CyHV-2). Deoxinivalenol (DON). Deoxinivalenol alto (H-DON). Deoxinivalenol bajo (L-DON).

Tabla 5. Recopilación de las características más importantes de los estudios encontrados sobre el efecto protector de PPSP contra micotoxinas en la interacción de la microbiota y la enfermedad renal. Para cada estudio, se describen la micotoxina, los PPSP, la muestra animal, la dosis de administración de micotoxinas, el tiempo de exposición, el ensayo experimental, los resultados y las referencias.

Micotoxina	PPSP	Animal /sexo	Dosis administrada	Tiempo de exposición	Diseño experimental	Resultados		Referencia
						Microbiota	Riñón	
OTA	Inulina	Pollos de engorde hembras	0,3 mg/kg de peso corporal 1 mL inulina/kg de peso corporal	2 semanas	Los pollos se dividieron aleatoriamente en 4 grupos: control, inulina, OTA y OTA + inulina Análisis bioquímico, examen histopatológico, índices antioxidantes y secuenciación génica	A nivel de filo, la suplementación con inulina (OTA + inulina) aumentó la proporción de Firmicutes y redujo aquella de Proteobacteria. A nivel de género, dicha suplementación incrementó <i>Lactobacillus</i> , <i>Ruminococcus</i> y <i>Escherichia</i> , mientras que redujo <i>Blautia</i> en comparación al grupo OTA	La exposición a inulina (OTA + inulina) mejoró los niveles de T-AOC, SOD, GSH y CAT, mientras que redujo los niveles de MDA, NLRP3, caspasa-1, IL-1 β y IL-18 respecto al grupo OTA	Wang et al., 2024c
OTA	Ácido fítico	Pollitos (no especificado)	0,4 mg OTA/kg de peso corporal Solución 0,1% ácido fítico en el alimento	2 semanas	Los pollitos se dividieron aleatoriamente en 4 grupos: control, ácido fítico, OTA, OTA + ácido fítico Análisis serológico y secuenciación genómica	A nivel de filo, la suplementación con ácido fítico (OTA + ácido fítico) incrementó Firmicutes y redujo Proteobacteria. A nivel de género, aumentó <i>Lactobacillus</i> y <i>Faecalibacterium</i> , mientras que redujo <i>Escherichia-Shigella</i> respecto al grupo OTA	El ácido fítico (OTA + ácido fítico) aumentó T-AOC, SOD, GSH, y CAT, mientras que redujo MDA y TNF- α en comparación al grupo OTA	Wang et al., 2024d
OTA	Extracto fermentado de jugo de tomate con <i>B. longum</i> F58-9, <i>B. bifidum</i> H22B664 y <i>B.</i>	Ratones (no especificado)	0,5 mg OTA/Kg de peso corporal 0,1 mL de postbiótico/kg de peso corporal	3 semanas	Se dividieron 32 ratones en cuatro grupos al azar: control, OTA, OTA + postbiótico y placebo Análisis bioquímico e histopatológico y secuenciación génica microbiana	A nivel de especie, la suplementación con postbiótico (OTA + postbiótico) aumentó <i>Bifidobacterium pseudolongum</i> y <i>Lactobacillus reuteri</i> , mientras que redujo <i>Lachnospiraceae bacterium</i> en comparación al grupo OTA	La suplementación con postbiótico (OTA + postbiótico) aumentó SOD y GSH, mientras que redujo MDA y TNF- α respecto al grupo OTA	Jiang et al., 2024

	<i>animalis</i> <i>H22B621</i>							
DON	<i>Clostridium</i> sp. <i>WJ06</i>	Cerdos machos y hembras	1,9 mg DON/Kg de alimento 30 mL de <i>Clostridium</i> sp. <i>WJ06</i> /día	5 semanas	Los cerdos fueron asignados aleatoriamente a tres grupos: control, DON y DON + <i>Clostridium</i> sp. <i>WJ06</i>	A nivel de filo, la suplementación con <i>Clostridium</i> sp. <i>WJ06</i> (DON + <i>Clostridium</i> sp. <i>WJ06</i>) aumentó Firmicutes, mientras que disminuyó Bacteroidetes y Proteobacteria. A nivel de género, incrementó <i>Lactobacillus</i> y <i>Streptococcus</i> , pero redujo <i>Coprococcus</i> , <i>Turicibacter</i> y <i>Alloprevotella</i> respecto al grupo DON	La exposición a <i>Clostridium</i> sp. <i>WJ06</i> (DON + <i>Clostridium</i> sp. <i>WJ06</i>) atenuó el aumento del peso relativo del riñón en comparación al grupo DON	Li et al., 2017

Bifidobacterium animalis H22B621 (*B. animalis* H22B621). *Bifidobacterium bifidum* H22B664 (*B. bifidum* H22B664). *Bifidobacterium longum* F58-9 (*B. longum* F58-9). Catalasa (CAT). Deoxinivalenol (DON). Glutation (GSH). Interleucina 1 beta (IL-1 β). Interleucina 18 (IL-18). Malondialdehído (MDA). Dominio de pirina de la familia NLR que contiene 3 (NLRP3). Ocratoxina A (OTA). Superóxido dismutasa (SOD). Capacidad antioxidante total (T-AOC). Factor de necrosis tumoral alfa (TNF- α).

1.3.5. Interacción microbiota-sistema inmune

La microbiota intestinal y los sistemas inmunológicos innato y adaptativo del huésped interactúan entre sí jugando un papel fundamental en el mantenimiento de la homeostasis intestinal y la inhibición de la inflamación (Yoo et al., 2020). La microbiota intestinal participa en la síntesis de vitaminas y otros productos metabólicos que median en la interacción entre las células del epitelio intestinal y las células inmunitarias. Como mecanismo de defensa, las células del epitelio intestinal sintetizan una barrera mucosa para separar la microbiota de las células inmunes del huésped y así, reducir la permeabilidad intestinal (Zhao & Maynard, 2022). Como consecuencia de una interacción deficiente entre la microbiota intestinal y el sistema inmunológico de las mucosas, puede haber un aumento de las bacteria gram negativas potencialmente patógenas y sus cambios metabólicos asociados, alterando la barrera epitelial y aumentando la susceptibilidad a las infecciones (Duraisingham et al., 2015). Estas alteraciones negativas en la composición microbiana – denominada disbiosis intestinal – pueden también causar una desregulación de las respuestas inmunitarias, provocando resistencia a la insulina, estrés oxidativo e inflamación. Con el tiempo, la disbiosis crónica junto con sus productos metabólicos a través de la barrera mucosa puede aumentar la prevalencia enfermedades autoinmunes (Miele et al., 2015).

1.3.5.2 Micotoxinas en la interacción microbiota-sistema inmune

Previamente, se ha demostrado que el BPA causa desequilibrios inmunitarios intestinales y sistémicos mediante una disminución de la frecuencia de células Th1/Th17 en la lámina propia, concomitante con un aumento de la respuesta inmunitaria Th1/Th17 esplénica (Malaisé et al., 2017). Por otra parte, se ha podido ver cómo la exposición a la acrilamida reduce la composición, riqueza y diversidad de la microbiota intestinal (Yue et al., 2022) y, además, reduce los componentes inmunitarios innatos, pero aumenta los niveles de inmunoglobulinas (Hashem et al., 2022).

Tabla 6. Recopilación de las características más importantes de los estudios encontrados sobre micotoxinas en la interacción de la microbiota y el sistema inmune. Para cada estudio, se describen la micotoxina, la muestra animal, la dosis de administración de micotoxinas, el tiempo de exposición, el ensayo experimental, los resultados y las referencias.

Micotoxina	Animal /sexo	Dosis administrada	Tiempo de exposición	Diseño experimental	Resultados		Referencia
					Microbiota	Sistema inmune	
AFB1	Ratones macho	25 µg AFB1/kg de peso corporal	4 semanas	Se asignaron aleatoriamente veinte ratones macho Balb/c a un grupo de control y a un grupo AFB1 Índice de bazo, análisis bioquímico e histopatológico y secuenciación génica de la microbiota	A nivel de filo, la AFB1 aumentó Firmicutes y redujo Bacteroidetes. A nivel de género, la AFB1 incrementó <i>Lactobacillus</i> y disminuyó <i>Allistipes</i> y <i>Helicobacter</i> en referencia al control	La puntuación histopatológica del bazo en el grupo AFB1 estaba regulada positivamente en comparación con el grupo de control. La exposición a AFB1 resultó en una regulación positiva significativa de la expresión del ARNm de IL-2 e IL-6 en el bazo y de las células CD4+, mientras que la expresión del ARNm de IL-10 fue regulado negativamente respecto al control	Chen et al., 2024
AFB1	Ratones machos y hembras	200-400 µg AFB1/kg de peso corporal	3 semanas	Los ratones fueron asignados aleatoriamente a dos grupos: control y AFB1 Análisis de citometría de flujo, ELISA y secuenciación génica microbiana	A nivel de filo, la exposición con AFB1 aumentó Bacteroidetes, mientras que redujo Firmicutes. A nivel de género, la AFB1 incrementó <i>Barnesiella</i> , <i>Bacteroides</i> y <i>Akkermansia</i> , pero disminuyó <i>Allistipes</i> en comparación al grupo control	La exposición a AFB1 produjo un aumento general del número de linfocitos en el intestino, una reducción de la expresión de genes epiteliales relacionados con la defensa microbiana, así como una disminución en la producción de citocinas por ILC2 en comparación al grupo control	Bastos-Amador et al., 2023
AFB1	Pollos de engorde machos y hembras	2.5 mg AFB1/kg	6 semanas	Los pollos de engorde se asignaron a cuatro tratamientos: control, AFB1, <i>Arthrospira platensis</i> y AFB1 + <i>Arthrospira platensis</i> Determinación población microbiana y respuesta inmune humoral	La población bacteriana total y las bacterias del ácido láctico en el íleon disminuyeron, mientras que las bacterias coliformes se redujeron tras la exposición a AFB1 respecto al grupo control	La AFB1 redujo la producción de anticuerpos contra los antígenos del virus de la enfermedad de Newcastle, el virus de la influenza y la enfermedad de la bronquitis infecciosa respecto al grupo control	Kasmani et al., 2023

DON	Polluelos macho	10 mg de DON/kg de alimento	6 semanas	Los polluelos se dividieron aleatoriamente en 3 grupos que: control, DON y DON + <i>L. plantarum</i> liofilizado JM113 Determinación linfocítica, análisis cuantitativo por RT-PCR de la expresión genética y secuenciación génica de la microbiota	A nivel de filo, la exposición con AFB1 aumentó Firmicutes y redujo Bacteroidetes y Proteobacteria. A nivel de género, el DON aumentó <i>Epsilonproteobacteria</i> respecto al grupo control	DON redujo significativamente el número de linfocitos T y linfocitos B de proliferación en sangre periférica. Además, DON disminuyó la expresión de ARNm de β -Defensina-8 y el receptor de Ig en el yeyuno en comparación al grupo control	Yang et al., 2020

Aflatoxina 1 (AFB1). Ácido ribonucleico mensajero (ARNm). Deoxinivalenol (DON). Enzimoinmunoanálisis de adsorción (ELISA). Inmunoglobulina (Ig). Interleucina 2 (IL-2). Interleucina 6 (IL-6). Interleucina 10 (IL-10). Células linfoides innatas intestinales tipo 2 (ILC2). Ocratoxina A (OTA). (Reacción en cadena de polimerasa de transcripción inversa (RT-PCR)).

Tabla 7. Recopilación de las características más importantes de los estudios encontrados sobre el efecto protector de PPSP contra micotoxinas en la interacción de la microbiota y el sistema inmune. Para cada estudio, se describen la micotoxina, los PPSP, la muestra animal, la dosis de administración de micotoxinas, el tiempo de exposición, el ensayo experimental, los resultados y las referencias.

Micotoxina	PPSP	Animal /sexo	Dosis administrada	Tiempo de exposición	Diseño experimental	Resultados		Referencia
						Microbiota	Sistema inmune	
AFB1	Curcumina	Patos (no especificado)	0,1 mg/kg de solución de AFB1 200-400 mg/kg de curcumina	3 semanas	Los patos se dividieron aleatoriamente en 4 grupos: control, AFB1, AFB1 + curcumina baja y AFB1 + curcumina alta Detección por PCR cuantitativa por fluorescencia en tiempo real y secuenciación génica de la microbiota	A nivel de filo, la exposición a curcumina (AFB1 + curcumina) aumentó Actinobacteria y disminuyó Firmicutes y Proteobacteria. A nivel de género, dicha exposición aumentó <i>Streptococcus</i> en comparación al grupo AFB1	La curcumina (AFB1 + curcumina baja/alta) redujo sustancialmente los niveles de proteína de NLRP3, GSDMD, caspasa-1 e IL-1 β en comparación al grupo AFB1	Pan et al., 2024
AFB1	<i>L. pentosus</i> HC-2	<i>Penaeus vannamei</i> (camarón) (no especificado)	500 μ g AFB1/kg alimento 5×10^8 CFU <i>L. pentosus</i> HC-2/g	6 semanas	Los camarones fueron aleatoriamente asignados en 3 grupos: control, AFB1 y AFB1+ <i>L. pentosus</i> HC-2 Análisis qPCR de genes relacionados con el sistema inmunológico y secuenciación génica microbiana	A nivel de filo, la suplementación con <i>L. pentosus</i> HC-2 (AFB1 + <i>L. pentosus</i> HC-2) aumentó Proteobacteria y redujo Firmicutes, Bacteroidetes y Actinobacteria. A nivel de género, la suplementación aumentó <i>Photobacterium</i> y <i>Vibrio</i> , mientras que disminuyó <i>Ruminococcus</i> y <i>Oscillospira</i> en comparación al grupo AFB1	La exposición a <i>L. pentosus</i> HC-2 (AFB1+ <i>L. pentosus</i> HC-2) llevó a un aumento de Rab, GST, dorsal, MLPM y Pro-PO, mientras que redujo ligeramente Relish en comparación al grupo AFB1	Fang et al., 2020
OTA	AST	Ratones (no especificado)	5 mg OTA/kg peso corporal	4 semanas	Los ratones se dividieron aleatoriamente en cuatro	A nivel de filo, la exposición a AST (OTA+AST) aumentó	En comparación al grupo OTA, la	Chen et al., 2021

			100 mg AST/kg peso corporal		grupos: control, OTA, AST y OTA+ AST Análisis histopatológico, bioquímico y genómico	Bacteroidetes, mientras que redujo Firmicutes y Proteobacteria en comparación al grupo OTA	suplementación con AST (OTA+AST) aumentó MUC2, Defa5, β -pD2 y las células caliciformes, pero redujo MUC1	
DON	Resveratrol	Lechones macho	3.8 mg DON/kg de alimento 300 mg resveratrol/kg de alimento	4 semanas	Los lechones fueron divididos aleatoriamente en 4 grupos: control, DON, resveratrol y DON + resveratrol Análisis bioquímico y secuenciación génica microbiana	A nivel de filo, la suplementación con resveratrol (DON+ resveratrol) aumentó Firmicutes, Bacteroidetes, mientras que redujo Proteobacteria. A nivel de género, la suplementación incrementó <i>Lactobacillus gasseri</i> y <i>Alloprevotella sp.</i> en comparación al grupo DON	La suplementación con resveratrol (DON + resveratrol) disminuyó significativamente la IL-1 β , TNF- α e IL-6 ileales respecto al grupo DON. Por su parte, dicha suplementación previno eficazmente la disminución inducida por DON en la expresión de los genes pBD-2, PG1-5 y MUC2	Qiu et al., 2022
DON	<i>L. plantarum JM113</i>	Pollos de engorde macho	10 mg DON/kg de alimento 1×10^9 CFU/ kg <i>L. plantarum JM113</i>	6 semanas	Los pollos de engorde fueron asignados aleatoriamente a 3 grupos: control, DON y DON + <i>L. plantarum JM113</i> Determinación bioquímica de los parámetros inmunes y secuenciación génica de la microbiota	A nivel de filo, la suplementación con <i>L. plantarum JM113</i> (DON + <i>L. plantarum JM113</i>) supuso un aumento de Bacteroidetes y una reducción de Firmicutes. A nivel de género, hubo un aumento de <i>Anaerostipes</i> y <i>Roseburia</i> respecto al grupo DON	La expresión duodenal de IL-10 e IL-1 e ileal de IgA aumentó significativamente como consecuencia de la exposición a <i>L. plantarum</i> (DON + <i>L. plantarum</i>) en comparación al grupo DON	Wu et al., 2018b

Aflatoxina B1 (AFB1). Astaxantina (AST). Defensina alfa 5 (Defa5). Deoxinivalenol (DON). Gasdermina D (GSDMD). Glutation S-transferasa (GST). Inmunoglobulina A (IgA). Interleucina 1 (IL-1). Interleucina 1 beta (IL-1 β). Interleucina 6 (IL-6). Interleucina 10 (IL-10). *Lactobacillus pentosus HC-2* (*L. pentosus HC-2*). *Lactobacillus plantarum JM113* (*L. plantarum JM113*). Péptido similar a la mucina (MLPM). Mucina 1 (MUC1). Mucina 2 (MUC2). Dominio de pirina de la familia NLR que contiene 3 (NLRP3). Ocratoxina A (OTA). Beta-defensina 2 porcina (pBD-2). Protegrina 5 (PG1-5). Profenoloxidasas (ProPO). Factor de necrosis tumoral alfa (TNF- α).

1.4 Técnicas avanzadas de análisis

1.4.1 Cromatografía líquida de alta resolución

La Cromatografía Líquida de Alta Resolución (HPLC, por sus siglas en inglés) es una técnica analítica ampliamente utilizada para la separación, identificación y cuantificación de compuestos en una mezcla. Se basa en el principio de la separación de las moléculas en una columna, gracias a la interacción entre la fase estacionaria (generalmente una sustancia sólida o gel) y la fase móvil (un líquido o gas que transporta la muestra a través de la columna). La fase estacionaria es el componente fijo de la columna, que puede estar compuesto por partículas pequeñas, de diferentes tamaños y propiedades, tales como sílice o polímeros. Esta fase tiene la función de interactuar con los analitos de manera que algunos de ellos se retienen en mayor o menor grado, dependiendo de su estructura química y afinidad. La fase móvil, es un disolvente o una mezcla de disolventes que se mueve a través de la columna, ayudando a arrastrar los analitos con ella. El tipo de fase móvil varía según la naturaleza de los analitos y la aplicación que se tenga en mente. Por lo general, se usan disolventes líquidos en HPLC, pero también se pueden utilizar gases en algunas técnicas específicas (como en la cromatografía de gases). La columna cromatográfica es el componente central de la técnica. En ella, la fase estacionaria está empaquetada en un tubo largo. A medida que la fase móvil fluye a través de la columna, los componentes de la mezcla se separan en función de su afinidad con la fase estacionaria. Esto se logra porque algunos compuestos se adsorben más fuertemente que otros, lo que hace que se muevan a diferentes velocidades (Skoog et al., 2001).

Al final de la columna se encuentra un detector que mide la cantidad de cada componente a medida que sale de la columna. Existen diferentes tipos de detectores, como el UV-VIS (ultravioleta-visible), el índice de refracción, la fluorescencia, entre otros.

El HPLC es esencial en el control de calidad de fármacos, asegurando que las concentraciones de los compuestos activos sean las correctas, para detectar contaminantes en alimentos y bebidas, como las micotoxinas, y pueden representar un riesgo para la salud humana. Además, permite la separación y análisis de compuestos en muestras

biológicas como sangre, orina y tejidos, incluyendo vitaminas, hormonas, metabolitos, etc., También el HPLC se emplea en la industria alimentaria para determinar aditivos, conservantes, colorantes y otros compuestos presentes en los productos.

Los componentes de un sistema HPLC incluyen, una bomba de HPLC, la cual se encarga de suministrar la fase móvil a alta presión a través de la columna. Esto permite que los analitos sean empujados a través de la fase estacionaria, el inyector que es el dispositivo donde se introduce la muestra. La inyección de la muestra puede ser automática o manual. La columna donde ocurre la separación. La columna está llena de la fase estacionaria y su tipo depende de la aplicación y de las características de los analitos. El detector que es el componente encargado de medir la presencia y concentración de los compuestos al salir de la columna. Existen detectores UV, de fluorescencia, de conductividad, entre otros. Además, un software que permite controlar todo el proceso y procesar los datos obtenidos del detector, generando un cromatograma que representa la separación de los analitos. El sistema de lectura muestra el resultado de la separación en forma de cromatograma. Este sistema se basa en una imagen que traduce visualmente la evolución, en función del tiempo, de un parámetro que depende de la concentración instantánea del soluto a la salida de la columna. Además, la posición de los picos sobre el eje del tiempo puede utilizarse con la finalidad de identificar los diferentes analitos que componen la muestra; así como las áreas que se sitúan bajo los picos sirven a modo de cuantificación de la concentración de cada especie (Skoog et al., 2001).

Además de su separación, el objetivo del uso de la cromatografía como método de análisis cuantitativo, consiste en conseguir una separación satisfactoria en un intervalo de tiempo adecuado. Para ello, diferentes métodos de cromatografía se han desarrollado. Algunos de ellos, incluyen cromatografía de afinidad, cromatografía en papel, cromatografía de permeación en gel, cromatografía en columna, cromatografía en capa fina, cromatografía de gases, cromatografía de intercambio iónico y HPLC.

La cromatografía líquida en columna se caracteriza por ser la técnica más antigua dentro del ámbito de la cromatografía. Sin embargo, su relevancia no ha estado reconocida hasta la década de los setenta, gracias al desarrollo de la HPLC (Cela et al., 2002). La técnica HPLC se distingue por su idoneidad para la separación de termolábiles o no volátiles, fácil adaptación a las determinaciones cuantitativas exactas, sensibilidad, y especialmente, por su gran aplicabilidad a sustancias como proteínas,

aminoácidos, hidrocarburos, plaguicidas, fármacos, etc. Estas características permiten que esta técnica sea de primordial interés en la industria, en la ciencia y en la sociedad en general (Cela et al., 2002). Su versatilidad permite que se adapte a diferentes tipos de análisis, lo que la convierte en una herramienta indispensable en laboratorios de control de calidad, investigación, farmacología y ciencias ambientales.

1.4.1.1 Cromatografía líquida de alta resolución acoplada a un detector de fluorescencia (HPLC-FLD).

La Cromatografía Líquida de Alta Resolución con Detección por Fluorescencia (HPLC-FLD) es una técnica que combina las ventajas del HPLC con un detector especializado en la fluorescencia de los analitos. En esta técnica, la muestra se separa en una columna cromatográfica, y los compuestos que poseen propiedades fluorescentes son excitados por una longitud de onda específica, emitiendo luz en una longitud de onda diferente. Esta emisión se mide con un detector de fluorescencia, proporcionando una señal altamente sensible que se puede correlacionar con la concentración del analito. La señal de fluorescencia detectada se convierte en una señal eléctrica, se procesa y se registra como un cromatograma. La representación cromatográfica supone la intensidad de la fluorescencia en relación con la concentración del compuesto, a diferentes longitudes de onda a medida que los componentes de la muestra eluyen de la columna cromatográfica. Las ventajas de HPLC-FLD:

- Alta sensibilidad: La fluorescencia es una técnica extremadamente sensible, lo que permite la detección de compuestos en concentraciones de traza, con una mejor relación señal-ruido en comparación con otros detectores.
- Especificidad: Los compuestos que tienen una alta emisión de luz fluorescente pueden ser detectados incluso en presencia de otras sustancias, lo que hace a la técnica ideal para analizar contaminantes o micotoxinas en muestras complejas.
- Aplicaciones en el análisis de micotoxinas: Muchos compuestos de interés en análisis alimentarios y farmacéuticos, como las aflatoxinas, son naturalmente fluorescentes o pueden derivarse para obtener fluorescencia, lo que permite su cuantificación precisa utilizando HPLC-FLD.

El proceso de separación en HPLC-FLD ocurre:

- Fase estacionaria: El componente de la columna HPLC que retiene temporalmente los analitos, lo que permite su separación basada en sus propiedades químicas y físicas, como la afinidad por la fase estacionaria.
- Fase móvil: Un líquido que transporta los analitos a través de la columna.
- Detector de fluorescencia: Después de la separación, el analito pasa por el detector FLD, que lo excita con luz de una longitud de onda específica. Si el analito tiene propiedades fluorescentes, emitirá luz en otra longitud de onda, que es medida por el detector.

El HPLC-FLD es utilizado para la detección de micotoxinas, como las aflatoxinas, en alimentos, ya que estas sustancias son fluorescentes y pueden detectarse de forma eficiente a través de esta técnica. Un ejemplo de aplicaciones de HPLC-FLD en micotoxinas: En el análisis de micotoxinas, como las aflatoxinas (AFB1, AFB2, AFG1, AFG2) y la OTA, los compuestos tienen la capacidad de emitir fluorescencia, lo que facilita su detección incluso en concentraciones muy bajas. Esto es crucial en el control de calidad de alimentos, donde estas micotoxinas representan un riesgo para la salud humana y animal. Con HPLC-FLD, es posible detectar y cuantificar múltiples micotoxinas simultáneamente en un solo análisis, lo que mejora la eficiencia del proceso analítico. Se han desarrollado métodos que permiten detectar simultáneamente hasta 12 tipos de estas (Soleimany et al., 2011).

Los principales componentes del sistema HPLC-FLD:

- Sistema de bombeo: Que impulsa la fase móvil a través de la columna cromatográfica a alta presión.
- Columna cromatográfica: Donde se produce la separación de los analitos.
- Detector de fluorescencia: Que capta la emisión fluorescente de los analitos separados.
- Software de análisis: Que interpreta los datos y genera el cromatograma.

En resumen, el HPLC-FLD es una técnica analítica poderosa, especialmente útil en el análisis de compuestos fluorescentes, como las micotoxinas, y es muy valorada por su alta sensibilidad y precisión. Se aplica ampliamente en diversos campos como la

industria alimentaria, farmacéutica y en estudios medioambientales, donde la detección de contaminantes a bajas concentraciones es crucial.

1.4.2 Citometría de flujo

En los últimos años el campo de la citómica se ha convertido en un área de gran impacto, sobre todo debido a la integración del conocimiento de la proteómica y de la genómica a la hora de estudiar los citomas, entendiéndose como aquellos sistemas celulares heterogéneos de los organismos pluricelulares (Bernas et al., 2006). Es considerada una disciplina científica de análisis celular cuyo objetivo es la definición del fenotipo molecular de las células individuales, como resultado de la interacción entre el genotipo y la exposición a factores internos y externos. El desarrollo de la citómica se ha podido desarrollar gracias a las nuevas y potentes tecnologías basadas en los análisis de células individuales, es decir, citometría de flujo (Valet, 2005).

La citometría de flujo comprende una serie de tecnologías capaces de analizar rápidamente ya sea células o bien partículas individuales en suspensión a medida que pasan por uno o varios láseres mientras. La suspensión consiste en una solución tamponada a base de sal. Cada partícula se analiza para determinar la dispersión de la luz visible y uno o varios parámetros de fluorescencia. La dispersión de la luz visible se mide en dos direcciones diferentes, la dirección hacia adelante (Forward Scatter o FSC), que puede indicar el tamaño relativo de la celda, y a 90° (Side Scatter o SSC), que indica la complejidad interna o granularidad de la celda. La dispersión de la luz es independiente de la fluorescencia. Las muestras se preparan para la medición de la fluorescencia mediante la transfección y expresión de proteínas fluorescentes (p. ej., proteína fluorescente verde, GFP), tinción con tintes fluorescentes ((p. ej., yoduro de propidio (PI), ADN)) o tinción con anticuerpos conjugados fluorescentemente (p. ej., CD3 FITC).

La citometría de flujo es una herramienta que tiene aplicaciones en múltiples disciplinas como biología molecular, biología del cáncer, monitoreo de enfermedades infecciosas, inmunología, virología. De hecho, es muy eficaz para el estudio del sistema

inmunológico y su respuesta ante enfermedades infecciosas y cáncer. Permite la caracterización simultánea de poblaciones heterogéneas de células de la sangre y de la médula ósea, así como de tejidos sólidos que pueden disociarse en células individuales, como ganglios linfáticos, bazo, tejidos mucosos, tumores sólidos, etc. Además del análisis de poblaciones de células, una aplicación importante de la citometría de flujo es la clasificación de células para su posterior análisis (McKinnon, 2018). Siguiendo lo expuesto por El-Hajjar et al. (2023), los componentes de un citómetro de flujo tradicional incluyen: Fluidos para procesamiento de muestras y clasificación de células

- Cámara de flujo: las células de la muestra se enfocan hidrodinámicamente y se presentan al láser.
- Óptica para la detección de fluorescencia
 - La luz se mueve a través de un sistema de espejos y filtros.
 - Las células se pueden medir en función de su tamaño, complejidad interna e intensidad de señal.
- Electrónica para procesamiento de señales, visualización y análisis de computadoras
 - El tubo fotomultiplicador convierte la luz en señales eléctricas.
 - La señal se amplifica y digitaliza.

1.4.3 Técnicas ómicas

Las ciencias ómicas abarcan tecnologías y enfoques colectivos para explorar las acciones y funciones de diversas moléculas que constituyen las células dentro de los organismos vivos. En las últimas décadas, las ómicas se han expandido desde la genómica inicial a una amplia gama de disciplinas biomoleculares destinadas a estudiar aspectos específicos considerados en su conjunto. En general, el principal objetivo de las ciencias ómicas es identificar, caracterizar y cuantificar todas las moléculas biológicas que intervienen en la estructura, función y dinámica de una célula, tejido u organismo. Muchas áreas de investigación pueden clasificarse como ómicas (Cimbalo et al., 2022). Como indica la Fig. 7, los ejemplos incluyen genómica,

proteómica, transcriptómica, metabolómica, lipidómica y epigenómica, que corresponden a análisis globales de genes, proteínas, ARN, metabolitos, lípidos y ADN metilado o proteínas histonas modificadas en los cromosomas, respectivamente (Micheel et al., 2012). Debido a que estas tecnologías se crearon con el objetivo de generar una cantidad masiva de datos en un solo experimento, han presentado un alto rendimiento sin precedentes en un corto periodo de tiempo. Gracias a las tecnologías ómicas, ahora es posible detectar niveles no solo cualitativos sino también cuantitativos de múltiples genes, transcripciones y proteínas simultáneamente. Además, gracias a la mayor sensibilidad, especificidad y precisión de la tecnología, se pueden identificar biomarcadores clave para la predicción de enfermedades mucho más rápido (Kim et al., 2017).

Desde una perspectiva molecular, cualquier evento tóxico refleja modificaciones a nivel de integridad y funcionalidad del ADN (nivel genómico), ARN (nivel transcriptómico), proteínas (nivel proteómico) y/o moléculas pequeñas (nivel metabolómico). A su vez, tales modificaciones se deben a interacciones moleculares primarias entre las formas toxicológicamente activas y el objetivo biológico respectivo. La investigación precisa de estos mecanismos moleculares y cómo afectan la homeostasis celular en los distintos niveles "ómicos" es en realidad obligatoria para comprender en profundidad la toxicidad de las micotoxinas. El caso de las toxinas de *Alternaria*, AOH y sus congéneres, proporciona un ejemplo de acción pleiotrópica a niveles moleculares, donde un solo tipo químico actúa sobre múltiples objetivos (Dellafiora & Dall'Asta, 2017).

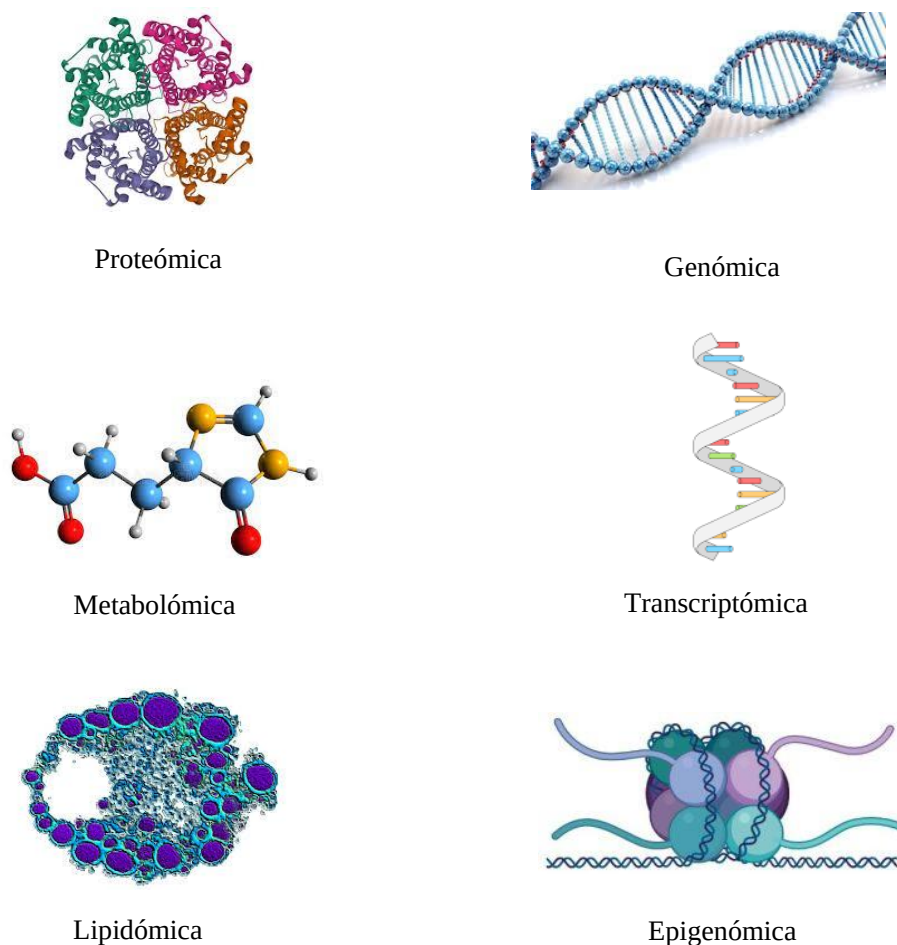


Figura 7. Representación de las ciencias ómicas más utilizadas: proteómica, genómica, metabolómica, transcriptómica, lipidómica y epigenómica.

1.4.3.1 ARNr 16S

Los ribosomas son orgánulos complejos, altamente especializados, cuya principal función es participar en el proceso de síntesis de proteínas. El ribosoma bacteriano tiene un coeficiente de sedimentación de 70S (expresado en unidades Svedberg), y se divide en dos subunidades, la subunidad grande (50S) y la subunidad pequeña (30S). Cada subunidad es un complejo ribonucleoproteico formado por una serie de proteínas ribosómicas y moléculas de ARNr específicas. Como indica la Fig. 8, la subunidad 50S contiene los ARNr 5S y 23S junto con unas 34 proteínas (L1-

L34; L, large), mientras que la subunidad 30S contiene el ARNr 16S y 21 proteínas diferentes (numeradas desde S1-S21, donde S procede de small) (Opron & Burton, 2018).

El ARN ribosómico (ARNr) 16S es la macromolécula más ampliamente utilizada en estudios de filogenia y taxonomía bacterianas. Su aplicación como cronómetro molecular fue propuesta por Carl Woese (Universidad de Illinois) a principios de la década de 1970 (Garrett, 2014).

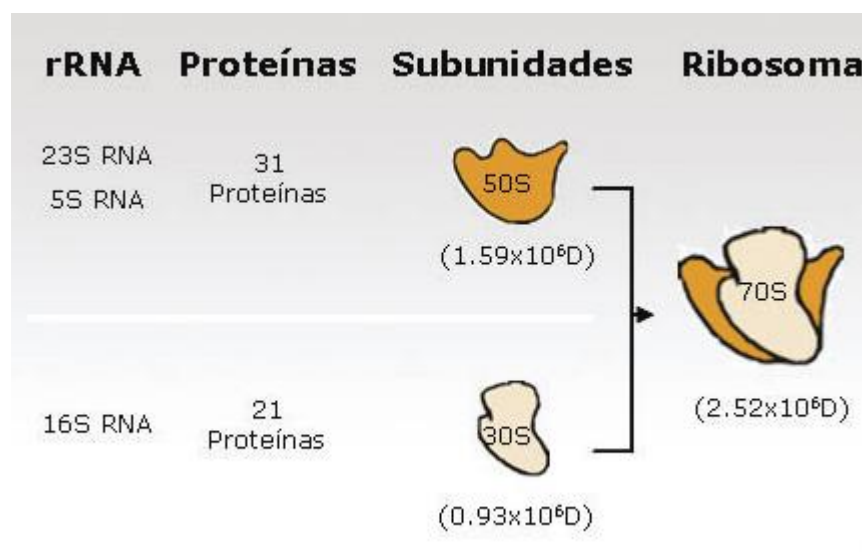


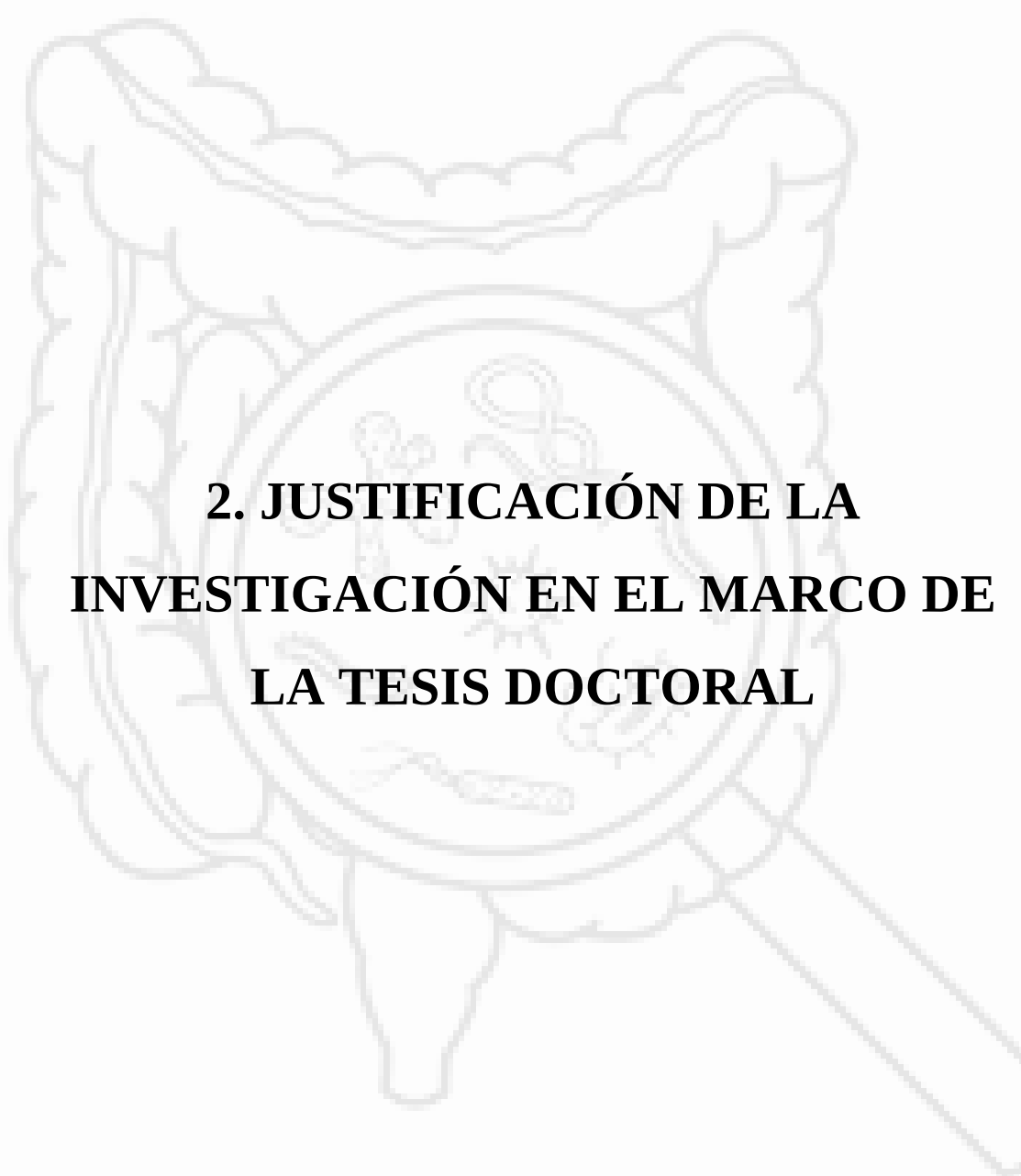
Figura 8. Ribosoma bacteriano de *Escherichia coli* (*E. coli*) adaptado de Opron & Burton, (2018). Se muestra un esquema de su estructura, y se indican las subunidades y macromoléculas que lo componen.

Pese a que existen cronómetros moleculares alternativos al ARNr 16S, de momento ninguno ha sido capaz de desplazarle. De hecho, esta macromolécula presenta una serie de características que la han convertido en el cronómetro molecular definitivo:

1. Se trata de una molécula muy antigua, presente en todas las bacterias actuales.

2. Tanto su estructura como su función se han mantenido constantes a lo largo del tiempo, ya que las alteraciones en la secuencia únicamente reflejan cambios aleatorios.
3. Información acerca de todos los procariotas y, junto con las variaciones en los ARNr 18S, a lo largo de toda la escala evolutiva, debido a que los cambios suceden lentamente. Además, los ARNr de la subunidad pequeña – a la que pertenece el ARNr 16s, sin embargo, presentan suficiente variabilidad para diferenciar tanto los organismos alejados como los más próximos.
4. Las fluctuaciones estadísticas son atenuadas por el tamaño relativamente largo de los ARNr 16S (1.500 nt).
5. Estructura secundaria conservada que puede servir de ayuda en las comparaciones, aportando una base para el alineamiento preciso.
6. Dado que resulta relativamente fácil secuenciar los ADNr 16S existen bases de datos amplias, en continuo crecimiento (Rodicio & del Carmen Mendoza, 2004).

Los genes bacterianos de ARNr 16S generalmente contienen nueve "regiones hipervariables" (V1-V9) que demuestran una notable diversidad de secuencias entre diferentes especies bacterianas y pueden usarse para la identificación de especies (Chakravorty et al., 2007). Sin embargo, las regiones hipervariables del ARNr 16S se caracterizan por presentar diferentes grados de diversidad de secuencia, por lo que ninguna región hipervariable sola permite distinguir entre todas las bacterias (Selim et al., 2005). Por ello, es conveniente llevar a cabo un análisis combinando, al menos, dos de estas regiones.



2. JUSTIFICACIÓN DE LA INVESTIGACIÓN EN EL MARCO DE LA TESIS DOCTORAL

2. JUSTIFICACIÓN DE LA INVESTIGACIÓN EN EL MARCO DE LA TESIS DOCTORAL

Las micotoxinas, especialmente la AFB1 y la OTA, representan un grave problema de salud pública y seguridad alimentaria debido a su alta toxicidad, su capacidad de bioacumulación y su impacto negativo en la salud humana y animal. A pesar de las numerosas estrategias desarrolladas para reducir su presencia en los alimentos y minimizar sus efectos adversos, sigue siendo necesario explorar nuevas alternativas eficaces, seguras y sostenibles. En este contexto, los compuestos bioactivos presentes en ciertos alimentos han demostrado un gran potencial como agentes protectores frente a la toxicidad de estas micotoxinas.

Investigaciones previas *in vitro* e *in vivo* han evidenciado el efecto preventivo y el potencial detoxificador de algunos de estos agentes protectores como el ajo de Voghiera, el FW y la P sobre la AFB1 y la OTA. Frangiamone *et al.* (2023) han demostrado *in vitro* cómo la toxicidad a nivel inmune producida por AFB1 y OTA se veía disminuida por la adición de FW y P. De hecho, Vila *et al.* (2025) han revelado *in vivo* que la inclusión de FW y P aumentaba la excreción fecal de ambas micotoxinas. Por su parte, el ajo de Voghiera también ha mostrado un efecto protector *in vitro* ante la exposición de ZEN y BEA. No obstante, aún existen muchas incógnitas sobre los mecanismos subyacentes de estos efectos protectores, así como sobre su interacción con otros factores biológicos, como es el caso de la microbiota intestinal.

Esta tesis doctoral profundiza en el estudio de la capacidad detoxificadora del ajo de Voghiera sobre la AFB1 en condiciones *in vitro*, introduciendo como elemento novedoso la simulación del tracto gastrointestinal humano. Este enfoque permite obtener una visión más precisa sobre la estabilidad y efectividad de sus compuestos bioactivos a lo largo del proceso digestivo, proporcionando información clave sobre su posible aplicación en la prevención de la toxicidad por micotoxinas.

Justificación de la investigación en el marco de la tesis doctoral

Además, esta investigación incorpora un aspecto innovador al analizar el papel de la microbiota intestinal en el metabolismo y la detoxificación de las micotoxinas. Se estudia no solo cómo la microbiota puede modular la toxicidad de las micotoxinas emergentes, sino también cómo la exposición a estas micotoxinas puede afectar la composición y el equilibrio del ecosistema microbiano intestinal. En este sentido, se recopila y analiza la información existente sobre la relación entre las micotoxinas emergentes y los efectos protectores de la microbiota, probióticos, prebióticos, sinbióticos y postbióticos, considerando su potencial para mitigar la toxicidad de estos contaminantes alimentarios.

Por otro lado, se evalúan los efectos beneficiosos de la P y el FW en la exposición a AFB1 y OTA, con especial énfasis en los cambios inducidos en el perfil de la microbiota intestinal. Como aspecto innovador, se introduce el análisis del dimorfismo sexual, evaluando posibles diferencias en la respuesta a la exposición a micotoxinas y su modulación por estos ingredientes bioactivos según el sexo. Esto permite comprender mejor la influencia de factores biológicos en la toxicidad y detoxificación de las micotoxinas, abriendo nuevas posibilidades en el desarrollo de estrategias de prevención.

En conjunto, los hallazgos de esta tesis doctoral contribuirán a ampliar el conocimiento sobre el papel de los ingredientes bioactivos en la dieta como agentes protectores frente a la toxicidad de la AFB1 y la OTA, proporcionando información valiosa para el desarrollo de nuevas estrategias de intervención basadas en la alimentación y la modulación de la microbiota.



3. OBJETIVOS

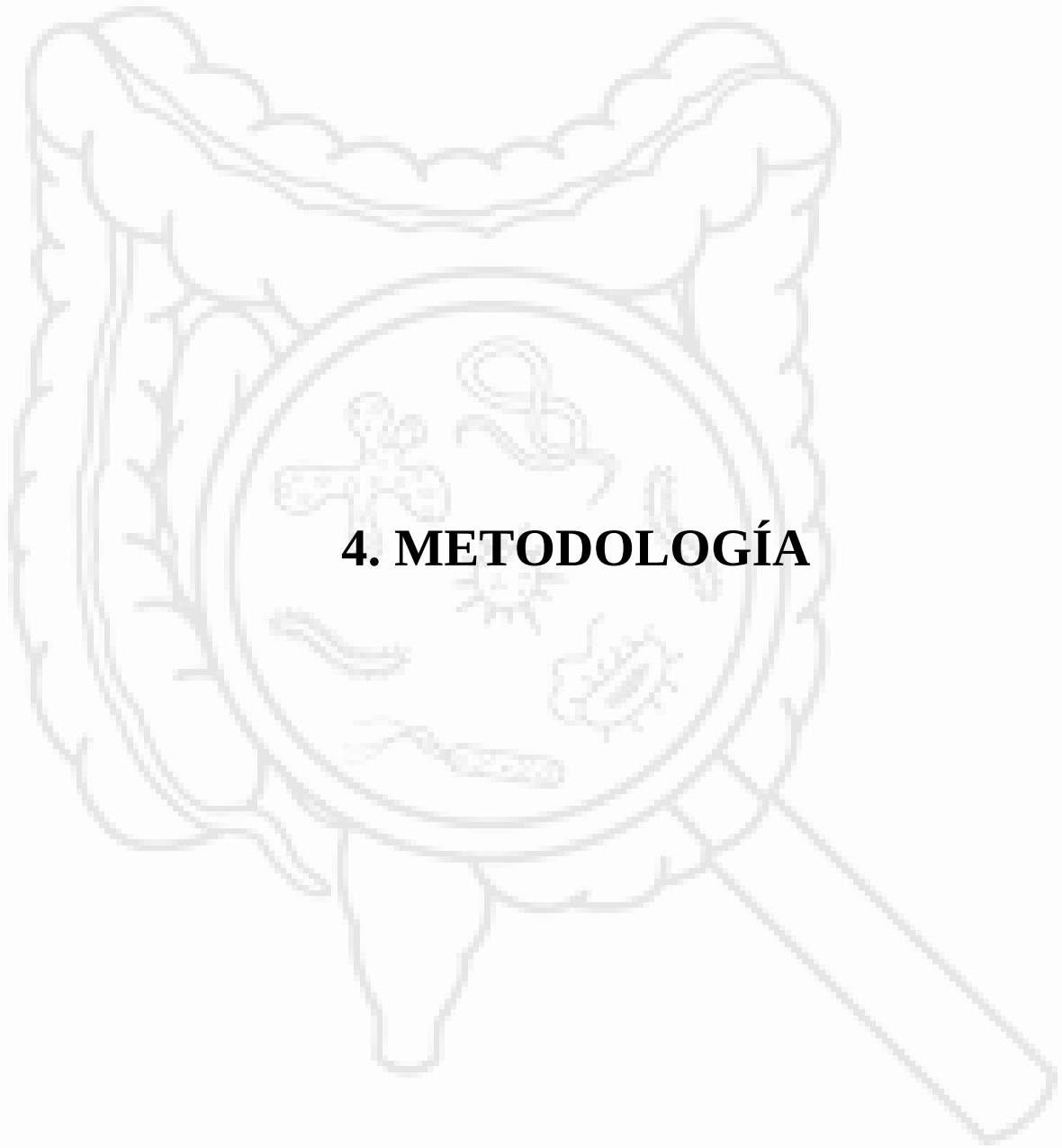
3. OBJETIVOS

El objetivo general de esta Tesis Doctoral es profundizar en el conocimiento de los mecanismos de toxicidad y los efectos toxicológicos de la AFB1 y la OTA, así como evaluar el potencial efecto protector de ingredientes bioactivos como el ajo de Voghiera (*Allium sativum* L. var. Voghiera), la P (*Cucurbita* spp.), el extracto de suero de leche de cabra fermentado (FW) y la microbiota intestinal. Para ello, se emplearán modelos *in vitro* e *in vivo*, combinados con enfoques ómicos y moleculares.

Para alcanzar este objetivo general, se plantean los siguientes objetivos específicos:

1. Evaluar el efecto protector del ajo de Voghiera (*Allium sativum* L. var. Voghiera) sobre la bioaccesibilidad y citotoxicidad de la AFB1, empleando modelos *in vitro* que simulan el tracto gastrointestinal humano y técnicas celulares avanzadas
2. Analizar el papel de la microbiota intestinal en la prevención de la toxicidad por micotoxinas, mediante la recopilación y revisión crítica de la literatura científica sobre los efectos protectores de la microbiota, los probióticos, prebióticos, sinbióticos y postbióticos frente a las micotoxinas emergentes.
3. Investigar los efectos de la P y el extracto de FW en la modulación del perfil de la microbiota intestinal ante la exposición a AFB1 y OTA en un modelo animal de ratas machos y hembras, considerando posibles diferencias asociadas al dimorfismo sexual.

4. METODOLOGÍA



4.1 Trabajo 1: *Allium sativum* L. var. *Voghiera* reduce la bioaccesibilidad y la citotoxicidad de la aflatoxina B1 *In Vitro*

4.1.1. Reactivos

La solución estándar AFB1 (pureza > 99%) se adquirió de Sigma-Aldrich (St. Louis, MO, EE. UU.). El tiocianato de potasio (KSCN), cloruro de potasio (KCl), ácido clorhídrico (HCl), dihidrógenofosfato de sodio (NaH_2PO_4), hidróxido de sodio (NaOH), sulfato de sodio (Na_2SO_4), cloruro de sodio (NaCl), hidrogenocarbonato de sodio (NaHCO_3), urea ($\text{CO}(\text{NH}_2)_2$), pepsina A (674 U mg^{-1} P7000), pancreatina (762 U mg^{-1} P1750), sales biliares (B8631) y α -amilasa (930 U mg^{-1} A3403) se adquirieron de Sigma-Aldrich (Madrid, España). El acetonitrilo (ACN) y el metanol (MeOH) se obtuvieron de Fisher Scientific (Madrid, España). El agua desionizada se obtuvo de un sistema de purificación de agua Milli-Q (Millipore, Bedford, MA, EE. UU.). El dimetilsulfóxido (DMSO) se adquirió de Fisher Scientific (Geel, Bélgica). Los ingredientes del pan se compraron en un supermercado local. El ajo fresco se conservó a 4 °C y se picó antes de su uso. Se obtuvieron el PI y un kit de reactivos de ADN CycleTEST™ PLUS de BD Biosciences (San Diego, CA, EE. UU.) para el ensayo del ciclo celular mediante citometría de flujo. El conjugado Anexina V-FITC se adquirió de Miltenyi y la sonda MitoTracker® se adquirió de Invitrogen (Waltham, MA, EE. UU.). El diacetato de 2',7'-diclorodihidrofluoresceína ($\text{H}_2\text{-DCFDA}$) y la solución salina tampón fosfato (PBS) se compraron de Sigma Chemical Co. (St. Louis, MO, EE. UU.).

4.1.2. Contaminación de la harina de maíz y producción de micotoxinas

El maíz fue contaminado por *A. flavus* ITEM 8111 adquirido de la Colección de Cultivos Microbianos Agroalimentarios del Instituto de Ciencias y Producción de Alimentos (ISPA, Bari, Italia). El hongo se mantuvo en condiciones ideales en el laboratorio para producir la micotoxina en la matriz alimentaria (maíz) en la que fue inoculado. En estas condiciones, la cepa utilizada sólo generó esta micotoxina y ninguna otra. Se introdujeron un total de 450 g de maíz por frasco de 1 L previamente esterilizado

y se prepararon dos frascos. La contaminación se realizó añadiendo 20 mL de suspensión de micelio y esporas en agua peptonada con Tween 80 de la cepa fúngica. Los frascos de vidrio se mantuvieron a temperatura ambiente durante un período de tres semanas. Cada 2-3 días se añadió 1 mL de agua destilada a los frascos para mantener las condiciones de humedad.

Después de tres semanas, los frascos se esterilizaron en un autoclave para eliminar la contaminación fúngica y el maíz se transformó en harina hasta lograr una total homogeneización, incluido el tamizado. Las muestras de maíz se mantuvieron a -20 °C (Escrivá et al., 2021). La harina de maíz se analizó mediante HPLC-FLD (como se informa en la Sección 4.1.6) para cuantificar AFB1 y descartar cualquier otra micotoxina.

4.1.3. Ingredientes del pan, elaboración de pan y horneado

Se elaboraron ocho panes combinando el ingrediente bioactivo y la AFB1, cuatro correspondientes al experimento 1 (E1) y otros cuatro correspondientes al experimento 2 (E2): un pan control (C), pan de ajo 2% (G), pan AFB1 (A), y pan AFB1 + ajo 2% (A+G). El pan control se preparó siguiendo la siguiente receta: 127 g de harina de trigo, 66 mL de agua mineral (37 °C), 8 g de levadura para productos de panadería (Levital, Sant Hilari Sacalm, España), 4 g de azúcar y 2,6 g de NaCl. Después de fusionar todos los ingredientes, se realizó una homogeneización en una máquina para hacer pan denominada SilverCrest SBB 850 A1 (Kompernass GMBH, Bo-chum, Alemania) durante 5 min, y se les dio forma en moldes (de aproximadamente 200 g), tapándose con un paño húmedo y siendo fermentados durante 1h a temperatura ambiente. Posteriormente, los panes se cubrieron con papel de aluminio y se hornearon a 180 °C durante 35 min en una mufla Memmert ULE 500 (Madrid, España). Luego, los panes se desmoldaron y se refrigeraron a temperatura ambiente durante 1h. Se prepararon panes enriquecidos modificando ligeramente la receta de control para producir pan de ajo al 2% (G y A+G). Finalmente, se prepararon panes contaminados con AFB1 reemplazando parte de la harina de trigo

con 5,9 g (E1~152 mg/kg) y 54,7 g (E2~197 mg/kg) de harina de maíz contaminada con AFB1 para obtener panes con baja y alta concentración - respectivamente. Esta diferencia en la concentración de AFB1 se utilizó para determinar si el ajo desempeñaba un papel importante en la bioaccesibilidad. Como se muestra en la Tabla 1, se obtuvieron ocho panes en total.

Tabla 1. Composición del pan en experimentos 1 y 2.

Ingredientes	C (g)		G (g)		A (g)		A + G (g)	
	E1	E2	E1	E2	E1	E2	E1	E2
Harina de trigo	127	127	124	124	121.1	72.3	118.1	69.3
NC maize flour	-	-	-	-	5.9	54.7	5.9	54.7
Agua	66	66	65	65	66	66	65	65
Sal	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6
Azúcar	4	4	4	4	4	4	4	4
Levadura fresca	8	8	8	8	8	8	8	8
Ajo	-	-	4	4	-	-	4	4
Cantidad total	207.6	207.6	207.6	207.6	207.6	207.6	207.6	207.6

C (Control). (G) Ajo. A (Aflatoxina B1). A + G (Aflatoxina B1 + ajo). C (No Contaminado). (n=3).

4.1.4. Modelo de digestión estática *in vitro*

El proceso de digestión constaba de tres fases: (oral, gástrica e intestinal), como se informó en trabajos anteriores (Escrivá et al., 2021). En resumen, se pusieron 10 g de pan en bolsas de plástico esterilizadas (500 ml) y se mezclaron con agua Milli-Q (a 37 °C) y 6 mL de saliva artificial (Millipore, Bedford, MA, EE. UU.). Para reproducir la fase oral, se utilizó un IUL Stomacher (IUL S.A, Barcelona, España) durante 30 s para simular el proceso de masticación. La saliva se preparó el día anterior (y se ajustó a pH = 6,8) mezclando 0,17 mL de NaCl (175,3 g/L), 1 mL de KSCN (20 g/L), 1 mL de NaH₂PO₄ (88,8 g/L), 2 mL de NaHCO₃ (84,7 g/L), 1 mL de KCl (89,6 g/L), 1 mL de Na₂SO₄ (57 g/L), 29 mg de α -amilasa, 0,8 mL de urea (25 g/L) y agua destilada para lograr un volumen de 100 mL. Una vez simulada la fase oral, se transfirió el contenido a un matraz Erlenmeyer topacio para realizar la fase gástrica. La mezcla se acidificó a pH= 2 con solución de HCl 6 N. Luego, se agregaron 14 mL de agua Milli-Q (a 37 °C) y 0,5 g de

solución de pepsina (1 g en 25 mL de HCl 0,1 N) para lograr 100 mL. Las muestras se incubaron durante 2 h a 37 °C en oscuridad y ligera agitación (1 g) utilizando un agitador orbital (Infors AG CH-4103, Bottmingen, Suiza). A continuación, se guardaron alícuotas gástricas (15 mL) para análisis posteriores y se realizó una digestión pancreática añadiendo 1,25 g de una mezcla de sales biliares/pancreatina (0,625 g de sales biliares y 0,1 g de pancreatina disueltos en 25 mL de NaHCO₃ 0,1 N)) a pH = 6,5 (NaHCO₃ 0,5 N). Los digeridos se incubaron como se detalló anteriormente (2 h a 37 °C en oscuridad y ligera agitación), finalmente se ajustó el pH a 7,2 (NaOH 0,5 N), las muestras se centrifugaron (226 x g durante 10 min a 4 °C), y el sobrenadante se recogió para obtener los digestos intestinales. Los experimentos se realizaron por triplicado y cuádruplicado.

4.1.5. Extracción de AFB1 de muestras de pan y digestión

Se colocaron un total de 5 g de maíz/trigo/harina de pan en tubos de centrífuga (50 mL). Luego se añadieron 25 mL de MeOH/H₂O (80/20). Se usó Ultraturrax (T 18 digital ULTRA-TURRAX®, Staufen, Alemania) durante 3 minutos para triturar las muestras después de centrifugarlas a 226 × g durante 5 minutos (Centrifuge 5810R, Eppendorf, Alemania). El sobrenadante se recogió y se filtró a través de un filtro de 0,22 µm (Phenomenex, Madrid, España). Todos los análisis se realizaron por triplicado (n = 3). Las muestras de digestión gástrica e intestinal se centrifugaron a 226 x g durante 10 min a 4 °C, se filtraron (filtro de 0,22 µm) (Phenomenex, Madrid, España), se diluyeron con MeOH (1:1) y se inyectaron en HPLC-FLD.

4.1.6. Determinación cuantitativa de AFB1 por HPLC-FLD

AFB1 se caracterizó mediante HPLC con un equipo Agilent serie 1100 (Agilent Technologies, Santa Clara, CA, EE. UU.) equipado con un muestreador automático, un desgasificador, una bomba cuaternaria y un detector FLD Agilent 1200 (Agilent Technologies, Santa Clara, CA, EE. UU.), utilizándose el software Agilent JP03924119 para el análisis de datos.

Se colocó un sistema de derivatización (reactor fotoquímico UVE™, LCTech, Jasco Analítica, Madrid, España, S.L.) entre la columna analítica y el detector FLD para mejorar la actividad fluorescente de AFB1. La separación cromatográfica se llevó a cabo utilizando una columna de fase inversa, 00F-4633-EO Kinetex EVO C18 (150 × 4,6 mm, 100 Å y tamaño de partícula de 5 µm) (Phenomenex, Palo Alto, CA, EE. UU.) a 40 °. La fase móvil isocrática estuvo compuesta por H2O/ACN/MeOH (60:10:30 v/v/v) con un caudal de 1 mL min⁻¹. El volumen de inyección fue de 20 µL para harinas y panes y 40 µL para digestiones gastrointestinales. Las longitudes de onda de excitación y emisión de AFB1 fueron $\lambda_{ex} = 365$ nm y $\lambda_{em} = 440$ nm.

4.1.7. Validación del método de determinación cuantitativa

La linealidad se evaluó mediante el coeficiente de regresión (r^2) de las curvas emparejadas con matrices de calibración. El límite de detección (LOD) y el límite de cuantificación (LOQ) se midieron mediante una comparación de las señales cromatográficas de muestras que contenían bajas concentraciones del compuesto con las de muestras en blanco y estableciendo la concentración mínima a la que el compuesto puede detectarse con precisión y medidos, respectivamente. Se consideraron relaciones señal-ruido de 10:1 y 3:1 para obtener el LOD y LOQ, respectivamente. La repetibilidad y la precisión intermedia se cuantificaron como la desviación estándar relativa de cuatro análisis realizados en cuatro días diferentes ($n = 4$) y cuatro análisis realizados el mismo día ($n = 4$), respectivamente.

Las curvas emparejadas de la matriz de harina y pan se prepararon fortificando, con un estándar de AFB1 (100 mg/L en MeOH), la solución extraída del blanco (pan con harina de trigo no contaminada) con diferentes concentraciones de AFB1 para obtener al menos 6 curvas de puntos de nivel. Los parámetros de validación para el método de determinación cuantitativa AFB1 (ecuación de regresión lineal, rango de linealidad, r^2 , LOD/LOQ) se describen en la Tabla 2.

Tabla 2. Parámetros de validación para el método de determinación cuantitativa de AFB1.

Matriz	Rango	R ²	Ecuación	LOD/LOQ (µg/L)
Harina de maíz	0.05-5 mg/L	0.9998	$y = \frac{516.44x}{4.7063}$	– 0.25/0.75
Pan	0.05-0.5 mg/L	0.9948	$y = 389.03 - 3.1217$	0.25/0.75
	0.05-5 mg/L	0.9999	$y = \frac{400.28x}{9.6478}$	– 0.25/0.75
Digerido gástrico	5-200 µg/L	0.9998	$y = \frac{0.7611x}{0.3598}$	– 0.25/0.75
	50-1500 µg/L	0.9996	$y = \frac{1134.4x}{14.942}$	– 0.25/0.75
Digerido intestinal	5-50 µg/L	0.9984	$y = \frac{0.8279x}{0.7944}$	– 0.25/0.75
	10-1500 µg/L	0.9994	$y = \frac{633.28x}{4.3939}$	– 0.25/0.75

LOD, límite de detección. LOQ, límite de cuantificación.

4.1.8. Estudio de bioaccesibilidad

Se calculó la bioaccesibilidad para las fases gástrica e intestinal. El cálculo de la bioaccesibilidad (%) de AFB1 se consideró como el porcentaje de micotoxinas del pan inicial que se detectó en las digestiones intestinales. La cantidad de micotoxina (µg) en 10 g de pan se obtuvo a partir de la concentración del pan (µg/kg) mediante factores de conversión ($\times 10/1000$). La cantidad de micotoxina (µg) en 100 mL de digerido se obtuvo a partir de la concentración del digerido (µg/L) mediante factores de conversión ($\times 100/1000$), como se puede ver en la siguiente fórmula.

$$\text{Bioaccesibilidad} = \text{concentración de digestión} \left(\frac{\mu\text{g}}{\text{L}} \right) \times \frac{1000}{\text{concentración de pan} \left(\frac{\mu\text{g}}{\text{kg}} \right)}$$

4.1.9. Cultivo celular

Las células de adenocarcinoma colorrectal humano, Caco-2 (ATCC HB-8065), se mantuvieron en DMEM suplementado con suero bovino fetal (FBS) al 10 %, estreptomicina 100 mg/mL y penicilina 100 U/ml. Para el cultivo de las células se utilizaron matraces de plástico de 75 cm² con tapón de rosca con filtro mantenidos en una atmósfera que contenía 5% de CO₂ a 95% de humedad relativa y 37 °C. Habitualmente se controlaba un número específico de subpases (de 15 a 20 pases) para mantener la homogeneidad genética. El medio de cultivo se cambió cada dos días. Las células T Jurkat (ATCC-TIB152) se mantuvieron en medio RPMI 1640 suplementado con FBS al 10 %, estreptomicina 100 mg/mL y penicilina 100 U/mL. Las condiciones de incubación fueron pH = 7,4, 37 °C bajo 5% de CO₂ y 95% de humedad constante. Habitualmente se controló un pequeño número de subpases (de 25 a 30 pases) para mantener la homogeneidad genética. El medio de cultivo se cambió cada dos días.

4.1.10. Ensayo de viabilidad celular

Las células Caco-2 se cultivaron en placas de cultivo de tejidos de 24 pocillos añadiendo 500 µL/pocillo de una suspensión de 2×10^6 células/mL. Se necesitaron doce placas, cuatro para cada grupo durante tres iteraciones cada una (24, 48 y 72 h). Cada 3 días se reemplazó el medio. Al cabo de 21 días, que es el tiempo necesario para que las células Caco-2 se diferencien, se realizó la exposición. La exposición consistió en diluciones en serie (no diluidas, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$ y $\frac{1}{32}$) de digestiones intestinales de E2, ya que contenía la concentración óptima de AFB1 necesaria para realizar los experimentos en contraste con E1, cuyos niveles de AFB1 eran muy bajos. Se probaron cuatro pocillos por condición. Para determinar la viabilidad de las células, se utilizó el ensayo de bromuro de 3-[4,5-dimetiltiazol-2-il]-2,5 difenil tetrazolio (MTT). Consiste en la reducción de la sal amarilla soluble de tetrazolio a un cristal insoluble de formazán violeta mediante una reacción independiente de las mitocondrias. Brevemente, después de los estudios de exposición con los digestos intestinales (24, 48 y 72 h), se eliminó el medio que contenía estos compuestos y las células de cada medio fresco bien recibido

que contenía MTT a 1 mg/mL. Las placas se envolvieron en papel de aluminio y se incubaron durante 4 h a 37 °C. Después de eso, se eliminó el medio que contenía MTT y la sal de formazán resultante se solubilizó en DMSO. La absorbancia se cuantificó a 620 nm utilizando un espectrómetro de absorción (Synergy™ H1, Berlín, Alemania).

4.1.11. Análisis del ciclo celular

Se sembró un total de 2×10^5 células T Jurkat/pocillo en placas de 6 pocillos y se incubaron utilizando los digestos intestinales diluidos al 10% en medio celular (C; G; A; A + G) durante 7 días (que representan AFB1 0,7 μ M). en los medios celulares de las exposiciones A y A+G), realizándose el experimento por cuadruplicado. La suspensión celular se vertió en un halcón de 15 mL y se centrifugó durante 5 minutos a 300 x g a temperatura ambiente. Se aspiró el sobrenadante, dejando aproximadamente 50 μ L de líquido residual en el tubo para evitar alterar el sedimento. Se siguió el protocolo del kit CycleTEST™ PLUS. Se añadió solución tampón (100 μ L) al sedimento celular y se resuspendió mediante agitación suave a baja velocidad. Se centrifugaron nuevamente durante 5 min a 300 x g a temperatura ambiente, repitiéndose este paso dos veces más. La tinción se realizó utilizando una solución de tinción de PI durante 10 minutos en el refrigerador en la oscuridad. Las muestras se analizaron mediante citometría de flujo con la configuración adecuada.

4.1.12. Análisis de la vía de apoptosis/necrosis

Las células se expusieron como se explica en la Sección 4.1.11. Las células se lavaron con 200 μ L de tampón de unión y se centrifugaron a 300 x g durante 10 min. El sobrenadante se aspiró completamente y el sedimento celular se resuspendió en 200 μ L de tampón de unión 1X. Siguiendo las instrucciones del fabricante, se agregaron a las células 10 μ L de anexina V-FITC. Las muestras se mezclaron y se incubaron durante 15 minutos en la oscuridad a temperatura ambiente. Después de centrifugar y aspirar completamente el sobrenadante nuevamente, el sedimento celular se resuspendió en 200 μ L de tampón de unión. Finalmente, se agregaron inmediatamente 5 μ L de solución de PI(100 μ g/mL) antes del análisis mediante citometría de flujo.

4.1.13. Análisis de ROS

Las células se expusieron como se explica en la Sección 4.1.11. La suspensión celular se centrifugó en un falcon de 15 mL (300 x g durante 5 min). Se añadió H₂DCFA (5 µM como concentración final) y las células se incubaron durante 20 minutos en la oscuridad a 37 °C. Los tubos se centrifugaron a 300 x g durante 5 minutos, se lavaron y se suspendieron en 300 µL de PBS. En este ensayo, se utilizó hidróperóxido de terbutilo (TBHP) (1 mM; 30 min) como control positivo.

4.1.14. Análisis de ROS mitocondriales

Las células se expusieron como se explica en la Sección 4.1.11. Las células se centrifugaron en un falcon de 15 mL (300 x g durante 5 min). Se añadió reactivo MitoSOX Red (1 mM como concentración final) en 500 µL de medio fresco y se incubó durante 20 minutos en la oscuridad a 37 °C. Los tubos se centrifugaron a 300 x g durante 5 minutos y se resuspendieron en 300 µL de PBS.

4.1.15. Análisis de masa mitocondrial

Las células se expusieron como se describe en la Sección 4.1.11, pero por triplicado. Se preparó una solución madre de la sonda MitoTracker utilizando DMSO, obteniendo una concentración de 100 µM. Los tubos se centrifugaron (300 x g durante 5 min) para obtener un sedimento celular y se aspiró el sobrenadante. Las células se resuspendieron suavemente en una solución de tinción precalentada (37 °C) que contenía MitoTracker® (100 nM) durante 20 minutos en la oscuridad a 37 °C. Una vez completada la tinción, las células se centrifugaron (300 x g durante 5 minutos) y se resuspendieron en 300 µL de PBS.

4.1.16. Configuración del citómetro

Todos los ensayos de citometría de flujo se llevaron a cabo utilizando el analizador MACSQuant 16 (Miltenyi Biotech GmbH, Bergisch Gladbach, Alemania). El número total de eventos por muestra fue de 20.000 en cada experimento. Se utilizaron láseres de color azul (488 nm), violeta (405 nm) y rojo (640 nm). Los resultados del ciclo celular se recogieron utilizando un filtro 579/34 PE B2. Los resultados de fluorescencia de Anexina V, MitoTracker™ y H2-DCFDA se recogieron utilizando un filtro FITC B1 525/50. Los resultados de fluorescencia de MitoSOX™ se recogieron utilizando un canal V4 con filtro 615/20.

4.1.17. Análisis estadístico de los datos

Los datos se expresaron como media $\pm$ DE de cuatro experimentos independientes. Mediante la prueba t de Student para muestras pareadas en Excel 2016 se realizó el análisis estadístico de los resultados. Se consideró estadísticamente significativo $p \leq 0,05$. La versión de cuantificación de Mac fue el software utilizado para los ensayos de citometría de flujo.

4.2 Trabajo 2: Micotoxinas emergentes y estrategias preventivas relacionadas con los cambios en la microbiota intestinal: probióticos, prebióticos y postbióticos: una revisión sistemática

4.2.1 Estrategia de búsqueda

El rigor científico de esta revisión sistemática y la minimización de posibles sesgos se garantizaron mediante el cumplimiento del protocolo de declaración de elementos de informe preferidos para revisiones sistemáticas y metanálisis (PRISMA) para la selección de títulos, resúmenes y textos completos (Page et al., 2021). Entre noviembre y diciembre de 2023 se realizó una búsqueda bibliográfica exhaustiva utilizando tres bases de datos (PubMed, Web of Science y Scopus). La búsqueda abarcó el período comprendido entre enero de 2012 y diciembre de 2023, con el objetivo de incluir datos recientes sobre micotoxinas emergentes en el contexto de la microbiota intestinal y los PPSP. Además, se consideraron otros términos relevantes, como “bacteria”, en combinación con “*in vitro*” o “*in vivo*” para capturar información adicional relevante.

La revisión sistemática se realizó utilizando las siguientes cadenas de búsqueda:

(1) Investigación refinada de Pubmed, Web of Science y Scopus. Período: todos los años.

(“Enniatinas” O “Enniatina A” O “Enniatina B” O “Enniatina A1” O “Beauvericina” O “ácido fusárico” O “Moniliformina” O “Fusarenon X” O “Aurofusarina” O “Fusaproliferina” O “Diacetoxiscirpenol” O “Éter monometílico de alternariol” O “Ácido tenuazónico” O “Altertoxina” O “Tentoxina” O “Citritina” O “Esterigmatocistina” O “Neosolaniol” O “Citreoviridina” O “Rugulosina” O “Fomopsina”) Y (“bacterias”) Y (“*in vitro*” O “*in vivo*”).

(2) Investigación refinada de Pubmed, Web of Science y Scopus. Período: 2012-2023

(“Enniatinas” O “Enniatina A” O “Enniatina B” O “Enniatina A1” O “Beauvericina” O “ácido fusárico” O “Moniliformina” O “Fusarenon X” O “Aurofusarina” O “Fusaproliferina” O “Diacetoxiscirpenol” O “Éter monometílico de alternariol” O “Ácido tenuazónico” O “Alttoxina” O “Tentoxina” O “Citritina” O “Esterigmatocistina” O “Neosolaniol” O “Citroviridina” O “Rugulosina” O “Fomopsina”) Y (“microbiota” O “prebióticos” O “probióticos” O “postbióticos”).

El criterio para incluir micotoxinas no reguladas en la lista se basó en Mihalache *et al.* (2023) y los informes emitidos por la Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) (AESAN, 2024). Es bien sabido que existen otros metabolitos no regulados que no se han considerado al realizar la investigación. No obstante, se suponía que el espectro cubierto sería suficiente para incluir todas las micotoxinas no reguladas.

4.2.2 Proceso de revisión sistemática

Entre los 811 informes encontrados a través de la investigación, 317 fueron recuperados de PubMed, 295 de Web of Science y 199 de Scopus. Después de un análisis inicial, se identificaron y eliminaron un total de 401 registros duplicados. Además, durante la selección de títulos y resúmenes, otros 381 registros fueron excluidos de la revisión sistemática por diferentes motivos: 124 artículos fueron descartados por centrarse únicamente en micotoxinas reguladas (AFB1, OTA, fumonisinas, etc.); 130 fueron rechazados porque incluían micotoxinas emergentes, pero no fueron evaluadas en combinación con los PPSP y 127 fueron descartados porque analizaron PPSP, pero no micotoxinas emergentes. Finalmente, un total de 29 se consideraron apropiados para su inclusión en la presente revisión sistemática, evaluados y clasificados en función de: la microbiota intestinal y los PPSP (Fig. 3). Los artículos seleccionados son clave para comprender más profundamente el papel de las micotoxinas emergentes en su combinación con la microbiota y los PPSP.

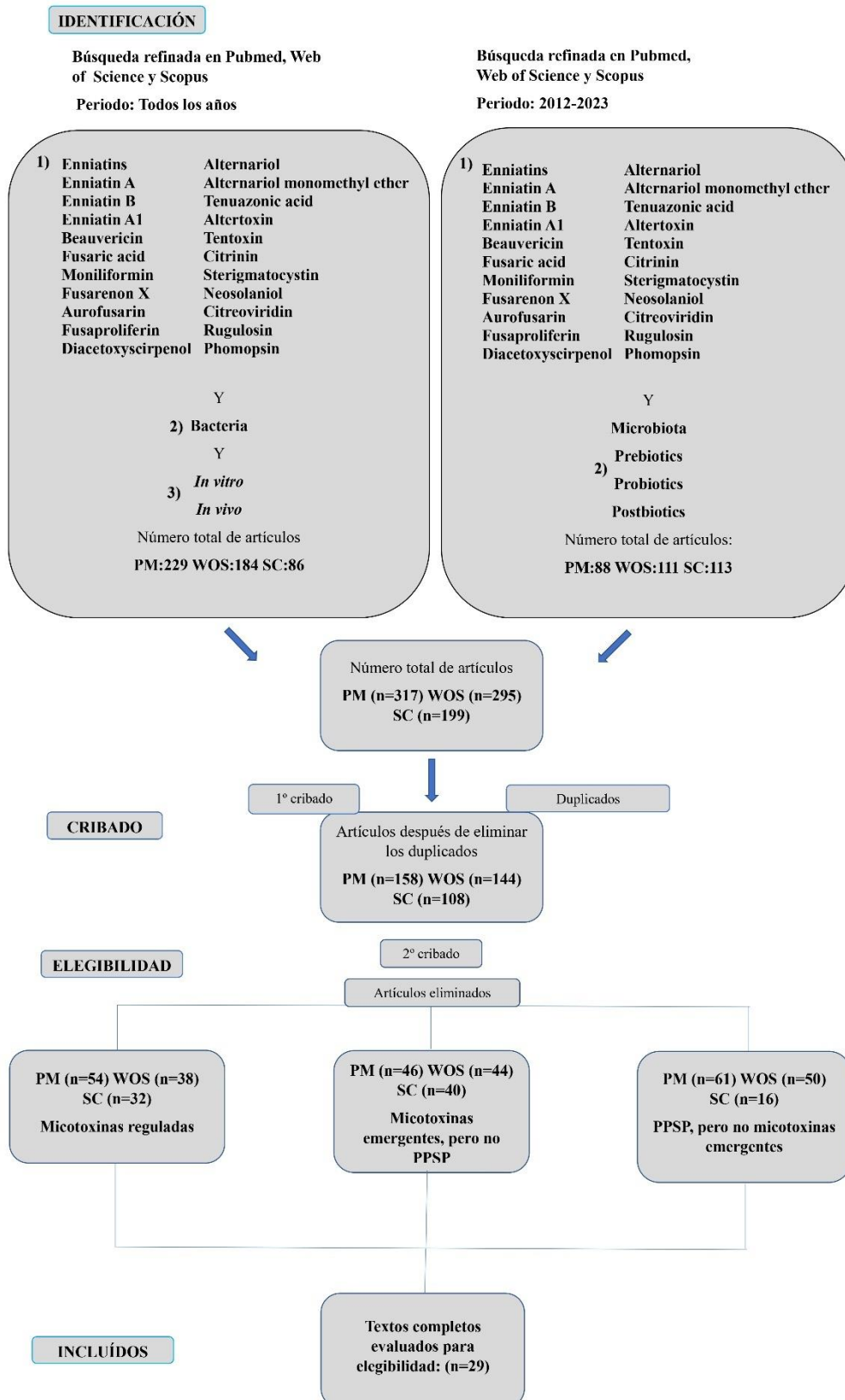


Figura 3. Representación gráfica del número total de artículos seleccionados a lo largo de las dos estrategias seguidas para la investigación bibliográfica utilizando las

combinaciones de palabras clave y el rango de años explicados. Número de muestras (n).
Pubmed – PM, Scopus – SC y Web of Science – WOS.

4.2.3 Conversión de datos

Todos los datos de los estudios analizados en esta revisión se convirtieron a $\mu\text{g kg}^{-1}$, mg kg^{-1} , ng mL^{-1} , $\mu\text{g mL}^{-1}$ o mg L^{-1} .

4.3 Trabajo 3: Efectos beneficiosos de la calabaza y el suero fermentado sobre el perfil de la microbiota fecal frente a la exposición a AFB1 y OTA en ratas Wistar

4.3.1. Diseño experimental y exposición a micotoxinas

Como previamente han descrito Vila-Donat *et al.* (2024), las ratas Wistar (n = 120) se dividieron aleatoriamente en doce grupos (10 ratas para cada uno, cinco hembras y cinco machos) y se expusieron por vía oral a doce dietas diferentes durante 28 días: 1) alimento control; 2) AFB1 (4,9 mg/kg), 3) OTA (6,0 mg/kg), 4) AFB1 (4,8 mg/kg) y OTA (6,4 mg/kg), 5) FW (1%) alimento, 6) FW (1%) y AFB1 (4,3 mg/kg), 7) FW (1%) y OTA (8,3 mg/kg), 8) FW (1%) con AFB1 (4,5 mg/kg) y OTA (7,5 mg/kg), 9) pienso FW (1%) y P (1%), 10) pienso FW (1%) y P (1%) y AFB1 (4,7 mg/kg), 11) Pienso FW (1%) y P (1%) y OTA (5,4 mg/kg) y 12) Pienso FW (1%) y P (1%), AFB1 (5,2 mg/kg) y OTA (8,8 mg/kg).

La elaboración de los panes se llevó a cabo en el laboratorio para preparar la dieta control y las demás dietas experimentales. Los ingredientes principales incluían harina de trigo, agua mineral, azúcar (sacarosa) y sal. Luego se prepararon alimentos contaminados y suplementados con modificaciones menores a la receta de control. Se recogieron muestras fecales semanalmente y cada animal se alojó individualmente en una jaula metabólica durante un período de 24 horas después de la exposición al alimento. Las muestras para este estudio se recolectaron específicamente durante la tercera semana del ensayo.

4.3.2 Extracción y purificación del ADN

La extracción del ADN se realizó utilizando 50 mg de heces de cada rata utilizando el kit ZymoBIOMICS DNA Miniprep (Zymo Research Corp., Irvine, CA, EE. UU.) de acuerdo con las instrucciones del fabricante. Para mejorar la eficiencia de la lisis celular, se utilizó la centrífuga Biospec Mini-BeadBeater-96 (BioSpec Products, Bartlesville, OK, EE. UU.) durante 1 min a velocidad máxima (2400 rpm) y 5 min de

reposo en hielo, repitiendo el ciclo 5 veces. La pureza y la cantidad de ADN se evaluaron espectrofotométricamente a través de un espectrofotómetro Nano UV/vis NeoDot (Neo Biotech, Nanterre, Francia) que mostró concentraciones entre 39,1 ng/μg y 163,2 ng/μg junto con proporciones de 260/280 nm y 260/230 nm alrededor. Un total de 103 muestras cumplieron con los requisitos de calidad y por lo tanto fueron secuenciadas (Tabla 1).

Tabla 1. Número de muestras de heces (n) secuenciadas de cada grupo y sexo (n=103). AFB1, aflatoxina B1. FW, suero fermentado. OTA, ocratoxina A. P, calabaza.

Grupo	Ratas macho (n)	Ratas hembra (n)
Control	5	5
AFB1	5	5
OTA	4	4
AFB1+OTA	5	5
FW	5	5
FW+AFB1	5	4
FW+OTA	2	1
FW+AFB1+OTA	5	5
FW+P	4	3
FW+P+AFB1	4	5
FW+P+OTA	4	3
FW+P+AFB1+OTA	5	5

4.3.3 Secuenciación del gen 16S rRNA

Las regiones variables V3 y V4 del gen 16S rDNA se amplificaron siguiendo el protocolo Illumina de preparación de la biblioteca de secuenciación metagenómica del gen 16S rDNA (Cod. 15044223 Rev. A). Los cebadores específicos de genes (PCR1_f: 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCA G3';

PCR1_r:5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGG TATCTAATCC-3') que contienen secuencias de nucleótidos sobresalientes del adaptador

Illumina se seleccionaron de acuerdo con Klindworth *et al.* (2013). Después de la amplificación del gen 16S rDNA, el paso de multiplexación se realizó utilizando el kit Nextera XT Index. Se ejecutó 1 µL del producto de PCR en un chip Bioanalyzer DNA 1000 (Agilent, Santa Clara, California, EE. UU.) para verificar el tamaño de los amplicones (~550 pb). Después de la verificación del tamaño, las bibliotecas se secuenciaron utilizando un experimento de extremo emparejado de 2 x 30¹ pb (kit de reactivos MiSeq v3) en un secuenciador MiSeq según las instrucciones del fabricante (Illumina, San Diego, California, EE. UU.).

4.3.4 Análisis bioinformático

Se utilizó el paquete de análisis de microbioma QIIME 2 (versión 2024.5) para analizar los datos demultiplexados proporcionados por la Unidad de Genómica (Bolyen *et al.*, 2019). Para el control de calidad de la secuencia y la construcción de tablas de características, se aplicó el complemento Dada2 (Callahan *et al.*, 2016). El programa Dada2 filtró secuencias ruidosas, corrigió errores en secuencias marginales, eliminó secuencias quiméricas, eliminó singletes, unió lecturas de extremos emparejados sin ruido y eliminó esas secuencias. El producto final es una tabla de variantes de secuencia de amplicones (ASV), un análogo de mayor resolución de la tabla de unidades taxonómicas operativas (OTU) tradicionales, que registra el número de veces que se observó cada variante exacta de la secuencia de amplicones en cada muestra. Los análisis de metacódigos de barras OTU y ASV arrojan resultados similares con respecto al examen de la diversidad y funcionalidad microbiana; el análisis basado en ASV puede capturar una diversidad microbiana intestinal ligeramente más detallada en comparación con el análisis basado en OTU (Inglis *et al.*, 2022). Los parámetros de recorte izquierdo fueron 0 bases hacia adelante y 10 hacia atrás. La longitud de truncamiento elegida fue 290 bases hacia adelante y 220 hacia atrás.

El siguiente paso fue la generación de un árbol para análisis de diversidad filogenética (PD), utilizando el canal *align-to-tree-mafft-fasttree* del complemento *q2-phylogeny* (Price *et al.* 2010). Los análisis de diversidad de QIIME 2 están disponibles a través del complemento *q2-diversity*, que admite el cálculo de métricas de diversidad alfa y beta, la aplicación de pruebas estadísticas relacionadas (Kruskal-Wallis) y la generación

de visualizaciones interactivas. La profundidad de muestreo elegida fue 18520, el valor más alto posible para retener más secuencias por muestra, excluyendo la menor cantidad de muestras posible. La diversidad α se muestra en el gráfico PD Faith, una medida cualitativa de la riqueza de la comunidad que incorpora relaciones filogenéticas entre los ASV (Faith, 1992). La diversidad β está representada por la trama Bray-Curtis y Jaccard Distance 3D Emperor, para medir la disimilitud de la comunidad.

El gráfico de rarefacción alfa fue producido por el visualizador de rarefacción alfa QUIIME diversity. Se utilizó para determinar si la riqueza de las muestras se había observado o secuenciado completamente. Si las líneas en el gráfico parecen acercarse a una pendiente de cero en cierta profundidad de muestreo a lo largo del eje x, eso sugiere que la recopilación de secuencias adicionales más allá de esa profundidad de muestreo probablemente no resulte en la observación de ASV adicionales.

Para explorar la composición taxonómica de las muestras, se entrenó un clasificador de características con q2-feature-classifier (Bokulich et al., 2018). Los clasificadores taxonómicos funcionan mejor cuando se entrenan en función de la preparación de muestras específicas y los parámetros de secuenciación, incluidos los cebadores que se utilizaron para la amplificación y la longitud de las lecturas de la secuencia. La secuencia de Greengenes2 utilizada fue 2022.10.backbone.full-length.fna.qza (McDonald et al., 2024). La secuencia del cebador directo aplicada fue 5'-GTCGGCAGCGTCAGATGTGTATAA -3' y la inversa, 5'-GGACTACHVGGGTATCTAAT 3'. La longitud mínima fue de 150 y la máxima de 300.

4.3.5 Análisis estadístico

El análisis y las gráficas de abundancia diferencial taxonómica (comparaciones entre grupos) se llevaron a cabo mediante análisis univariado clásico utilizando la prueba de Kruskal-Wallis mediante IBM SPSS Statistics 29.0.2.0 (IBM Corp, Armonk, NY, EE. UU.). Se desarrollaron ecuaciones de Bray-Curtis para el estudio de la diversidad β . Se probó la normalidad de todos los datos mediante la prueba de Shapiro-Wilk y posteriormente se analizaron mediante los métodos estadísticos apropiados. Un valor de $p < 0,05$ se consideró estadísticamente significativo.



5. TRABAJOS PUBLICADOS

5.1 Artículo 1: Efecto del ajo *Allium sativum* L. var. Voghiera sobre la bioaccesibilidad y citotoxicidad de la aflatoxina B1 *in vitro*

Foods

Allium sativum* L. var. Voghiera Reduces Aflatoxin B1 Bioaccessibility and Cytotoxicity *In Vitro

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Abstract

The present work focuses on the evaluation of AFB1's bioaccessibility and cytotoxicity *in vitro* using bread (naturally contaminated) enriched or not enriched with fresh Voghiera garlic (2%). Two different experiments were carried out: experiment 1 (E1), with low-AFB1-concentration breads (1.6–1.7 mg/kg); and experiment 2 (E2), with high-AFB1-concentration breads (96.4–102.7 mg/kg). Eight breads were prepared, four for E1 (experiment 1) and another four for E2 (experiment 2), with each experiment having a control group (C), a garlic-enriched group (2%) (G), an AFB1 group (A), and an AFB1 + garlic group (A + G). Simulated digestion was performed on each type of bread, and gastric and intestinal digests were obtained. AFB1 content in flours, baked bread, and gastric and intestinal digests was measured by High-Performance Liquid Chromatography coupled to Fluorescence Detection. The results demonstrate dose-dependent AFB1 bioaccessibility and that the presence of garlic contributed to its reduction in both doses (7–8%). Moreover, garlic's presence in AFB1-contaminated bread increased cell viability (9–18%) in differentiated Caco-2 cells and mitigated the arrest of S and G2/M phases provoked by AFB1 on Jurkat T cells and reduced apoptosis/necrosis, cellular reactive oxygen species (ROS), and mitochondrial ROS by 16%, 71%, and 24% respectively. The inclusion of garlic as a functional ingredient helped relieve the presence and effects of AFB1.

Keywords: mycotoxin, garlic, prebiotic, Jurkat T cells, *in vitro* digestion, flow cytometr

1. Introduction

Aflatoxins (AFs) are secondary metabolites produced by mycotoxigenic fungi belonging to *Aspergillus* genera which contaminate a wide variety of crops, such as cereals, oilseeds, tree nuts, and spices. Among them, aflatoxin B1 (AFB1) is the most studied due to its severe carcinogenicity for humans and animals [1]. In fact, the International Agency for Research on Cancer classified AFB1 as a human carcinogen belonging to Group 1 due to its ability to form DNA adducts [2]. Different *in vitro* and *in vivo* studies have demonstrated its acute and chronic toxicity, which can lead to the onset of liver damage, nutritional dysfunctions, immunosuppression, and cancer [3,4,5].

As mycotoxins are major contaminants of cereals, it has been found that AFB1 contaminates feeds universally [6]. It has been shown that in 87.5% of samples found to contain AFB1, its level was higher than the allowed level, which was 2 µg/kg from 2006 to 2023 [7]. Despite treatments applied during the production process, the natural occurrence of this mycotoxin has been observed in commercial bread loaves [8], as well as in maize and in wheat bread [9,10]. Consequently, to enhance AFB1 reduction in bakery products, the use of bio-preservatives in their formulation could be a good alternative to conventional procedures [11]. Following this trend, the addition of functional ingredients from various food matrices rich in bioactive compounds has been previously investigated to assess AFB1 bioaccessibility reduction through simulated gastrointestinal digestion models [12, 13,14,15,16]. Likewise, the cytoprotective effect of some natural compounds against mycotoxin was demonstrated by using different cell models [17,18].

Among others, garlic plant (*Allium sativum* L.) has shown to exert multiple health benefits such as prevention and treatment of cardiovascular diseases, antioxidant activity, and antimicrobial effects [19] The variety “Voghiera” is a typical Italian ecotype of garlic cultivated in a particular area of north-east of Voghiera (Ferrara)

in Emilia-Romagna region and classified as a Protected Designation of Origin (PDO) product. Its phytochemical analysis has reported the isolation of diverse saponins such as furostanol, voghierosides (A1/A2-E1/E2), spirostanol and eugenol diglycosides, which possess important antimicrobial activities [20]. In addition, its role in mitigate the evolution of cancer progression was recently investigated [21].

Thus, the aim of this study was to evaluate the possible beneficial effect of Voghiera garlic on bread in terms of modifying AFB1's bioaccessibility and cytotoxicity by using Caco-2 cells, which are widely used as a prototype of the intestinal epithelial barrier [22], and Jurkat T human cells, which are also broadly used in *in vitro* studies of T cytokines, receptor expression, and cell signal transduction lines [23].

1. Materials and Methods

2.1. Reagents

AFB1 standard solution (purity > 99%) was acquired from Sigma-Aldrich (St. Louis, MO, USA). Potassium thiocyanate (KSCN), potassium chloride (KCl), hydrochloric acid (HCl), sodium dihydrogen phosphate (NaH₂PO₄), sodium hydroxide (NaOH), sodium sulfate (Na₂SO₄), sodium chloride (NaCl), sodium hydrogen carbonate (Na-HCO₃), urea (CO(NH₂)₂), pepsin A (674 U mg⁻¹ P7000), pancreatin (762 U mg⁻¹ P1750), bile salts (B8631), and α -amylase (930 U mg⁻¹ A3403) were purchased from Sigma-Aldrich (Madrid, Spain). Acetonitrile (ACN) and methanol (MeOH) were obtained from Fisher Scientific (Madrid, Spain). Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Dimethyl sulfoxide (DMSO) was acquired from Fisher Scientific (Geel, Belgium). Bread ingredients were purchased at a local supermarket. Fresh garlic was preserved at 4 °C and minced before use. Propidium iodide (PI) and a cycleTEST™ PLUS DNA Reagent Kit were obtained from BD Biosciences (San Diego, CA, USA) for the cell cycle assay by flow cytometry.

The Annexin V–FITC conjugate was acquired from Miltenyi and the MitoTracker® probe was purchased from Invitrogen (Waltham, MA, USA). 2',7'-dichlorodihydrofluorescein diacetate (H2-DCFDA) and phosphate buffer saline (PBS) were bought from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Maize Flour Contamination and Mycotoxin Production

Maize was contaminated by *A. flavus* ITEM 8111 acquired from the Agro-Food Micro-bial Culture Collection of the Institute of Sciences and Food Production (ISPA, Bari, Italy). The fungus was kept under ideal conditions in the laboratory to produce the mycotoxin in the food matrix (maize) in which it was inoculated. Under these conditions, the strain used only generated this mycotoxin and not any others. A total of 450 g of maize was introduced per 1 L previously sterilized jar, and two jars were prepared. The contamination was carried out by adding 20 mL of mycelium suspension and spores in peptone water with Tween 80 of the fungal strain. Glass jars were maintained at room temperature for a period of three weeks. Every 2–3 days, 1 mL of distilled water was added to the jars to maintain the humidity conditions.

After three weeks, the jars were autoclaved to eliminate fungal contamination and the maize was transformed into flour until total homogenization was achieved, including sieving. The maize samples were kept at –20 °C [12]. The maize flour was analyzed via High-Performance Liquid Chromatography coupled to Fluorescence Detection (HPLC-FLD) (as reported in Section 2.6) to quantify AFB1 and discard any other mycotoxins.

2.3. Bread Ingredients, Bread Making, and Baking

Eight breads were made combining the bioactive ingredient and the AFB1, four corresponding to experiment 1 (E1) and another four corresponding to experiment 2 (E2): a control bread (C), 2% garlic bread (G), AFB1 bread (A), and AFB1 + 2% garlic bread (A + G). Control bread was prepared using the following recipe: 127 g of wheat flour, 66 mL of mineral water (37 °C), 8 g of yeast for bakery products (Levital, Sant Hilari Sacalm,

Spain), 4 g of sugar, and 2.6 g of NaCl. After merging all the ingredients, homogenization was conducted in a SilverCrest Bread Maker SBB 850 A1 (Kompernass GMBH, Bochum, Germany) for 5 min, and they were shaped in molds (approximately 200 g), covered using a damp cloth, and fermented for 1 h at room temperature. Afterwards, the breads were covered with silver foil and baked at 180 °C for 35 min in a Memmert ULE 500 muffle furnace (Madrid, Spain). Then, the breads were unmolded and refrigerated at room temperature for 1 h. Enriched breads were prepared by slightly modifying the control recipe to produce 2% garlic bread (G and A + G). Finally, breads contaminated with AFB1 were prepared by replacing some of the wheat flour with 5.9 g (E1~152 mg/kg) and 54.7 g (E2~197 mg/kg) of maize flour contaminated with AFB1 to obtain low-concentration and high-concentration breads, respectively. This difference in AFB1 concentration was used to determine if it played an important role in bioaccessibility. As shown in Table 1, eight breads were obtained in total.

Table 1. Bread composition in experiments 1 and 2.

Ingredient	C (g)		G (g)		A (g)		A + G (g)	
	E1	E2	E1	E2	E1	E2	E1	E2
Wheat flour	127	127	124	124	121.1	72.3	118.1	69.3
NC maize flour	-	-	-	-	5.9	54.7	5.9	54.7
Water	66	66	65	65	66	66	65	65
Salt	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6
Sugar	4	4	4	4	4	4	4	4
Fresh yeast	8	8	8	8	8	8	8	8
Garlic	-	-	4	4	-	-	4	4
Total quantity	207.6	207.6	207.6	207.6	207.6	207.6	207.6	207.6

C (control). E1 (experiment 1). E2 (experiment 2). (G) garlic. A (Aflatoxin B1). A + G (Aflatoxin B1 + garlic). C (contaminated). - (no content) (n = 3).

2.4. *In Vitro* Static Digestion Model

The digestion process consisted of three phases: (oral, gastric, and intestinal), as re-reported in previous work [12]. In short, 10 g of bread was put in sterilized plastic bags (500 mL) and mixed with Milli-Q water (at 37 °C) and 6 mL of artificial saliva (Millipore, Bedford, MA, USA). To reproduce the oral phase, an IUL Stomacher (IUL S.A,

Barcelona, Spain) was used for 30 s to simulate the mastication process. Saliva was prepared the day before (and adjusted to pH = 6.8) by mixing 0.17 mL of NaCl (175.3 g/L), 1 mL of KSCN (20 g/L), 1 mL of NaH₂PO₄ (88.8 g/L), 2 mL of NaHCO₃ (84.7 g/L), 1 mL of KCl (89.6 g/L), 1 mL of Na₂SO₄ (57 g/L), 29 mg of α -amylase, 0.8 mL of urea (25 g/L), and distilled water to achieve a volume of 100 mL. Once the oral phase was simulated, the content was transferred to a topaz Erlenmeyer flask to carry out the gastric phase. The mixture was acidified to pH = 2 with 6 N HCl solution. Then, 14 mL of Milli-Q water (at 37 °C) and 0.5 g of pepsin solution (1 g in 25 mL of 0.1 N HCl) were added to achieve 100 mL. Samples were incubated for 2 h at 37 °C under darkness and slight agitation (1 g) using an orbital shaker (Infors AG CH-4103, Bottmingen, Switzerland). Following that, gastric aliquots (15 mL) were kept for further analysis and pancreatic digestion was conducted by adding 1.25 g of a bile salt/pancreatin mixture (0.625 g of bile salts and of 0.1 g of pancreatin dissolved in 25 mL of 0.1 N NaHCO₃) at pH = 6.5 (0.5 N NaHCO₃). Digests were incubated as formerly detailed (2 h at 37 °C in darkness and slight agitation), the pH was finally adjusted to 7.2 (0.5 N NaOH), the samples were centrifuged (226× g for 10 min at 4 °C), and the supernatant was gathered to obtain the intestinal digests. The experiments were performed in triplicate and quadruplicate.

2.5. AFB1 Extraction from Bread and Digest Samples

A total of 5 g of maize/wheat/bread flour was placed in centrifuge tubes (50 mL). Then, 25 mL of MeOH/H₂O (80/20) was added. Ultraturrax (T 18 digital ULTRATURRAX®, Staufen, Germany) was used for 3 min to crush the samples after being centrifuged at 226× g for 5 min (Centrifuge 5810R, Eppendorf, Germany). The supernatant was collected and filtered through a 0.22 μ m filter (Phenomenex, Madrid, Spain). All analyses were performed in triplicate (n = 3). Gastric and intestinal digest samples were centrifuged at 226× g for 10 min at 4 °C, filtered (0.22 μ m filter) (Phenomenex, Madrid, Spain), diluted with MeOH (1:1), and injected in HPLC-FLD.

2.6. Quantitative Determination of AFB1 by HPLC-FLD

AFB1 was characterized using HPLC with an Agilent 1100 series (Agilent Technologies, Santa Clara, CA, USA) furnished with an automatic sampler, a degasser, a quaternary pump, and an FLD detector Agilent 1200 (Agilent Technologies, Santa Clara, CA, USA), and Agilent Software JP03924119 was used for data analysis.

A derivatization system (UVETM Photochemical reactor, LCTech, Jasco Analitica, Madrid, Spain, S.L.) was placed between the analytical column and the FLD detector to improve the fluorescent activity of AFB1. The chromatographic separation was carried out by using a reverse phase column, 00F-4633-EO Kinetex EVO C18 (150 × 4.6 mm, 100 Å, and particle size of 5 µm) (Phenomenex, Palo Alto, CA, USA) at 40 °C. The isocratic mobile phase was composed of H₂O/ACN/MeOH (60:10:30 v/v/v) with a flow rate of 1 mL min⁻¹. The injection volume was 20 µL for flour and breads and 40 µL for gastrointestinal digests. The AFB1 excitation and emission wavelengths were $\lambda_{ex} = 365$ nm and $\lambda_{em} = 440$ nm.

2.7. Quantitative Determination Method Validation

Linearity was assessed by means of the coefficient of regression (r^2) of calibration matrix-matched curves. The limit of detection (LOD) and limit of quantification (LOQ) were measured through a comparison of the chromatographic signals from samples containing low concentrations of the compound to those of blank samples and establishing the minimum concentration at which the compound can be accurately detected and measured, respectively. Signal-to-noise ratios of 10:1 and 3:1 was considered to obtain the LOD and LOQ, respectively. Repeatability and intermediate precision were quantified as the relative standard deviation of four analyses performed on four different days ($n = 4$) and four analyses performed on the same day ($n = 4$), respectively.

Flour and bread matrix-matched curves were prepared by fortifying, with an AFB1 standard (100 mg/L in MeOH), the extracted solution from the blank (bread with uncontaminated wheat flour) with different AFB1 concentrations to obtain at least 6 level

point curves. Validation parameters for the AFB1 quantitative determination method (linear regression equation, linearity range, r^2 , LOD/LOQ) are described in Table 2.

Table 2. Validation parameters for AFB1 quantitative determination method. LOD, limit of detection. LOQ, limit of quantification.

Matrices	Linearity range	R^2	Linear regression equation	LOD/LOQ ($\mu\text{g/L}$)
Maize flour	0.05-5 mg/L	0.9998	$y = 516.44x - 4.7063$	0.25/0.75
Bread	0.05-0.5 mg/L	0.9948	$y = 389.03 - 3.1217$	0.25/0.75
	0.05-5 mg/L	0.9999	$y = 400.28x - 9.6478$	0.25/0.75
Gastric digest	5-200 $\mu\text{g/L}$	0.9998	$y = 0.7611x - 0.3598$	0.25/0.75
	50-1500 $\mu\text{g/L}$	0.9996	$y = 1134.4x - 14.942$	0.25/0.75
Intestinal digest	5-50 $\mu\text{g/L}$	0.9984	$y = 0.8279x - 0.7944$	0.25/0.75
	10-1500 $\mu\text{g/L}$	0.9994	$y = 633.28x - 4.3939$	0.25/0.75

2.8. Bioaccessibility Study

Bioaccessibility was calculated for the gastric and intestinal phases. The calculation of AFB1 bioaccessibility (%) was considered as the percentage of mycotoxin from the initial bread that was detected in the intestinal digests. The quantity of mycotoxin (μg) in 10 g of bread was obtained from bread concentration ($\mu\text{g/kg}$) by conversion factors ($\times 10/1000$). The quantity of mycotoxin (μg) in 100 mL of digest was obtained from digest concentration ($\mu\text{g/L}$) by conversion factors ($\times 100/1000$), as can be seen in the formula below.

$$\text{Bioaccessibility} = \text{digest concentration } (\mu\text{g/L}) \times 1000 / \text{bread concentration } (\mu\text{g/kg})$$

2.9. Cell Culture

Human colorectal adenocarcinoma cells, Caco-2 (ATCC HB-8065), were kept in DMEM supplemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin, and 100 U/mL penicillin. To culture cells, 75 cm² plastic flasks with filter screw caps kept in a in an atmosphere containing 5% CO₂ at 95% relative humidity and 37 °C were used. A specific number of sub-passages (from 15 to 20 passages) were habitually controlled to maintain genetic homogeneity. The culture medium was changed every two days.

Jurkat T cells (ATCC-TIB152) were kept in RPMI 1640 medium supplemented with 10% FBS, 100 mg/mL streptomycin, and 100 U/mL penicillin. The conditions of incubation were pH = 7.4, 37 °C under 5% CO₂, and 95% constant humidity. A small number of sub-passages (from 25 to 30 passages) were habitually controlled to maintain genetic homogeneity. The culture medium was changed every two days.

2.10. Cell Viability Assay

Caco-2 cells were cultured in 24-well tissue culture plates by adding 500 µL/well of a suspension of 2×10^6 cells/mL. Twelve plates were needed, four for each group for three iterations each (24, 48, and 72 h). Every 3 days, the medium was replaced. After 21 days, which is the time necessary for Caco-2 cells to differentiate, the exposure was carried out. The exposure consisted of serial dilutions (non-diluted, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, and $\frac{1}{32}$) of intestinal digests from E2, since it contained the optimal AFB1 concentration needed to conduct the experiments in contrast to E1, whose AFB1 levels were very low. Four wells per condition were tested.

To determine the viability of cells, the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was used. This consists of the reduction of yellow soluble tetrazolium salt to an insoluble purple formazan crystal through a reaction independent of the mitochondria. Briefly, after exposure studies with the intestinal digests (24, 48, and 72 h), the medium containing these compounds was eliminated, and the cells of each well-received fresh medium containing MTT at 1 mg/mL. The plates were wrapped in foil and incubated for 4 h at 37 °C. After that, the medium containing the

MTT was removed, and the resulting formazan salt was solubilized in DMSO. The absorbance was quantified at 620 nm using an absorption spectrometer (Synergy™ H1, Berlin, Germany).

2.11. Cell Cycle Analysis

A total of 2×10^5 Jurkat T cells/well was seeded in 6-well plates and incubated using the intestinal digests diluted to 10% in cell media (C; G; A; A + G) for 7 days (representing 0.7 μ M AFB1 in the cell media of A and A + G exposures), with the experiment carried out in quadruplicate. The cell suspension was poured into a 15 mL falcon and centrifuged for 5 min at $300\times$ g at room temperature. The supernatant was aspirated, leaving about 50 μ L of residual fluid in the tube to avoid disturbing the pellet. The CycleTEST™ PLUS kit protocol was followed. Buffer solution (100 μ L) was added to the cell pellet and resuspended by gentle vortexing at low speed. They were again centrifuged for 5 min at $300\times$ g at room temperature, and this step was repeated two more times. Staining was conducted by using PI stain solution for 10 min in the refrigerator in the dark. Samples were analyzed by flow cytometry with the appropriate settings.

2.12. Apoptosis/Necrosis Pathway Analysis

Cells were exposed as explained in Section 2.11. Cells were washed with 200 μ L of binding buffer and centrifuged at $300\times$ g for 10 min. The supernatant was completely aspirated, and the cell pellet was resuspended in 200 μ L of $1\times$ binding buffer. Following the fabricant instructions, 10 μ L of Annexin V–FITC was added to the cells. The samples were mixed and incubated for 15 min in the dark at room temperature. After centrifuging and aspirating the supernatant completely again, the cell pellet was resuspended in 200 μ L of binding buffer. Finally, 5 μ L of PI (100 μ g/mL) solution was immediately added prior to analysis by flow cytometry.

2.13. ROS Analysis

Cells were exposed as explained in Section 2.11. The cell suspension was centrifuged in a 15 mL falcon (300× g for 5 min). H₂DCFDA (5 μM as the final concentration) was added, and cells were incubated for 20 min in the dark at 37 °C. The tubes were centrifuged at 300× g for 5 min, washed, and suspended in 300 μL of PBS. In this assay, tert-Butyl hydroperoxide (TBHP) (1 mM; 30 min) was used as the positive control.

2.14. Mitochondrial ROS Analysis

Cells were exposed as explained in Section 2.11. Cells were centrifuged in a 15 mL falcon (300× g for 5 min). MitoSOX Red reagent was added (1 mM as the final concentration) in 500 μL of fresh medium and incubated for 20 min in the dark at 37 °C. The tubes were centrifuged at 300× g for 5 min and resuspended in 300 μL of PBS.

2.15. Mitochondrial Mass Analysis

Cells were exposed as described in Section 2.11, but in triplicate. A stock solution of MitoTracker probe was prepared by using DMSO, obtaining a concentration of 100 μM. The tubes were centrifuged (300× g for 5 min) to obtain a cell pellet and the supernatant was aspirated. The cells were resuspended gently in prewarmed (37 °C) staining solution containing the MitoTracker® (100 nM) for 20 min in the dark at 37 °C. After staining was completed, cells were centrifuged (300× g for 5 min) and resuspended in 300 μL of PBS.

2.16. Cytometer Settings

All flow cytometry assays were carried out using the MACSQuant 16 analyzer (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). The total number of events per sample was 20,000 in each experiment. Blue (488 nm), violet (405 nm), and red (640 nm) lasers were used. Cell cycle results were collected using a 579/34 PE B2 filter. The fluorescence results of Annexin V, MitoTrackerTM, and H2-DCFDA were collected using a 525/50 FITC B1 filter. Fluorescence results of MitoSOXTM were collected using a V4 channel with 615/20 filter.

2.17. Statistical Analysis of the Data

Data were expressed as mean $\pm$ SD of four independent experiments. By using Student's t-test for paired samples in Excel 2016, statistical analysis of the results was performed. $p \leq 0.05$ was considered statistically significant. Macs quantify version was the software used for flow cytometry assays.

3. Results

3.1. AFB1 Bioaccessibility

The data regarding the matrix-matched curves, linearity range, regression coefficients (r^2), equations, and limits of detection and quantification obtained in HPLC-FLD for the maize flours, bread, and simulated digests are shown in Table 2.

The concentration of AFB1 in naturally contaminated maize flour was 151.9 ± 1.4 mg/kg for E1 and 197.1 ± 3.8 mg/kg for E2. After baking, the AFB1 concentrations obtained for breads in E1 were 1.6 ± 0.1 mg/kg and 1.7 ± 0.1 mg/kg for A and A + G,

respectively. In E2, the AFB1 concentrations of breads were 96.4 ± 9.6 mg/kg and 102.7 ± 4.4 mg/kg for A and A + G, respectively. Neither bread C or G showed AF content in E1 or in E2. The AFB1 concentration in bread was not measured before baking since the flours used as ingredients for making the bread had already been analyzed (Section 2.5 and Section 2.6). Regarding the gastric digests, the mean AFB1 concentrations were 0.176 ± 0.005 μ M (A) and 0.191 ± 0.005 μ M (A + G) for E1 and 3.934 ± 0.823 μ M (A) and 3.861 ± 0.006 μ M (A + G) for E2. In the intestinal digests, the AFB1 concentrations were 0.181 ± 0.002 μ M and 0.162 ± 0.001 μ M in E1 and 8.621 ± 0.021 μ M and 7.742 ± 0.031 μ M in E2 for A and A + G, respectively.

AFB1 bioaccessibility ranged from 29% to 39% in E1 and from 14% to 30% in E2 (Figure 1). Regarding E1, AFB1 bioaccessibility was $39 \pm 3\%$ (A) and $38 \pm 1\%$ (A + G) for the gastric digest and $37 \pm 1\%$ (A) and $29 \pm 1\%$ (A + G) for the intestinal digest. In E2, the results were $16 \pm 1\%$ (A) and $14 \pm 1\%$ (A + G) for the gastric digest and $30 \pm 1\%$ (A) and $23 \pm 1\%$ (A + G) for the intestinal digest.

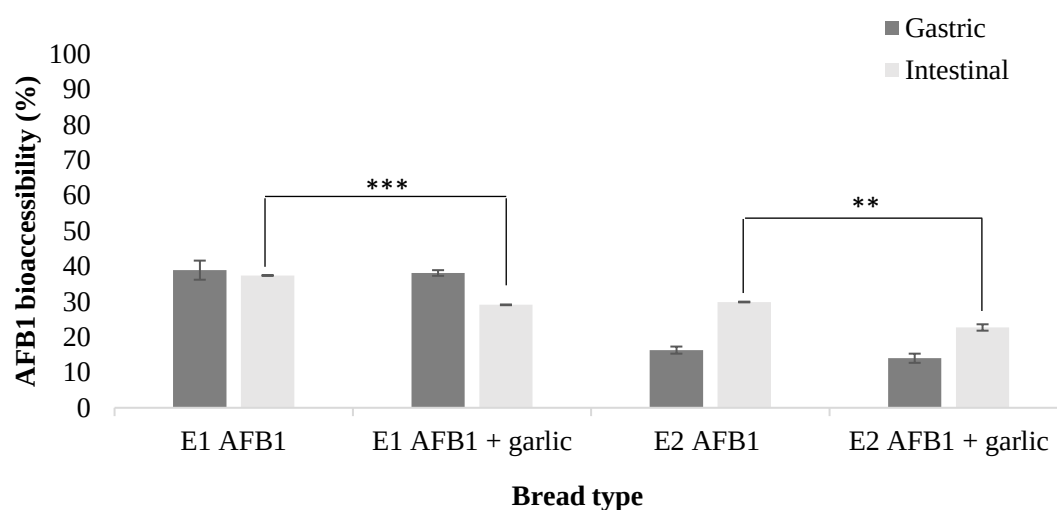


Figure 1. Bioaccessibility comparison referred to experiment 1 and 2. Significant differences are indicated as, $p < 0.01$ (**), $p < 0.001$ (***). E1, experiment 1. E2, experiment 2.

3.2 Cell viability

In order to evaluate the cell viability, the MTT assay was performed. Control cells exposed to control bread intestinal digests were considered as 100% viability for calculations. No differences were observed between non-exposed cells and control cells. The addition of garlic (A+G) in contrast to the AFB1 intestinal digest (A) contributed to increase significantly the cell viability at 24 h using non diluted digests and at 48 h (10-13%) and 72 h (11-18%) in all dilutions tested. Table 3 collects the cell viability percentages when exposure to both A+G and A intestinal digests, respectively.

Table 3. Cell viability of AFB1 and AFB1 + garlic exposures at different times and dilutions. Cell viability (%) results obtained in differentiated Caco-2 cells after exposure to AFB1 and AFB1-garlic bread intestinal digests.

		Cell viability (%)				
Exposure time		24 h		48 h		72 h
Intestinal digest		<i>AFB1</i>	<i>AFB1-garlic</i>	<i>AFB1</i>	<i>AFB1-garlic</i>	<i>AFB1</i>
<i>1/32</i>		79±4	86±2	74±4	86±4*	73±1
<i>1/16</i>		76±5	84±6	72±5	85±4*	72±9
<i>1/8</i>		76±7	81±3	72±1	83±5**	66±2
<i>1/4</i>		76±3	80±2	69±4	80±3*	64±8
<i>1/2</i>		73±6	79±7	68±4	78±4**	63±6
<i>No dilution</i>		70±4	79±6*	66±5	77±4**	62±4

p≤0.05 (*). p≤0.01 (**). p≤0.001 (***).

3.3 Flow cytometry analysis

3.3.1 Cell cycle

To evaluate the impact of the different digests bread in Jurkat cell cycle, the PI dye was used. As Figure 2 indicates, cell cycle analysis was performed for every condition at the different phases. For the four conditions (C, G, A, A+G) at every cell phase, the results were the next, respectively: 19, 19, 16 and 20% for the Sub-G0/G1 phase; 46, 46, 47 and 47% for G0/G1; 20, 19, 23 and 19% for S and 13, 12, 13 and 12% for G2/M.

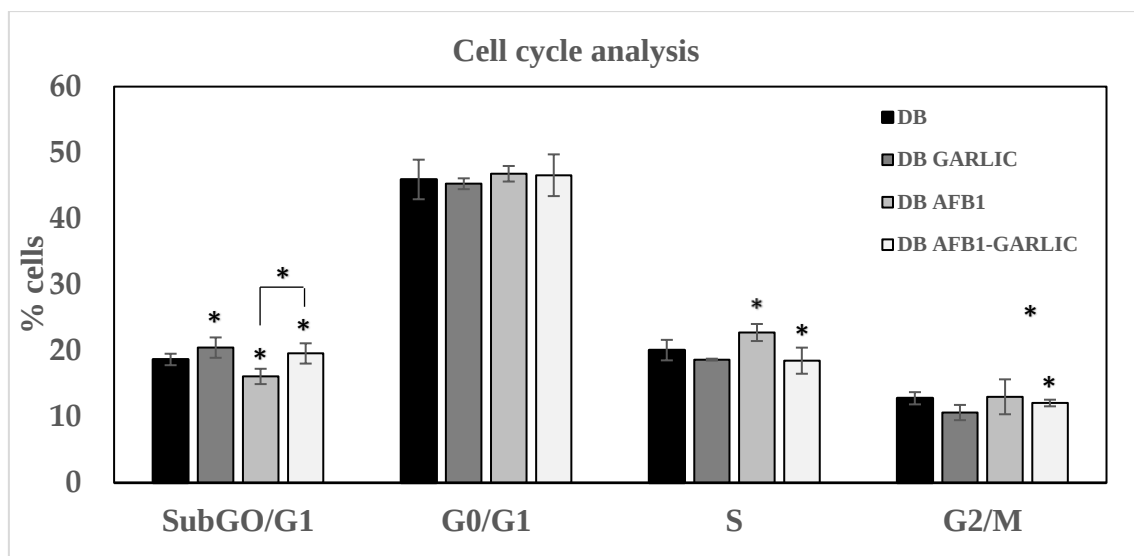


Figure 2. Effect of intestinal digests on Jurkat cell cycle by flow cytometry. Cell exposure for 7 days to 10% v/v control bread intestinal digest; garlic bread intestinal digest; AFB1 bread intestinal digest; and AFB1+garlic 2% bread intestinal digest. AFB1 concentration on both AFB1 and AFB1+garlic exposures were 0.7 μ M. $p < 0.05$ (*).

3.3.2 Apoptosis/necrosis after intestinal digests exposure

Flow cytometry was used to assess the impact of the different bread digests on apoptosis/necrosis processes. Living cells accounted for the 93% for C, 90% for G, 53% A and 69% A+G; dead ones the 1, 1, 6 and 4%; early apoptosis ones the 2, 4, 12 and 9% and late apoptosis ones the 4, 6, 30 and 18%, respectively (Figure 3A). A+G exposure significantly increased live cells percentage and reduced early and late apoptotic Jurkat T cells compared with A exposure. Gating for Annexin V⁻/PI⁻ (live), Annexin V⁺/PI⁻ (early apoptosis), Annexin V⁻/PI⁺ (necrosis), and Annexin V⁺/PI⁺ (late apoptosis) populations is shown in Figure 3B.

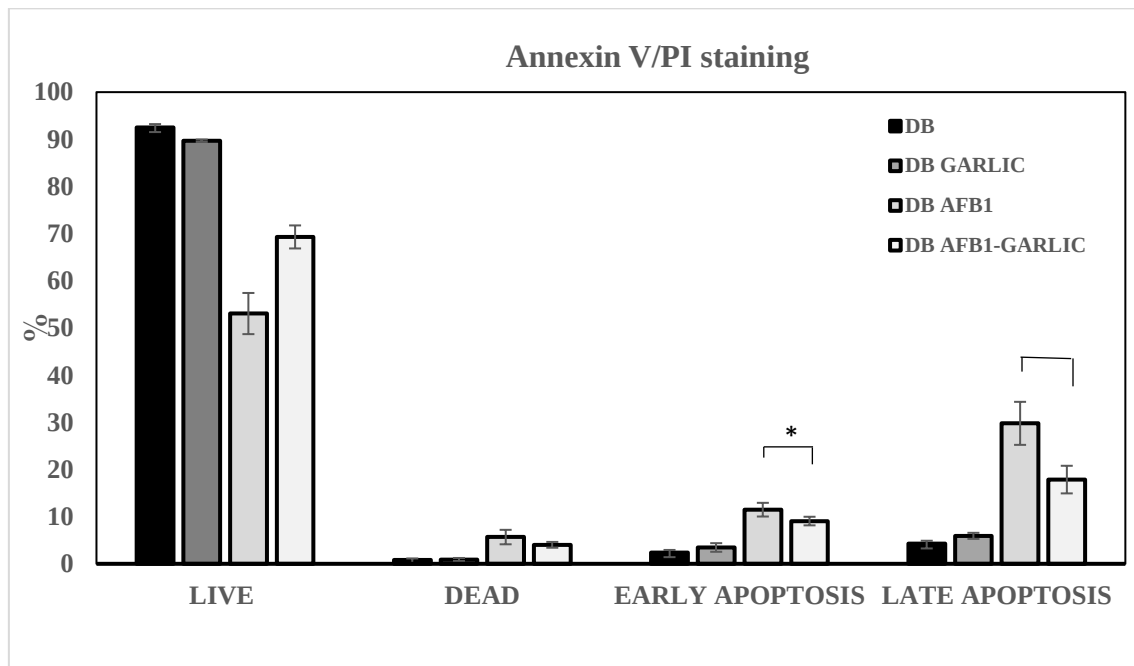
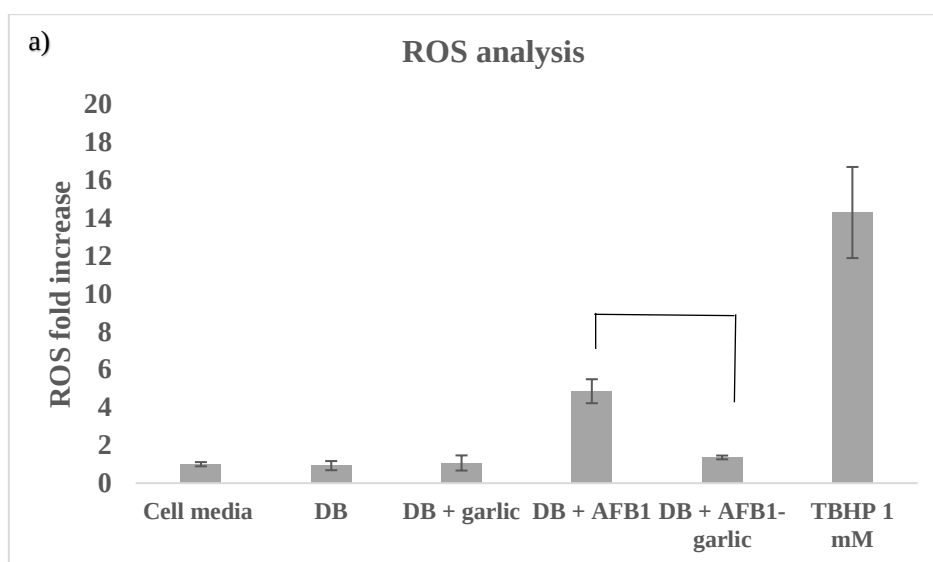


Figure 1. Effect of bread intestinal digest on apoptosis/necrosis by flow cytometry. Significant differences from the control indicated as Jurkat T cells were exposed for 7 days to digested bread (DB); digested bread garlic (DB garlic); AFB1 (0.7 μ M) bread digest; and AFB1 (0.7 μ M) + garlic bread digest. 7 μ M. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

3.3.3 Effect of intestinal digests exposure on ROS

From the lowest to the highest value, the control condition revealed a 1 ROS fold increase (FI) as well as the garlic condition while the AFB1-garlic showed 2 FI, the AFB1 5 FI and 1 mM of THBP (a compound which enhances the ROS production and used as a positive control) revealed 14.3 FI. The results indicate that AFB1 contributes to an increase in ROS production, while the presence of garlic diminishes the AFB1 effect (A+G) (Figure 4A).

In order to detect mitochondrial superoxide production in Jurkat cells after exposures, MitoSOX Red dye was used. As Figure 4B indicates, the mitochondrial ROS analysis was normalized where the digests bread condition (control) scored a 100% of intensity. From the lowest to the highest value, the AFB1-garlic condition showed a 111 % of intensity, the garlic condition evidenced 117 % and AFB1 condition revealed 135 %. The results show that the presence of AFB1 enhances the production of ROS at a mitochondrial level (A), while the addition of garlic attenuates this effect (A+G).



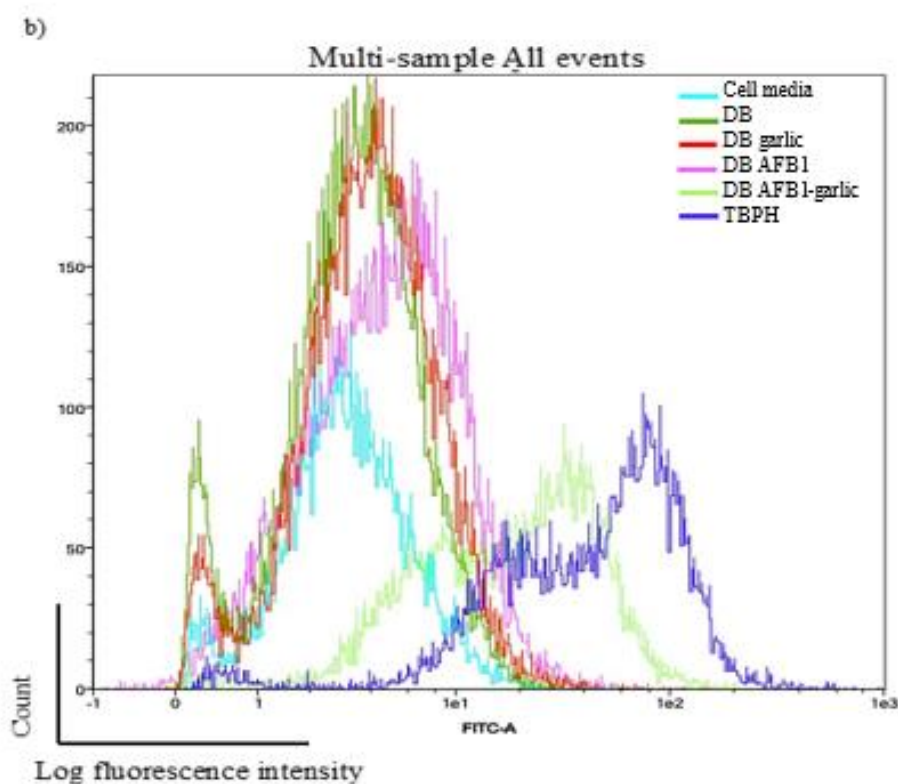
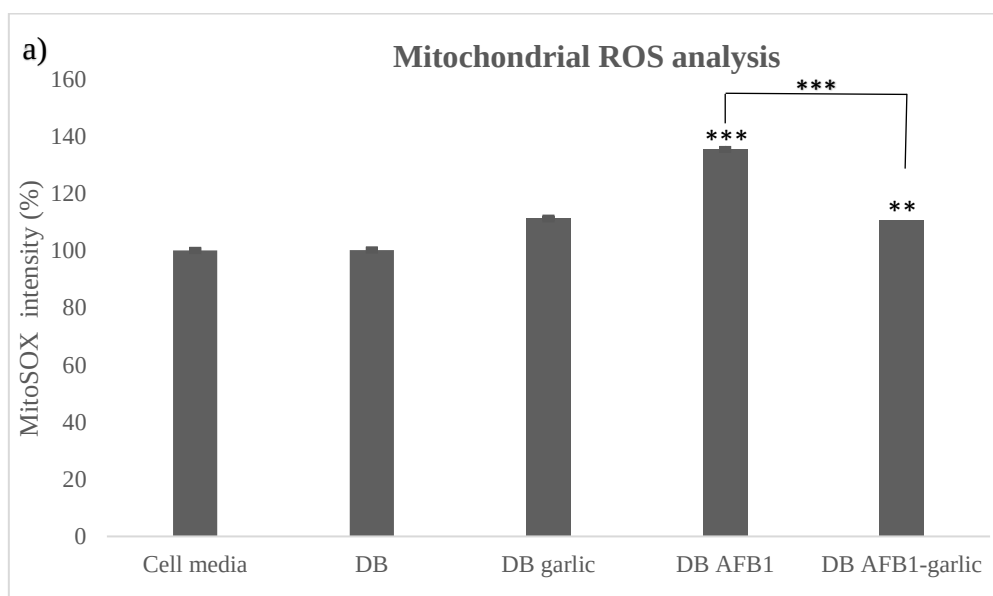


Figure 4. Bar plot showing ROS production in Jurkat T cells exposed for 7 days to cell media, digest bread (DB); DB garlic; DB AFB1; DB AFB1-garlic in relation to DB. AFB1 concentration on both DB AFB1 and DB AFB1-garlic treatments was 0.7 μ M. ROS: reactive oxygen species; TBHP: tert-Butyl hydroperoxide. $p < 0.001$ (***).



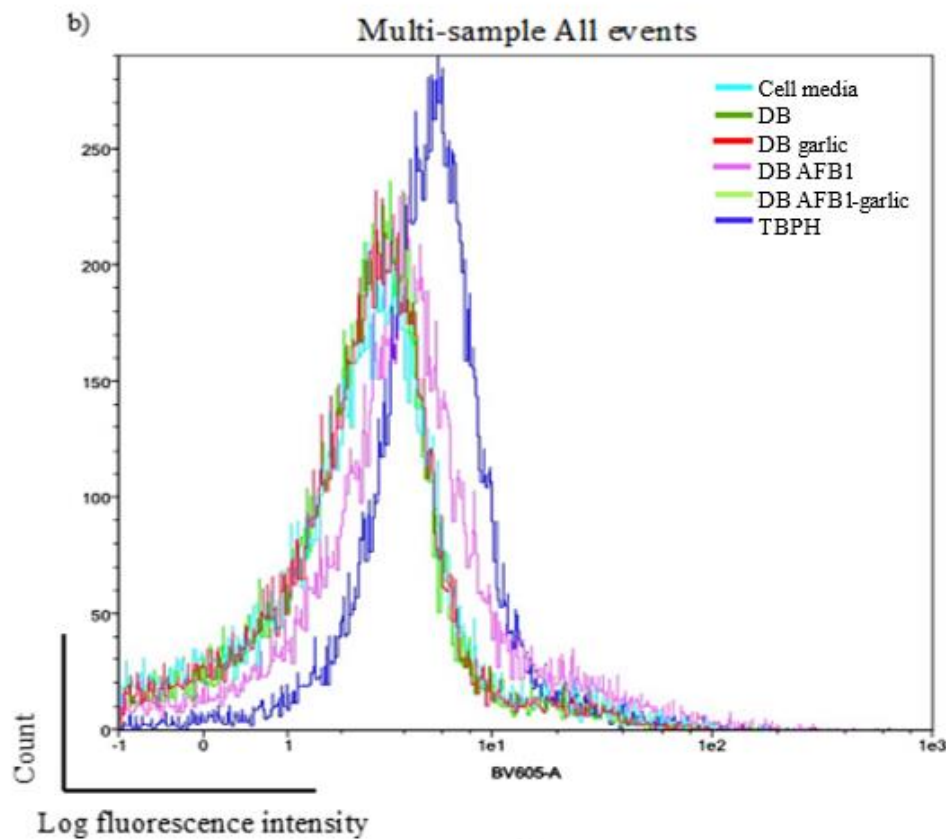


Figure 5. Effect of intestinal bread digests (0.7 μ M) in relation to control on mitochondrial ROS by flow cytometry. Significant differences from the control indicated as $p < 0.01$ (**), $p < 0.001$ (***). a) Bar plots showing Jurkat T cells exposed for 7 days to cell media (cells not treated), digest bread (DB); DB garlic; DB AFB1; DB AFB1-garlic; negative control and then stained with MitoSOX for flow cytometry detection. AFB1 concentration on both DB AFB1 and DB AFB1-garlic treatments was 0.7 μ M. b) Fluorescence representation obtained by overlaying the single plot of each condition by flow cytometry.

3.6.3 Mitochondrial mass

To evaluate the effects of the different digests on the mitochondrial mass was used the MitoTracker® dye. As shown in Figure 6, the bars indicate the results of each treatment which stem from taking as a reference the C treatment as a 100%. A+G

condition shows the lowest value (88%), followed by A (92%) and G (98%), while the C one reveals the highest mitochondrial mass (100%).

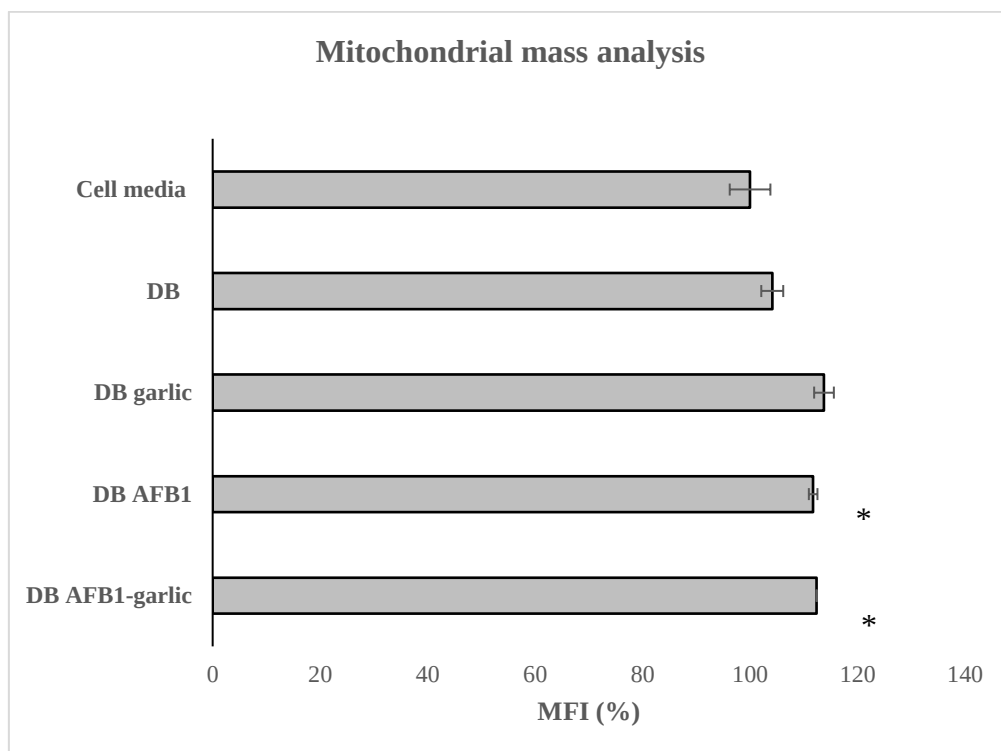


Figure 6. Effect of intestinal bread digests (0.7 μ M) in relation to control on mitochondrial mass by flow cytometry. Significant differences from the control indicated as $p < 0.05$ (*). Jurkat T cells were exposed to each treatment for 7 days to cell media (cells not treated), digest bread (DB); DB garlic; DB AFB1; DB AFB1-garlic and then fixed and stained with MitoTracker® for flow cytometry detection. AFB1 concentration on both DB AFB1 and DB AFB1-garlic treatments was 0.7 μ M. CTRL: Control; MFI: Median fluorescent intensity.

4. DISCUSSION

In the last years, AFB1 bioaccessibility has been studied in different food matrices including or not functional ingredients. The inclusion of functional ingredients as a strategy to reduce AFB1 exposure has offered promising results. Escrivá *et al.* [12] measured AFB1 bioaccessibility by using AFB1 contaminated wheat flour in the bread recipe and achieving concentrations of 78–164 μ g AFB1/kg bread with and without 1% of goat milk whey, fermented whey or lyophilized pumpkin. After performing a human

simulated digestion, the intestinal AFB1 bioaccessibility was reduced 64% when including 1% of goat milk whey in the bread recipe, 57% with 1% fermented whey and 74% with 1% lyophilized pumpkin. In this work, the intestinal AFB1 bioaccessibility reduction when including the garlic is 7-8%, which may seem small compared with the reduction reported by Escrivá *et al.* [12]. Nevertheless, the total AFB1 bioaccessible fraction when including functional ingredients is smaller in this work using garlic. The main difference between these two studies and the AFB1 bioaccessible final percentages belongs to the result related to AFB1 bioaccessibility in AFB1 contaminated bread without functional ingredients. For Escrivá *et al.* [12] the result is 114% while this work it is 38-29%.

Other bioaccessibility studies have used AFB1 fortification with commercial AFB1 standards, including the mycotoxin in the food product after its production instead of simulating a natural fungal contamination of the raw ingredients. It has been reported that fortification enhances the release of the bioactive compounds [14]. In the only one published article comparable to this work, Llorens *et al.* [15] results regarding intestinal AFB1 bioaccessibility were similar to the ones found in this study, including or not red beetroot to bread.

Cell viability studies exposing differentiated Caco-2 cells to AFB1 have been conducted [20,21]. Gao *et al.* [24], by using the CCK-8 assay, showed that Caco-2 cells exposure to AFB1 (1.6-25.6 μM) led to a cell viability decrease in a dose-dependent manner at 48 h. Zhang *et al.* [25] showed that Caco-2 cells exposure to 0.032-3.2 μM AFB1 also decreased cell viability in a dose-dependent manner at all times by using the MTT technique. In accordance with literature, the present study also shows a cell viability decrease due to the exposure to AFB1. Not only are different the techniques used, except for Zhang *et al.* [25], but also the mycotoxin AFB1 concentration and time.

Protective effects of garlic on differentiated Caco-2 cells have been studied previously. Engdal & Nilsen [26] showed that natto K2 and green tea were characterized by increasing cell viability in Caco-2 cells, while garlic had no significant effects (0.017-0.17 % p/v) after 90 min of exposure. Eguchi *et al.* [27] revealed that that aqueous garlic extract decreased cell viability to <35% after 24 h exposure at 5-25% (v/v). The sample used for cell exposure was the supernatant, while the pellet, which might still contain a considerable fraction of garlic, was discarded. In this experiment, the Voghiera garlic added as functional ingredient to bread suffered a cooking process and an *in vitro*

digestion. The garlic concentration in bread was 2% (w/v), but after the *in vitro* digestion and media dilution was 0.002% (w/v). According to Bhatt & Patel [28], while cooking reduces drastically the total amount of phenolic compounds, *in vitro* gastrointestinal digestion provides garlic extracts with much higher antioxidant potential than using organic solvents.

The toxic effects triggered by AFB1 in cells and tissues are principally provoked through inhibition of cell proliferation and cell cycle arrest, apoptosis, oxidative stress, endoplasmic reticulum stress and autophagy [29]. Huang *et al.* [30] showed that in IRM-32 neuroblastoma cell line, AFB1 induced significant S-phase arrest at concentrations of 6.4 μ M and 19.2 μ M for 24 h or 48 h. Zhu *et al.* [31] revealed a S-phase arrest in HepG2 cells exposed to 0-48 μ M of AFB1 for 24 h, especially the arrest was significant when 32 and 48 μ M AFB1. Liu *et al.* [32] showed that AFB1 exposure induced G0/G1 arrest in F344 rat hepatocytes when administering 100 or 200 μ g/kg AFB1 for 28 days. In accordance with these previous studies, this study showed that AFB1 exposure can promote a possible arrest of cell cycle at S phase while the addition of garlic decreases S phase (23 vs 19%) similar to control, by limiting significantly an eventual cell cycle arrest.

Regarding apoptosis, 3D4/21 cells apoptosis rate increased in AFB1 dose-dependent manner [33]. In accordance with these results, this work found that exposure to AFB1 increases the number of apoptotic and necrotic cells, while the addition of garlic attenuates its effects. Comparing garlic vs AFB1 and garlic exposures, the percentage of apoptotic cells reached its peak in the AFB1 one (41%) including cells in early and late apoptosis, while there was a decrease when combining AFB1 and garlic (27%) (Figure 3). It was clearly shown that AFB1 enhances cell apoptosis and that garlic attenuates AFB1-promoting apoptosis in Jurkat-T cells.

Liu & Wang [34] showed that in primary broiler hepatocytes different AFB1 concentrations (0.5-5 μ M) for 6 h led to ROS generation in a significant and concentration-dependent manner. Wang *et al.* [35] also revealed that when exposing broiler cardiomyocytes to different AFB1 concentrations (0.5-5 μ M) for 12 h resulted in a significant increase of intracellular ROS production. Furthermore, it was observed that *Cetraria islandica* methanol extract caused an increase in the activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) and a decrease in MDA

level in human lymphocytes *in vitro* [36]. The protective effect of garlic regarding oxidative processes has been also documented. Not only does garlic contribute to the decrease ROS levels, but also to mitigate other proinflammatory parameters such as SOD, catalase (CAT), and GPx activities [37,38,39,40]. In accordance with the previous literature, this work showed that AFB1 increased five times the ROS production in comparison to the control while the addition of garlic attenuated the effect of AFB1, reporting similar values to the control. This study confirms the capacity of garlic to lower ROS promoted by AFB1 in Jurkat-T cells for the first time.

Research about AFB1 impact specifically on mitochondrial ROS is scarce in literature. However, there are others mitochondrial parameters, such as ATP production, mitochondrial membrane permeability and respiratory capacity, which may be good indicators of ROS production status [41]. Chen *et al.* [42] revealed that AFB1 reduced mitochondrial respiratory capacity and ATP production in HepG2 and Caco-2 cells, which are related with cell dysfunctions. Liu *et al.* [41] showed that AFB1 increased mitochondrial membrane permeability, which would result in a release of proteins such as cytochrome c to activate caspase cascade and programmed cell death. Similarly, the present study revealed that AFB1 exposure increased mitochondrial ROS production (35% more) when compared to AFB1 and garlic one (11% more), confirming the antioxidant effect of garlic against AFB1 also at the mitochondrial level (Figure 4).

Mitochondrial changes in response to different stimuli can be assessed by using mitochondrial dyes to track mitochondrial mass and volume [43]. AFB1 has revealed to diminish the mitochondrial mass in fish exposed to 5-10 μ M of AFB1 for 7-8 days and mice fed with 0.375-1.5 mg/kg of AFB1 for 30 days [44,45]. However, no significant differences were found in Jurkat cells in this study, by suggesting that neither AFB1 nor garlic could affect the mass of this organelle (Figure 6). Overall, these results confirm the toxicity *in vitro* of AFB1 at very low concentration and long exposure.

5. CONCLUSION

Garlic presence contributed to reduce AFB1 bioaccessibility in bread (7-8 %). Garlic also revealed to increase cell viability (9-18 %). Flow cytometry showed that garlic mitigates the toxic effects of AFB1 in Jurkat-T cells on cell cycle by preventing the arrest at S phase, on apoptosis/necrosis, cellular and mitochondrial ROS production. Therefore, the antigenotoxic, antiapoptotic, and antioxidant effect of garlic was demonstrated. Nevertheless, further investigations are needed to confirm the protective role of garlic *in vivo* and its probable application in food industry to counteract the possible AFB1 food contamination at low levels and potential human toxicity.

ACKNOWLEDGMENTS

The authors are very grateful to the Spanish Ministry of Science and Innovation for the financial support (PID2022-140722OB-I00).

Author Contributions

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

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5.2 Artículo 2: Micotoxinas emergentes y estrategias preventivas relacionadas con los cambios en la microbiota intestinal: probióticos, prebióticos y postbióticos: una revisión sistemática

Food and function

Emerging mycotoxins and preventive strategies related to gut microbiota changes: probiotics, prebiotics, and postbiotics - a systematic review

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Abstract

Recent research has focused on the involvement of the gut microbiota in various diseases, where probiotics, prebiotics, synbiotics, and postbiotics (PPSP) exert beneficial effects through modulation of the microbiome. This systematic review aims to provide insight into the interplay among emerging mycotoxins, gut microbiota, and PPSP. The review was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. In this review, unregulated yet highly recurrent mycotoxins are classified as emerging mycotoxins. The most frequently observed mycotoxins included those from the *Fusarium* genus—enniatins (n = 11) and beauvericin (n = 11)—and the *Alternaria* genus—alternariol monomethyl ether, altertoxin, and tentoxin (n = 10). Among probiotics, the most studied genera were *Lactobacillus*, *Bifidobacterium*, and the yeast *Saccharomyces cerevisiae*. Inulin and cellulose were the most found prebiotics. Data on synbiotics and postbiotics are scarce. Studies have shown that both the gut microbiota and PPSP can detoxify and mitigate the harmful effects of emerging mycotoxins. PPSP not only reduced mycotoxin bioaccessibility, but also counteracted their detrimental effects by activating health-promoting pathways such as short-chain fatty acid production, genoprotection, and reduction of oxidative stress. However, both quantitative and qualitative data remain limited, indicating a need for further *in vivo* and long-term studies. The formulation of PPSP as functional foods, feeds, or nutraceuticals should be considered a preventive strategy against the toxicity of emerging mycotoxins, for which, there is no established regulatory framework.

Keywords: non-regulated mycotoxins; microbiome; probiotic; prebiotic; functional food

1. Introduction

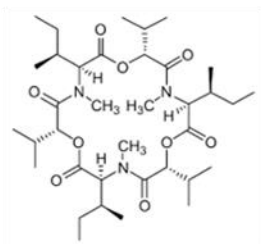
Mycotoxins are secondary metabolites produced by various species of fungi, primarily belonging to the genera *Aspergillus*, *Penicillium*, and *Fusarium*. They are estimated to be present in up to 80% of food and feed. Animal feed contamination is particularly concerning, as in developed countries, up to 70% of cereal harvest—a primary source of mycotoxins—is utilized in the daily diet of animals. Mycotoxin regulation for feed is less restrictive than for food. These toxins pose significant public health risks due to their widespread occurrence and their toxic properties in both animals and humans. They can induce immunotoxicity, neurotoxicity, hepatotoxicity, nephrotoxicity, reproductive and developmental toxicity, and carcinogenicity [1].

Currently, the legislative framework on maximum limits on mycotoxins is established by the recently updated Commission Regulation (EU) 2023/915 of April 25, for aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), aflatoxin M1 (AFM1), ochratoxin A (OTA), patulin (PAT), deoxynivalenol (DON), zearalenone (ZEN), fumonisin B1 (FB1), fumonisin B2 (FB2) and citrinin (CIT), the last one being applied only in food supplements [2]. In addition, there are other recent recommendations for emerging or unregulated mycotoxins such as alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA) and the trichothecenes T-2 and HT-2 toxins (T-2 and HT-2) [3,4].

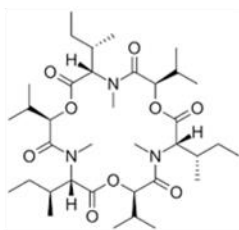
The term “emerging mycotoxins” was first introduced in 2008 and initially referred primarily to *Fusarium* metabolites such as fusaproliferin (FP), beauvericin (BEA), enniatins (ENs), and moniliformin (MON). However, more recent scientific publications have defined emerging mycotoxins as those that are neither routinely detected nor regulated by legislation, despite growing evidence of their prevalence. This work is focused on the 22 emerging mycotoxins included in Fig. 1 and 2 that have been recognized for over 20 years, continue to be widespread, and are not yet subject to regulatory oversight, even though their toxic effects are increasingly documented. The mycotoxins covered in this review are: enniatin A (ENA), enniatin (ENB), enniatin A1 (ENA1), enniatin B1 (ENB1), BEA, fusaric acid (FA), MON, fusarenon X (FX),

aurofusarin (AUR), FP, diacetoxyscirpenol (DAS), altuene (ALT), AME, TeA, altertoxin (ATX), tentoxin (TEN), CIT, sterigmatocystin (STE), neosolaniol (NEO), citreoviridin, rugulosin and phomopsin.

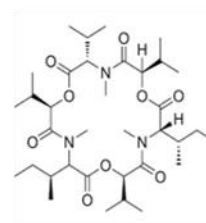
Fusarium



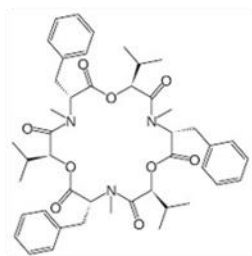
Enniatin A



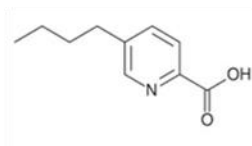
Enniatin B



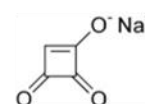
Enniatin A1



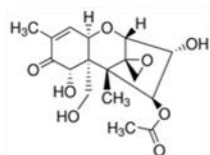
Beauvericin



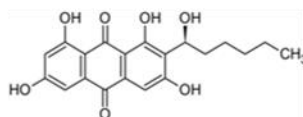
Fusaric acid



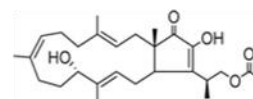
Moniliformin



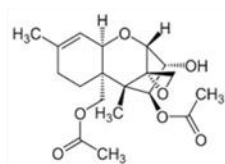
Fusarenon X



Aurofusarin

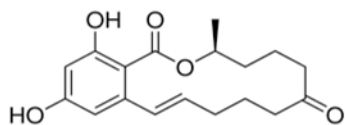


Fusaproliferin

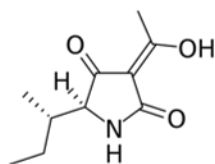


Diacetoxyscirpenol

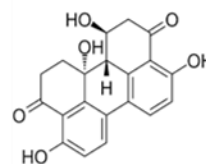
Figure 1. Chemical structures of *Fusarium* emerging mycotoxins included in the bibliographic search (n=10)

Alternaria

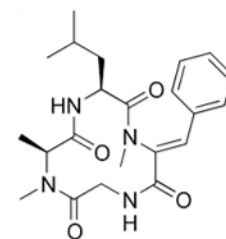
Alternariol monomethyl ether



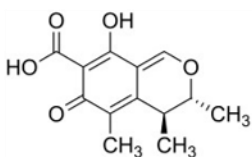
Tenuazonic acid



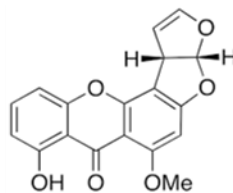
Altertoxin



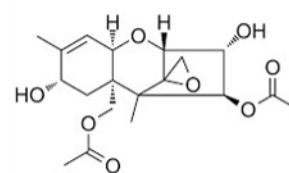
Tentoxin

Monascus

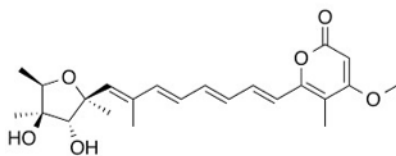
Citrinin



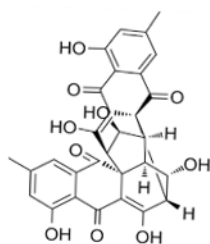
Sterigmatocystin



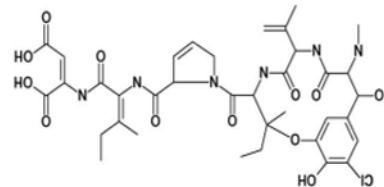
Neosolaniol

Penicillium

Citreoviridin

Diaporthe

Rugulosin



Phomopsis

Figure 2. Chemical structures of *Alternaria*, *Monascus*, *Penicillium* and *Diaporthe* emerging mycotoxins included in the bibliographic search (n=10).

To better understand the relevance of these emerging mycotoxins, the occurrence and co-occurrence of most of them have been studied by Mihalache *et al.* (2023) [5], who evidenced 38 different combinations in foodstuff (maize, rice, wheat, and flours). These combinations changed from binary mixtures (AME + AOH or AME + TeA) to include 12 different emerging mycotoxins (AOH + AME + TeA + TEN + ENA1 + ENB + ENB + BEA + MON + DAS + NIV + STE). Furthermore, the rising of new data regarding their presence in foodstuff has been highly relevant to enhance multiple risk assessment

methodologies, developing future evidence-based regulations and public health [5]. As shown in Table 1, these emerging mycotoxins are of significant concern due to their involvement in various severe health issues, including cell apoptosis, chromosomal abnormalities, acute cardiac distress, genotoxicity, hemorrhages in legs, skull, and feet, liver injury, cardiovascular collapse, chlorosis, embryotoxicity, teratogenesis, cancer, liver cirrhosis, hepatocellular carcinoma, Shoshin-kakke (acute cardiac beriberi), fatty degeneration, liver cell necrosis and cell death. Although not all emerging mycotoxins were considered in this research, the spectra covered are expected to encompass most of them.

Table 1. Emerging mycotoxins, food sources, sample, dose administration, exposure time, target organ, toxicological mechanism, damage, and references

Mycotoxin	Food sources	Sample	Dose administration	Exposure time	Target organ	Toxicological mechanism	Damages	References
<u>Fusarium</u>								
ENA	Wheat, barley, maize, and rice.	Caco-2 and SH-SY5Y cells	0.66 - 66.70 µg/mL	24-48h	Intestine, liver	Generation of ROS and mitochondrial depolarization	Apoptosis	[8,9]
ENB	Wheat, barley, maize, and rice.	Caco-2 and SH-SY5Y cells	0.63 - 63.60 µg/mL	24-48h	Intestine, liver	Generation of ROS and mitochondrial depolarization	Apoptosis	[8,9]
ENA1	Wheat, barley, maize, and rice.	Caco-2 and SH-SY5Y cells	0.66 - 66.70 µg/mL	24-48h	Intestine, liver	Generation of ROS and NADPH oxidase activation	Apoptosis	[8,9]
ENB1	Wheat, barley, maize, and rice	Caco-2 and SH-SY5Y cells	0.63 - 63.60 µg/mL	24-48h	Intestine, liver	Generation of ROS and NADPH oxidase activation	Apoptosis	[9]
BEA	Wheat, maize, nuts, and coffee	Caco-2 cells	1.17 - 19.6 µg/mL	24-72h	Intestine, liver	Generation of ROS	Apoptosis	[10]
FA	Wheat, barley, corn, and rice	<i>Allium cepa</i> L. bulbs and HepG2 cells	0.18-1.8 µg/mL	24-168h	Liver	Inhibition of cytochrome oxidase, decrease in ATP synthesis, change in the electrochemical gradient in the plasma membrane and increase in electrolyte loss.	Chromosomal abnormalities	[11,12]
MON	Maize, wheat	Male Sprague-Dawley rats	500 µg/mL	28 days	Heart	Pyruvate substitution and carbohydrate metabolism interference.	Death and acute cardiac distress	[13]

FX	Wheat, barley, and cereal-based products	Male and female mice	3-15 mg/kg b.w.	48h	Liver, kidney, spleen	Inhibition of protein and DNA synthesis	Apoptosis	[14,15]
AUR	Maize, barley, oats, and wheat	CHO-K1 cell line	0.57--5.7 µg/mL	48h	Intestine	Enhanced levels of p53 protein	Genotoxicity	[16]
FP	Maize	Chicken embryo	0.45-2.22 µg/mL	21 days	Chicken embryos	Reduction in cell viability	Hemorrhages on the surfaces of legs, skull, and feet.	[17,18]
DAS	Wheat, corn, rice, and maize	Newborn chickens and Wistar rats	1-3 mg/kg bw	6-24h	Liver, gallbladder, and small intestine	DNA synthesis and protein biosynthesis inhibitor and heterophil extracellular traps release	Liver injury	[19,20]
Apicidin	NR	HepG2 cells	0.06-62.3 µg/mL	24h	Liver	Citotoxicity	Cell death	[21]

Alternaria

AME	Wheat and its derivatives, rapeseed oil and peas	Male Sprague-Dawley rats	7.35 µg/kg bw/day	28 days	Liver, kidney, spleen	DNA adducts and production of ROS.	Genotoxicity	[21]
TeA	Tomatoes, apples, beer, and cereal foods	Mice	238 µg/kg/day	56 days	Liver, kidney	Generation of ROS	Cardiovascular collapse and gastrointestinal hemorrhage	[22]
ATX	Cereals, oilseeds, fruits, and vegetables	Male Sprague-Dawley rats	5.51 µg/kg. w/day	28 days	Liver, kidney, spleen	Nuclear factor erythroid-derived 2-like 2/antioxidant response element (Nrf2/ARE)	Genotoxicity	[16,23]
TEN	Rice	Male Sprague Dawley rats	0.4 – 41.45 pg/mL	10 min	Liver	ATP synthesis reduction	Chlorosis	[24,25]

Monascus

Trabajos publicados

CIT	Cheese, sake, and soy sauce	ProTox-II in humans	105 mg/kg weight	24h	Kidney	Inhibition of malate dehydrogenase, glutamate dehydrogenase and ATP synthase	Genotoxic, embryotoxic, teratogenic, carcinogenic,	[26]
<u>Aspergillus</u>								
STE	Maize and peanuts	HepG2 cells	0.16–2.27 µg/mL	24-48h	Liver, kidney	Generation of ROS	Liver cirrhosis hepatocellular carcinoma	[27]
NEO	Maize, wheat, and oats	Porcine Langerhans cells	143.4 mg/mL	24h	Leydig cells	Decrease in the ATP content, overproduction of ROS, reduction in mitochondrial membrane potential	Cellular apoptosis	[28]
Emodin	NR	HepG2 cells	0.027-27µg/mL	24h	Liver	Citotoxicity	Cell death	[21]
<u>Penicillium</u>								
Citreoviridin	Maize, pecan nuts, and wheat products	Mice	1-10 mg/L	15 days	Heart	Inhibition of mitochondrial ATPase	Shoshin-kakke (acute cardiac beriberi)	[29]
Rugulosin	Rice	Mice	67 mg/kg 1 b.w.	21 days	Liver	Inhibition <i>in vivo</i> and <i>in vitro</i> of DNA replication, transcription, and reparation	Fatty degeneration and liver cell necrosis	[30,31]
Cyclopiazonic acid	Corn, peanuts and cheese	Caco-2 cells	0.014– 42 ng/mL	24- 48h	Intestine	Citotoxicity	Cell death	[33]
<u>Diaporthe</u>								
Phomopsin	Lupins	Sheep	0.5 mg/kg	4 days	Liver	Cell cycle arrest	Cell death	[32,33]
<u>Phoma</u>								

Trabajos publicados

Cytochalasin B	Potatoes	EL4	2 mg/mL	4h	Blood	DNA fragmentation	Apoptosis	[36]
Cytochalasin D	Potatoes	Mice	0.3 mg/kg	17 days	Neural tube	Teratogenicity	Fetal death	[37]
Cytochalasin E	Potatoes	Brine shrimp	0.01-5 µg/mL	16-24h	NR	Citotoxicity	Cell death	[38]

Deoxyribonucleic acid (DNA). Alternariol monomethyl ether (AME). Adenosine triphosphate (ATP). Aurofusarin (AUR). Beauvericin (BEA). Citrinin (CIT). Diacetoxyscirpenol (DAS). Enniatin A (ENA). Enniatin B (ENB). Enniatin A1 (ENA1). Fusaric acid (FA). Moniliformin (MON) Nicotinamide adenine dinucleotide phosphate oxidase (NADPH). NF-E2-related factor 2 /antioxidant responsive element (Nrf2/ARE). Reactive oxygen species (ROS). Tenuazonic acid (TeA). Tentoxin (TEN).

Furthermore, climate change is anticipated to become a major environmental concern in the 21st century. Higher temperatures combined with extreme precipitation or prolonged droughts increase the stress experienced by plants, making all plant-based foods more susceptible to fungal infection and mycotoxin contamination. *Fusarium* mycotoxins are expected to shift towards Northern Europe, while *Aspergillus* species will primarily affect southern and central Europe. Indeed, it is estimated that in Europe, over the next 50–100 years, mycotoxins will become a significant concern. This includes not only the most common mycotoxins, such as aflatoxins and OTA, but also emerging *Fusarium* mycotoxins like ENs and BEA [6,7].

Recent research has focused on the involvement of the gut microbiota in a considerable number of diseases such as anxiety, depression, inflammatory bowel disease, dementia, Parkinson's disease, diabetes, obesity, and cancer [39-43]. To understand how important the gut microbiota is, it should be said that there are around 26 600 human genes, whereas bacterial genes are more than 4 000 000 in our body. What is more, the bacterial density in the colon is around 10^{11} – 10^{12} mL⁻¹, being the highest density in any ecosystem on Earth [44]. On the other hand, there are some factors, such as diet [45], stress [46], exercise [47], and medication [48], having a notable impact on gut microbiota modulation. Among these factors, this systematic review focuses on emerging mycotoxins and probiotics, prebiotics, synbiotics and postbiotics (PPSP); PPSP is a term recently coined by Li *et al.* (2021) [49].

PPSP has shown to regulate the abundances of some intestinal bacteria such as *Akkermansia*, *Bacteroidetes*, *Blautia*, *Bifidobacteria*, *Bifidobacterium* (B.) and gut microbial metabolites, such as lactic acid, acetate, butyrate and propionate, while suppressing the bile acid pools and decreasing the production of trimethylamine N-oxide and lipopolysaccharides [49]. The significance of PPSP, mainly probiotics and prebiotics, gave rise to the International Scientific Association for Probiotics and Prebiotics (ISAPP) in 2002. ISAPP is a non-profit organization that works to advance the science of probiotics, prebiotics and related substances, such as synbiotics, postbiotics and fermented foods [50], contributing to a better understanding of how biotics are connected to health and disease. Regarding definitions, prebiotics are described as “a substrate that is selectively utilized by host microorganisms, conferring a health benefit” [51]. Traditionally, they have been defined as “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited

number of bacterial species already resident in the colon, thus attempting to improve host health” (Hill et al., 2014) Certainly, probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” [52]. Recently, there has been an emerging trend to investigate synbiotics and postbiotics. Synbiotics are considered “a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host”, whereas postbiotics are described as “a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host” [53,54].

It has been shown that there are three main strategies for alleviating the harm caused by mycotoxins transmitted by microorganisms: (I) directly degrading mycotoxins in food, thanks to microbial pretreatment, and decreasing toxin intake; (II) by boosting probiotic colonization, the creation of bacterial-toxin complexes is suppressed, consequently inhibiting toxin absorption in the intestinal tract; and (III) by modulating the intestinal microecology with probiotics or prebiotics, enhancing the intestinal barrier, remodeling the intestinal microflora, improving intestinal toxicity, and decreasing toxin penetration [55]. Previously, the interactions between the gut microbiota and regulated mycotoxins [6,56] and the detoxifying capacity of PPSP on some mycotoxins have been studied [57,61]. Notwithstanding, the interplay among emerging mycotoxins, gut microbiota and PPSP still remains a framework to systemize.

The aim of this systematic review is to shed light on the potential of PPSP in mitigating the harmful effects of emerging mycotoxins. In addition, it aims to enhance our understanding of the reciprocal relationship between emerging mycotoxins and the gut microbiota, elucidating how emerging mycotoxins can influence the gut microbiota and vice versa. Furthermore, this review aims to contribute to future research endeavors by proposing the development of new functional foods and/or nutraceuticals as a strategy for mitigating toxicity associated with emerging mycotoxins.

2. Materials and methods

2.1 Search strategy

The scientific rigor of this systematic review and the minimization of potential bias were ensured through adherence to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement protocol for screening titles, abstracts, and full texts [62]. A comprehensive literature search was conducted in November–December 2023, utilizing three databases (PubMed, Web of Science, and Scopus). The search encompassed the period from January 2012 to December 2023, aiming to include recent data on emerging mycotoxins within the context of the gut microbiota and PPSP. In addition, other relevant terms, such as “bacteria”, were considered in combination with “*in vitro*” or “*in vivo*” to capture additional relevant information

The systematic review was conducted using the following search strings:

(1) Refined research of Pubmed, Web of Science and Scopus. Period: all years.

(“Enniatins” OR “Enniatin A” OR “Enniatin B” OR “Enniatin A1” OR “Beauvericin” OR “Fusaric acid” OR “Moniliformin” OR “Fusarenon X” OR “Aurofusarin” OR “Fusaproliferin” OR “Diacetoxyscirpenol” OR “Alternariol monomethyl ether” OR “Tenuazonic acid” OR “Altoxin” OR “Tentoxin” OR “Citritin” OR “Sterigmatocystin” OR “Neosolaniol” OR “Citroviridin” OR “Rugulosin” OR “Phomopsis”) AND (“bacteria”) AND (“*in vitro*” OR “*in vivo*”).

(2) Refined research of Pubmed, Web of Science and Scopus. Period: 2012–2023.

("Enniatins" OR "Enniatin A" OR "Enniatin B" OR "Enniatin A1" OR "Beauvericin" OR "Fusaric acid" OR "Moniliformin" OR "Fusarenon X" OR "Aurofusarin" OR "Fusaproliferin" OR "Diacetoxyscirpenol" OR "Alternariol monomethyl ether" OR "Tenuazonic acid" OR "Altertoxin" OR "Tentoxin" OR "Citritin" OR "Sterigmatocystin" OR "Neosolaniol" OR "Citreoviridin" OR "Rugulosin" OR "Phomopsis") AND ("microbiota" OR "prebiotics" OR "probiotics" OR "postbiotics").

The criteria to include non-regulated mycotoxins in the list was based on Mihalache *et al.* (2023) and the reports issued by the Spanish Agency for Food Safety and Nutrition (AESAN) [5,35]. It is well known that there are other non-regulated metabolites not been considered when conducting the research. Notwithstanding, it was supposed that the spectra covered would be enough to include all the non-regulated mycotoxins.

2.2 Systematic review process

Among the 811 reports found through the research, 317 were retrieved from PubMed, 295 from Web of Science and 199 from Scopus. After an initial analysis, a total of 401 duplicate records were identified and removed. In addition, during the screening of titles and abstracts, another 381 records were excluded from the systematic review because of different reasons: 124 articles were discarded because they only focused on regulated mycotoxins (AFB1, OTA, fumonisins, etc.); 130 were rejected since they included emerging mycotoxins, but they were not assessed in combination with PPSP and 127 were discarded because they analyzed PPSP, but no emerging mycotoxins. Finally, a total of 29 were considered appropriate for inclusion in the present systematic review, assessed and classified based on: the gut microbiota, probiotics, prebiotics, synbiotics and postbiotics (Fig. 3). The selected articles are key to comprehend more deeply the role of the emerging mycotoxins in their combination with microbiota and PPSP.

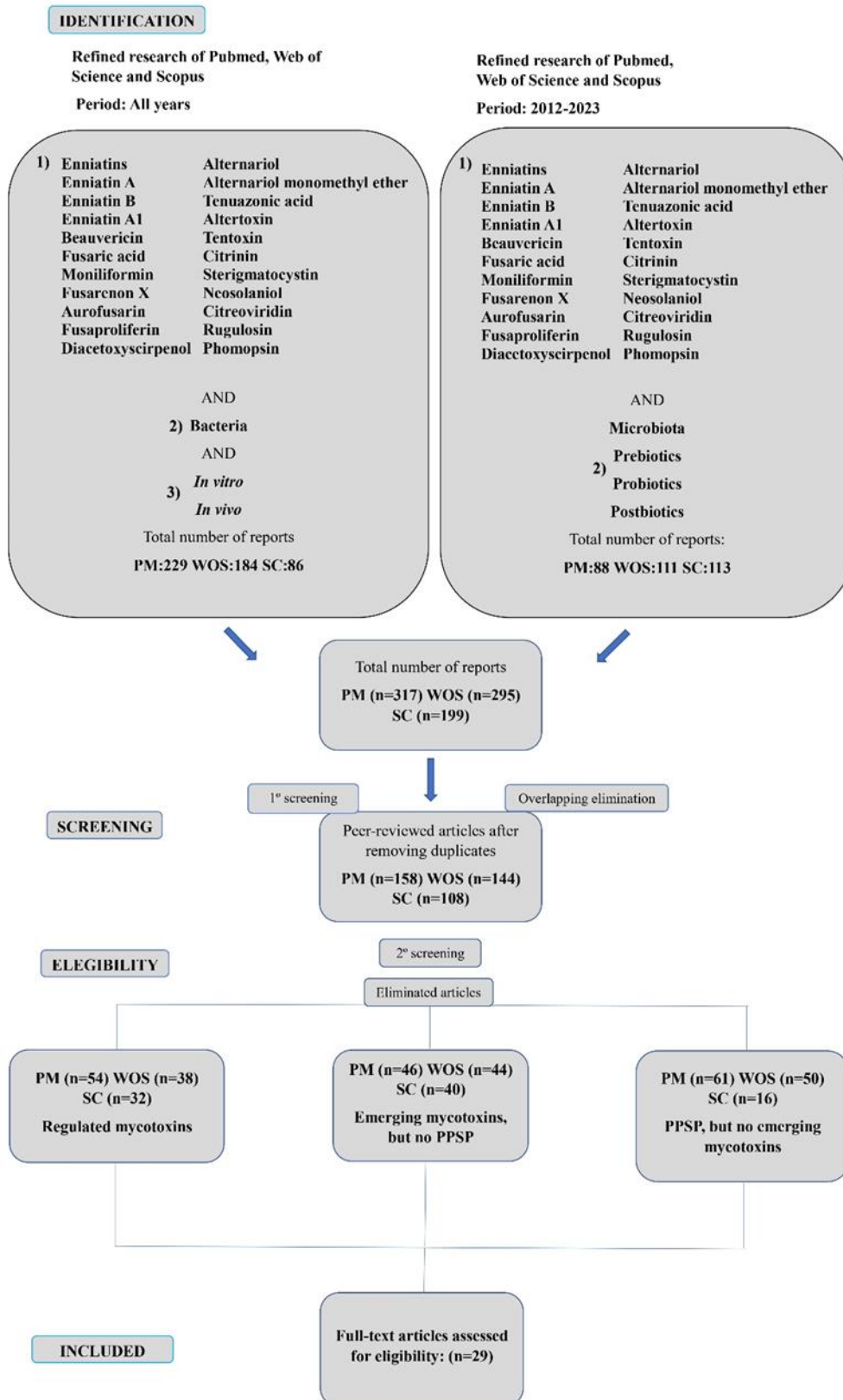


Figure 3. Graphical representation of the total number of articles screened throughout the two strategies followed for the bibliographic research using the keyword combinations

and range of years explained. Number of samples (n). Pubmed – PM, Scopus – SC and Web of Science – WOS.

2.3 Data conversion

All data from the studies discussed in this review were converted to $\mu\text{g kg}^{-1}$, mg kg^{-1} , ng mL^{-1} , $\mu\text{g mL}^{-1}$ or mg L^{-1} .

3. Results

3.1 Mycotoxins and the gut microbiota

The bidirectionality between regulated mycotoxins and the gut microbiota has been previously described in mice, pigs, rats, broilers, turkey, and ducks. DON has revealed to up-regulate the relative abundance of pathogens highly related to chronic intestinal diseases at different taxonomical levels (phylum, family, and genus). OTA has disrupted the structure and diversity of gut microbial communities and AFB1 to alter gut microbiota-dependent organic acid metabolism. Meanwhile, some gut microbiota strains, such as probiotics *L. plantarum* and *Bacillus shackletonii*, have also shown mitigation effects by metabolizing, binding, and eliminating these regulated mycotoxins [6,7,56].

3.1.1 *In vitro* detoxification of emerging mycotoxins by the gut microbiota

Rumen microbiota has been found to completely detoxify NIV at normal pH after 24 h of incubation, whereas it partially degrades ENB up to 72% after 48 h. In both cases, this degradation depended on microbial activity and was influenced by pH [63]. Hedman and Pettersson (1997) [64] described that the capacity of cow gastrointestinal microflora to metabolize NIV has exerted the same trend. It was shown that 78–82% of anaerobically incubated NIV for 48 h was transformed into de-epoxy-NIV by gastrointestinal

microbiota in the fluid rumen [64]. DAS, also produced by the genera *Fusarium*, has revealed to be deacetylated into 15-monoacetoxyscirpenol (15-MAS) and scirpentriol (SCP) as an additional metabolite by human fecal microbiota, being of utmost importance since deacetylation of mycotoxins results in reduced toxicity, for 15-MAS has been reported to be 8 times less toxic than both DAS and SCP [65].

The metabolism of *Alternaria* mycotoxins such as AOH, AME and ALT by the microbiota present in feces from three human volunteers has also been studied. As a control for the activity of the fecal microbiota, the isoflavone daidzein was incubated with the fecal cultures and was transformed to its expected metabolites. In contrast, no metabolites of AOH, AME and ALT were detected in the fecal cultures from the same volunteers, showing that the gut microbiota did not possess the capacity to metabolize these compounds. Interestingly, AOH was noted to bind non-covalently to the surface of bacteria where the type and composition of this bacterial cell surface played a notable role in the magnitude of this binding [66]. An *Alternaria alternata* extract ($5 \mu\text{g mL}^{-1}$), containing among others, AOH, AME, altertoxin-II (ATX-II), stemphytoxin III (STTX-III) and alterperyleneol (ALP), all of which are known as genotoxic, was used to assess the impact of short-term fecal incubation on the DNA-damaging effects. It was shown that microorganisms suppressed the DNA-strand-breaking potential promoted by the applied extract. Besides, in fresh fecal samples, even before anaerobic incubation, the concentrations of most of the tested mycotoxins (AOH, AME, ATX-II, STTX-III, and ALP) were lower in those containing microorganisms, especially fecal matter, implying that microbes were engaged in mycotoxin detoxification [67].

3.1.2 Emerging mycotoxins and the gut microbiota *in vivo*

Novak *et al.* (2021) [68] fed weaning piglets with different diets for a 14 day-period: DON, ENB + ENB1 + BEA and the four mycotoxins combined. Those piglets receiving the ENs + BEA diet were characterized by possessing a fecal microbiome with a lower diversity compared to the control group, and also compared to the ENs + BEA + DON diet. *L. amylovorus*, which is known to exhibit positive probiotic activities in weaning piglets, was reduced only in the ENs + BEA-fed group, but not in the ENs + BEA + DON one. Furthermore, the relative abundance of *L. reuteri*, which has shown to

contribute to diarrhea treatment in piglets by reducing *Cryptosporidium parvum*, was increased in the ENs + BEA-fed group and, to a lesser extent, in the ENs + BEA + DON-fed group, compared to the control and the DON ones [68].

The ability of the gastrointestinal microflora of pig and chicken to metabolize NIV was studied by Hedman & Pettersson. (1997) [64]. On one hand, before pigs were fed with NIV, this mycotoxin was not de-epoxidated or metabolized in any way. However, when the pigs received either 2.5 or 5 $\mu\text{g g}^{-1}$ NIV for one week, 97.1% of NIV in feces from five of six pigs was in the de-epoxy-NIV form, while the concentration of unaltered NIV in feces was 0.08 $\mu\text{g g}^{-1}$. On the other hand, after three weeks on the NIV feed, even the pig that after one week could not metabolize NIV, was now able to form de-epoxides. In the feces from this pig, 90% of NIV was as de-epoxy-NIV, the concentration of it being NIV 0.17 $\mu\text{g g}^{-1}$. Regarding chicken, it was revealed that de-epoxy-NIV was not detected in any samples of feces collected after three weeks of feeding with either 2.5 or 5 $\mu\text{g g}^{-1}$ NIV. Interestingly, another unidentified metabolite of NIV was found in all feces samples, except from one bird fed with 5 $\mu\text{g g}^{-1}$ NIV (Hedman & Pettersson, 1997) [64]. All the data relating emerging mycotoxins and gut microbiota are shown in Fig. 4 and Table 2. These studies were primarily conducted *in vitro* using feces from humans, pigs, and chickens, or in cow rumen fluids. The most frequently encountered emerging mycotoxins were ENs, BEA, NIV, and *Alternaria* mycotoxins (AOH, AME, ALTX-I, and ALT).

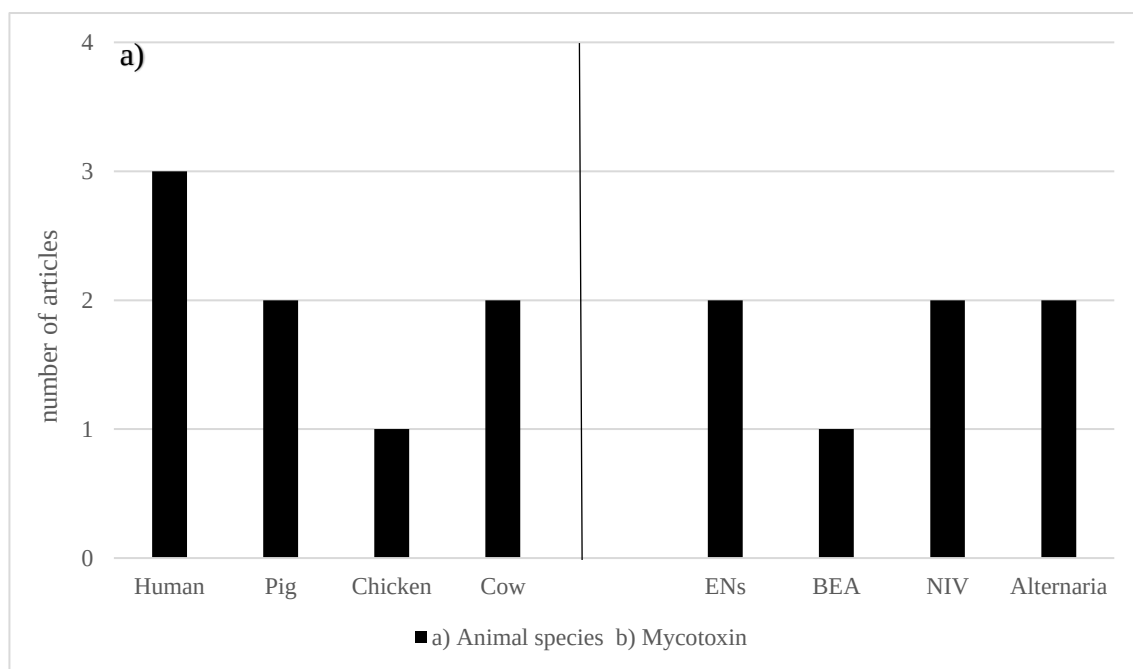


Figure 4. Graphical bars of the gut microbiota publications (n = 6) according to (a) the animal species and (b) emerging mycotoxins studied. Beauvericin – BEA, enniatins – ENs and nivalenol – NIV.

Table 2. Collection of the most important characteristics of the studies found regarding emerging mycotoxins and the gut microbiota *in vitro* and *in vivo*. For each study, the studied mycotoxin, type of sample, mycotoxin dose administration, exposure time, experimental assay, results and references are described.

Mycotoxin	Sample	Dose administration	Exposure time	Experimental assay	Results	References
<i>In vitro</i>						
ENB NIV	Cow rumen fluid	1 mg/kg ENB 60 mg/kg NIV	0-48 h	<i>In vitro</i> rumen simulation	ENB degradation up to 72% after 48 h and NIV completely detoxified at normal pH (6.8) after 24 h of incubation. In both cases, the degradation was dependent of the microbial activity.	[63]
NIV	Cow rumen fluid	2 µg/mL	48 h	Anaerobic fecal culture	78–82% of incubated NIV was transformed into de-epoxy-NIV by gastrointestinal microbiota in the fluid rumen. Anaerobic incubation of NIV with cow rumen fluid produced de-epoxide at a high proportion.	[64]
DAS	Human feces	0.7 ng/mL	2-72 h	Fecal batch culture	DAS was deacetylated into 15-MAS and SCP as an additional metabolite by human fecal microbiota.	[65]
AOH AME ALT	Human feces	12.9 µg/mL	72 h	Anaerobic fecal culture	AOH, AME and ALT were not metabolized by gut bacteria. AOH bounded non-covalently to bacteria in function of the type and composition of its surface.	[66]
AOH AME ATX-II	Human feces	50 µg/mL	48 h	Fecal anaerobic incubation	Microorganisms suppressed the DNA-strand-breaking potential promoted by the applied extract. AOH, AME, ATX-II, STTX-III, and ALP were lower in the samples containing microorganisms,	[67]

STTX-III ALP					especially fecal matter, implying that microbes were engaged in detoxification.	
Mycotoxin	Sample	Mycotoxin dose administration	Exposure time	Experimental assay	Results	References
<i>In vivo</i>						
ENB ENB1 BEA	Weaning piglets' feces	1.345 mg/kg (ENB) 1.830 mg/kg (ENB1) 2.570-3.578 mg/kg (BEA)	14 days	Metagenomic analysis of fecal microbiome	<i>L. amylovorus</i> , was reduced only in the BEA-fed group, but not in the BEA + DON group. The relative abundance of <i>L. reuteri</i> was increased in the BEA group and, to a lesser extent, in the BEA + DON group compared to the control and the DON group.	[68]
NIV	Pig and chicken feces	2.5-5 mg/kg	1-3 weeks	Chemical analysis of feces	After one week, 97.1% of NIV was in the de-epoxy-NIV form in feces from five of six pigs. After three weeks, also this pig was now able to form de-epoxides (90%). No de-epoxide of NIV, but another unidentified metabolite was found in feces from chicken fed with 2.5 or 5 µg/g NIV for three weeks	[64]

Alternariol (AOH). Alternariol methyl ether (AME). Alterperyleneol (ALP). Altertoxin I (ATX-I). Alterperyleneol (ALP). Altuene (ALT). Beauvericin (BEA). Diacetoxyscirpenol (DAS). Deoxynivalenol (DON). Enniatin B (ENB). *Lactobacillus* (L). Nivalenol (NIV). Scirpentriol (SCP). Stemphyliotoxin III (STTX-III). 15-monoacetylscirpenol (15-MAS). Both SCP and 15-MAS are the products of DAS deacetylation.

3.2 Emerging mycotoxins and probiotics

Studies *in vitro* and *in vivo* have shown the ability of probiotic cultures to bind to and/or even degrade many toxic substances, mitigating their toxicity [64,69]. Not only has it been reported that single strain or combination of lactic acid bacteria (LAB) are very useful for removal of heavy metals (copper, lead, cadmium, chromium and arsenic) and cyanotoxins (microcystin-LR, -RR and -LF), but also regarding mycotoxins (AFB1, AFB2, AFM1, aflatoxin M2 (AFM2), AFG1, AFG2, PAT, OTA, DON, FB1 and FB2), 3-acetyldeoxynivalenol, NIV, HT-2 and T-2, ZEN and its derivatives, etc., and their harmful effects [58,61,70].

3.2.1 Emerging mycotoxins and probiotics *in vitro*

The strains *L. johnsonii* CECT 289, *L. rhamnosus* CECT 288, *L. plantarum* CECT 220, *L. reuteri* CECT 725, *L. casei* CECT 475, *B. breve* CECT 4839 T, *B. adolescentis* CECT 5781 T, *B. bifidum* CECT 870 T and *B. longum* CECT 4551 have exerted to degrade different types of ENs during a simulated gastrointestinal digestion process for 4 h. It was reported that all the bacteria reduced ENA, ENA1, ENB and ENB1 mean bioaccessibility (ranging from 21.0% to 31.2%) in comparison with the control (33.4%–39.6%) [71]. The antimicrobial activity of *B. longum*, *B. bifidum*, *B. breve*, *B. adolescentis*, *L. rhamnosus*, *L. casei*–*casei*, *L. plantarum*, *L. paracasei*, *L. ruminis*, *Streptococcus thermophilus*, twenty-two strains of *Saccharomyces cerevisiae* and nine of *Bacillus subtilis* has been assessed against the minor *Fusarium* mycotoxin ENs (ENA, ENA1, ENA2, ENB, ENB1 and ENB4). The most active mycotoxins were ENA1 and ENB, mainly against *B. adolescentis* 5871 and *S. thermophilus*. Conversely, ENA1 and ENB1 have been reported to inhibit the growth of the cultivated *B. subtilis* strain with a minimum inhibitory concentration (MIC) of 16 and 8 $\mu\text{g mL}^{-1}$, respectively, and to be active against *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Enterococcus faecalis* with MIC values ranging from 2 to 8 $\mu\text{g mL}^{-1}$ [73]. Another nine bacterial strains characteristic of the gastrointestinal tract like *B. longum*, *B. bifidum*, *B. breve*, *B. adolescentis*, *L. rhamnosus*, *L. casei*–*casei*, *S. thermophilus*, *L. ruminis*, *L. casei* and twenty-two strains

of *S. cerevisiae* have been studied to conduct EN metabolism. The fermentation processes were carried out in a liquid medium of De Man Rogosa Sharpe under anaerobic conditions and in a potato dextrose broth, and the degradation of the EN bioactive compounds was assessed in a food system made of naturally contaminated wheat flour. The results showed ENA, ENA1, ENB and ENB1 degradation ranging from 5.2% to 99.5%. The highest reduction was caused by *S. thermophilus* CECT 986 in ENA and the lowest by *B. breve* CECT 4839 in ENB. Furthermore, degradation products were identified for all the studied mycotoxins in both the liquid medium and the food system [74].

Apart from ENs, the interaction between BEA and probiotics is also well documented. Nine yeast strains of *S. cerevisiae* (LO9, YE-2, YE5, YE-6, YE-4, A34, A17, A42 and A08) have shown a BEA mean degradation of 86.2%. The highest degradation activity (98.8%) was due to *S. cerevisiae* LO-9, whereas the lowest (39.7%) by *S. cerevisiae* LS100 [75]. The protective effect of *L. acidophilus* against BEA toxicity has been assessed on a Caco-2 cell line. It was revealed that, in the presence of BEA, *L. acidophilus* significantly increased cell viability at 12 h and 24 h [76]. *L. paracasei* CECT 277, *L. casei* CECT 4180, *L. rhamnosus* CECT 278T, *L. plantarum* CECT 220, *L. ruminis* CECT 4061T, *L. casei casei* CECT 277, *B. breve* CCT 4839T, *B. adolescentis* CECT 5781T, *B. bifidum* CECT 870T, *B. longum* CECT 4551, *Corynebacterium vitaeruminis* CECT 537, *Eubacterium crispatus* CECT 4840 and *S. cerevisiae* CECT 1324 have proven to exert a notable impact on BEA bioaccessibility. The highest bioaccessibility decrease was carried out by *B. longum* showing 45.4%, whereas the lowest data were revealed with the strain *L. rhamnosus* (27.5%) [77]. Other 13 bacterial strains belonging to the gastrointestinal tract (*B. longum*, *B. bifidum*, *B. breve*, *B. adolescentis*, *L. rhamnosus*, *L. casei*–*casei*, *L. plantarum*, *Eubacterium crispatus*, *Salmonella fecalis*, *Salmonella thermophilus*, *L. ruminis*, *L. casei* and *L. animalis*) were fermented under anaerobic conditions in the liquid medium of De Man–Rogosa–Sharpe agar for 4 h, 12 h, 16 h, 24 h and 48 h at 37 °C. It was revealed that BEA reduction ranged from 66.5% to 83.1%, being the highest decrease caused by the strain *L. casei casei* [78].

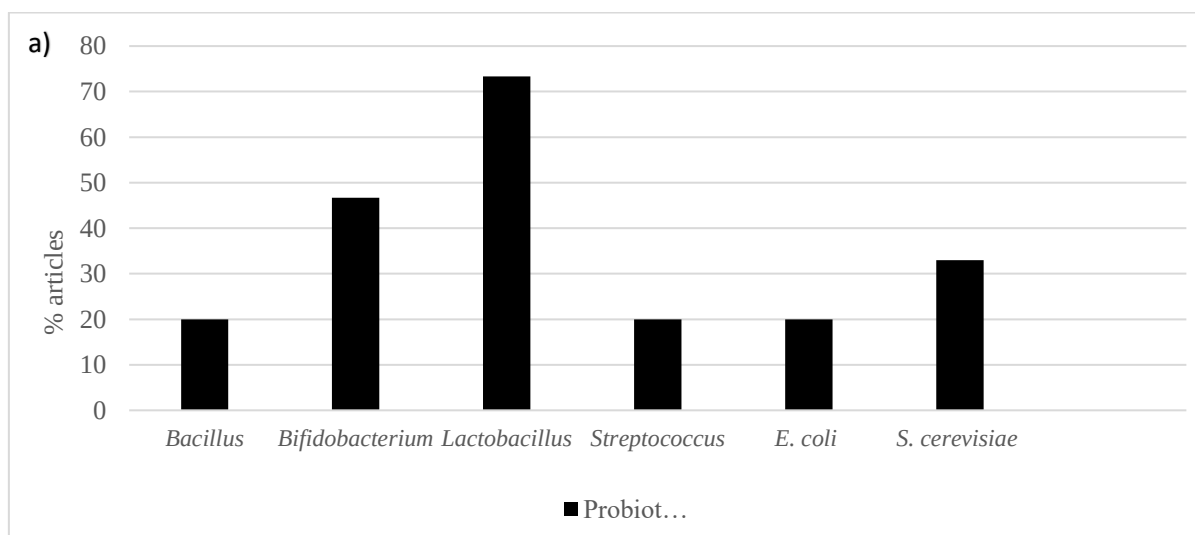
Other *Fusarium* mycotoxins have been studied in combination with probiotics showing similar results. *E. coli* (ATCC 25922), *Salmonella enterica* serovar typhimurium 3389–1 (DT12), *Staphylococcus aureus* (ATCC 29213), *L. acidophilus* (DSMZ 20079), *L. acidophilus* (20079), *L. salivarius* (20555), *L. sobrius* (16698), *B. longum* subsp.

longum Reuter (20219) and *B. breve* (20213) received a fast digital time-lapse microscopic method to assess AUR, BEA, ENs and FA antibacterial effects after 6 h of cultivation. The most potent compound was AUR against *L. acidophilus*, whereas BEA, ENs and FA were characterized by having weak antibiotic effects [79]. *Burkholderia ambifaria* T16, a naturally beneficial bacterium with proven biocontrol properties but potential pathogenic risk, has shown to inhibit the growth of several *Fusarium* species and possess the unique ability to degrade FA as a carbon, nitrogen and energy source, as well as the capacity to detoxify FA in barley seedlings [80]. Different *S. cerevisiae*, *Lactobacillus* and *Bacillus* strains were tested for their ability to degrade different mycotoxins. Among the tested mycotoxins, DAS was proven to possess an inhibitory effect on the growth of almost all *Lactobacillus* and *Bacillus* strains, whereas no effect on yeast strains could be observed [81]. The human microbiome characteristic bacteria *Prevotella copri* has revealed to deacetylate 51.3% of DAS to 15-MAS after 48 h [82].

The same trends have also been reported with *Alternaria* mycotoxins in Ge *et al.* (2017) [83]. *L. brevis* 20023 (LAB-20023) cells have revealed to adsorb TeA from aqueous solution, demonstrating that polysaccharides and protein were important components of the LAB cell wall and were involved in TeA removal. The lowest and highest absorption rates were 12.5% and 90.1%, respectively [83]. The effects of a complex extract of *Alternaria* mycotoxins (AME, ALT, TeA, TEN, ATX-I, ATX-II, ALP, STTX-III, altenusin and altersetin (AST)) were studied in combination with some typical bacterial strains of the gut microbiome such as *Bacteroides caccae*, *Bacteroides eggerthii*, *Bacteroides thetaiotaomicron*, *Bacteroides vulgatus*, *Parabacteroides distasonis*, *E. coli*, *Clostridium innocuum*, *Alistipes finegoldii*, *Alistipes timonensis*, *Ruminococcus bicirculans*, *Akkermansia muciniphila*, *L. hominis*, *B. longum* and *B. sp.* The results showed that both Gram types were able to adsorb AOH, AME, and AST, and it was seen that Gram-negative bacteria had higher adsorptive abilities. Notwithstanding, this absorption was not observed for neither ATX-I nor ALP. Interestingly, the mycotoxin tendency to accumulate within bacterial pellets, especially in those of Gram-negative strains, was connected to their lipophilic profile [84].

3.2.2 Emerging mycotoxins and probiotics *in vivo*

To study the influence of fermentation on feed acidity and microbiological parameters, two different diets were prepared: (i) non-fermented basal feed and (ii) fermented basal feed. A fermented basal diet was characterized by including some microbial starters (*L. uvarum* LUHS245, *L. casei* LUHS210, *Pediococcus acidilactici* LUHS29 and *Pediococcus pentosaceus* LUHS183). Piglets were fed using these diets for 36 days and feces microbiota, growth performance and health were evaluated. Mycotoxin biotransformation was quantified, including masked mycotoxins, in feed and piglet fecal samples. The analysis showed that AME and ALT were found in 61-day-old control piglets' feces and in fermented feed samples. However, AME was not found in treated piglets' feces. *Prevotella* was the most prevalent genus in both groups, but the prevalence of *Lactobacillus* was 6-fold higher in treated animals compared to the control ones (23.7 vs. 3.9%). After the exposure, in the fermented feed group animals' blood, triglycerides and serum high-density lipoprotein cholesterol were notably higher, but the levels of glucose, alkaline phosphatase, urea, thyroxine, glucose and potassium were significantly decreased ($p \leq 0.05$) in comparison with the control group [85]. Fig. 5 and Table 3 show all the data concerning emerging mycotoxins and probiotics.



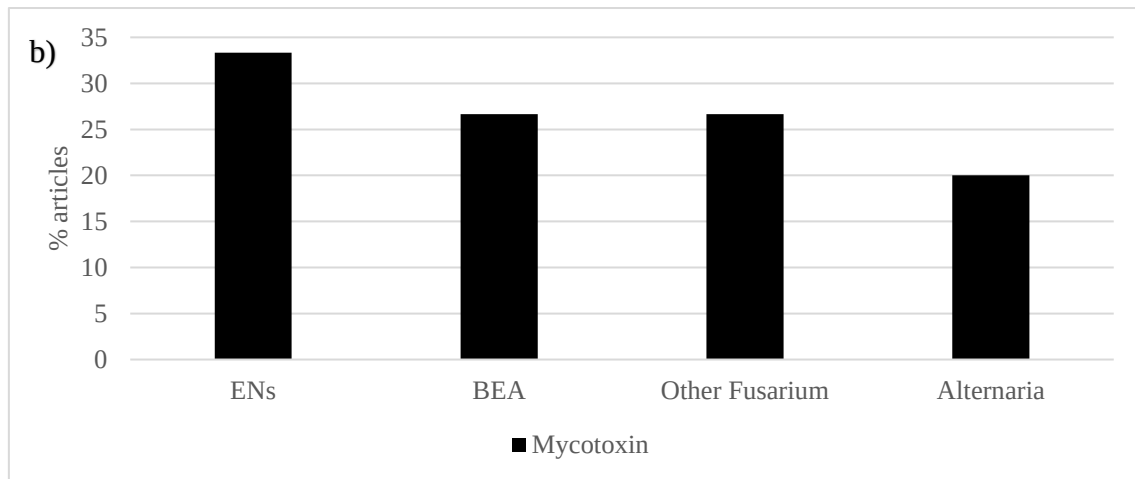


Figure 5. Graphical bars of the probiotic publications ($n = 15$) according to the (a) microbial species and (b) the emerging mycotoxins studied. In the reports, *Bacillus*, *Bifidobacterium*, *Lactobacillus*, *Streptococcus*, *E. coli* and *S. cerevisiae* were the most recurrent probiotics, whereas enniatins, beauvericin, other *Fusarium* mycotoxins and *Alternaria* mycotoxins were the most used emerging mycotoxins. Beauvericin – BEA, enniatins – ENs, *Escherichia coli* – *E. coli* and *Saccharomyces cerevisiae* – *S. cerevisiae*.

Table 3. Collection of the most important characteristics of the studies found regarding the emerging mycotoxins and probiotics *in vitro* and *in vivo*. For each study, the studied mycotoxin, type of probiotic, mycotoxin dose administration, exposure time, experimental assay, results and references are described.

Mycotoxin	Probiotic		Dose administration	Exposure time	Experimental assay	Results	References
<i>In vitro</i>							
ENA ENA1 ENB ENB1	<i>L. johnsonii</i> CECT 289 <i>L. rhamnosus</i> CECT <i>L. plantarum</i> CECT 220 <i>L. reuteri</i> CECT 725 <i>L. casei</i> CECT 47	<i>B. breve</i> CECT 4839 T <i>B. adolescentis</i> CECT 5781 T <i>B. bifidum</i> CECT 870 T <i>B. longum</i> CECT 4551	Not reported	4 h	Dynamic gastrointestinal <i>in vitro</i> digestion	ENA, ENA1, ENB and ENB1 mean bioaccessibility reduced ranging from 21% to 31.2% comparing to control (33.4% - 39.6%)	[71]
ENA ENA1 ENA2 ENB ENB1 ENB4	<i>B. longum</i> <i>B. bifidum</i> <i>B. breve</i> <i>B. adolescentis</i> <i>L. rhamnosus</i> <i>L. casei-casei</i> , <i>L. plantarum</i> <i>L. paracasei</i>	<i>L. ruminis</i> , <i>Streptococcus thermophilus</i> 22 strains of <i>S. cerevisiae</i> 9 strains of <i>Bacillus subtilis</i> .	0.2–20.000 ng per disc	24 h	Antimicrobial analyses carried out the disc-diffusion method	ENA1 and ENB were the most actives against <i>B. adolescentis</i> 5871, <i>S. thermophilus</i> , 2 strains of <i>Lactobacillus</i> and 2 other strains of <i>Bifidobacterium</i> .	[72]

EN1 ENB1	<i>Bacillus subtilis</i> 168 <i>trpC2</i> <i>Staphylococcus aureus</i> ATCC 29213 <i>Streptococcus pneumoniae</i> ATCC 49619 <i>E. coli</i> ATCC 25922 <i>Staphylococcus aureus</i> 25697 <i>Enterococcus faecalis</i> UW 268 <i>Pseudomonas aeruginosa</i> B 63230		2–8 µg/mL	3 weeks	Cocultivation media	EN1 and ENB1 inhibited the growth of <i>B. subtilis</i> and were also active against <i>Staphylococcus aureus</i> , <i>Streptococcus pneumoniae</i> , and <i>Enterococcus faecalis</i>	[73]
ENA ENA1 ENB ENB1	<i>B. longum</i> <i>B. bifidum</i> <i>B. breve</i> <i>B. adolescentes</i> <i>L. rhamnosus</i> <i>L. casei-casei</i>	<i>Streptococcus. Termofilus</i> <i>L. ruminis</i> <i>L. casei</i> 22 strains of <i>S. cerevisiae</i>	Not reported	48 h	<i>In vitro</i> degradation in anaerobic atmosphere	ENA, ENA1, ENB and ENB1 degradation ranging from 5.2% to 99.5%	[74]
BEA	<i>S. cerevisiae</i> LO9 <i>S. cerevisiae</i> YE-2 <i>S. cerevisiae</i> YE5 <i>S. cerevisiae</i> YE-6 <i>S. cerevisiae</i> YE-4 <i>S. cerevisiae</i> YE5	<i>S. cerevisiae</i> YE-6 <i>S. cerevisiae</i> A34 <i>S. cerevisiae</i> A17 <i>S. cerevisiae</i> A42 <i>S. cerevisiae</i> A08	5 mg/L; 5 mg/Kg	48-72 h	Biological degradations under aerobic conditions in the liquid medium of PDB	BEA mean degradation of 86.2%. The highest degradation activity (98.8%) was due to the strain of <i>S. cerevisiae</i> LO-9, whereas the lowest to <i>LS100</i> (39.7%)	[75]

BEA	<i>L. acidophilus</i>		0.8- 15.7 µg/mL	24-72 h	Cell viability assay	<i>L. acidophilus</i> significantly increased cell viability at 12 h and 24 h in the presence of BEA	[76]
BEA	<i>L. paracasei</i> CECT 277 <i>L. casei</i> CECT 4180 <i>L. rhamnosus</i> CECT 278T <i>L. plantarum</i> CECT 220 <i>L. ruminis</i> CECT 4061T <i>L. casei casei</i> CECT 277 <i>B. breve</i> CECT 4839T	<i>B. adolescentes</i> CECT 5781T <i>B. bifidum</i> CECT 870T <i>B. longum</i> CECT 4551 <i>Corynebacterium vitaeruminis</i> CECT 537 <i>Eubacterium crispatus</i> CECT 4840 <i>S. cerevisinas</i> CECT 1324	5-25 mg/kg	48 h	<i>In vitro</i> dynamic digestion model	BEA bioaccessibility highest reduction (45.4%) was carried out by <i>B. longum</i> , whereas the lowest bioaccessibility data was revealed with the strain of <i>L. rhamnosus</i> , with a 27.5%	[77]
BEA	<i>B. longum</i> <i>B. bifidum</i> <i>B. breve</i> <i>B. adolescentes</i> <i>L. rhamnosus</i> <i>L. casei-casei</i> <i>L. plantarum</i>	<i>Eubacterium crispatus</i> <i>Salmonella fecalis</i> <i>Salmonella termofilus</i> <i>L. ruminis</i> <i>L. casei</i> <i>L. animalis</i>	5 mg/L	4-48 h	Fermentations in the liquid medium of MRS	BEA reduction ranged from 66.5% to 83.1%, being the highest decrease value by the strain of <i>L. casei-casei</i> .	[78]
AUR ENs	<i>E. coli</i> (ATCC 25922)	<i>L. Acidophilus</i> (DSMZ 20079) <i>L. acidophilus</i> (20079)	1.1-146.1 µg/mL 1.4-174.6 µg/mL	6 h	Fast digital time-lapse microscopic method	The most potent compound was AUR against <i>L. acidophilus</i> . Meanwhile, BEA, ENs and FA were characterized by having weak antibiotic effects.	[79]

BEA FA	<i>Salmonella enterica</i> serovar <i>typhimurium</i> 3389-1 (DT12) <i>Staphylococcus aureus</i> (ATCC 29213)	<i>L. salivarius</i> (20555) <i>L. sobrius</i> (16698) <i>B. longum</i> subsp. <i>longum</i> Reuter (20219) <i>B. breve</i> (20213)	1.6-200.7 µg/mL 0.2-29.7 µg/mL				
FA	<i>Burkholderia ambifaria</i> T16		0.06 mg/mL	72 h	<i>In vitro</i> detoxification	<i>Burkholderia ambifaria</i> has been shown to inhibit the growth of several <i>Fusarium</i> species and possess the unique ability to use the mycotoxin FA as sole C, N and energy source, as well as the capacity to detoxify FA in barley seedlings.	[80]
DAS	<i>L. acidophilus</i> 1A <i>L. acidophilus</i> 4A <i>L. helveticus</i> 2A <i>L. bulgaricus</i> 3A	<i>L. Bulgaricus</i> 5A <i>Bacillus licheniformis</i> <i>Bacillus subtilis</i> <i>S. cerevisiae</i>	0.5 µg/mL	48 h	Biodegradation <i>in vitro</i> in agar plates	DAS appeared to possess an inhibitory effect on the growth of almost all <i>Lactobacilli</i> and <i>Bacilli</i> strains, whereas no effect on yeast strains could be observed.	[81]
DAS	<i>Akkermansia muciniphila</i> DSM 22959 <i>B. adolescentis</i> DSM 20083 <i>Bacteroides thetaiotaomicron</i> DSM 2079 <i>Prevotella copri</i> DSM 18205	<i>Butyrivibrio fibrisolvens</i> 16/4 <i>Coprococcus</i> sp. ART55/1 <i>Eubacterium rectale</i> DSM 17629 (A1-86) <i>Roseburia intestinalis</i> L1-82 <i>Lactiplantibacillus plantarum</i> NCIMB 7220 <i>Faecalibacterium</i>	0.7 ng/mL	0-48 h	Anaerobic incubation	The human microbiome characteristic bacteria <i>Prevotella copri</i> revealed to de-acetylated the 51.3% of DAS to 15-MAS after 48 h.	[82]

	<i>Faecalitalea cylindroides</i> T2-87, <i>Anaerobutyricum hallii</i> DSM 3353 <i>Anaerostipes hadrus</i> SSC/2 <i>Blautia obeum</i> A2-162	<i>prausnitzii</i> A2-165 <i>Enterococcus mundtii</i> DSM 4838					
TeA	<i>L. brevis</i> 20023 (LAB-20023)		0.5 µg/mL	12 h	Adsorption	<i>L. brevis</i> 20023 (LAB-20023) cells have revealed to adsorb TeA from 12.5% to 90.1%.	[83]
AOH AME AST ATX-I ALP	<i>Bacteroides caccae</i> <i>Bacteroides eggerthii</i> <i>Bacteroides thetaiotaomicron</i> , <i>Bacteroides vulgatus</i> , <i>Parabacteroides distasonis</i> <i>E. coli</i> <i>Clostridium innocuum</i> (CI)	<i>Alistipes finegoldii</i> <i>Alistipes timonensis</i> <i>Ruminococcus bicirculans</i> <i>Akkermansia muciniphila</i> , <i>L. hominis</i> <i>B. longum</i> <i>B. sp.</i>	0.5- 50 µg/mL	24 h	Absorption assay	Both Gram types were able to adsorb AOH, AME, and AST. Gram-negative bacteria showed higher adsorptive capacities. This tendency was not observed for neither ATX-I nor ALP	[84]
Mycotoxin	Probiotic		Mycotoxin dose administration	Exposure time	Experimental assay	Results	References
In vivo							
AME ALT	<i>L. uvarum</i> LUHS245 <i>L. casei</i> LUHS210		10.1-17.06 µg/kg	36 days	Fermentation/biotransformation	AME and ALT were found in 61-day-old control piglets' feces and in fermented	[85]

	<i>Pentosaceus acidilactici</i> LUHS29				feed samples. However, AME was not found in probiotics treated piglets' feces.	
	<i>Pediococcus pentosaceus</i> LUHS183					

Alternariol methyl ether (AME). Alterperyleneol (ALP). Altertoxin I (ATX-I). Alterperyleneol (ALP). Altuene (ALT). Altersetin (AST). Aurofusarin (AUR). Beauvericin (BEA). *Bifidobacterium* (B.). Diacetoxyscirpenol (DAS). Enniatin A (ENA). Enniatin A1 (ENA1). Enniatin A2 (ENA2). Enniatin B (ENB). Enniatin B1 (ENB). Enniatin B4 (ENB4). Enniatins (ENs). Fusaric acid (FA). Lactic acid bacteria (LAB). *Lactobacillus* (L.). Minimal inhibitory concentrations (MICs). De Man–Rogosa–Sharpe agar (MRS). Potato Dextrose Broth (PDB). *Saccharomyces* (S.). Tenuazonic acid (TeA).

3.3 Mycotoxins and prebiotics

Prebiotics have been described as a potent agent to prevent metabolic disease through microbiome modulation [50]. K-carrageenan has revealed to be a promising fiber, thanks to ZEN bioaccessibility reduction, obtaining values lower than 20%. Lactose from fermented whey has shown to protect from AFB1 and OTA toxicity [86,87]. Pectin has exerted to decrease DON, HT-2, and T-2 (50–88%) bioaccessibility [59]. Garlic, whose prebiotic content is notable, has shown to reduce AFB1 cytotoxicity [88]. It has also been reported that flavonoids play an important role in protecting against the toxicity of mycotoxins like OTA, PAT, and DON in different experimental models [89,90].

3.3.1 Emerging mycotoxins and prebiotics *in vitro*

As Fig. 6 shows, *Fusarium* mycotoxins, ENs and BEA are the most studied ones. Inulin (ranging from 1.5% to 10%) has revealed to decrease the bioaccessibility of ENA, ENA1, ENB and ENB1 in a dose-dependent manner in a digestion model [91]. Cellulose (1% and 5%) has exerted to reduce ENA, ENA1, ENB and ENB1 bioaccessibility at a mean of 26.9% and 44.7%, respectively. Meanwhile, in the case of BEA, the bioaccessibility decrease ranged from 60% to 80% [71]. Inulin and fructooligosaccharides (FOS) (1% and 5%), in a static gastrointestinal digestion model, have shown to reduce ENA, ENA1, ENB, ENB1 and BEA bioaccessibility. The lowest bioaccessibility was caused by adding the inulin 5% (5.7%) and FOS 5% enriched samples (3.9%) for ENA and ENA1, respectively [92].

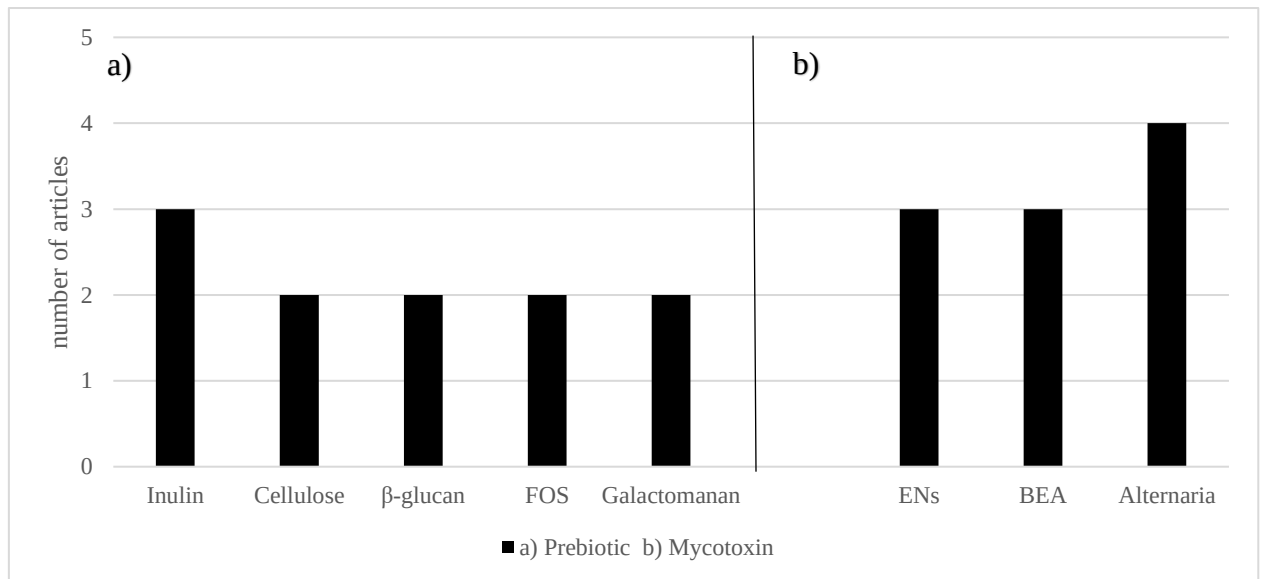


Figure 6. Graphical bars of the prebiotics (n = 9) according to (a) the prebiotic type and (b) the emerging mycotoxins studied. Beauvericin – BEA, enniatins – ENs and fructooligosaccharides – FOS. In the studies, inulin, cellulose, β-glucan, FOS and galactomannan were the most used prebiotics, whereas ENs, BEA and *Alternaria* mycotoxins were the most recurrent emerging mycotoxins.

BEA bioaccessibility was decreased, ranging from 15.7% to 60.5%, compared to the control (93.2%), with the addition of the following prebiotics: galactomannan 5%, glucomannan high-molecular weight (HMW) 1%, glucomannan HMW 5%, glucomannan fine powder 1%, glucomannan fine powder 5%, citrus fiber 1%, citrus fiber 5%, bamboo fiber 1%, bamboo fiber 5%, carrot fiber 1%, carrot fiber 5%, pie fiber 1%, pie fiber 1%, β-glucan 1%, β-glucan 5%, xilan 1%, xilan 5%, cellulose HMW 1%, cellulose HMW 5%, cellulose medium-molecular weight (MMW) 1% and cellulose MMW 5% (Roig et al., 2014). BEA has also shown to be decreased by β-1,3 glucan, chitosan LMW, chitosan MMW, FOS, galactomannan, inulin, and pectin at both concentrations (1% and 5%). Each sample was contaminated, reaching a BEA concentration of both 5 and 25 mg L⁻¹. While the mean control bioaccessibility was 92.6% and 90.0% for both BEA concentrations, respectively; when adding prebiotics, it was 13.2% and 50.4%, respectively, galactomannan being the most reducing one [93].

Similar results have been obtained with *Alternaria* mycotoxins. AOH, AME, ATX-II, STTX-III and ALP have been found to be decreased, already before anaerobic incubation, in the samples containing particulate fecal matter. These samples were

supposed to be also composed of indigestible fiber fractions [67]. The antifungal effects of the volatile compound 2-phenylethyl isothiocyanate (2-PEITC) against *A. alternata* and its mycotoxins TEN, AOH, AME, and ALT were assessed. 2-PEITC is found in some vegetables and contributes to gastrointestinal health [94]. After a 2 h treatment, these four mycotoxins were extracted and detected by high performance liquid chromatography-tandem mass spectrometry (HPLC-TOF-ESI-MS) in the mycelium of *A. alternata*. The concentrations of TEN, AOH, AME, and ALT at the MIC of 2-PEITC-treated groups were only 27%, 90%, 90%, and 88% of the corresponding control groups, respectively [95]. The anthocyanidin delphinidin ($30.3 \mu\text{g mL}^{-1}$) has exerted to strongly antagonize the genotoxic properties of ATX-II by significantly suppressing the level of DNA strand breaks by up to 75% in HT-29 colon carcinoma cells [84]. Conversely, AOH and AME degradation has been assessed after cinnamaldehyde treatment in centrifuge tubes under rotary shaking conditions (150 rpm) at 25 °C for 120 min. It was found that AOH and AME degradation rates were 16.8% and 7.3%, respectively [96]. The results are shown in Table 4.

Table 4. Collection of the most important characteristics of the studies found concerning the emerging mycotoxins and prebiotics *in vitro*. For each study, the studied mycotoxin, type of prebiotic, mycotoxin dose administration, exposure time, experimental assay, results and references are described

Mycotoxin	Prebiotic	Dose administration	Exposure time	Experimental assay	Results	References
<i>In vitro</i>						
ENA ENA1 ENB ENB1	Inulin (1.5% - 10%)	1.02- 2.04 mg/mL	8 h	<i>In vitro</i> digestion model	ENA, ENA1, ENB and ENB1 bioaccessibility reduction in a dose-dependent manner in an inulin concentration ranging from 1.5% to 10%.	[91]
ENA ENA1 ENB ENB1 BEA	Cellulose (1% and 5%)	Not reported	4 h	<i>In vitro</i> digestion model	Cellulose (1% and 5%) decreased ENA, ENA1, ENB and ENB1 bioaccessibility a mean of 26.9% and 44.7%, respectively. BEA bioaccessibility reduction ranged from 60% to 80%.	[71]
ENA ENA1 ENB ENB1	Inulin and FOS (1% and 5%)	Not reported	4 h	Static gastrointestinal <i>in vitro</i> digestion model	Highest reductions carried out when adding enriched inulin 5% (5.7%) and FOS 5% (3.9%) for ENA and ENA1, respectively.	[92]

BEA							
BEA	Galactomannan 1-5% Glucomannan HMW 1%-5% Glucomannan FP 1%-5% Citrus fiber 1%-5% Bamboo fiber 1%-5% Carrot fiber 1%-5%	Pie fiber 1%-5% β -Glucan 1%-5% Xilan 1-5% Cellulose HMW 1-5% Cellulose MMW 1-5%	5-25 mg/kg	52 h	<i>In vitro</i> digestion model	BEA bioaccessibility reduction ranging from 15.7% to 60.5% comparing to control (93.2%).	[77]
BEA	β -1,3 glucan Chitosan LMW Chitosan MMW FOS	Galactomannan Inulin Pectin (All 1% and 5%)	5 mg/kg	52 h	<i>In vitro</i> digestion model	BEA mean bioaccessibility decrease when adding prebiotics (13.2% and 50.4%, respectively for 1% ad 5%), being galactomannan the most reducing one (5.2%)	[94]
AOH AME ATX-II STTX-III ALP	Particulate fecal matter supposed to be composed also by indigestible fiber fractions		50 μ g/mL	48 h	Fecal incubation <i>in vitro</i>	AOH, AME, ATX-II, STTX-III, and ALP reduction by samples supposed to be composed also by indigestible fiber fractions reduce.	[67]
TEN AOH AME	2-PEITC		0.1-1.38 mg/kg	2 h	Qualitative analysis of performed using a mass spectrometer	TEN, AOH, AME, and ALT concentrations of 2-PEITC-treated groups were only 27%, 90%, 90%, and 88% of the corresponding control groups, respectively.	[95]

ALT				after incubation for the MIC.		
ATX-II	Delphinidin	0.35 µg/mL	24 h	Coupled SRB/WST-1 cytotoxicity assays	ATX-II genotoxic properties were antagonized by delphinidin (30.3 µg/mL) by significantly suppressing the level of DNA strand breaks by up to 75% in HT-29 colon carcinoma cells	[89]
AOH AME	Cinnamaldehyde	0.81-0.82 µg/mL	2 h	Degradation assay	AOH and AME degradation rate was 16.8% and 7.3%, respectively, after 120 min of cinnamaldehyde treatment	[96]

Alternariol (AOH). Alternariol monomethyl ether (AME). Alterperyleneol (ALP). Altertoxin II (ATX-II). Altuene (ALT). Beauvericin (BEA). Enniatin A (ENA). Enniatin A1 (ENA1). Enniatin B (ENB). Enniatin B1 (ENB). Fructooligosaccharides (FOS). High-molecular weight (HMW). Low-molecular weight (LMW). Medium-molecular weight (MMW). Minimum inhibitory concentration (MIC). Sulforhodamine B/water-soluble tetrazolium salt (SRB/WST-1). Tentoxin (TEN). 2-phenylethyl isothiocyanate (2-PEITC). Stemphyliotoxin III (STTX-III).

3.4 Mycotoxins and synbiotics

The use of synbiotics has been previously proved to be effective in the removal of PAT [97,98]. The deleterious effects of OTA have also been reversed by using beneficial synbiotics possessing the ability to reduce the number of potential pathogens in the digestive tract and by increasing the activity of α -glucosidase and α -galactosidase, while decreasing the activity of potentially harmful fecal enzymes (β -glucosidase, β -galactosidase and β -glucuronidase) [60,99].

3.4.1 Emerging mycotoxins and synbiotics

The bioaccessibility of ENs (ENA, ENA1, ENB, and ENB1) and BEA was analyzed by using an *in vitro* static and dynamic simulated gastrointestinal digestion system, imitating the digestive physiological conditions until the colonic compartment. Inulin and FOS at two concentrations (1% and 5%) were first added, and afterwards, the colonic microbial fermentation was carried out by using *L. paracasei* CECT 277, *L. casei* CECT 4180, *L. rhamnosus* CECT 278T, *L. plantarum* CECT 220, *L. ruminis* CECT 4061T, *L. casei casei* CECT 277, *B. breve* CECT 4839T, *B. adolescentis* CECT 5781T, *B. bifidum* CECT 870T, *B. longum* CECT 4551, *Corynebacterium vitaeruminis* CECT 537, *Eubacterium crispatus* CECT 4840 and *S. cerevisiae* CECT 1324. The bioaccessibility of EN and BEA was shown to be higher in the synbiotic prebiotic static model (6.2%–44.9% and 46.7%–61.1%, respectively) than that found in the dynamic one (23.0%–68.9% and 76.2–91.0%, respectively) [92]. Similar results have been revealed in Meca *et al.* (2012) [93], where the fibers β -1,3 glucan, chitosan LMW, chitosan MMW, FOS, galactomannan, inulin and pectin (1% and 5%) were combined with the bacteria *L. animalis* CECT 4060T, *L. casei* CECT 4180, *L. casei rhamnosus* CECT 278T, *L. plantarum* CECT 220, *L. ruminis* CECT 4061T, *L. casei-casei* CECT 277, *B. breve* CECT 4839T, *B. adolescentis* CECT 5781T, *B. bifidum* CECT 870T, *Corynebacterium vitaeruminis* CECT 537, *Streptococcus faecalis* CECT 407, *Eubacterium crispatus* CECT 4840, and *S. cerevisiae* CECT 1324. *In vitro* mean bioaccessibility values of BEA (at both experimental concentrations: 5 and 25 mg L⁻¹) by using synbiotics were 12.9% and

42.4%, respectively – 5.3 and 28.2-fold higher in comparison with the data evidenced with the prebiotic sample treated with duodenal digestion [93].

3.5 Mycotoxins and postbiotics

Postbiotics are considered to have positive effects on human health [50]. Yeast cell wall extract (YCWE) and a post-biotic yeast cell wall-based blend (PYCW) have revealed to reduce the adverse effects related to serum biochemistry, liver function, immune response, altered cecal short chain fatty acids (SCFAs), goblet cell counts and architecture of the intestinal villi. These results were found studying commercial broilers fed with a diet containing DON, T-2 and ZEN at 3.0 mg kg^{-1} , $104 \text{ } \mu\text{g kg}^{-1}$ and $79 \text{ } \mu\text{g kg}^{-1}$, respectively [57]. Some characteristic bacterial enzymes, which isolated are considered postbiotics, have exerted to remove AFB1, OTA, DON and ZEN [63]. Butyrate, one of the most studied gut microbiota metabolites, has shown to reverse the alterations caused by DON by blocking some genes involved in reactive oxygen species and tumor necrosis factor alpha mediated pathways [100]. Likewise, AFB1 and ZEN have been reported to reduce the content of SCFAs (butyrate, propionate and acetate) [101,102].

3.5.1 Emerging mycotoxins and postbiotics

The utilization of postbiotics (PYCW) for addressing emerging mycotoxins was first documented in Xu *et al.* (2023) [103]. In contrast to inorganic clay-based adsorbents characterized by presenting some limitations, such as the possible contamination with other detrimental compounds (dioxins, polychlorinated biphenyls, and heavy metals), organic adsorbents such as PYCW have shown to be more efficient and safe product substitutes [104]. In this study, an absorption bioassay was conducted exposing BEA (0.5, 2, 5, 20, 40 and $63 \text{ } \mu\text{g mL}^{-1}$) and CIT (0.5, 1, 10, 40, 50 and $68 \text{ } \mu\text{g mL}^{-1}$) to PYCW at different concentrations for 2 h. The results showed that PYCW presented an overall mean adsorption efficiency of 70.1% for BEA and 35.1% for CIT [103].

4. Discussion

The interactions along the gut are very complex due to the quantity of factors, which play a notable role in its physiology, so that there are times where it is so likely not to know completely which specific pathways or molecules are the consequence of a particular fact. However, there are some established correlations which help us to better understand how biological systems work. One of these strong correlations is the preventive role of PPSP and the gut microbiota in disease and its health-promotion effects [49]. As has already been said [62], PPSP and the gut microbiota possess the capacity to bind and metabolize a vast quantity of substances entering the gastrointestinal tract. Therefore, despite theoretically reducing beneficial compounds, PPSP can also decrease the exposure to detrimental ones, such as toxins, including emerging mycotoxins.

As presented, European regulations only encompass a small set of mycotoxins (AFB1, AFB2, AFG1, AFG2, AFM1, OTA, PAT, DON, ZEN, FB1, FB2, CIT, AOH, AME, TeA, T-2 and HT-2 and ergoalkaloids) [2]. Due to their regulation, these mycotoxins tend to be more studied – also including their interactions with the gut microbiota and PPSP. In fact, studies show that studied mycotoxins disrupt the gut microbiota, while concomitant intake of PPSP mitigates the effects of the mycotoxins and contributes to maintain gut microbiota homeostasis [56,58,90,99,101]. Although similar trends are expected with unregulated mycotoxins, it has to be carried out as a one-on-one mycotoxin analysis, since every mycotoxin possesses a specific chemical structure and a different response in the environment where it is located [62]. Actually, there are some 400 known mycotoxins, so the vast majority of them are not still regulated and need a more profound comprehension [1]. Furthermore, it should not be forgotten that mycotoxins tend to be found in combinations, so the most common mixtures found in food and feed are the ones interesting to be tested.

4.1 Emerging mycotoxins and the gut microbiota

Regarding the interaction between the gut microbiota and emerging mycotoxins, studies have revealed a clear tendency: the gut microbiota contributes to emerging

mycotoxin detoxification through binding and/or metabolization [63-65,67] and emerging mycotoxins alter the gut microbiota composition [68]. Despite being the trend confirmed by these studies, the difficulty stems from the heterogeneity of the analyzed samples. The microbiota exhibits significant variability, with the microbiome differing by species. In humans, microbiota changes are influenced by factors such as the level of urbanization of the location and even the time of day within the same individual [105-107]. For example, rumen microbiome is characterized by possessing many enzymes involved in the *de novo* synthesis of vitamin B12 [108].

According to experimental feasibility, it has been shown that gut microbiota samples are obtained from feces in most of the experiments, since the fecal microbiota is more accessible than the colon one. Interestingly, it has been revealed that colon microbiota has a different profile than the fecal one, the microbiome in the large intestine being much more diverse than those of the small intestine and feces. For instance, while in the large intestine there were 4080 operational taxonomic units, in the feces only 443 were found [109]. Indeed, bacterial biomass also changes along the gastrointestinal tract and the feces: duodenum (10^3 bacteria per g), jejunum (10^4 g⁻¹), ileum (10^7 g⁻¹), colon (10^{12} g⁻¹) and feces (5^{10} g⁻¹) [110,111]. In addition, short-term trials are likely to hide that long-term mycotoxin exposure may lead to the development of the ability to degrade these mycotoxins despite being unable to do it in the first moment [64].

There are other limitations that should be highlighted. Although in Debevere *et al.* (2020) [63] the incubation time (48 h) was in line with other experiments studying mycotoxin degradation *in vitro*, it is considerably longer than the *in vivo* retention time of the digesta in the rumen, implying that under normal physiological conditions the results would be different. Despite Crudo *et al.* (2020) [67] having shown that AOH, AME, ATX-II, STTX-III and ALP were lower in the samples containing microorganisms than in those without them, it should be noted that the sample size was very small ($n = 4$). In this study, all donors shared some characteristics: they were healthy omnivorous volunteers (two males and two females) ranging from 26 to 34 years old and with a normal body mass index ($18.5\text{--}24.9$ kg m⁻²), none of them had been treated with antibiotics, probiotics, and prebiotics for the previous three months and they did not present previous intestinal diseases. However, the entire diet, playing a notable role in gut metabolism and in the interactions with mycotoxins, was not considered. Moreover, as mentioned in the same article, there are other limitations such as the short-term trial (3 h) and the fact that

the growth media used might alter the initial microbial composition of the feces, contributing to the growth of some microorganisms at the expense of others. Conversely, in Novak *et al.* (2021) [68], only the mycotoxins ENB, ENB1, BEA and DON were assessed in weaning piglets' feces in contrast to the control. It was shown that *L. amylovorus* and *L. reuteri*, both beneficial microbes, were up-regulated and down-regulated, respectively, when adding BEA in comparison with DON and the control. Furthermore, the combination of ENs and BEA increased the total protein synthesis, but the addition of DON had an inhibitory effect [68]. Although it is important to know the interactions among these four mycotoxins, the real conditions are more complex, since a meal may contain a variety of contaminants, so the results under normal conditions are so likely to differ from these ones. While microbes could metabolize emerging mycotoxins, these toxins can also influence the composition of the microbiome, creating a potential double-edged sword scenario. In addition, it is important to consider the varying doses of emerging mycotoxins used in experiments, ranging from nanograms to milligrams.

Viewing mycotoxins as toxic compounds or as potential drugs is indeed a question that arises. On the one hand, they can reduce beneficial microorganisms, such as *Lactobacillus*, *S. ceverisiae* and *Bacillus* [72,73,79,81] whose depletion is generally connected to disease promotion [112]. On the other hand, they can play a role as antibiotics by eliminating microorganisms involved in diseases [112-116]. For instance, AOH and AME have exerted to be promising inhibitors of glycogen-synthase-kinase 3, being an important target in drug discovery. In fact, this enzyme has revealed to participate in signaling pathways of type 2 diabetes, cancer, neurological disorders, and other diseases [117]. In addition, ergotamine with its α -adrenoreceptor-blocking mode of action has also been used for acute migraine treatments for over 50 years [118]. Further investigation is needed in the field to clarify the results and assess the real implications of these emerging mycotoxins towards health and diseases, also taking into consideration that the dose and the type of mycotoxin may be decisive in the health or disease-promoting outcomes.

4.2 Emerging mycotoxins and probiotics

The bidirectional fluxes between probiotics and emerging mycotoxins have been widely confirmed. A vast quantity of probiotics (*Lactobacillus*, *Bifidobacterium* and *S. cerevisiae*) has shown to reduce emerging mycotoxins' bioaccessibility (ENs, BEA and *Alternaria*) [74-78,92,93]. At the same time, emerging mycotoxins have exerted to alter probiotic compositions (*B. subtilis* and *Staphylococcus aureus*) [72,73,79,81]. This bioaccessibility decrease may be due to the bacterial mycotoxin binding capacity [83,84] and/or their ability to contribute to detoxification by originating derivatives characterized by possessing less toxicity [82,83]. However, in most of these studies, probiotics are not assessed in combination, but one by one and using *in vitro* conditions. Only Vadopalas *et al.* (2020) [85] assessed probiotics' effects on emerging mycotoxins under *in vivo* conditions. In this study, not only did the addition of some microbial starters modified some blood parameters, but also contributed to modulate the gut microbiota and detoxify AME. Despite these beneficial effects, it should be noted that the experiment only lasted two months and the piglets' organs were not fully mature. Combining probiotics and working *in vivo* would be a more realistic approach to the human environment, since there are a lot of microorganisms that form a part of the gut microbiota [119]. What is more, it is very well documented that the interplay along the gut concerns not only different bacteria, but also fungi, archaea, viruses, and helminths [120].

4.3 Emerging mycotoxins and prebiotics

It has been clearly shown that prebiotics reduce emerging mycotoxin bioaccessibility. Although the term prebiotic is very vast, the collected data cover a high quantity of these types of compounds at a different concentration [67,71,77,91-93,95,96]. As in the probiotic case, there are two mechanisms explaining this reduction. The first one is the capacity of prebiotics to bind emerging mycotoxins, and the second one is the ability to metabolize them, and it is very likely to have both processes working at the same time. However, most studies do not consider an analysis of secondary metabolites of emerging mycotoxins, so this metabolization cannot be fully confirmed. Regarding

mycotoxin reduction, if the metabolites have not been degraded, it is assumed that they have bound to the structures of the prebiotics, although they are also subject to metabolization anyway. Conversely, apart from reducing bioaccessibility, prebiotics can also directly suppress the detrimental effects of mycotoxins. As it is shown in Aichinger *et al.* (2018), the anthocyanidin delphinidin blocks significantly ATX-II genotoxic properties by suppressing the level of DNA strand breaks [89]. The action of these prebiotics may derive from blocking the mycotoxin, preventing its action or from having a beneficial agonist impact on some physiological pathways. Furthermore, it should also be noted that in all these experiments, prebiotics are evaluated only one by one. The reality of the human diet includes a wide combination of them, so more research needs to be carried out taking this complexity into account.

4.4 Emerging mycotoxins, synbiotics and postbiotics

The role of synbiotics in the modulation of emerging mycotoxins has been assessed only in two studies [92,93]. In both, the results showed that the addition of synbiotics increased mycotoxin bioaccessibility when compared to prebiotics. It has been hypothesized that probiotics degraded prebiotics, so that the binding ability of the last ones was lost. Although it may be possible, it cannot be extended as a generality, since only small fractions of prebiotics and probiotics were used in these two trials, and they do not represent either all probiotics or complete prebiotics. Conversely, those synbiotics applied to non-emerging mycotoxins reveal opposite results. For instance, Zoghi *et al.* (2017) [97] showed that the removal of PAT by probiotic bacteria from apple juice depended significantly on the FOS content (as a prebiotic) and the addition of ascorbic acid. Under the best conditions, 91.3% of the initial patulin concentration was removed from juice for 6 weeks [97]. Zoghi *et al.* (2019) also reported that by adding FOS, ascorbic and citric acid to apple juice inoculated with *L. plantarum*, the efficiency of PAT removal was improved to 95.9% for 6 weeks cold storage [98]. On the other hand, despite being supposed to enhance clinical outcomes compared to probiotics or prebiotics alone due to their synergistic effect, synbiotics are very difficult to formulate and few have shown to possess clinical efficacy. It is also worth mentioning that most clinical trials are not characterized by including relevant probiotic or prebiotic controls, and many have not

performed relevant microbiota analyses [121]. Research needs to be expanded to more (emerging) mycotoxins in combination with synbiotics.

Finally, postbiotics have shown to bind, metabolize and alleviate the harmful effects of some emerging and non-emerging mycotoxins [57,63,100,103], as well as these mycotoxins have revealed to alter the composition of some postbiotics [101,102]. Notwithstanding, it is needed to extend these experiments to more (emerging) mycotoxins and to more postbiotics, since only the combination of PYCW with BEA and CIT has been assessed. In fact, it will be very interesting to focus on the interplay between (emerging) mycotoxins and SCFAs.

4.5 Interplay between emerging mycotoxins, the gut microbiota and PPSP

Although the results have revealed a clear trend, data on this interaction have only been found in 12 of the 20 emerging mycotoxins investigated. However, apart from these 20 ones, other emerging mycotoxins were found. The collected data encompassed a lot of experimental differences (mycotoxin/biotic type and concentration, species, time exposure, assay type, etc.), making the comparisons difficult among them. As Fig. 7 illustrates, the interaction between the gut microbiota and emerging mycotoxins involves metabolization and binding processes. The role of PPSP in relation to emerging mycotoxins primarily revolves around colonization (probiotics) and modulation (all). Furthermore, there exists a two-way communication between microbiota and PPSP, where the gut microbiota can degrade and metabolize PPSP, activating secondary bioactive compounds that may contribute to the detoxification and/or alleviation of emerging mycotoxins. Similarly, PPSP can enhance certain gut microbiota strains involved in the removal of emerging mycotoxins. Indeed, this reciprocal relationship can also be applied to the entire spectrum of PPSP, as the addition of probiotics, prebiotics, and synbiotics may enhance the production of certain postbiotics (such as short-chain fatty acids), and vice versa.

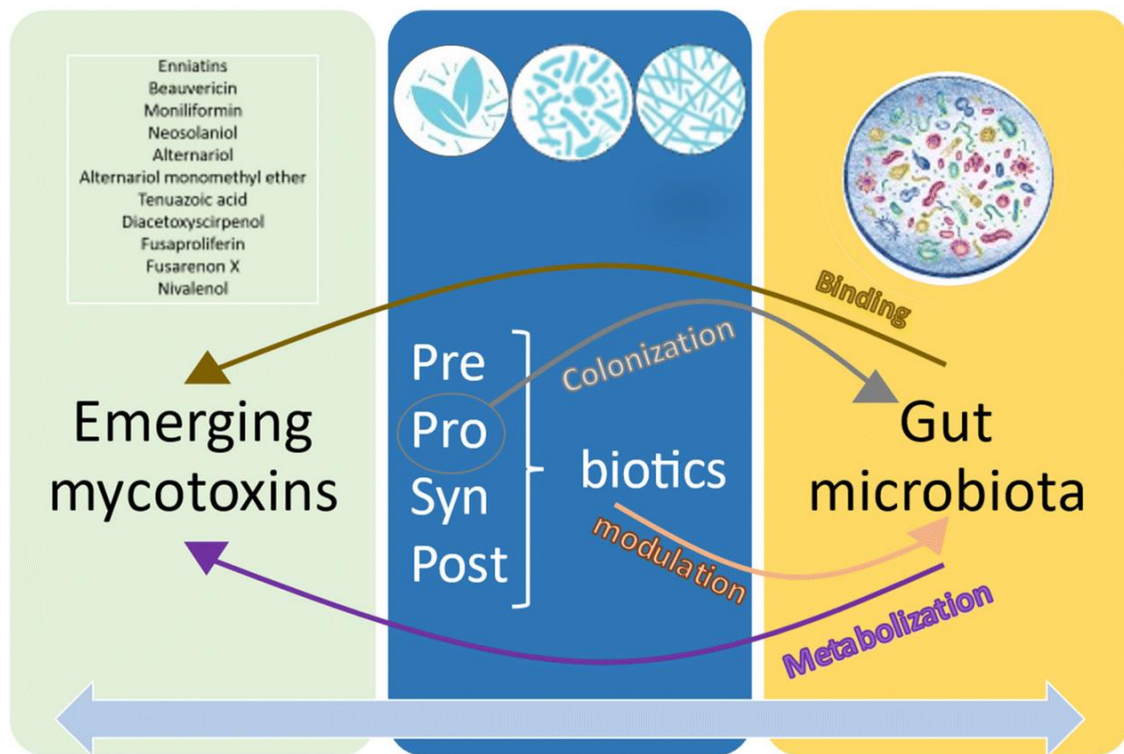


Figure 7. Detoxification processes of the emerging mycotoxins by probiotics, prebiotics, synbiotics and postbiotics (PPSP) and the gut microbiota. Emerging mycotoxins are metabolized and bound by the gut microbiota. At the same time, PPSP contributes to colonize and modulate the gut microbiota, and also have an indirect impact on the metabolism of the emerging mycotoxins.

5. Conclusions

Emerging mycotoxins have been implicated in numerous harmful biological processes. Despite their diverse nature, studies have demonstrated that the gut microbiota and PPSP can detoxify and/or alleviate the adverse effects resulting from exposure to these emerging mycotoxins. ENs, BEA, NIV, and *Alternaria* mycotoxins were among the most frequently encountered emerging mycotoxins. The genera *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces* were the most extensively studied probiotics, while inulin, cellulose, β -glucan, FOS, and galactomannan were among the most evaluated prebiotics. Data on synbiotics and postbiotics were limited. Evidence clearly demonstrates the existence of interplay between the gut microbiota and emerging mycotoxins, as well as between PPSP and emerging mycotoxins, involving processes

such as metabolization, colonization, modulation, and binding. In addition, connections between the gut microbiota and PPSP, as well as within the same PPSP, were identified, all contributing to the alleviation of the effects of the emerging mycotoxins. However, quantitative and qualitative data remain limited. Further *in vivo* and long-term trials are necessary to better comprehend the complexity of these interactions in both animals and humans. The formulation of PPSP as functional food and nutraceuticals should be considered a preventive strategy against the toxicity of emerging mycotoxins in human diets and animal feed.

Author contributions

Á. Lázaro: writing – original draft, methodology, investigation and validation. P. Vila-Donat: methodology, investigation, and review and editing. L. Manyes: investigation, review and editing, project administration, and conceptualization.

Acknowledgements

This publication is part of the project PID2022-140722OB-I00 funded by MCIU/AEI/AEI/10.13039/501100011033/FEDER, EU.

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5.3 Artículo 3: Evaluación de los efectos beneficiosos de la calabaza y el suero fermentado sobre el perfil de la microbiota intestinal frente a la exposición a AFB1 y OTA en ratas Wistar

**Effects of pumpkin and fermented whey on fecal microbiota profile
against AFB1 and OTA exposure in Wistar rats**

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Abstract

Mycotoxins have been pointed out to perturb the gut microbiota performance. Bioactive compounds have been recently used as a new food strategy to diminish mycotoxins bioaccessibility and prevent their toxic effects on human and animal health. Male and female Wistar rats were exposed orally to twelve different diets containing aflatoxin B1 (AFB1) and/or ochratoxin A (OTA) including or not fermented whey (FW) and pumpkin (P) for 28 days. Fecal microbiota using 16S rRNA gene sequencing and subsequent metagenomics analysis were analyzed to study the effect of 28 day-exposure through diet of contaminated and enriched feed. QIIME 2 microbiome analysis package (version 2024.5) was used to analyze the demultiplexed data. Mycotoxins-functional ingredients combination contributed more to microbial phylogenetic faith α -diversity rather than the functional ingredients alone, while the same combination reported a microbial α -diversity enhancement in comparison to the mycotoxins alone. Proteobacteria phylum was reduced in rat samples fed with contaminated diets (AFB1, OTA and AFB1+OTA), while there was an increase – although not in all groups - when adding the functional ingredients. The main difference between the sexes was found in FW+AFB1+OTA group, with males (25%) showing higher % of Proteobacteria than females (1.86%). PD (Phylogenetic diversity) faith only focuses on microbial genetic (dis)similarity, not considering the biological function. *Morganella morganii*, a Proteobacteria found in some groups presents anticancer activity, but it is also related to inflammatory bowel disease and colorectal cancer. To sum up, both mycotoxins and functional ingredients trigger changes in the microbiota profile of Wistar rats in a sex-specific manner.

Keywords: mycotoxins; probiotics; feces; microbiota; sexual dimorphism

1. Introduction

Despite more than 400 mycotoxins have been identified, the most toxic and studied are aflatoxin B1 (AFB1) and ochratoxin A (OTA), accounting for a notable economic and public health concern. Even at lower concentrations, these mycotoxins can affect human and animal health by disrupting nutrient digestion, absorption, metabolism, and normal physiology (Manyes & Font, 2023).

AFB1 is a secondary metabolite produced by the *Aspergillus* genera, which is a mycotoxigenic fungi contaminating a wide diversity of foodstuff such as rice, maize, milk, meat, pistachios, nuts, seeds, and spices. AFB1 is very well documented because of its serious carcinogenicity for both humans and animals (EFSA, 2020). In fact, the International Agency for Research on Cancer (IARC) classified AFB1 as a human carcinogen belonging to Group 1 due to its participation in DNA adducts construction. Its acute and chronic toxicity have been displayed through several *in vitro* and *in vivo* studies connecting to immunosuppression, nutritional dysfunctions, cancer, and liver damage (Cimbalo et al., 2020; Marchese et al., 2018). OTA is another world-pervasive mycotoxin which is largely produced by *Aspergillus* and *Penicillium* species. Literature shows that OTA-exposure can enhance DNA damage, oxidative stress, inflammation and cell death. Moreover, IARC classified OTA in the group 2B as possibly carcinogenic to humans. Typical foods contaminated by OTA are cereals, meat, dried fruits, wine and spices (Cimbalo et al., 2020; Frangiamone et al., 2022).

According to the Commission Regulation (EU) 2023/915 of April 25, 2023 legislative framework, the regulated mycotoxins maximum limits in humans are established, among them, those belonging to AFB1 and OTA. The EU maxim limits established for AFB1 are 8 µg/kg for groundnuts (peanuts) and other oilseeds, hazelnuts and Brazil nuts; 5 mg/kg for cereals and products derived from cereals with some exceptions, and 2 µg/kg for almonds, pistachios and apricot kernels. The maximum limits for OTA are 20 µg/kg for marshmallow and dandelion roots (dried); 5 mg/kg for unprocessed cereal grains; 3 mg/kg for products derived from unprocessed cereal grains; 20 µg/kg for orange blossoms (dried) for use in herbal infusions or in coffee substitutes;

and 80 µg/kg for liquorice extract for use in food in particular beverages and confectionary.

Mycotoxins have been pointed out to perturb the gut microbiota performance. It has been described that AFB1 disturbs gut microbiota-dependent organic acid metabolism and OTA changes the structure and diversity of gut microbial communities, mainly because of the alteration of the short-chain fatty acids (SCFA) metabolism (Lázaro et al., 2024). (Lázaro et al., 2024). The gut microbiota has emerged as a key factor in a substantial quantity of diseases such as diabetes, obesity, dementia, Parkinson, inflammatory bowel disease, anxiety, depression and cancer (Ancona et al., 2021; Daily et al., 2021; Menozzi et al., 2021; Micic et al., 2023; Moughnyeh et al., 2021). Microbiota has been coined as the last human organ whose complexity of interactions is under current research (Baquero et al., 2012). There are some factors, such as diet, antibiotics, species, age and sex playing an important role on gut microbiota modulation (Hasan & Yang, 2019; Valeri & Endres, 2021). Although microbiota may be only understood as bacteria, since they are the most numerous, but also fungi, archaea, viruses and helminths belong to it (Vemuri et al., 2020). In order to better comprehend the relevance of the microbes, it should be highlighted that, whereas there are only 26600 human genes, more than 4,000,000 bacteria genes can be found in human body. Furthermore, bacteria highest density in any ecosystem on earth is found in the human colon (10^{11} - 10^{12} / mL) (Sender et al., 2016).

Bioactive compounds have been recently used as a new food strategy to diminish mycotoxins bioaccessibility and prevent their toxic effects on human and animal health (Frangiamone et al., 2023). Also, it has been shown that bioactive compounds have a positive impact on the health (the intestine and the whole body) of host animals by enhancing the abundance of beneficial bacteria and decreasing the abundance of harmful bacteria (Chen et al., 2022). Pumpkin (P) is a well-known ingredient containing polyunsaturated fatty acids, tocopherols, carotenoids, delta-7-sterols, tryptophan and many other phytochemicals. This multifunctional ingredient is characterized by presenting some benefits such as anti-inflammatory, antimicrobial antioxidant, and anti-ulcerative properties, improving spermatogenesis, wound healing and treatment of benign prostatic hyperplasia (Batool et al., 2022). In fact, pumpkin components have shown to have a beneficial effect on human gut microbiota by enhancing (SCFA) - and SCFA producing bacteria, such as *Akkermansia*, *Bacteroides*, and *Lachnospiraceae_NK4A136*

group (Yan et al., 2025). Milk whey, a cheese by-product with high nutritional values, is an excellent source of lactose, proteins, small amounts of vitamins and minerals, and an optimal substrate for lactic acid bacteria fermentation (Gupta & Prakash, 2017), which originates the fermented whey (FW). Some of its benefits are related to the improvement of urinary function, urinary tract symptoms, systemic oxidative stress, and protection against bacterial infection (Ausmees et al., 2018; Cordeiro et al., 2019). Furthermore, it has been revealed that FW augments butyrate and propionate production, one of the most important mechanisms through which improves the gut microbiota profile (Smith et al., 2025).

Previously, it has been reported that AFB1 and OTA alter gut microbiota in mice, sheep, broilers, hens, ducklings and rats (Lázaro et al., 2024). Interestingly, sex hormones have recently appeared to be potent drivers of microbiota regulation (Valeri & Endres, 2021). However, there are no studies which focus on microbiota sex differences when exposing animals to AFB1 and OTA. In fact, a human study analyzing the composition of the gut microbiota during adolescence revealed higher fecal microbiota dissimilarity in teenager (13–17 years) twin pairs with opposite sex in comparison to twins with the same sex (Yatsunenکو et al., 2012). What is more, rats subjected to adult ovariectomy or neonatal androgenization, as a model of menopause, reported changes in gut microbiota composition (Moreno-Indias et al., 2016).

Therefore, the aim of the present study was to investigate the effects on the gut microbiota profile after AFB1 and OTA oral chronic exposure and the possible beneficial effects provided by fermented whey and pumpkin feed enrichment considering the interplay of sexual dimorphism in Wistar rats.

2. Material and methods

2.1 Experimental design and mycotoxin exposures

As Vila-Donat *et al.* (2024) previously described, Wistar rats (n = 120) were randomly divided into twelve groups (10 rats for each, five females, and five males) and exposed orally to twelve different diets during 28 days: 1) control feed; 2) AFB1 (4.9

mg/kg), 3) OTA (6.0 mg/kg), 4) AFB1 (4.8 mg/kg) and OTA (6.4 mg/kg), 5) FW (1%) feed, 6) FW (1%) and AFB1 (4.3 mg/kg), 7) FW (1%) and OTA (8.3 mg/kg), 8) FW (1%) with AFB1 (4.5 mg/kg) and OTA (7.5 mg/kg), 9) FW (1%) and pumpkin (1%) feed, 10) FW (1%) and pumpkin (1%) feed and AFB1 (4.7 mg/kg), 11) FW (1%) and pumpkin (1%) feed and OTA (5.4 mg/kg) and 12) FW (1%) and pumpkin (1%) feed, AFB1 (5.2 mg/kg) and OTA (8.8 mg/kg).

Vila-Donat et al. (2024) estimated the mycotoxin ingestion in these rats, reporting values of 176-387 µg/kg bw for AFB1 and 162-462 µg/kg bw for OTA. While real-world exposure levels are generally lower (1.26 ng AFB1/kg bw and 4.1 ng OTA/kg bw), (Hoteit et al., 2024), higher concentrations were used in this study to obtain clearer results.

Feed production was carried out in the laboratory to prepare the control diet and the other experimental diets. The main ingredients included wheat flour, mineral water, sugar (sucrose), and salt. Contaminated and supplemented feeds were then prepared with minor modifications to the control recipe. Fecal samples were collected weekly, with each animal housed individually in a metabolic cage for a 24-hour period following feed exposure. Samples for this study, were collected specifically during the third week of the trial.

2.2 DNA extraction and purification

DNA extraction was performed using 50 mg of feces from each rat using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research Corp., Irvine, CA, USA) according to the instructions of the manufacturer. To enhance the efficiency of cell lysis, Biospec Mini-BeadBeater-96 centrifuge (BioSpec Products, Bartlesville, OK, USA) was used 1 min at maximum speed (2400 rpm) and 5 min rest in ice repeating the cycle 5 times. The purity and quantity of DNA were spectrophotometrically assessed through a NeoDot UV/vis Nano Spectrophotometer (Neo Biotech, Nanterre, France) showing concentrations between 39.1 ng/µg and 163.2 ng/µg along with 260/280 nm and 260/230 nm ratios around 2. A total of 103 samples met the quality requirements and were therefore sequenced (Table 1).

Table 1. Number of feces samples (n) sequenced from each group and sex (n=103). AFB1, aflatoxin B1. FW, fermented whey. OTA, ochratoxin A. P, pumpkin.

Group exposure	male rats (n)	female rats (n)
Control	5	5
AFB1	5	5
OTA	4	4
AFB1+OTA	5	5
FW	5	5
FW+AFB1	5	4
FW+OTA	2	1
FW+AFB1+OTA	5	5
FW+P	4	3
FW+P+AFB1	4	5
FW+P+OTA	4	3
FW+P+AFB1+OTA	5	5

2.3 16S rRNA gene sequencing

Variable V3 and V4 regions of the 16S rDNA gene were amplified following the 16S rDNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15044223 Rev. A). Gene-specific primers (PCR1_f: 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCA G3';PCR1_r:5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHV GGGTATCTAATCC-3') containing Illumina adapter overhang nucleotide sequences were selected according to Klindworth *et al.* (2013). After 16S rDNA gene amplification, the multiplexing step was performed using Nextera XT Index Kit. 1 µL of the PCR product was run on a Bioanalyzer DNA 1000 chip (Agilent, Santa Clara, California, USA) to verify the amplicons size (~550 bp). After size verification the libraries were sequenced using a 2 × 301 pb paired-end run (MiSeq Reagent kit v3) on a MiSeq Sequencer according to manufacturer's instructions (Illumina, San Diego, California, USA).

2.4 Bioinformatic analysis

QIIME 2 microbiome analysis package (version 2024.5) was used to analyze the demultiplexed data provided by the Genomics Unit (Bolyen et al., 2019). For sequence quality control and feature table construction, Dada2 plugin was applied (Callahan et al, 2016). Dada2 filtered out noisy sequences, corrected errors in marginal sequences, removed chimeric sequences, removed singletons, joined denoised paired-end reads, and dereplicated those sequences. The end product is an amplicon sequence variant (ASV) table, a higher-resolution analogue of the traditional operational taxonomic units (OTUs) table, which records the number of times each exact amplicon sequence variant was observed in each sample. OTU and ASV metabarcoding analyses yield similar results with regard to examining microbial diversity and functionality, ASV-based analysis is able to capture slightly more detailed gut microbial diversity compared to OTU-based analysis (Inglis et al., 2022). Trimming left parameters were 0 bases forward and 10 reverse. Truncation length chosen was 290 bases forward and 220 reverse.

The next step was the generation of a tree for phylogenetic diversity (PD) analyses, using align-to-tree-mafft-fasttree pipeline from the q2-phylogeny plugin (Price et al., 2010). QIIME 2's diversity analyses are available through the q2-diversity plugin, which supports computing alpha and beta diversity metrics, applying related statistical test (Kruskal-Wallis), and generating interactive visualizations. Sampling depth chosen was 18520, the value that was as high as possible to retain more sequences per sample, while excluding as few samples as possible. Alpha diversity is shown by Faith's PD plot, a qualitative measure of community richness that incorporates phylogenetic relationships between the ASVs (Faith, 1992). Beta diversity is represented by Bray–Curtis and Jaccard Distance 3D Emperor plot, to measure of community dissimilarity.

Alpha rarefaction plot was produced by QIIMEdiversity alpha-rarefaction visualizer. It was used to determine if the richness of the samples had been fully observed or sequenced. If the lines in the plot appear to approach a slope of zero at some sampling depth along the x-axis, that suggests that collecting additional sequences beyond that sampling depth would not be likely to result in the observation of additional ASVs.

To explore the taxonomic composition of the samples, a feature classifier was trained with q2-feature-classifier (Bokulich et al., 2018). Taxonomic classifiers perform best when they are trained based on the specific sample preparation and sequencing parameters, including the primers that were used for amplification and the length of the sequence reads. The Greengenes2 sequence used was 2022.10.backbone.full-length.fna.qza (McDonald et al., 2024). Forward primer sequence applied was 5'-GTCGGCAGCGTCAGATGTGTATAA -3' and reverse, 5'-GGACTACHVGGGTATCTAAT 3'. Minimum length was 150 and maximum, 300.

2.5 Statistical analysis

Taxonomic differential abundance analysis and plots (comparisons among groups) were carried out by classical univariate analysis using Kruskal–Wallis test by IBM SPSS Statistics 29.0.2.0 (IBM Corp, Armonk, NY, USA). Equations for Bray-Curtis were developed for the study of beta diversity. All data were tested for normality using the Shapiro-Wilk test and subsequently analyzed using the appropriate statistical methods. A p-value of <0.05 was considered statistically significant.

3. Results

The fecal microbiota from 12 groups of control and exposed rats were analyzed using 16S rRNA gene sequencing and subsequent metagenomic analysis to study the effects of 28-day exposure to contaminated and enriched feed through the diet. Alpha rarefaction was used to determine if the richness of the samples has been fully observed or sequenced. The sequencing depth number represents the unique sequencing reads that align to a region in a reference genome or de novo assembly. The curves of the 12 groups reached a plateau, indicating that the sequencing depth used in this study was adequate in evaluating the microbial diversity of each sample and in precisely describing the fecal microbial communities. After denoising the samples using Dada2 plugin (Callahan et al, 2016), 1,907,560 (32%) features were retained in 103 (100%) samples at 18520 sampling depth.

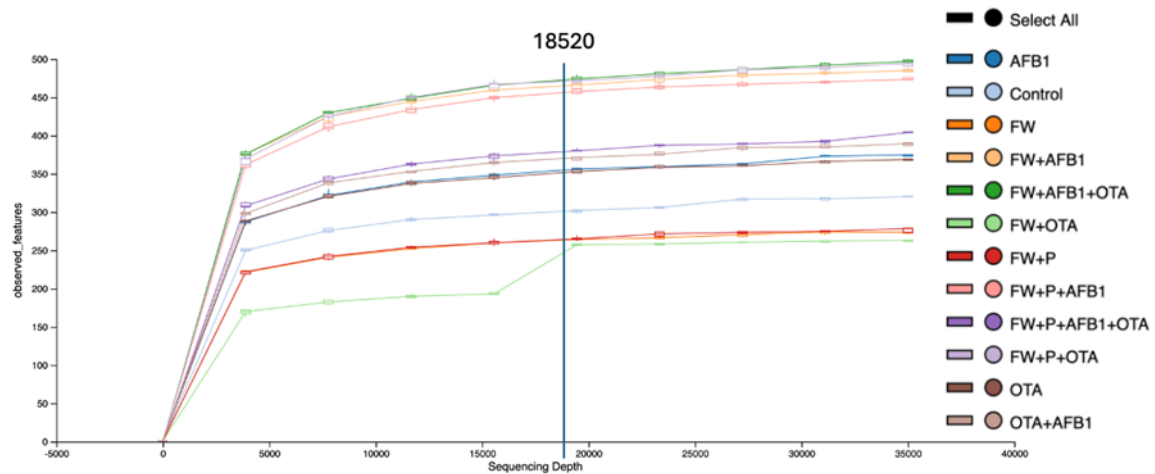


Figure 1. Rarefaction curves of observed features for each group. The x-axis represents the number of sequences per sample and the y-axis refers to the observed number of features. The plateau indicates that the fecal microbial community was fully detected with the sequencing depth used. AFB1, aflatoxin B1. FW, fermented whey. OTA, ochratoxin A. P, pumpkin.

3.1 Diversity analysis

3.1.1 Alpha diversity results

Faith's PD is the sum of the branch lengths on the minimum spanning tree linking a set of terminal taxa to the root. So, a higher number means more branches, that is to say, more diversity. There were significant statistical differences interesting to highlight in figure 2 after applying the Kruskal-Wallis test regarding diet exposure. Groups FW+P+OTA and FW+AFB1+OTA showed more diversity than control ($p=0.019$ in both cases). Likewise, microbiota in FW+AFB1 ($p=0.002$) and FW+AFB1+OTA ($p=0.001$) were more diverse than in FW. The group FW+AFB1+OTA ($p=0.028$) showed a richer diversity than FW+OTA. Finally, FW+P+OTA was more diverse than OTA ($p=0.049$) and FW+OTA ($p=0.030$). In the supplementary material, some differences including sex variable can be observed. Only two groups showed significant differences between males and females, AFB1 ($p=0.016$) and FW+P+AFB1+OTA ($p=0.009$).

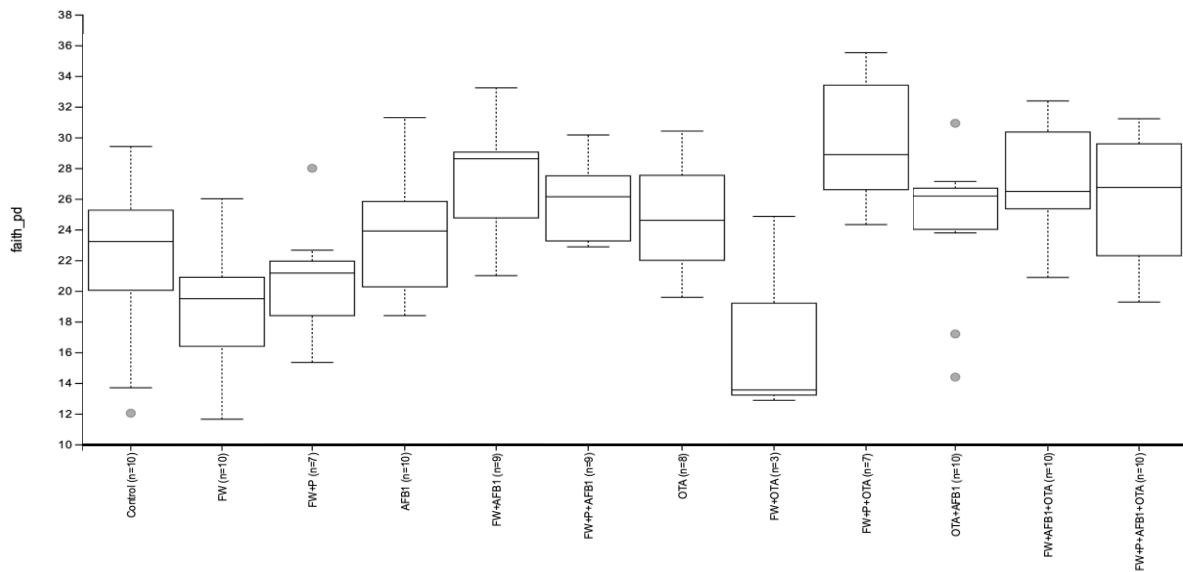


Figure 2. Faith's phylogenetic diversity values by diet. AFB1, aflatoxin B1. FW, fermented whey. OTA, ochratoxin A. P, pumpkin.

Microbial evenness in the context of the gut microbiome is about how fairly the different types of bacteria share space in the gut. Evenness is higher when species are present in similar proportions, that is to say, the same relative abundance. This parameter was not affected neither by diet nor sex.

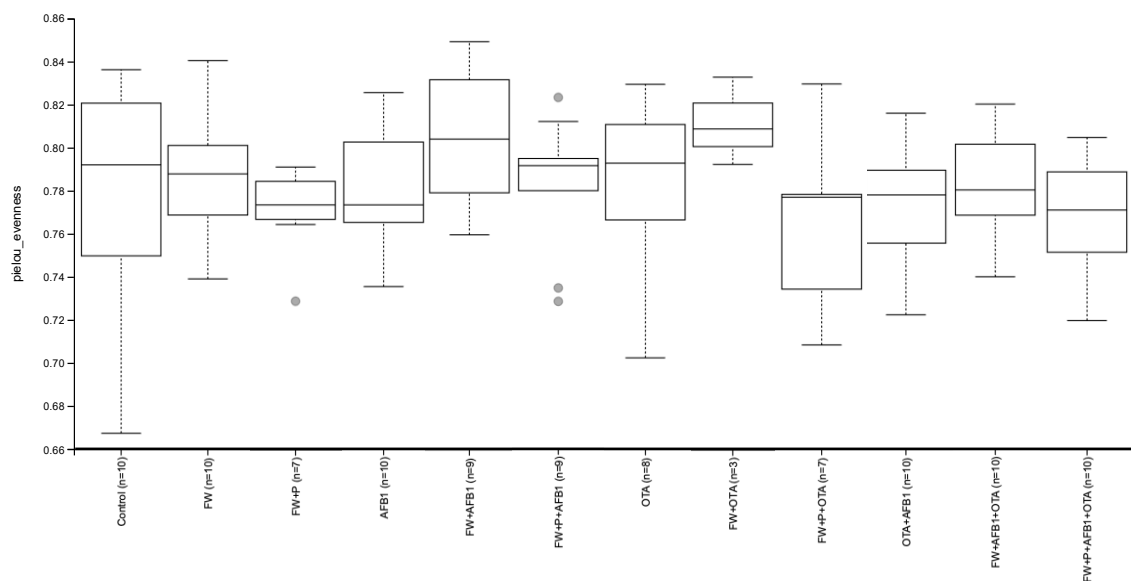


Figure 3. Evenness values by diet. AFB1, aflatoxin B1. FW, fermented whey. OTA, ochratoxin A. P, pumpkin.

3.1.1 Beta diversity

Bray–Curtis dissimilarity is a statistic test used to quantify the dissimilarity in species composition between two different sites. It ranges from 0 to 1. If both samples share the same number of microbes, at the same abundance, their "dissimilarity" will equal zero. The Bray-Curtis dissimilarity is based on occurrence data (abundance), while the Jaccard distance is based on presence/absence data. Jaccard measures how similar samples are based on the presence or absence of species, without considering their abundance. It also ranges from 0 to 1, where 0 means no species are shared between samples, and 1 means they share the exact same species. Axis represent principal components (PC) in the plots.

Principal component (PC) analysis plot based on Bray–Curtis dissimilarity (Figure 4A) showed that fecal microbial communities from FW+AFB1, FW+AFB1+OTA and AFB1+OTA were more similar to each other than FW, FW+P, FW+OTA, AFB1, OTA and control, which are separated by PC1 and PC2. The second similar group of fecal microbial communities that we can observe is composed by FW+P+AFB1 and FW+P+AFB1+OTA, separated from the first one by PC1. Some differences in abundance can be observed but are not enough to confirm a beneficial effect from FW and FW+P intake in microbiota.

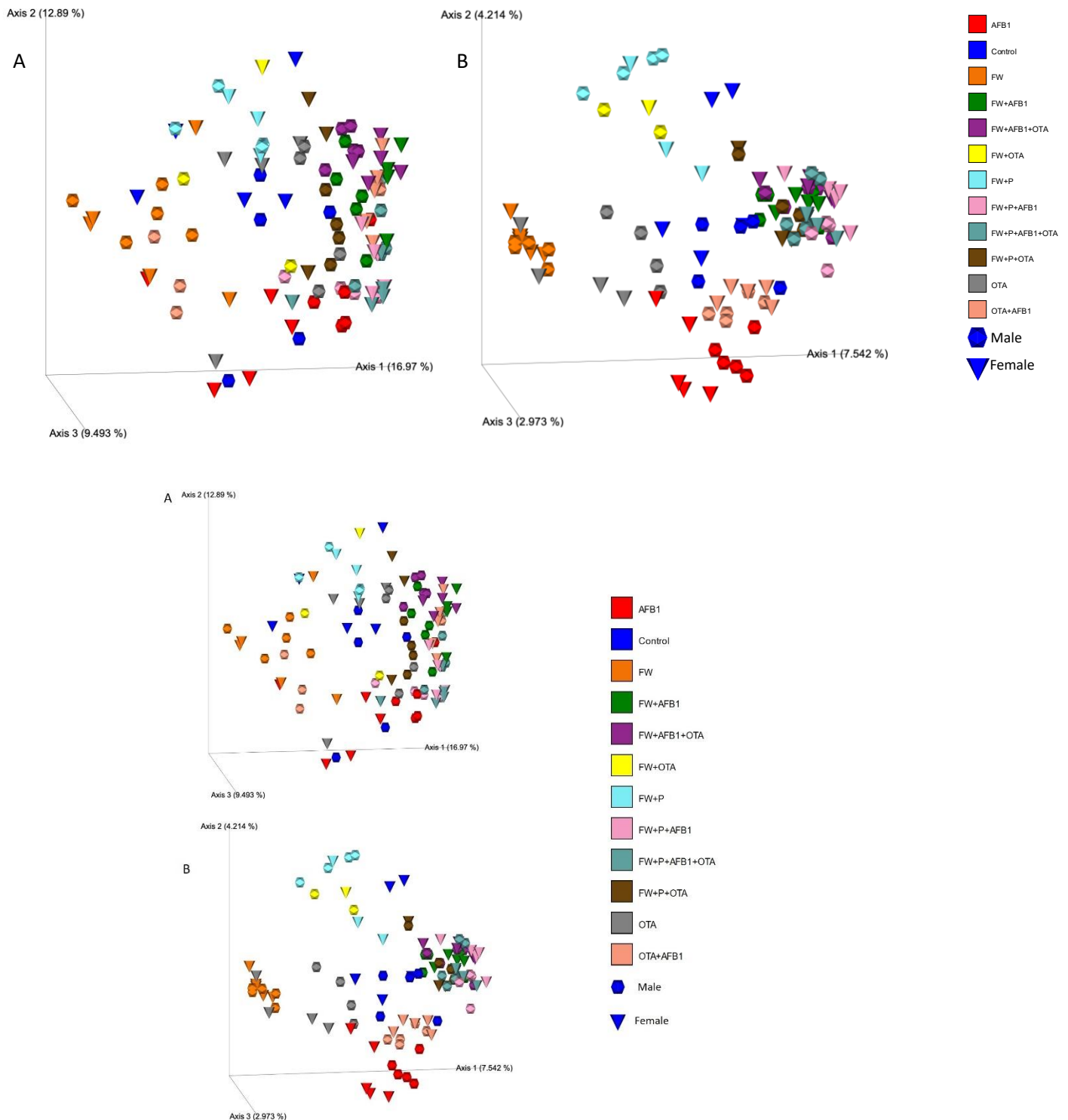


Figure 4. Bray-Curtis (A) and Jaccard (B) dissimilarity by diet and sex in microbial community rat feces. AFB1, aflatoxin B1. FW, fermented whey. OTA, ochratoxin A. P, pumpkin.

Regarding Jaccard PCoA plot, less differences among samples from the same diet group can be observed compared to Bray-Curtis plot. Control samples remain at the middle of the plot, while FW and FW+P are at two different edges. Fecal samples from FW, AFB1, OTA, FW+OTA and AFB1+OTA did not share the same microbial species, and they were clearly separated mostly by PC2. The diet groups more alike in their microbial composition were FW+AFB1, FW+AFB1+OTA, FW+P+AFB1, FW+P+OTA, and FW+P+AFB1+OTA. This result evidences how functional ingredients influence fecal microbiota composition, making it more similar to control group at PC2 level when feed is contaminated by mycotoxins. Even FW+OTA, which is not aligned with the rest of groups including functional ingredients, certainly exhibited different microbial species than groups fed with contaminated diets without functional ingredients.

3.1 Taxonomic differential abundances

To explain more detailed changes in the fecal microbial structure induced by mycotoxins and/or functional ingredients exposure, the relative abundance at the phylum level was determined. Figure 5 shows the relative abundance (%) of phylum per sample and figure 6 shows it per diet group, including statistical differences. The main difference between sexes can be found in group FW+AFB1+OTA, with males (25%) showing higher % of Proteobacteria than females (1.86%).

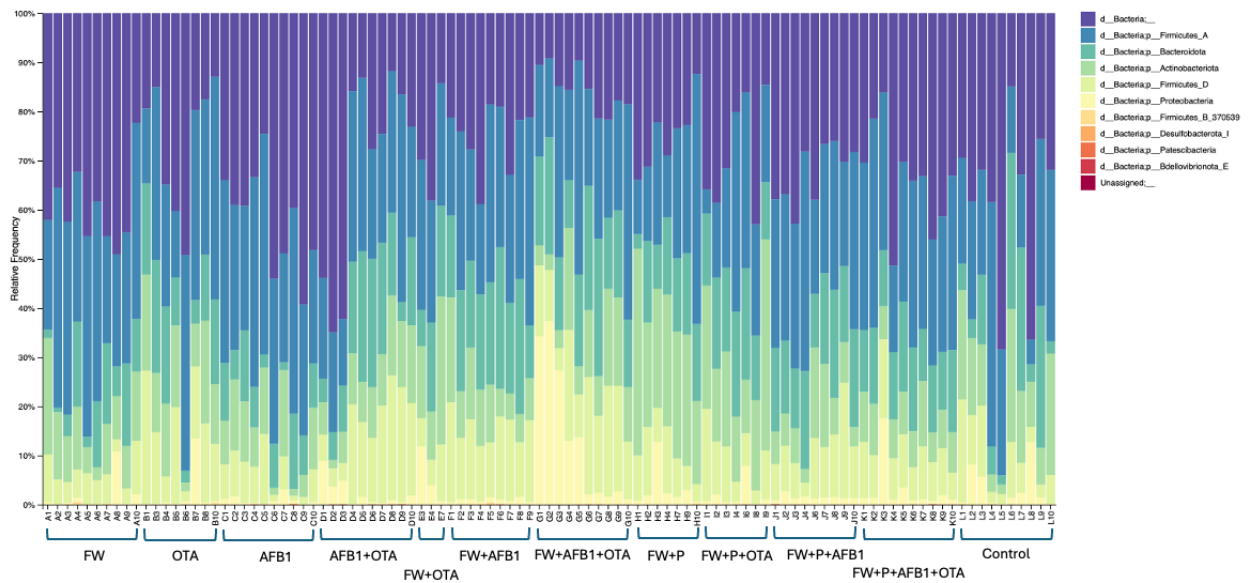


Figure 5. Microbiota relative abundance at phylum level per sample. Numbers from 1 to 5 represent males and from 6 to 10, females. Color-coded bar plot shows the relative abundance of bacterial phyla across the different samples. AFB1, aflatoxin B1. FW, fermented whey. OTA, ochratoxin A. P, pumpkin.

For a more detailed statistical analysis of the taxonomical results, the five most prevalent bacteria phylum were evaluated based on the diet. In the first place, Shapiro-Wilk test was chosen to study normality, showing Firmicutes D and Actinobacteria data as normal. For these, general linear model and Tukey post hoc test was applied. For Bacteroidota, Firmicutes A and Proteobacteria, Kruskal-Wallis test was chosen (Figure 6). Firmicutes A and D were not altered by the mycotoxins' ingestion or the inclusion of functional ingredients in the feed compared to control. FW+P addition to the diet provoked a significant increase of Actinobacteria in fecal microbiota. It is important to highlight that feed enrichment with FW and FW+P, when contaminated with AFB1, significantly increased the percentage of Bacteroidota in fecal samples compared to the AFB1-contaminated diet. In general, a reduction in Proteobacteria phylum in rat samples fed with contaminated diets (AFB1, OTA and AFB1+OTA) was observed. When these contaminated diets included functional ingredients, in all cases Proteobacteria increased its percentage, even if not in all groups the increasement was statistically significant.

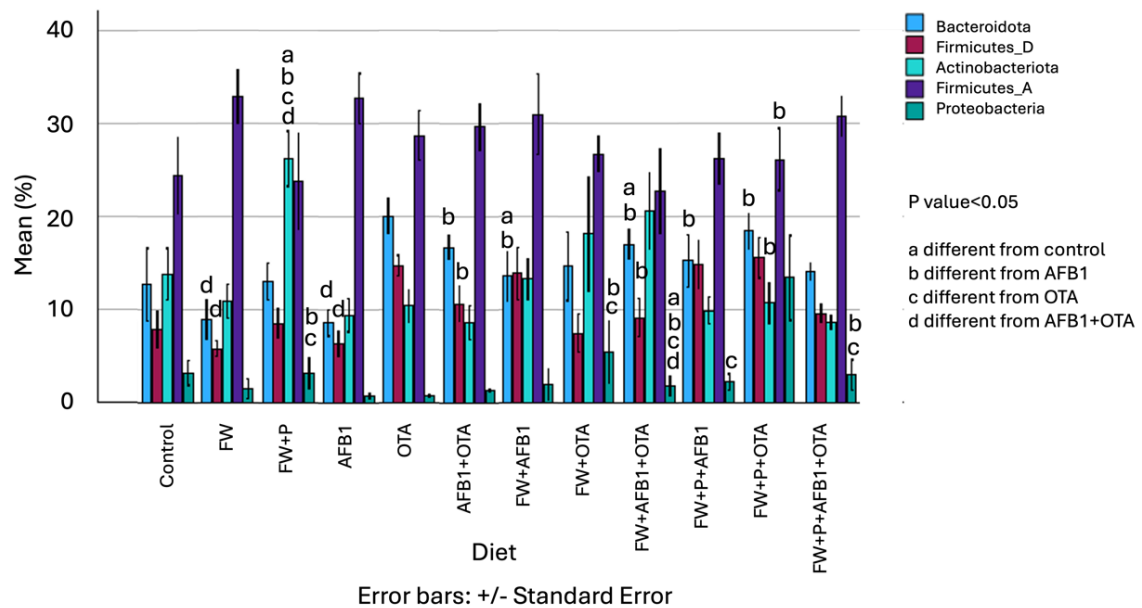


Figure 6. Microbiota relative abundance (%) at phylum level by diet group. AFB1, aflatoxin B1. FW, fermented whey. OTA, ochratoxin A. P, pumpkin.

The Firmicutes/Bacteroidota (F/B) ratio modulation by diet and sex was evaluated (Figure 7). The main differences can be observed between sexes in the rats non-exposed to mycotoxins. Between control groups, the difference between male and female was not significant but a tendency was detected, while for FW and FW+P groups the differences between sexes were statistically significant. This was not observed when mycotoxins were present, decreasing the F/B ration for both sexes.

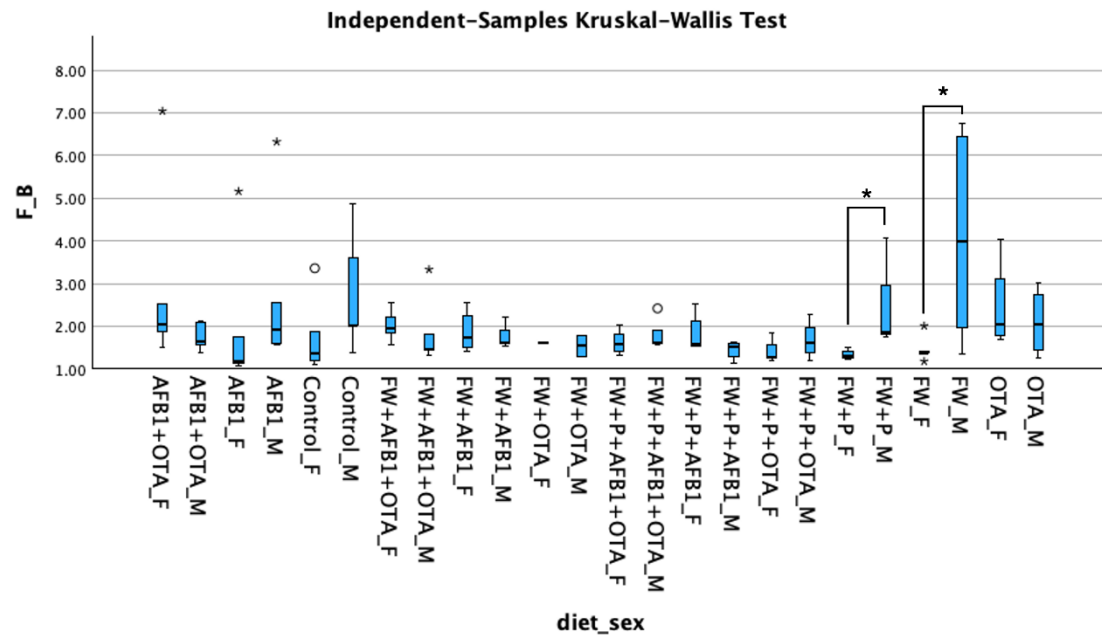


Figure 7. Boxplot representing the Firmicutes/Bacteroidota ratio by diet and sex group. Kruskal-Wallis statistical test applied. * p value < 0.05. AFB1, aflatoxin B1. F, female. FW, fermented whey. M, male. OTA, ochratoxin A. P, pumpkin.

Regarding the genus and species, the presence of genus *Morganella*, represented by *Morganella morganii*, was the only microorganism which totally correlated with the diet provided to the rats (Figure 8). It was the most frequent in control rats, followed by all groups, including FW+P and mycotoxin. When comparing sexes fed with the same diet, *Morganella morganii* was always more prevalent in females than in males, especially in OTA group. The other two most occurrent genus were *Cryptobacteroides*, represented by *Cryptobacteroides* sp900544195 and *Floccifex* by *Floccifex porci*.

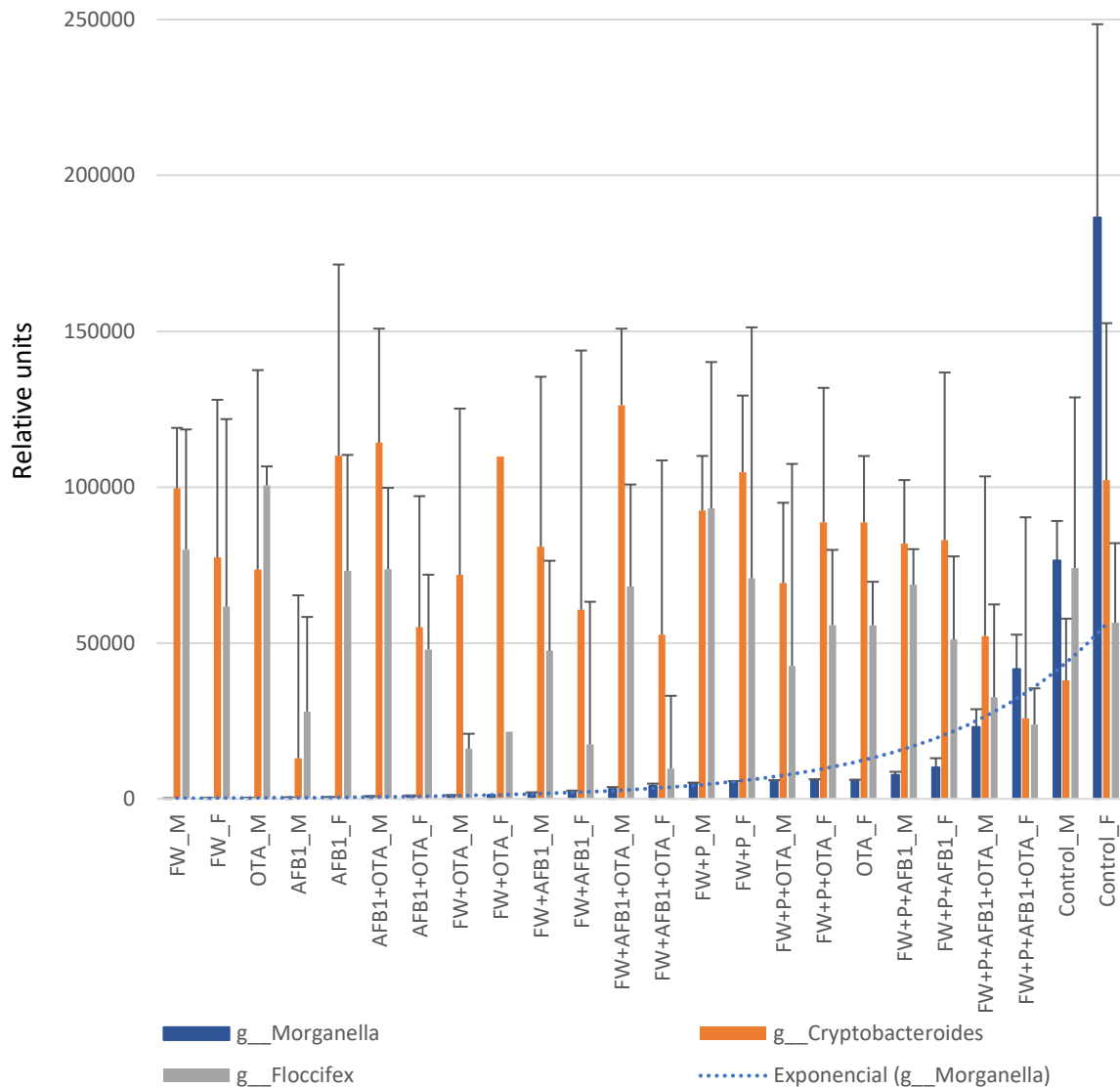


Figure 8. Barplot representing the three most predominant genus in feces microbiota by diet and sex group. Error bars represent standard error. F, female. FW, fermented whey. M, male. P, pumpkin. AFB1, aflatoxin B1. OTA, ochratoxin A.

4. Discussion

The study aimed to investigate the effects of AFB1 and OTA on the gut microbiota, both individually and in combination, using fecal samples from rats exposed to diets contaminated with these mycotoxins for 28 days, with or without the inclusion of functional ingredients (FW and P). Previous studies have tested other mycotoxins for their

effects on gut microbiota using rat models, such as deoxynivalenol (Miró-Abella et al., 2018) and zearalenone (Zhang et al., 2020).

The dose of the mycotoxins in the diets was selected based on the results on previous *in vivo* experiments (Cimbalo et al., 2020). The animals exposed to these twelve diets did not show any clinical sign of toxicity (weekly observations) during the study (Vila-Donat et al., 2024). Both sexes were included in the interpretation of the results, as it is important to consider sex in studies examining the health impacts of functional foods or ingredients intended to improve gut health (Shastri et al., 2015).

It was observed that groups containing a mix of the functional ingredients and mycotoxins (FW+AFB1 and FW+AFB1+OTA) showed more PD faith value than groups only containing the functional ingredient (FW). Moreover, FW+AFB1+OTA also reported a higher PD faith value than FW+OTA. However, as expected, FW+P+OTA revealed a higher PD faith value than both groups, OTA and FW+OTA. On the one hand, it is shown that the combination of mycotoxins-functional ingredients contributes more to microbial diversity rather than the functional ingredients alone. On the other hand, the oral exposure to mycotoxins-functional ingredients combination reported a microbial diversity enhancement in comparison to the mycotoxins alone.

This synergism may be explained by the fact that, although some beneficial bacteria – such as *Lactobacillus* strains – have shown to reduce AFB1 and OTA (Ragoubi et al., 2021), these mycotoxins also possess the ability to decrease harmful bacteria such as *Enterococcus faecalis*, *E. coli* and *Staphylococcus aureus* (Heller et al., 1975; Tiwari et al., 1986). Microbial diversity is often associated with healthy status, while pathological conditions tend to show a decrease in diversity (Hou et al., 2022). In fact, it has been found that certain combinations of species display much greater colonization resistance to pathogens together than alone. One specie alone is not sufficient to strongly impact pathogen growth, but rather a combination of species is required to block nutrient access (Spragge et al., 2023). Despite PD faith is one of the most used α -diversity parameters, it should be noticed that it only focuses on microbial genetic similarity, not taking into consideration the biologically functional similarity and/or diversity.

4.1 Taxonomic differential abundances

Firmicutes, Actinobacteria and Bacteroidota were the major phyla of gut microbiota in feces observed in this study, followed by Proteobacteria. The highest proportion of Actinobacteria was found in FW+P fecal samples. Regarding other taxonomic differential abundances, there was a non-significant reduction in Proteobacteria phylum in rat samples fed with contaminated diets (AFB1, OTA and AFB1+OTA) compared to control, while an increase – although not in all groups - when adding the functional ingredients. In humans, an increasing amount of data identifies Proteobacteria as a possible and more homogeneous microbial signature of disease (Rizzatti et al., 2017). Indeed, analysis of microbiota composition in children has demonstrated a gradual increase in Proteobacteria between healthy, obese, and children with nonalcoholic steatohepatitis (Zhu et al., 2013). Moreover, Proteobacteria seems to be implicated in cardiovascular diseases, as there is evidence that high levels of Proteobacteria are present in atherosclerosis patients (Shen et al., 2021).

The main difference between the sexes was found in the group FW+AFB1+OTA, with males (25%) showing higher % of Proteobacteria than females (1.86%). Indeed, it has been shown that Proteobacteria proportion is higher in male birds than female ones (Yan et al., 2022). Another study conducted in fish (*Largemouth bronze gudgeon*) reported the same trend, since the phylum profile in the intestine of males revealed that phylum Proteobacteria was dominant, in contrast to female where phyla *Tenericutes* was the most abundant one (Li et al., 2016). However, Zhao *et al.* (2019) reported that female giant pandas harbored a higher abundance of phylum Proteobacteria than male ones with the contribution from genera *Escherichia/Shigella*.

According to these results, it could be thought that, apart from the effects induced by diet, sex plays an important role in augmenting the Proteobacteria ratio. Yet, testosterone and dihydrotestosterone – two hormones more abundant in males than in females, have shown to diminish the Proteobacteria relative abundance in contrast to control in mice. Therefore, the higher abundance of Proteobacteria in males should not be directly related to the sex condition. What is more, there could be more biological pathways to be investigated in which males are more sensitive to the bioactive compounds than females.

It is important to mention the higher presence of Proteobacteria species *Morganella morganii* in the feces of animals fed with control and FW+P enriched diets, contaminated or not with AFB1 and OTA, compared to the diets only enriched with FW or contaminated with mycotoxins and not enriched with functional ingredients at all. In rats, this microorganism is involved in the biotransformation of se-methylselenocysteine (MeSeCys) into dimethyldiselenide and dimethylselenide. The biotransformation into dimethyldiselenide modifies the efficiency of MeSeCys as Se source but also produces a potential compound expected for pharmacological effects, such as anticancer activity. The study concluded that gut microflora plays a role in the biotransformation of MeSeCys, and seems to support efficient metabolism in animals (Takahashi et al., 2023). However, *Morganella morganii* has also been connected to tumor abscess formation in humans (Chen & Lin, 2011). In fact, it has been revealed that *Morganella morganii* is enriched in the gut microbiota of inflammatory bowel disease and colorectal cancer patients, producing a previously undescribed family of genotoxins, known as indolimines, which are characterized by enhancing intestinal permeability and colon tumorigenesis in gnotobiotic mice (Hernández-Castro et al., 2024). These contradictory results are also found in another species such as *Ruminococcus gnavus*, which has been associated both positively and negatively with an increasing number of intestinal and extraintestinal diseases from inflammatory bowel diseases to neurological disorders (Croston et al., 2023). Extrapolation of results from rats to humans and vice versa in microbiota studies might be performed and interpreted carefully.

Besides, the addition of FW+P to the diet increased the abundance of *Actinobacteria* in the fecal microbiota. It should be stressed that *Actinobacteria* is a phylum accounting for very different genera. Both *Mycobacterium* – connected with tuberculosis, leprosy, pulmonary disease, skin and soft tissue disease, musculoskeletal infections and disseminated disease (Rakotoarisaona et al., 2024), and *Bifidobacterium* – used as therapeutic agents for gastrointestinal disorders and other pathologies - belong to *Actinobacteria* (Tojo et al., 2014). In fact, it was shown that OTA increased *Corynebacterium* – a genus of opportunistic pathogen bacteria belonging to *Actinobacteria* - in males, but when adding the functional ingredients (FW+P+OTA/ FW+OTA) *Corynebacterium* relative abundance was decreased (Belmares et al., 2007; Lo et al., 2015; Yatera & Mukae, 2020).

4.2 F/B ratio

Most studies have demonstrated that *Bacteroidota* in fecal microbiota exhibit pro-inflammatory properties due to endotoxins and influence cytokine production, contributing to inflammatory bowel disease (IBD). Furthermore, *Firmicutes* bacteria exhibit anti-inflammatory effects and can alleviate the progression of IBD (Stojanov et al., 2020). In a gastrointestinal disorder study using male rats, the control group showed the highest F/B ratio, confirming the correlation of the ratio with a healthy profile (Wu et al., 2021). The results obtained using FW and P point to a higher effect of functional ingredients in increasing F/B ratio in rat males rather than females, but the presence of mycotoxins suppressed this potentially beneficial outcome. On the contrary, in humans, literature indicates that the F/B ratio is significantly increased in females compared to males in a project carried out in Ukraine population (Koliada et al., 2021). However, in a Spanish study, there were no sex differences in the microbiota diversity, phylum level, overall composition, and the Firmicutes/Bacteroidetes ratio. (Haro et al., 2016). Interestingly, a study analyzing gut microbiota from polycystic ovary syndrome patients showed that testosterone increased Bacteroidetes and decreased Firmicutes (Matsushita et al., 2022), which is in accordance with the Ukraine population. Anyway, apart from the F/B ratio, it should be paid more attention to the microbiota genus or species, since a phylum is characterized by containing a complexity of bacteria playing contradictory roles in the balance of health and disease.

4.3 Comparing other results obtained with the same animals

Gut microbiota dysbiosis has been associated with liver and kidney disease (Raj et al., 2020). Like our experiment, Frangiamone *et al.* (2023b) studied the effects of the twelve same groups of diet (AFB1, OTA and/or functional ingredients) on liver and kidney damage biomarkers expression. It was found that in males' liver, the most damaging exposure was AFB1 by reducing CPS1 expression - indicator of non-alcoholic fatty liver disease (Eriksen et al., 2019), which was totally reversed by FW administration. This physiological improvement by exposing them to the functional ingredient should

theoretically be related to a better gut flora. However, in our study not only AFB1 led to a Proteobacteria decrease, but also the FW supplementation (FW+OTA) augmented Proteobacteria abundance. These contradictory results are striking because it has been hypothesized and confirmed that a rise in the Proteobacteria phylum proportion was the most prominent alteration in gut-liver axis-induced hepatic steatosis (Vasques-Monteiro et al., 2021). On the other hand, OTA was the most damaging in kidney by upregulating kidney injury molecule-1 (KIM-1). Also, as in the liver case, FW administration mitigated mycotoxins induced KIM-1 upregulation (Frangiamone et al., 2023b). However, in our study, when comparing OTA vs FW+OTA exposures, FW addition surprisingly revealed to increase Proteobacteria, a phylum related to chronic kidney disease (Voroneanu et al., 2023). It can be concluded that the beneficial effects of functional ingredients on F/B ratio and at other antioxidant levels influence more the positive outcome of their intake than the changes observed in Proteobacteria proportions.

Finally, Vila-Donat *et al.* (2024) quantified AFB1 and OTA in rat urine and showed the impact of dietary bioactive ingredients on mycotoxin excretion. The results revealed that the urinary AFB1 levels of the group that ingested FW+AFB1+OTA were significantly higher than those of the group without FW (AFB1+OTA). A metagenomic analysis has revealed that diverse microorganisms, mainly belonging to the phyla Actinobacteria and Proteobacteria, possess key functional enzymes involved in the initial step of AFB1 biotransformation (Hu et al., 2024). P has a notable content in vitamin E, which has been related to AFB1 excretion through its metabolization (Abdel-Hamid & Firgany, 2015). Also, both FW and P include a lot of phenolic compounds (p-hydroxybenzoic acid, catechin, chlorogenic acid). These compounds have been considered as microbial toxin detoxification agents (Saricaoglu et al., 2022). Additionally, FW contains probiotic strains, such as *Bifidobacterium* and *Lactobacillus*, which have been shown to degrade both AFB1 and OTA (Vila-Donat et al., 2025). Moreover, LAB through their cell wall interactions, can absorb AFB1 by binding it to their cell wall components, including polysaccharides and peptidoglycans, reducing the bioavailability and systemic absorption of the toxin (Haskard et al., 2001). These mechanisms underscore the potential of bioactive ingredients in supporting mycotoxin detoxification, especially in diets rich in plant-based foods as seen in Mediterranean patterns. The most important effects of the bioactive compounds on gut microbiota in rats are indicated in Table 2.

Table 2. Summary of the most important effects of the bioactive compounds on gut microbiota in rats. AFB1, aflatoxin B1. FW, fermented whey. OTA, ochratoxin A. P, pumpkin. PD, phylogenetic diversity.

Parameters	Results
α-diversity (Faith's PD)	FW+P+OTA and FW+AFB1+OTA > control
	FW+AFB1 and FW+AFB1+OTA > FW
	FW+AFB1+OTA > FW+OTA
	FW+P+OTA > OTA
Taxonomic differential abundance	The main difference between sexes can be found in group FW+AFB1+OTA, with males (25%) showing higher percentage of Proteobacteria than females (1.86%).
	A reduction in Proteobacteria phylum in rat samples fed with contaminated diets (AFB1, OTA and AFB1+OTA) was observed. When these contaminated diets included functional ingredients, in all cases Proteobacteria increased the percentage.
Firmicutes/Bacteroidetes ratio	FW and FW+P groups presented statistically significant differences between sexes.
<i>Morganella morganii</i> abundance	<i>Morganella morganii</i> was always more prevalent in females than in males, especially in OTA group.

5. Conclusions

In this work, alpha diversity changes regarding Faith's PD were more related to diet, functional ingredients and mycotoxins exposure, than to sex. On the contrary, microbial evenness was not altered by any of these parameters. Beta diversity plots showed a cluster of contaminated diets and another cluster grouping the contaminated diets enriched with functional ingredients. Control diets enriched with functional ingredients changed fecal microbial communities in different aspects and were displayed in the plots separately from the control and the two previously mentioned clusters. In general, it can be observed a reduction in Proteobacteria phylum in samples of rat fed with contaminated diets and *Morganella morganii* was the most occurrent species in control rats, followed by all groups including functional ingredients and mycotoxins. Finally, diet did not modify the F/B ratio, while significant differences were found between sexes in FW and FW+P diets. It can be concluded that mycotoxin exposure

through diet triggers changes in the microbiota of rats, but also does the inclusion of functional ingredients. Moreover, the sex of the animals leads to different outcomes.

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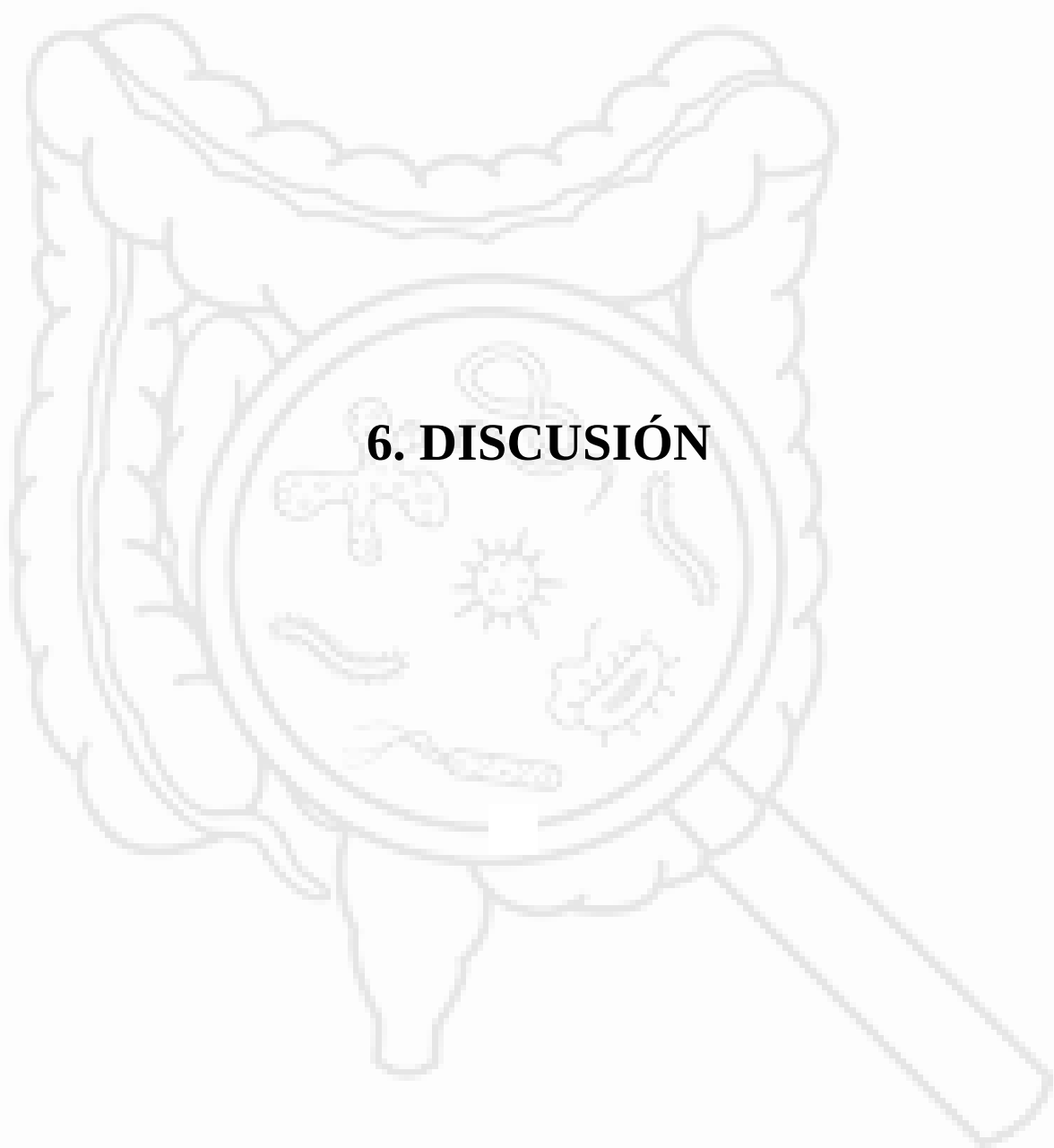
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6. DISCUSIÓN

6. Discusión general

Para alcanzar los objetivos planificados en la presente Tesis Doctoral, en primer lugar, se ha evaluado el posible efecto beneficioso del ajo Voghiera sobre el pan en términos de modificación de la bioaccesibilidad y citotoxicidad de AFB1 mediante el uso de células Caco-2. Posteriormente, se ha realizado una revisión bibliográfica con la finalidad de arrojar luz sobre el potencial de los PPSP para mitigar los efectos nocivos de las micotoxinas emergentes. Además, de modo que se pueda mejorar la comprensión de la relación recíproca entre las micotoxinas emergentes y la microbiota intestinal, dilucidando cómo las micotoxinas emergentes pueden influir en la microbiota intestinal y viceversa. Finalmente, se han investigado los efectos sobre el perfil de la microbiota intestinal, después de la exposición crónica oral a AFB1 y OTA y los posibles efectos beneficiosos proporcionados por el enriquecimiento del alimento con FW y P considerando la interacción del dimorfismo sexual en ratas Wistar.

6.1 Artículo 1

En los últimos años se ha estudiado la bioaccesibilidad de AFB1 en diferentes matrices alimentarias que incluyen o no ingredientes funcionales. La inclusión de ingredientes funcionales como estrategia para reducir la exposición a AFB1 ha ofrecido resultados prometedores. Escrivá *et al.* (2021) midieron la bioaccesibilidad de AFB1 utilizando harina de trigo contaminada con AFB1 en una receta de pan logrando concentraciones de 78 a 164 μg de AFB1/kg en el pan con y sin de suero de leche de cabra, suero fermentado o calabaza liofilizada (1%). Después de realizar una digestión humana simulada, la bioaccesibilidad intestinal de AFB1 se redujo un 64% al incluir 1% de suero de leche de cabra en la receta del pan, un 57% con 1% de suero fermentado y un 74% con 1% de calabaza liofilizada. En este trabajo, la reducción de la bioaccesibilidad intestinal de AFB1 al incluir el ajo es del 7-8%, lo que puede parecer pequeño en comparación con la reducción reportada por Escrivá *et al.* (2021). Sin embargo, la fracción bioaccesible total de AFB1 al incluir ingredientes funcionales es menor en este trabajo utilizando ajo. La principal diferencia entre estos dos estudios y los porcentajes finales bioaccesibles de AFB1 pertenece al resultado relacionado con la bioaccesibilidad

de AFB1 en pan contaminado con AFB1 sin ingredientes funcionales. Para Escrivá *et al.* (2021) el resultado es del 114% mientras que en este trabajo es del 38-29%.

Otros estudios de bioaccesibilidad han utilizado la fortificación de AFB1 con estándares comerciales de AFB1, incluyendo la micotoxina en el producto alimenticio después de su producción en lugar de simular una contaminación fúngica natural de los ingredientes crudos. Se ha visto cómo la fortificación mejora la liberación de compuestos bioactivos (Kabak y Ozbey, 2012). En el único artículo publicado comparable a este trabajo, Llorens *et al.* (2022) hallaron resultados similares a los del estudio con ajo respecto a la bioaccesibilidad intestinal de AFB1, ya fuese incluyendo o no remolacha roja al pan.

Se han realizado estudios de viabilidad celular que exponen células Caco-2 diferenciadas a AFB1 (Gao *et al.*, 2021; Zhang *et al.*, 2015). Gao *et al.* (2021), mediante el uso del ensayo CCK-8, mostraron que la exposición de las células Caco-2 a AFB1 (1,6-25,6 μM) condujo a una disminución de la viabilidad celular de manera dependiente de la dosis a las 48 h. Zhang *et al.* (2015) demostraron que la exposición de células Caco-2 a AFB1 (0,032-3,2 μM) también disminuía la viabilidad celular de forma dosis dependiente en todo momento mediante el uso de la técnica MTT. De acuerdo con la literatura, el presente estudio también muestra una disminución de la viabilidad celular debido a la exposición a AFB1. Sin embargo, se ha de tener en cuenta que, no sólo son diferentes las técnicas utilizadas - excepto Zhang *et al.* (2015) - sino también la concentración de AFB1 y el tiempo de exposición.

Los efectos protectores del ajo sobre las células Caco-2 diferenciadas se han estudiado previamente. Engdal & Nilsen (2008) demostraron que el natto K2 y el té verde se caracterizaron por aumentar la viabilidad celular en las células Caco-2, mientras que el ajo no tuvo efectos significativos (0,017-0,17 % p/v) después de 90 min de exposición. Eguchi *et al.* (2005) revelaron que el extracto acuoso de ajo disminuía la viabilidad celular a <35 % después de 24 h de exposición al 5-25 % (v/v). La muestra utilizada para la exposición celular fue el sobrenadante, mientras que se descartó el sedimento, que aún podría contener una fracción considerable de ajo. En este experimento, el ajo de Voghiera añadido como ingrediente funcional al pan sufrió un proceso de cocción y digestión *in vitro*. La concentración de ajo en el pan fue del 2% (p/v), pero después de la digestión *in vitro* y la dilución del medio fue del 0,002% (p/v). Según Bhatt y Patel. (2013), mientras

que la cocción reduce drásticamente la cantidad total de compuestos fenólicos, la digestión gastrointestinal *in vitro* proporciona extractos de ajo con un potencial antioxidante mucho mayor que el uso de disolventes orgánicos.

Los efectos tóxicos desencadenados por AFB1 en células y tejidos se provocan principalmente mediante la inhibición de la proliferación celular y la detención del ciclo celular, la apoptosis, el estrés oxidativo, el estrés del retículo endoplásmico y la autofagia (Li et al., 2022). Huang *et al.* (2020) demostraron que en la línea celular de neuroblastoma IRM-32, AFB1 indujo una detención significativa de la fase S en concentraciones de 6,4 μM y 19,2 μM durante 24 h o 48 h. Zhu *et al.* (2015) revelaron una detención de la fase S en células HepG2 expuestas a 0-48 μM de AFB1 durante 24 h, especialmente la detención fue significativa cuando se utilizaron 32 y 48 μM de AFB1. Liu *et al.* (2015) revelaron que la exposición a AFB1 inducía la detención de G0/G1 en hepatocitos de rata F344 cuando se administraban 100 o 200 $\mu\text{g/kg}$ de AFB1 durante 28 días. De acuerdo con estos estudios previos, este estudio demostró que la exposición a AFB1 puede promover una posible detención del ciclo celular en la fase S, mientras que la adición de ajo disminuye la fase S (23 frente a 19%) de manera similar al control, al limitar significativamente una eventual detención del ciclo celular.

Con respecto a la apoptosis, se ha visto cómo la tasa de apoptosis de las células 3D4/21 aumenta de manera dependiente de la dosis de AFB1 (Hou et al., 2018). De acuerdo con estos resultados, este trabajo encontró que la exposición a AFB1 incrementa el número de células apoptóticas y necróticas, mientras que la adición de ajo atenúa sus efectos. Comparando la exposición a AFB1 versus AFB1 + ajo, el porcentaje de células apoptóticas alcanzó su máximo en la AFB1 (41%), incluidas las células en apoptosis temprana y tardía, mientras que hubo una disminución al combinar AFB1 y ajo (27%) (Fig. 3). Se demostró claramente que AFB1 mejora la apoptosis celular y que el ajo atenúa la apoptosis que promueve AFB1 en células Jurkat-T.

Liu y Wang (2016) demostraron que, en hepatocitos primarios de pollos de engorde, diferentes concentraciones de AFB1 (0,5-5 μM) durante 6 h condujeron a la generación de ROS de una manera significativa y dependiente de la concentración. Wang *et al.* (2017b) también revelaron que la exposición de cardiomiocitos de pollo de engorde a diferentes concentraciones de AFB1 (0,5-5 μM) durante 12 h resultó en un aumento significativo de la producción de ROS intracelular. Además, se observó que el extracto

metanólico de *Cetraria islandica* provocó un aumento en la actividad de la superóxidodismutasa (SOD) y la glutathion peroxidasa (GPx) y una disminución en el nivel de MDA en linfocitos humanos *in vitro* (Kotan et al., 2011). También se ha documentado el efecto protector del ajo frente a los procesos oxidativos. El ajo no solo contribuye a disminuir los niveles de ROS, sino también a mitigar otros parámetros proinflamatorios como las actividades SOD, catalasa (CAT) y GPx (Abdel-Tawwab et al., 2021; Al-Fayyadh et al., 202; Kohda et al., 2013; Ilmawati et al., 2019). De acuerdo con la literatura previa, este trabajo demostró que la AFB1 incrementó cinco veces la producción de ROS en comparación con el control, mientras que la adición de ajo atenuó el efecto de AFB1, reportando valores similares al control. Este estudio confirma por primera vez la capacidad del ajo para reducir las ROS promovidas por AFB1 en las células Jurkat-T.

La investigación sobre el impacto de AFB1 - específicamente en el ROS mitocondrial - es escasa en la literatura. Sin embargo, existen otros parámetros mitocondriales, como la producción de ATP, la permeabilidad de la membrana mitocondrial y la capacidad respiratoria, que pueden ser buenos indicadores del estado de producción de ROS (Liu et al., 2014). Chen *et al.* (2022) revelaron que AFB1 reducía la capacidad respiratoria mitocondrial y la producción de ATP en células HepG2 y Caco-2, que están relacionadas con disfunciones celulares. Liu *et al.* (2015) demostraron que AFB1 aumentaba la permeabilidad de la membrana mitocondrial, lo que daría lugar a una liberación de proteínas como el citocromo c para activar la cascada de caspasas y la muerte celular programada. De manera similar, el presente estudio reveló que la exposición a AFB1 aumentaba la producción de ROS mitocondrial (35% más) en comparación con AFB1 y ajo (11% más), lo que confirma el efecto antioxidante del ajo contra AFB1 también a nivel mitocondrial.

Los cambios mitocondriales en respuesta a diferentes estímulos se pueden evaluar mediante el uso de tintes mitocondriales para rastrear la masa y el volumen mitocondriales (Monteiro et al., 2020). Se ha revelado que la AFB1 disminuye la masa mitocondrial en peces expuestos a 5-10 μ M de AFB1 durante 7-8 días y ratones alimentados con 0,375-1,5 mg/kg de AFB1 durante 30 días (Cheng et al., 2019; Xu et al., 2021). Sin embargo, no se encontraron diferencias significativas en las células Jurkat-T en este estudio, al sugerir que ni AFB1 ni el ajo podrían afectar la masa de este orgánulo (Fig. 6). En general, estos resultados confirman la toxicidad *in vitro* de AFB1 en concentraciones muy bajas y

exposición prolongada. Este experimento permite seguir investigando en la importancia del tracto gastrointestinal en la bioaccesibilidad y posterior efecto de estos tóxicos.

6.2 Artículo 2

A tenor del estudio anterior, se ha de comentar que las interacciones a lo largo del intestino son muy complejas debido a la cantidad de factores que juegan un papel notable en su fisiología, de modo que hay ocasiones en que es muy probable no saber del todo qué vías o moléculas concretas son consecuencia de un hecho concreto. Sin embargo, existen algunas correlaciones establecidas que nos ayudan a comprender mejor cómo funcionan los sistemas biológicos. Una de estas fuertes correlaciones es el papel preventivo de los PPSP y la microbiota intestinal en las enfermedades y sus efectos beneficiosos sobre la salud (Li et al., 2021). Como ya se ha dicho, los PPSP y la microbiota intestinal poseen la capacidad de unirse y metabolizar una gran cantidad de sustancias que ingresan al tracto gastrointestinal. Además, a pesar de reducir teóricamente los compuestos beneficiosos, los PPSP también puede disminuir la exposición a aquellos perjudiciales, como las toxinas, incluidas las micotoxinas emergentes (Guerre, 2020).

Tal como se presenta, las regulaciones europeas solo abarcan un pequeño conjunto de micotoxinas (AFB1, AFB2, AFG1, AFG2, AFM1, OTA, PAT, DON, ZEN, FB1, FB2, CIT, AOH, AME, TeA, T-2 y HT-2 y ergocalcoides) (Comisión Europea, 2023). Debido a su regulación, estas micotoxinas tienden a ser más estudiadas, incluidas sus interacciones con la microbiota intestinal y los PPSP. De hecho, los estudios muestran que estas micotoxinas estudiadas alteran la microbiota intestinal, mientras que la ingesta concomitante de PPSP mitiga los efectos de las micotoxinas y contribuye a mantener la homeostasis de la microbiota intestinal (Cai et al., 2022; Frangiamone et al., 2024; Kudupoje et al., 2022; López-Ruiz et al., 2023; Markowiak et al., 2019). Aunque se esperan tendencias similares con las micotoxinas no reguladas, se debe realizar un análisis de estas micotoxinas emergentes una a una, puesto que cada micotoxina posee una estructura química específica y una respuesta diferente en el ambiente donde se encuentra (Guerre, 2020). En realidad, existen unas 400 micotoxinas conocidas, por lo que la gran mayoría de ellas aún no están reguladas y necesitan una comprensión más profunda. Además, no hay que olvidar que las micotoxinas tienden a encontrarse en combinación

con otras, por lo que las mezclas más comunes que se encuentran en los alimentos y piensos son los que interesa probar.

6.2.1 Micotoxinas emergentes y microbiota intestinal

En cuanto a la interacción entre la microbiota intestinal y las micotoxinas emergentes, los estudios han revelado una tendencia clara: la microbiota intestinal contribuye a la desintoxicación de las micotoxinas emergentes mediante su unión y/o metabolización (Crudo et al., 2020; Daud et al., 2020; Debevere et al., 2020; Hedman & Pettersson, 1997) y las micotoxinas emergentes alteran la composición de la microbiota intestinal. (Novak et al., 2021). A pesar de ser la tendencia confirmada por estos estudios, la dificultad surge de la heterogeneidad de las muestras analizadas. La microbiota exhibe una variabilidad significativa. De hecho, esta difiere según la especie. En los humanos, los cambios en la microbiota están influenciados por factores como el nivel de urbanización del lugar e incluso la hora del día dentro de un mismo individuo (Subramanian et al., 2022; Thaïss et al., 2015; Trinh et al., 2018). Por ejemplo, la microbiota ruminal se caracteriza por poseer muchas enzimas involucradas en la síntesis *de novo* de vitamina B12 (Seshadri et al., 2018).

Según la viabilidad experimental, se ha demostrado que en la mayoría de los experimentos las muestras de microbiota intestinal se obtienen a partir de heces, ya que la microbiota fecal es más accesible que la del colon. Curiosamente, se ha revelado que la microbiota del colon tiene un perfil diferente al de las heces, siendo la microbiota del intestino grueso mucho más diversa que aquella del intestino delgado y heces. Por ejemplo, mientras que en el intestino grueso había 4080 unidades taxonómicas operativas, en las heces solo se encontraron 443 (Ahn et al., 2023). De hecho, la biomasa bacteriana también cambia a lo largo del tracto gastrointestinal y las heces: duodeno (10^3 bacterias por g), yeyuno (10^4 g⁻¹), íleon (10^7 g⁻¹), colon (10^{12} g⁻¹) y heces (5^{10} g⁻¹) (Campos et al., 2015; Heeney et al., 2018). Además, es probable que los ensayos a corto plazo oculten que a largo plazo, la exposición a micotoxinas puede conducir al desarrollo de la capacidad de degradar estas micotoxinas a pesar de no poder hacerlo en el primer momento (Hedman y Pettersson, 1997).

Hay otras limitaciones que conviene destacar. Aunque en Debevere *et al.* (2020) el tiempo de incubación (48 h) estuvo en línea con otros experimentos que estudian la degradación de micotoxinas *in vitro*, es considerablemente más largo que el tiempo de retención *in vivo* de la digestión en el rumen, lo que implica que en condiciones fisiológicas normales los resultados serían diferentes. A pesar de que Crudo *et al.* (2020) hayan demostrado que AOH, AME, ATX-II, STTX-III y ALP eran menores en las muestras que contenían microorganismos que en las que no los tenían, cabe señalar que el tamaño de la muestra fue muy pequeño ($n = 4$). En este estudio, todos los donantes compartían algunas características: eran voluntarios omnívoros sanos (dos hombres y dos mujeres) con edades comprendidas entre 26 y 34 años y con un índice de masa corporal normal ($18,5\text{-}24,9 \text{ kg m}^{-2}$), ninguno de ellos tenía habían sido tratados con antibióticos, probióticos y prebióticos durante los tres meses anteriores y no presentaban enfermedades intestinales previas. Sin embargo, no se consideró la dieta completa, desempeñando esta un papel notable en el metabolismo intestinal y en las interacciones con las micotoxinas. Además, como se menciona en el mismo artículo, existen otras limitaciones como el ensayo de corta duración (3 h) y el hecho de que los medios de crecimiento utilizados podrían alterar la composición microbiana inicial de las heces, contribuyendo al crecimiento de algunos microorganismos a expensas de los demás. Por el contrario, en Novak *et al.* (2021) solo se evaluaron las micotoxinas ENB, ENB1, BEA y DON en las heces de lechones destetados en comparación con el control. Se demostró que *L. amylovorus* y *L. reuteri*, ambos microbios beneficiosos, estaban regulados positiva y negativamente, respectivamente, cuando se agregaba BEA en comparación con DON y el control. Además, la combinación de enniatinas y BEA aumentó la síntesis total de proteínas, pero la adición de DON tuvo un efecto inhibitorio (Novak *et al.*, 2021). Aunque es importante conocer las interacciones entre estas cuatro micotoxinas, las condiciones reales son más complejas, ya que una comida puede contener una variedad de componentes, por lo que es muy probable que los resultados en condiciones normales difieran de estos. Si bien los microbios podrían metabolizar las micotoxinas emergentes, estas toxinas también pueden influir en la composición de la microbiota, creando un posible escenario de arma de doble filo. Además, es importante considerar las distintas dosis de micotoxinas emergentes utilizadas en los experimentos, que van desde nanogramos hasta miligramos.

Ver las micotoxinas como compuestos tóxicos o como fármacos potenciales es, de hecho, una cuestión emergente. Por un lado, estas pueden reducir microorganismos beneficiosos, como *Lactobacillus*, *S. cerevisiae* y *Bacillus* (Böhm et al., 2000; Ola et al., 2013; Roig et al., 2014; Sondergaard et al., 2016) cuya reducción está generalmente relacionada con la promoción de enfermedades (Thanh et al., 2020). Por otro lado, pueden desempeñar un papel como antibióticos eliminando microorganismos implicados en enfermedades (Frangiamone et al., 2023; Thanh et al., 2020; Wang et al., 2019; Wiese et al., 2016; Wu et al., 2018). Por ejemplo, AOH y AME han demostrado ser inhibidores prometedores de la glucógeno-sintasa-quinasa 3, siendo un objetivo importante en el descubrimiento de fármacos. De hecho, se ha revelado que esta enzima participa en las vías de señalización de la diabetes tipo 2, el cáncer, los trastornos neurológicos y otras enfermedades (Vemuri et al., 2020). Además, la ergotamina, con su modo de acción bloqueador de los receptores adrenérgicos α , se ha utilizado también para el tratamiento de la migraña aguda durante más de 50 años (Gómez Quintero et al., 2022). Se necesita más investigación en el campo para aclarar los resultados y evaluar las implicaciones reales de estas micotoxinas emergentes para la salud y las enfermedades, teniendo en cuenta también que la dosis y el tipo de micotoxina pueden ser decisivas en los resultados promotores ya bien sea de la salud o de la enfermedad.

6.2.2 Micotoxinas emergentes y probióticos

Los flujos bidireccionales entre probióticos y micotoxinas emergentes han sido ampliamente confirmados. Se ha demostrado que una gran cantidad de probióticos (*Lactobacillus*, *Bifidobacterium* y *S. cerevisiae*) reducen la bioaccesibilidad de las micotoxinas emergentes (EN, BEA y *Alternaria*) (Mallebrera et al., 2013; Manzini et al., 2015; Meca et al., 2012; Meca et al., 2013; Roig et al., 2013; Salim et al., 2020). Al mismo tiempo, las micotoxinas emergentes alteran las composiciones de los probióticos (*B. subtilis* y *Staphylococcus aureus*) (Böhm et al., 2000; Ola et al., 2013; Roig et al., 2014; Sondergaard et al., 2016). Esta disminución de la bioaccesibilidad puede deberse a la capacidad de unión de las cepas bacterianas a las micotoxinas (Crudo et al., 2021; Ge et al., 2017) y/o a su capacidad para contribuir a la detoxificación al originar derivados caracterizados por poseer menor toxicidad (Daud et al., 2020; Ge et al., 2017). Sin

embargo, en la mayoría de estos estudios los probióticos no se evalúan combinados, sino uno a uno y en condiciones *in vitro*. Sólo Vadopalas *et al.* (2020) evaluaron los efectos de los probióticos sobre las micotoxinas emergentes en condiciones *in vivo*. En este estudio, la adición de algunos iniciadores microbianos no solo modificó algunos parámetros sanguíneos, sino que también contribuyó a modular la microbiota intestinal y desintoxicar la AME. A pesar de estos efectos beneficiosos, cabe señalar que el experimento sólo duró dos meses y los órganos de los lechones no estaban completamente maduros. La combinación de probióticos y las condiciones *in vivo* sería una aproximación más realista al entorno humano, ya que hay muchos microorganismos que forman parte de la microbiota intestinal (Mulac & Humpf, 2011). Es más, está muy bien documentado que la interacción a lo largo del intestino afecta no solo a diferentes bacterias, sino también a hongos, arqueas, virus y helmintos (Frangiamone *et al.*, 2023).

6.2.3 Micotoxinas emergentes y prebióticos

Se ha demostrado claramente que los prebióticos reducen la bioaccesibilidad de las micotoxinas emergentes. Aunque el término prebiótico es muy amplio, los datos recopilados abarcan una gran cantidad de este tipo de compuestos en diferentes concentraciones (Crudo *et al.*, 2020; Ferrer *et al.*, 2015; Mallebrera *et al.*, 2013; Manzini *et al.*, 2015; Meca *et al.*, 2012b; Meca *et al.*, 2012c; *al.*, Salim *et al.*, 2020; Xu *et al.*, 2018; Zhang *et al.*, 2020). Como en el caso de los probióticos, existen dos mecanismos que explican esta reducción. El primero es la capacidad de los prebióticos para unirse a las micotoxinas emergentes, mientras que el segundo es la capacidad de metabolizarlas, siendo muy probable que ambos procesos funcionen al mismo tiempo. Sin embargo, la mayoría de los estudios no consideran un análisis de metabolitos secundarios de micotoxinas emergentes, por lo que esta metabolización no puede confirmarse completamente. En cuanto a la reducción de micotoxinas, si los metabolitos no se han degradado, se supone que se han unido a las estructuras de los prebióticos, aunque de todos modos también están sujetos a metabolización. Por el contrario, además de reducir la bioaccesibilidad, los prebióticos también pueden suprimir directamente los efectos perjudiciales de las micotoxinas. Como se muestra en Aichinger *et al.* (2018), la antocianidina delfinidina bloquea significativamente las propiedades genotóxicas de

ATX-II al suprimir el nivel de roturas de la cadena de ADN (Aichinger et al., 2018). La acción de estos prebióticos puede derivar del bloqueo de los efectos de las micotoxinas emergentes, impidiendo su acción o bien teniendo un impacto agonista beneficioso sobre algunas vías fisiológicas. Además, cabe señalar también que en todos estos experimentos los prebióticos se evalúan sólo uno por uno. Sin embargo, la realidad de la dieta humana incluye una amplia combinación de ellos, por lo que es necesario realizar más investigaciones teniendo en cuenta esta complejidad.

6.2.4 Micotoxinas emergentes, sinbióticos y postbióticos

El papel de los sinbióticos en la modulación de las micotoxinas emergentes se ha evaluado solo en dos estudios (Manzini et al., 2015; Meca et al., 2012b). En ambos, los resultados mostraron que la adición de sinbióticos aumentó la bioaccesibilidad de las micotoxinas en comparación con los prebióticos. Se ha planteado la hipótesis de que los probióticos degradaban a los prebióticos, de forma que estos últimos pueden perder su capacidad de unión. Aunque puede ser posible, no se puede extender como una generalidad, ya que en estos dos ensayos solo se utilizaron pequeñas fracciones de prebióticos y probióticos, y no representan la totalidad de los probióticos y prebióticos. Por el contrario, los sinbióticos aplicados a micotoxinas no emergentes revelan resultados opuestos. Por ejemplo, Zoghi *et al.* (2017) demostró que la eliminación de PAT por bacterias probióticas del jugo de manzana dependía significativamente del contenido de FOS (prebiótico) y de la adición de ácido ascórbico. En las mejores condiciones, el 91,3% de la concentración inicial de patulina se eliminó del jugo durante 6 semanas (Zoghi et al., 2017). Zoghi *et al.* (2019) también informaron que al agregar FOS, ácido ascórbico y cítrico al jugo de manzana inoculado con *L. plantarum*, la eficiencia de la eliminación de PAT mejoró al 95,9 % durante 6 semanas de almacenamiento en frío. Por otra parte, a pesar de que se supone que los sinbióticos mejoran los resultados clínicos en comparación con los probióticos o prebióticos solos debido a su sinergia, estos son muy difíciles de formular y pocos han demostrado poseer eficacia clínica. También cabe mencionar que la mayoría de los ensayos clínicos no se caracterizan por incluir controles probióticos o prebióticos relevantes, y muchos no han realizado análisis de microbiota relevantes

(Vemuri et al., 2020). Es necesario ampliar la investigación a más micotoxinas (emergentes) en combinación con sinbióticos.

Finalmente, se ha demostrado que los postbióticos se unen, metabolizan y alivian los efectos nocivos de algunas micotoxinas emergentes y no emergentes (Debevere et al., 2020; Petrova et al., 2022; Xu et al., 2023; Zong et al., 2023), así como estas micotoxinas han revelado alterar la composición de algunos postbióticos (Cai et al., 2022; Zhou et al., 2018). No obstante, es necesario ampliar estos experimentos a más micotoxinas (emergentes) y a más postbióticos, ya que sólo se ha evaluado la combinación de PYCW con BEA y CIT. De hecho, será muy interesante centrarse en la interacción entre las micotoxinas (emergentes) y los AGCC.

6.3 Artículo 3

Debido a la escasez de bibliografía, el estudio *in vivo* llevado a cabo tuvo como objetivo investigar los efectos de AFB1 y OTA en la microbiota intestinal, tanto individualmente como en combinación, utilizando muestras fecales de ratas expuestas a dietas contaminadas con estas micotoxinas durante 28 días, con o sin la inclusión de ingredientes funcionales (FW y P). Estudios anteriores han probado otras micotoxinas para determinar sus efectos sobre la microbiota intestinal utilizando modelos de ratas, como en el caso del DON (Miró-Abella et al., 2018) y la ZEN (Zhang et al., 2020b).

La dosis de micotoxinas en las dietas se seleccionó en función de los resultados de experimentos *in vivo* previos (Cimbalo et al., 2020). Los animales expuestos a estas doce dietas no mostraron ningún signo clínico de toxicidad (observaciones semanales) durante el estudio (Vila-Donat et al., 2024). Se incluyeron ambos sexos en la interpretación de los resultados, ya que es importante tener en cuenta el sexo en los estudios que examinan los impactos en la salud de los alimentos funcionales o ingredientes destinados a mejorar la salud intestinal (Shastri et al., 2015).

Se observó que los grupos que contenían una mezcla de ingredientes funcionales y micotoxinas (FW+AFB1 y FW+AFB1+OTA) mostraron un mayor valor de PD faith que los grupos que solo contenían el ingrediente funcional (FW). Además, FW+AFB1+OTA también informó un valor de PD faith más alto que FW+OTA. Sin embargo, como se esperaba, FW+P+OTA reveló un valor de PD faith más alto que ambos

grupos OTA y FW+OTA. Por un lado, está demostrado que la combinación micotoxinas-ingredientes funcionales contribuye más a la diversidad microbiana que los ingredientes funcionales por sí solos. Por otro lado, la exposición oral a la combinación de micotoxinas e ingredientes funcionales informó una mejora de la diversidad microbiana en comparación con las micotoxinas solas.

Esta sinergia puede explicarse por el hecho de que, aunque algunas bacterias beneficiosas, como las cepas de *Lactobacillus*, han demostrado reducir la AFB1 y la OTA (Ragoubi et al., 2021), estas micotoxinas también poseen la capacidad de disminuir bacterias dañinas como *Enterococcus faecalis*, *E. coli* y *Staphylococcus aureus* (Heller et al., 1975; Tiwari et al., 1986). La diversidad microbiana a menudo se asocia con un buen estado de salud, mientras que las condiciones patológicas tienden a mostrar una disminución en la diversidad (Hou et al., 2022). De hecho, se ha descubierto que ciertas combinaciones de especies muestran una resistencia a la colonización de patógenos mucho mayor juntas que solas. Una especie por sí sola no es suficiente para impactar fuertemente el crecimiento de patógenos, sino que se requiere una combinación de especies para bloquear el acceso a los nutrientes (Spragge et al., 2023). A pesar de que la PD faith es uno de los parámetros de diversidad α más utilizados, debe tenerse en cuenta que solo se centra en la similitud genética microbiana, sin tener en cuenta la similitud y/o diversidad biológicamente funcional.

6.3.1 Abundancias diferenciales taxonómicas

Firmicutes, Actinobacteria y Bacteriodota fueron los principales filos de microbiota intestinal en las heces observados en este estudio, seguidos por Proteobacteria. La mayor proporción de Actinobacteria se encontró en muestras fecales de FW+P. Respecto a otras abundancias diferenciales taxonómicas, hubo una reducción no significativa del filo Proteobacteria en muestras de ratas alimentadas con dietas contaminadas (AFB1, OTA y AFB1+OTA) respecto al control, mientras que un aumento - aunque no en todos los grupos - al añadir las abundancias de ingredientes funcionales. En humanos, una cantidad cada vez mayor de datos identifica al filo Proteobacteria como un posible y más homogéneo signo microbiano de enfermedad (Rizzatti et al., 2017). De hecho, el análisis de la composición de la microbiota en niños ha demostrado un aumento

gradual de Proteobacteria entre niños sanos, obesos y con esteatohepatitis no alcohólica (Zhu et al., 2013). Además, Proteobacteria parece estar implicadas en las enfermedades cardiovasculares, ya que hay evidencia de que hay niveles elevados de este filo presentes en pacientes con aterosclerosis (Shen et al., 2021).

La principal diferencia entre sexos se encontró en el grupo FW+AFB1+OTA, presentando los machos (25%) mayor % de Proteobacteria que las hembras (1,86%). De hecho, se ha demostrado que la proporción de Proteobacteria es mayor en los machos que en las hembras (Yan et al., 2022). Otro estudio realizado en peces (*Coreius guichenoti*) reportó la misma tendencia, ya que el perfil del filo en el intestino de los machos reveló que el filo Proteobacteria era dominante, a diferencia de las hembras donde el filo Tenericutes era el más abundante (Li et al., 2016). Sin embargo, Zhao *et al.* (2019) informaron que las hembras de panda gigante albergaban una mayor abundancia del filo Proteobacteria que los machos con la contribución de los géneros *Escherichia/Shigella*.

Según estos resultados, podría pensarse que, además de los efectos inducidos por la dieta, el sexo desempeña un papel importante en el aumento de la proporción de Proteobacteria. Sin embargo, se ha demostrado que la testosterona y la dihidrotestosterona, dos hormonas más abundantes en machos que en hembras, disminuyen la abundancia relativa de Proteobacteria en ratones a diferencia del grupo control. Por lo tanto, la mayor abundancia de Proteobacteria en machos no debería estar directamente relacionada con la condición sexual. Es más, podrían investigarse más vías biológicas en las que los machos sean más sensibles a los compuestos bioactivos que las hembras.

Es importante mencionar la mayor presencia de la especie integrante del filo Proteobacteria denominada *Morganella morganii* en las heces de animales alimentados con dietas control y enriquecidas con FW+P, contaminadas o no con AFB1 y OTA, en comparación con las dietas solo enriquecidas con FW o contaminadas con micotoxinas y no enriquecido con ingredientes funcionales en absoluto. En ratas, este microorganismo participa en la biotransformación de S-metilselenocisteína (MeSeCys) en dimetildiselenuro y dimetilselenuro. La biotransformación en dimetildiselenuro modifica la eficiencia de MeSeCys como fuente de Se, pero también produce un compuesto con un potencial aparentemente farmacológico, como la actividad anticancerígena. El estudio concluyó que la microflora intestinal desempeña un papel en la biotransformación de

MeSeCys y parece favorecer un metabolismo eficiente en los animales (Takahashi et al., 2023). De hecho, se ha revelado que *Morganella morganii* aumenta en la microbiota intestinal de pacientes con la EII y cáncer colorrectal, produciendo una familia de genotoxinas no descrita anteriormente, conocidas como indolimas, que se caracterizan por aumentar la permeabilidad intestinal y la tumorigénesis del colon en ratones gnotobióticos. (Hernández-Castro et al., 2024). Además, *Morganella morganii* también se ha relacionado con la formación de abscesos tumorales en humanos (Chen et al., 2011). Estos resultados contradictorios también se encuentran en otra especie como *Ruminococcus gnavus*, que se ha asociado tanto positiva como negativamente con un número cada vez mayor de enfermedades intestinales y extraintestinales, desde enfermedades inflamatorias intestinales hasta trastornos neurológicos (Crost et al., 2023). Sin embargo, la extrapolación de los resultados de ratas a humanos y viceversa en estudios de microbiota podría realizarse e interpretarse con cuidado.

Además, la adición de FW+P a la dieta aumentó la abundancia del filo Actinobacteria en la microbiota fecal. Cabe destacar que Actinobacteria es un filo que abarca géneros muy diferentes. Tanto *Mycobacterium*, relacionado con la tuberculosis, lepra, enfermedades pulmonares, enfermedades de la piel y los tejidos blandos, infecciones musculoesqueléticas y enfermedades diseminadas (Rakotoarisaona et al., 2024), como *Bifidobacterium*, utilizado como agentes terapéuticos para trastornos gastrointestinales y otras patologías, pertenecen a Actinobacteria (Tojo et al., 2014). De hecho, se demostró que la OTA aumentó *Corynebacterium* – un género de bacterias patógenas oportunistas pertenecientes a Actinobacteria – en los machos, pero al agregar los ingredientes funcionales (FW+P+OTA/ FW+OTA) la abundancia relativa de *Corynebacterium* disminuyó (Belmares et al., 2007; Lo et al., 2015; Yatera & Mukae, 2020).

6.3.2 Ratio F/B

La mayoría de los estudios han demostrado que los Bacteroidota en la microbiota fecal exhiben propiedades proinflamatorias debido a las endotoxinas e influyen en la producción de citoquinas, lo que contribuye a la EII. Además, las bacterias pertenecientes al filo Firmicutes exhiben efectos antiinflamatorios y puede aliviar la progresión de la EII

(Stojanov et al, 2020). En un estudio de trastornos gastrointestinales con ratas macho, el grupo de control mostró la proporción Firmicutes/Bacteroidetes (F/B) más alta, lo que confirma la correlación de la proporción con un perfil más saludable (Wu et al., 2021b). Los resultados obtenidos utilizando FW y P apuntan a un mayor efecto de los ingredientes funcionales en el aumento del ratio F/B en ratas macho que en hembras, pero la presencia de micotoxinas suprimió este resultado potencialmente beneficioso. Por el contrario, en humanos, la literatura indica que el ratio F/B aumentó significativamente en las mujeres en comparación con los hombres en un proyecto llevado a cabo en la población de Ucrania (Koliada et al., 2021). Sin embargo, en un estudio español, no se observaron diferencias de género en la diversidad de la microbiota, el nivel de filo, la composición general ni la proporción Firmicutes/Bacteroidetes (Haro et al., 2016). Curiosamente, un estudio que analizó la microbiota intestinal de pacientes con SOP mostró que la testosterona aumentó la proporción de Bacteroidetes y disminuyó la de Firmicutes (Matsushita et al., 2022), lo cual coincide con la población ucraniana. En cualquier caso, además de la proporción F/B, se debería prestar más atención al género o especie de la microbiota, ya que un filo se caracteriza por contener una complejidad de bacterias que desempeñan funciones contradictorias en el equilibrio entre la salud y la enfermedad.

6.3.3 Comparación de otros resultados obtenidos con los mismos animales

La disbiosis de la microbiota intestinal se ha asociado con enfermedades hepáticas y renales (Raj et al., 2020). De manera similar a nuestro experimento, Frangiamone *et al.* (2023b) estudiaron los efectos de estos doce grupos de dieta (AFB1, OTA y/o ingredientes funcionales) sobre la expresión de biomarcadores de daño hepático y renal. Se encontró que, en el hígado de los hombres, la exposición más dañina fue aquella de la AFB1 al reducir la expresión de CPS1, siendo este un indicador de la enfermedad del hígado graso no alcohólico (Eriksen et al., 2019), que fue totalmente revertida con la administración de FW. Esta mejora fisiológica al exponerlos al ingrediente funcional debería, en teoría, estar relacionada con una mejor flora intestinal. Sin embargo, en nuestro estudio no solo la AFB1 condujo a una disminución del filo Proteobacteria, sino que también la suplementación con FW (FW+OTA) aumentó la abundancia de Proteobacteria. Estos resultados contradictorios son sorprendentes porque se ha planteado

la hipótesis y se ha confirmado que un aumento en la proporción del filo Proteobacteria fue la alteración más destacada en la esteatosis hepática inducida por el eje intestino-hígado (Vasques-Monteiro et al., 2021). Por otro lado, la OTA fue la más dañina en el riñón al regular positivamente la molécula 1 de lesión renal (KIM-1). Además, como en el caso del hígado, la administración de FW mitigó la regulación positiva de KIM-1 inducida por las micotoxinas (Frangiamone et al., 2023b). Sin embargo, en nuestro estudio, al comparar las exposiciones a OTA con FW+OTA, la adición de FW sorprendentemente reveló un aumento de Proteobacteria, un filo relacionado con la enfermedad renal crónica (Voroneanu et al., 2023). Por tanto, se puede concluir que los efectos beneficiosos de los ingredientes funcionales sobre la relación F/B y otros niveles de antioxidantes influyen más en el resultado positivo de su ingesta que los cambios observados en las proporciones de Proteobacteria.

Finalmente, Vila-Donat *et al.* (2024) cuantificaron AFB1 y OTA en orina de ratas y mostraron el impacto de los ingredientes bioactivos de la dieta en la excreción de micotoxinas. Los resultados revelaron que los niveles urinarios de AFB1 del grupo que ingirió FW+AFB1+OTA fueron significativamente más altos que los del grupo sin FW (AFB1+OTA). La calabaza tiene un contenido notable de vitamina E, que se ha relacionado con la excreción de AFB1 a través de su metabolización (Abdel-Hamid y Firgany, 2015). Además, tanto el FW como la P incluyen una gran cantidad de compuestos fenólicos (ácido p-hidroxibenzoico, catequina, ácido clorogénico). Estos compuestos se han considerado agentes de desintoxicación de toxinas microbianas (Saricaoglu et al., 2022). Además, el FW contiene cepas probióticas, como *Bifidobacterium* y *Lactobacillus*, que han demostrado degradar tanto AFB1 como OTA (Vila-Donat et al., 2025). Además, las LAB, a través de sus interacciones con la pared celular, pueden absorber AFB1 uniéndola a sus componentes, incluyendo polisacáridos y peptidoglicanos, reduciendo la biodisponibilidad y la absorción sistémica de la toxina. Este mecanismo subraya el potencial de estos ingredientes bioactivos para favorecer la desintoxicación de micotoxinas, especialmente en dietas ricas en alimentos de origen vegetal, como se observa en los patrones mediterráneos.

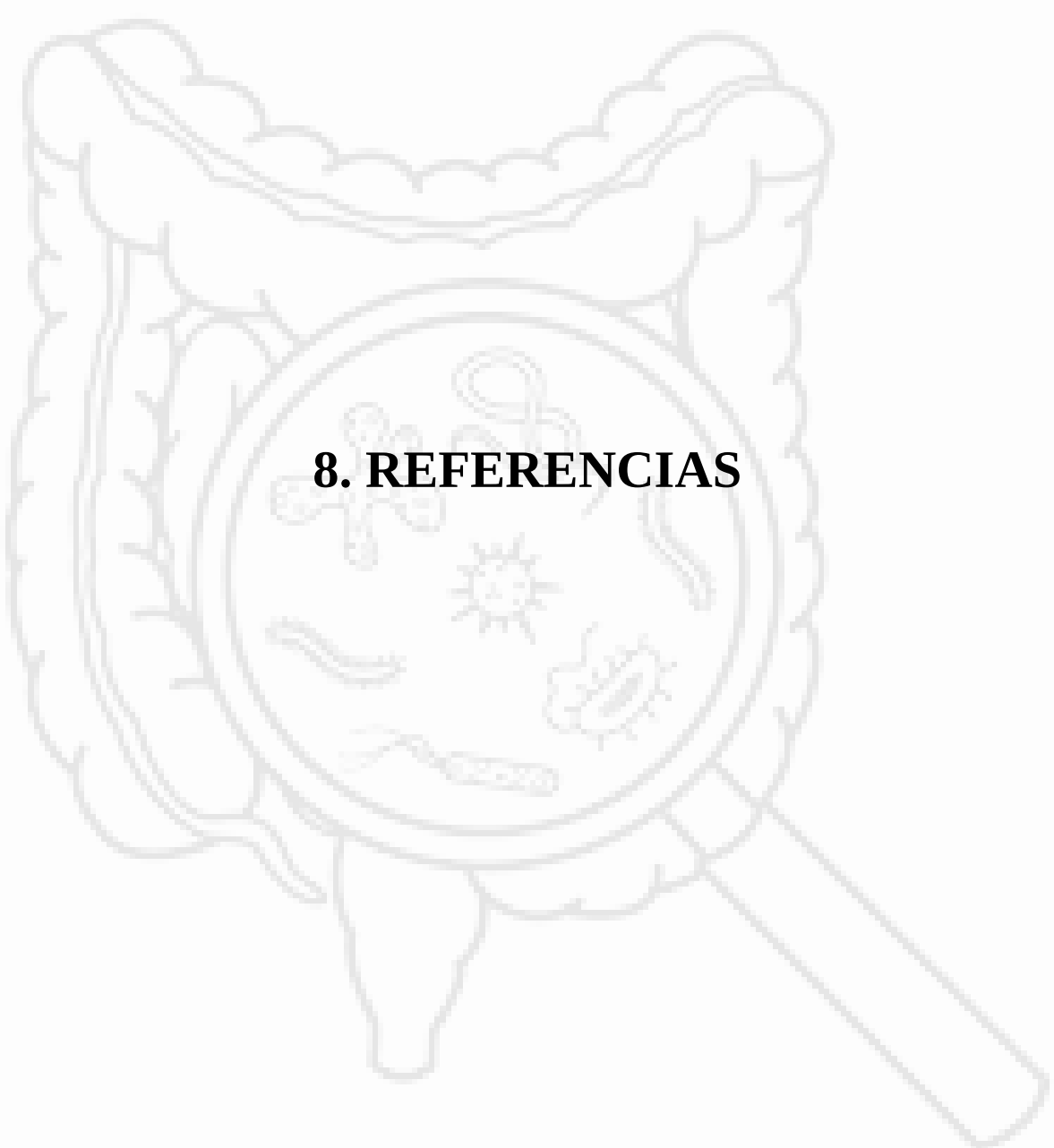


7.CONCLUSIONES

1. La presencia de ajo contribuyó a reducir la bioaccesibilidad de la AFB1 en el pan (7–8%). También se reveló que el ajo aumenta la viabilidad celular (9-18%). La citometría de flujo mostró que el ajo mitiga los efectos tóxicos de AFB1 en las células Jurkat-T en el ciclo celular al prevenir la detención de la fase S, la apoptosis/necrosis y la producción de ROS celular y mitocondrial. Por tanto, se demostraron los efectos antígenotóxicos, antiapoptóticos y antioxidantes del ajo. Sin embargo, se necesitan más investigaciones para confirmar el papel protector del ajo *in vivo* y su probable aplicación en la industria alimentaria como prebiótico para contrarrestar la posible contaminación alimentaria por AFB1 a niveles bajos y su potencial toxicidad en humanos.
2. Además de la AFB1 junto a otras micotoxinas reguladas, las micotoxinas emergentes también han estado implicadas en numerosos procesos biológicos dañinos. A pesar de su naturaleza diversa, los estudios han demostrado que la microbiota intestinal y los PPSP pueden desintoxicar y/o aliviar los efectos adversos resultantes de la exposición a estas micotoxinas emergentes. Las micotoxinas enniatinas, BEA, NIV y *Alternaria* se encuentran entre las micotoxinas emergentes encontradas con mayor frecuencia. Los géneros *Lactobacillus*, *Bifidobacterium* y *Saccharomyces* fueron los probióticos más estudiados, mientras que la inulina, la celulosa, el β -glucano, el FOS y el galactomanano se encuentran entre los prebióticos más evaluados. Los datos sobre sinbióticos y postbióticos fueron limitados. La evidencia demuestra claramente la existencia de una interacción entre la microbiota intestinal y las micotoxinas emergentes, así como entre los PPSP y las micotoxinas emergentes, involucrando procesos como la metabolización, la colonización, la modulación y la unión. Además, se identificaron conexiones entre la microbiota intestinal y los PPSP, así como dentro del mismo PPSP, lo que contribuye a aliviar los efectos de las micotoxinas emergentes. Sin embargo, los datos cuantitativos y cualitativos siguen siendo limitados. Se necesitan más ensayos *in vivo* y a largo plazo para comprender mejor la complejidad de estas interacciones tanto en animales como en humanos. La formulación de PPSP como alimentos funcionales y nutracéuticos

debe considerarse como una estrategia preventiva contra la toxicidad de las micotoxinas emergentes en la dieta humana y la alimentación animal.

3. En el ensayo *in vivo* llevado a cabo los cambios en la diversidad α con respecto a la PD faith estuvieron más relacionados con la dieta, los ingredientes funcionales y la exposición a micotoxinas que con el sexo. Por el contrario, ninguno de estos parámetros alteró la uniformidad microbiana. Los gráficos de diversidad β mostraron un grupo de dietas contaminadas y otro grupo que agrupaba las dietas contaminadas enriquecidas con ingredientes funcionales. Las dietas de control enriquecidas con ingredientes funcionales cambiaron las comunidades microbianas fecales en diferentes aspectos y se mostraron en las parcelas por separado del control y de los dos grupos mencionados anteriormente. En general, se puede observar una reducción en el filo Proteobacteria en muestras de ratas alimentadas con dietas contaminadas, siendo *Morganella morganii* la especie más frecuente en las ratas control, seguida de todos los grupos, incluidos los ingredientes funcionales y las micotoxinas. Finalmente, la dieta no modificó el ratio F/B, mientras que se encontraron diferencias significativas entre sexos en las dietas FW y FW+P. Se puede concluir que la exposición a micotoxinas a través de la dieta, así como la inclusión de ingredientes funcionales, desencadenan cambios en la microbiota de las ratas. Además, el sexo de los animales conduce a resultados diferentes.
4. Los efectos beneficiosos de los ingredientes funcionales se deben seguir investigando, no solo a la hora de promover la salud humana frente a los efectos adversos de la AFB1 y OTA, sino también ofrecer a la industria alimentaria estrategias para el desarrollo de alimentos funcionales y nutraceuticos que puedan mitigar el problema global de la contaminación por micotoxinas.



8. REFERENCIAS

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AGRADECIMIENTOS



El agradecimiento es uno de los actos humanos más verdaderos. Básicamente, porque reconoce una realidad: hemos recibido, se nos ha dado, no por nuestros méritos, sino por el mero hecho de existir, de ser nosotros mismos.

Esto no quiere decir que el esfuerzo personal, la constancia, el trabajo, las virtudes o los defectos no determinen lo que tenemos o lo que somos. No todo está ya predeterminado desde que nacemos, sino que la libertad humana tiene una de las últimas palabras. Ahora bien, no somos omnipotentes. De hecho, no nos solemos salir con la nuestra, puesto que aquello que viene es muchísimo más importante que aquello que va. Sin embargo, la naturaleza de nuestras acciones tiene un eco importante en todo aquello que nos sucede. La moralidad se carga de atracción gravitatoria.

Unas acciones que en demasiadas ocasiones no reflejan nuestra realidad. En un mundo que intenta conocer cada vez más – y que está muy bien, se olvida de que lo que verdaderamente nos salva es el saber. Podemos creer que contemplamos la realidad con total nitidez, pero esto no es así. ¿Cuántas veces hemos valorado un momento con el paso del tiempo? ¿Cuántas veces nos hemos arrepentido de no haber emprendido tal o cual acción por miedo o vergüenza? ¿Cuántas veces hemos dejado pasar el tren que nos llevaba a casa?

Muchas veces – por no decir cada una de ellas – no somos totalmente conscientes de las repercusiones de nuestras acciones. Es normal, somos humanos. Ser limitados es algo que forma parte intrínseca de nuestro ser. Dentro de cada acción, no solo se halla nuestra persona, sino que hay otros rostros que vienen al encuentro. Unos rostros que pueden ser familiares, conocidos o personas que jamás habíamos visto. Hay mucha diversidad, pero lo que une a todos es que cada uno nos da algo que jamás olvidaremos. Por eso, el agradecimiento siempre ha de estar presente.

Agradecer implica humildad. Somos consciente de que no nos valemos por nosotros mismos. Sin los demás, no llegamos ya a buen puerto, ni tan siquiera podemos avistar el puerto. No solo somos el resultado de nuestra genética o virtud, sino que aquellos que pasan por nuestras vidas también dejan huellas imborrables en nosotros. Las personas transforman. Incluso aquellas huellas que nos gustaría borrar de nuestra mente y corazón constituyen una catapulta hacia el crecimiento personal.

Creer también supone tomar decisiones. No podemos quedarnos con todo. Seríamos infelices. Elegir implica necesariamente un proceso de selección-exclusión.

Pese a que pueda parecer un contrasentido, la libertad no se halla en la multitud de opciones, sino en abrazar que la unicidad de esa opción elegida abraza el todo. Además, elegir libremente es un regalo que se nos ha dado. Solo los humanos tenemos la verdadera capacidad de elegir aquello que nos da plenitud, independientemente de la carga emocional o de las adversidades que pueda presentar cada momento.

Cada acción es un acto de fe. Un pie se halla en el suelo, mientras que el otro se eleva hacia el cielo. Tengamos la edad que tengamos, no tenemos el control total sobre lo que hacemos. Podemos madurar y coger experiencia, pero el más anciano es igual de niño – o incluso más, que aquel que se adentra en la mayoría de edad.

Había una vez un chico de 24 años que entró en el despacho de un profesor. Después de haber estudiado Farmacia y Nutrición Humana y Dietética, le preguntó acerca de continuar sus estudios haciendo el doctorado. Él era un mar de dudas, sueños e ingenuidad. El profesor le habló de lo que suponía iniciarse en ello. El chico escuchó atentamente, pero entendió lo que pudo. Uno estaba de salida, el otro de entrada. La diferencia de edad era abismal. Sin embargo, había algo en común que se respiraba en el ambiente. Quizás, nunca ambos lo lleguen a saber del todo. Somos un misterio hasta para nosotros mismos.

Una vez acabó el doctorado, el chico se había dado cuenta de que era totalmente otro. Habían pasado algo menos de 3 años, pero parecían 30. Había vivido mucho. El enriquecimiento había sido inimaginable. Pensaba acerca de todo lo sucedido y no podía evitar sentirse invadido por ese romanticismo que sacude violentamente todo tiempo y espacio. Agradecía todo lo que le había sido dado.

Comenzaba por sus padres, que le habían dado el maravilloso don de la vida, aquel que compartía con su hermana. No podía olvidar a sus abuelos, en especial, a su abuelo Guillermo. Un hombre que le recordaba que no somos de este mundo. Tampoco podían faltar aquellos familiares que habían jugado un papel más que importante, sobre todo, en los momentos más difíciles. Los amigos tampoco podían faltar. Algunos se unieron en los párvulos, otros a lo largo del colegio y, ya más tarde, aquellos de la etapa universitaria. Los años de la universidad le habían transformado completamente. Él iba hacia Damasco, pero un rostro se interpuso en su camino.

No podía pararse ahí. Se acordaba también de todos los profesores que habían formado parte de su instrucción, desde los 3 años hasta esos momentos. Desde la señorita

Pilar hasta las directoras Lara y Pilar. Los compañeros tampoco podían faltar, había aprendido tanto gracias a ellos. A veces, el compañero era indistinguible del profesor.

Dejando aquello más cercano y concreto, también se acordaba de su patria, de la que se sentía profundamente orgulloso. No era un orgullo que escondiera aquellas partes más oscuras ni que se enarbolara únicamente en la bandera, sino un orgullo que venía de saberse caminando a hombros de gigantes. Se sabía portador de un legado épico. Por último, dio gracias a Dios.

Después de cerrar los ojos mientras suspiraba, el chico se dio la vuelta y decidió abandonar el lugar que le había visto crecer a lo largo de 9 años. En ese mismo momento, se acordó de la conversación que mantuvo con aquel profesor antes de comenzar el doctorado. El tiempo se paró, había descubierto aquello misterioso que se hallaba en el ambiente ese día. No dudó en coger papel y boli y escribirle al profesor:

Querido amigo

Soñaba tanto con este momento

El fracaso me recordó quién era

Sé que es hora de volar

Tan solo te pido una cosa

Volvamos a casa

Article

Allium sativum L. var. Voghiera Reduces Aflatoxin B1 Bioaccessibility and Cytotoxicity *In Vitro*

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Abstract: The present work focuses on the evaluation of AFB1's bioaccessibility and cytotoxicity *in vitro* using bread (naturally contaminated) enriched or not enriched with fresh Voghiera garlic (2%). Two different experiments were carried out: experiment 1 (E1), with low-AFB1-concentration breads (1.6–1.7 mg/kg); and experiment 2 (E2), with high-AFB1-concentration breads (96.4–102.7 mg/kg). Eight breads were prepared, four for E1 (experiment 1) and another four for E2 (experiment 2), with each experiment having a control group (C), a garlic-enriched group (2%) (G), an AFB1 group (A), and an AFB1 + garlic group (A + G). Simulated digestion was performed on each type of bread, and gastric and intestinal digests were obtained. AFB1 content in flours, baked bread, and gastric and intestinal digests was measured by High-Performance Liquid Chromatography coupled to Fluorescence Detection. The results demonstrate dose-dependent AFB1 bioaccessibility and that the presence of garlic contributed to its reduction in both doses (7–8%). Moreover, garlic's presence in AFB1-contaminated bread increased cell viability (9–18%) in differentiated Caco-2 cells and mitigated the arrest of S and G2/M phases provoked by AFB1 on Jurkat T cells and reduced apoptosis/necrosis, cellular reactive oxygen species (ROS), and mitochondrial ROS by 16%, 71%, and 24% respectively. The inclusion of garlic as a functional ingredient helped relieve the presence and effects of AFB1.

Keywords: mycotoxin; garlic; prebiotic; Jurkat T cells; *in vitro* digestion; flow cytometry



Citation: Lázaro, Á.; Frangiamone, M.; Maietti, A.; Cimbalo, A.; Vila-Donat, P.; Manyes, L. *Allium sativum* L. var. Voghiera Reduces Aflatoxin B1 Bioaccessibility and Cytotoxicity *In Vitro*. *Foods* **2024**, *13*, 487. <https://doi.org/10.3390/foods13030487>

Academic Editors: Ana Isabel Prieto and Silvia Pichardo

Received: 10 January 2024

Revised: 29 January 2024

Accepted: 1 February 2024

Published: 2 February 2024



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1. Introduction

Aflatoxins (AFs) are secondary metabolites produced by mycotoxigenic fungi belonging to *Aspergillus* genera which contaminate a wide variety of crops, such as cereals, oilseeds, tree nuts, and spices. Among them, aflatoxin B1 (AFB1) is the most studied due to its severe carcinogenicity for humans and animals [1]. In fact, the International Agency for Research on Cancer classified AFB1 as a human carcinogen belonging to Group 1 due to its ability to form DNA adducts [2]. Different *in vitro* and *in vivo* studies have demonstrated its acute and chronic toxicity, which can lead to the onset of liver damage, nutritional dysfunctions, immunosuppression, and cancer [3–5].

As mycotoxins are major contaminants of cereals, it has been found that AFB1 contaminates feeds universally [6]. It has been shown that in 87.5% of samples found to contain AFB1, its level was higher than the allowed level, which was 2 µg/kg from 2006 to 2023 [7]. Despite treatments applied during the production process, the natural occurrence of this mycotoxin has been observed in commercial bread loaves [8], as well as in maize and in wheat bread [9,10]. Consequently, to enhance AFB1 reduction in bakery products, the use of bio-preservatives in their formulation could be a good alternative to conventional procedures [11]. Following this trend, the addition of functional ingredients from various food matrices rich in bioactive compounds has been previously investigated to assess AFB1

bioaccessibility reduction through simulated gastrointestinal digestion models [12–16]. Likewise, the cytoprotective effect of some natural compounds against mycotoxin was demonstrated by using different cell models [17,18].

Among others, garlic plant (*Allium sativum* L.) has been shown to exert multiple health benefits, such as the prevention and treatment of cardiovascular diseases, antioxidant activity, and antimicrobial effects [19]. The variety “Voghiera” is a typical Italian eco-type of garlic cultivated in a particular area north-east of Voghiera (Ferrara) in the Emilia-Romagna region and classified as a Protected Designation of Origin product. Phytochemical analysis of this garlic has been used for the isolation of several saponins, such as furostanol, voghierosides (A1, A2, E1, E2), spirostanol, and eugenol diglycosides, which possess important antimicrobial activities [20]. In addition, its role in mitigating the evolution of cancer progression was recently investigated [21].

Thus, the aim of this study was to evaluate the possible beneficial effect of Voghiera garlic on bread in terms of modifying AFB1's bioaccessibility and cytotoxicity by using Caco-2 cells, which are widely used as a prototype of the intestinal epithelial barrier [22], and Jurkat T human cells, which are also broadly used in *in vitro* studies of T cytokines, receptor expression, and cell signal transduction lines [23].

2. Materials and Methods

2.1. Reagents

AFB1 standard solution (purity > 99%) was acquired from Sigma-Aldrich (St. Louis, MO, USA). Potassium thiocyanate (KSCN), potassium chloride (KCl), hydrochloric acid (HCl), sodium dihydrogen phosphate (NaH_2PO_4), sodium hydroxide (NaOH), sodium sulfate (Na_2SO_4), sodium chloride (NaCl), sodium hydrogen carbonate (Na-HCO_3), urea ($\text{CO}(\text{NH}_2)_2$), pepsin A (674 U mg^{-1} P7000), pancreatin (762 U mg^{-1} P1750), bile salts (B8631), and α -amylase (930 U mg^{-1} A3403) were purchased from Sigma-Aldrich (Madrid, Spain). Acetonitrile (ACN) and methanol (MeOH) were obtained from Fisher Scientific (Madrid, Spain). Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Dimethyl sulfoxide (DMSO) was acquired from Fisher Scientific (Geel, Belgium). Bread ingredients were purchased at a local supermarket. Fresh garlic was preserved at 4°C and minced before use. Propidium iodide (PI) and a cycleTEST™ PLUS DNA Reagent Kit were obtained from BD Biosciences (San Diego, CA, USA) for the cell cycle assay by flow cytometry. The Annexin V-FITC conjugate was acquired from Miltenyi and the MitoTracker® probe was purchased from Invitrogen (Waltham, MA, USA). 2',7'-dichlorodihydrofluorescein diacetate ($\text{H}_2\text{-DCFDA}$) and phosphate buffer saline (PBS) were bought from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Maize Flour Contamination and Mycotoxin Production

Maize was contaminated by *A. flavus* ITEM 8111 acquired from the Agro-Food Microbial Culture Collection of the Institute of Sciences and Food Production (ISPA, Bari, Italy). The fungus was kept under ideal conditions in the laboratory to produce the mycotoxin in the food matrix (maize) in which it was inoculated. Under these conditions, the strain used only generated this mycotoxin and not any others. A total of 450 g of maize was introduced per 1 L previously sterilized jar, and two jars were prepared. The contamination was carried out by adding 20 mL of mycelium suspension and spores in peptone water with Tween 80 of the fungal strain. Glass jars were maintained at room temperature for a period of three weeks. Every 2–3 days, 1 mL of distilled water was added to the jars to maintain the humidity conditions.

After three weeks, the jars were autoclaved to eliminate fungal contamination and the maize was transformed into flour until total homogenization was achieved, including sieving. The maize samples were kept at -20°C [12]. The maize flour was analyzed via High-Performance Liquid Chromatography coupled to Fluorescence Detection (HPLC-FLD) (as reported in Section 2.6) to quantify AFB1 and discard any other mycotoxins.

2.3. Bread Ingredients, Bread Making, and Baking

Eight breads were made combining the bioactive ingredient and the AFB1, four corresponding to experiment 1 (E1) and another four corresponding to experiment 2 (E2): a control bread (C), 2% garlic bread (G), AFB1 bread (A), and AFB1 + 2% garlic bread (A + G). Control bread was prepared using the following recipe: 127 g of wheat flour, 66 mL of mineral water (37 °C), 8 g of yeast for bakery products (Levital, Sant Hilari Sacalm, Spain), 4 g of sugar, and 2.6 g of NaCl. After merging all the ingredients, homogenization was conducted in a SilverCrest Bread Maker SBB 850 A1 (Kompernass GMBH, Bo-chum, Germany) for 5 min, and they were shaped in molds (approximately 200 g), covered using a damp cloth, and fermented for 1 h at room temperature. Afterwards, the breads were covered with silver foil and baked at 180 °C for 35 min in a Memmert ULE 500 muffle furnace (Madrid, Spain). Then, the breads were unmolded and refrigerated at room temperature for 1 h. Enriched breads were prepared by slightly modifying the control recipe to produce 2% garlic bread (G and A + G). Finally, breads contaminated with AFB1 were prepared by replacing some of the wheat flour with 5.9 g (E1~152 mg/kg) and 54.7 g (E2~197 mg/kg) of maize flour contaminated with AFB1 to obtain low-concentration and high-concentration breads, respectively. This difference in AFB1 concentration was used to determine if it played an important role in bioaccessibility. As shown in Table 1, eight breads were obtained in total.

Table 1. Bread composition in experiments 1 and 2.

Ingredient (g)	C		G		A		A + G	
	E1	E2	E1	E2	E1	E2	E1	E2
Wheat flour	127	127	124	124	121.1	72.3	118.1	69.3
C maize flour	-	-	-	-	5.9	54.7	5.9	54.7
Water	66	66	65	65	66	66	65	65
Salt	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6
Sugar	4	4	4	4	4	4	4	4
Fresh yeast	8	8	8	8	8	8	8	8
Garlic	-	-	4	4	-	-	4	4
Total quantity	207.6	207.6	207.6	207.6	207.6	207.6	207.6	207.6

C (control). E1 (experiment 1). E2 (experiment 2). (G) garlic. A (Aflatoxin B1). A + G (Aflatoxin B1 + garlic). C (contaminated). - (no content) (*n* = 3).

2.4. In Vitro Static Digestion Model

The digestion process consisted of three phases: (oral, gastric, and intestinal), as reported in previous work [12]. In short, 10 g of bread was put in sterilized plastic bags (500 mL) and mixed with Milli-Q water (at 37 °C) and 6 mL of artificial saliva (Millipore, Bedford, MA, USA). To reproduce the oral phase, an IUL Stomacher (IUL S.A, Barcelona, Spain) was used for 30 s to simulate the mastication process. Saliva was prepared the day before (and adjusted to pH = 6.8) by mixing 0.17 mL of NaCl (175.3 g/L), 1 mL of KSCN (20 g/L), 1 mL of NaH₂PO₄ (88.8 g/L), 2 mL of NaHCO₃ (84.7 g/L), 1 mL of KCl (89.6 g/L), 1 mL of Na₂SO₄ (57 g/L), 29 mg of α -amylase, 0.8 mL of urea (25 g/L), and distilled water to achieve a volume of 100 mL. Once the oral phase was simulated, the content was transferred to a topaz Erlenmeyer flask to carry out the gastric phase. The mixture was acidified to pH = 2 with 6 N HCl solution. Then, 14 mL of Milli-Q water (at 37 °C) and 0.5 g of pepsin solution (1 g in 25 mL of 0.1 N HCl) were added to achieve 100 mL. Samples were incubated for 2 h at 37 °C under darkness and slight agitation (1 g) using an orbital shaker (Infors AG CH-4103, Bottmingen, Switzerland). Following that, gastric aliquots (15 mL) were kept for further analysis and pancreatic digestion was conducted by adding 1.25 g of a bile salt/pancreatin mixture (0.625 g of bile salts and of 0.1 g of pancreatin dissolved in 25 mL of 0.1 N NaHCO₃) at pH = 6.5 (0.5 N NaHCO₃). Digests were incubated as formerly detailed (2 h at 37 °C in darkness and slight agitation), the pH was finally adjusted to 7.2 (0.5 N NaOH), the samples were centrifuged (226× *g*

for 10 min at 4 °C), and the supernatant was gathered to obtain the intestinal digests. The experiments were performed in triplicate and quadruplicate.

2.5. AFB1 Extraction from Bread and Digest Samples

A total of 5 g of maize/wheat/bread flour was placed in centrifuge tubes (50 mL). Then, 25 mL of MeOH/H₂O (80/20) was added. Ultraturrax (T 18 digital ULTRA-TURRAX®, Staufen, Germany) was used for 3 min to crush the samples after being centrifuged at 226 × g for 5 min (Centrifuge 5810R, Eppendorf, Germany). The supernatant was collected and filtered through a 0.22 µm filter (Phenomenex, Madrid, Spain). All analyses were performed in triplicate (*n* = 3). Gastric and intestinal digest samples were centrifuged at 226 × g for 10 min at 4 °C, filtered (0.22 µm filter) (Phenomenex, Madrid, Spain), diluted with MeOH (1:1), and injected in HPLC-FLD.

2.6. Quantitative Determination of AFB1 by HPLC-FLD

AFB1 was characterized using HPLC with an Agilent 1100 series (Agilent Technologies, Santa Clara, CA, USA) furnished with an automatic sampler, a degasser, a quaternary pump, and an FLD detector Agilent 1200 (Agilent Technologies, Santa Clara, CA, USA), and Agilent Software JP03924119 was used for data analysis.

A derivatization system (UVE™ Photochemical reactor, LCTech, Jasco Analitica, Madrid, Spain, S.L.) was placed between the analytical column and the FLD detector to improve the fluorescent activity of AFB1. The chromatographic separation was carried out by using a reverse phase column, 00F-4633-EO Kinetex EVO C18 (150 × 4.6 mm, 100 Å, and particle size of 5 µm) (Phenomenex, Palo Alto, CA, USA) at 40 °C. The isocratic mobile phase was composed of H₂O/ACN/MeOH (60:10:30 *v/v/v*) with a flow rate of 1 mL min⁻¹. The injection volume was 20 µL for flour and breads and 40 µL for gastrointestinal digests. The AFB1 excitation and emission wavelengths were λ_{ex} = 365 nm and λ_{em} = 440 nm.

2.7. Quantitative Determination Method Validation

Linearity was assessed by means of the coefficient of regression (*r*²) of calibration matrix-matched curves. The limit of detection (LOD) and limit of quantification (LOQ) were measured through a comparison of the chromatographic signals from samples containing low concentrations of the compound to those of blank samples and establishing the minimum concentration at which the compound can be accurately detected and measured, respectively. Signal-to-noise ratios of 10:1 and 3:1 was considered to obtain the LOD and LOQ, respectively. Repeatability and intermediate precision were quantified as the relative standard deviation of four analyses performed on four different days (*n* = 4) and four analyses performed on the same day (*n* = 4), respectively.

Flour and bread matrix-matched curves were prepared by fortifying, with an AFB1 standard (100 mg/L in MeOH), the extracted solution from the blank (bread with uncontaminated wheat flour) with different AFB1 concentrations to obtain at least 6 level point curves. Validation parameters for the AFB1 quantitative determination method (linear regression equation, linearity range, *r*², LOD/LOQ) are described in Table 2.

Matrix-matched calibration curves were obtained by spiking blank digest (gastric and intestinal) with the AFB1 standard (10 mg/L in MeOH) at different concentrations to obtain at least 6 level point curves (Table 2).

2.8. Bioaccessibility Study

Bioaccessibility was calculated for the gastric and intestinal phases. The calculation of AFB1 bioaccessibility (%) was considered as the percentage of mycotoxin from the initial bread that was detected in the intestinal digests. The quantity of mycotoxin (µg) in 10 g of bread was obtained from bread concentration (µg/kg) by conversion factors (×10/1000).

The quantity of mycotoxin (μg) in 100 mL of digest was obtained from digest concentration ($\mu\text{g/L}$) by conversion factors ($\times 100/1000$), as can be seen in the formula below.

$$\text{Bioaccessibility} = \text{digest concentration } (\mu\text{g/L}) \times 1000 / \text{bread concentration } (\mu\text{g/kg})$$

Table 2. Validation parameters for AFB1 quantitative determination method.

Matrices	Linearity Range	r^2	Linear Regression Equation	LOD/LOQ ($\mu\text{g/L}$)
Maize flour	0.05–5 mg/L	0.9998	$y = 516.44x - 4.7063$	0.25/0.75
Bread	0.05–0.5 mg/L	0.9948	$y = 389.03 - 3.1217$	0.25/0.75
	0.05–5 mg/L	0.9999	$y = 400.28x - 9.6478$	0.25/0.75
Gastric digest	5–200 $\mu\text{g/L}$	0.9998	$y = 0.7611x - 0.3598$	0.25/0.75
	50–1500 $\mu\text{g/L}$	0.9996	$y = 1134.4x - 14.942$	0.25/0.75
Intestinal digest	5–50 $\mu\text{g/L}$	0.9984	$y = 0.8279x - 0.7944$	0.25/0.75
	10–1500 $\mu\text{g/L}$	0.9994	$y = 633.28x - 4.3939$	0.25/0.75

LOD, limit of detection. LOQ, limit of quantification. r^2 , regression coefficient. Mean $\pm$ standard deviation ($n = 4$).

2.9. Cell Culture

Human colorectal adenocarcinoma cells, Caco-2 (ATCC HB-8065), were kept in DMEM supplemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin, and 100 U/mL penicillin. To culture cells, 75 cm² plastic flasks with filter screw caps kept in a in an atmosphere containing 5% CO₂ at 95% relative humidity and 37 °C were used. A specific number of sub-passages (from 15 to 20 passages) were habitually controlled to maintain genetic homogeneity. The culture medium was changed every two days.

Jurkat T cells (ATCC-TIB152) were kept in RPMI 1640 medium supplemented with 10% FBS, 100 mg/mL streptomycin, and 100 U/mL penicillin. The conditions of incubation were pH = 7.4, 37 °C under 5% CO₂, and 95% constant humidity. A small number of sub-passages (from 25 to 30 passages) were habitually controlled to maintain genetic homogeneity. The culture medium was changed every two days.

2.10. Cell Viability Assay

Caco-2 cells were cultured in 24-well tissue culture plates by adding 500 μL /well of a suspension of 2×10^6 cells/mL. Twelve plates were needed, four for each group for three iterations each (24, 48, and 72 h). Every 3 days, the medium was replaced. After 21 days, which is the time necessary for Caco-2 cells to differentiate, the exposure was carried out. The exposure consisted of serial dilutions (non-diluted, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, and $\frac{1}{32}$) of intestinal digests from E2, since it contained the optimal AFB1 concentration needed to conduct the experiments in contrast to E1, whose AFB1 levels were very low. Four wells per condition were tested.

To determine the viability of cells, the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was used. This consists of the reduction of yellow soluble tetrazolium salt to an insoluble purple formazan crystal through a reaction independent of the mitochondria. Briefly, after exposure studies with the intestinal digests (24, 48, and 72 h), the medium containing these compounds was eliminated, and the cells of each well-received fresh medium containing MTT at 1 mg/mL. The plates were wrapped in foil and incubated for 4 h at 37 °C. After that, the medium containing the MTT was removed, and the resulting formazan salt was solubilized in DMSO. The absorbance was quantified at 620 nm using an absorption spectrometer (SynergyTM H1, Berlin, Germany).

2.11. Cell Cycle Analysis

A total of 2×10^5 Jurkat T cells/well was seeded in 6-well plates and incubated using the intestinal digests diluted to 10% in cell media (C; G; A; A + G) for 7 days (representing

0.7 μM AFB1 in the cell media of A and A + G exposures), with the experiment carried out in quadruplicate. The cell suspension was poured into a 15 mL falcon and centrifuged for 5 min at $300\times g$ at room temperature. The supernatant was aspirated, leaving about 50 μL of residual fluid in the tube to avoid disturbing the pellet. The CycleTESTTM PLUS kit protocol was followed. Buffer solution (100 μL) was added to the cell pellet and resuspended by gentle vortexing at low speed. They were again centrifuged for 5 min at $300\times g$ at room temperature, and this step was repeated two more times. Staining was conducted by using PI stain solution for 10 min in the refrigerator in the dark. Samples were analyzed by flow cytometry with the appropriate settings.

2.12. Apoptosis/Necrosis Pathway Analysis

Cells were exposed as explained in Section 2.11. Cells were washed with 200 μL of binding buffer and centrifuged at $300\times g$ for 10 min. The supernatant was completely aspirated, and the cell pellet was resuspended in 200 μL of $1\times$ binding buffer. Following the fabricant instructions, 10 μL of Annexin V-FITC was added to the cells. The samples were mixed and incubated for 15 min in the dark at room temperature. After centrifuging and aspirating the supernatant completely again, the cell pellet was resuspended in 200 μL of binding buffer. Finally, 5 μL of PI (100 $\mu\text{g}/\text{mL}$) solution was immediately added prior to analysis by flow cytometry.

2.13. ROS Analysis

Cells were exposed as explained in Section 2.11. The cell suspension was centrifuged in a 15 mL falcon ($300\times g$ for 5 min). H_2DCFDA (5 μM as the final concentration) was added, and cells were incubated for 20 min in the dark at 37°C . The tubes were centrifuged at $300\times g$ for 5 min, washed, and suspended in 300 μL of PBS. In this assay, tert-Butyl hydroperoxide (TBHP) (1 mM; 30 min) was used as the positive control.

2.14. Mitochondrial ROS Analysis

Cells were exposed as explained in Section 2.11. Cells were centrifuged in a 15 mL falcon ($300\times g$ for 5 min). MitoSOX Red reagent was added (1 mM as the final concentration) in 500 μL of fresh medium and incubated for 20 min in the dark at 37°C . The tubes were centrifuged at $300\times g$ for 5 min and resuspended in 300 μL of PBS.

2.15. Mitochondrial Mass Analysis

Cells were exposed as described in Section 2.11, but in triplicate. A stock solution of MitoTracker probe was prepared by using DMSO, obtaining a concentration of 100 μM . The tubes were centrifuged ($300\times g$ for 5 min) to obtain a cell pellet and the supernatant was aspirated. The cells were resuspended gently in prewarmed (37°C) staining solution containing the MitoTracker[®] (100 nM) for 20 min in the dark at 37°C . After staining was completed, cells were centrifuged ($300\times g$ for 5 min) and resuspended in 300 μL of PBS.

2.16. Cytometer Settings

All flow cytometry assays were carried out using the MACSQuant 16 analyzer (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). The total number of events per sample was 20,000 in each experiment. Blue (488 nm), violet (405 nm), and red (640 nm) lasers were used. Cell cycle results were collected using a 579/34 PE B2 filter. The fluorescence results of Annexin V, MitoTrackerTM, and $\text{H}_2\text{-DCFDA}$ were collected using a 525/50 FITC B1 filter. Fluorescence results of MitoSOXTM were collected using a V4 channel with 615/20 filter.

2.17. Statistical Analysis of the Data

Data were expressed as mean $\pm$ SD of four independent experiments. By using Student's *t*-test for paired samples in Excel 2016, statistical analysis of the results was performed. $p \leq 0.05$ was considered statistically significant. Macs quantify version was the software used for flow cytometry assays.

3. Results

3.1. AFB1 Bioaccessibility

The data regarding the matrix-matched curves, linearity range, regression coefficients (r^2), equations, and limits of detection and quantification obtained in HPLC-FLD for the maize flours, bread, and simulated digests are shown in Table 2.

The concentration of AFB1 in naturally contaminated maize flour was 151.9 ± 1.4 mg/kg for E1 and 197.1 ± 3.8 mg/kg for E2. After baking, the AFB1 concentrations obtained for breads in E1 were 1.6 ± 0.1 mg/kg and 1.7 ± 0.1 mg/kg for A and A + G, respectively. In E2, the AFB1 concentrations of breads were 96.4 ± 9.6 mg/kg and 102.7 ± 4.4 mg/kg for A and A + G, respectively. Neither bread C or G showed AF content in E1 or in E2. The AFB1 concentration in bread was not measured before baking since the flours used as ingredients for making the bread had already been analyzed (Sections 2.5 and 2.6). Regarding the gastric digests, the mean AFB1 concentrations were 0.176 ± 0.005 μ M (A) and 0.191 ± 0.005 μ M (A + G) for E1 and 3.934 ± 0.823 μ M (A) and 3.861 ± 0.006 μ M (A + G) for E2. In the intestinal digests, the AFB1 concentrations were 0.181 ± 0.002 μ M and 0.162 ± 0.001 μ M in E1 and 8.621 ± 0.021 μ M and 7.742 ± 0.031 μ M in E2 for A and A + G, respectively.

AFB1 bioaccessibility ranged from 29% to 39% in E1 and from 14% to 30% in E2 (Figure 1). Regarding E1, AFB1 bioaccessibility was $39 \pm 3\%$ (A) and $38 \pm 1\%$ (A + G) for the gastric digest and $37 \pm 1\%$ (A) and $29 \pm 1\%$ (A + G) for the intestinal digest. In E2, the results were $16 \pm 1\%$ (A) and $14 \pm 1\%$ (A + G) for the gastric digest and $30 \pm 1\%$ (A) and $23 \pm 1\%$ (A + G) for the intestinal digest.

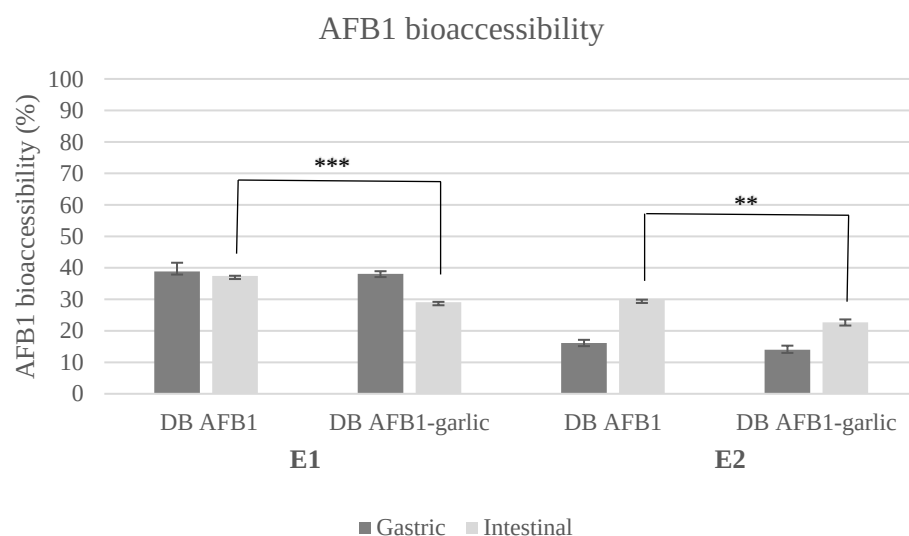


Figure 1. Bioaccessibility bar plot for experiments 1 and 2. Significant differences are indicated as $p < 0.01$ (**), $p < 0.001$ (***). E1, experiment 1. E2, experiment 2. DB, bread digest. Mean $\pm$ standard deviation ($n = 3$).

3.2. Cell Viability

To evaluate the cell viability, an MTT assay was performed. Cells exposed to control bread intestinal digests were considered to have 100% viability for the calculations. No differences were observed between non-exposed cells and control cells. The addition of garlic (A + G) in contrast to the AFB1 intestinal digest (A) significantly increased the cell viability at 24 h in non-diluted digests and at 48 h (10–13%) and 72 h (11–18%) in all dilutions tested. Table 3 presents the cell viability percentages and statistical analysis of cells exposed to A + G and A intestinal digests.

Table 3. Cell viability of AFB1 and AFB1 + garlic samples at different times and dilutions. Cell viability (%) results obtained for differentiated Caco-2 cells after exposure to AFB1 and AFB1–garlic bread intestinal digests.

Cell Viability (%)						
Exposure Time	24 h		48 h		72 h	
Intestinal Digest	<i>AFB1</i>	<i>AFB1–Garlic</i>	<i>AFB1</i>	<i>AFB1–Garlic</i>	<i>AFB1</i>	<i>AFB1–Garlic</i>
1/32	79 ± 4	86 ± 2	74 ± 4	86 ± 4 *	73 ± 1	87 ± 6 ***
1/16	76 ± 5	84 ± 6	72 ± 5	85 ± 4 *	72 ± 9	83 ± 2 **
1/8	76 ± 7	81 ± 3	72 ± 1	83 ± 5 **	66 ± 2	83 ± 3 *
1/4	76 ± 3	80 ± 2	69 ± 4	80 ± 3 *	64 ± 8	82 ± 7 ***
1/2	73 ± 6	79 ± 7	68 ± 4	78 ± 4 **	63 ± 6	81 ± 3 *
<i>No dilution</i>	70 ± 4	79 ± 6 *	66 ± 5	77 ± 4 **	62 ± 4	79 ± 3 *

$p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***). Mean ± standard deviation ($n = 4$).

3.3. Flow Cytometry Analysis

3.3.1. Cell Cycle

To evaluate the impact of the different digest breads on the Jurkat T cell cycle, PI dye was used. As Figure 2 indicates, cell cycle analysis was performed for every condition during the different phases. For the four conditions (C, G, A, A + G), at every cell phase, the results were, respectively: 19, 19, 16, and 20% for the Sub-G0/G1 phase; 46, 46, 47, and 47% for G0/G1; 20, 19, 23, and 19% for S; and 13, 12, 13, and 12% for G2/M.

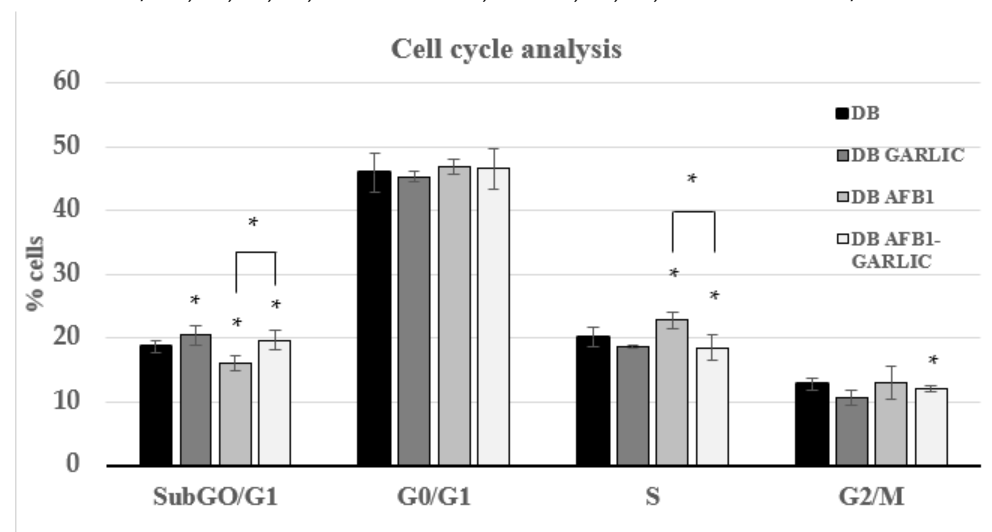


Figure 2. Effect of intestinal digests on Jurkat T cell cycle as assessed by flow cytometry. Cells were exposed for 7 days to bread digest (DB); garlic bread digest (garlic DB); AFB1 bread digest (0.7 μ M) (AFB1 DB); and AFB1 (0.7 μ M) + garlic (AFB1–garlic DB) bread digest. AFB1 concentration after both AFB1 and AFB1 + garlic exposures was 0.7 μ M. $p < 0.05$ (*). Mean ± standard deviation ($n = 4$).

3.3.2. Apoptosis/Necrosis after Intestinal Digest Exposure

Flow cytometry was used to assess the impact of the different bread digests on apoptosis/necrosis processes. Living cells accounted for 93% for C, 90% for G, 53% for A, and 69% for A + G; dead ones 1, 1, 6, and 4%; early apoptosis ones 2, 4, 12, and 9%; and late apoptosis ones 4, 6, 30, and 18%, respectively (Figure 3). A + G exposure significantly increased the live cell percentage and reduced early, and late apoptotic Jurkat T cells compared with A exposure. Annexin V–/PI– (live), Annexin V+/PI– (early apoptosis), Annexin V–/PI+ (necrosis), and Annexin V+/PI+ (late apoptosis) populations are shown in Figure 3.

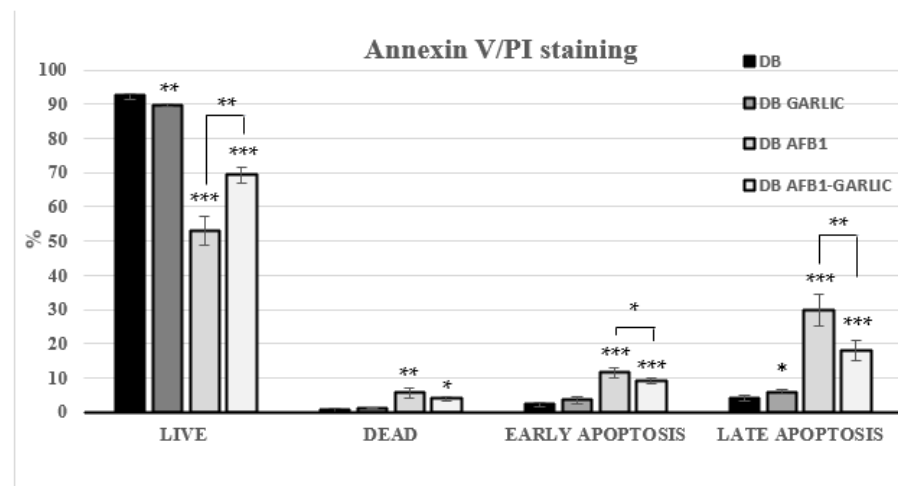


Figure 3. Effect of bread intestinal digest on apoptosis/necrosis as assessed by flow cytometry. Significant differences from the control, indicated by Jurkat T cells, compared with samples exposed for 7 days to bread digest (DB); bread digest garlic (garlic DB); AFB1 (0.7 μ M) bread digest; and AFB1 (0.7 μ M) + garlic bread digest. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). Mean $\pm$ standard deviation ($n = 4$).

3.3.3. Effect of Intestinal Digest Exposure on ROS

From the lowest to the highest value, the control condition as well as the garlic condition revealed a 1-fold increase (FI) in ROS, while the AFB1–garlic condition showed a 2 FI, the AFB1 condition a 5 FI, and 1 mM THBP (a compound which enhances ROS production and was used as a positive control) revealed a 14.3 FI. The results indicate that AFB1 contributes to an increase in ROS production, while the presence of garlic diminishes the AFB1 effect (A + G) (Figure 4a,b).

To detect mitochondrial superoxide production in Jurkat T cells after exposures, MitoSOX Red dye was used. As Figure 5a,b indicate, the mitochondrial ROS analysis was normalized when the bread digest condition (control) showed 100% intensity. From the lowest to the highest value, the AFB1–garlic condition showed 111% intensity, the garlic condition evidenced 117%, and AFB1 condition revealed 135%. The results show that the presence of AFB1 enhances the production of ROS at a mitochondrial level (A), while the addition of garlic attenuates this effect (A + G).

3.3.4. Mitochondrial Mass

To evaluate the effects of the different digests on the mitochondrial mass, MitoTracker[®] dye was used. As shown in Figure 6, the bars indicate the results of each exposure, which stem from taking as a reference the C exposure as 100%. The A + G condition showed the lowest value (88%), followed by A (92%) and G (98%), while the C one revealed the highest mitochondrial mass (100%).

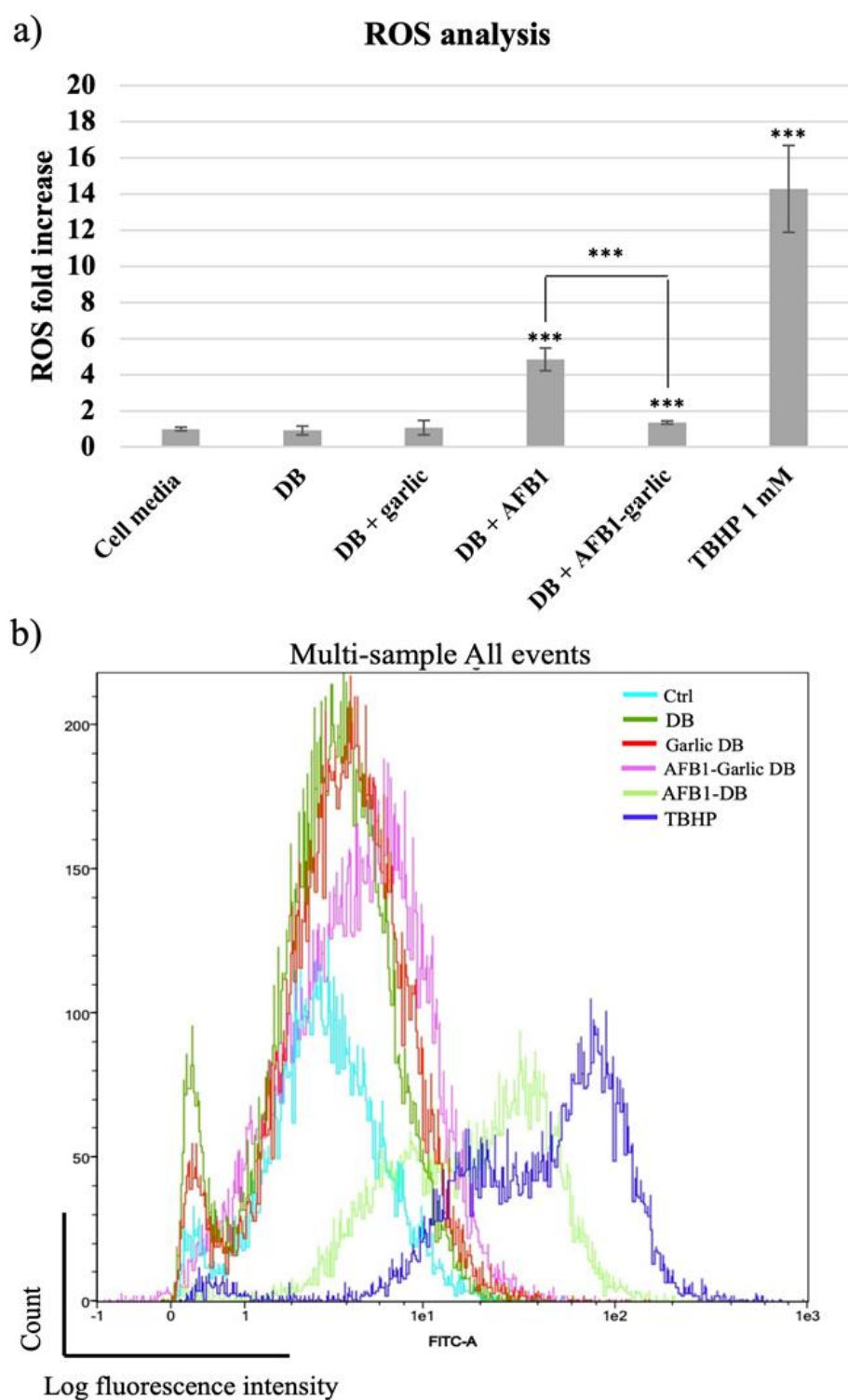


Figure 4. (a) Bar plot showing ROS production in Jurkat T cells exposed for 7 days to cell media, bread digest (DB); garlic DB; AFB1 DB; and AFB1–garlic DB in relation to DB. AFB1 concentration in both AFB1 DB and AFB1–garlic DB conditions was 0.7 μ M. (b) Fluorescence representation obtained by overlaying the single plot of each condition by flow cytometry. ROS: reactive oxygen species; TBHP: tert-Butyl hydroperoxide. $p < 0.001$ (***). Mean $\pm$ standard deviation ($n = 4$).

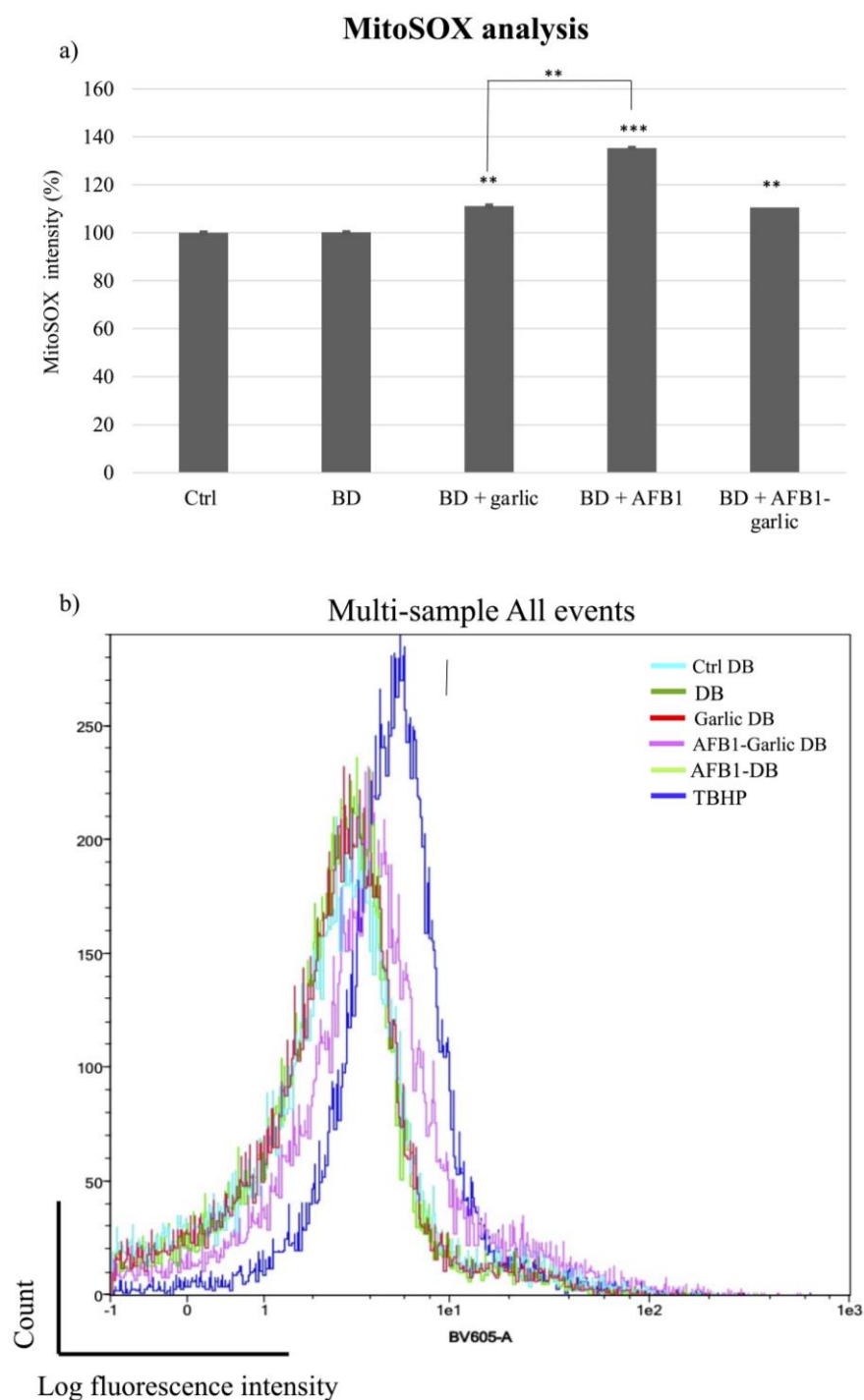


Figure 5. Effect of intestinal bread digests (0.7 μ M) compared with control on mitochondrial ROS as assessed by flow cytometry. Significant differences from the control indicated as $p < 0.01$ (**), $p < 0.001$ (***). (a) Bar plots showing Jurkat T cells exposed for 7 days to control (non-exposed cells), digested bread (DB), garlic DB, AFB1 DB, AFB1 + garlic DB, and the negative control, and then stained with MitoSOX for flow cytometry detection. AFB1 concentration for both A and A + G exposures was 0.7 μ M. (b) Fluorescence representation obtained by overlaying the single plot of each condition by flow cytometry. Mean $\pm$ standard deviation ($n = 4$).

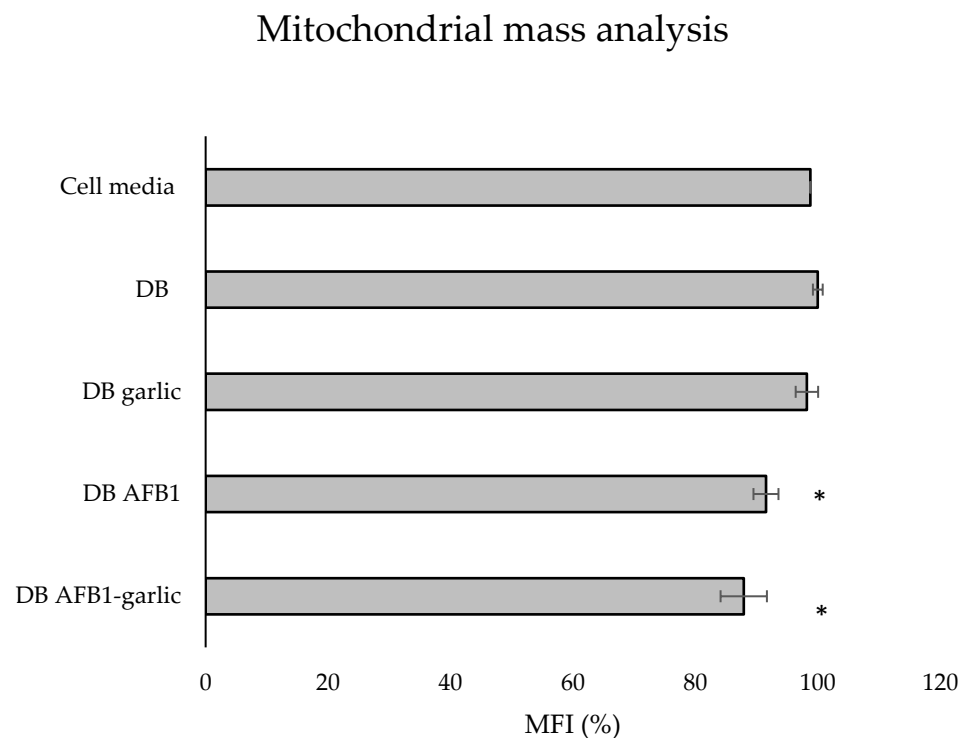


Figure 6. Effect of intestinal bread digests (0.7 μ M) compared with control on mitochondrial mass as assessed by flow cytometry. Significant differences from the control indicated as $p < 0.05$ (*). Jurkat T cells were exposed to each exposure for 7 days (non-exposed cells), digested bread (DB), garlic DB, AFB1 DB, AFB1–garlic DB, and then fixed and stained with MitoTracker[®] for flow cytometry detection. AFB1 concentration for both AFB1 DB and AFB1–garlic DB exposures was 0.7 μ M. MFI: median fluorescent intensity. Mean $\pm$ standard deviation ($n = 3$).

4. Discussion

In the last few years, AFB1 bioaccessibility has been studied in different food matrices by including functional ingredients. The inclusion of functional ingredients as a strategy to reduce exposure to AFB1 has offered promising results. Escrivá et al. [12] measured AFB1 bioaccessibility by using AFB1-contaminated wheat flour in the bread recipe, achieving concentrations of 78–164 μ g AFB1/kg bread with and without 1% goat milk whey, fermented whey, or lyophilized pumpkin. After performing simulated human digestion, the intestinal AFB1 bioaccessibility was reduced by 64% when including 1% goat milk whey in the bread recipe, 57% with 1% fermented whey, and 74% with 1% lyophilized pumpkin. In this work, the intestinal AFB1 bioaccessibility reduction when including garlic was 7–8%, which may seem small compared with the reduction reported by Escrivá et al. [12]. Nevertheless, the total AFB1 bioaccessible fraction when including functional ingredients was smaller in this work, which used garlic. The main difference between these two studies is the AFB1 bioaccessibility result for AFB1-contaminated bread without functional ingredients. For Escrivá et al. [12], the result was 114%, while in this work, it was 38–29%. This may be caused by the different AFB1 concentrations used.

Other bioaccessibility studies have used AFB1 fortification with commercial AFB1 standards, including integrating mycotoxin in the food product after its production instead of simulating natural fungal contamination of the raw ingredients. It has been reported that fortification enhances the release of bioactive compounds [14]. In the only published article comparable to this work, Llorens et al. [15] found intestinal AFB1 bioaccessibility values like the ones found in this study for both bread with the addition of red beetroot (10–45%) and bread without this addition.

Cell viability studies exposing differentiated Caco-2 cells to AFB1 have been conducted [20,21]. Gao et al. [24], by using the CCK-8 assay, showed that Caco-2 cell exposure

to AFB1 (1.6–25.6 μM) led to a cell viability decrease in a dose-dependent manner at 48 h. Zhang et al. [25] showed that Caco-2 cell exposure to 0.032–3.2 μM AFB1 also decreased cell viability in a dose-dependent manner by using the MTT technique. In accordance with the literature, the present study also showed a cell viability decrease due to the exposure to AFB1. Not only are the techniques used different, except for Zhang et al. [25], but the mycotoxin AFB1 concentration and time also varied.

The protective effects of garlic on differentiated Caco-2 cells have been studied previously. Engdal and Nilsen [26] showed that natto K2 and green tea were characterized by their ability to increase cell viability in Caco-2 cells, while garlic had no significant effects (0.017–0.17% p/v) after 90 min of exposure. Eguchi et al. [27] revealed that aqueous garlic extracts decreased cell viability to <35% after 24 h of exposure at 5–25% (v/v). The sample used for cell exposure was the supernatant, while the pellet, which might still contain a considerable fraction of garlic, was discarded. In this experiment, the Voghiera garlic added as a functional ingredient to bread underwent a cooking process and an *in vitro* digestion. The garlic concentration in the bread was 2% (w/v), but after the *in vitro* digestion and media dilution, it was 0.002% (w/v). According to Bhatt and Patel [28], while cooking drastically reduces the total amount of phenolic compounds, *in vitro* gastrointestinal digestion provides garlic extracts with much higher antioxidant potential than can be obtained using organic solvents.

The toxic effects triggered by AFB1 on cells and tissues are principally provoked through the inhibition of cell proliferation and cell cycle arrest, apoptosis, oxidative stress, endoplasmic reticulum stress, and autophagy [29]. Huang et al. [30] showed that in the IRM-32 neuroblastoma cell line, AFB1 induced significant S-phase arrest at concentrations of 6.4 μM and 19.2 μM for 24 h and 48 h. Zhu et al. [31] revealed S-phase arrest in HepG2 cells exposed to 0–48 μM AFB1 for 24 h; the arrest was especially significant at 32 and 48 μM AFB1. Liu et al. [32] showed that AFB1 exposure induced G0/G1 arrest in F344 rat hepatocytes when administering 100 or 200 $\mu\text{g/kg}$ AFB1 for 28 days. In accordance with these previous studies, this study showed that AFB1 exposure can promote a possible arrest of the cell cycle in the S phase, while the addition of garlic decreases the S phase (23 vs. 19%), like the control, by significantly limiting eventual cell cycle arrest.

Regarding apoptosis, 3D4/21 cells' apoptosis rate increased in a AFB1-dose-dependent manner [33]. In accordance with these results, this work found that exposure to AFB1 increases the number of apoptotic and necrotic cells, while the addition of garlic attenuates its effects. Comparing garlic vs. AFB1 and garlic exposures, the percentage of apoptotic cells reached its peak in the AFB1 one (41%), including cells in early and late apoptosis, while there was a decrease in the combined AFB1 and garlic (27%) sample (Figure 3). It was clearly shown that AFB1 enhances cell apoptosis, and that garlic attenuates AFB1-promoting apoptosis in Jurkat T cells.

Liu and Wang [34] showed that in primary broiler hepatocytes, exposure of samples to different AFB1 concentrations (0.5–5 μM) for 6 h led to significant ROS generation in a concentration-dependent manner. Wang, Xu, Yu, and Xu [35] also revealed that exposing broiler cardiomyocytes to different AFB1 concentrations (0.5–5 μM) for 12 h resulted in a significant increase in intracellular ROS production. Furthermore, it was observed that *Cetraria islandica* methanol extract caused an increase in the activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) and a decrease in MDA levels in human lymphocytes *in vitro* [36]. The protective effect of garlic regarding oxidative processes has also been documented. Not only does garlic contribute to the decrease in ROS levels, but it also mitigates other proinflammatory parameters, such as SOD, catalase (CAT), and GPx activities [37–40]. In accordance with the previous literature, this work showed that AFB1 increased ROS production five times in comparison to the control, while the addition of garlic attenuated the effect of AFB1, resulting in similar values to the control. This study confirms the capacity of garlic to lower ROS via AFB1 in Jurkat T cells for the first time.

Research on AFB1's impact, specifically on mitochondrial ROS, is scarce in the literature. However, there are other mitochondrial parameters, such as ATP production,

mitochondrial membrane permeability, and respiratory capacity, which may be good indicators of ROS production status [41]. Chen et al. [42] revealed that AFB1 reduced mitochondrial respiratory capacity and ATP production in HepG2 and Caco-2 cells, which are related to cell dysfunctions. Liu et al. [41] showed that AFB1 increased mitochondrial membrane permeability, which would result in a release of proteins such as cytochrome c to activate the caspase cascade and programmed cell death. Similarly, the present study revealed that AFB1 exposure increased mitochondrial ROS production (by 35%) when compared with AFB1 and garlic one (11% more), confirming the antioxidant effect of garlic against AFB1 at the mitochondrial level also (Figure 4).

Mitochondrial changes in response to different stimuli can be assessed by using mitochondrial dyes to track mitochondrial mass and volume [43]. AFB1 has been revealed to diminish mitochondrial mass in fish exposed to 5–10 μ M AFB1 for 7–8 days and mice fed with 0.375–1.5 mg/kg AFB1 for 30 days [44,45]. However, no significant differences were found in Jurkat T cells in this study, suggesting that neither AFB1 nor garlic can affect the mass of this organelle (Figure 6). Overall, these results confirm the toxicity *in vitro* of AFB1 at very low concentrations and long exposure times.

5. Conclusions

Garlic's presence contributed to reduced AFB1 bioaccessibility in bread (7–8%). Garlic was also revealed to increase cell viability (9–18%). Flow cytometry showed that garlic mitigates the toxic effects of AFB1 in Jurkat T-cells on the cell cycle by preventing the arrest of the S phase, on apoptosis/necrosis, and on cellular and mitochondrial ROS production. Therefore, the antigenotoxic, antiapoptotic, and antioxidant effects of garlic were demonstrated. Nevertheless, further investigations are needed to confirm the protective role of garlic *in vivo* and its probable application in the food industry as prebiotic to counteract possible AFB1 food contamination at low levels and potential human toxicity.

Author Contributions: Conceptualization, L.M.; methodology, L.M. and P.V.-D.; software, M.F., A.C. and P.V.-D.; formal analysis, Á.L. and M.F.; investigation, Á.L., M.F. and A.C.; data curation, M.F., L.M. and A.C.; writing—original draft preparation, Á.L.; writing—review and editing, L.M., A.M. and P.V.-D.; visualization, M.F., L.M. and A.M.; supervision, L.M. and P.V.-D.; project administration, L.M. and A.M.; funding acquisition, L.M. All authors have read and agreed to the published version of the manuscript.

Funding: The publication is part of the project PID2022-140722OB-I00, funded by MCIU/AEI/10.13039/501100011033/FEDER, EU.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained in the article.

Conflicts of Interest: The authors declare no conflicts of interest.

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REVIEW

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Cite this: *Food Funct.*, 2024, **15**, 8998

Emerging mycotoxins and preventive strategies related to gut microbiota changes: probiotics, prebiotics, and postbiotics – a systematic review†

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Recent research has focused on the involvement of the gut microbiota in various diseases, where probiotics, prebiotics, synbiotics, and postbiotics (PPSP) exert beneficial effects through modulation of the microbiome. This systematic review aims to provide insight into the interplay among emerging mycotoxins, gut microbiota, and PPSP. The review was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. In this review, unregulated yet highly recurrent mycotoxins are classified as emerging mycotoxins. The most frequently observed mycotoxins included those from the *Fusarium* genus—enniatins ($n = 11$) and beauvericin ($n = 11$)—and the *Alternaria* genus—alternariol monomethyl ether, altertoxin, and tentoxin ($n = 10$). Among probiotics, the most studied genera were *Lactobacillus*, *Bifidobacterium*, and the yeast *Saccharomyces cerevisiae*. Inulin and cellulose were the most found prebiotics. Data on synbiotics and postbiotics are scarce. Studies have shown that both the gut microbiota and PPSP can detoxify and mitigate the harmful effects of emerging mycotoxins. PPSP not only reduced mycotoxin bioaccessibility, but also counteracted their detrimental effects by activating health-promoting pathways such as short-chain fatty acid production, genoprotection, and reduction of oxidative stress. However, both quantitative and qualitative data remain limited, indicating a need for further *in vivo* and long-term studies. The formulation of PPSP as functional foods, feeds, or nutraceuticals should be considered a preventive strategy against the toxicity of emerging mycotoxins, for which, there is no established regulatory framework.

Received 12th April 2024,
 Accepted 17th August 2024
 DOI: 10.1039/d4fo01705f
rsc.li/food-function

1. Introduction

Mycotoxins are secondary metabolites produced by various species of fungi, primarily belonging to the genera *Aspergillus*, *Penicillium*, and *Fusarium*. They are estimated to be present in up to 80% of food and feed. Animal feed contamination is particularly concerning, as in developed countries, up to 70% of cereal harvest—a primary source of mycotoxins—is utilized in the daily diet of animals. Mycotoxin regulation for feed is less restrictive than for food. These toxins pose significant public health risks due to their widespread occurrence and their toxic properties in both animals and humans. They can induce immunotoxicity, neurotoxicity, hepatotoxicity, nephrotoxicity, reproductive and developmental toxicity, and carcinogenicity.¹

Currently, the legislative framework on maximum limits on mycotoxins is established by the recently updated Commission Regulation (EU) 2023/915 of April 25, for aflatoxin B1 (AFB1),

aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), aflatoxin M1 (AFM1), ochratoxin A (OTA), patulin (PAT), deoxynivalenol (DON), zearalenone (ZEA), fumonisin B1 (FB1), fumonisin B2 (FB2) and citrinin (CIT), the last one being applied only in food supplements.² In addition, there are other recent recommendations for emerging or unregulated mycotoxins such as alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA) and the trichothecenes T-2 and HT-2 toxins (T-2 and HT-2).^{3,4}

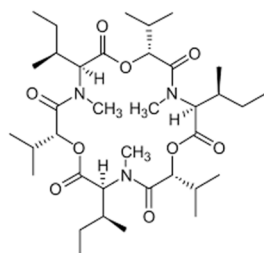
The term “emerging mycotoxins” was first introduced in 2008 and initially referred primarily to *Fusarium* metabolites such as fusaproliferin (FP), beauvericin (BEA), enniatins (ENs), and moniliformin (MON). However, more recent scientific publications have defined emerging mycotoxins as those that are neither routinely detected nor regulated by legislation, despite growing evidence of their prevalence. This work is focused on the 22 emerging mycotoxins included in Fig. 1 and 2 that have been recognized for over 20 years, continue to be widespread, and are not yet subject to regulatory oversight, even though their toxic effects are increasingly documented. The mycotoxins covered in this review are: enniatin A (ENA), enniatin (ENB), enniatin A1 (ENA1), enniatin B1 (ENB1), BEA,

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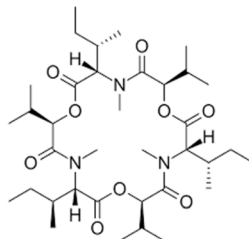
† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d4fo01705f>



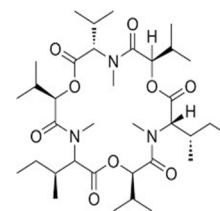
Fusarium



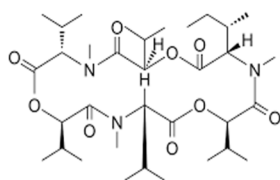
Enniatin A



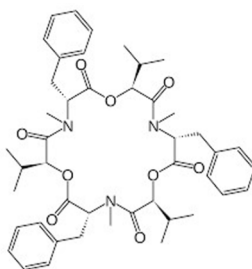
Enniatin B



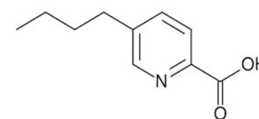
Enniatin A1



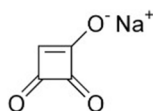
Enniatin B1



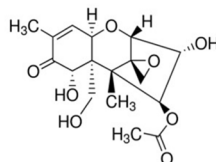
Beauvericin



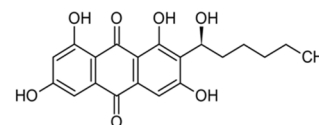
Fusaric acid



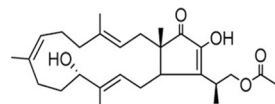
Moniliformin



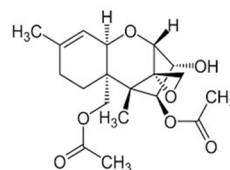
Fusarenon X



Aurofusarin



Fusaproliferin



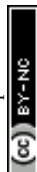
Diacetoxyscirpenol

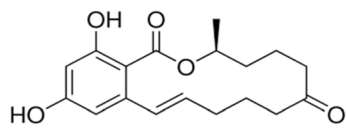
Fig. 1 Chemical structures of the emerging mycotoxins of *Fusarium* included in the bibliographic search ($n = 11$).

fusaric acid (FA), MON, fusarenon X (FX), aurofusarin (AUR), FP, diacetoxyscirpenol (DAS), altuene (ALT), AME, TeA, altertoxin (ATX), tentoxin (TEN), CIT, sterigmatocystin (STE), neosolaniol (NEO), citreoviridin, rugulosin and phomopsis.

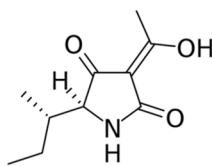
To better understand the relevance of these emerging mycotoxins, the occurrence and co-occurrence of most of them have been studied by Mihalache *et al.* (2023),⁵ who evidenced 38

different combinations in foodstuff (maize, rice, wheat, and flours). These combinations changed from binary mixtures (AME + AOH or AME + TeA) to include 12 different emerging mycotoxins (AOH + AME + TeA + TEN + ENA1 + ENB + ENB + BEA + MON + DAS + NIV + STE). Furthermore, the rising of new data regarding their presence in foodstuff has been highly relevant to enhance multiple risk assessment methodologies,

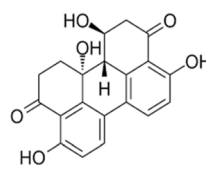


Alternaria

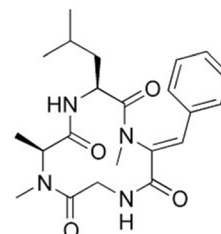
Alternariol monomethyl ether



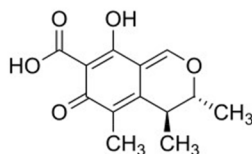
Tenuazonic acid



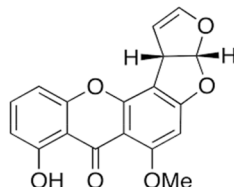
Altertoxin



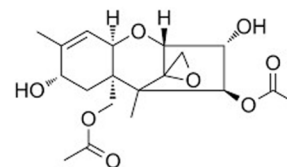
Tentoxin

Monascus

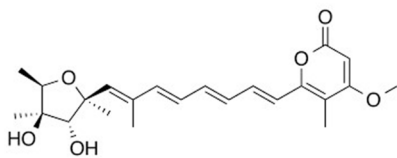
Citrinin



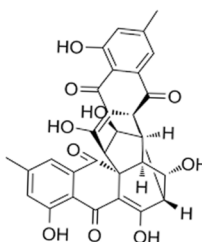
Sterigmatocystin



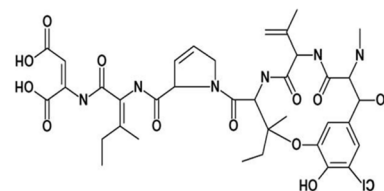
Neosolanin

Penicillium

Citreoviridin

Diaporthe

Rugulosin



Phomopsis

Fig. 2 Chemical structures of the emerging mycotoxins of *Alternaria*, *Monascus*, *Penicillium* and *Diaporthe* included in the bibliographic search ($n = 10$).

developing future evidence-based regulations and public health.⁵ As shown in Table 1, these emerging mycotoxins are of significant concern due to their involvement in various severe health issues, including cell apoptosis, chromosomal abnormalities, acute cardiac distress, genotoxicity, hemorrhages in legs, skull, and feet, liver injury, cardiovascular collapse, chlorosis, embryotoxicity, teratogenesis, cancer, liver cirrhosis, hepatocellular carcinoma, Shoshin-kakke (acute cardiac beriberi), fatty degeneration, liver cell necrosis and cell death. Although not all emerging mycotoxins were considered in this research, the spectra covered are expected to encompass most of them.

Furthermore, climate change is anticipated to become a major environmental concern in the 21st century. Higher

temperatures combined with extreme precipitation or prolonged droughts increase the stress experienced by plants, making all plant-based foods more susceptible to fungal infection and mycotoxin contamination. *Fusarium* mycotoxins are expected to shift towards Northern Europe, while *Aspergillus* species will primarily affect southern and central Europe. Indeed, it is estimated that in Europe, over the next 50–100 years, mycotoxins will become a significant concern. This includes not only the most common mycotoxins, such as aflatoxins and OTA, but also emerging *Fusarium* mycotoxins like ENs and BEA.^{6,7}

Recent research has focused on the involvement of the gut microbiota in a considerable number of diseases such as anxiety, depression, inflammatory bowel disease, dementia,



Table 1 Emerging mycotoxins, food sources, sample, dose administration, exposure time, target organ, toxicological mechanism, damage, and references

Mycotoxin	Food sources	Sample	Dose administration	Exposure time	Target organ	Toxicological mechanism	Damage	Ref
<i>Fusarium</i>								
ENA	Wheat, barley, maize, and rice	Caco-2 and SH-SY5Y cells	0.66–66.70 $\mu\text{g mL}^{-1}$	24–48 h	Intestine, liver	Generation of ROS and mitochondrial depolarization	Apoptosis	8 and 9
ENB	Wheat, barley, maize, and rice	Caco-2 and SH-SY5Y cells	0.63–63.60 $\mu\text{g mL}^{-1}$	24–48 h	Intestine, liver	Generation of ROS and mitochondrial depolarization	Apoptosis	8 and 9
ENA1	Wheat, barley, maize, and rice	Caco-2 and SH-SY5Y cells	0.66–66.70 $\mu\text{g mL}^{-1}$	24–48 h	Intestine, liver	Generation of ROS and NADPH oxidase activation	Apoptosis	8 and 9
ENB1	Wheat, barley, maize, and rice	Caco-2 and SH-SY5Y cells	0.63–63.60 $\mu\text{g mL}^{-1}$	24–48 h	Intestine, liver	Generation of ROS and NADPH oxidase activation	Apoptosis	9
BEA	Wheat, maize, nuts, and coffee	Caco-2 cells	1.17–19.6 $\mu\text{g mL}^{-1}$	24–72 h	Intestine, liver	Generation of ROS	Apoptosis	10
FA	Wheat, barley, corn, and rice	<i>Allium cepa</i> L. bulbs and HepG2 cells	0.18–1.8 $\mu\text{g mL}^{-1}$	24–168 h	Liver	Inhibition of cytochrome oxidase, decrease in ATP synthesis, change in the electrochemical gradient in the plasma membrane and increase in electrolyte loss	Chromosomal abnormalities	11 and 12
MON	Maize, wheat	Male Sprague-Dawley rats	500 $\mu\text{g mL}^{-1}$	28 days	Heart	Pyruvate substitution and carbohydrate metabolism interference	Death and acute cardiac distress	13
FX	Wheat, barley, and cereal-based products	Male and female mice	3–15 mg per kg b.w.	48 h	Liver, kidneys, spleen	Inhibition of protein and DNA synthesis	Apoptosis	14 and 15
AUR	Maize, barley, oats, and wheat	CHO-K1 cell line	0.57–5.7 $\mu\text{g mL}^{-1}$	48 h	Intestine	Enhanced levels of p53 protein	Genotoxicity	16
FP	Maize	Chicken embryo	0.45–2.22 $\mu\text{g mL}^{-1}$	21 days	Chicken embryos	Reduction in cell viability	Hemorrhages on the surfaces of legs, skull, and feet	17 and 18
DAS	Wheat, corn, rice, and maize	Newborn chickens and Wistar rats	1–3 mg per kg bw	6–24 h	Liver, gallbladder, and small intestine	DNA synthesis and protein biosynthesis inhibitor and heterophil extracellular trap release	Liver injury	19 and 20
Apicidin	NR	HepG2 cells	0.06–62.3 $\mu\text{g mL}^{-1}$	24 h	Liver	Cytotoxicity	Cell death	21
<i>Alternaria</i>								
AME	Wheat and its derivatives, rapeseed oil and peas	Male Sprague-Dawley rats	7.35 $\mu\text{g per kg bw per day}$	28 days	Liver, kidneys, spleen	DNA adducts and production of ROS	Genotoxicity	21
TeA	Tomatoes, apples, beer, and cereal foods	Mice	238 $\mu\text{g per kg per day}$	56 days	Liver, kidneys	Generation of ROS	Cardiovascular collapse and gastrointestinal hemorrhage	22
ATX	Cereals, oilseeds, fruits, and vegetables	Male Sprague-Dawley rats	5.51 $\mu\text{g per kg w per day}$	28 days	Liver, kidneys, spleen	Nuclear factor erythroid-derived 2-like 2/antioxidant response element (Nrf2/ARE)	Genotoxicity	16 and 23



Table 1 (Contd.)

Mycotoxin	Food sources	Sample	Dose administration	Exposure time	Target organ	Toxicological mechanism	Damage	Ref
TEN	Rice	Male Sprague Dawley rats	0.4–41.45 pg mL ⁻¹	10 min	Liver	ATP synthesis reduction	Chlorosis	24 and 25
<i>Monascus</i> CIT	Cheese, sake, and soy sauce	ProTox-II in humans	105 mg per kg weight	24 h	Kidneys	Inhibition of malate dehydrogenase, glutamate dehydrogenase and ATP synthase	Genotoxic, embryotoxic, teratogenic, carcinogenic	26
<i>Aspergillus</i> STE	Maize and peanuts	HepG2 cells	0.16–2.27 µg mL ⁻¹	24–48 h	Liver, kidneys	Generation of ROS	Liver cirrhosis hepatocellular carcinoma	27
NEO	Maize, wheat, and oats	Porcine Langerhans cells	143.4 mg mL ⁻¹	24 h	Leydig cells	Decrease in the ATP content, overproduction of ROS, reduction in mitochondrial membrane potential	Cellular apoptosis	28
Emodin	NR	HepG2 cells	0.027–27 µg mL ⁻¹	24 h	Liver	Cytotoxicity	Cell death	21
<i>Penicillium</i> Citreoviridin	Maize, pecan nuts, and wheat products	Mice	1–10 mg L ⁻¹	15 days	Heart	Inhibition of mitochondrial ATPase	Shoshin-kakke (acute cardiac beriberi)	29
Rugulosin	Rice	Mice	67 mg per kg b.w.	21 days	Liver	Inhibition <i>in vivo</i> and <i>in vitro</i> of DNA replication, transcription, and reparation	Fatty degeneration and liver cell necrosis	30 and 31
Cyclopiazonic acid	Corn, peanuts and cheese	Caco-2 cells	0.014–42 ng mL ⁻¹	24–48 h	Intestine	Cytotoxicity	Cell death	33 and 34
<i>Diaporthe</i> Phomopsis	Lupins	Sheep	0.5 mg kg ⁻¹	4 days	Liver	Cell cycle arrest	Cell death	32 and 33
<i>Phoma</i> Cytochalasin B	Potatoes	EL4	2 mg mL ⁻¹	4 h	Blood	DNA fragmentation	Apoptosis	36
Cytochalasin D	Potatoes	Mice	0.3 mg kg ⁻¹	17 days	Neural tube	Teratogenicity	Fetal death	37
Cytochalasin E	Potatoes	Brine shrimp	0.01–5 µg mL ⁻¹	16–24 h	NR	Cytotoxicity	Cell death	38

Deoxyribonucleic acid (ADN), alternariol monomethyl ether (AME), adenosine triphosphate (ATP), altertoxin (ATX), aurofusarin (AUR), beauvericin (BEA), body weight (b.w.), citrinin (CIT), diacetoxyscirpenol (DAS), enniatin A (ENA), enniatin A1 (ENA1), enniatin B (ENB), enniatin B (ENB), fusaproliferin (FP), fusarenol X (FX), fusaric acid (FA), moniliformin (MON), neosolaniol (NEO), nicotinamide adenine dinucleotide phosphate oxidase (NADPH), NF-E2-related factor 2/antioxidant responsive element (Nrf2/ARE), not reported (NR), reactive oxygen species (ROS), sterigmatocystin (STE), tenuazonic acid (TeA), and tentoxin (TEN).

Parkinson's disease, diabetes, obesity, and cancer.^{39–43} To understand how important the gut microbiota is, it should be said that there are around 26 600 human genes, whereas bacterial genes are more than 4 000 000 in our body. What is more, the bacterial density in the colon is around 10¹¹–10¹² mL⁻¹, being the highest density in any ecosystem on Earth.⁴⁴ On the other hand, there are some factors, such as diet,⁴⁵ stress,⁴⁶ exercise⁴⁷ and medication,⁴⁸ having a notable impact on gut microbiota modulation. Among these factors, this systematic review focuses on emerging mycotoxins and probiotics, prebiotics, synbiotics and postbiotics (PPSP); PPSP is a term recently coined by Li *et al.* (2021).⁴⁹

PPSP has shown to regulate the abundances of some intestinal bacteria such as *Akkermansia*, *Bacteroidetes*, *Blautia*, *Bifidobacteria*, *Bifidobacterium* (B.) and gut microbial metabolites, such as lactic acid, acetate, butyrate and propionate, while suppressing the bile acid pools and decreasing the production of trimethylamine *N*-oxide and lipopolysaccharides.⁴⁹ The significance of PPSP, mainly probiotics and prebiotics, gave rise to the International Scientific Association for Probiotics and Prebiotics (ISAPP) in 2002. ISAPP is a non-profit organization that works to advance the science of probiotics, prebiotics and related substances, such as synbiotics, postbiotics and fermented foods,⁵⁰ contributing to a better under-



standing of how biotics are connected to health and disease. Regarding definitions, prebiotics are described as “a substrate that is selectively utilized by host microorganisms, conferring a health benefit”.⁵¹ Traditionally, they have been defined as “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, thus attempting to improve host health”.⁵² Certainly, probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”.⁵² Recently, there has been an emerging trend to investigate synbiotics and postbiotics. Synbiotics are considered “a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host”, whereas postbiotics are described as “a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host”.^{53,54}

It has been shown that there are three main strategies for alleviating the harm caused by mycotoxins transmitted by microorganisms: (I) directly degrading mycotoxins in food, thanks to microbial pretreatment, and decreasing toxin intake; (II) by boosting probiotic colonization, the creation of bacterial-toxin complexes is suppressed, consequently inhibiting toxin absorption in the intestinal tract; and (III) by modulating the intestinal microecology with probiotics or prebiotics, enhancing the intestinal barrier, remodeling the intestinal microflora, improving intestinal toxicity, and decreasing toxin penetration.⁵⁵ Previously, the interactions between the gut microbiota and regulated mycotoxins^{6,56} and the detoxifying capacity of PPSP on some mycotoxins^{57–61} have been studied. Notwithstanding, the interplay among emerging mycotoxins, gut microbiota and PPSP still remains a framework to systemize.

The aim of this systematic review is to shed light on the potential of PPSP in mitigating the harmful effects of emerging mycotoxins. In addition, it aims to enhance our understanding of the reciprocal relationship between emerging mycotoxins and the gut microbiota, elucidating how emerging mycotoxins can influence the gut microbiota and *vice versa*. Furthermore, this review aims to contribute to future research endeavors by proposing the development of new functional foods and/or nutraceuticals as a strategy for mitigating toxicity associated with emerging mycotoxins.

2. Materials and methods

2.1 Search strategy

The scientific rigor of this systematic review and the minimization of potential bias were ensured through adherence to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement protocol for screening titles, abstracts, and full texts.⁶² A comprehensive literature search was conducted in November–December 2023, utilizing three databases (PubMed, Web of Science, and Scopus). The search

encompassed the period from January 2012 to December 2023, aiming to include recent data on emerging mycotoxins within the context of the gut microbiota and PPSP. In addition, other relevant terms, such as “bacteria”, were considered in combination with “*in vitro*” or “*in vivo*” to capture additional relevant information

The systematic review was conducted using the following search strings:

(1) Refined research of Pubmed, Web of Science and Scopus. Period: all years.

(“Enniatins” OR “Enniatin A” OR “Enniatin B” OR “Enniatin A1” OR “Beauvericin” OR “Fusaric acid” OR “Moniliformin” OR “Fusarenon X” OR “Aurofusarin” OR “Fusaproliferin” OR “Diacetoxyscirpenol” OR “Alternariol monomethyl ether” OR “Tenuazonic acid” OR “Altartoxin” OR “Tentoxin” OR “Citritin” OR “Sterigmatocystin” OR “Neosolaniol” OR “Citroviridin” OR “Rugulosin” OR “Phomopsis”) AND (“bacteria”) AND (“*in vitro*” OR “*in vivo*”).

(2) Refined research of Pubmed, Web of Science and Scopus. Period: 2012–2023.

(“Enniatins” OR “Enniatin A” OR “Enniatin B” OR “Enniatin A1” OR “Beauvericin” OR “Fusaric acid” OR “Moniliformin” OR “Fusarenon X” OR “Aurofusarin” OR “Fusaproliferin” OR “Diacetoxyscirpenol” OR “Alternariol monomethyl ether” OR “Tenuazonic acid” OR “Altartoxin” OR “Tentoxin” OR “Citritin” OR “Sterigmatocystin” OR “Neosolaniol” OR “Citroviridin” OR “Rugulosin” OR “Phomopsis”) AND (“microbiota” OR “prebiotics” OR “probiotics” OR “postbiotics”).

The criteria to include non-regulated mycotoxins in the list was based on Mihalache *et al.* (2023) and the reports issued by the Spanish Agency for Food Safety and Nutrition (AESAN).^{5,35} It is well known that there are other non-regulated metabolites not been considered when conducting the research. Notwithstanding, it was supposed that the spectra covered would be enough to include all the non-regulated mycotoxins.

2.2 Systematic review process

Among the 811 reports found through the research, 317 were retrieved from PubMed, 295 from Web of Science and 199 from Scopus. After an initial analysis, a total of 401 duplicate records were identified and removed. In addition, during the screening of titles and abstracts, another 381 records were excluded from the systematic review because of different reasons: 124 articles were discarded because they only focused on regulated mycotoxins (AFB1, OTA, fumonisins, *etc.*); 130 were rejected since they included emerging mycotoxins, but they were not assessed in combination with PPSP and 127 were discarded because they analyzed PPSP, but no emerging mycotoxins. Finally, a total of 29 were considered appropriate for inclusion in the present systematic review, assessed and classified based on: the gut microbiota, probiotics, prebiotics, synbiotics and postbiotics (Fig. 3). The selected articles are key to comprehend more deeply the role of the emerging mycotoxins in their combination with microbiota and PPSP.



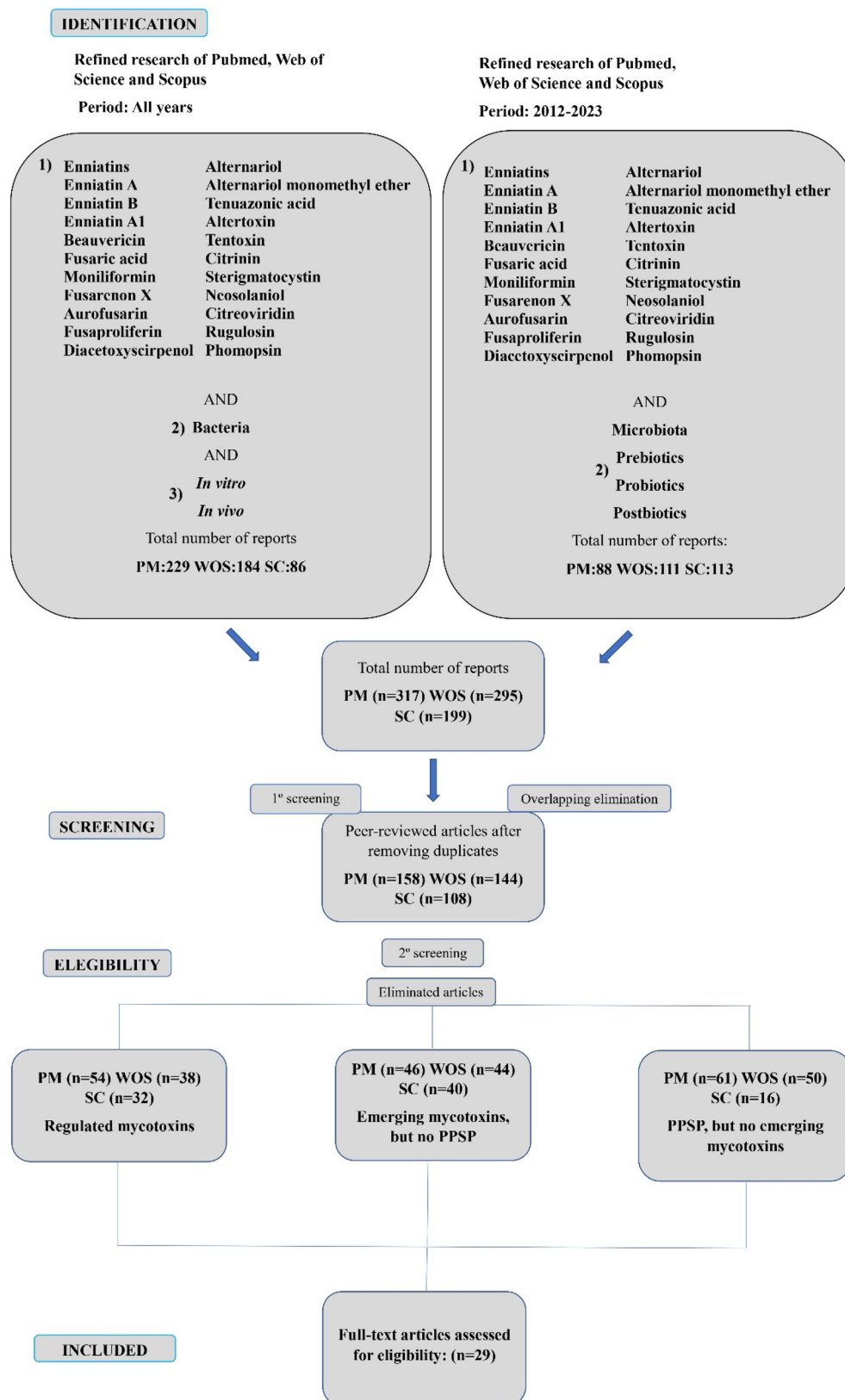


Fig. 3 Graphical representation of the total number of articles screened throughout the two strategies followed for the bibliographic research using the keyword combinations and range of years explained. Number of samples (*n*). Pubmed – PM, Scopus – SC and Web of Science – WOS.



2.3 Data conversion

All data from the studies discussed in this review were converted to $\mu\text{g kg}^{-1}$, mg kg^{-1} , ng mL^{-1} , $\mu\text{g mL}^{-1}$ or mg L^{-1} .

3. Results

3.1 Mycotoxins and the gut microbiota

The bidirectionality between regulated mycotoxins and the gut microbiota has been previously described in mice, pigs, rats, broilers, turkey, and ducks. DON has revealed to up-regulate the relative abundance of pathogens highly related to chronic intestinal diseases at different taxonomical levels (phylum, family, and genus). OTA has disrupted the structure and diversity of gut microbial communities and AFB1 to alter gut microbiota-dependent organic acid metabolism. Meanwhile, some gut microbiota strains, such as probiotics *Lactobacillus plantarum* and *Bacillus shackletonii*, have also shown mitigation effects by metabolizing, binding, and eliminating these regulated mycotoxins.^{6,7,56}

3.1.1 *In vitro* detoxification of emerging mycotoxins by the gut microbiota. Rumen microbiota has been found to completely detoxify NIV at normal pH after 24 h of incubation, whereas it partially degrades ENB up to 72% after 48 h. In both cases, this degradation depended on microbial activity and was influenced by pH.⁶³ Hedman and Pettersson (1997)⁶⁴ described that the capacity of cow gastrointestinal microflora to metabolize NIV has exerted the same trend. It was shown that 78–82% of anaerobically incubated NIV for 48 h was transformed into de-epoxy-NIV by gastrointestinal microbiota in the fluid rumen.⁶⁴ DAS, also produced by the genera *Fusarium*, has revealed to be deacetylated into 15-monoacetoxyscirpenol (15-MAS) and scirpentriol (SCP) as an additional metabolite by human fecal microbiota, being of utmost importance since deacetylation of mycotoxins results in reduced toxicity, for 15-MAS has been reported to be 8 times less toxic than both DAS and SCP.⁶⁵

The metabolism of *Alternaria* mycotoxins such as AOH, AME and ALT by the microbiota present in feces from three human volunteers has also been studied. As a control for the activity of the fecal microbiota, the isoflavone daidzein was incubated with the fecal cultures and was transformed to its expected metabolites. In contrast, no metabolites of AOH, AME and ALT were detected in the fecal cultures from the same volunteers, showing that the gut microbiota did not possess the capacity to metabolize these compounds. Interestingly, AOH was noted to bind non-covalently to the surface of bacteria where the type and composition of this bacterial cell surface played a notable role in the magnitude of this binding.⁶⁶ An *Alternaria alternata* extract ($5 \mu\text{g mL}^{-1}$), containing among others, AOH, AME, altertoxin-II (ATX-II), stemphytoxin III (STTX-III) and alterperyleneol (ALP), all of which are known as genotoxic, was used to assess the impact of short-term fecal incubation on the DNA-damaging effects. It was shown that microorganisms suppressed the DNA-strand-breaking potential promoted by the applied extract. Besides, in fresh fecal samples, even before anaerobic incubation, the con-

centrations of most of the tested mycotoxins (AOH, AME, ATX-II, STTX-III, and ALP) were lower in those containing microorganisms, especially fecal matter, implying that microbes were engaged in mycotoxin detoxification.⁶⁷

3.1.2 Emerging mycotoxins and the gut microbiota *in vivo*. Novak *et al.* (2021)⁶⁸ fed weaning piglets with different diets for a 14 day-period: DON, ENB + ENB1 + BEA and the four mycotoxins combined. Those piglets receiving the ENs + BEA diet were characterized by possessing a fecal microbiome with a lower diversity compared to the control group, and also compared to the ENs + BEA + DON diet. *L. amylovorus*, which is known to exhibit positive probiotic activities in weaning piglets, was reduced only in the ENs + BEA-fed group, but not in the ENs + BEA + DON one. Furthermore, the relative abundance of *L. reuteri*, which has shown to contribute to diarrhea treatment in piglets by reducing *Cryptosporidium parvum*, was increased in the ENs + BEA-fed group and, to a lesser extent, in the ENs + BEA + DON-fed group, compared to the control and the DON ones.⁶⁸

The ability of the gastrointestinal microflora of pig and chicken to metabolize NIV was studied by Hedman and Pettersson (1997).⁶⁴ On one hand, before pigs were fed with NIV, this mycotoxin was not de-epoxidated or metabolized in any way. However, when the pigs received either 2.5 or $5 \mu\text{g g}^{-1}$ NIV for one week, 97.1% of NIV in feces from five of six pigs was in the de-epoxy-NIV form, while the concentration of unaltered NIV in feces was $0.08 \mu\text{g g}^{-1}$. On the other hand, after three weeks on the NIV feed, even the pig that after one week could not metabolize NIV, was now able to form de-epoxides. In the feces from this pig, 90% of NIV was as de-epoxy-NIV, the concentration of it being NIV $0.17 \mu\text{g g}^{-1}$. Regarding chicken, it was revealed that de-epoxy-NIV was not detected in any samples of feces collected after three weeks of feeding with either 2.5 or $5 \mu\text{g g}^{-1}$ NIV. Interestingly, another unidentified metabolite of NIV was found in all feces samples, except from one bird fed with $5 \mu\text{g g}^{-1}$ NIV.⁶⁴ All the data relating emerging mycotoxins and gut microbiota are shown in Fig. 4 and Table 2. These studies were primarily conducted *in vitro* using feces from humans, pigs, and chickens, or in cow rumen fluids. The most frequently encountered emerging mycotoxins were ENs, BEA, NIV, and *Alternaria* mycotoxins (AOH, AME, ALT-X-I, and ALT).

3.2 Emerging mycotoxins and probiotics

Studies *in vitro* and *in vivo* have shown the ability of probiotic cultures to bind to and/or even degrade many toxic substances, mitigating their toxicity.^{64,69} Not only has it been reported that single strain or combination of lactic acid bacteria (LAB) are very useful for removal of heavy metals (copper, lead, cadmium, chromium and arsenic) and cyanotoxins (microcystin-LR, -RR and -LF), but also regarding mycotoxins (AFB1, AFB2, AFM1, aflatoxin M2 (AFM2), AFG1, AFG2, PAT, OTA, DON, FB1 and FB2), 3-acetyldeoxynivalenol, NIV, HT-2 and T-2, ZEA and its derivatives, *etc.*, and their harmful effects.^{58,61,70}

3.2.1 Emerging mycotoxins and probiotics *in vitro*. The strains *L. johnsonii* CECT 289, *L. rhamnosus* CECT 288,



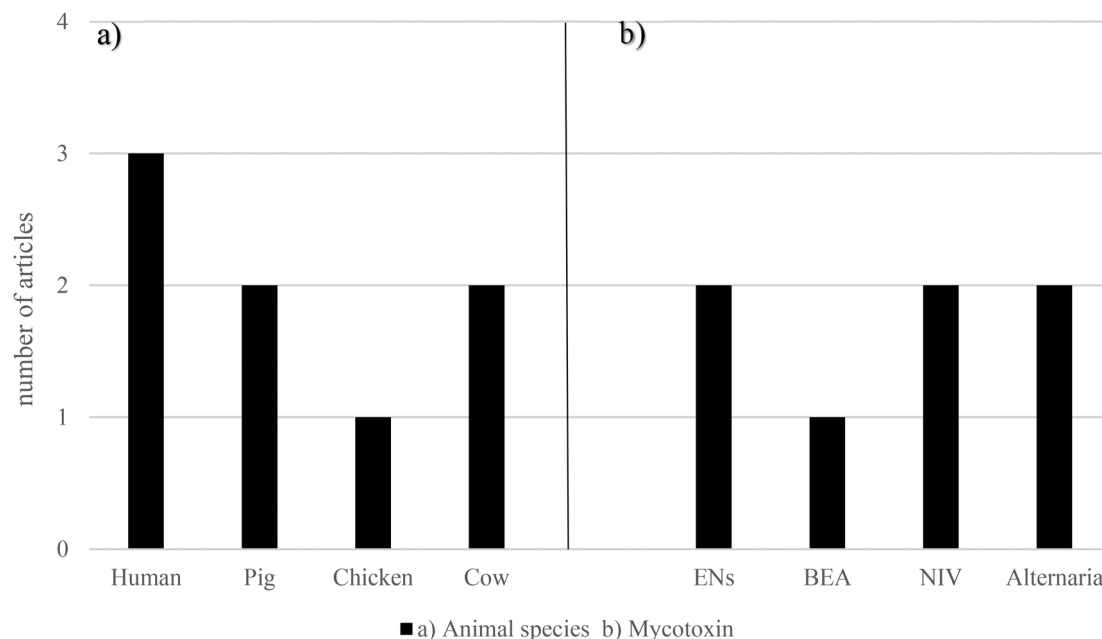


Fig. 4 Graphical bars of the gut microbiota publications ($n = 6$) according to (a) the animal species and (b) emerging mycotoxins studied. Beauvericin – BEA, enniatins – ENs and nivalenol – NIV.

L. plantarum CECT 220, *L. reuteri* CECT 725, *L. casei* CECT 475, *B. breve* CECT 4839 T, *B. adolescentis* CECT 5781 T, *B. bifidum* CECT 870 T and *B. longum* CECT 4551 have exerted to degrade different types of ENs during a simulated gastrointestinal digestion process for 4 h. It was reported that all the bacteria reduced ENA, ENA1, ENB and ENB1 mean bioaccessibility (ranging from 21.0% to 31.2%) in comparison with the control (33.4%–39.6%).⁷¹ The antimicrobial activity of *B. longum*, *B. bifidum*, *B. breve*, *B. adolescentis*, *L. rhamnosus*, *L. casei-casei*, *L. plantarum*, *L. paracasei*, *L. ruminis*, *Streptococcus thermophilus*, twenty-two strains of *Saccharomyces cerevisiae* and nine of *Bacillus subtilis* has been assessed against the minor *Fusarium* mycotoxin ENs (ENA, ENA1, ENA2, ENB, ENB1 and ENB4). The most active mycotoxins were ENA1 and ENB, mainly against *B. adolescentis* 5871 and *S. thermophilus*. Conversely, ENA1 and ENB1 have been reported to inhibit the growth of the cultivated *B. subtilis* strain with a minimum inhibitory concentration (MIC) of 16 and 8 $\mu\text{g mL}^{-1}$, respectively, and to be active against *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Enterococcus faecalis* with MIC values ranging from 2 to 8 $\mu\text{g mL}^{-1}$.⁷³ Another nine bacterial strains characteristic of the gastrointestinal tract like *B. longum*, *B. bifidum*, *B. breve*, *B. adolescentis*, *L. rhamnosus*, *L. casei-casei*, *S. thermophilus*, *L. ruminis*, *L. casei* and twenty-two strains of *S. cerevisiae* have been studied to conduct EN metabolism. The fermentation processes were carried out in a liquid medium of De Man Rogosa Sharpe under anaerobic conditions and in a potato dextrose broth, and the degradation of the EN bioactive compounds was assessed in a food system made of naturally contaminated wheat flour. The results showed ENA, ENA1, ENB and ENB1 degradation ranging from 5.2% to 99.5%. The

highest reduction was caused by *S. thermophilus* CECT 986 in ENA and the lowest by *B. breve* CECT 4839 in ENB. Furthermore, degradation products were identified for all the studied mycotoxins in both the liquid medium and the food system.⁷⁴

Apart from ENs, the interaction between BEA and probiotics is also well documented. Nine yeast strains of *S. cerevisiae* (LO9, YE-2, YE5, YE-6, YE-4, A34, A17, A42 and A08) have shown a BEA mean degradation of 86.2%. The highest degradation activity (98.8%) was due to *S. cerevisiae* LO-9, whereas the lowest (39.7%) by *S. cerevisiae* LS100.⁷⁵ The protective effect of *L. acidophilus* against BEA toxicity has been assessed on a Caco-2 cell line. It was revealed that, in the presence of BEA, *L. acidophilus* significantly increased cell viability at 12 h and 24 h.⁷⁶ *L. paracasei* CECT 277, *L. casei* CECT 4180, *L. rhamnosus* CECT 278T, *L. plantarum* CECT 220, *L. ruminis* CECT 4061T, *L. casei casei* CECT 277, *B. breve* CCT 4839T, *B. adolescentis* CECT 5781T, *B. bifidum* CECT 870T, *B. longum* CECT 4551, *Corynebacterium vitaeruminis* CECT 537, *Eubacterium crispatus* CECT 4840 and *S. cerevisiae* CECT 1324 have proven to exert a notable impact on BEA bioaccessibility. The highest bioaccessibility decrease was carried out by *B. longum* showing 45.4%, whereas the lowest data were revealed with the strain *L. rhamnosus* (27.5%).⁷⁷ Other 13 bacterial strains belonging to the gastrointestinal tract (*B. longum*, *B. bifidum*, *B. breve*, *B. adolescentis*, *L. rhamnosus*, *L. casei-casei*, *L. plantarum*, *Eubacterium crispatus*, *Salmonella fecalis*, *Salmonella thermophilus*, *L. ruminis*, *L. casei* and *L. animalis*) were fermented under anaerobic conditions in the liquid medium of De Man–Rogosa–Sharpe agar for 4 h, 12 h, 16 h, 24 h and 48 h at 37 °C. It was revealed that BEA reduction ranged from 66.5% to





Table 2 Collection of the most important characteristics of the studies found regarding emerging mycotoxins and the gut microbiota *in vitro* and *in vivo*. For each study, the studied mycotoxin, type of sample, mycotoxin dose administration, exposure time, experimental assay, results and references are described

Mycotoxin	Sample	Dose administration	Exposure time	Experimental assay	Results	Ref.
<i>In vitro</i>						
ENB	Cow rumen fluid	1 mg kg ⁻¹ ENB	0–48 h	<i>In vitro</i> rumen simulation	ENB degradation up to 72% after 48 h and NIV completely detoxified at normal pH (6.8) after 24 h of incubation. In both cases, the degradation was dependent of the microbial activity	63
NIV	Cow rumen fluid	60 mg kg ⁻¹ NIV	48 h	Anaerobic fecal culture	78–82% of incubated NIV was transformed into de-epoxy-NIV by the gastrointestinal microbiota in the fluid rumen	64
DAS	Human feces	0.7 ng mL ⁻¹	2–72 h	Fecal batch culture	Anaerobic incubation of NIV with cow rumen fluid produced de-epoxide at a high proportion DAS was deacetylated into 15-MAS and SCP as an additional metabolite by human fecal microbiota	65
AOH	Human feces	12.9 µg mL ⁻¹	72 h	Anaerobic fecal culture	AOH, AME and ALT were not metabolized by the gut bacteria. AOH bound non-covalently to the bacteria in function according to the type and composition of its surface	66
AME						
ALT						
AOH	Human feces	50 µg mL ⁻¹	48 h	Fecal anaerobic incubation	Microorganisms suppressed the DNA-strand-breaking potential promoted by the applied extract. AOH, AME, ATX-II, STTX-III, and ALP were lower in the samples containing microorganisms, especially fecal matter, implying that microbes were engaged in detoxification	67
AME						
ATX-II						
STTX-III						
ALP						

Mycotoxin	Sample	Mycotoxin dose administration	Exposure time	Experimental assay	Results	Ref.
<i>In vivo</i>						
ENB	Weaning piglets' feces	1.345 mg kg ⁻¹ (ENB)	14 days	Metagenomic analysis of the fecal microbiome	<i>L. amylovorus</i> was reduced only in the BEA-fed group, but not in the BEA + DON group	68
ENB1		1.830 mg kg ⁻¹ (ENB1)			The relative abundance of <i>L. reuteri</i> was increased in the BEA group and, to a lesser extent, in the BEA + DON group compared to the control and the DON group	
BEA		2.570–3.578 mg kg ⁻¹ (BEA)				
NIV	Pig and chicken feces	2.5–5 mg kg ⁻¹	1–3 weeks	Chemical analysis of feces	After one week, 97.1% of NIV was in the de-epoxy-NIV form in the feces from five of six pigs. After three weeks, this pig was now able to form de-epoxides (90%)	64
					No de-epoxide of NIV, but another unidentified metabolite was found in the feces from chicken fed with 2.5 or 5 µg g ⁻¹ NIV for three weeks	

Alternariol (AOH), alternariol methyl ether (AME), alterperyleneol (ALP), alterperyleneol I (ATX-I), alterperyleneol II (ATX-II), alterperyleneol III (STTX-III), alterperyleneol IV (DON), enniatin B (ENB), *Lactobacillus* (L), nivalenol (NIV), scirpentriol (SCP), stemphytoxin III (STTX-III), and 15-monoacetylscripenol (15-MAS); both SCP and 15-MAS are the products of DAS deacetylation.

83.1%, being the highest decrease caused by the strain *L. casei casei*.⁷⁸

Other *Fusarium* mycotoxins have been studied in combination with probiotics showing similar results. *Escherichia coli* (ATCC 25922), *Salmonella enterica* serovar typhimurium 3389-1 (DT12), *Staphylococcus aureus* (ATCC 29213), *L. acidophilus* (DSMZ 20079), *L. acidophilus* (20079), *L. salivarius* (20555), *L. sobrius* (16698), *B. longum* subsp. *longum* Reuter (20219) and *B. breve* (20213) received a fast digital time-lapse microscopic method to assess AUR, BEA, ENs and FA antibacterial effects after 6 h of cultivation. The most potent compound was AUR against *L. acidophilus*, whereas BEA, ENs and FA were characterized by having weak antibiotic effects.⁷⁹ *Burkholderia ambifaria* T16, a naturally beneficial bacterium with proven biocontrol properties but potential pathogenic risk, has shown to inhibit the growth of several *Fusarium* species and possess the unique ability to degrade FA as a carbon, nitrogen and energy source, as well as the capacity to detoxify FA in barley seedlings.⁸⁰ Different *S. cerevisiae*, *Lactobacillus* and *Bacillus* strains were tested for their ability to degrade different mycotoxins. Among the tested mycotoxins, DAS was proven to possess an inhibitory effect on the growth of almost all *Lactobacillus* and *Bacillus* strains, whereas no effect on yeast strains could be observed.⁸¹ The human microbiome characteristic bacteria *Prevotella copri* has revealed to deacetylate 51.3% of DAS to 15-MAS after 48 h.⁸²

The same trends have also been reported with *Alternaria* mycotoxins in Ge *et al.* (2017).⁸³ *L. brevis* 20023 (LAB-20023) cells have revealed to adsorb TeA from aqueous solution, demonstrating that polysaccharides and protein were important components of the LAB cell wall and were involved in TeA removal. The lowest and highest absorption rates were 12.5% and 90.1%, respectively.⁸³ The effects of a complex extract of *Alternaria* mycotoxins (AME, ALT, TeA, TEN, ATX-I, ATX-II, ALP, STTX-III, altenusin and altersetin (AST)) were studied in combination with some typical bacterial strains of the gut microbiome such as *Bacteroides caccae*, *Bacteroides eggerthii*, *Bacteroides thetaiotaomicron*, *Bacteroides vulgatus*, *Parabacteroides distasonis*, *E. coli*, *Clostridium innocuum*, *Alistipes finegoldii*, *Alistipes timonensis*, *Ruminococcus bicirculans*, *Akkermansia muciniphila*, *L. hominis*, *B. longum* and *B. sp.* The results showed that both Gram types were able to adsorb AOH, AME, and AST, and it was seen that Gram-negative bacteria had higher adsorptive abilities. Notwithstanding, this absorption was not observed for neither ATX-I nor ALP. Interestingly, the mycotoxin tendency to accumulate within bacterial pellets, especially in those of Gram-negative strains, was connected to their lipophilic profile.⁸⁴

3.2.2 Emerging mycotoxins and probiotics *in vivo*. To study the influence of fermentation on feed acidity and microbiological parameters, two different diets were prepared: (i) non-fermented basal feed and (ii) fermented basal feed. A fermented basal diet was characterized by including some microbial starters (*L. uvarum* LUHS245, *L. casei* LUHS210, *Pediococcus acidilactici* LUHS29 and *Pediococcus pentosaceus* LUHS183). Piglets were fed using these diets for 36 days and feces microbiota,

growth performance and health were evaluated. Mycotoxin biotransformation was quantified, including masked mycotoxins, in feed and piglet fecal samples. The analysis showed that AME and ALT were found in 61-day-old control piglets' feces and in fermented feed samples. However, AME was not found in treated piglets' feces. *Prevotella* was the most prevalent genus in both groups, but the prevalence of *Lactobacillus* was 6-fold higher in treated animals compared to the control ones (23.7 vs. 3.9%). After the exposure, in the fermented feed group animals' blood, triglycerides and serum high-density lipoprotein cholesterol were notably higher, but the levels of glucose, alkaline phosphatase, urea, thyroxine, glucose and potassium were significantly decreased ($p \leq 0.05$) in comparison with the control group.⁸⁵ Fig. 5 and Table 3 show all the data concerning emerging mycotoxins and probiotics.

3.3 Mycotoxins and prebiotics

Prebiotics have been described as a potent agent to prevent metabolic disease through microbiome modulation.⁵⁰ K-carrageenan has revealed to be a promising fiber, thanks to ZEA bioaccessibility reduction, obtaining values lower than 20%. Lactose from fermented whey has shown to protect from AFB1 and OTA toxicity.^{86,87} Pectin has exerted to decrease DON, HT-2, and T-2 (50–88%) bioaccessibility.⁵⁹ Garlic, whose prebiotic content is notable, has shown to reduce AFB1 cytotoxicity.⁸⁸ It has also been reported that flavonoids play an important role in protecting against the toxicity of mycotoxins like OTA, PAT, and DON in different experimental models.^{89,90}

3.3.1 Emerging mycotoxins and prebiotics *in vitro*. As Fig. 6 shows, *Fusarium* mycotoxins, ENs and BEA are the most studied ones. Inulin (ranging from 1.5% to 10%) has revealed to decrease the bioaccessibility of ENA, ENA1, ENB and ENB1 in a dose-dependent manner in a digestion model.⁹¹ Cellulose (1% and 5%) has exerted to reduce ENA, ENA1, ENB and ENB1 bioaccessibility at a mean of 26.9% and 44.7%, respectively. Meanwhile, in the case of BEA, the bioaccessibility decrease ranged from 60% to 80%.⁷¹ Inulin and fructooligosaccharides (FOS) (1% and 5%), in a static gastrointestinal digestion model, have shown to reduce ENA, ENA1, ENB, ENB1 and BEA bioaccessibility. The lowest bioaccessibility was caused by adding the inulin 5% (5.7%) and FOS 5% enriched samples (3.9%) for ENA and ENA1, respectively.⁹²

BEA bioaccessibility was decreased, ranging from 15.7% to 60.5%, compared to the control (93.2%), with the addition of the following prebiotics: galactomannan 5%, glucomannan high-molecular weight (HMW) 1%, glucomannan HMW 5%, glucomannan fine powder 1%, glucomannan fine powder 5%, citrus fiber 1%, citrus fiber 5%, bamboo fiber 1%, bamboo fiber 5%, carrot fiber 1%, carrot fiber 5%, pie fiber 1%, pie fiber 1%, β -glucan 1%, β -glucan 5%, xilan 1%, xilan 5%, cellulose HMW 1%, cellulose HMW 5%, cellulose medium-molecular weight (MMW) 1% and cellulose MMW 5%.⁷² BEA has also shown to be decreased by β -1,3 glucan, chitosan LMW, chitosan MMW, FOS, galactomannan, inulin, and pectin at both concentrations (1% and 5%). Each sample was contaminated, reaching a BEA concentration of both 5 and 25 mg L⁻¹.



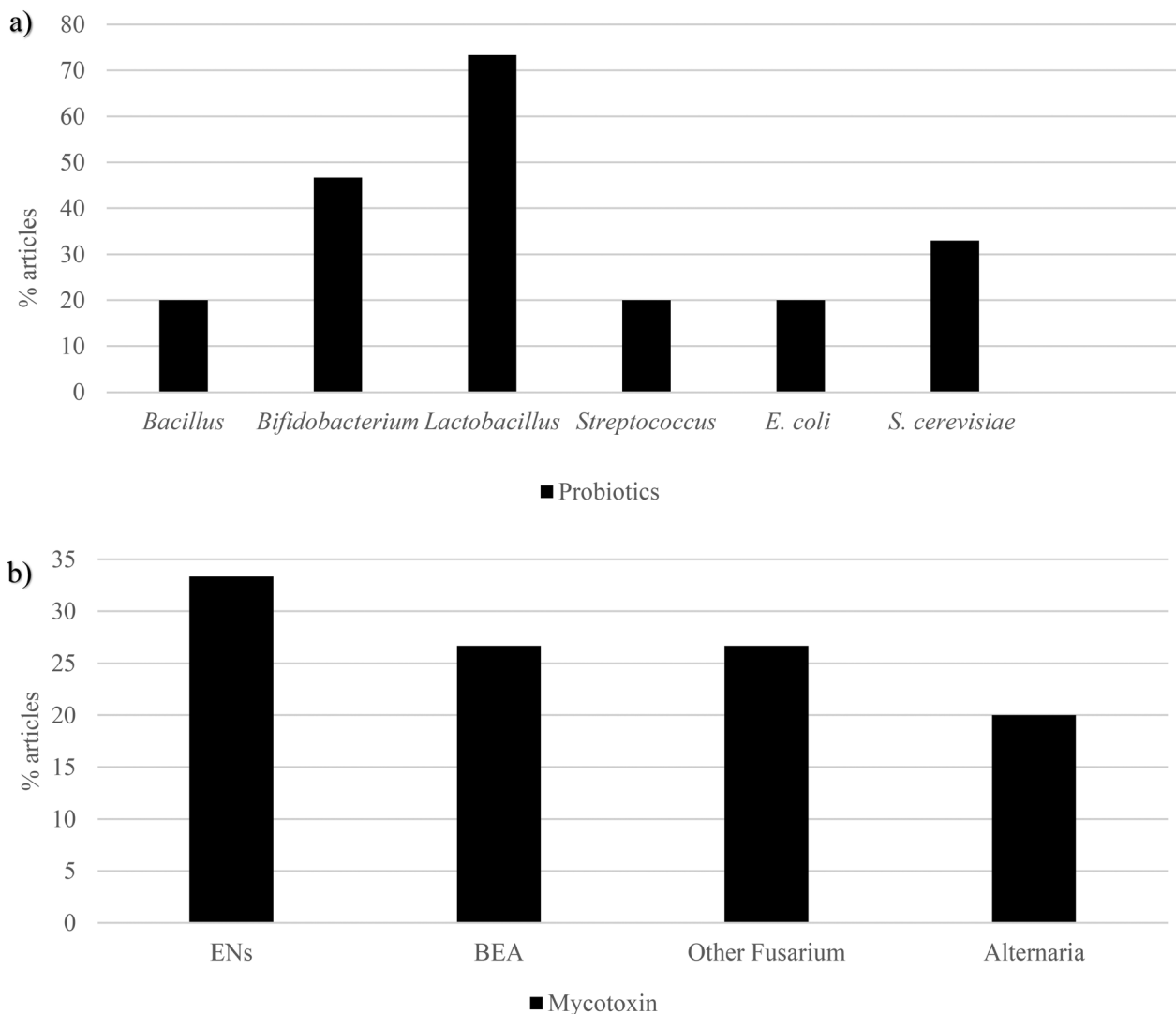


Fig. 5 Graphical bars of the probiotic publications ($n = 15$) according to the (a) microbial species and (b) the emerging mycotoxins studied. In the reports, *Bacillus*, *Bifidobacterium*, *Lactobacillus*, *Streptococcus*, *E. coli* and *S. cerevisiae* were the most recurrent probiotics, whereas enniatins, beauvericin, other *Fusarium* mycotoxins and *Alternaria* mycotoxins were the most used emerging mycotoxins. Beauvericin – BEA, enniatins – ENs, *Escherichia coli* – *E. coli* and *Saccharomyces cerevisiae* – *S. cerevisiae*.

While the mean control bioaccessibility was 92.6% and 90.0% for both BEA concentrations, respectively; when adding prebiotics, it was 13.2% and 50.4%, respectively, galactomannan being the most reducing one (5.2%).⁹³

Similar results have been obtained with *Alternaria* mycotoxins. AOH, AME, ATX-II, STTX-III, and ALP have been found to be decreased, already before anaerobic incubation, in the samples containing particulate fecal matter. These samples were supposed to be also composed of indigestible fiber fractions.⁶⁷ The antifungal effects of the volatile compound 2-phenylethyl isothiocyanate (2-PEITC) against *A. alternata* and its mycotoxins TEN, AOH, AME, and ALT were assessed. 2-PEITC is found in some vegetables and contributes to gastrointestinal health.⁹⁴ After a 2 h treatment, these four mycotoxins were extracted and detected by high performance liquid chromatography-tandem mass spectrometry (HPLC-TOF-ESI-MS) in the

mycelium of *A. alternata*. The concentrations of TEN, AOH, AME, and ALT at the MIC of 2-PEITC-treated groups were only 27%, 90%, 90%, and 88% of the corresponding control groups, respectively.⁹⁵ The anthocyanidin delphinidin (30.3 $\mu\text{g mL}^{-1}$) has exerted to strongly antagonize the genotoxic properties of ATX-II by significantly suppressing the level of DNA strand breaks by up to 75% in HT-29 colon carcinoma cells.⁸⁴ Conversely, AOH and AME degradation has been assessed after cinnamaldehyde treatment in centrifuge tubes under rotary shaking conditions (150 rpm) at 25 °C for 120 min. It was found that AOH and AME degradation rates were 16.8% and 7.3%, respectively.⁹⁶ The results are shown in Table 4.

3.4 Mycotoxins and synbiotics

The use of synbiotics has been previously proved to be effective in the removal of PAT.^{97,98} The deleterious effects of



Table 3 Collection of the most important characteristics of the studies found regarding the emerging mycotoxins and probiotics *in vitro* and *in vivo*. For each study, the studied mycotoxin, type of probiotic, mycotoxin dose administration, exposure time, experimental assay, results and references are described

Mycotoxin	Probiotic	Dose administration	Exposure time	Experimental assay	Results	Ref.
<i>In vitro</i>						
ENA	<i>L. johnsonii</i> CECT 289	<i>B. breve</i> CECT 4839 T	Not reported	4 h	Dynamic gastrointestinal <i>in vitro</i> digestion	71
ENA1	<i>L. rhamnosus</i> CECT	<i>B. adolescentis</i> CECT 5781 T				
ENB	<i>L. plantarum</i> CECT 220	<i>B. bifidum</i> CECT 870 T				
ENB1	<i>L. reuteri</i> CECT 725 <i>L. casei</i> CECT 47	<i>B. longum</i> CECT 4551				
ENA	<i>B. longum</i>	<i>L. ruminis</i>	0.2–20.000 ng per disc	24 h	Antimicrobial analyses carried out the disc-diffusion method	72
ENA1	<i>B. bifidum</i>	<i>Streptococcus thermophilus</i>				
ENA2	<i>B. breve</i>	22 strains of <i>S. cerevisiae</i>				
ENB	<i>B. adolescentis</i>	9 strains of <i>Bacillus subtilis</i> .				
ENB1	<i>L. rhamnosus</i>					
ENB4	<i>L. casei-casei</i> <i>L. plantarum</i> <i>L. paracasei</i>					
EN1	<i>Bacillus subtilis</i> 168 trpC2		2–8 µg mL ⁻¹	3 weeks	Cocultivation media	73
ENB1	<i>Staphylococcus aureus</i> ATCC 29213 <i>Streptococcus pneumoniae</i> ATCC 49619 <i>E. coli</i> ATCC 25922 <i>Staphylococcus aureus</i> 25697 <i>Enterococcus faecalis</i> UW 268 <i>Pseudomonas aeruginosa</i> B 63230					
ENA	<i>B. longum</i>	<i>Streptococcus thermophilus</i>	Not reported	48 h	<i>In vitro</i> degradation under an anaerobic atmosphere	74
ENA1	<i>B. bifidum</i>	<i>L. ruminis</i>				
ENB	<i>B. breve</i>	<i>L. casei</i>				
ENB1	<i>B. adolescentis</i>	22 strains of <i>S. cerevisiae</i>				
BEA	<i>L. rhamnosus</i> <i>L. casei-casei</i> <i>S. cerevisiae</i> LO9 <i>S. cerevisiae</i> YE-2 <i>S. cerevisiae</i> YE5 <i>S. cerevisiae</i> YE-6 <i>S. cerevisiae</i> YE-4 <i>S. cerevisiae</i> YE5	<i>S. cerevisiae</i> YE-6 <i>S. cerevisiae</i> A34 <i>S. cerevisiae</i> A17 <i>S. cerevisiae</i> A42 <i>S. cerevisiae</i> A08	5 mg L ⁻¹ ; 5 mg kg ⁻¹	48–72 h	Biological degradations under aerobic conditions in the liquid medium of PDB	75
BEA	<i>L. acidophilus</i>		0.8–15.7 µg mL ⁻¹	24–72 h	Cell viability assay	76
BEA	<i>L. paracasei</i> CECT 277 <i>L. casei</i> CECT 4180 <i>L. rhamnosus</i> CECT 278T <i>L. plantarum</i> CECT 220 <i>L. ruminis</i> CECT 4061T <i>L. casei casei</i> CECT 277 <i>B. breve</i> CECT 4839T	<i>B. adolescentis</i> CECT 5781T <i>B. bifidum</i> CECT 870T <i>B. Longum</i> CECT 4551 <i>Corynebacterium vitaeruminis</i> CECT 537 <i>Eubacterium crispatus</i> CECT 4840 <i>S. cerevisinas</i> CECT 1324	5–25 mg kg ⁻¹	48 h	<i>In vitro</i> dynamic digestion model	77



Table 3 (Contd.)

Mycotoxin	Probiotic		Dose administration	Exposure time	Experimental assay	Results	Ref.
BEA	<i>B. longum</i> <i>B. bifidum</i> <i>B. breve</i> <i>B. adolescentis</i> <i>L. rhamnosus</i> <i>L. casei-casei</i> <i>L. plantarum</i>	<i>Eubacterium crispatus</i> , <i>Salmonella fecalis</i> and <i>Salmonella thermophilus</i> <i>L. ruminis</i> <i>L. casei</i> <i>L. animalis</i>	5 mg L ⁻¹	4–48 h	Fermentations in the liquid medium of MRS	BEA reduction ranged from 66.5% to 83.1%, the highest decrease value being that caused by the strain of <i>L. casei-casei</i>	78
AUR	<i>E. coli</i> (ATCC 25922)	<i>L. acidophilus</i> (DSMZ 20079)	1.1–146.1 µg mL ⁻¹	6 h	Fast digital time-lapse microscopic method	The most potent compound was AUR against <i>L. acidophilus</i> . Meanwhile, BEA, ENs and FA were characterized by having weak antibiotic effects	79
ENs	<i>Salmonella enterica</i> serovar typhimurium 3389-1 (DT12)	<i>L. acidophilus</i> (20079)	1.4–174.6 µg mL ⁻¹				
BEA	<i>Staphylococcus aureus</i> (ATCC 29213)	<i>L. salivarius</i> (20555)	1.6–200.7 µg mL ⁻¹				
FA		<i>L. sobrius</i> (16698) <i>B. longum</i> subsp. <i>longum</i> Reuter (20219) <i>B. breve</i> (20213)	0.2–29.7 µg mL ⁻¹				
FA	<i>Burkholderia ambifaria</i> T16		0.06 mg mL ⁻¹	72 h	<i>In vitro</i> detoxification	<i>Burkholderia ambifaria</i> has been shown to inhibit the growth of several <i>Fusarium</i> species and possess the unique ability to use the mycotoxin FA as the sole C, N and energy sources, as well as the capacity to detoxify FA in barley seedlings	80
DAS	<i>L. acidophilus</i> 1A <i>L. acidophilus</i> 4A <i>L. helveticus</i> 2A <i>L. bulgaricus</i> 3A	<i>L. bulgaricus</i> 5A <i>Bacillus licheniformis</i> <i>Bacillus subtilis</i> <i>S. cerevisiae</i>	0.5 µg mL ⁻¹	48 h	Biodegradation <i>in vitro</i> in agar plates	DAS appeared to possess an inhibitory effect on the growth of almost all <i>Lactobacillus</i> and <i>Bacillus</i> strains, whereas no effect was observed on yeast strains	81
DAS	<i>Akkermansia muciniphila</i> DSM 22959 <i>B. adolescentis</i> DSM 20083 <i>Bacteroides thetaiotaomicron</i> DSM 2079 <i>Prevotella copri</i> DSM 18205 <i>Faecalitalea cylindroides</i> T2-87 <i>Anaerobutyricum hallii</i> DSM 3353 <i>Anaerostipes hadrus</i> SSC/2 <i>Blautia obeum</i> A2-162	<i>Butyrivibrio fibrisolvens</i> 16/4 <i>Coprococcus</i> sp. ART55/1 <i>Eubacterium rectale</i> DSM 17629 (A1-86) <i>Roseburia intestinalis</i> L1-82 <i>Lactiplantibacillus plantarum</i> NCIMB 7220 <i>Faecalibacterium prausnitzii</i> A2-165 <i>Enterococcus mundtii</i> DSM 4838	0.7 ng mL ⁻¹	0–48 h	Anaerobic incubation	The human microbiome characteristic bacteria <i>Prevotella copri</i> revealed to deacetylate 51.3% of DAS to 15-MAS after 48 h	82
TeA	<i>L. brevis</i> 20023 (LAB-20023)		0.5 µg mL ⁻¹	12 h	Adsorption	<i>L. brevis</i> 20023 (LAB-20023) cells have revealed to adsorb TeA from 12.5% to 90.1%	83



Table 3 (Contd.)

Mycotoxin	Probiotic		Dose administration	Exposure time	Experimental assay	Results	Ref.
AOH	<i>Bacteroides caccae</i>	<i>Alistipes finegoldii</i>	0.5–50 $\mu\text{g mL}^{-1}$	24 h	Absorption assay	Both Gram types were able to adsorb AOH, AME, and AST. Gram-negative bacteria showed higher adsorptive capacities. This tendency was not observed for neither ATX-I nor ALP	84
AME	<i>Bacteroides eggerthii</i>	<i>Alistipes timonensis</i>					
AST	<i>Bacteroides thetaiotaomicron</i>	<i>Ruminococcus bicirculans</i>					
ATX-I	<i>Bacteroides vulgatus</i>	<i>Akkermansia muciniphila</i>					
ALP	<i>Parabacteroides distasonis</i>	<i>L. hominis</i>					
	<i>E. coli</i>	<i>B. Longum</i>					
	<i>Clostridium innocuum</i> (CI)	<i>B. sp.</i>					

Mycotoxin	Probiotic	Mycotoxin dose administration	Exposure time	Experimental assay	Results	Ref.
<i>In vivo</i>						
AME	<i>L. uvarum</i> LUHS245	10.1–17.06 $\mu\text{g kg}^{-1}$	36 days	Fermentation/biotransformation	AME and ALT were found in 61-day-old control piglets' feces and in fermented feed samples. However, AME was not found in probiotic-treated piglets' feces	85
ALT	<i>L. casei</i> LUHS210					
	<i>Pentoseaceus acidilactici</i> LUHS29					
	<i>Pediococcus pentosaceus</i> LUHS183					

Alternariol methyl ether (AME), alterperyleneol (ALP), altertoxin I (ATX-I), alterperyleneol (ALP), altuene (ALT), altersetin (AST), aurofusarin (AUR), beauvericin (BEA), *Bifidobacterium* (B), diacetoxyscirpenol (DAS), enniatin A (ENA), enniatin A1 (ENA1), enniatin A2 (ENA2), enniatin B (ENB), enniatin B1 (ENB), enniatin B4 (ENB4), enniatins (ENS), *Escherichia coli* (*E. coli*), fusaric acid (FA), lactic acid bacteria (LAB), *Lactobacillus* (L), minimal inhibitory concentrations (MICs), De Man–Rogosa–Sharpe agar (MRS), potato dextrose broth (PDB), *Saccharomyces* (S.), and tenuazonic acid (TeA).

OTA have also been reversed by using beneficial synbiotics possessing the ability to reduce the number of potential pathogens in the digestive tract and by increasing the activity of α -glucosidase and α -galactosidase, while decreasing the activity of potentially harmful fecal enzymes (β -glucosidase, β -galactosidase and β -glucuronidase).^{60,99}

3.4.1 Emerging mycotoxins and synbiotics. The bioaccessibility of ENs (ENA, ENA1, ENB, and ENB1) and BEA was analyzed by using an *in vitro* static and dynamic simulated gastrointestinal digestion system, imitating the digestive physiological conditions until the colonic compartment. Inulin and FOS at two concentrations (1% and 5%) were first added, and afterwards, the colonic microbial fermentation was carried out by using *L. paracasei* CECT 277, *L. casei* CECT 4180, *L. rhamnosus* CECT 278T, *L. plantarum* CECT 220, *L. ruminis* CECT 4061T, *L. casei casei* CECT 277, *B. breve* CECT 4839T, *B. adolescentis* CECT 5781T, *B. bifidum* CECT 870T, *B. longum* CECT 4551, *Corynebacterium vitaeruminis* CECT 537, *Eubacterium crispatus* CECT 4840 and *S. cerevisiae* CECT 1324. The bioaccessibility of EN and BEA was shown to be higher in the synbiotic prebiotic static model (6.2%–44.9% and 46.7%–61.1%, respectively) than that found in the dynamic one (23.0%–68.9% and 76.2–91.0%, respectively).⁹² Similar results have been revealed in Meca *et al.* (2012),⁹³ where the fibers β -1,3 glucan, chitosan LMW, chitosan MMW, FOS, galactomannan, inulin and pectin (1% and 5%) were combined with the bacteria *L. animalis*

CECT 4060T, *L. casei* CECT 4180, *L. casei rhamnosus* CECT 278T, *L. plantarum* CECT 220, *L. ruminis* CECT 4061T, *L. casei-casei* CECT 277, *B. breve* CECT 4839T, *B. adolescentis* CECT 5781T, *B. bifidum* CECT 870T, *Corynebacterium vitaeruminis* CECT 537, *Streptococcus faecalis* CECT 407, *Eubacterium crispatus* CECT 4840, and *S. cerevisiae* CECT 1324. *In vitro* mean bioaccessibility values of BEA (at both experimental concentrations: 5 and 25 mg L^{-1}) by using synbiotics were 12.9% and 42.4%, respectively – 5.3 and 28.2-fold higher in comparison with the data evidenced with the prebiotic sample treated with duodenal digestion.⁹³

3.5 Mycotoxins and postbiotics

Postbiotics are considered to have positive effects on human health.⁵⁰ Yeast cell wall extract (YCWE) and a post-biotic yeast cell wall-based blend (PYCW) have revealed to reduce the adverse effects related to serum biochemistry, liver function, immune response, altered cecal short chain fatty acids (SCFAs), goblet cell counts and architecture of the intestinal villi. These results were found studying commercial broilers fed with a diet containing DON, T-2 and ZEA at 3.0 mg kg^{-1} , 104 $\mu\text{g kg}^{-1}$ and 79 $\mu\text{g kg}^{-1}$, respectively.⁵⁷ Some characteristic bacterial enzymes, which isolated are considered postbiotics, have exerted to remove AFB1, OTA, DON and ZEA.⁶³ Butyrate, one of the most studied gut microbiota metabolites, has shown to reverse the alterations caused by DON by blocking



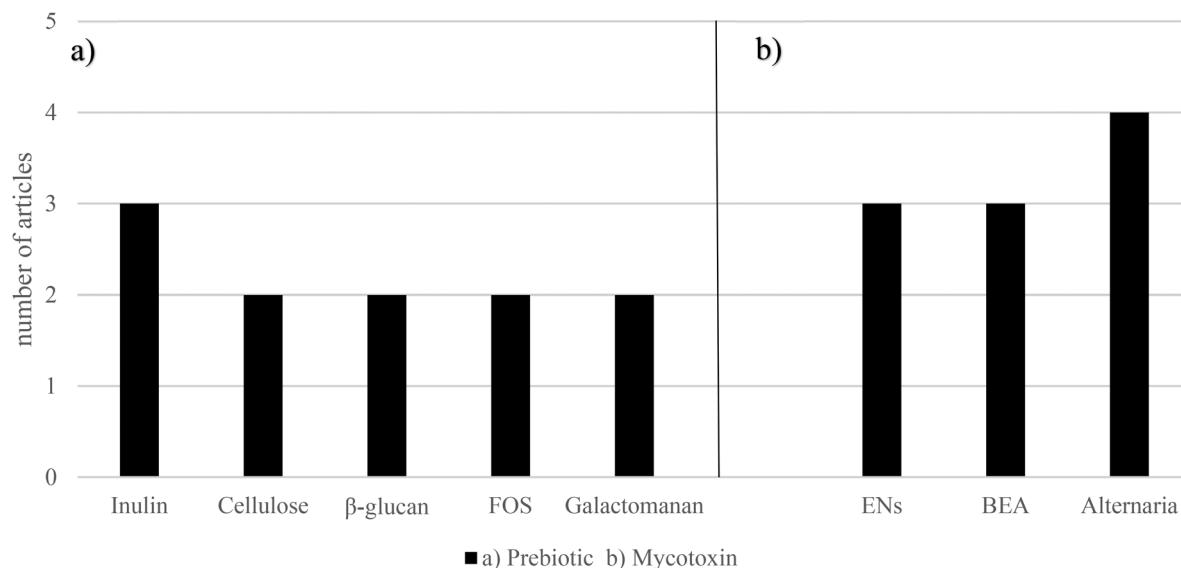


Fig. 6 Graphical bars of the prebiotics ($n = 9$) according to (a) the prebiotic type and (b) the emerging mycotoxins studied. Beauvericin – BEA, enniatins – ENs and fructooligosaccharides – FOS. In the studies, inulin, cellulose, β -glucan, FOS and galactomannan were the most used prebiotics, whereas ENs, BEA and *Alternaria* mycotoxins were the most recurrent emerging mycotoxins.

some genes involved in reactive oxygen species and tumor necrosis factor alpha mediated pathways.¹⁰⁰ Likewise, AFB1 and ZEA have been reported to reduce the content of SCFAs (butyrate, propionate and acetate).^{101,102}

3.5.1 Emerging mycotoxins and postbiotics. The utilization of postbiotics (PYCW) for addressing emerging mycotoxins was first documented in Xu *et al.* (2023).¹⁰³ In contrast to inorganic clay-based adsorbents characterized by presenting some limitations, such as the possible contamination with other detrimental compounds (dioxins, polychlorinated biphenyls, and heavy metals), organic adsorbents such as PYCW have shown to be more efficient and safe product substitutes.¹⁰⁴ In this study, an absorption bioassay was conducted exposing BEA (0.5, 2, 5, 20, 40 and 63 $\mu\text{g mL}^{-1}$) and CIT (0.5, 1, 10, 40, 50 and 68 $\mu\text{g mL}^{-1}$) to PYCW at different concentrations for 2 h. The results showed that PYCW presented an overall mean adsorption efficiency of 70.1% for BEA and 35.1% for CIT.¹⁰³

4 Discussion

The interactions along the gut are very complex due to the quantity of factors, which play a notable role in its physiology, so that there are times where it is so likely not to know completely which specific pathways or molecules are the consequence of a particular fact. However, there are some established correlations which help us to better understand how biological systems work. One of these strong correlations is the preventive role of PPSP and the gut microbiota in disease and its health-promotion effects.⁴⁹ As has already been said,⁶² PPSP and the gut microbiota possess the capacity to bind and metabolize a vast quantity of substances entering the gastrointestinal tract. Therefore, despite theoretically reducing ben-

eficial compounds, PPSP can also decrease the exposure to detrimental ones, such as toxins, including emerging mycotoxins.

As presented, European regulations only encompass a small set of mycotoxins (AFB1, AFB2, AFG1, AFG2, AFM1, OTA, PAT, DON, ZEA, FB1, FB2, CIT, AOH, AME, TeA, T-2 and HT-2 and ergoalkaloids).² Due to their regulation, these mycotoxins tend to be more studied – also including their interactions with the gut microbiota and PSPP. In fact, studies show that studied mycotoxins disrupt the gut microbiota, while concomitant intake of PSPP mitigates the effects of the mycotoxins and contributes to maintain gut microbiota homeostasis.^{56,58,90,99,101} Although similar trends are expected with unregulated mycotoxins, it has to be carried out as a one-on-one mycotoxin analysis, since every mycotoxin possesses a specific chemical structure and a different response in the environment where it is located.⁶² Actually, there are some 400 known mycotoxins, so the vast majority of them are not still regulated and need a more profound comprehension.¹ Furthermore, it should not be forgotten that mycotoxins tend to be found in combinations, so the most common mixtures found in food and feed are the ones interesting to be tested.

4.1 Emerging mycotoxins and the gut microbiota

Regarding the interaction between the gut microbiota and emerging mycotoxins, studies have revealed a clear tendency: the gut microbiota contributes to emerging mycotoxin detoxification through binding and/or metabolism^{63–65,67} and emerging mycotoxins alter the gut microbiota composition.⁶⁸ Despite being the trend confirmed by these studies, the difficulty stems from the heterogeneity of the analyzed samples. The microbiota exhibits significant variability, with the microbiome differing by species. In humans, microbiota changes are influenced by factors such as the level of urbaniz-



Table 4 Collection of the most important characteristics of the studies found concerning the emerging mycotoxins and prebiotics *in vitro*. For each study, the studied mycotoxin, type of prebiotic, mycotoxin dose administration, exposure time, experimental assay, results and references are described

Mycotoxin	Prebiotic		Dose administration	Exposure time	Experimental assay	Results	Ref.
<i>In vitro</i> ENA ENA1 ENB ENB1	Inulin (1.5%–10%)		1.02–2.04 mg mL ⁻¹	8 h	<i>In vitro</i> digestion model	ENA, ENA1, ENB and ENB1 bioaccessibility reduction in a dose-dependent manner at an inulin concentration ranging from 1.5% to 10%	91
ENA ENA1 ENB ENB1 BEA ENA ENA1 ENB ENB1 BEA	Cellulose (1% and 5%)		Not reported	4 h	<i>In vitro</i> digestion model	Cellulose (1% and 5%) decreased ENA, ENA1, ENB and ENB1 bioaccessibility a mean of 26.9% and 44.7%, respectively BEA bioaccessibility reduction ranged from 60% to 80%	71
ENA ENA1 ENB ENB1 BEA ENA ENA1 ENB ENB1 BEA	Inulin and FOS (1% and 5%)		Not reported	4 h	Static gastrointestinal <i>in vitro</i> digestion model	Highest reductions carried out when adding enriched inulin 5% (5.7%) and FOS 5% (3.9%) for ENA and ENA1, respectively	92
BEA	Galactomannan 1–5% Glucomannan HMW 1%–5% Glucomannan FP 1%–5% Citrus fiber 1%–5% Bamboo fiber 1%–5% Carrot fiber 1%–5%	Pie fiber 1%–5% β-Glucan 1%–5% Xilan 1–5% Cellulose HMW 1–5% Cellulose MMW 1–5%	5–25 mg kg ⁻¹	52 h	<i>In vitro</i> digestion model	BEA bioaccessibility reduction ranging from 15.7% to 60.5% comparing to control (93.2%)	77
BEA	β-1,3 glucan Chitosan LMW Chitosan MMW FOS	Galactomannan Inulin Pectin (All 1% and 5%)	5 mg kg ⁻¹	52 h	<i>In vitro</i> digestion model	BEA mean bioaccessibility decrease when adding prebiotics (13.2% and 50.4%, respectively for 1% ad 5%), galactomannan being the most reducing one (5.2%)	94
AOH AME ATX-II STTX-III ALP TEN AOH AME ALT	Particulate fecal matter supposed to be composed also by indigestible fiber fractions		50 µg mL ⁻¹	48 h	Fecal incubation <i>in vitro</i>	AOH, AME, ATX-II, STTX-III, and ALP reduction by samples supposed to be also composed of indigestible fiber fractions	67
	2-PEITC		0.1–1.38 mg kg ⁻¹	2 h	Qualitative analysis performed using a mass spectrometer after incubation for the MIC	TEN, AOH, AME, and ALT concentrations of 2-PEITC-treated groups were only 27%, 90%, 90%, and 88% of the corresponding control groups, respectively	95
ATX-II	Delphinidin		0.35 µg mL ⁻¹	24 h	Coupled SRB/WST-1 cytotoxicity assays	ATX-II genotoxic properties were antagonized by delphinidin (30.3 µg mL ⁻¹) by significantly suppressing the level of DNA strand breaks by up to 75% in HT-29 colon carcinoma cells	89
AOH AME	Cinnamaldehyde		0.81–0.82 µg mL ⁻¹	2 h	Degradation assay	AOH and AME degradation rate was 16.8% and 7.3%, respectively, after 120 min of cinnamaldehyde treatment	96

Alternariol (AOH), alternariol monomethyl ether (AME), alterperyleneol (ALP), altertoxin II (ATX-II), altuene (ALT), beauvericin (BEA), enniatin A (ENA), enniatin A1 (ENA1), enniatin B (ENB), enniatin B1 (ENB), fructooligosaccharides (FOS), high-molecular weight (HMW), low-molecular weight (LMW), medium-molecular weight (MMW), minimum inhibitory concentration (MIC), sulforhodamine B/water-soluble tetrazolium salt (SRB/WST-1), tentoxin (TEN), 2-phenylethyl isothiocyanate (2-PEITC), and stemphyliotoxin III (STTX-III).



ation of the location and even the time of day within the same individual.^{105–107} For example, rumen microbiome is characterized by possessing many enzymes involved in the *de novo* synthesis of vitamin B12.¹⁰⁸

According to experimental feasibility, it has been shown that gut microbiota samples are obtained from feces in most of the experiments, since the fecal microbiota is more accessible than the colon one. Interestingly, it has been revealed that colon microbiota has a different profile than the fecal one, the microbiome in the large intestine being much more diverse than those of the small intestine and feces. For instance, while in the large intestine there were 4080 operational taxonomic units, in the feces only 443 were found.¹⁰⁹ Indeed, bacterial biomass also changes along the gastrointestinal tract and the feces: duodenum (10^3 bacteria per g), jejunum (10^4 g⁻¹), ileum (10^7 g⁻¹), colon (10^{12} g⁻¹) and feces (5^{10} g⁻¹).^{110,111} In addition, short-term trials are likely to hide that long-term mycotoxin exposure may lead to the development of the ability to degrade these mycotoxins despite being unable to do it in the first moment.⁶⁴

There are other limitations that should be highlighted. Although in Devere *et al.* (2020)⁶³ the incubation time (48 h) was in line with other experiments studying mycotoxin degradation *in vitro*, it is considerably longer than the *in vivo* retention time of the digesta in the rumen, implying that under normal physiological conditions the results would be different. Despite Crudo *et al.* (2020)⁶⁷ having shown that AOH, AME, ATX-II, STTX-III and ALP were lower in the samples containing microorganisms than in those without them, it should be noted that the sample size was very small ($n = 4$). In this study, all donors shared some characteristics: they were healthy omnivorous volunteers (two males and two females) ranging from 26 to 34 years old and with a normal body mass index ($18.5\text{--}24.9$ kg m⁻²), none of them had been treated with antibiotics, probiotics, and prebiotics for the previous three months and they did not present previous intestinal diseases. However, the entire diet, playing a notable role in gut metabolism and in the interactions with mycotoxins, was not considered. Moreover, as mentioned in the same article, there are other limitations such as the short-term trial (3 h) and the fact that the growth media used might alter the initial microbial composition of the feces, contributing to the growth of some microorganisms at the expense of others. Conversely, in Novak *et al.* (2021),⁶⁸ only the mycotoxins ENB, ENB1, BEA and DON were assessed in weaning piglets' feces in contrast to the control. It was shown that *L. amylovorus* and *L. reuteri*, both beneficial microbes, were up-regulated and down-regulated, respectively, when adding BEA in comparison with DON and the control. Furthermore, the combination of ENs and BEA increased the total protein synthesis, but the addition of DON had an inhibitory effect.⁶⁸ Although it is important to know the interactions among these four mycotoxins, the real conditions are more complex, since a meal may contain a variety of contaminants, so the results under normal conditions are so likely to differ from these ones. While microbes could metabolize emerging mycotoxins, these toxins can also influence the composition of the microbiome, creating a potential

double-edged sword scenario. In addition, it is important to consider the varying doses of emerging mycotoxins used in experiments, ranging from nanograms to milligrams.

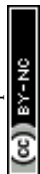
Viewing mycotoxins as toxic compounds or as potential drugs is indeed a question that arises. On the one hand, they can reduce beneficial microorganisms, such as *Lactobacillus*, *S. cerevisiae* and *Bacillus*,^{72,73,79,81} whose depletion is generally connected to disease promotion.¹¹² On the other hand, they can play a role as antibiotics by eliminating microorganisms involved in diseases.^{112–116} For instance, AOH and AME have exerted to be promising inhibitors of glycogen-synthase-kinase 3, being an important target in drug discovery. In fact, this enzyme has revealed to participate in signaling pathways of type 2 diabetes, cancer, neurological disorders, and other diseases.¹¹⁷ In addition, ergotamine with its α -adrenoreceptor-blocking mode of action has also been used for acute migraine treatments for over 50 years.¹¹⁸ Further investigation is needed in the field to clarify the results and assess the real implications of these emerging mycotoxins towards health and diseases, also taking into consideration that the dose and the type of mycotoxin may be decisive in the health or disease-promoting outcomes.

4.2 Emerging mycotoxins and probiotics

The bidirectional fluxes between probiotics and emerging mycotoxins have been widely confirmed. A vast quantity of probiotics (*Lactobacillus*, *Bifidobacterium* and *S. cerevisiae*) has shown to reduce emerging mycotoxins' bioaccessibility (ENs, BEA and *Alternaria*).^{74–78,92,93} At the same time, emerging mycotoxins have exerted to alter probiotic compositions (*B. subtilis* and *Staphylococcus aureus*).^{72,73,79,81} This bioaccessibility decrease may be due to the bacterial mycotoxin binding capacity^{83,84} and/or their ability to contribute to detoxification by originating derivatives characterized by possessing less toxicity.^{82,83} However, in most of these studies, probiotics are not assessed in combination, but one by one and using *in vitro* conditions. Only Vadopalas *et al.* (2020)⁸⁵ assessed probiotics' effects on emerging mycotoxins under *in vivo* conditions. In this study, not only did the addition of some microbial starters modified some blood parameters, but also contributed to modulate the gut microbiota and detoxify AME. Despite these beneficial effects, it should be noted that the experiment only lasted two months and the piglets' organs were not fully mature. Combining probiotics and working *in vivo* would be a more realistic approach to the human environment, since there are a lot of microorganisms that form a part of the gut microbiota.¹¹⁹ What is more, it is very well documented that the interplay along the gut concerns not only different bacteria, but also fungi, archaea, viruses, and helminths.¹²⁰

4.3 Emerging mycotoxins and prebiotics

It has been clearly shown that prebiotics reduce emerging mycotoxin bioaccessibility. Although the term prebiotic is very vast, the collected data cover a high quantity of these types of compounds at a different concentration.^{67,71,77,91–93,95,96} As in the probiotic case, there are two mechanisms explaining this reduction. The first one is the capacity of prebiotics to bind



emerging mycotoxins, and the second one is the ability to metabolize them, and it is very likely to have both processes working at the same time. However, most studies do not consider an analysis of secondary metabolites of emerging mycotoxins, so this metabolization cannot be fully confirmed. Regarding mycotoxin reduction, if the metabolites have not been degraded, it is assumed that they have bound to the structures of the prebiotics, although they are also subject to metabolization anyway. Conversely, apart from reducing bioaccessibility, prebiotics can also directly suppress the detrimental effects of mycotoxins. As it is shown in Aichinger *et al.* (2018), the anthocyanidin delphinidin blocks significantly ATX-II genotoxic properties by suppressing the level of DNA strand breaks.⁸⁹ The action of these prebiotics may derive from blocking the mycotoxin, preventing its action or from having a beneficial agonist impact on some physiological pathways. Furthermore, it should also be noted that in all these experiments, prebiotics are evaluated only one by one. The reality of the human diet includes a wide combination of them, so more research needs to be carried out taking this complexity into account.

4.4 Emerging mycotoxins, synbiotics and postbiotics

The role of synbiotics in the modulation of emerging mycotoxins has been assessed only in two studies.^{92,93} In both, the results showed that the addition of synbiotics increased mycotoxin bioaccessibility when compared to prebiotics. It has been hypothesized that probiotics degraded prebiotics, so that the binding ability of the last ones was lost. Although it may be possible, it cannot be extended as a generality, since only small fractions of prebiotics and probiotics were used in these two trials, and they do not represent either all probiotics or complete prebiotics. Conversely, those synbiotics applied to

non-emerging mycotoxins reveal opposite results. For instance, Zoghi *et al.* (2017)⁹⁷ showed that the removal of PAT by probiotic bacteria from apple juice depended significantly on the FOS content (as a prebiotic) and the addition of ascorbic acid. Under the best conditions, 91.3% of the initial patulin concentration was removed from juice for 6 weeks.⁹⁷ Zoghi *et al.* (2019) also reported that by adding FOS, ascorbic and citric acid to apple juice inoculated with *L. plantarum*, the efficiency of PAT removal was improved to 95.9% for 6 weeks cold storage.⁹⁸ On the other hand, despite being supposed to enhance clinical outcomes compared to probiotics or prebiotics alone due to their synergistic effect, synbiotics are very difficult to formulate and few have shown to possess clinical efficacy. It is also worth mentioning that most clinical trials are not characterized by including relevant probiotic or prebiotic controls, and many have not performed relevant microbiota analyses.¹²¹ Research needs to be expanded to more (emerging) mycotoxins in combination with synbiotics.

Finally, postbiotics have shown to bind, metabolize and alleviate the harmful effects of some emerging and non-emerging mycotoxins^{57,63,100,103} as well as these mycotoxins have revealed to alter the composition of some postbiotics.^{101,102} Notwithstanding, it is needed to extend these experiments to more (emerging) mycotoxins and to more postbiotics, since only the combination of PYCW with BEA and CIT has been assessed. In fact, it will be very interesting to focus on the interplay between (emerging) mycotoxins and SCFAs.

4.5 Interplay between emerging mycotoxins, the gut microbiota and PSPP

Although the results have revealed a clear trend, data on this interaction have only been found in 12 of the 20 emerging mycotoxins investigated. However, apart from these 20 ones,

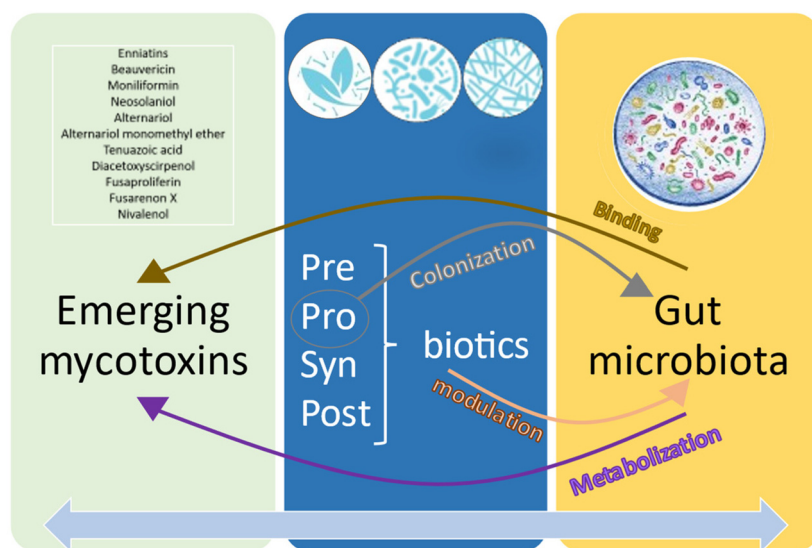


Fig. 7 Detoxification processes of the emerging mycotoxins by probiotics, prebiotics, synbiotics and postbiotics (PPSP) and the gut microbiota. Emerging mycotoxins are metabolized and bound by the gut microbiota. At the same time, PPSP contributes to colonize and modulate the gut microbiota, and also have an indirect impact on the metabolism of the emerging mycotoxins.



other emerging mycotoxins were found. The collected data encompassed a lot of experimental differences (mycotoxin/biotic type and concentration, species, time exposure, assay type, *etc.*), making the comparisons difficult among them. As Fig. 7 illustrates, the interaction between the gut microbiota and emerging mycotoxins involves metabolization and binding processes. The role of PPSP in relation to emerging mycotoxins primarily revolves around colonization (probiotics) and modulation (all). Furthermore, there exists a two-way communication between microbiota and PPSP, where the gut microbiota can degrade and metabolize PPSP, activating secondary bioactive compounds that may contribute to the detoxification and/or alleviation of emerging mycotoxins. Similarly, PPSP can enhance certain gut microbiota strains involved in the removal of emerging mycotoxins. Indeed, this reciprocal relationship can also be applied to the entire spectrum of PPSP, as the addition of probiotics, prebiotics, and synbiotics may enhance the production of certain postbiotics (such as short-chain fatty acids), and *vice versa*.

5 Conclusions

Emerging mycotoxins have been implicated in numerous harmful biological processes. Despite their diverse nature, studies have demonstrated that the gut microbiota and PPSP can detoxify and/or alleviate the adverse effects resulting from exposure to these emerging mycotoxins. *ENS*, *BEA*, *NIV*, and *Alternaria* mycotoxins were among the most frequently encountered emerging mycotoxins. The genera *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces* were the most extensively studied probiotics, while inulin, cellulose, β -glucan, FOS, and galactomannan were among the most evaluated prebiotics. Data on synbiotics and postbiotics were limited. Evidence clearly demonstrates the existence of interplay between the gut microbiota and emerging mycotoxins, as well as between PPSP and emerging mycotoxins, involving processes such as metabolization, colonization, modulation, and binding. In addition, connections between the gut microbiota and PPSP, as well as within the same PPSP, were identified, all contributing to the alleviation of the effects of the emerging mycotoxins.

However, quantitative and qualitative data remain limited. Further *in vivo* and long-term trials are necessary to better comprehend the complexity of these interactions in both animals and humans. The formulation of PPSP as functional food and nutraceuticals should be considered a preventive strategy against the toxicity of emerging mycotoxins in human diets and animal feed.

Author contributions

Á. Lázaro: writing – original draft, methodology, investigation and validation. P. Vila-Donat: methodology, investigation, and review and editing. L. Manyes: investigation, review and editing, project administration, and conceptualization.

Data availability

The data supporting this article have been included as part of the ESI.†

Conflicts of 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.

Acknowledgements

This publication is part of the project PID2022-140722OB-I00 funded by MCIU/AEI/AEI/10.13039/501100011033/FEDER, EU.

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To cite this article: Álvaro Lázaro, Pilar Gómez-Ramírez, Pilar Vila-Donat, Alessandra Cimbalo & Lara Manyes (08 Apr 2025): Effects of pumpkin and fermented whey on fecal microbiota profile against AFB1 and OTA exposure in Wistar rats, Toxicology Mechanisms and Methods, DOI: [10.1080/15376516.2025.2484636](https://doi.org/10.1080/15376516.2025.2484636)

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RESEARCH ARTICLE



Effects of pumpkin and fermented whey on fecal microbiota profile against AFB1 and OTA exposure in Wistar rats

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ABSTRACT

Mycotoxins perturb the gut microbiota performance. Bioactive compounds have been recently used as a new food strategy to diminish mycotoxins bioaccessibility and prevent their toxic effects on human and animal health. Male and female Wistar rats were exposed orally to twelve different diets containing aflatoxin B1 (AFB1) and/or ochratoxin A (OTA) with or without fermented whey (FW) and pumpkin (P) for 28 days. Fecal microbiota using 16S rRNA gene sequencing and subsequent metagenomics analysis were analyzed to study the effect of 28-day exposure through diet of contaminated and enriched feed. QIIME 2 microbiome analysis package (version 2024.5) was used to analyze the demultiplexed data. Mycotoxins-functional ingredients combination contributed more to microbial phylogenetic faith α -diversity rather than the functional ingredients alone, while the same combination reported a microbial α -diversity enhancement in comparison to the mycotoxins alone. Proteobacteria phylum was reduced in rat samples fed with contaminated diets (AFB1, OTA, and AFB1+OTA), while there was an increase—although not in all groups—when adding the functional ingredients. The main difference between the sexes was found in FW+AFB1+OTA group, with males (25%) showing higher % of *Proteobacteria* than females (1.86%). Phylogenetic diversity faith only focuses on microbial genetic (dis)similarity, not considering the biological function. *Morganella morganii*, a *Proteobacteria* found in some groups presents anticancer activity, but it is also related to inflammatory bowel disease and colorectal cancer. To sum up, both mycotoxins and functional ingredients trigger changes in the microbiota profile of Wistar rats in a sex-specific manner.

ARTICLE HISTORY

Received 16 January 2025

Revised 20 March 2025

Accepted 21 March 2025

KEYWORDS

Mycotoxins; probiotics; feces; microbiota; sexual dimorphism

1. Introduction

Despite more than 400 mycotoxins have been identified, the most toxic and studied are aflatoxin B1 (AFB1) and ochratoxin A (OTA), accounting for a notable economic and public health concern. Even at lower concentrations, these mycotoxins can affect human and animal health by disrupting nutrient digestion, absorption, metabolism, and normal physiology (Manyes and Font 2023). AFB1 is a secondary metabolite produced by the *Aspergillus* genera, which is a mycotoxigenic fungi contaminating a wide diversity of foodstuff such as rice, maize, milk, meat, pistachios, nuts, seeds, and spices. AFB1 is very well documented because of its serious carcinogenicity for both humans and animals (EFSA Panel on Contaminants in the Food Chain (CONTAM) et al. 2020). In fact, the International Agency for Research on Cancer (IARC) classified AFB1 as a human carcinogen belonging to Group 1 due to its participation in DNA adducts construction. Its acute and chronic toxicity have been displayed through several *in vitro* and *in vivo* studies connecting to immunosuppression, nutritional dysfunctions, cancer, and liver damage (Marchese et al. 2018; Cimbalo et al. 2020). OTA is another world-pervasive

mycotoxin which is largely produced by *Aspergillus* and *Penicillium* species. Literature shows that OTA exposure can enhance DNA damage, oxidative stress, inflammation, and cell death. Moreover, IARC classified OTA in the group 2B as possibly carcinogenic to humans. Typical foods contaminated by OTA are cereals, meat, dried fruits, wine, and spices (Cimbalo et al. 2020; Frangiamone et al. 2022).

According to the Commission Regulation (EU) 2023/915 of 25 April 2023 legislative framework, the regulated mycotoxins maximum limits in humans are established, among them, those belonging to AFB1 and OTA (European Commission 2023). The EU maxim limits established for AFB1 are 8 µg/kg for groundnuts (peanuts) and other oilseeds, hazelnuts, and Brazil nuts; 5 mg/kg for cereals and products derived from cereals with some exceptions, and 2 µg/kg for almonds, pistachios, and apricot kernels. The maximum limits for OTA are 20 µg/kg for marshmallow and dandelion roots (dried); 5 mg/kg for unprocessed cereal grains; 3 mg/kg for products derived from unprocessed cereal grains; 20 µg/kg for orange blossoms (dried) for use in herbal infusions or in coffee substitutes; and 80 µg/kg for licorice extract for use in food in particular beverages and confectionary.

Mycotoxins have been pointed out to perturb the gut microbiota performance. It has been described that AFB1 disturbs gut microbiota-dependent organic acid metabolism and OTA changes the structure and diversity of gut microbial communities, mainly because of the alteration of the short-chain fatty acids (SCFA) metabolism (Lázaro et al. 2024). The gut microbiota has emerged as a key factor in a substantial quantity of diseases such as diabetes, obesity, dementia, Parkinson, inflammatory bowel disease, anxiety, depression, and cancer (Ancona et al. 2021; Daily et al. 2021; Menozzi et al. 2021; Moughnyeh et al. 2021; Micic et al. 2023). Microbiota has been coined as the last human organ whose complexity of interactions is under current research (Baquero and Nombela 2012). There are some factors, such as diet, antibiotics, species, age, and sex playing an important role on gut microbiota modulation (Hasan and Yang 2019; Valeri and Endres 2021). Although microbiota may be only understood as bacteria, since they are the most numerous, but also fungi, archaea, viruses, and helminths belong to it (Vemuri et al. 2020). To better comprehend the relevance of the microbes, it should be highlighted that, whereas there are only 26 600 human genes, more than 4 000 000 bacteria genes can be found in human body. Furthermore, bacteria highest density in any ecosystem on earth is found in the human colon (10^{11} – 10^{12} /mL) (Sender et al. 2016).

Bioactive compounds have been recently used as a new food strategy to diminish mycotoxins bioaccessibility and prevent their toxic effects on human and animal health (Frangiamone et al. 2023a). Also, it has been shown that bioactive compounds have a positive impact on the health (the intestine and the whole body) of host animals by enhancing the abundance of beneficial bacteria and decreasing the abundance of harmful bacteria (Chen et al. 2022). Pumpkin (P) is a well-known ingredient containing polyunsaturated fatty acids, tocopherols, carotenoids, delta-7-sterols, tryptophan, and many other phytochemicals. This multifunctional ingredient is characterized by presenting some benefits such as anti-inflammatory, antimicrobial antioxidant, and anti-ulcerative properties, improving spermatogenesis, wound healing, and treatment of benign prostatic hyperplasia (Batoool et al. 2022). In fact, pumpkin (P) components have shown to have a beneficial effect on human gut microbiota by enhancing SCFA, which increase the population some types of bacteria, such as *Akkermansia*, *Bacteroides*, and *Lachnospiraceae_NK4A136* group (Yan et al. 2025). Milk whey, a cheese by-product with high nutritional values, is an excellent source of lactose, proteins, small amounts of vitamins and minerals, and an optimal substrate for lactic acid bacteria fermentation (Gupta and Prakash 2017), which originates the fermented whey (FW). Some of its benefits are related to the improvement of urinary function, urinary tract symptoms, systemic oxidative stress, and protection against bacterial infection (Ausmees et al. 2018; Cordeiro et al. 2019). Furthermore, it has been revealed that FW augments butyrate and propionate production, one of the most important mechanisms through which improves the gut microbiota profile (Smith et al. 2020).

Previously, it has been reported that AFB1 and OTA alter gut microbiota in mice, sheep, broilers, hens, ducklings, and

rats (Lázaro et al. 2024). Interestingly, sex hormones have recently appeared to be potent drivers of microbiota regulation (Valeri and Endres 2021). However, there are no studies which focus on microbiota sex differences when exposing animals to AFB1 and OTA. In fact, a human study analyzing the composition of the gut microbiota during adolescence revealed higher fecal microbiota dissimilarity in teenager (13–17 years) twin pairs with opposite sex in comparison to twins with the same sex (Yatsuneneko et al. 2012). What is more, rats subjected to adult ovariectomy or neonatal androgenization, as a model of menopause, reported changes in gut microbiota composition (Moreno-Indias et al. 2016).

Therefore, the aim of the present study was to investigate the effects on the gut microbiota profile after AFB1 and OTA oral chronic exposure and the possible beneficial effects provided by FW and P feed enrichment considering the interplay of sexual dimorphism in Wistar rats.

2. Material and methods

2.1. Experimental design and mycotoxin exposures

As Vila-Donat et al. (2024) previously described, Wistar rats ($n=120$) were randomly divided into twelve groups (10 rats for each, five females, and five males) and exposed orally to twelve different diets during 28 days: 1) control feed; 2) AFB1 (4.9 mg/kg), 3) OTA (6.0 mg/kg), 4) AFB1 (4.8 mg/kg) and OTA (6.4 mg/kg), 5) FW (1%) feed, 6) FW (1%) and AFB1 (4.3 mg/kg), 7) FW (1%) and OTA (8.3 mg/kg), 8) FW (1%) with AFB1 (4.5 mg/kg) and OTA (7.5 mg/kg), 9) FW (1%) and P (1%) feed, 10) FW (1%) and P (1%) feed and AFB1 (4.7 mg/kg), 11) FW (1%) and P (1%) feed and OTA (5.4 mg/kg) and 12) FW (1%) and P (1%) feed, AFB1 (5.2 mg/kg) and OTA (8.8 mg/kg).

Vila-Donat et al. (2024) estimated the mycotoxin ingestion in these rats, reporting values of 176–387 $\mu\text{g/kg}$ bw for AFB1 and 162–462 $\mu\text{g/kg}$ bw for OTA. While real-world exposure levels are generally lower (1.26 ng AFB1/kg bw and 4.1 ng OTA/kg bw) (Hoteit et al. 2024), higher concentrations were used in this study to obtain clearer results.

Feed production was carried out in the laboratory to prepare the control diet and the other experimental diets. The main ingredients included wheat flour, mineral water, sugar (sucrose), and salt. Contaminated and supplemented feeds were then prepared with minor modifications to the control recipe. Fecal samples were collected weekly, with each animal housed individually in a metabolic cage for a 24-hour period following feed exposure. Samples for this study, were collected specifically during the third week of the trial.

2.2. DNA extraction and purification

DNA extraction was performed using 50 mg of feces from each rat using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research Corp., Irvine, CA, USA). To enhance the efficiency of cell lysis, Biospec Mini-BeadBeater-96 centrifuge (BioSpec Products, Bartlesville, OK, USA) was used 1 min at maximum speed (2400 rpm) and 5 min rest in ice repeating the cycle 5 times. The purity and quantity of DNA were spectrophotometrically assessed through a NeoDot UV/vis Nano

Spectrophotometer (Neo Biotech, Nanterre, France) showing concentrations between 39.1 ng/μg and 163.2 ng/μg along with 260/280 nm and 260/230 nm ratios around 2. A total of 103 samples met the quality requirements and were therefore sequenced (Table 1).

2.3. 16S rRNA gene sequencing

Variable V3 and V4 regions of the 16S rDNA gene were amplified following the 16S rDNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15044223 Rev. A). Gene-specific primers containing Illumina adapter overhang nucleotide sequences were selected according to Klindworth et al. (2013). After 16S rDNA gene amplification, the multiplexing step was performed using Nextera XT Index Kit. One microliter of the PCR product was run on a Bioanalyzer DNA 1000 chip (Agilent, Santa Clara, California, USA) to verify the amplicons size (~550 bp). After size verification, the libraries were sequenced using a 2 × 301 pb paired-end run (MiSeq Reagent kit v3) on a MiSeq Sequencer according to manufacturer's instructions (Illumina, San Diego, California, USA).

2.4. Bioinformatic analysis

QIIME 2 microbiome analysis package (version 2024.5) was used to analyze the demultiplexed data provided by the Genomics Unit (Bolyen et al. 2019). For sequence quality control and feature table construction, Dada2 plugin was applied (Callahan et al. 2016). Dada2 filtered out noisy sequences, corrected errors in marginal sequences, removed chimeric sequences, removed singletons, joined denoised paired-end reads, and dereplicated those sequences. The end product is an amplicon sequence variant (ASV) table, a higher-resolution analogue of the traditional operational taxonomic units (OTUs) table, which records the number of times each exact amplicon sequence variant was observed in each sample. OTU and ASV metabarcoding analyses yield similar results with regard to examining microbial diversity and functionality, ASV-based analysis is able to capture slightly more detailed gut microbial diversity compared to OTU-based analysis (Inglis et al. 2022). Trimming left parameters were 0

bases forward and 10 reverse. Truncation length chosen was 290 bases forward and 220 reverse.

The next step was the generation of a tree for phylogenetic diversity (PD) analyses, using align-to-tree-mafft-fasttree pipeline from the q2-phylogeny plugin (Price et al. 2010). QIIME 2's diversity analyses are available through the q2-diversity plugin, which supports computing alpha and beta diversity metrics, applying related statistical test (Kruskal-Wallis), and generating interactive visualizations. Sampling depth chosen was 18 520, the value that was as high as possible to retain more sequences per sample, while excluding as few samples as possible. Alpha diversity is shown by Faith's PD plot, a qualitative measure of community richness that incorporates phylogenetic relationships between the ASVs (Faith 1992). Beta diversity is represented by Bray-Curtis and Jaccard Distance 3D Emperor plot, to measure of community dissimilarity.

Alpha rarefaction plot was produced by QIIME diversity alpha-rarefaction visualizer. It was used to determine if the richness of the samples had been fully observed or sequenced. If the lines in the plot appear to approach a slope of zero at some sampling depth along the x-axis, that suggests that collecting additional sequences beyond that sampling depth would not be likely to result in the observation of additional ASVs.

To explore the taxonomic composition of the samples, a feature classifier was trained with q2-feature-classifier (Bokulich et al. 2018). Taxonomic classifiers perform best when they are trained based on the specific sample preparation and sequencing parameters, including the primers that were used for amplification and the length of the sequence reads. The Greengenes2 sequence used was 2022.10. backbone.full-length.fna.qza (McDonald et al. 2024). Forward primer sequence applied was 5'-GTCGGCAGCGTCAGATGTGTATAA -3' and reverse, 5'-GGACTACHVGGGTATCTAAT 3'. Minimum length was 150 and maximum, 300.

2.5. Statistical analysis

Taxonomic differential abundance analysis and plots (comparisons among groups) were carried out by classical univariate analysis using Kruskal-Wallis test by IBM SPSS Statistics 29.0.2.0 (IBM Corp, Armonk, NY, USA). Equations for Bray-Curtis were developed for the study of beta diversity. All data were tested for normality using the Shapiro-Wilk test and subsequently analyzed using the appropriate statistical methods. A *p*-value of <0.05 was considered statistically significant.

3. Results

The fecal microbiota from 12 groups of control and exposed rats were analyzed using 16S rRNA gene sequencing and subsequent metagenomic analysis to study the effects of 28-day exposure to contaminated and enriched feed through the diet. Alpha rarefaction was used to determine if the richness of the samples has been fully observed or sequenced. The sequencing depth number represents the unique sequencing reads that align to a region in a reference genome or de novo assembly. The curves of the 12 groups

Table 1. Number of feces samples (*n*) sequenced from each group and sex (*n* = 103).

Group exposure	Male rats (<i>n</i>)	Female rats (<i>n</i>)
Control	5	5
AFB1	5	5
OTA	4	4
AFB1 + OTA	5	5
FW	5	5
FW+AFB1	5	4
FW+OTA	2	1
FW+AFB1 + OTA	5	5
FW+P	4	3
FW+P + AFB1	4	5
FW+P + OTA	4	3
FW+P + AFB1 + OTA	5	5

AFB1, aflatoxin B1; FW, fermented whey; OTA, ochratoxin A; P, pumpkin.

reached a plateau, indicating that the sequencing depth used in this study was adequate in evaluating the microbial diversity of each sample and in precisely describing the fecal microbial communities. After denoising the samples using Dada2 plugin (Callahan et al. 2016), 1 907 560 (32%) features were retained in 103 (100%) samples at 18 520 sampling depth (Figure 1).

3.1. Diversity analysis

3.1.1. Alpha diversity results

Faith's PD is the sum of the branch lengths on the minimum spanning tree linking a set of terminal taxa to the root. So, a higher number means more branches, that is to say, more diversity. There were significant statistical

differences interesting to highlight in Figure 2 after applying the Kruskal-Wallis test regarding diet exposure. Groups FW+P+OTA and FW+AFB1+OTA showed more diversity than control ($p=0.019$ in both cases). Likewise, microbiota in FW+AFB1 ($p=0.002$) and FW+AFB1+OTA ($p=0.001$) were more diverse than in FW. The group FW+AFB1+OTA ($p=0.028$) showed a richer diversity than FW+OTA. Finally, FW+P+OTA was more diverse than OTA ($p=0.049$) and FW+OTA ($p=0.030$). In the Supplementary Material, some differences including diet (Figure S1) and diet and sex (Figure S2) variables can be observed. Only two groups showed significant differences between males and females, AFB1 ($p=0.016$) and FW+P+AFB1+OTA ($p=0.009$).

Microbial evenness in the context of the gut microbiome is about how fairly the different types of bacteria share space

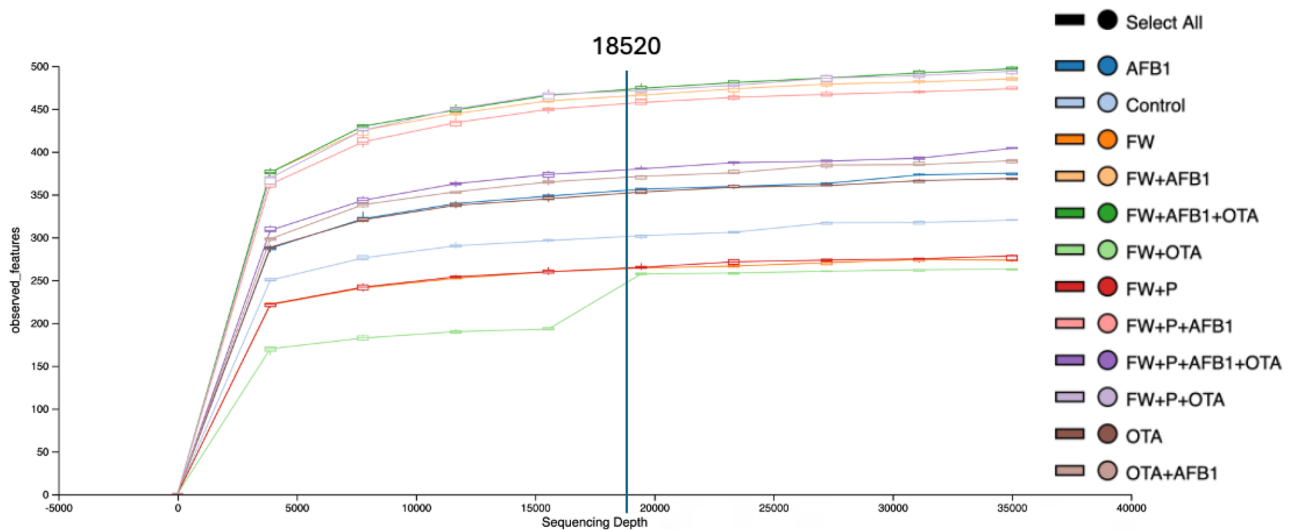


Figure 1. Rarefaction curves of observed features for each group. The x-axis represents the number of sequences per sample and the y-axis refers to the observed number of features. The plateau indicates that the fecal microbial community was fully detected with the sequencing depth used. AFB1, aflatoxin B1; FW, fermented whey; OTA, ochratoxin A; P, pumpkin.

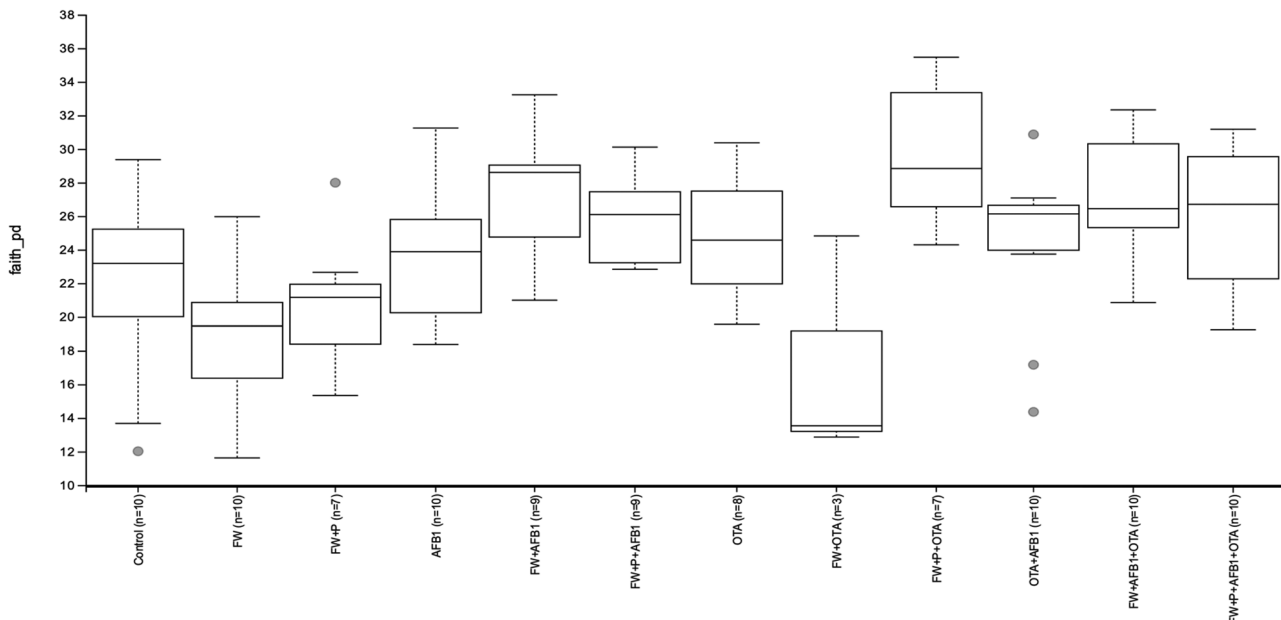


Figure 2. Faith's phylogenetic diversity values by diet. AFB1, aflatoxin B1; FW, fermented whey; OTA, ochratoxin A; P, pumpkin.

in the gut. Evenness is higher when species are present in similar proportions, that is to say, the same relative abundance. This parameter was not affected neither by diet nor sex (Figure 3).

3.1.2. Beta diversity

Bray–Curtis dissimilarity is a statistic test used to quantify the dissimilarity in species composition between two different sites. It ranges from 0 to 1. If both samples share the same number of microbes, at the same abundance, their ‘dissimilarity’ will equal zero. The Bray–Curtis dissimilarity is based on occurrence data (abundance), while the Jaccard distance is based on presence/absence data. Jaccard measures how similar samples are based on the presence or absence of species, without considering their abundance. It also ranges from 0 to 1, where 0 means no species are shared between samples, and 1 means they share the exact same species. Axis represents principal components (PC) in the plots.

Principal component (PC) analysis plot based on Bray–Curtis dissimilarity (Figure 4a) showed that fecal microbial communities from FW+AFB1, FW+AFB1+OTA, and AFB1+OTA were more similar to each other than FW, FW+P, FW+OTA, AFB1, OTA and control, which are separated by PC1 and PC2. The second similar group of fecal microbial communities that we can observe is composed by FW+P+AFB1 and FW+P+AFB1+OTA, separated from the first one by PC1. Some differences in abundance can be observed but are not enough to confirm a beneficial effect from FW and FW+P intake in microbiota.

Regarding Jaccard PCoA plot (Figure 4b), less differences among samples from the same diet group can be observed compared to Bray–Curtis plot. Control samples remain at the middle of the plot, while FW and FW+P are at two different edges. Fecal samples from FW, AFB1, OTA, FW+OTA, and AFB1+OTA did not share the same microbial species, and they

were clearly separated mostly by PC2. The diet groups more alike in their microbial composition were FW+AFB1, FW+AFB1+OTA, FW+P+AFB1, FW+P+OTA, and FW+P+AFB1+OTA. This result evidences how functional ingredients influence fecal microbiota composition, making it more similar to control group at PC2 level when feed is contaminated by mycotoxins. Even FW+OTA, which is not aligned with the rest of groups including functional ingredients, certainly exhibited different microbial species than groups fed with contaminated diets without functional ingredients.

3.2. Taxonomic differential abundances

To explain more detailed changes in the fecal microbial structure induced by mycotoxins and/or functional ingredients exposure, the relative abundance at the phylum level was determined. Figure 5 shows the relative abundance (%) of phylum per sample and Figure 6 shows it per diet group, including statistical differences. The main difference between sexes can be found in group FW+AFB1+OTA, with males (25%) showing higher percentage of *Proteobacteria* than females (1.86%).

For a more detailed statistical analysis of the taxonomical results, the five most prevalent bacteria phylum were evaluated based on the diet. In the first place, Shapiro–Wilk test was chosen to study normality, showing *Firmicutes* D and *Actinobacteria* data as normal. For these, general linear model and Tukey post hoc test was applied. For *Bacteroidota*, *Firmicutes* A, and *Proteobacteria*, Kruskal–Wallis test was chosen (Figure 6). *Firmicutes* A and D were not altered by the mycotoxins’ ingestion or the inclusion of functional ingredients in the feed compared to control. FW+P addition to the diet provoked a significant increase of *Actinobacteria* in fecal microbiota. It is important to highlight that feed enrichment with FW and FW+P, when contaminated with AFB1,

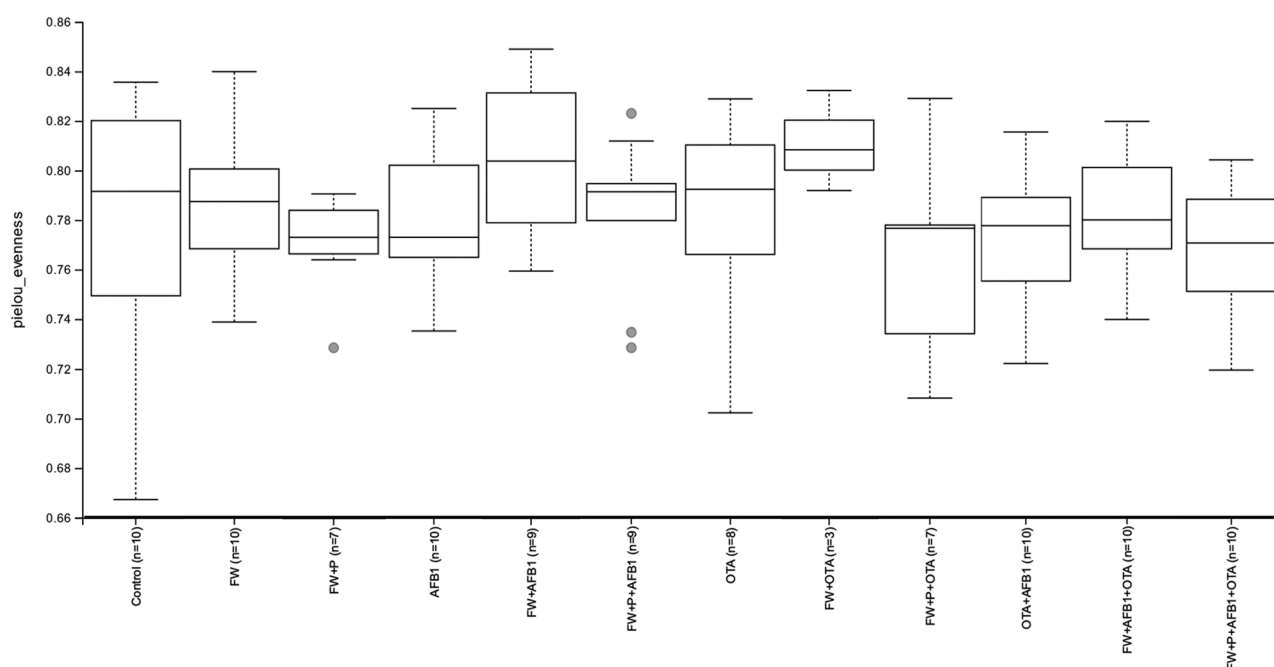


Figure 3. Evenness values by diet. AFB1, aflatoxin B1; FW, fermented whey; OTA, ochratoxin A; P, pumpkin.

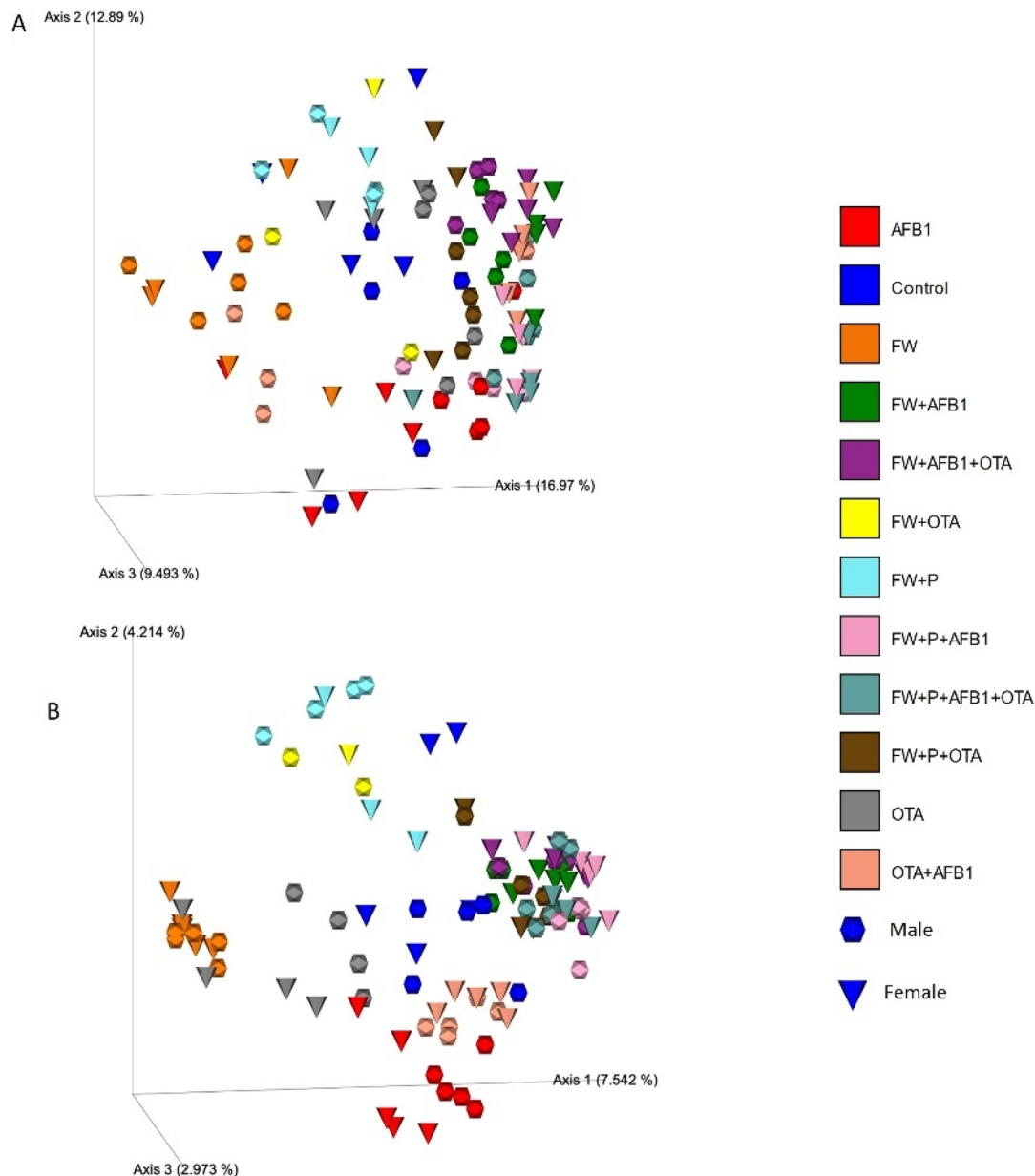


Figure 4. Bray-Curtis (a) and Jaccard (b) dissimilarity by diet and sex in microbial community rat feces. AFB1, aflatoxin B1; FW, fermented whey; OTA, ochratoxin A; P, pumpkin.

significantly increased the percentage of *Bacteroidota* in fecal samples compared to the AFB1-contaminated diet. In general, a reduction in *Proteobacteria* phylum in rat samples fed with contaminated diets (AFB1, OTA, and AFB1+OTA) was observed. When these contaminated diets included functional ingredients, in all cases *Proteobacteria* increased its percentage, even if not in all groups the increase was statistically significant.

The *Firmicutes/Bacteroidota* (F/B) ratio modulation by diet and sex was evaluated (Figure 7). The main differences can be observed between sexes in the rats non-exposed to mycotoxins. Between control groups, the difference between male and female was not significant but a tendency was detected, while for FW and FW+P groups the differences between sexes were statistically significant. This was not observed when mycotoxins were present, decreasing the F/B ration for both sexes.

Regarding the genus and species, the presence of genus *Morganella*, represented by *Morganella morganii*, was the only microorganism which totally correlated with the diet provided to the rats (Figure 8). It was the most frequent in control rats, followed by all groups, including FW+P and mycotoxin. When comparing sexes fed with the same diet, *Morganella morganii* was always more prevalent in females than in males, especially in OTA group. The other two most current genus were *Cryptobacteroides*, represented by *Cryptobacteroides* sp900544195, and *Floccifex* by *Floccifex porci*.

4. Discussion

The study aimed to investigate the effects of AFB1 and OTA on the gut microbiota, both individually and in combination, using fecal samples from rats exposed to diets contaminated with these mycotoxins for 28 days, with or without the

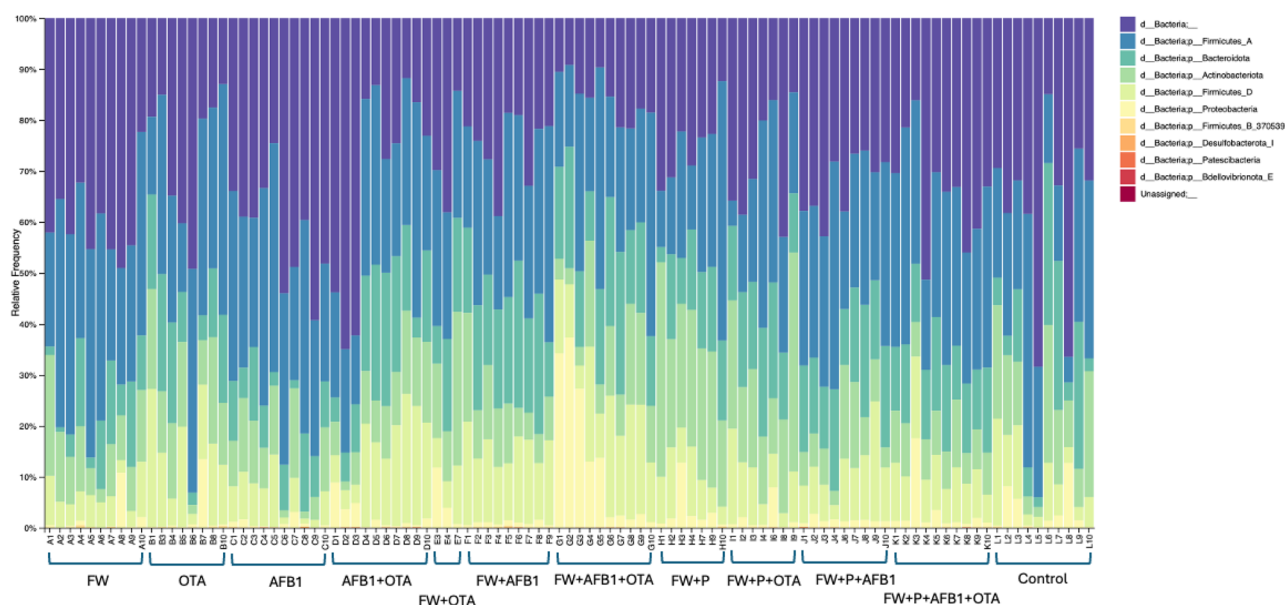


Figure 5. Microbiota relative abundance at phylum level per sample. Numbers from 1 to 5 represent males and from 6 to 10, females. Color-coded bar plot shows the relative abundance of bacterial phyla across the different samples. AFB1, aflatoxin B1; FW, fermented whey; OTA, ochratoxin A; P, pumpkin.

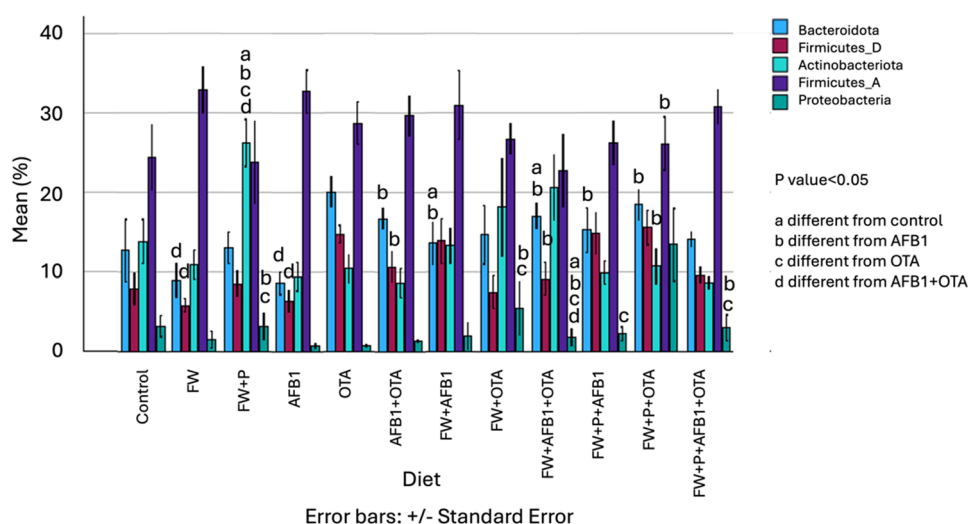


Figure 6. Microbiota relative abundance (%) at phylum level by diet group. AFB1, aflatoxin B1; FW, fermented whey; OTA, ochratoxin A; P, pumpkin.

inclusion of functional ingredients (FW and P). Previous studies have tested other mycotoxins for their effects on gut microbiota using rat models, such as deoxynivalenol (Miró-Abella et al. 2018) and zearalenone (Zhang et al. 2020).

The dose of the mycotoxins in the diets was selected based on the results on previous *in vivo* experiments (Cimbalo et al. 2020). The animals exposed to these twelve diets did not show any clinical sign of toxicity (weekly observations) during the study (Vila-Donat et al. 2024). Both sexes were included in the interpretation of the results, as it is important to consider sex in studies examining the health impacts of functional foods or ingredients intended to improve gut health (Shastri et al. 2015).

It was observed that groups containing a mix of the functional ingredients and mycotoxins (FW+AFB1 and FW+AFB1+OTA) showed more PD faith value than groups only containing the functional ingredient (FW). Moreover,

FW+AFB1+OTA also reported a higher PD faith value than FW+OTA. However, as expected, FW+P+OTA revealed a higher PD faith value than both groups OTA and FW+OTA. On the one hand, it is shown that the combination of mycotoxins-functional ingredients contributes more to microbial diversity rather than the functional ingredients alone. On the other hand, the oral exposure to mycotoxins-functional ingredients combination reported a microbial diversity enhancement in comparison to the mycotoxins alone.

This synergism may be explained by the fact that, although some beneficial bacteria—such as *Lactobacillus* strains—have shown to reduce AFB1 and OTA (Ragoubi et al. 2021), these mycotoxins also possess the ability to decrease harmful bacteria such as *Enterococcus faecalis*, *Escherichia coli* and *Staphylococcus aureus* (Heller et al. 1975; Tiwari et al. 1986). Microbial diversity is often associated with healthy status, while pathological conditions tend to show a decrease in

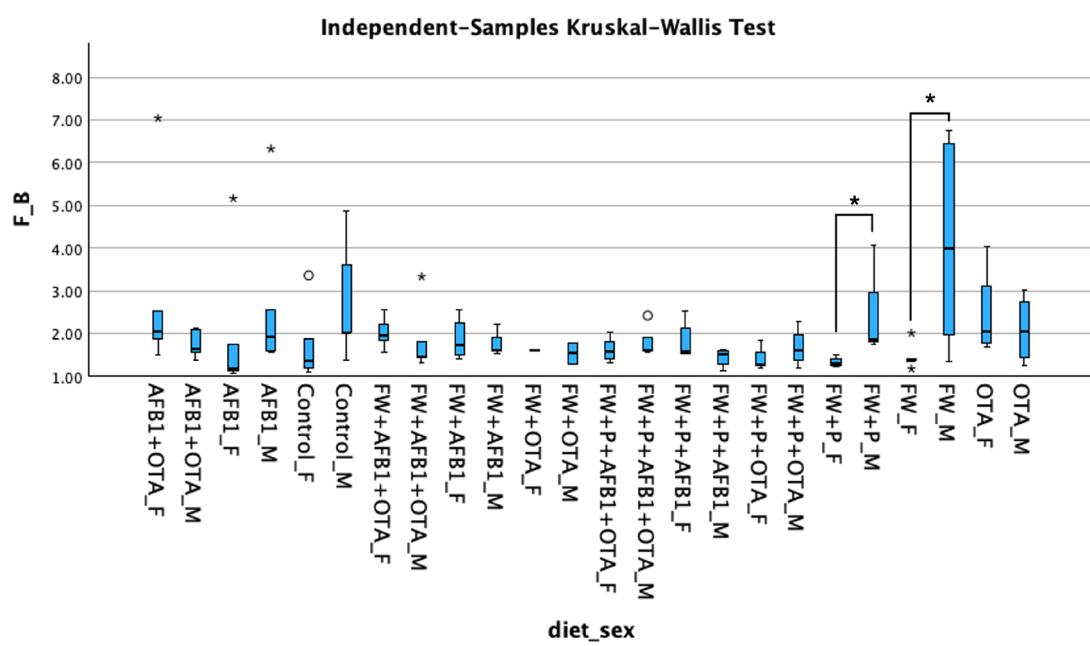


Figure 7. Boxplot representing the *Firmicutes/Bacteroidota* ratio by diet and sex group. Kruskal-Wallis statistical test applied. * p -value < 0.05. AFB1, aflatoxin B1; F, female; FW, fermented whey; M, male; OTA, ochratoxin A; P, pumpkin.

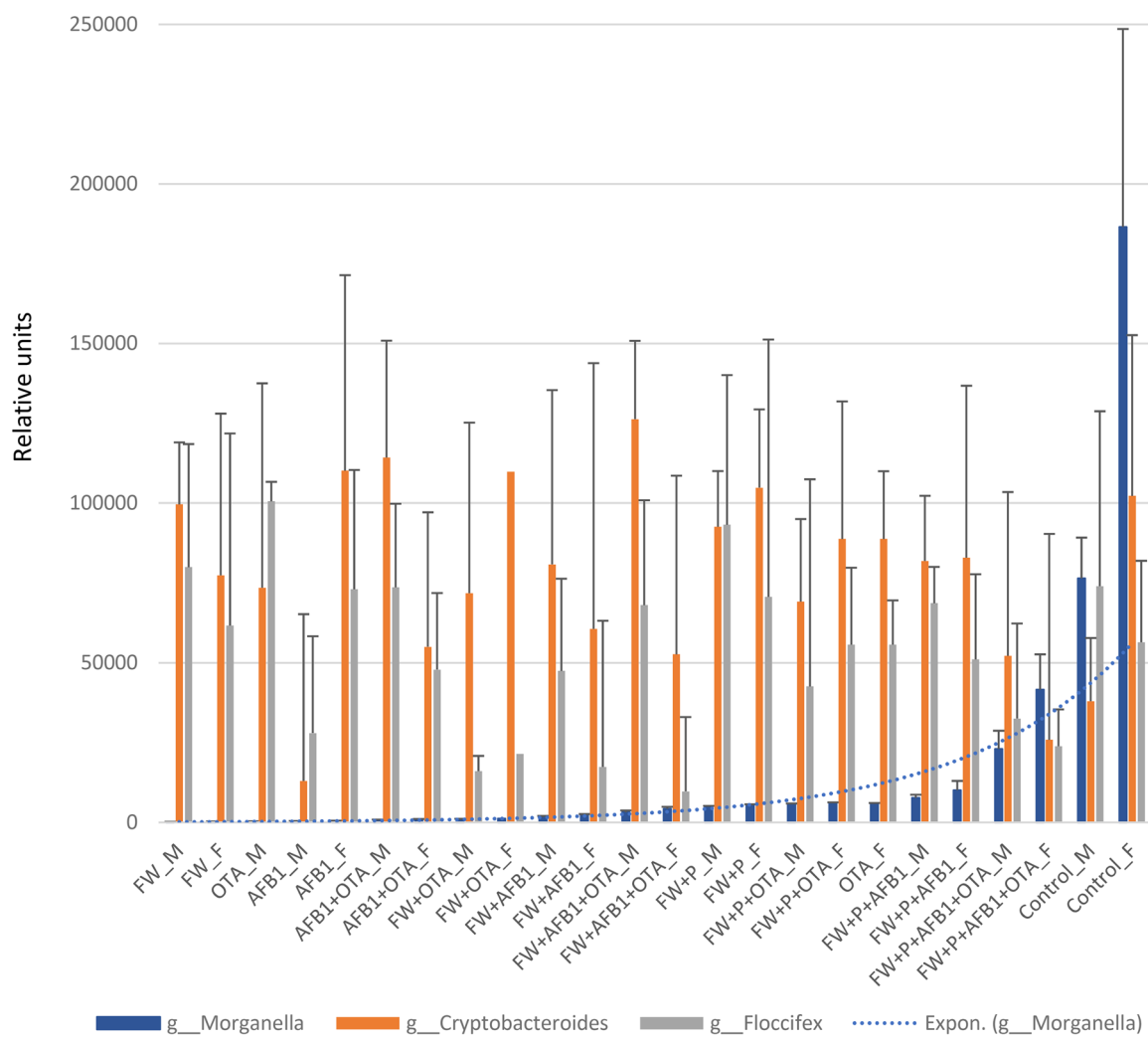


Figure 8. Barplot representing the three most predominant genus in feces microbiota by diet and sex group. Error bars represent standard error. F, female; FW, fermented whey; M, male; P, pumpkin; AFB1, aflatoxin B1; OTA, ochratoxin A.

diversity (Hou et al. 2022). In fact, it has been found that certain combinations of species display much greater colonization resistance to pathogens together than alone. One species alone is not sufficient to strongly impact pathogen growth, but rather a combination of species is required to block nutrient access (Spragge et al. 2023). Despite PD faith is one of the most used α -diversity parameters, it should be noticed that it only focuses on microbial genetic similarity, not taking into consideration the biologically functional similarity and/or diversity.

4.1. Taxonomic differential abundances

Firmicutes, *Actinobacteria*, and *Bacteroidota* were the major phyla of gut microbiota in feces observed in this study, followed by *Proteobacteria*. The highest proportion of *Actinobacteria* was found in FW+P fecal samples. Regarding other taxonomic differential abundances, there was a non-significant reduction in *Proteobacteria* phylum in rat samples fed with contaminated diets (AFB1, OTA, and AFB1+OTA) compared to control, while an increase was found—although not in all groups—when adding the functional ingredients. In humans, an increasing amount of data identifies *Proteobacteria* as a possible and more homogeneous microbial signature of disease (Rizzatti et al. 2017). Indeed, analysis of microbiota composition in children has demonstrated a gradual increase in *Proteobacteria* between healthy, obese, and children with nonalcoholic steatohepatitis (Zhu et al. 2013). Moreover, *Proteobacteria* seems to be implicated in cardiovascular diseases, as there is evidence that high levels of *Proteobacteria* are present in atherosclerosis patients (Shen et al. 2021).

The main difference between the sexes was found in the group FW+AFB1+OTA, with males (25%) showing higher % of *Proteobacteria* than females (1.86%). Indeed, it has been shown that *Proteobacteria* proportion is higher in male birds than female ones (Yan et al. 2022). Another study conducted in fish (*Largemouth bronze gudgeon*) reported the same trend, since the phylum profile in the intestine of males revealed that phylum *Proteobacteria* was dominant, in contrast to females where phylum *Tenericutes* was the most abundant one (Li et al. 2016). However, Zhao et al. (2019) reported that female giant pandas harbored a higher abundance of phylum *Proteobacteria* than male ones with the contribution from genera *Escherichia/Shigella*.

According to these results, it could be thought that, apart from the effects induced by diet, sex plays an important role in augmenting the *Proteobacteria* ratio. Yet, testosterone and dihydrotestosterone—two hormones more abundant in males than in females, have shown to diminish the *Proteobacteria* relative abundance in contrast to control in mice. Therefore, the higher abundance of *Proteobacteria* in males should not be directly related to the sex condition. What is more, there could be more biological pathways to be investigated in which males are more sensitive to the bioactive compounds than females.

It is important to mention the higher presence of *Proteobacteria* species *Morganella morganii* in the feces of animals fed with control and FW+P enriched diets,

contaminated or not with AFB1 and OTA, compared to the diets only enriched with FW or contaminated with mycotoxins and not enriched with functional ingredients at all. In rats, this microorganism is involved in the biotransformation of se-methylselenocysteine (MeSeCys) into dimethyldiselenide and dimethylselenide. The biotransformation into dimethyldiselenide modifies the efficiency of MeSeCys as Se source but also produces a potential compound expected for pharmacological effects, such as anticancer activity. The study concluded that gut microflora plays a role in the biotransformation of MeSeCys, and seems to support efficient metabolism in animals (Takahashi et al. 2023). However, *Morganella morganii* has also been connected to tumor abscess formation in humans (Chen and Lin 2012). In fact, it has been revealed that *Morganella morganii* is enriched in the gut microbiota of inflammatory bowel disease (IBD) and colorectal cancer patients, producing a previously undescribed family of genotoxins, known as indolimines, which are characterized by enhancing intestinal permeability and colon tumorigenesis in gnotobiotic mice (Hernández-Castro et al. 2024). These contradictory results are also found in another species such as *Ruminococcus gnavus*, which has been associated both positively and negatively with an increasing number of intestinal and extraintestinal diseases from inflammatory bowel diseases to neurological disorders (Croft et al. 2023). Extrapolation of results from rats to humans and vice versa in microbiota studies might be performed and interpreted carefully.

Besides, the addition of FW+P to the diet increased the abundance of *Actinobacteria* in the fecal microbiota. It should be stressed that *Actinobacteria* is a phylum accounting for very different genera. Both *Mycobacterium*—connected with tuberculosis, leprosy, pulmonary disease, skin and soft tissue disease, musculoskeletal infections, and disseminated disease (Rakotoarisaona et al. 2024), and *Bifidobacterium*—used as therapeutic agents for gastrointestinal disorders and other pathologies—belong to *Actinobacteria* (Tojo et al. 2014). In fact, it was shown that OTA increased *Corynebacterium*—a genus of opportunistic pathogen bacteria belonging to *Actinobacteria*—in males, but when adding the functional ingredients (FW+P+OTA/FW+OTA) *Corynebacterium* relative abundance was decreased (Belmares et al. 2007; Lo et al. 2015; Yatera and Mukae 2020).

4.2. F/B ratio

Most studies have demonstrated that *Bacteroidota* in fecal microbiota exhibit pro-inflammatory properties due to endotoxins and influence cytokine production, contributing to IBD. Furthermore, *Firmicutes* bacteria exhibit anti-inflammatory effects and can alleviate the progression of IBD (Stojanov et al. 2020). In a gastrointestinal disorder study using male rats, the control group showed the highest F/B ratio, confirming the correlation of the ratio with a healthy profile (Wu et al. 2021). The results obtained using FW and P point to a higher effect of functional ingredients in increasing F/B ratio in rat males rather than females, but the presence of mycotoxins suppressed this potentially beneficial outcome. On the contrary, in humans, literature indicates that the F/B ratio is

Table 2. Summary of the most important effects of the bioactive compounds on gut microbiota in rats.

Parameters	Results
α -diversity (PD faith)	FW+P+OTA and FW+AFB1+OTA > control FW+AFB1 and FW+AFB1+OTA > FW FW+AFB1+OTA > FW+OTA FW+P+OTA > OTA
Taxonomic differential abundance	The main difference between sexes can be found in group FW+AFB1+OTA, with males (25%) showing higher percentage of <i>Proteobacteria</i> than females (1.86%). A reduction in <i>Proteobacteria</i> phylum in rat samples fed with contaminated diets (AFB1, OTA, and AFB1+OTA) was observed. When these contaminated diets included functional ingredients, in all cases <i>Proteobacteria</i> increased the percentage.
Firmicutes/Bacteroidetes ratio	FW and FW+P groups presented statistically significant differences between sexes.
<i>Morganella morganii</i> abundance	<i>Morganella morganii</i> was always more prevalent in females than in males, especially in OTA group.
α -diversity (faith's PD)	FW+P+OTA and FW+AFB1+OTA > control FW+AFB1 and FW+AFB1+OTA > FW FW+AFB1+OTA > FW+OTA
Taxonomic differential abundance	The main difference between sexes can be found in group FW+AFB1+OTA, with males (25%) showing higher percentage of <i>Proteobacteria</i> than females (1.86%).

AFB1, aflatoxin B1; FW, fermented whey; OTA, ochratoxin A; P, pumpkin; PD, phylogenetic diversity.

significantly increased in females compared to males in a project carried out in Ukraine population (Koliada et al. 2021). However, in a Spanish study, there were no sex differences in the microbiota diversity, phylum level, overall composition, and the F/B ratio (Haro et al. 2016). Interestingly, a study analyzing gut microbiota from polycystic ovary syndrome patients showed that testosterone increased *Bacteroidetes* and decreased *Firmicutes* (Matsushita et al. 2022), which is in accordance with the Ukraine population. Anyway, apart from the F/B ratio, it should be paid more attention to the microbiota genus or species, since a phylum is characterized by containing a complexity of bacteria playing contradictory roles in the balance of health and disease.

4.3. Comparing other results obtained with the same animals

Gut microbiota dysbiosis has been associated with liver and kidney disease (Raj et al. 2020). Frangiamone et al. (2023b) studied the effects of the twelve same groups of diet (AFB1, OTA, and/or functional ingredients) on liver and kidney damage biomarkers expression. It was found that in males' liver, the most damaging exposure was AFB1 by reducing CPS1 expression—biomarker for nonalcoholic fatty liver disease (Eriksen et al. 2019), which was totally reversed by FW administration. This physiological improvement by exposing them to the functional ingredient should theoretically be related to a better gut flora. However, in our study not only AFB1 led to a *Proteobacteria* decrease, but also the FW supplementation (FW+OTA) augmented *Proteobacteria* abundance. These contradictory results are striking because it has been hypothesized and confirmed that a rise in the *Proteobacteria* phylum

proportion was the most prominent alteration in gut-liver axis-induced hepatic steatosis (Vasques-Monteiro et al. 2021). On the other hand, OTA diet was the most damaging for the kidney by upregulating kidney injury molecule-1 (KIM-1). Also, as in the liver case, FW administration mitigated mycotoxins induced KIM-1 upregulation (Frangiamone et al. 2023b). However, in our study, when comparing OTA vs FW+OTA exposures, FW addition surprisingly revealed to increase *Proteobacteria*, a phylum related to chronic kidney disease (Voroneanu et al. 2023). It can be concluded that the beneficial effects of functional ingredients on F/B ratio and at other antioxidant levels influence more the positive outcome of their intake than the changes observed in *Proteobacteria* proportions.

Finally, Vila-Donat et al. (2024) quantified AFB1 and OTA in rat urine and showed the impact of dietary bioactive ingredients on mycotoxin excretion. The results revealed that the urinary AFB1 levels of the group that ingested FW+AFB1+OTA were significantly higher than those of the group without FW (AFB1+OTA). P has a notable content in vitamin E, which has been related to AFB1 excretion through its metabolism (Abdel-Hamid and Firgany 2015). Also, both FW and P include a lot of phenolic compounds (p-hydroxybenzoic acid, catechin, chlorogenic acid). These compounds have been considered as microbial toxin detoxification agents (Saricaoglu et al. 2023). Additionally, FW contains probiotic strains, such as *Bifidobacterium* and *Lactobacillus*, which have been shown to degrade both AFB1 and OTA (Vila-Donat et al. 2025). Moreover, LAB through their cell wall interactions, can absorb AFB1 by binding it to their cell wall components, including polysaccharides and peptidoglycans, reducing the bioavailability and systemic absorption of the toxin (Haskard et al. 2001). These mechanisms underscore the potential of bioactive ingredients in supporting mycotoxin detoxification, especially in diets rich in plant-based foods as seen in Mediterranean patterns. The most important effects of the bioactive compounds on gut microbiota in rats are indicated in Table 2.

5. Conclusions

In this work, alpha diversity changes regarding Faith's PD were more related to diet, functional ingredients, and mycotoxins exposure, than to sex. On the contrary, microbial evenness was not altered by any of these parameters. Beta diversity plots showed a cluster of contaminated diets and another cluster grouping the contaminated diets enriched with functional ingredients. Control diets enriched with functional ingredients changed fecal microbial communities in different aspects and were displayed in the plots separately from the control and the two previously mentioned clusters. In general, it can be observed a reduction in *Proteobacteria* phylum in samples of rat fed with contaminated diets and *Morganella morganii* was the most occurrent species in control rats, followed by all groups including functional ingredients and mycotoxins. Finally, diet did not modify the F/B ratio, while significant differences were found between sexes in FW and FW+P diets. It can be concluded that mycotoxin exposure through diet triggers changes in the microbiota of

rats, but also does the inclusion of functional ingredients. Moreover, the sex of the animals leads to different outcomes.

Ethical statement

The animal study protocol was approved by the Ethics Committee of University of Valencia (protocol code 2021/VSC/PEA/0112—17 June 2021).

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This research was funded by Spanish Ministry of Science, Innovation and Universities for the project PID2022-140722OB-I00, MCIU/AEI/10.13039/501100011033/FEDER, EU and for the postdoctoral grant of Alessandra Cimbalo 'Margarita Salas' (MS21-165).

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its Supplementary Materials.

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