AMANDA LOPES HASUDA

AVALIAÇÃO DA TOXICIDADE DE DESOXINIVALENOL E MICOTOXINAS EMERGENTES EM SUÍNOS

Londrina
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Tese apresentada ao Programa de Pós-graduação em Ciência Animal (Área de concentração em Sanidade Animal) da Universidade Estadual de Londrina - UEL, como requisito parcial para a obtenção do título de Doutor.

Orientadora: Profª. Drª. Ana Paula Frederico Rodrigues Loureiro Bracarense

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“Existem muitas hipóteses em ciência que estão erradas. Isso é perfeitamente aceitável, elas são a abertura para achar as que estão certas.” (Carl Sagan)

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RESUMO

O desoxinivalenol (DON) é uma micotoxina que causa danos intestinais por meio do estresse ribotóxico e oxidativo. Algumas micotoxinas detectadas recentemente são chamadas de micotoxinas emergentes, entre elas a beauvericina (BEA) e as eniatinas (ENNs). No entanto, dada a falta de estudos *in vivo,* o risco de BEA e ENNs para a saúde animal e humana não pode ser confirmado. Portanto, o objetivo desse trabalho foi verificar os efeitos tóxicos de DON, BEA e ENNs em suínos. Para isso, foram realizados três experimentos. O primeiro avaliou o efeito da ingestão de DON (2,5 mg/kg) sozinho (grupo DON), uma mistura de BEA (2,5 mg/k) e ENN B + ENN B1 (1,35 mg/kg) (grupo EB) e uma associação entre todas as micotoxinas (2, 3,5 e 1,8 mg/kg, respectivamente) (grupo EB + DON) em 32 leitões por 14 dias. O sangue dos animais foi colhido 7 e 14 dias após o início dos tratamentos para obtenção do plasma para avaliações hepáticas (atividade de enzimas e metabolismo hepático) e intestinais (i-FABP, ZON e IGF-1). Os animais foram eutanasiados para obter amostras de jejuno, cólon, fígado e linfonodos. Todos os fragmentos de órgãos foram processados para análise histológica e a expressão de genes no jejuno e fígado. As fezes foram colhidas ao final do experimento para análise do microbioma. A ingestão de EB + DON diminuiu o ganho de peso dos animais. As atividades das enzimas hepáticas diminuíram após 14 dias em leitões recebendo dieta EB + DON em comparação com leitões recebendo dieta controle. Todas as dietas contaminadas com as micotoxinas levaram a alterações histológicas moderadas a graves no jejuno, fígado e linfonodos. A análise metagenômica revelou que a ingestão de EB diminuiu a diversidade da microbiota intestinal. O segundo e o terceiro experimentos utilizaram uma associação do modelo *in vitro* (células HepG2) e o modelo *ex vivo*, com uma técnica de corte de precisão (PCLS) do fígado. Foram avaliados a viabilidade celular, os biomarcadores hepáticos, a morfologia hepática e a expressão de diversos genes. Para avaliar a citotoxicidade, as células foram incubadas em concentrações crescentes (0-100 µM). Para as demais análises, os dois modelos foram expostos ao DON (10 µM) ou às micotoxinas emergentes (BEA, ENN A1/B/B1, emodina - EMO, apicidina - API e aurofusarina - AFN) (10 µM). No segundo experimento, o DON diminuiu a viabilidade celular, induziu alterações teciduais, apoptose e modificou a expressão de genes inflamatórios e de fatores de transcrição de forma semelhante em ambos os modelos. Os biomarcadores hepáticos não mostraram alteração, e a expressão dos genes apoptóticos e do estresse oxidativo alterou-se apenas *in vitro*. Os resultados demonstram que o fígado é um alvo relevante para observar a resposta inflamatória devido à superexpressão de fatores de transcrição em ambos os modelos. Os parâmetros avaliados *in vitro* e *ex vivo* mostraram perfis semelhantes, apesar de algumas diferenças na intensidade da expressão gênica. No terceiro experimento, a maioria das micotoxinas foram citotóxicas e alteraram os níveis de genes relacionados à inflamação, fator de transcrição e metabolismo hepático *in vitro*. Nos explantes, apenas ENN B1 foi capaz de causar alteração morfológica significativa, mas sem mudanças nos níveis plasmáticos dos bioquímicos hepáticos. A ENN B1 também alterou poucos genes relacionados à inflamação, estresse oxidativo e proteínas de junção. No geral, nossos resultados fornecem novos *insights* sobre o impacto das micotoxinas emergentes sozinhas ou com DON no desempenho, saúde intestinal e parâmetros imunológicos

em suínos. Também fornecem dados adicionais sobre os efeitos tóxicos do DON e das micotoxinas emergentes no fígado e mostram que explantes de fígado são uma ferramenta relevante para avaliar a toxicologia de contaminantes alimentares.

Palavras-chave: Fígado. Jejuno. Micotoxinas não regulamentadas. Tricotecenos. *Ex vivo*. RT-qPCR.

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ABSTRACT

The deoxynivalenol (DON) is a mycotoxin that cause intestinal damage through ribotoxic and oxidative stress. Some recently detected mycotoxins are called emerging mycotoxins, among them beauvericin (BEA) and enniatins (ENNs). However, given the lack of in vivo studies, the risk of BEA and ENNs to animal and human health cannot be confirmed. Therefore, this work aimed to verify the toxic effects of DON, BEA, and ENNs in swine. For this, three experiments were carried out. The first evaluated the effect of the ingestion of DON (2.5 mg/kg) alone (DON group), a mixture of BEA (2.5 mg/k) and ENN B + ENN B1 (1.35 mg/kg) (EB group) and an association between all mycotoxins (2, 3.5 and 1.8 mg/kg, respectively) (EB + DON group) in 32 piglets for 14 days. The animals' blood was collected 7 and 14 days after the start of the treatments to obtain plasma for liver (enzyme activity and liver metabolism) and intestinal (i-FABP, ZON and IGF-1) evaluations. Animals were euthanized to obtain jejunum, colon, liver, and lymph node samples. All the organ fragments were processed for histological analysis and gene expression in the jejunum and liver. Feces were collected at the end of the experiment for microbiome analysis. The ingestion of EB + DON decreased the animals' weight gain. Liver enzyme activities decreased after 14 days in piglets receiving the EB + DON diet compared to piglets receiving the control diet. All diets contaminated with mycotoxins led to moderate to severe histological changes in the jejunum, liver, and lymph nodes. The metagenomic analysis revealed that EB ingestion decreased the intestinal microbiota diversity. The second and third experiments used an association of the *in vitro* model (HepG2 cells) and the *ex vivo* model, with a precision-cut technique (PCLS) of the liver. Cell viability, liver biomarkers, liver morphology, and the expression of several genes were evaluated. To assess cytotoxicity, cells were incubated at increasing concentrations (0-100 µM). For the other analyses, both models were exposed to DON (10 µM) or to emerging mycotoxins (BEA, ENN A1/B/B1, emodin - EMO, apicidin – API, and aurofusarin - AFN) (10 µM). In the second experiment, DON decreased cell viability, induced tissue alterations, apoptosis and similarly modified the expression of genes related to inflammation and transcription factor in both models. Liver biomarkers did not show changes, and the expression of apoptotic genes and oxidative stress changed only *in vitro*. The results demonstrate that the liver is a relevant target to observe the inflammatory response due to the overexpression of transcription factors in both models. The parameters evaluated *in vitro* and *ex vivo* showed similar profiles, despite some differences in the intensity of gene expression. In the third experiment, most of the mycotoxins were cytotoxic and changed the levels of genes related to inflammation, transcription factor and hepatic metabolism *in vitro*. In explants, only ENN B1 was able to cause significant morphological change, but without changes in plasmatic levels of liver biochemicals. ENN B1 also altered few genes related to inflammation, oxidative stress and junction proteins. Overall, our results provide new insights into the impact of emerging mycotoxins alone or with DON on animal performance, gut health, and immune parameters in pigs. They also provide additional data on the effects of DON or the emerging mycotoxins in the liver and show that liver explants are a relevant tool to assess the toxicology of food contaminants.

Keywords: Liver. Jejunum. Non-regulated mycotoxins. Trichothecenes. *Ex vivo*. RT-qPCR.

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LISTA DE ABREVIATURAS E SIGLAS

3ADON	3–acetil–DON
15ADON	15–acetil–DON
ABTS	ácido 2,2'-azino-bis-3-etilbenzotiazolina-6-sulfônico
AOH	alternariol
ASC	ascladiol
BEA	beauvericina
Ca^{2+}	íon cálcio
Caco-2	linhagem celular contínua de células de heterogêneas de adenocarcinoma colorretal epitelial humano
CAT	catalase
CCF-STTG1	linhagem celular de astrocitoma humano estabelecido a partir de um astrocitoma grau IV
CCL20	ligante quimiocina 20
CCRF-CEM	linhagem celular de leucemia linfoblástica aguda humana
CHO-K1	linhagem celular de ovário de hamster chinês
COX2	ciclo-oxigenase 2
CT-26	linhagem celular de adenocarcinoma de cólon de camundongo
CTAO	capacidade total antioxidante
D3G	3–O–glicosídeo–DON
D3GA	DON–3–glicuronídeo
D15GA	DON–15–glicuronídeo
DE_{50}	dose efetiva
DOM-1	de–epóxi–DON
DON	desoxinivalenol
EFSA	Autoridade Europeia para a Segurança dos Alimentos
ENNs	eniatinas
ENN A	eniatina A
ENN A1	eniatina A1
ENN B	eniatina B
ENN B1	eniatina B1
ERN	espécies reativas de nitrogênio
ERO	espécies reativas de oxigênio
FB	fumonisina
FB1	fumonisina B1

FRAP	capacidade plasmática de redução do ferro
FUS	fusaproliferina
FX	fusarenona X
GCLM	subunidade reguladora da glutamato-cisteína ligase
GPx	glutationa peroxidase
GR	glutationa redutase
GSH	glutationa
H295R	linhagem celular de adenocarcinoma humano
H4IIE	linhagem celular de hepatoma de rato
HBMEC	linhagem celular de células endoteliais microvasculares de cérebro humano
HDP	peptídeos de defesa do hospedeiro
HepG2	linhagem celular de câncer de fígado humano
HIF-1α	fator 1-alfa induzível por hipóxia
HL-60	linhagem celular de humano com leucemia promielocítica aguda
HMOX	heme-oxigenase 1
IC_{50}	concentração inibitória
IDT	ingestão diária tolerável
IFN-γ	interferon gama
Ig	imunoglobulina
IgA	imunoglobulina A
IL	interleucina
IL1α	interleucina 1 alfa
IL1β	interleucina 1 beta
IL6	interleucina 6
IL8	Interleucina 8
IL10	interleucina 10
IPEC-J2	linhagem celular de epitélio intestinal de suíno
KB-3-1	linhagem celular de carcinoma primário do colo do útero de uma mulher negra de 31 anos
Keap1	proteína 1 associada à ECH do tipo Kelch
MAPKs	proteínas quinases ativadas por mitógeno
MDA	malondialdeído
MON	moniliformina
N_2	nitrogênio
NAC	N-acetilcisteína
NF-$_K$B	fator de transcrição nuclear kappa B

NIV	nivalenol
NQO1	NAD(P)H desidrogenase quinona 1
Nrf2	fator nuclear 2 relacionado ao eritroide 2
NSCLC A549	linhagem celular de carcinoma pulmonar humano de células não pequenas
O_2	oxigênio
ORAC	capacidade de absorbância de radicais de oxigênio
OTA	ocratoxina A
PAT	patulina
PBCEC	linhagem celular de células endoteliais capilares de cérebro de suíno
PC	peso corporal
PK15	linhagem celular de rim de suíno
PLHC-1	linhagem celular de carcinoma hepatocelular de peixe *Poeciliopsis lucida*
RAW 265.7	linhagem celular de células murinas de macrófagos
RTH-149	linhagem celular de hepatoma de truta arco-íris
SCF	Comitê Científico para Alimentos da Comissão Europeia
SOD	superóxido dismutase
STE	esterigmatocistina
TEAC	capacidade antioxidante equivalente ao trolox
TEER	resistência elétrica transepitelial
TNFα	fator de necrose tumoral alfa
TRAP	potencial antioxidante reativo total
TFF3	fator trifólio 3
U-937	linhagem celular de leucemia mieloide pró-monocítica humana
V79	linhagem celular do pulmão de um hamster chinês normal
ZEA	zearalenona

SUMÁRIO

1 INTRODUÇÃO

Vários fungos podem produzir micotoxinas, principalmente os gêneros *Aspergillus, Penicilium, Alternaria, Fusarium* e *Claviceps*. Em torno de 300 a 400 metabólitos dos fungos se enquadram como micotoxinas. Eles contaminam vários tipos de grãos, como trigo, cevada, centeio, aveia, milho e arroz. Por isso, em animais alimentados à base de grãos, esses metabólitos podem afetar o organismo, levando à manifestação clínica de lesões nos diversos tecidos (BRYDEN, 2012).

O desoxinivalenol (DON) é uma micotoxina do grupo dos tricotecenos, produzida por fungos do gênero *Fusarium*, principalmente *F. graminearum*. O DON permanece estável em temperaturas muito altas (170 a 350°C) e está presente em grande parte das amostras de alimentos à base de grãos. Em torno de 90% das amostras de grãos estão contaminadas com DON e ele também é um potencial marcador da ocorrência de outras micotoxinas (GROVE, 1993; SOBROVA *et al.*, 2010). A toxicidade do DON está associada à sua capacidade de inibir a síntese proteica ao se ligar na subunidade 60s do ribossomo. Além do estresse ribotóxico, o DON também é capaz de causar estresse oxidativo e disfunção mitocondrial nas células intestinais, levando a alterações morfológicas e de função em diversos órgãos (PESTKA; SMOLINSKI, 2005; WANG *et al.*, 2021).

A beauvericina (BEA) é encontrada em cereais como trigo, aveia, cevada e centeio, em uma prevalência de contaminação que pode variar de 12 a 100%, mas geralmente a concentração não ultrapassa 100 µg/kg. Porém, em outros cereais como arroz, o nível de contaminação pode chegar a 26.300 µg/kg (FRAEYMAN *et al.*, 2017; MALLEBRERA *et al.*, 2018). As eniatinas (ENNs) também contaminam cereais como trigo, aveia, cevada e centeio, mas com alta frequência de ocorrência (96 a 100%). Os níveis de contaminação geralmente variam entre 41–569 µg/kg, mas ocasionalmente podem ultrapassar 1000 µg/kg (FRAEYMAN *et al.*, 2017; PROSPERINI *et al.*, 2017). Ambos os metabólitos são considerados micotoxinas emergentes, que por definição são micotoxinas que não são rotineiramente avaliadas, nem possuem uma regulamentação, mas tem alta frequência de ocorrência (JESTOI, 2008).

O intestino é considerado um órgão-alvo para a ação das micotoxinas,

pois, como é a primeira barreira de defesa contra agentes patogênicos e substâncias tóxicas, ele é exposto à altas concentrações das micotoxinas presentes nos alimentos (BOUHET; OSWALD, 2005; PINTON; OSWALD, 2014). Além do intestino, o fígado também pode ser considerado um órgão de interesse para a ação das micotoxinas, pois é o principal órgão de detoxificação do organismo (TENNANT; CENTER, 2008; TOLOSA *et al.*, 2021).

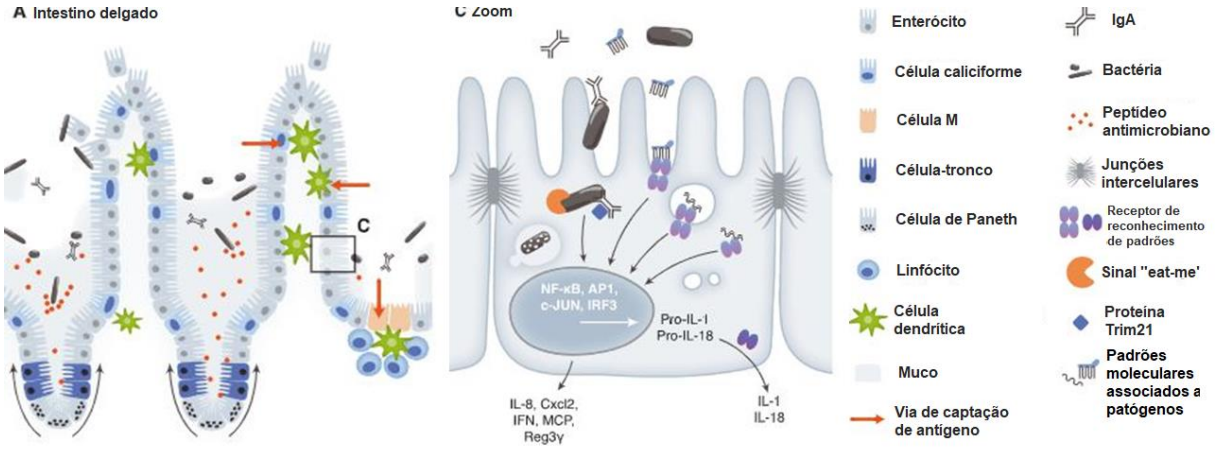
A Autoridade Europeia para a Segurança dos Alimentos (EFSA) expressou preocupação com a saúde humana e animal em relação à ingestão crônica dessas micotoxinas emergentes (EFSA, 2014). Como esses cereais estão presentes em concentrações elevadas na alimentação animal e humana, há grande interesse com possíveis consequências à ingestão aguda ou crônica de alimentos contaminados com micotoxinas. Algumas, como o DON, já têm seus efeitos bem conhecidos em órgãos como o intestino, mas para as micotoxinas ditas emergentes é limitado o conhecimento do impacto delas na saúde humana e animal, por isso há necessidade de mais estudos *in vivo* e *ex vivo* para determinar se a ingestão de BEA e ENNs é nociva para a saúde.

2 REFERENCIAL TEÓRICO

2.1. MORFOLOGIA DO INTESTINO DELGADO

O intestino delgado é divido em três partes: duodeno, jejuno e íleo. É responsável pela absorção de nutrientes, absorção e secreção de eletrólitos e água, secreção de imunoglobulinas (Ig) e mucina, além de ser uma barreira de proteção contra possíveis patógenos (Figura 1) (HORNBUCKLE; SIMPSON; TENNANT, 2008; LALLÈS *et al.*, 2004).

Figura 1 – Mecanismos de defesa na barreira intestinal. A – superfície epitelial do intestino delgado. C – Aumento de um enterócito. Receptores de reconhecimento padrão (PRR) detectam a presença de microrganismos na superfície epitelial e no citoplasma, estimulando uma resposta celular transcricional como a produção de citocinas.



Fonte: Adaptado de Zhang, Hornef, Dupont et al. (2015)

É composto por várias estruturas como as vilosidades intestinais, que são projeções da mucosa contendo uma camada de epitélio colunar e lâmina própria (composta de tecido conjuntivo frouxo), medindo cerca de 0,5 a 1,5mm de altura. Nesse epitélio pode-se encontrar dois tipos celulares: os enterócitos e células caliciformes. O primeiro tipo são células colunares altas com uma borda em escova no seu ápice, formando assim as microvilosidades, que aumentam a superfície de absorção. A célula M é um tipo especializado de enterócito presente no epitélio associado aos folículos linfoides que recobrem a região das placas de Peyer, tendo a função de facilitar a apresentação de antígenos no lúmen intestinal para o tecido linfoide adjacente. Já as células caliciformes estão presentes entre os enterócitos e

em menor quantidade no duodeno do que no jejuno e íleo, sua principal função é a produção de mucina (JUNQUEIRA; CARNEIRO, 2008; ZACHARY; MCGAVIN, 2013).

Entre as vilosidades, na região de lâmina própria, existem pequenas glândulas tubulares simples que são chamadas de criptas de Lieberkühn, ou somente criptas, que secretam enzimas e hormônios. As criptas são formadas por enterócitos e células caliciformes (ambas na parte superior da cripta), células de Paneth e células-tronco. As células de Paneth são células exócrinas localizadas na parte basal das criptas e produzem lisozimas e defesinas, duas enzimas de defesa contra patógenos, mas não se sabe com certeza se esse tipo celular está presente nos suínos. Além disso, outros mecanismos de defesa do intestino delgado são a presença de linfócitos intraepiteliais e das junções intercelulares oclusivas (JUNQUEIRA; CARNEIRO, 2008; ZACHARY; MCGAVIN, 2013; ZHANG; HORNEF; DUPONT, 2015).

A mucina secretada pelas células caliciformes forma uma camada de muco que impede que as bactérias se aproximem do epitélio intestinal e essa camada também é enriquecida com peptídeos antimicrobianos e proteínas produzidos nas células de Paneth, conferindo atividade antibacteriana local. As células nas vilosidades apresentam junções intercelulares, principalmente, ocludinas e claudinas, junções aderentes (E-caderinas) e desmossomos. Essa forte adesão intercelular cria uma barreira na mucosa intestinal. No entanto, patógenos e as micotoxinas podem alterar a integridade dessas junções bem como mediadores inflamatórios podem influenciar essa permeabilidade (PINTON *et al.*, 2009; ZHANG; HORNEF; DUPONT, 2015).

Além da barreira física, os enterócitos expressam receptores de reconhecimento de padrões (PRR) que reconhecem padrões moleculares associados a patógenos (PAMP), além de produzir diversas citocinas (IL-1β, IL6, IL8, IFN-γ e TNFα) que vão recrutar e ativar células do sistema imune (STADNYK, 2002). Porém, resultados demonstraram que as micotoxinas, em especial o desoxinivalenol, são capazes de modular essa resposta imune tanto no intestino quanto de maneira sistêmica (ALASSANE-KPEMBI *et al.*, 2017b; BOUHET; OSWALD, 2005; GAUTHIER *et al.*, 2013; OSWALD *et al.*, 2005; PINTON *et al.*, 2008).

2.2. MORFOLOGIA DO FÍGADO

O fígado é revestido por uma fina cápsula de tecido conjuntivo, sua unidade funcional é o lóbulo hepático e em suínos eles são separados entre si por uma camada de tecido conjuntivo (EKATAKSIN; WAKE, 1991). Essa unidade funcional é composta por hepatócitos, células epiteliais cuboides, ligados por junções intercelulares, organizadas em cordões ou trabéculas. Esses cordões estão arranjados em uma distribuição radial ao redor da veia central. O espaço entre esses cordões contém canalículos biliares, capilares chamados de sinusoides hepáticos, que são compostos de uma camada descontínua de células endoteliais fenestradas, e macrófagos ou células de Kupffer (representando aproximadamente 15-35% das células do fígado). Na periferia dos lóbulos hepáticos encontram-se vasos sanguíneos, vasos linfáticos e ductos biliares (EKATAKSIN; WAKE, 1991; GISSEN; ARIAS, 2015; JUNQUEIRA; CARNEIRO, 2008).

Ao contrário de outros órgãos, o fígado recebe sangue de duas fontes distintas, a artéria hepática (30-40%) e a veia portal hepática (60-70%), que drena o sangue do intestino delgado para o fígado. Danos no epitélio intestinal podem resultar na translocação de patógenos do intestino para o sangue portal. A ausência de infecção sistêmica quando essa translocação ocorre é uma prova da função imunológica desempenhada pelo fígado (KUBES; JENNE, 2018). Os canalículos biliares coletam a bile secretada pelos hepatócitos e a drenam para os ductos biliares, que se juntam para formar o ducto hepático, que junto com os ductos pancreático e cístico desembocam no duodeno para secretar a bile no intestino delgado (ZACHARY; MCGAVIN, 2013; STEADMAN; BRAUNFELD; PARK, 2019).

O fígado desempenha várias funções e é essencial para o metabolismo de nutrientes, detoxificação e excreção de substâncias tóxicas, síntese de proteínas e o processo de absorção e digestão de lipídios e vitaminas lipossolúveis. Além disso, o fígado possuí capacidade regenerativa, por conta disso, manifestações de falência hepática, como concentração diminuída especificamente de albumina e ácidos biliares e aumento de amônia, só são observadas quando 70% ou mais do parênquima está comprometido (TENNANT; CENTER, 2008). As enzimas hepáticas são excelentes marcadores de lesão hepatocelular e colestase. As enzimas aspartato aminotransferase (AST), alanina aminotransferase (ALT), sorbitol desidrogenase

(SDH) e lactato desidrogenase (LDH) estão presentes no citoplasma dos hepatócitos e seu extravasamento está associado à ruptura da membrana dos hepatócitos ou quando há alteração de permeabilidade por lesão subletal. Quando ocorre o dano às membranas, elas são imediatamente extravasadas e caem na circulação sanguínea. A fosfatase alcalina (FA) e a γ–glutamiltranspeptidase (GGT) são enzimas produzidas na membrana das células epiteliais biliares e em menor extensão nos hepatócitos associados aos sinusoides e canalículos biliares. A presença de alguma obstrução do fluxo biliar estimula a produção dessas enzimas, que pode demorar algumas horas após o início do estímulo (PAVENTI; PIZZUTO; PASSARELLA, 2017; TENNANT; CENTER, 2008; THRALL *et al.*, 2015).

Além do intestino, o fígado é um dos primeiros órgãos a entrar em contato com potenciais patógenos e contaminantes de alimentos, como as micotoxinas (KUBES; JENNE, 2018). Estudos já demonstraram que o desoxinivalenol é capaz de alterar a morfológica hepática, causar apoptose e modular a expressão de vários genes no fígado de animais (BRACARENSE *et al.*, 2017; MIKAMI *et al.*, 2010; REDDY *et al.*, 2018; SKIEPKO *et al.*, 2020). Também afeta a viabilidade dos hepatócitos, promove a expressão de fatores de transcrição e induz o estresse oxidativo e a apoptose em culturas celulares hepáticas (NIELSEN et al., 2009; SUN, LV-HUI et al., 2015; YANG, JUN et al., 2019; DARWISH et al., 2020).

2.3. MODELO *EX VIVO*

Um modelo que tem sido bastante utilizado no estudo do efeito das micotoxinas é o modelo *ex vivo*, que consiste na cultura de um fragmento de tecido (explante) em meio propício. No intestino, essa técnica foi descrita pela primeira vez em 1969 por Browning e Trier em um modelo de cultivo do intestino humano (COOPER; WILSON; FEIGHERY, 2015; RUSSO *et al.*, 2016). Além disso, o uso de um modelo animal, como o suíno, apresenta bons resultados para extrapolação em humanos (HELKE; SWINDLE, 2013).

Uma vantagem dessa técnica em relação ao modelo *in vitro* é que o cultivo celular não consegue reproduzir a morfologia da mucosa intestinal presente no modelo *in vivo* e nem a interação complexa dos mecanismos inflamatórios. O modelo

de cultivo de explantes tem uma resposta bem específica e é preferível porque preserva a interação entre diferentes tipos e compartimentos celulares e utiliza menos animais no experimento (RUSSO *et al.*, 2016). Ele é utilizado como uma ferramenta intermediária entre animais e humanos para estudar a homeostase do órgão-alvo e seu comportamento em condições fisiológicas e patológicas (MARESCA *et al.*, 2018).

Os explantes de intestino de animais são os modelos mais próximos do intestino humano do que os modelos *in vitro*, pois contém todos os tipos de células epiteliais normalmente presentes na mucosa. Além das células epiteliais intestinais, os explantes contêm células do sistema imunológico, fibroblastos e células nervosas entéricas que também desempenham papéis importantes na fisiologia intestinal (MARESCA *et al.*, 2018). Eles são preparados a partir de segmentos intestinais, que são cortados com o auxílio de *punch* cirúrgico e os fragmentos teciduais mantidos em meio de cultura. A manutenção deles pode ser por diferentes abordagens: cultura na interface ar-líquido, cultura de imersão em poços ou em uma câmara de Ussing, que é um dispositivo que permite acesso específico aos compartimentos apical/luminal ou aos compartimentos basolaterais/serosais do tecido (RANDALL; TURTON; FOSTER, 2011).

Já para estudos de fisiologia, patologia, metabolismo e toxicidade de drogas no fígado, a técnica de cultivo de hepatócitos isolados de ratos é a mais utilizada. No entanto, em 1980, Krumdieck, Santos e Ho descreveram um novo instrumento para preparar fatias finas de fígado cortadas com precisão e de maneira reprodutível. A espessura dessas fatias de fígado é fina o suficiente para permitir o suprimento de oxigênio e nutrientes para as camadas celulares internas. Os hepatócitos dessas fatias retêm sua membrana e polarização intracelular, ao contrário dos hepatócitos isolados. A técnica de fatias de corte de precisão promove o uso mais eficiente do tecido e não depende do uso de enzimas proteolíticas que potencialmente danificam as células, como é o caso do isolamento dos hepatócitos. Porém, há a necessidade de adquirir equipamentos específicos, como um fatiador Krumdieck ou fatiador Brendel-Vitron, que aumentam o seu custo (DE GRAAF *et al.*, 2010).

A técnica *ex vivo* possui algumas limitações. Alguns tecidos, como o intestino, não podem ser incubados por um longo período (> 4-6 horas), pois têm sua

integridade tecidual comprometida (COOPER; WILSON; FEIGHERY, 2015; KOLF-CLAUW *et al.*, 2009). Mas para outros tecidos, como o ovariano, esse período de incubação pode ser mais longo, até 48 horas (GEREZ; DESTO; BRACARENSE, 2017). Também não é viável a utilização desse modelo em estudos de triagem de larga escala (MARESCA *et al.*, 2018). Mas, mesmo com essas limitações, o cultivo de explantes ainda é preferível porque tem menor custo menor do que estudos *in vivo* e apresenta resultados que podem ser extrapolados para o organismo vivo (RUSSO *et al.*, 2016).

Devido a necessidade de se utilizar cada vez menos animais em experimentos, pelos aspectos éticos e de bem-estar, essa técnica está cada vez mais em evidência, mostrando-se eficaz nos estudos sobre os efeitos das micotoxinas no organismo (KOLF-CLAUW *et al.*, 2009). Diversos estudos com explantes intestinais, avaliando os efeitos das micotoxinas, já foram relatados (ALASSANE-KPEMBI *et al.*, 2017a; GEREZ *et al.*, 2021; LUCIOLI *et al.*, 2013; MAIDANA *et al.*, 2016; PIERRON *et al.*, 2016; SILVA *et al.*, 2014, 2019). No entanto, estudos sobre os efeitos das micotoxinas nos explantes hepáticos são escassos (AJANDOUZ *et al.*, 2016).

O modelo *ex vivo*, apesar de ter se mostrado altamente relevante para estudos de fisiologia, toxicidade e metabolismo de drogas e xenobióticos em animais e humanos, foi subutilizado nas últimas décadas e ganhou destaque apenas recentemente. Seu uso é interessante também quando em conjunto com outros modelos *in vitro* para a avaliação de risco de contaminantes tóxicos em alimentos humanos (MARESCA *et al.*, 2018).

2.4. MICOTOXINAS

Micotoxinas são metabólitos secundários produzidos por fungos encontrados em alimentos consumidos por animais e humanos, que podem causar doenças ao alterar a imunidade dos indivíduos (OSWALD *ET AL.*, 2005). As principais vias de contaminação são por ingestão, inalação e contato na pele. A metabolização das micotoxinas no organismo pode levar ao seu acúmulo em diferentes tecidos e, consequentemente, podem entrar na cadeia alimentar por meio de produtos de origem

animal, como carne, leite ou ovos contaminados (MARIN *ET AL.*, 2013).

Os principais gêneros de fungos que produzem micotoxinas são: *Alternaria, Claviceps, Aspergillus, Fusarium* e *Penicilium*. Sendo os três últimos de maior importância, pois a maioria das micotoxinas pode ser produzida por esses três gêneros (BRYDEN, 2012). As principais micotoxinas são: aflatoxinas, ocratoxina A (OTA), tricotecenos (tipo A: toxina T-2 e tipo B: desoxinivalenol), zearalenona (ZEA), fumonisinas (B1 e B2), alcaloides do ergot, alternariol (AOH) e as micotoxinas emergentes (MARIN *et al.*, 2013).

Algumas micotoxinas emergentes como beauvericina (BEA), eniatinas (ENNs), fusaproliferina (FUS) e moniliformina (MON) também são produzidas por fungos do gênero *Fusarium* (JESTOI, 2008). Elas são consideradas emergentes, pois são micotoxinas com alta frequência de ocorrência e com o avanço de técnicas de detecção é possível identificar metabólitos que antes não eram identificados e não são regulamentadas (FRAEYMAN *et al.*, 2017). Como ainda foram pouco estudadas e os estudos de toxicidade *in vivo* sobre essas micotoxinas são escassos, não há nenhum tipo de regulamentação quanto aos limites máximos toleráveis que essas micotoxinas podem apresentar nos alimentos.

Estimativas demonstraram que 60–80% dos grãos de cereais no mundo estão contaminados com algum tipo de micotoxina (ESKOLA *et al.*, 2020). Em 2021, a Pesquisa Mundial de Micotoxinas da BIOMIN, empresa de saúde e nutrição animal, demonstrou que das 16.164 amostras de alimentos para animais colhidos em 74 países, 64% das amostras estavam contaminadas com mais de uma micotoxina. Na América do Sul e Europa, as duas micotoxinas mais frequentes são as fumonisinas (FB) e o desoxinivalenol (DON). Em 618 amostras originárias de 68 países, 10 em cada 10 amostras de rações acabadas e matérias-primas estavam contaminadas com toxinas do gênero *Fusarium* e dessas amostras, 96% delas estavam contaminadas com 10 ou mais metabólitos (BIOMIN, 2021).

Sabe-se que algumas micotoxinas são capazes de alterar a barreira intestinal (BOUHET; OSWALD, 2005). Os tricotecenos, entre eles o DON, são as principais micotoxinas a causar danos na integridade da barreira intestinal, com

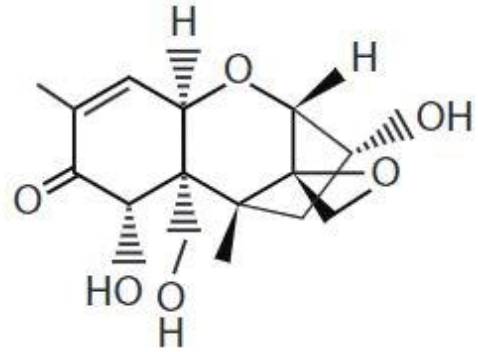
comprometimento das células de revestimento epitelial e das proteínas de junção intercelular, enquanto a patulina (PAT) afeta diretamente a monocamada das células epiteliais no intestino (AKBARI *et al.*, 2017). Isso pode levar ao aumento da permeabilidade, por conta da diminuição da resistência elétrica transepitelial (TEER), que pode ter como consequência a translocação de patógenos ou outras substâncias (AKBARI *et al.*, 2017; BOUHET; OSWALD, 2005; LUO *et al.*, 2019; PINTON; OSWALD, 2014).

Porém, os efeitos tóxicos das micotoxinas emergentes ainda não foram bem estudados. A maioria dos estudos na literatura envolvem micotoxinas já regulamentadas, como o DON e outros tricotecenos, fumonisinas, ocratoxina A, zearalenona e patulina, principalmente em intestino e órgãos linfoides (ALASSANE-KPEMBI et al., 2017a, 2017b; BRACARENSE et al., 2017; GEREZ et al., 2015; LEWCZUK et al., 2016a; LUO et al., 2019; MAIDANA et al., 2016; PIERRON et al., 2018; PINTON; OSWALD, 2014; SILVA et al., 2014, 2019).

2.4.1. Desoxinivalenol

O desoxinivalenol (DON) é o principal representante da família dos tricotecenos tipo B. O DON foi isolado pela primeira vez em cevada contaminada por fungos do gênero *Fusarium* no Japão (MOROOKA *et al.*, 1972). O DON é um composto orgânico polar e seu nome químico é 12,13-epoxi3α,7α,15-trihidroxitricotec-9-en-8-one e contém em sua molécula três radicais hidroxila (–OH) livres (Figura 2), que estão associados com sua toxicidade (SOBROVA *et al.*, 2010).

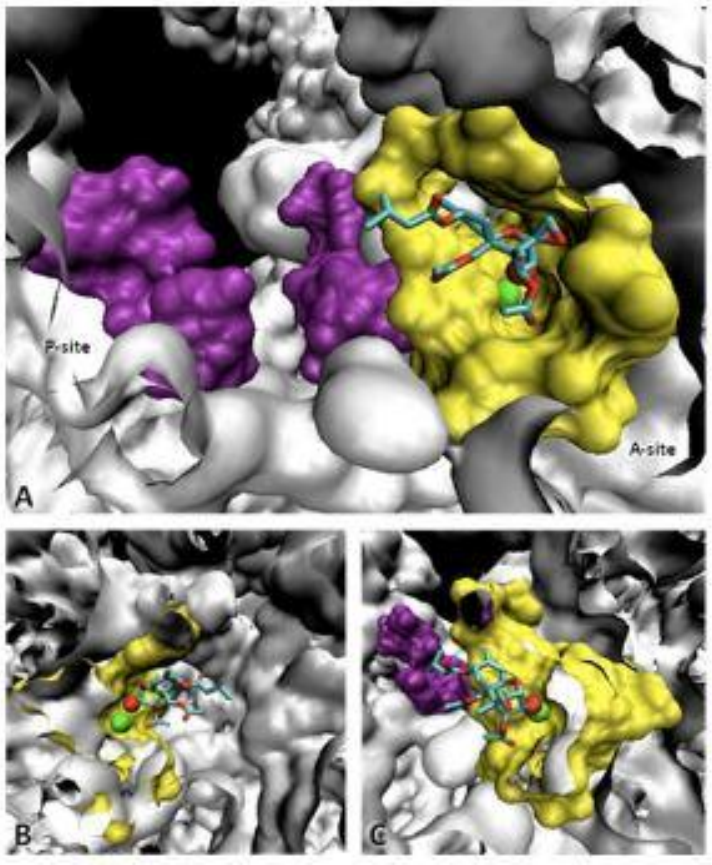
Figura 2 – Estrutura química do desoxinivalenol. Molécula com três radicais hidroxila (–OH) livres.



Fonte: Sobrova et al. (2010)

O DON inibe a síntese proteica ao se ligar no sítio A do centro da proteína peptidil-transferase da subunidade 60S do ribossomo (Figura 3) e interfere na tradução de proteínas (PESTKA, 2010b). O DON se encaixa no bolso do sítio A da subunidade 60S do ribossomo, e seu terceiro radical –OH está associado a um átomo de magnésio e estabilizado por outros nucleotídeos (PIERRON *et al.*, 2016). A ligação de DON no ribossomo vai gerar uma resposta de estresse ribotóxico, resultando na ativação de proteínas quinases ativadas por mitógeno (MAPKs) (PESTKA, 2010a). A via de sinalização das MAPKs regula diversos processos biológicos, entre eles a apoptose e a imunidade (PESTKA; SMOLINSKI, 2005). A ativação das MAPKs pelo DON leva à ativação de fatores de transcrição (NIELSEN *et al.*, 2009; SUN *et al.*, 2014; YUAN *et al.*, 2018) que vão levar à produção e secreção de citocinas, expressão de ciclo-oxigenase 2 (COX2) e apoptose (KINSER *et al.*, 2005; MOON, 2002).

Figura 3 – Interação entre o local de ligação da subunidade 60S do ribossomo e DON. A – vista frontal, B – vista ortogonal direita, C – vista ortogonal esquerda. Sítios P (roxo) e A (amarelo) da subunidade 60S. Radicais hidroxila (vermelho) de DON (azul). Átomo de magnésio dentro do bolso do sítio A (verde).



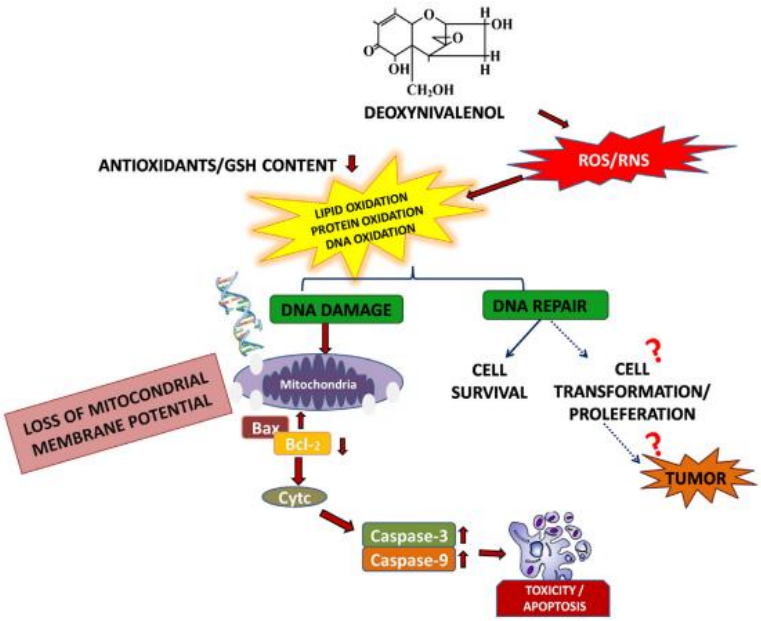
Fonte: Pierron et al. (2016)

A disfunção mitocondrial também desenvolve um papel fundamental

na toxicidade induzida por DON, principalmente no intestino (WANG *et al.*, 2021). Essa disfunção ocorre por causa do estresse oxidativo na organela. A oxidação é um processo que ocorre em todos os organismos aeróbios durante processos fisiológicos. Nesse processo, há formação de uma pequena quantidade de substâncias químicas altamente reativas que são chamadas de radicais livres. Esses radicais livres são moléculas que contém um ou mais elétrons desemparelhados na camada orbital externa de átomos de oxigênio (O_2) ou nitrogênio (N_2) e podem ser denominadas como espécies reativas de oxigênio (ERO ou ROS) e espécies reativas de nitrogênio (ERN ou RNS) (BARREIROS; DAVID, 2006; MCMICHAEL, 2007; PAVELESCU, 2015; RUSSO; BRACARENSE, 2016).

O estresse oxidativo ocorre quando o equilíbrio está prejudicado e a quantidade de ERO ultrapassa a quantidade de antioxidantes, levando a danos celulares com consequentes lesões teciduais, sendo comum em órgãos com alta demanda e metabolismo energéticos. Isso ocorre por conta de mudanças oxidativas em macromoléculas celulares, morte celular por apoptose ou necrose que geram danos estruturais nos tecidos (BARREIROS; DAVID, 2006; BIRBEN *et al.*, 2012; LYKKESFELDT; SVENDSEN, 2007; PUPPEL; KAPUSTA; KUCZYNSKA, 2014). No proposto mecanismo de ação de estresse oxidativo mediado pelo DON (Figura 4) há um aumento na produção de ERO e ERN e uma diminuição nos compostos antioxidantes (SOD, CAT, GSH, HMOX1), levando à oxidação de lipídios, proteínas e do DNA. Em seguida, a via intrínseca da apoptose será ativada, porque o excesso de ERO leva à perda do potencial de membrana mitocondrial e, consequentemente, à liberação do citocromo C, desencadeando a ativação das caspases 9 e 3 que promovem a apoptose (MISHRA *et al.*, 2014; WANG *et al.*, 2021).

Figura 4 – Mecanismo de ação do estresse oxidativo mediado por DON na mitocôndria. Produção de espécies reativas de oxigênio (ROS) que levam à danos oxidativos e perda do potencial de membrana mitocondrial, com liberação de citocromo C (Cytc) no citoplasma e ativação das caspases 9 e 3.



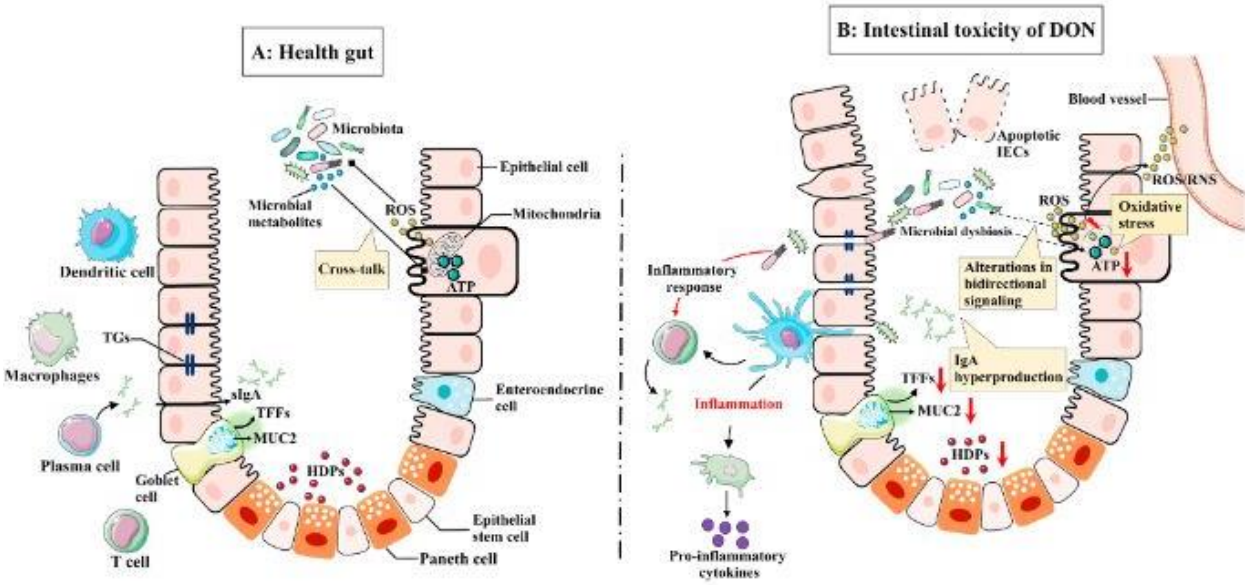
Fonte: Mishra et al. (2014)

Os mecanismos gerais de defesa intestinal para eliminar bactérias da superfície epitelial contam com a liberação de mucinas e fator trifólio 3 (TFF3) pelas células caliciformes, secreção de peptídeos de defesa do hospedeiro (HDP) pelas células de Paneth e de Imunoglobulina A (IgA) pelos plasmócitos (WANG *et al.*, 2021).

Além disso, como a renovação do epitélio intestinal e a manutenção da integridade das junções intercelulares são dependentes de energia, a mitocôndria é cada vez mais reconhecida como essencial na manutenção da saúde intestinal (NOVAK; MOLLEN, 2015). A disfunção mitocondrial caracterizada pela perda das capacidades respiratórias, geração reduzida de ATP e superprodução de ERRO, foi demonstrada em células intestinais expostas ao DON (FAVERO *et al.*, 2018; GUO *et al.*, 2019; KALAISELVI *et al.*, 2013; KANG *et al.*, 2019). O excesso de ERO leva ao início precoce da apoptose e ao fornecimento insuficiente de ATP. A apoptose substancial das células epiteliais intestinais comprometem a integridade das junções intercelulares, e consequentemente da barreira intestinal, levando à translocação de bactérias e antígenos luminais (Figura 5). Assim, há inflamação, com aumento na produção de IgA. Já a secreção de mucinas, TFF3 e HDP é inibida pela exposição ao DON e há uma diminuição de células caliciformes (GEREZ *et al.*, 2015; WANG *et al.*,

2021).

Figura 5 – Mecanismo de ação da toxicidade de DON na mitocôndria de células intestinais. A – Intestino com saúde normal, B – Intestino após a ingestão de alimentos contaminados com DON.



Fonte: Wang et al. (2021)

Na União Europeia, as diretrizes regulatórias estabeleceram limites máximos de DON para a alimentação completa de leitões em 0,9 mg/kg, e em matérias-primas, como cereais e produtos de milho, em 8 e 12 mg/kg, respectivamente (EUROPEAN COMMISSION, 2006). Ainda, na Europa, em 2002 foi estabelecida a ingestão diária tolerável (IDT) provisória para humanos para DON de 1 µg/kg de peso corporal (PC)/dia pelo Comitê Científico para Alimentos (SCF). No Brasil, não há legislação para níveis de contaminação por micotoxinas em rações animais. Já para humanos, a legislação prevê limites máximos toleráveis (LMT) de 200 µg/kg para alimentos infantis e 750 a 2.000 µg/kg, variando de acordo com o tipo de grão e beneficiamento utilizado (BRASIL, 2021). Em 2017, a EFSA estabeleceu o risco para a presença de DON e seus compostos acetilados em alimentos e rações na saúde humana e animal. Para humanos, o risco não é considerado um problema grave de saúde. E para suínos, o risco de efeitos agudos para a saúde é baixo com base nas concentrações dietéticas estimadas (EFSA *et al.*, 2017).

Em 2021, na América do Sul e na Europa as duas micotoxinas mais frequentes foram o desoxinivalenol (46% e 59%, respectivamente) e as fumonisinas (66% e 49%, respectivamente) (BIOMIN, 2021). Estudos demonstraram que DON está

entre as 10 micotoxinas mais frequentes na ração para suínos, com prevalência de 77–88% sob uma concentração mediana de 193 µg/kg, e que DON é um potencial marcador da presença de outras micotoxinas, pois a co-ocorrência de DON com outras micotoxinas é muito comum, especialmente com ZEA (97%), BEA (89%) e ENNs (89–93%) (KHOSHAL *et al.*, 2019; NOVAK *et al.*, 2019; SOBROVA *et al.*, 2010).

Além disso, uma crescente preocupação está surgindo por conta das mudanças climáticas. Observou-se que o perfil das espécies de *Fusarium* que produzem micotoxinas está mudando no continente europeu (MORETTI; PASCALE; LOGRIECO, 2019). Na Espanha, uma meta-análise em amostras de trigo descreveu uma alta exposição ao DON, especialmente em bebês e crianças abaixo de 3 anos de idade, que tiveram uma ingestão de 175% e 119% da IDT, respectivamente. Pão e produtos derivados foram os principais contribuintes para exposição ao DON em todas as faixas etárias, com valores de IDT variando de 12% a 34% (NARVÁEZ *et al.*, 2022).

2.4.1.1 Toxicocinética

Todas as espécies estudadas possuem algum grau de sensibilidade ao DON, e as espécies mais estudadas estão classificados em ordem decrescente de sensibilidade: suínos > camundongos > ratos > aves ≈ ruminantes (PRELUSKY *et al.*, 1994).

A diferença entre elas se deve principalmente ao diferente tipo de metabolização a que o DON é submetido em cada espécie, pois aves e ruminantes possuem bactérias comensais que são capazes de transformar rapidamente o DON em um derivado de-epóxido (DOM-1) não-tóxico antes de chegar ao intestino delgado e ser absorvido (MARESCA, 2013). Outros derivados do DON também podem ser formados por fungos (derivados acetilados: 3ADON e 15ADON) e plantas (D3G), ou por animais (D3GA e D15GA) pelo processo de glicuronidação hepática (MARESCA, 2013). O D3GA também não apresenta toxicidade, já que sua molécula não consegue se ligar no sítio A da subunidade 60S ribossomal (PIERRON *et al.*, 2016).

Não se sabe exatamente como o DON entra nas células, mas é provavelmente através de um mecanismo de difusão passivo (SERGENT *et al.*, 2006). Ainda não existem estudos identificando a entrada dos derivados de DON nas células,

e supõe-se que os derivados que passaram pela metabolização hepática têm menor capacidade de entrar nas células, por terem maior polaridade e massa molecular que o DON (MARESCA, 2013).

Em suínos, o DON é rapidamente absorvido no intestino delgado e atinge o pico de concentração plasmática em 15–30 minutos, sendo que em torno de 82% de todo DON ingerido é absorvido pelos intestinos (PESTKA; SMOLINSKI, 2005). Porém, a concentração máxima no plasma não excede 25% da dose ingerida e o DON possuí uma meia-vida plasmática de 4 horas (ERIKSEN; PETTERSSON; LINDBERG, 2003). Quanto à sua excreção, a maior parte parece ser eliminada na forma de D3GA, D15GA, DON, glicuronídeo-DOM1 e DOM-1. Em suínos, 68% do total de DON ingerido é eliminado na urina como DON ou D3GA/D15GA, o restante é eliminado nas fezes na forma de DOM-1 e DON (80% e 20%, respectivamente) (MARESCA, 2013).

2.4.1.2 Estudos *in vitro*

Estudos *in vitro* têm sido amplamente realizados para estudar os efeitos das micotoxinas em diversos tipos celulares (DARWISH *et al.*, 2020; KATIKA *et al.*, 2012; MAYER *et al.*, 2017; NOVAK *et al.*, 2018; WENTZEL *et al.*, 2017). Um dos principais parâmetros avaliados nesse modelo de estudo é a viabilidade celular. Os resultados encontrados na literatura já demonstraram que o DON diminui a viabilidade celular em vários tipos celulares. O DON apresentou citotoxicidade para células hepáticas de carcinoma humano (HepG2) após 24 horas em concentrações variando de 1-10 µM (DARWISH *et al.*, 2020; FERNÁNDEZ-BLANCO *et al.*, 2018; MAYER *et al.*, 2017). Uma concentração inibitória (IC_{50}) de 41,4 µM foi encontrada em HepG2, mas em hepatócitos primários de humanos e hepatócitos de ratos (BRL 3A) tiveram uma IC_{50} de 6 e 13 µM, respectivamente. Esses resultados mostram que células saudáveis são mais sensíveis ao DON (KÖNIGS *et al.*, 2008; SUN *et al.*, 2015). A partir de doses intermediárias (4-16 µM), o DON também causou citotoxicidade nos hepatócitos de suínos e em co-culturas de hepatócitos + células de Kupffer (DÖLL *et al.*, 2009).

Levando em consideração a sensibilidade do tipo celular, as células intestinais aparentam ser mais sensíveis ao DON do que as células hepáticas. Após 24 horas, a viabilidade celular estava diminuída com doses baixas de DON (0,5 e 0,9

μM) em células intestinais de suínos IPEC-J2 e IPEC-1 (YANG *et al.*, 2019). E após 48 horas houve uma inibição na viabilidade de 50% das células com 1 e 3 μM em células intestinais humanas (Caco-2) e em IPEC-1 (KHOSHAL *et al.*, 2019; PIERRON *et al.*, 2016).

O DON é capaz de alterar a imunidade no órgão em que se encontra (PAYROS *et al.*, 2016). Os leucócitos são importantes alvos da ação imunomoduladora de DON. Em linfócitos do baço de suínos e linfócitos circulantes de humanos houve citotoxicidade de maneira dose-dependente ao DON em concentrações que variaram de 0,02 – 25 μM por 24 horas; nos linfócitos do baço a IC_{50} foi de 5 μM após 48 horas e nos linfócitos circulantes a IC_{50} foi de 0,3 μM após 24 horas (REN *et al.*, 2017; YANG *et al.*, 2014). O DON também inibiu a proliferação de linfócitos circulantes de humanos e suínos, e a IC_{50} foi de 0,4 e 0,5 μM, respectivamente (TARANU *et al.*, 2010). Portanto, linfócitos circulantes parecem ser mais sensíveis ao DON do que linfócitos do baço. Já em neutrófilos de suínos, concentrações de 0,5–10 μM por até 24 horas diminuíram a secreção de interleucina 8 (IL8), a quimiotaxia e a capacidade de fagocitose dessas células. A ação do DON nos neutrófilos foi mediada pela fosforilação da MAPK p38 nos primeiros 30 minutos de exposição (GAUTHIER *et al.*, 2013). O contrário foi encontrado em macrófagos expostos ao DON, em que houve aumento na secreção de interleucina 8 (IL8) e fator de necrose tumoral alfa (TNF-α) (SUGITA-KONISHI; PESTKA, 2001).

No intestino, o DON aumenta a resposta inflamatória. Em células IPEC-1 expostas à 10 μM por 24 horas, houve aumento na expressão de várias citocinas (IL8, IL1α, IL1β e TNF-α) (CANO *et al.*, 2013). E em células Caco-2 expostas por 24 horas à 2,5-100 μM, o aumento na secreção de interleucinas (IL) foi diretamente afetada pelo DON, que também potencializou o efeito de interleucina 1 β (IL1β) na secreção de IL8, diminuiu a resistência elétrica transepitelial (TEER) e permitiu o aumento na translocação de bactérias intestinais (MARESCA *et al.*, 2008). No mesmo tipo celular, o DON (30 μM) também aumentou a atividade do fator nuclear κ B (NF-κB) e a secreção de IL8 e esse efeito foi acentuado com a presença de uma estimulação inflamatória (VAN DE WALLE *et al.*, 2008).

O estresse oxidativo induzido por DON causa danos em proteínas, lipídios e DNA, sendo um dos seus mecanismos de toxicidade (MISHRA *et al.*, 2014).

Em linfócitos de humanos (0,2-1,7 μM de DON por 24h) houve danos ao DNA, aumento na expressão de ERO e na peroxidação lipídica e diminuição na expressão de um gene antioxidante (HMOX1) (YANG *et al.*, 2014). Em células HepG2, a exposição à 2,5-60 μM por até 24 horas aumentou a geração de ERO, os danos ao DNA e os níveis de enzimas antioxidantes (CAT, SOD e GPx), além de diminuir os níveis do antioxidante glutationa (GSH) (BODEA et al., 2009; ZHANG et al., 2009). Efeitos semelhantes foram relatados em HepG2 expostas à 0,1-5 μM por 24 horas, em que o DON também causou uma regulação negativa nas enzimas antioxidantes reguladas pela via Keap1-Nrf2 (DARWISH *et al.*, 2020). A apoptose mediada pelo estresse oxidativo que o DON promove nas células, foi verificada pelo aumento de atividade de caspase-3, também esteve presente em HepG2 e hepatócitos primários de humanos expostos à 0,1-100 μM de DON por até 48 horas (KÖNIGS *et al.*, 2008). Em hepatócitos de ratos BRL 3, o DON, em concentrações de 1-4 μM por 24 horas, diminuiu a expressão do gene anti-apoptótico Bcl-2 e aumentou a expressão dos genes caspase-3 e Bax e a produção de proteínas (p53) envolvida na apoptose (SUN *et al.*, 2015).

Em células intestinais HT-69, após 24 horas de exposição à 0,8-1,7 μM de DON, houve aumento na geração de ERO e ERN, alteração do estado antioxidante e indução de COX2 e NF-κB que levam à apoptose (KALAISELVI *et al.*, 2013; KRISHNASWAMY; DEVARAJ; PADMA, 2010). O DON, em concentrações de 0,9-13,5 μM por 24 horas, também foi capaz de regular negativamente a via Keap1-Nrf2 em células IPEC-J2, além de aumentar o número de células apoptóticas (YANG *et al.*, 2019). E nesse mesmo tipo celular, a exposição de 0,7-20 μM de DON por 24 horas diminuiu os níveis de enzimas antioxidantes (CAT e GPx), aumentou a geração de ERO e a expressão de genes inflamatórios (IL1β, IL6 e TNF-α) e apoptóticos (caspase-3/8/9) (KANG *et al.*, 2019).

2.4.1.3 Estudos *in vivo* e *ex vivo*

A morfologia tecidual é um importante parâmetro para verificar os efeitos tóxicos das substâncias e é uma das vantagens dos modelos *in vivo* e *ex vivo* em relação ao modelo *in vitro*. Em baixas doses, DON (12 μg/kg de PC) e/ou ZEA (40 μg/kg de PC), fornecidos na ração por seis semanas, não afetou a morfologia do duodeno de suínos. A espessura da mucosa e o comprimento das vilosidades

permaneceram inalterados, porém o número de linfócitos presentes no epitélio das vilosidades foi significativamente maior do que no grupo controle (LEWCZUK *et al.*, 2016b). Em leitões recém-nascidos (mães consumiram 9,5 mg/kg DON por cinco semanas) e suínos adultos (4,6 mg/kg por quatro semanas), não houve alteração da morfologia hepática (RENNER *et al.*, 2017; TIEMANN *et al.*, 2008).

Já a ingestão dessas mesmas micotoxinas, em doses realistas de DON (1,5 ou 3 mg/kg), além do nivalenol (NIV) em associação ou sozinhas por quatro semanas, induziu alterações histológicas sistêmicas, afetando o fígado, intestinos (jejuno e íleo) e órgão linfoides de suínos. No fígado as principais alterações foram desorganização dos cordões de hepatócitos, vacuolização citoplasmática e megalocitose. No intestino houve atrofia e fusão de vilosidades e edema de lâmina própria multifocalmente, assim como houve diminuição de células caliciformes em ambas as regiões do intestino. Em linfonodos mesentéricos e em baço as alterações foram leves e consistiram principalmente em depleção folicular e apoptose de linfócitos (GEREZ *et al.*, 2015).

Alterações compatíveis também foram observadas no fígado de leitões alimentados com DON (3 mg/kg) e/ou FB (6 mg/kg) na dieta por cinco semanas apresentou desorganização dos cordões de hepatócitos, megalocitose e vacuolização citoplasmática (GRENIER *et al.*, 2011). Da mesma forma, em ratos, a ingestão de DON (1,75 ou 11,4 mg/kg) por quatro semanas causou desorganização dos cordões hepáticos, vacuolização citoplasmática moderada, presença de infiltrado inflamatório e megalocitose (BRACARENSE *et al.*, 2017). Nos dois estudos a proliferação de hepatócitos estava aumentada nos animais que receberam micotoxinas em baixas doses, reforçando a hipótese de que uma baixa concentração de DON ativa a via das MAPKs para aumentar a sobrevivência celular (BRACARENSE *et al.*, 2017; GRENIER *et al.*, 2011). A associação de DON a um metal traço, o cádmio, na ração de ratos por quatro semanas também provocou atrofia e fusão das vilosidades, edema intersticial, achatamento dos enterócitos apicais e redução de profundidade de cripta, porém não causou alteração no comprimento de vilosidade (LUO *et al.*, 2019).

Em aves, a ingestão por três semanas (7,5 mg/kg) diminuiu a altura de vilosidades e a profundidade de criptas tanto no duodeno, como no jejuno (OSSELAERE *et al.*, 2013). A morfologia intestinal de aves alimentadas com 19,3

mg/kg por uma semana também causou atrofia de vilosidades, edema e vacuolização de enterócitos, edema intersticial, infiltrado inflamatório e aderência de bactérias às vilosidades. Aumentou a profundidade de cripta e o número de células caliciformes e linfócitos intraepiteliais. No fígado, também aumentou o escore lesional, com desorganização trabecular, infiltrado inflamatório, congestão, vacuolização citoplasmática dos hepatócitos, megalocitose e necrose (SOUZA *et al.*, 2020). No entanto, a ingestão por seis semanas de uma dieta contaminada com DON (10 mg/kg) promoveu poucas alterações, com diminuição da altura e espessura de vilosidades em duodeno e jejuno (AWAD *et al.*, 2006).

A exposição de explantes de jejuno de suínos ao DON e/ou fumonisina B1 (FB1) também levou a alterações histológicas como atrofia e fusão das vilosidades de maneira multifocal a difusa, perda do epitélio apical das vilosidades, vacuolização celular, achatamento dos enterócitos, diminuição do comprimento de vilosidade e no número de células caliciformes nas vilosidades e em região de cripta. Mas quando associadas a um antioxidante, o ácido fítico, o escore dessas lesões diminuiu, indicando que o ácido fítico tem um efeito protetor contra essas micotoxinas (SILVA *et al.*, 2014, 2019). Também se observou que os efeitos de fusarenona X (FX) foram mais prejudiciais para a estrutura jejunal do que o DON, embora ambas as micotoxinas causaram lesão nas células intestinais. A principal alteração observada foi a fusão de vilosidades, mas também foram visualizadas alterações como: células epiteliais cuboides, edema de lâmina própria, atrofia das vilosidades e desnudamento apical com perda de enterócitos (ALASSANE-KPEMBI *et al.*, 2017b)

Em relação à imunidade dos animais, o DON é capaz de alterar a resposta imune do organismo, promovendo aumento de imunoglobulinas e citocinas (OSWALD *et al.*, 2005). O aumento desses mediadores inflamatórios induzido por DON já foi descrito em vários órgãos como intestino, baço, linfonodos, fígado, rins e pulmões (PESTKA, 2010a). O aumento na expressão de citocinas (IL1α, IL1β, IL6, IL8, IL10, TNFα, NF-κB e CCL20) foi observado no jejuno, fígado e baço de leitões alimentados por 1 semana com 3 mg/kg (BRACARENSE *et al.*, 2020) e no jejuno de leitões alimentados com 0,5 nmol/kg PC por três semanas ou 1 nmol/kg PC por duas semanas (PIERRON *et al.*, 2018) e 3 mg/kg por 5 semanas (BRACARENSE *et al.*, 2012), no fígado de suínos alimentados com 1 mg/kg por seis semanas (CHEN *et al.*, 2008) e nos explantes intestinais de suínos expostos à 0,05-40 μM por 4-5 horas

(ALASSANE-KPEMBI *et al.*, 2017b; PIERRON *et al.*, 2016).

Assim como nas células, estudos já comprovaram que DON é capaz de alterar o estado redox do organismo de animais *in vivo*. A ingestão de 4 mg/kg por duas semanas em leitões levou ao aumento de danos ao DNA de linfócitos e à diminuição da capacidade total antioxidante (CTAO) plasmática (FRANKIČ; SALOBIR; REZAR, 2008), e quando consumido por três semanas, também diminuiu a CTAO e outros antioxidantes (GSH, GPx e SOD) e aumentou os níveis de ERO e a peroxidação lipídica (MDA) (TANG; YUAN; LIAO, 2021). Em camundongos alimentados com 2,4 mg/kg de PC por quatro semanas, houve diminuição de antioxidantes (CAT e GPx) e aumento na expressão de genes que participam da proteção ao estresse oxidativo (Keap1, Nrf2, HMOX1, NQO1 e GCLM) e são ativados em baixos níveis de estresse oxidativo (BAI *et al.*, 2021).

Mesmo as aves, sendo animais mais resistentes ao DON, tiveram alterações no estado redox após o consumo de concentrações altas de DON. Quando ingerida uma concentração de 7,5 mg/kg por três semanas, o DON diminuiu a expressão de genes antioxidantes (HMOX e HIF-1α) no fígado e aumentou a expressão de um gene de oxidação tardio (XOR) no jejuno e fígado (OSSELAERE *et al.*, 2013). A ingestão de 10 mg/kg por cinco semanas também levou ao aumento da peroxidação lipídica (TBARS) e danos ao DNA de linfócitos (AWAD *et al.*, 2014; OSSELAERE *et al.*, 2013). Nos animais alimentados com 19,3 mg/kg por duas semanas, houve redução da capacidade antioxidante (GSH, FRAP e ABTS) e aumento do estresse oxidativo (TBARS e NBT) no intestino e fígado (SOUZA *et al.*, 2020).

A apoptose *in vivo* induzida por DON foi confirmada pela imuno-histoquímica de caspase-3 no fígado de leitões injetados com 1 mg/kg de PC (MIKAMI *et al.*, 2010) e de camundongos alimentados com 5 mg/kg por duas semanas (SUN *et al.*, 2014), nos linfonodos de leitões alimentados com 1,5 mg/kg por quatro semanas (GEREZ *et al.*, 2015) e de ratos alimentados com 1,75 ou 11,4 mg/kg por até quatro semanas (BRACARENSE *et al.*, 2017) e em explantes intestinais de suínos expostos à 10 μM por 4 horas (SILVA *et al.*, 2014). No entanto, em explantes ovarianos de suínos expostos à 10 μM de DON por 48 horas, não se observou apoptose (GEREZ *et al.*, 2021).

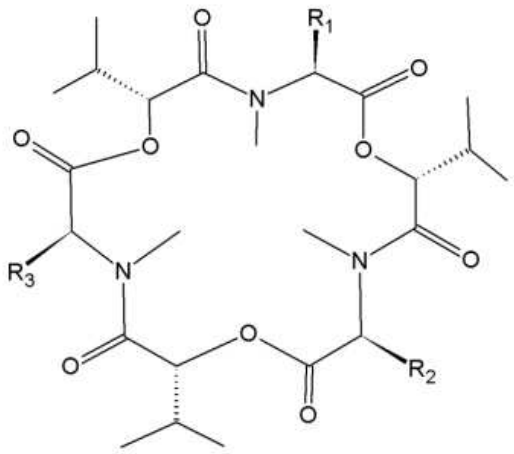
2.4.2 Micotoxinas Emergentes

Micotoxinas emergentes são micotoxinas que não são determinadas rotineiramente e nem possuem uma regulamentação. Porém, sua incidência está aumentando rapidamente (VACLAVIKOVA *et al.*, 2013). Podem contaminar uma grande variedade de alimentos e são consideradas emergentes, pois, com o desenvolvimento de técnicas avançadas, como cromatografia líquida associada à espectrometria de massa, foi possível identificar diferentes tipos de micotoxinas que não eram detectadas antes da existência desses métodos (FRAEYMAN *et al.*, 2017).

A beauvericina (BEA) e as eniatinas (ENNs) são exemplos dessas micotoxinas emergentes. Elas são hexadepsipeptídeos que contém três N-metil aminoácidos e três grupos de hidroxiácido em sua estrutura (Figura 6) (URBANIAK; WAŚKIEWICZ; STĘPIEŃ, 2020). A BEA foi isolada pela primeira vez em 1969 a partir do fungo *Beauveria bassiana* (HAMILL *et al.*, 1969) e as ENNs foram descobertas a partir de culturas de *Fusarium oxysporum* (GÄUMANN; ROTH; ETTLINGER, 1947), mas tanto a BEA quanto as ENNs também são produzidas a partir de várias espécies de *Fusarium,* como *F. acuminatum, F. avenaceum, F. oxysporum, F. poae, F. sporotrichioides, F. sambucinum and F. tricinctum*.

No total, existem 27 tipos de ENNs já descritas, mas os tipos mais frequentes são ENN A, ENN A1, ENN B e ENN B1. A ENN A possuí três radicais isoleucina e a ENN B tem três radicais valina, enquanto a ENN A1 tem um radical valina e dois isoleucina e a ENN B1 possuí dois radicais valina e um radical isoleucina (FRAEYMAN *et al.*, 2017; JESTOI, 2008).

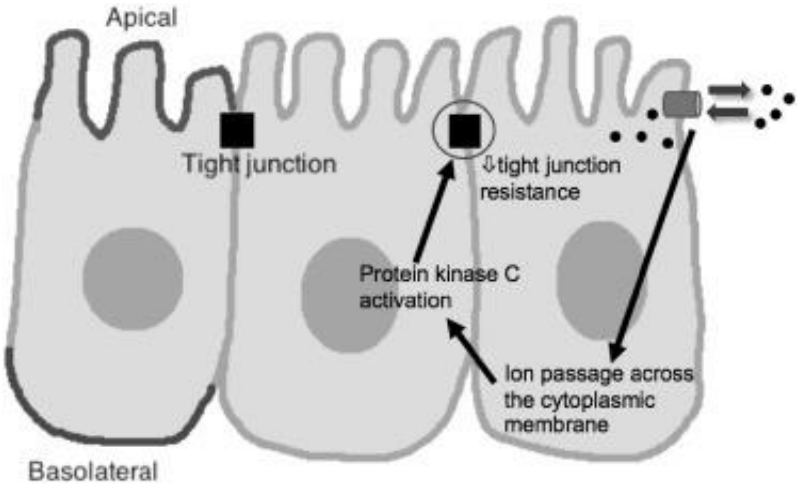
Figura 6 – Estrutura química da beauvericina e eniatinas (A, A1, B e B1). Beauvericina, R1 = R2 = R3: fenilmetil; Eniatina A, R1 = R2 = R3: –$CH(CH_3)CH_2CH_3$; Eniatina A1, R1 = R2: –$CH(CH_3)CH_2CH_3$, R3: –$CH(CH_3)_2$; Eniatina B, R1 = R2 = R3: –$CH(CH_3)_2$; Eniatina B1, R1 = R2: –$CH(CH_3)_2$, R3 = –$CH(CH_3)CH_2CH_3$;



Fonte: Fraeyman et al. (2017)

Sabe-se que sua toxicidade está relacionada às suas propriedades ionóforas, pois tanto BEA quanto as ENNs são capazes de transportar cátions monovalentes (K^+) e divalentes (Ca^{2+}) através da membrana celular, levando a alterações tóxicas por causar uma desproporção intracelular nos íons transportados, afetando a homeostase celular e provavelmente diminuindo a resistência entre as junções intercelulares no intestino (Figura 7) (BERTERO; SPICER; CALONI, 2018; FRAEYMAN *et al.*, 2017; JESTOI, 2008; MALLEBRERA *et al.*, 2018; PROSPERINI *et al.*, 2017).

Figura 7 – Mecanismo de ação de BEA e ENNs nas junções intercelulares de enterócitos. Transporte de cátions através de poros formados na membrana celular.



Fonte: Bertero et al. (2018)

As ENNs têm alta seletividade por íons potássio (K^+) e em mitocôndrias de ratos, promovem um aumento no influxo de K^+ para a matriz mitocondrial, causando edema da organela. O aumento de cálcio (Ca^{2+}) intracelular também pode ocorrer porque a capacidade de retenção de cálcio na matriz mitocondrial está diminuída na presença de BEA e ENNs. Esse aumento de Ca^{2+} pode induzir a apoptose, pois esse íon é responsável por ativar a via das caspases (JOW *et al.*, 2004; TONSHIN *et al.*, 2010).

BEA e ENNs já foram detectados em uma variedade de alimentos ou rações originários de vários países (URBANIAK; WAŚKIEWICZ; STĘPIEŃ, 2020). A BEA é encontrada em cereais como trigo, aveia, cevada e centeio, com prevalência que pode variar de 12 a 100%, mas geralmente a concentração não ultrapassa 100 µg/kg. Porém, em outros cereais, como arroz, os níveis de contaminação podem chegar a 26.300 µg/kg (FRAEYMAN *et al.*, 2017; MALLEBRERA *et al.*, 2018). As ENNs também contaminam cereais como trigo, aveia, cevada e centeio, com prevalência de 96 a 100%. Os níveis de contaminação geralmente variam entre 41-569 µg/kg, mas ocasionalmente podem ultrapassar 1.000 µg/kg (FRAEYMAN *et al.*, 2017; PROSPERINI *et al.*, 2017).

Estudos com muitas amostras e análises de várias micotoxinas revelaram uma alta prevalência de micotoxinas emergentes (GRUBER-DORNINGER *et al.*, 2017; KHOSHAL *et al.*, 2019; NOVAK *et al.*, 2019). No entanto, existem poucos estudos, a maioria *in vitro*, sobre os efeitos dessas micotoxinas na saúde animal e humana e nenhuma legislação ou recomendação foi desenvolvida até o momento. O que existe na literatura é uma opinião científica para avaliação de riscos para BEA e ENNs e a conclusão é de que essa avaliação não pode ser realizada devido à falta de dados sobre a toxicidade *in vivo* dessas micotoxinas (EFSA, 2014).

2.4.2.1 Toxicocinética

Não existem muitos estudos de toxicocinética para BEA (MALLEBRERA *et al.*, 2018) e com os resultados encontrados na literatura não foi possível delinear um perfil tempo-concentração em suínos após administração oral, pois apenas em 2 momentos a concentração plasmática de BEA ultrapassou limite de quantificação (DEVREESE *et al.*, 2013).

Com a administração crônica (4 semanas) de ENN A em ratos, foi possível detectar aumento nos níveis dessa micotoxina no soro a partir da segunda semana de tratamento, mas ela não foi detectada nas fezes ou urina em nenhum momento. A hipótese apontada seria que a ENN A sofre bioacumulação em alguns órgãos e, por consequência, é encontrada no soro (JUAN *et al.*, 2014). Outro estudo com fases *in vitro* e *in vivo* sobre a toxicocinética de ENN A em ratos também foi conduzido. Os autores verificaram que a ENN A teve alta ligação às proteínas plasmáticas (99%), alta depuração hepática, sofreu metabolização principalmente pela via da CYP3A4 e a administração oral de 5 mg/kg de ENN A obteve uma biodisponibilidade oral absoluta de 47% (BHATERIA *et al.*, 2022).

Após a administração intraperitoneal de BEA e ENN B, por três e dois dias, respectivamente, ficou demonstrado que BEA estava presente em vários tecidos (músculo, intestinos, gordura, cérebro, rim e fígado) e no soro na forma não metabolizada e em concentrações baixas. Também foi verificado que a BEA sofre bioacumulação no fígado desses animais, com sua concentração sendo 18 vezes maior que a de ENN B nesse tecido (RODRÍGUEZ-CARRASCO *et al.*, 2016).

Pela via oral, a biodisponibilidade varia de acordo com a espécie (FRAEYMAN *et al.*, 2017; MALLEBRERA *et al.*, 2018; PROSPERINI *et al.*, 2017). A ENN B1 em suínos tem biodisponibilidade de aproximadamente 91%, mas em frangos a ENN B e B1 não são muito absorvidas, com biodisponibilidade de 11% e 5%, respectivamente (DEVREESE *et al.*, 2014; FRAEYMAN *et al.*, 2016).

Essas diferenças entre as espécies podem ser explicadas pela biotransformação que essas micotoxinas sofrem no organismo dos animais, esse processo gera metabólitos hidroxilados e carboxilados. Em suínos, após administração oral, foi encontrada maior razão metabólito/ENN B1 do que após administração intravenosa, indicando que há um metabolismo pré-sistêmico atuando nessa espécie (IVANOVA *et al.*, 2017). Em frangos não foram encontradas evidências de que esse metabolismo pré-sistêmico atue de forma diferente do metabolismo sistêmico, já que há baixa concentração plasmática dos metabólitos após administração oral (FRAEYMAN *et al.*, 2017).

2.4.2.2 Estudos *in vitro*

Muitos dos estudos descritos na literatura com micotoxinas emergentes foram realizado com experimentos *in vitro*. A tabela 1 traz as informações a respeitos dos principais efeitos dessas micotoxinas em diferentes tipos celulares.

Os efeitos citotóxicos para BEA e ENNs já foram demonstrados em vários estudos *in vitro* em diversos tipos celulares (FRAEYMAN *et al.*, 2017, 2018b; JUAN-GARCÍA *et al.*, 2019; MALLEBRERA *et al.*, 2018; PROSPERINI *et al.*, 2013a, 2013b, 2017).

Em células Caco-2 tratadas com BEA, a IC_{50} foi equivalente à 24,6 e 12,7 µM após 24 e 48 horas de exposição, respectivamente. E em células HT-29 a IC_{50} correspondeu a 15 e 9,7 µM após 24 e 48 horas, respectivamente (PROSPERINI *et al.*, 2013a). Ainda nas células Caco-2, outras micotoxinas também foram testadas. A exposição combinada à ENN B, DON e alternariol (AOH) comprovou que a viabilidade celular diminuiu na seguinte ordem: (DON + AOH + ENN B) > (DON + AOH) > (ENN B + AOH) > (DON + ENN B) (FERNÁNDEZ-BLANCO; FONT; RUIZ, 2016).

Em três tipos celulares diferentes, células Caco-2, HT-29 e HepG2, a citotoxicidade das ENNs (A, A1, B, B1, B4 e J3) (0-30 µM) foi testada por 24 e 48 horas, e entre todas, a ENN A1 foi a mais citotóxica (IC_{50} 9,1-12,3 µM e 1,4-2,7 após 24 e 48 horas, respectivamente) e as ENNs A, B1 e B4 foram as menos citotóxicas entre todas (MECA; FONT; RUIZ, 2011). Em outro estudo com células HepG2 e Balb 3T3, a menor dose efetiva (DE_{50}), em que 50% das foram afetadas, foi equivalente à 0,50 µM de ENN B em HepG2, indicando que esse tipo celular é mais sensível à ENN B (JONSSON *et al.*, 2016).

Nesse mesmo tipo celular, verificou-se que a exposição tanto de BEA e ocratoxina A (OTA) isoladamente quanto em conjunto foi citotóxica para essas células, com BEA sendo mais tóxica que OTA quando comparadas isoladamente e quando misturadas a toxicidade ocorreu de maneira dose-dependente (JUAN-GARCÍA *et al.*, 2019). Já em células de hepatoma de rato (H4IIE) e células hepáticas de peixes (RTH-149 e PLHC-1), em que foram analisadas a citotoxicidade de BEA, DON e OTA, a micotoxina com menor toxicidade foi OTA, enquanto BEA e DON

mostraram toxicidade similar nessas células (GARCÍA-HERRANZ *et al.*, 2019).

Um tratamento com ENN B e outras micotoxinas em células pulmonares (V79) demonstrou que a ENN B produziu citotoxicidade e a IC_{50} encontrada foi de 4 µM, sendo inferior apenas à IC_{50} de DON, que foi equivalente à 0,8 µM (FÖLLMANN; BEHM; DEGEN, 2009).

A citotoxicidade também foi verificada em células ovarianas CHO-K1, as micotoxinas BEA (2,2-10,7 µM), patulina (PAT) (2,9 µM) e esterigmatocistina (STE) (12,5-25 µM) foram testadas isoladamente e em combinação. Após 24-72 horas, as combinações de 2 ou 3 micotoxinas tiveram efeito citotóxico dose-dependente, agindo de maneira sinérgica (ZOUAOUI *et al.*, 2016). Já nas células de origem mieloide de humanos, HL-60 e U-937, o tratamento com BEA (0,1-300 µM) por 4 ou 24 horas causou diminuição da viabilidade celular dose e tempo dependente (CALÒ *et al.*, 2004).

736 Tabela 1. Efeitos das micotoxinas BEA e ENNs em estudos realizados em diferentes tipos celulares.

Linhagem celular	Micotoxina	Dose	Tempo de exposição	Principais efeitos	Referência
A549	BEA	1-30 μM	24 h	BEA induziu a apoptose de maneira concentração e tempo dependentes, perda do potencial de membrana mitocondrial e aumentou a liberação de citocromo C mitocondrial e a ativação da caspase 3.	(LIN *et al*., 2005)
Blastocistos de camundongos	ENN B1	1-10 μM	24 h	Diminuição da viabilidade celular, induziu estresse oxidativo intracelular e aumentou a apoptose.	(HUANG *et al*., 2019)
Caco-2	BEA	3,125-25 μM	24-72 h	Diminuição da viabilidade celular, aumento da geração de ERO, indução de apoptose, perda do potencial de membrana mitocondrial e parada do ciclo celular em fase G2/M.	(PROSPERINI *et al*., 2013a)
Caco-2	ENN (A, A1, B e B1)	0.5-15 μM	24-72 h	Diminuição da viabilidade celular na seguinte ordem: ENN A = ENN A1 > ENN B1 > ENN B. Aumento da geração de ERO e peroxidação lipídica. Induziu apoptose, danos ao DNA, perda do potencial de membrana mitocondrial e parada do ciclo celular nas fases G2/M (24h) e S (72h).	(PROSPERINI *et al*., 2013b)
Caco-2	ENN B	1-25 μM	24 h	Perda de funcionalidade lisossômica, parada do ciclo celular na fase G2/M e aumento da produção intracelular de ERO.	(IVANOVA *et al*., 2012)
Caco-2	ENN B, DON e AOH	0,312-10 μM (ENN B e DON) e 1,85-90 μM (AOH).	24-72 h	Diminuição da viabilidade celular de maneira dose-dependente. Combinações terciárias produziram uma diminuição menor em comparação com combinações binárias.	(FERNÁNDEZ-BLANCO *et al*., 2016)
Caco-2 e HT-29	BEA e FUS	0,6-30 μM	24-48 h	Apenas BEA foi citotóxica para essas linhagens celulares. A biodisponibilidade de BEA foi de 50,1-54,3%, enquanto a biodisponibilidade de FUS foi de 80,2-83,2%.	(PROSPERINI *et al*., 2012)
Caco-2, HL-60, A549, GLC-4, SW-1573, KB-3-1, KBC-1, MDA-MB-231 e A549	BEA e ENN	0,5-10 μM	24-72 h	Houve citotoxicidade por ENN e BEA e ambas as micotoxinas interagiram de forma potente com as funções de transporte ABCB1 e ABCG2.	(DORNETSHUBER *et al*., 2009b)
Caco-2, HT-29 e HepG2	ENN (A, A1, B, B1, B4 e J3)	0-30 μM	24-48 h	ENN A1 foi a mais citotóxica, enquanto ENN A, B1 e B4 foram menas citotóxicas entre as eniatinas testadas.	(MECA *et al*., 2011)
CCRF-CEM	BEA	1-10 μM	24 h	Aumento da atividade da caspase-3 de maneira dose-dependente. A maior dose aumentou a liberação de citocromo C da mitocôndria em um padrão tempo-dependente.	(JOW *et al*., 2004)

Células da granulosa de bovinos	BEA e FB1	0-10 μM	48 h	BEA inibiu a proliferação celular e diminuiu fortemente a produção de esteroides. FB1 inibiu fracamente a produção de estradiol e não influenciou os efeitos de BEA.	(ALBONICO *et al*., 2017)
Células da granulosa de suínos	BEA and AOH	0,3-20 μM	24 h	Na ausência do fluido folicular (FF), a viabilidade celular diminuiu para ambas as micotoxinas. A presença do FF com AOH manteve a viabilidade celular, mas o FF com BEA não teve efeito citoprotetor.	(SANTOS *et al*., 2015)
Células dendríticas humanas e macrófagos	BEA, ENN B e MON	0,1-80 μM (BEA e MON) e 0,1-10 μM (ENN B)	2-8 d	BEA e ENN B foram citotóxicas para essas células. O processo de diferenciação de monócitos em células dendríticas imaturas não foi perturbado. Células dendríticas expostas a BEA e ENN B apresentaram aumento da secreção de IL10 e diminuição da capacidade de endocitose.	(FICHEUX *et al*., 2013)
CHO-K1	BEA	0.625, 1.25, 2.5, 5, 10, and 20 μM	24-72 h	Diminuição da viabilidade celular de maneira tempo-dependente, parada do ciclo celular em fase G0/G1, danos ao DNA e aumento da atividade de enzimas antioxidantes.	(MALLEBRERA *et al*., 2016)
CHO-K1	BEA	0.1-5 μM	24 h	Aumento das atividades de GST e GPx e diminuição das atividades de GR e GSH após 24h. N-acetilcisteína (NAC) é um eliminador eficaz de BEA.	(MALLEBRERA *et al*., 2014)
CHO-K1	BEA	0.1-5 μM	24-72 h	O pré-tratamento com resveratrol (RSV) antes da exposição a BEA causou um efeito citoprotetor e diminuiu a produção de ERO. A BEA sozinha induziu a peroxidação lipídica aumentando os níveis de MDA, mas o pré-tratamento com RSV diminuiu os níveis de MDA.	(MALLEBRERA *et al*., 2015)
CHO-K1	BEA, PAT e STE	0,75-20 μM (BEA), 0,049-6,25 μM (PAT) e 1,56-75 μM (STE)	24-72 h	A associação de 2 ou 3 micotoxinas diminuiu a viabilidade celular de forma dose-dependente. Valores de IC_{50} de 2,9 μM para PAT, 10,7 (24 h) a 2,2 μM (72 h) e de 25 (24 h) a 12,5 μM (72 h) para BEA e STE, respectivamente.	(ZOUAOUI *et al*., 2016)
CHO-K1	BEA, PAT e ZEA	1-100 μM (BEA), 0,2-25 (PAT) e 1,5-150 μM (ZEA)	2-48 h	Todas as micotoxinas foram citotóxicas de maneira dose-dependente. Aumento da produção intracelular de ERO e de MDA após a exposição a BEA e PAT de maneira dependente da concentração e do tempo.	(FERRER *et al*., 2009)
Fibroblastos embrionários de camundongo	ENN B1	0,625–40 μmol/L	24 h	Desestabilização do complexo LAMP-2 na membrana lisossômica, resultando na alcalinização dos lisossomos, vazamento de componentes associados à autofagia mediada por chaperonas no citosol.	(OLIVEIRA *et al*., 2019)

H295R e células de Leydig neonatal de suínos	ENN B	0,01–100m μM	24-72 h	Diminuição da viabilidade celular em ambos os tipos de celulares na maior dose (100 uM). Parada do ciclo celular na fase S e diminuição na produção de progesterona, testosterona e cortisol.	(KALAYOU *et al*., 2015)
H4IIE, RTH-149 e PLHC-1	BEA, DON, e OTA	0,00076-25 μM (BEA) e 0,003-100 μM (DON e OTA)	24 h	As linhagens celulares de peixes e mamíferos foram muito sensíveis às micotoxinas. Diminuição da viabilidade celular na seguinte ordem: BEA = DON > OTA. Todas as micotoxinas mostraram um forte efeito antagonista no receptor da tireoide. BEA teve um efeito antagonista fraco no receptor de androgênio e OTA produziu uma curva dose-responsiva bifásica no receptor de estrogênio.	(GARCÍA-HERRANZ *et al*., 2019)
HepG2	BEA e ENN (A1 e B1)	1,5-3 μM	24-72 h	Parada do ciclo celular em G1, diminuição do percentual apoptótico-necrótico de células e perda do potencial de membrana mitocondrial.	(JUAN-GARCÍA *et al*., 2015)
HepG2	BEA e OTA	0-25 μM (BEA) e 0-100 μM (OTA)	24-72 h	Citotoxicidade de BEA foi maior que de OTA. Efeitos aditivos e sinérgicos com a associação das duas micotoxinas. Parada do ciclo celular em fase G0/G1 para a associação.	(JUAN-GARCÍA *et al*., 2019)
HepG2 e Balb 3T	ENN B	1,5-100 μM	24-48 h	Diminuição da viabilidade celular e alteração do metabolismo energético.	(JONSSON *et al.*, 2016)
HepG2, H4IIE, Hct116 e C6	BEA	0,1-25 μM	4-24 h	Alta citotoxicidade para todas as linhagens celulares. Em células H4IIE, induziu apoptose, inibiu a atividade de NF-kB e modulou MAPKs. Nas células C6, causou morte celular por necrose.	(WÄTJEN *et al*., 2014)
HL-60 e U-937	BEA	0,1-300 μM	4-24 h	Diminuição da viabilidade celular tempo e dose-dependentes.	(CALÒ *et al*., 2004)
HL-60, A549, GLC-4 e KB-3-1	BEA e ENN	0,5-10 μM	24 h	Diversos ensaios celulares e moleculares indicaram que o estresse oxidativo não contribuiu para a citotoxicidade induzida por ENN e BEA. A micotoxinas induziram atividade antioxidante moderada.	(DORNETSHUBER *et al*., 2009a)
IPEC-J2	BEA e ENN (A, A1, B e B1)	0-100 μM	24 h	As células foram altamente sensíveis à BEA e ENN A. A ENN B foi a micotoxina menos citotóxica entre todas as micotoxinas testadas.	(FRAEYMAN *et al*., 2018)
IPEC-J2	BEA, ENN (A, A1, B e B1), API, AFN, DON, MON, rubrofusarina, equisetina e bikaverina	0-10 μM	24-72 h	Diminuição da resistência elétrica transepitelial (TEER) na seguinte ordem: API > ENN B> BEA = DON > ENN B1 > ENN A > AFN = ENN A1 > moniliformina = equisetina = bikaverina = rubrofusarina. Nenhum efeito citotóxico foi observado após 72h.	(SPRINGLER *et al*., 2016)

Jukart-T	BEA	1,5-5 μM	24 h	Danos mitocondriais, afetando a cadeia respiratória e levando a apoptose através da cascata de caspases.	(ESCRIVÁ *et al*., 2018)
Jukart-T	BEA e ENN B	1-15 μM	24-72 h	BEA diminuiu a viabilidade celular a partir de 3 μM, enquanto ENN B diminuiu a viabilidade celular apenas com 15 μM. Apenas BEA apresentou distúrbios do ciclo celular e células apoptóticas e apoptóticas/necróticas aumentadas.	(MANYES *et al*., 2018)
Jukart-T	BEA e ENN B	0,1-1,5 μM	24 h	Alterações transcricionais a nível mitocondrial foram observadas após a co-exposição a BEA e ENN B, incluindo a regulação negativa de genes relacionados à atividade antioxidante.	(ESCRIVÁ *et al*., 2019)
Jukart-T	ENN B	1,5-5 μM	24 h	Genes envolvidos em processos biológicos, funções moleculares e vias relacionadas ao metabolismo mitocondrial e respiração foram significativamente alterados.	(ALONSO-GARRIDO *et al*., 2018)
Oócitos, células cumulus e embriões de suínos	BEA	0-10 μM	44 h	BEA foi tóxica para embriões, oócitos e células cumulus em concentrações superiores a 0,5 μM, mas exerceu efeitos diferentes nos três tipos celulares.	(SCHOEVERS *et al*., 2016)
PBCEC, HBMEC e CCF-STTG1	ENN B e B1	0,1-10 μM	48 h	As células CCF-STTG1 foram mais sensíveis que as células endoteliais, e apenas ENN B aumentou a atividade da caspase-3 nas células CCF-STTG1. O transporte das micotoxinas através da barreira hematoencefálica revelou altas taxas de influxo para ambas as micotoxinas.	(KRUG *et al*., 2018)
PK15	BEA, FB1 e OTA	0,05, 0,5 e 5 μg/ml	24-48 h	Diminuição da viabilidade celular e aumento dos níveis de GSH e TBARS de maneira concentração e tempo-dependentes. O tratamento combinado com BEA, FB1 e OTA resultou em efeitos aditivos, especialmente após uma exposição de 24 h.	(KLARIĆ *et al*., 2007)
RAW264.7	BEA	0-4,5 μM	24 h	BEA inibiu a resposta inflamatória de macrófagos pela inibição da via do fator de transcrição NF-kB.	(YOO *et al*., 2017)
V79	ENN B, DON, PAT, OTA, ZEA e CIT	0,1–100 μM	24-48 h	Diminuição da viabilidade celular na seguinte ordem: DON ENN B > PAT > OTA > ZEA > CIT. Nenhum potencial genotóxico significativo da ENN B foi revelado. Fragmentação nuclear induzida por ENN B em baixas concentrações submicromolares.	(FÖLLMANN *et al*., 2009)

Alternariol (AOH), citrinina (CIT), desoxinivalenol (DON), fumonisina B1 (FB1), fusaproliferina (FUS), moniliformina (MON), ocratoxina A (OTA), patulina (PAT), esterigmatocistina (STE), zearalenona (ZEA).

Em células IPEC-J2 de suínos, foi testado a resistência elétrica transepitelial (TEER), e os resultados indicaram que a TEER foi maior para ENN B, seguido de BEA > ENN B1 > ENN A > ENN A1 (SPRINGLER *et al.*, 2016). As células IPEC-J2 também apresentaram maior sensibilidade à BEA e ENN A, tendo menor viabilidade celular após 24h de exposição a essas micotoxinas em várias doses, enquanto na exposição à ENN B, em alta concentração (100 μM), as células apresentavam viabilidade de 83% após 24 horas (FRAEYMAN *et al.*, 2018b).

Em células HepG2, os efeitos de BEA, ENN A1 e B1 após 24-72 horas foram avaliados. BEA e ENN A1 causaram a interrupção do potencial de membrana mitocondrial e ENN B1 promoveu a parada do ciclo celular em fase G1 de maneira tempo dependente (JUAN-GARCÍA *et al.*, 2015).

Ainda em relação ao ciclo celular, nas células Caco-2, as ENN A e A1 apresentaram maior toxicidade que ENN B1 e BEA, sendo que ENN B teve o menor efeito citotóxico nessas células. ENN A, A1 e B1 induziram danos ao DNA e parada do ciclo celular na fase G_2/M nas concentrações 1,5 e 3 μM (PROSPERINI *et al.*, 2013b).

A exposição à BEA também causou parada do ciclo celular nas fases G_0/G_1 de células HepG2, aumentou a apoptose, alterou o potencial de membrana mitocondrial e causou danos ao DNA de células CHO-K1 (JUAN-GARCÍA *et al.*, 2015; MALLEBRERA *et al.*, 2016). Já em células HepG2 tratadas com BEA e/ou OTA, houve acúmulo de células nas fases G_0/G_1 e S para OTA e a combinação de BEA + OTA, mas para BEA isoladamente não houve acúmulo celular, demonstrando que a combinação das duas teve um efeito aditivo e sinérgico (JUAN-GARCÍA *et al.*, 2019).

Em doses de 2,5-10 μM, BEA foi capaz de produzir alterações cromossômicas e micronúcleos em linfócitos de humanos e células IPEC-J2 (PROSPERINI *et al.*, 2013a; SPRINGLER *et al.*, 2016). Em fibroblastos embrionários de camundongo, a exposição à ENN B1 (0,625–40 μmol/L) por 24 horas desestabilizou o complexo LAMP-2 na membrana lisossômica, o que resultou na alcalinização dos lisossomos e vazamento de componentes associados à autofagia mediada por chaperonas no citoplasma dessas células (OLIVEIRA *et al.*, 2019).

Em uma linhagem de linfócitos T (Jurkat), BEA teve maior toxicidade do que ENN B, a exposição a essas micotoxinas variou de 24 a 72 horas. BEA também provocou alterações no ciclo celular e nas mitocôndrias, enquanto ENN B não causou mudanças no ciclo celular e as alterações mitocondriais ocorreram apenas em doses mais altas (15 µM) e com maior tempo de exposição (72 horas) (MANYES *et al.*, 2018).

BEA e ENNs também possuem efeito imunomodulador (FRAEYMAN *et al.*, 2017; MALLEBRERA *et al.*, 2018; PROSPERINI *et al.*, 2017), aumentando a secreção de interleucina 10 (IL-10) e interferindo na migração de células dendríticas. A fagocitose de macrófagos também foi prejudicada pela exposição a essas micotoxinas (FICHEUX; SIBIRIL; PARENT-MASSIN, 2013). A exposição por 24 horas com BEA também inibiu a resposta inflamatória de macrófagos RAW 265.7, por inibir a via do fator de transcrição NF-$_{K}$B (YOO; KIM; CHO, 2017).

A ENN B alterou significativamente a expressão de genes em células Jurkat. Mais de 245 genes, entre eles os genes de processos metabólicos, como cadeia de transporte elétrico, fosforilação oxidativa e respiração celular, estavam alterados (ALONSO-GARRIDO *et al.*, 2018). Nessas células, o tratamento com BEA também alterou a expressão de vários genes envolvidos no metabolismo celular. Entre eles, 77 genes responsáveis pela cadeia respiratória foram inibidos, 21 genes de apoptose e 12 genes relacionados com a atividade das caspases tiveram maior expressão, principalmente das caspases 8, 9 e 10 (ESCRIVÁ *et al.*, 2018).

Quando considerada uma associação entre BEA + ENN B em células Jukart, essa associação apresentou menos alterações na expressão de genes mitocondriais do que a exposição isolada de cada micotoxina. No entanto, a associação provou ser mais prejudicial para o estado oxidativo, uma vez que esse tratamento diminuiu a expressão de genes relacionados com a atividade antioxidante e aumentou a expressão de genes oxidantes (ESCRIVÁ *et al.*, 2019).

Estudos demonstraram que BEA e ENNs estimulam a produção de ERO, que levam a alterações oxidativas como a peroxidação lipídica e diminuição de glutationa (GSH) em diferentes tipos celulares (IVANOVA *et al.*, 2012; KLARIĆ *et al.*, 2007; PROSPERINI *et al.*, 2013a, 2013b). A peroxidação lipídica após exposição à BEA, FB1 e OTA, em células animais, foi comprovada primeiramente em células

renais PK-15. Após 24 horas, OTA causou aumento da peroxidação lipídica e após 48 horas isso também ocorreu com BEA e FB1. A diminuição de GSH foi detectada em 24 horas e uma combinação das três micotoxinas teve efeito aditivo (KLARIĆ *et al.*, 2007).

Em células CHO-K1 e HepG2 também foram reportados aumento da produção de ERO e da atividade de superóxido dismutase (SOD) e catalase (CAT) assim como aumento da peroxidação lipídica nessas células e diminuição dos níveis de enzimas antioxidantes (KLARIĆ *et al.*, 2007; MALLEBRERA *et al.*, 2015, 2016; MALLEBRERA; FONT; RUIZ, 2014). Quando comparada com PAT e ZEA, a BEA teve citotoxicidade intermediária, sendo PAT a mais tóxica e ZEA a que causou menos danos em células CHO-K1, essa citotoxicidade foi associada à formação de ERO e peroxidação lipídica (FERRER *et al.*, 2009).

Quanto ao uso de diferentes substâncias antioxidantes, em um estudo foi testado o efeito de N-acetilcisteína (NAC) sobre células CHO-K1 expostas à BEA, os resultados mostraram que NAC foi capaz de impedir a diminuição de enzimas antioxidantes pela BEA (MALLEBRERA; FONT; RUIZ, 2014). Outro tratamento, utilizando resveratrol, antes da exposição à BEA, foi capaz de diminuir a citotoxicidade, a formação de ERO e a peroxidação lipídica em células CHO-K1 (MALLEBRERA *et al.*, 2015). Em doses intravenosas de 5 mg/kg em camundongos, ocorreu aumento na produção de ERO, que levou à apoptose dos blastocistos. Mas quando foi associado um tratamento com NAC, houve interrupção efetiva na produção de ERO (HUANG; WANG; CHAN, 2019).

No entanto, em células cancerígenas de útero (KB-3-1) e de leucemia promielocítica (HL-60), a BEA exerceu um efeito antioxidante, diminuindo a produção de ERO e os danos observados pela intensidade na cauda do teste de cometa (DORNETSHUBER *et al.*, 2009a). Em células Jurkat, não foram encontradas alterações oxidativas após 72 horas de exposição à BEA e ENN B, mas BEA produziu mais quebra de DNA, evidenciada na cauda do teste de cometa (MANYES *et al.*, 2018). Por conta desses resultados controversos e dos poucos estudos sobre estresse oxidativo com essas micotoxinas estarem disponíveis, mais estudos em relação a esse assunto são desejáveis.

A apoptose celular após exposição à BEA foi confirmada em várias linhagens celulares (DORNETSHUBER *et al.*, 2009b; KLARIĆ *et al.*, 2007; MALLEBRERA *et al.*, 2015; PROSPERINI *et al.*, 2013a; WÄTJEN *et al.*, 2014). Alguns dos principais reguladores da apoptose são as caspases e ficou demonstrado que BEA é capaz de aumentar a atividade da caspase-3 (JOW *et al.*, 2004), aumentando a taxa de apoptose por meio de alterações morfológicas e fragmentação do DNA nas células. Na linhagem celular de leucemia linfoblástica (CCRF-CEM), a ativação da caspase-3, por conta da liberação do citocromo C mitocondrial, foi o principal indutor da apoptose (JOW *et al.*, 2004), já em células PK15 e Jurkat, houve ativação das caspases 3 e 7 (MANYES *et al.*, 2018; WÄTJEN *et al.*, 2014).

Também foi constatado que a apoptose induzida por BEA é regulada pelo equilíbrio entre a expressão de genes pró (Bax, Bad e BaK) e anti-apoptóticos (Bcl-2) em células cancerígenas pulmonares (NSCLC A549) (LIN *et al.*, 2005b). Os genes da família Bcl-2 também são importantes na manutenção da integridade da membrana mitocondrial, quando as células foram expostas à BEA, houve diminuição do potencial de membrana das mitocôndrias, indicando que isso deve ocorrer para que haja liberação do citocromo C no citosol (JOW *et al.*, 2004; LIN *et al.*, 2005a; MALLEBRERA *et al.*, 2015; PROSPERINI *et al.*, 2013a, 2013b).

Em células do sistema nervoso de humanos (HBMEC e CCF-STTG1) e de suínos (PBCEC), as ENN B e B1 foram capazes de atravessar a barreira hematoencefálica e atingir o parênquima cerebral. Dentre os três tipos celulares, CCF-STTG1 apresentou maior sensibilidade para as micotoxinas e apenas ENN B causou aumento da atividade da caspase-3 (KRUG *et al.*, 2018).

Estudos sugerem que BEA e ENNs exercem citotoxicidade no sistema reprodutivo. O tratamento com BEA prejudicou o desenvolvimento de oócitos e embriões de suínos (SANTOS *et al.*, 2015; SCHOEVERS *et al.*, 2016). Foi comprovado que BEA afetou de maneira diferente células cumulus, oócitos e embriões de suínos, por isso, acredita-se que o mecanismo de toxicidade seja diferente nos vários estágios embrionários (SCHOEVERS *et al.*, 2016). Em blastocistos de camundongos, a ENN B1 (1-10 μM) aumentou a taxa de apoptose e a reabsorção embrionário pós-implantação. Quando administrada via intravenosa (1-5 mg/kg/d) por quatro dias, a ENN B1 causou apoptose dos blastocistos por danos

oxidativos e prejudicou o desenvolvimento embrionário, com degradação do embrião (HUANG; WANG; CHAN, 2019).

Em células da granulosa de bovinos, a BEA inibiu a síntese de estradiol e progesterona nas concentrações 3 e 10 µM, na dose 10 µM houve diminuição marcante do número células da granulosa, suprimindo a expressão dos genes CYP19A1 e CYP11A1 (ALBONICO *et al.*, 2017). Além disso, a ENN B suprimiu a secreção de cortisol, testosterona e progesterona em células de adenocarcinoma adrenocortical de humanos da linhagem H295R, em doses maiores que 10 µM as células de Leydig também reduziram a produção de testosterona e estradiol (KALAYOU *et al.*, 2015).

Elas também exercem efeito antimicrobiano em micro-organismos patogênicos e probióticos. A BEA inibe o crescimento de bactérias patogênicas como *Escherichia coli, Enterococcus faecium, Salmonella enterica, Shigella dysenteriae, Listeria monocytogenes, Yersinia enterocolitica, Clostridium perfringens* e *Pseudomonas aeruginosa* (MECA *et al.*, 2010). Enquanto as ENN A, A1 e B1 são capazes de inibir o crescimento de micro-organismos como: *Streptococcus thermophilus, Bifidobacterium* spp. e *Lactobacillus* spp. (ROIG *et al.*, 2014).

2.4.2.3 Estudos *in vivo*

A administração por três dias de doses altas (50, 100 ou 200 mg/kg peso vivo) de BEA ou ENN B por gavagem oral induziu apenas efeitos tóxicos leves no fígado de camundongos (MARANGHI *et al.*, 2018). A ingestão de uma dieta contaminada com 2.352 µg/kg de ENN B, por três semanas, não causou efeitos negativos na morfologia intestinal, nem lesões histológicas no fígado de frangos de corte. Além disso, a taxa de transferência de ENN B para o tecido hepático nesses animais foi baixo, o que indica que o consumo de frangos contaminados com ENN B não é o principal contribuinte para a contaminação alimentar em humanos (FRAEYMAN *et al.*, 2018a).

Estudos *in vivo* confirmaram a capacidade imunomoduladora de BEA e ENNs em roedores. Em ratos alimentados com ração contaminada por ENN A (465 mg/kg) por quatro semanas, houve modulação na expressão de antígenos de superfície de linfócitos no sangue periférico, aumento do número de células $CD4^+$ e

diminuição da citotoxicidade de células $CD8^+$ (JUAN *et al.*, 2014). Em camundongos com colite induzida, a administração intraperitoneal de BEA (2-4 mg/kg) por 1 semana induziu a apoptose de linfócitos T e diminuiu a secreção de TNF-α e interferon gama (IFN-γ), BEA também atenuou a inflamação induzida e impediu a formação de alterações histopatológicas no cólon dos animais tratados (WU *et al.*, 2013).

As ENNs também são capazes de levar a alterações a nível transcricional. Em ratos Wistar, a administração orofaringeal aguda (8 h) de ENNs (A, A1, B e B1) em dose única em concentrações moderadas (256, 353, 540 e 296 µg/mL de ENN A, A1, B e B1, respectivamente) ou altas (513, 706, 1.021 e 593 µg/mL de ENN A, A1, B e B1, respectivamente) levou a alterações transcricionais no estômago, cólon, fígado e rim desses animais de genes relacionados a cadeia de transporte de elétrons, apoptose, estresse oxidativo, inflamação e integridade da barreira intestinal (CIMBALO *et al.*, 2021).

Outro efeito exercido por BEA e as ENNs é o seu potencial anticarcinogênico, com a indução da apoptose e a interrupção da sinalização da via das MAPKs em células de hepatoma H4IIE (WÄTJEN *et al.*, 2014) e o envolvimento dos genes p53 e p21 na apoptose de diversos tipos de células tumorais humanas (DORNETSHUBER *et al.*, 2007). Esse potencial também foi demonstrado *in vivo* em camundongos, a ENN B teve grande efeito sinérgico com uma droga inibidora de quinases (Sorafenib®) contra o câncer cervical (DORNETSHUBER-FLEISS *et al.*, 2015).

Em camundongos enxertados com células cancerígenas de intestinos (CT-26) ou de útero (KB-3-1), o grupo tratado com BEA teve tumores menores em relação ao grupo não tratado, além disso, constatou-se que houve necrose tecidual e acúmulo de BEA nas regiões tumorais desses animais (HEILOS *et al.*, 2017). O papel anticarcinogênico dessas micotoxinas deve ser mais explorado a fim de que elas se tornem componentes importantes na terapia de tumores.

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4 HIPÓTESE

H_0: A exposição a doses realistas de DON, BEA e ENNs não causa alterações tóxicas no fígado e intestino de suínos.

H_1: A exposição a doses realistas de DON, BEA e ENNs causa alterações tóxicas no fígado e intestino de suínos.

5 OBJETIVOS

5.1 OBJETIVO GERAL

Verificar os efeitos tóxicos de DON e das micotoxinas emergentes BEA e ENNs em suínos.

5.2 OBJETIVOS ESPECÍFICOS

Avaliar a performance animal em suínos alimentados com dieta contaminada com DON, BEA e ENNs.

Avaliar aspectos histológicos do intestino, fígado e linfonodos de suínos alimentados com dieta contaminada com DON, BEA e ENNs.

Identificar alterações nos parâmetros plasmáticos e na expressão de genes no intestino e fígado de suínos tratados com DON, BEA e ENNs.

Identificar alterações na microbiota intestinal de suínos tratados com DON, BEA e ENNs.

Avaliar a viabilidade celular de células hepáticas humanas expostas ao DON e às micotoxinas emergentes.

Avaliar o aspecto histológico do novo modelo de explantes de fígado de suínos cortados com precisão expostos ao DON e às micotoxinas emergentes.

Identificar alterações na expressão de genes dos explantes de fígado de suínos e células hepáticas humanas expostos ao DON e às micotoxinas emergentes.

6 ARTIGO A – EFFECTS OF FUSARIUM METABOLITES BEAUVERICIN AND ENNIATINS ALONE OR IN MIXTURE WITH DEOXYNIVALENOL ON WEANING PIGLETS

Effects of *Fusarium* metabolites beauvericin and enniatins alone or in mixture with deoxynivalenol on weaning piglets

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Abstract: The impact of the *Fusarium*-derived metabolites beauvericin, enniatin B and B1 (EB) alone or in combination with deoxynivalenol (DON) was investigated in 28-29 days old weaning piglets over a time period of 14 days. The co-application of EB and DON (EB+DON) led to a significant decrease in the weight gain of the animals. Liver enzyme activities in plasma were significantly decreased at day 14 in piglets receiving the EB+DON-containing diets compared to piglets receiving the control diet. All mycotoxin-contaminated diets led to moderate to severe histological lesions in the jejunum, the liver and lymph nodes. Shotgun metagenomics revealed a significant effect of EB-application on the gut microbiota. Our results provide novel insights into the harmful impact of emerging mycotoxins alone or with DON on the performance, gut health and immunological parameters in pigs.

Keywords: biomarker of effect, cyclic hexadepsipeptides, fungal metabolites, gut microbiota, histological alterations, mycotoxins

1. Introduction

Cyclic hexadepsipeptides such as enniatins (ENNs) and beauvericin (BEA) are secondary fungal metabolites produced by different fungal genera, but mainly by *Fusarium* species and are considered

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as emerging mycotoxins (Jestoi, 2008b; Urbaniak et al., 2020). Over the past decade, these ionophoric and lipophilic compounds have been the subject of controversy in the literature due to their many detrimental and beneficial effects (Caloni et al., 2020; Gruber-Dorninger et al., 2017b; Křížová et al., 2021). On the one hand, they showed cytotoxic, oxidative, proinflammatory and genotoxic effects in numerous cell lines and impaired the gut barrier function of intestinal cells (Albonico et al., 2016; Juan-García et al., 2015; Khoshal et al., 2019; Manyes et al., 2018; Novak et al., 2019; Prosperini et al., 2013; Springler et al., 2016). On the other side, they are also known to possess antibacterial, antifungal and insecticidal properties (Olleik et al., 2019; Urbaniak et al., 2020).

As they occur ubiquitously in feed and food samples, the European Food and Safety Agency (EFSA) published a scientific opinion on their risk as early as 2014, but no regulations or guidelines have been established to date (EFSA, 2014). This is mainly a result of the lack of *in vivo* experiments and long-exposure studies in mammals. The main challenges to perform such *in vivo* trials is the lack of naturally contaminated culture material and the economic feasibility to obtain an affordable amount of those toxins. BEA, enniatin B (ENN B) and enniatin B1 (ENN B1) were detected to 83%, 71% and 69%, respectively, of 1113 feed samples recently analyzed (Kovalsky et al., 2016). Another study found a similarly high prevalence of 82 % for ENN B and ENN B1, and 67% for BEA in 1141 finished feed samples for swine (Novak et al., 2019). Furthermore, several studies have been published showing different occurrence of those mycotoxins in different geographic regions, different feed commodities and feed destined for different species (Hietaniemi et al., 2016; Juan et al., 2016; Mahnine et al., 2011; Serrano et al., 2013). Concentrations of ENNs and BEA vary from low µg/kg to high mg/kg ranges, depending on feed type, climate, country and weather conditions (Medina et al., 2017; Santini et al., 2012; Urbaniak et al., 2020). Furthermore, co-occurrence with more investigated and regulated *Fusarium* mycotoxins, such as deoxynivalenol (DON), was already described (Khoshal et al., 2019; Spanic et al., 2020; Yoshinari et al., 2016). DON is one of the most prevent mycotoxins detected in 55% to 95% of analyzed feed stuff depending on the region with several described negative effects on animals' health, performance, and immune system (Holanda

and Kim, 2021). Among the known 29 analogues of ENNs, ENN B and B1 seem to occur more often than the others (Novak et al., 2019; Reisinger et al., 2019).

The aim of this study was to investigate the short-term effects of the emerging mycotoxins BEA, ENN B and ENN B1 alone (EB), and together with the regulated mycotoxin DON (EB+DON) on the performance and feed consumption as well as on the intestine and liver of piglets. We chose 28-29 days old piglets for the study, as they are in a critical phase during the first weeks after weaning (Campbell et al., 2013) and thus, are more susceptible to mycotoxins and infectious diseases (Pierron et al., 2016a). Due to the known antibiotic properties of the used emerging mycotoxins, we further investigated their impact on the fecal microbiome by applying a shotgun metagenomics approach. Finally, we analyzed the effect of the mycotoxins on several clinical blood parameters, gene expression in liver and jejunum tissue, as well as histology of liver, intestine and lymph nodes.

2. Materials and Methods

2.1. *Chemical and reagents*

Culture material containing 4.67 g/kg beauvericin and 2.50 g/kg enniatins was prepared by Dr. Roman Labudá from the BiMM institute (Bioactive Microbial Metabolites, UFT Tulln, Austria). In brief, 200 g corn kernels were mixed with 200 mL deionized water and soaked for 60 min under room temperature (23 °C ± 1 °C) in polypropylene plastic bags (200 x 300 mm, Roth) before autoclaving at standard conditions (121 °C for 20 min). The autoclaved corn material was then transferred into disposable PD 1200 Microboxes (Nevele, Belgium). A *Fusarium oxysporum* strain RL 108 (personal collection of Dr. Roman Labudá) was used as a production organism. Inoculation was performed with 100 µL conidial suspension (1.0 x 10^5 conidia/mL) per box, followed by cultivation in darkness at 25 °C for 4 weeks. At the end of cultivation, the cultures were exposed to 75 °C for 2 hours (closed boxes) and consequently to 70 °C (open boxes) for the next 4 days till complete dryness. Heat deactivation and drying was performed in a thermostat with forced air circulation (Binder, Germany). The dried cultures were then transferred into a 30 L capacity bag, thoroughly mixed and crushed into smaller pieces. The material was then ground in the mill (Retsch GM 200, Germany), sieved, and

further homogenized before final sampling. During the milling process, from every portion of freshly ground material (ca. 100 g), several grams were taken as aliquots and all such aliquots were consequently mixed (homogenized) before preparing the final representative sample. The final representative sample (10 g) in duplication was then transferred into 250 mL capacity Erlenmeyer flasks and extracted with 100 mL of solvent mix (ACN:H_2O:AcOH, 70:29:1) by rotary agitating at 160 rpm for 60 min. An aliquot of 100 µL was taken, diluted, and filtered for LC-MS/MS analysis.

Culture material with a DON concentration of 15.48 g/kg was obtained by Romer Labs (Tulln, Austria) and produced according to a publication from the year 1994 (ALTPETER; POSSELT, 1994).

The LC-MS grade eluents used within the analytical method were purchased as followed: acetonitrile (VWR chemicals, USA), methanol (Honeywell Riedel-de-Haën™, Germany), formic acid (Honeywell Riedel-de-Haën™, Germany) and ammonium formate (Merck, Germany).

2.2. *Animal and study design*

All procedures related to the animal experiment were performed according to Austrian law and following the European Guidelines for the Care and Use of Animals for Research Purpose. The experiment was approved by the office of the Lower Austrian Region Government, Group of Agriculture and Forestry, Department of Agricultural Law (approval code LF1-TVG-57/015-2019) and carried out at the Center of Applied Animal Nutrition (BIOMIN Holding GmbH, Tulln, Austria).

Thirty-two crossbred piglets (Breed Ö-HYB-F1 [Landrace x Large White] x Pietrain, 28-29 days old, average weight 7.32 ± 0.72 kg, mixed sex) were obtained from a local swine producer. Piglets were randomly grouped pairwise in metabolic cages (8 animals/group), had free access to water and were allowed to acclimatize for five days. Feed was provided twice daily in the morning and evening for *ad libitum* intake. Prior to the start of the experiment, the feed was mixed with the respective contaminated culture material and tested for mycotoxin contamination by a multi-toxin LC-MS/MS method (Sulyok et a*l.*, 2020).

After an adaptation period of 5 days, piglets received control feed; DON-contaminated feed; enniatin B, B1 and beauvericin (EB)-contaminated diet or an EB+DON-contaminated diet for a period of 14 days (for mycotoxin concentrations see table 1).

Table 1. Mycotoxin concentration in the respective diet

Group \ Toxin	BEA	ENN B + B1	DON	ZEN	FB1
Control	4	161	92	<LOQ	<LOQ
DON	5	186	2524	342	<LOQ
EB	2570	1345	93	<LOQ	<LOQ
EB + DON	3578	1830	2034	263	<LOQ

Concentrations in µg/kg
<LOQ = below limit of quantification

Blood of each piglet was collected after 7 days (D7) and 14 days (D14) on the experimental diets, respectively. Samples were aliquoted and stored at -80 °C until analysis. For blood sampling, about 8 mL full blood was taken with Li-Heparin tubes (S-Monovette 9 mL, Sarstedt, cat. no. 02.1065) and immediately cooled to 4 °C. After transfer to the laboratory, samples were centrifuged at 2,000 x g for 20 min at 4 °C and plasma was aliquoted á 500 µL to labeled 1.5 mL tubes and frozen to -80°C. Determination of the respective parameters was done within the next 2 months.

Feces samples were taken at the end of the trial from individual piglets during euthanasia (D15/16). About 1 g were transferred into a homemade nucleic acid preservation buffer (NAP) and stored at room temperature for microbiome analysis.

2.3. *Metagenomic analysis*

DNA from individual fecal samples was extracted using the QIAamp PowerFecal kit (Qiagen, Crawley, West Sussex, UK) which as shown in a recent study, has minimum effects on the microbial community structure and promising results in terms of the DNA integrity for pig feces samples (Wegl et al., 2021). Briefly, 0.25 mg of digesta/fecal sample was added to the dry bead tube containing 750 µL of bead solution and gently vortexed. C1 solution was added, briefly vortexed, and incubated at 65 °C for 10 min. Samples were shaken in Mo Bio Vortex Adapter Genie2 at maximum speed for 5 min. Samples were centrifuged at 13,000 × g for 1 min, the supernatant transferred to the 2 mL collection tube and the remainder of the protocol was followed as recommended by the manufacturer. All samples were eluted in 100 µL 10 mM Tris-buffer pH 8.0 after being incubated for 5 min for maximum elution efficiency. The total DNA concentration in each extract were determined

fluorometrically using QubitTM dsDNA BR assay kit on a QubitTM 2.0 fluorometer (InvitrogenTM, Unites States) and purity was assessed via 260/280 and 260/230 absorbance ratios determined via spectrophotometry (NanoDrop® ND-1000, Thermo Fisher Scientific, United States).

For the size-selected ONT library, 600 ng of genomic DNA was used and quality was controlled using Agilent 4200 TapeStation. The DNA was sheared using Covaris g-Tubes to generate >7-8 kb fragments (Covaris, Inc., Woburn, Ma, USA). After clean-up, DNA was repaired and end-prepared using the NEBNext FFPE DNA Repair kit (New England BioLabs, Ipswich, MA, USA). AMPure XP beads were added to the repaired DNA and incubated on a Hula mixer at RT for 30 min, followed by two washes with 70% EtOH. Beads were then resuspended with 61 µL of nuclease-free (NF) water and incubated on a Hula mixer at RT for 30 min. Then, 61 µL of the eluate was transferred into a clean 1.5 ml Eppendorf tube. The resulting DNA was quantified using the Qubit HS DNA kit. Adapter ligation and clean-up was performed using the Ligation Sequencing Kit SQK-LSK109 (Oxford Nanopore Technologies, Oxford, United Kingdom) with a slightly changed protocol. In brief, ligation buffer, NEBNext Quick T4 DNA ligase, and adapter mix were added to the repaired DNA and incubated at RT for 10 min and then overnight at 4°C. The ligated sample was purified using 100 µL of AMPure XP beads during a 30 min incubation at RT on the Hula mixer, two bead washing steps using the kit-provided wash buffer and resuspension of the beads in 40 µL of elution buffer at RT for 30 min on the Hula mixer. Then, 40 µL of the eluate was transferred into a clean 1.5 mL tube. The library was then sequenced on a MK1C device (software version 20.03) using R10 flow cell sequencing chemistry, resulting in 11,653,936 1D reads with the quality score >8. Taxonomic profiles of the demultiplexed reads and taxa relative abundance estimated were generated using Kraken2 (Wood et al., 2019) and Bracken (Lu et al., 2017). The R-packages phyloseq (v. 1.30.0) (Mcmurdie and Holmes, 2013), was used for microbiota data handling and calculating alpha (observed richness and Shannon index) and beta diversity.

2.4. *Histological analysis*

After euthanasia, samples of jejunum, colon, liver, and lymph nodes were collected from all treatments and fixed in 10% buffered formalin for histopathological evaluation. After fixation, the

tissues were dehydrated in a graded series of alcohol, cleared and diaphanized by xylol, and embedded in paraffin for histological sections. Afterwards, 5-µm sections were stained with hematoxylin and eosin or alcian blue for histological analysis. Histological changes were evaluated using a lesion score scale, considering the injury intensity as described by Terciolo et al. (2019). The lesion score was established by considering the degree of severity (severity factor) and the extent of each lesion (according to intensity or observed frequency, scored from 0 to 3). For each lesion, the score of the extent was multiplied by the severity factor.

The morphometric analysis of the number of goblet cells and villi height were adapted from Bracarense *et al*. (2012). Goblet cells were counted randomly in ten fields per slide at 60X magnification. Villi height was measured randomly on ten villi using a MOTIC Image Plus 2.0 MLw image analysis system (MOTIC Image Plus Motic Instruments, Richmond, Canada).

2.5. *Immunoassays and biochemical analysis*

A human free IGF-I/IGF-1 immunoassay (Cat. No. DG100B, R&D Systems, Minneapolis, USA), a human FABP2/I-FABP immunoassay (Quantikine® ELISA, Cat. No. DFBP20, R&D Systems, Minneapolis, USA) and a porcine zonulin immunoassay (Cat. No. MBS2607498, MyBioSource, San Diego, USA) were used. The detection limits are indicated as 0.01 ng/mL, 3.63 pg/mL and 0.5 ng/mL, respectively. Determination of the respective protein was done according to the manufacturer's protocol using lithium-heparin plasma taken on D7 and D14. Plasma biochemistry was determined at D7 and D14 at GenoToul-Anexplo platform (Toulouse, France) with a Pentra 400 Clinical Chemistry benchtop analyzer (Horiba, Les Ulis, France).

2.6. *Expression of jejunal and liver mRNA by real-time PCR*

RT-qPCR assays were performed as previously described (Maruo et al., 2018). Primers obtained from previous studies or designed using PrimerQuest® software were purchased from Sigma (Table 2). Data were analyzed with the LinRegPCR 2016.2 program. The expression values of the genes of interest were normalized against three housekeeping genes: hypoxanthine phosphoribosyltransferase

154 1 (HPRT1), topoisomerase (DNA) II beta (TOP2B) and hydroxymethylbilane synthase (HMBS) and

155 validated with NormFinder software. Gene expression was expressed relatively to the control group.

156 **Table 2.** Primer sequences of genes used for qRT-PCR analysis of the jejunum (J) and liver (L)

Target gene		Primer sequence (5'-3')	mRNA	Reference
Nitric oxide synthase 2 (NOS2)	F R	GAGAGGCAGAGGCTTGAGAC TGGAGGAGCTGATGGAGTAG	ENSSSCT00000065180.2	present study
Interferon gamma (IFNG)	F R	TGGTAGCTCTGGGAAACTGAATG GGCTTTGCGCTGGATCTG	ENSSSCT00070017081.1	(Gourbeyre et al., 2015)
Apoptosis inducing factor mitochondria associated 1 (AIFM1)	F R	ATCATCTGCTCCAGAAGGA AGTGCCTCCACCAATGA	ENSSSCT00000013838.4	present study
Superoxide Dismutase 1 (SOD1)	F R	ATCATGGATTCCATGTCCATCAG GGACCTGCACTGGTACAGCC	ENSSSCG00000021355	present study
Tumor protein p53 (TP53)	F R	AAAAGAAGAAGCCACTGGATGG GTTCACGCCCACGGATCT	ENSSSCT00000019534.4	present study
Tumor Necrosis Factor alpha (TNFA)	F R	ACTGCACTTCGAGGTTATCGG GGCGACGGGCTTATCTGA	ENSSSCT00070048157.1	(Gourbeyre et al., 2015)
Toll-like receptor 9 (TLR9)	F R	CACGACAGCCGAATAGCAC GGGAACAGGGAGCAGAGC	ENSSSCT00000012516.5	present study
Interleukin 1A (IL1A)	F R	GCCAATGACACAGAAGAAGA ATGCACTGGTGGTTGATG	ENSSSCT00000008863.3	(Pierron et al., 2016b)
Nuclear Factor Kappa B (NFkB)	F R	CCTCCACAAGGCAGCAAATAG TCCACACCGCTGTCACAGA	ENSSSCT00000033438	(Maruo et al., 2018)
Mitogen-activated protein kinase 8 (MAPK8)	F R	GTGGAATCAAGCACCTTCACTCT GGGCTTTAAGTCCCGATGAATA	ENSSSCT00000036610.1	present study
Transforming growth factor beta 1 (TGFB1)	F R	GGATACCAACTACTGCTTCAG GGTTCATGAATCCACTTCCA	ENSSSCT00000036469.3	(Gourbeyre et al., 2015)
Interleukin 10 (IL10)	F R	GGCCCAGTGAAGAGTTTCTTTC CAACAAGTCGCCCATCTGGT	ENSSSCT00000017049.5	(Pierron et al., 2016b)
Interleukin 8 (IL8)	F R	GCTCTCTGTGAGGCTGCAGTTC AAGGTGTGGAATGCGTATTTATGC	ENSSSCG00000008953	(Cano et al., 2013)
Peptide YY (PYY)	F R	CTGCGCCACTACCTCAACCT GGGAAGAGCAGTTTGGAGAGAA	ENSSSCT00000030078.3	present study
Cholecystokinin (CCK)	F R	AAAGCACCTTCTGGCCGAGT GGTCACTTATTCTGTGGCTGGG	ENSSSCT00000012346.4	present study
Insulin like growth factor 1 (IGF1)	F R	TTCAGTTCGTGTGCGGAGAC CGTACCCTGTGGGCTTGTTG	ENSSSCT00000083279.1	present study
Ghrelin (GHRL)	F R	AGAAGACAGTGGTGAGGTGGAAG TGAACCGGATTTCCAGCTTG	ENSSSCT00000012660	present study
Insulin like growth factor binding protein 1 (IGFBP1)	F R	ACCGACATCAAGAAGTGGAAGG CACTTTGTAGAGTTCTCGCTGGC	ENSSSCT00000018209.3	present study
Gastric inhibitory polypeptide (GIP)	F R	CTCCTGGCAGTGGCGCTA GTGGAATCTGGAGTGACCCTCT	ENSSSCT00000030856.3	present study
Leptin (LEP)	F R	TTTCACACATGCAGTCTGTCTCC AAGTCCAAACCGGTGACCCT	ENSSSCT00015057529.1	present study
Interleukin 1B (IL1B)	F R	ATGCTGAAGGCTCTCCACCTC TTGTTGCTATCATCTCCTTGCAC	ENSSSCT00070040964.1	(Maruo et al., 2018)
Interleukin 6 (IL6)	F R	TTCACCTCTCCGGACAAAACTG TCTGCCAGTACCTCCTTGCTGT	ENSSSCT00000023544.3	(Gourbeyre et al., 2015)

Caspase 3 (CASP3)	F	ATAATAAGAACTTTGATAAAAATACCGGAATG	NM_214131.1	present study
	R	TCCACATCTGTACCAGATCGACAT		
Oxidative stress induced growth inhibitor 1 (OSGIN)	F	CATTGGCAACGGTCCC	ENSSSCT00000066181.2	present study
	R	TATAGCCTGAGAGCAGGTAG		
NADH dehydrogenase subunit 2 (MT-ND2)	F	GCCTCCACTATCAGGATTTATG	ENSSSCT00000019664.4	present study
	R	GGAGTAGGCTAGTCGTATGT		
Mitochondrially encoded cytochrome c oxidase III (MT-CO3)	F	GCACTAGGCGTATACTTCAC	ENSSSCT00000019677.1	present study
	R	CCCTGTAGCCACAAAGAAA		
Mitochondrially encoded cytochrome c oxidase I (MT-CO1)	F	CTTCCACCATCCTTCCTATTAC	ENSSSCT00000019670.1	present study
	R	GGGCTAAGTTTCCAGCTAAA		
ATP synthase F0 subunit 6 (MT-ATP6)	F	CACCCACCACACAACTATC	ENSSSCT00070061678.1	present study
	R	GTGTTCCTTGTGGTAGAAA		
Cytochrome b (MT-CYB)	F	TAGGAGACCCAGACAACTAC	ENSSSCT00000019689.3	present study
	R	GTAGAATAGCGTAGGCGAATAA		
Mitochondrially Encoded NADH Dehydrogenase 1 (MT-ND1)	F	CATACCCACGATTCCGATAC	ENSSSCT00000019660.4	present study
	R	AGGGAGTGAGATGTGTCATA		
RNA, Ribosomal 45S Cluster 2 (MT-RNR2)	F	TCCAGGTCGGTTTCTATCT	NC_026992.1	present study
	R	GGTAGGTCCCATTTCTCTTG		
Hypoxanthine phosphoribosyltransferase 1 (HPRT1)	F	CTGACCTGCTGGATTACA	ENSSSCT00065066899.1	present study
	R	CCCGTTGACTGGTCATTA		
Topoisomerase (DNA) II beta (TOP2B)	F	AAGGGCGAGAGGTCAATGAT	ENSSSCT00015080370.1	(Park et al., 2015)
	R	ACATCTTCTCGTTCTTGCGC		
Hydroxymethylbilane synthase (HMBS)	F	AGGATGGGCAACTCTACCTG	ENSSSCT00000060506.2	(Wang et al., 2018)
	R	GATGGTGGCCTGCATAGTCT		

2.7. *Statistical analysis*

Performance and feed conversion data were evaluated in the software R-3.5. Boxplots were used to visually inspect the data distribution, variability, and outliers. The model assumptions (normality, homoscedasticity, and independence) were inspected via the residual plots. Mixed Effects Models and Generalized Least Squares with subsequent multiple comparisons were used as indicated to test for differences between groups. The significance level was defined as $p < 0.05$, and $p \geq 0.05$ to <0.10 were considered as tendencies. All histology statistical analyses were performed using GraphPad Prism 9.0.2 software (GraphPad Software Inc., La Jolla, USA). Data were expressed as mean ± SEM (standard error of the mean) for normal distribution or median and interquartile range when data were not normally distributed. They were submitted to statistical analysis, using normality (Shapiro–Wilk) and homogeneity (Bartlett) tests. Significant differences were assessed by one-way ANOVA followed by Tukey's test for parametric data and by Kruskal-Wallis test followed by Dunn's test for non-parametric data. Statistical analysis for all other results were performed by GraphPad Prism 7.05 (GraphPad Software Inc., La Jolla, USA). Values were analyzed for normality (Shapiro–Wilk) as

well as homogeneity of variance (Levene Statistics). Normally distributed homogeneous data were analyzed by one-way ANOVA (Dunnett's multiple comparison test). If normal distribution was violated, the Kruskal-Wallis Test was used. Outliers were identified by Grubbs' test and removed, if applicable.

3. Results

3.1. *Growth performance*

During the entire study period, the mean body weight gain significantly differed between the groups (Mixed Effects Model, $p = 0.006$). Compared to the control, the weight gain was significantly lower in the EB+DON group (Mixed Effects Model, multiple comparisons, $p = 0.039$) and tended to be lower in the DON group ($p = 0.062$) (Figure 1A). The DON group did differ significantly in the feed consumption per cage during the entire study period if compared to the control group ($p = 0.001$). In the EB-DON group, a slight decrease in feed consumption was determined as well (by trend, $p = 0.076$) (Figure 1B).

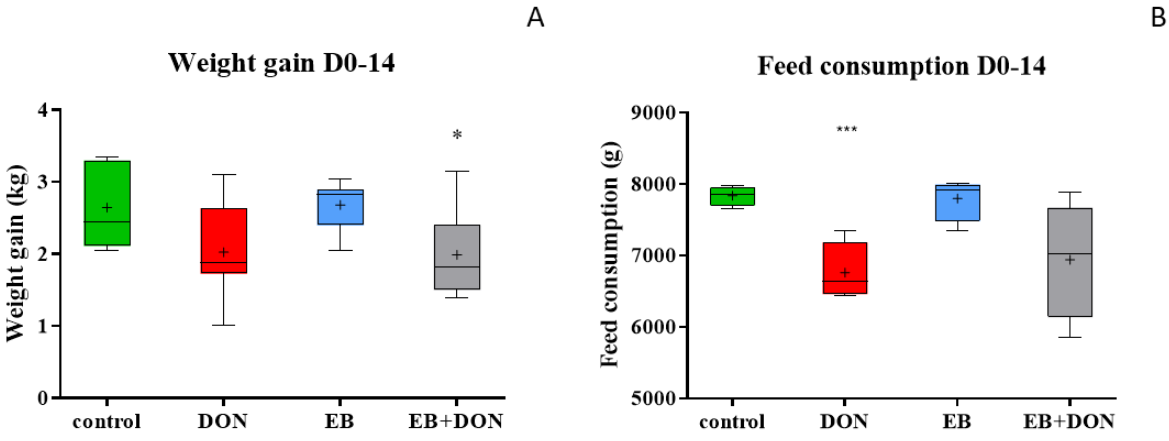


Figure 1. Boxplot of the body weight gain of piglets (**A**; n = 8) and feed consumption of two piglets per cage (**B**; n = 4) during a time period of D0-14. The box represents the interquartile range (IQR: 50 % of data are found between Q1 to Q3). A line within the box indicates the median. The lines/whiskers outside the box extend by Q1 - 1.5 × IQR (25 % of data) and Q3 + 1.5 × IQR (25 % of data). The + indicates the mean of the group. Significant differences to the control are indicated with * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.2. *Plasma parameters*

Plasma biochemical parameters revealing liver injury (activities of alkaline phosphatase, ALP; alanine aminotransferase, ALT; aspartate aminotransferase, AST), liver function (concentrations of albumin and total protein), lipid profile, kidney function and metabolism were assessed at D7 and D14 (Table 3).

On D7, a significant increase in albumin concentration was seen in piglets that received either of the EB-containing diets from 445.1 ± 31.4 and 453.4 ± 26.9 µmol/L, respectively compared to 404.6 ± 24.7 µmol/L in the control. Additionally, total protein was higher in the EB group (55.9 ± 3.0 g/L) compared to the control (50.3 ± 3.9 g/L). Plasma calcium was slightly, but significantly higher in piglets that received the EB+DON diet (2.46 ± 0.12 mmol/L) compared to the control (2.30±0.09 mmol/L).

On D14, the liver enzymes ALP, AST and ALT were affected by the mycotoxin-contaminated diets (Table 3). The most remarkable results were observed in the EB+DON group with a decrease of in 21 - 30% for each of the three enzymes compared to the control group.

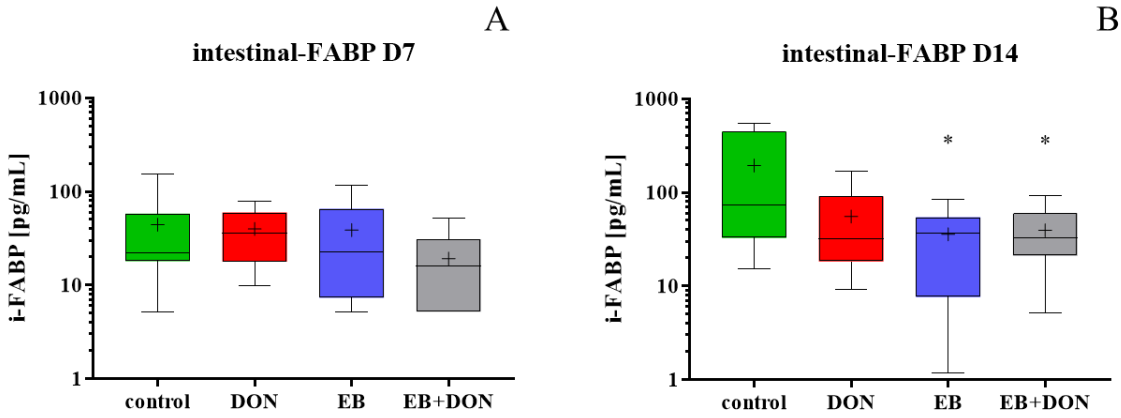
Table 3. Plasma biochemical analysis at D7 (left side) and D14 (right side)

	Animal groups (time and diet)							
	Control D7	**DON D7**	**EB D7**	**EB+DON D7**	**Control D14**	**DON D14**	**EB D14**	**EB+DON D14**
Alkaline phosphatase (U/L)	313.8±91.5	231.2±51.4	238.6±68.5	236.5±86.7	410.1±91.5	293.6±51.0*	326.3±94.8	*288.6±95.7**
Alanine aminotransferase (U/L)	32.6±5.3	31.6±4.3	31.4±7.1	31±6.8	57.8±9.4	49.4±8.35	45.6±5.9*	*45.8±9.4**
Aspartate aminotransferase (U/L)	33.1±8.5	34.8±5.9	31.8±7.6	28.3±5.3	49.3±7.4	46.8±6.0	40.8±6.0	*38.6±5.7**
Albumin (µmol/L)	404.6±24.7	437.5±38.2	*445.1±31.4**	*453.4±26.9**	430±42.7	453.4±18.0	443.2±14.6	461.4±28.2
Total proteins (g/L)	50.3±3.9	52.2±3.3	*55.9±3.0**	52.5±2.3	48.4±3.6	48.9±3.2	48.8±2.3	48.6±2.1
Urea (mmol/L)	0.95±0.21	1.90±1.27	1.91±1.14	1.74±0.68	0.82±0.173	0.86±0.28	0.55±0.12	1.06±0.3
Glucose PAP (mmol/L)	6.05±0.53	5.75±0.39	5.96±0.49	5.66±0.33	6.43±0.46	6.20±0.58	6.45±0.74	5.93±0.48
Cholesterol (mmol/L)	1.81±0.24	1.84±0.34	1.97±0.28	1.96±0.29	2.05±0.27	2.01±0.25	1.92±0.41	1.95±0.27
HDL (mmol/L)	0.97±0.13	0.94±0.14	1.06±0.14	1.05±0.14	1.15±0.11	1.13±.0.12	1.08±0.23	1.13±0.17
LDL (mmol/L)	0.65±0.12	0.71±0.20	0.73±0.13	0.71±0.15	0.79±0.14	0.77±0.18	0.74±0.16	0.71±0.10
Triglycerides (mmol/L)	0.47±0.05	0.43±0.08	0.70±0.36	0.49±0.10	0.4±0.10	0.40±0.08	0.35±0.06	0.48±0.14
Creatinine (µmol/L)	117.9±19.2	124.1±18.8	118.7±12.8	110.3±14.7	120.8±16.2	123.4±24.9	113.7±10.1	120.1±16.3
Uric acid (µmol/L)	13.9±7.5	15.0±6.8	15.9±8.8	17.4±6.5	19.4±14.7	18.6±13.6	18.9±10.9	17.5±9.2
Calcium (mmol/L)	2.30±0.09	2.36±0.05	2.39±0.07	*2.46±0.12**	2.64±0.16	2.64±0.11	2.59±0.13	2.55±0.13
Phosphorus (mmol/L)	2.24±0.10	2.22±0.19	2.32±0.15	2.39±0.20	2.77±0.16	2.84±0.17	2.80±0.22	2.85±0.24

Values are means ± SD. Comparison between control group and animals exposed to contaminated diets. n=8; Significant differences are indicated with * $p \leq 0.05$ (marked in red and italics).

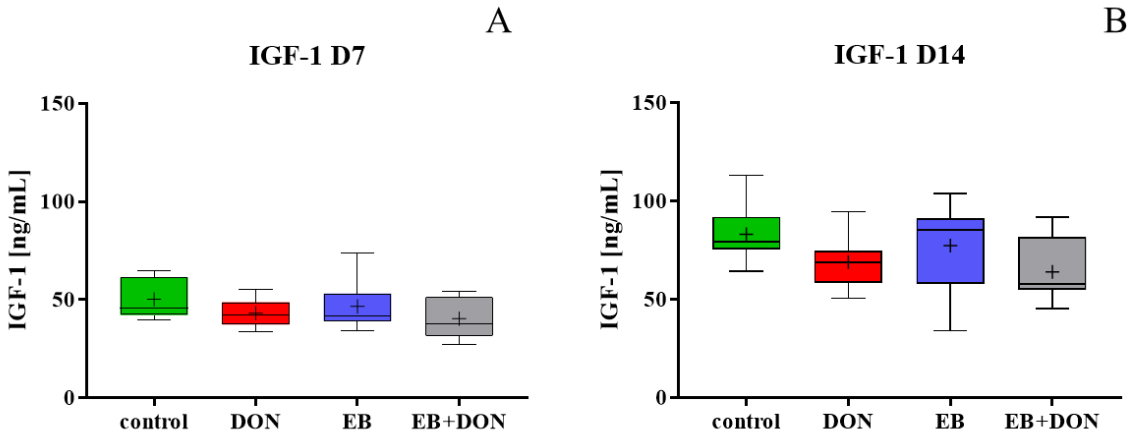
239 Potential biomarkers for gut health and growth, such as the intestinal-fatty acid binding protein (i-
240 FABP), the tight junction protein regulator zonulin (ZON) and the insulin-like growth factor 1 (IGF-1)
241 were analyzed in heparin plasma samples taken on D7 and D14 (Figures 2 – 4).

242 As shown in Figure 2, the i-FABP concentration was significantly decreased in the EB group (p =
243 0.028) and the EB+DON group (p = 0.032) on D14 compared to the control. This protein was also
244 reduced in the DON-fed group, but not significantly (p =0.059). IGF-1 protein, which might be a
245 parameter for growth reduction, was not significantly affected by the different diets on any sampling
246 day. However, p-value between control and EB+DON group was 0.092 on D14, which could indicate a
247 declining trend. Likewise, a decrease of in IGF-1 mean values in the DON group on both days is evident,
248 from 50.1 ng/mL (control) to 43.1 ng/mL (D7) and from 83.1 ng/mL (control) to 68.9 ng/mL,
249 respectively (Figure 3). ZON, described as permeability marker in chronical bowel diseases, did not
250 vary between the groups, although a decreasing trend was seen in the DON group (p = 0.099) on D14
251 (Figure 4).



252

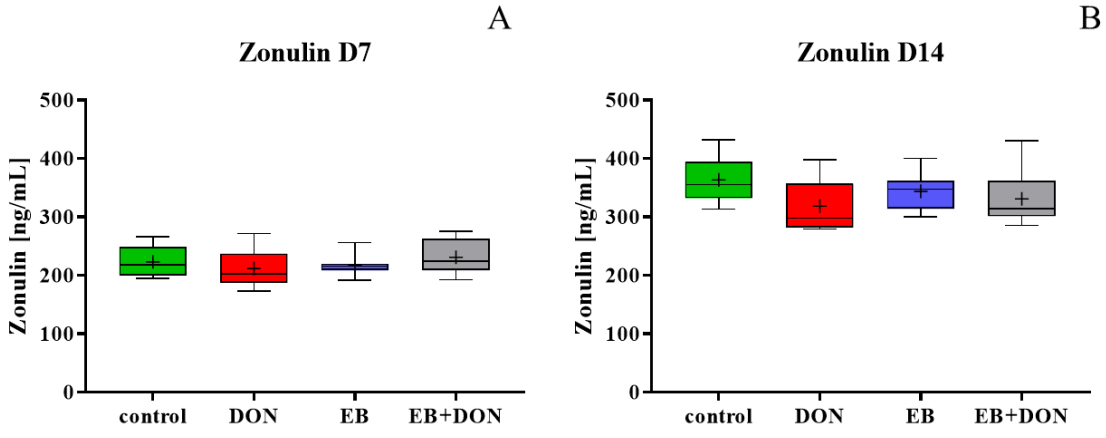
Figure 2. Intestinal-fatty acid binding protein (pg/mL) measured in heparin plasma samples from D7 (A) and D14 (B); n = 8; Significant differences are indicated with * $p \leq 0.05$.



253

254 **Figure 3**. IGF-1 concentration (ng/mL) measured in heparin plasma samples from D7 (A) and D14 (B);
255 n = 8.

256
257



258

Figure 4. Zonulin concentration (ng/mL) measured in heparin plasma samples from D7 (A) and D14 (B); n= = 8.

3.3. *mRNA expression in jejunum and liver tissue*

The mRNA expression of genes related to immune response (NOS2, IFNG, AIFM1, SOD1, TP53, TNFA, TLR9, IL1A, NFKB1, MAPK, TGFB1, IL10, IL8) as well as to satiety and growth (PYY, CCK, IGF1, GHRL, IGFBP1, GIP, LEP) was analyzed in the jejunum. The results are presented in Figure 5. Expression profile of most genes showed a slight, but not significant upregulation in the DON group or the EB+DON group.

In the liver tissue, the expression of genes coding for inflammatory mediators (IL1B, AIFM1, TP53, TNFA, SOD1, TGFB1, IL6, CASP3, OSGIN1) and of mitochondrial genes (MT-ND2, MT-CO3, MT-CO1, MT-ATP6, MT-CYB, MT-ND1, MT-RNR2) was affected but to a lesser extent (Figure 6). Oxidative Stress Induced Growth Inhibitor 1 (OSGIN1) tended to be up-regulated in the two groups receiving DON.

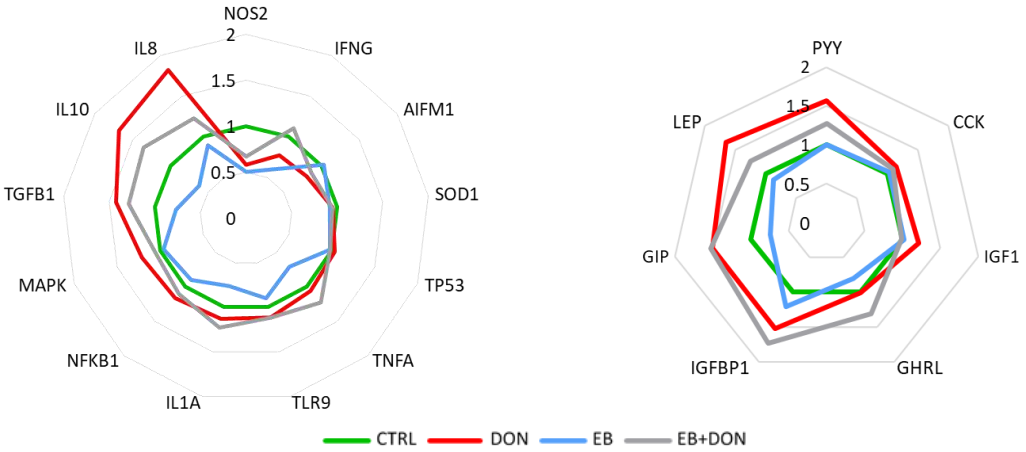


Figure 5. mRNA expression of selected genes in the jejunum of animals fed a control or contaminated diet. Mean values of gene expression levels for the control group are set to 1.

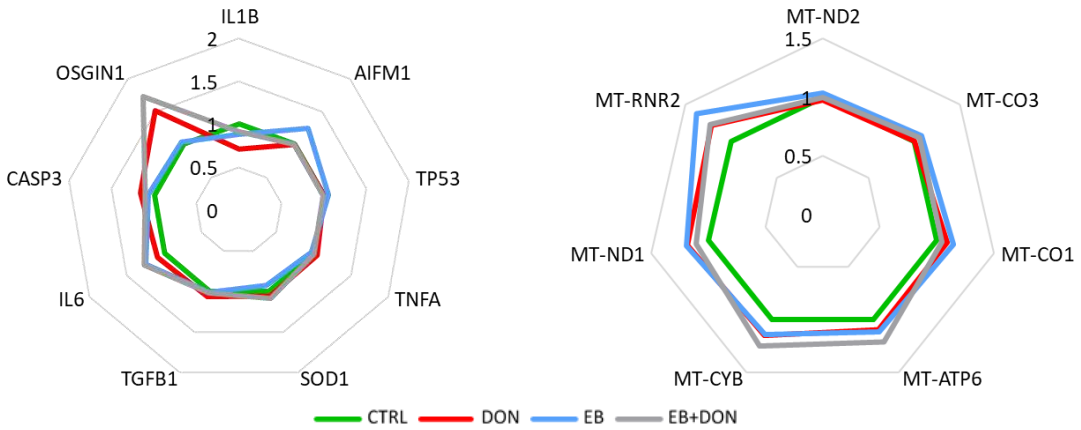


Figure 6. mRNA expression of selected genes in the liver of animals fed a control or contaminated diet. Mean values of gene expression levels for the control group are set to 1.

3.4. *Histology*

In jejunum, a significant increase ($p = 0.007$) in the lesion score was detected in animals fed the multi-toxin contaminated diet (EB+DON) compared to the control (Figure 7A). Control animals showed well-delineated villi lined by columnar enterocytes (Figure 7B), while changes such as lymphatic vessel dilation and interstitial edema with mild intensity were also observed in these animals. Animals fed the DON-contaminated diet showed villi atrophy, as well as flattening and cytoplasmic vacuolation of enterocytes (Figure 7C). In animals that received the EB diet, cytoplasmic vacuolation of enterocytes was the most frequently observed change (Figure 7D). Animals receiving the EB+DON-contaminated diet showed mainly villi atrophy and vacuolation of enterocytes (Figure 7E). A significant decrease in the number of goblet cells in the jejunum was observed in DON-fed animals ($p = 0.029$, Figure 8A). Furthermore, villi height was decreased in the DON- ($p = 0.008$) and EB+DON-fed ($p = 0.022$) groups (Figure 8B).

Colon samples were analyzed, however, no significant difference was observed in the lesion score between treatments (Figure 9A). The control group showed a normal histological structure (Figure 9B). The main histological findings in pigs fed the mycotoxin-contaminated diets were edema of the lamina propria, flattening and cytoplasmic vacuolation of apical enterocytes as well as focal areas of enterocytes necrosis (Figure 9C-E).

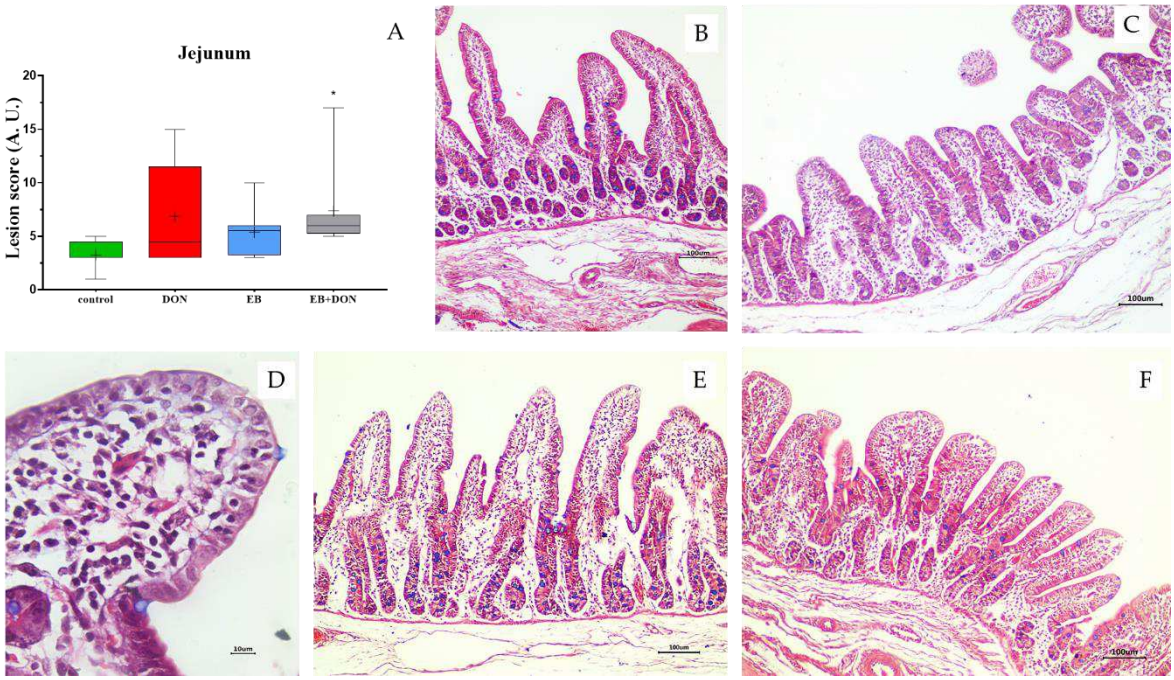


Figure 7. Lesion score of jejunum tissue (**A**, n = 8) and histological images of experimental groups. Control (**B**) showing a normal aspect of villi; DON (**C**) with villi atrophy and edema of lamina propria; EB (**D**) showing enterocyte flattening; EB+DON showing edema of lamina propria (**E**) and villi atrophy (**F**). Alcian blue staining. Scale bar 100 µm (B, C, E, F), 10 µm (D). Significant differences are indicated with * $p \leq 0.05$.

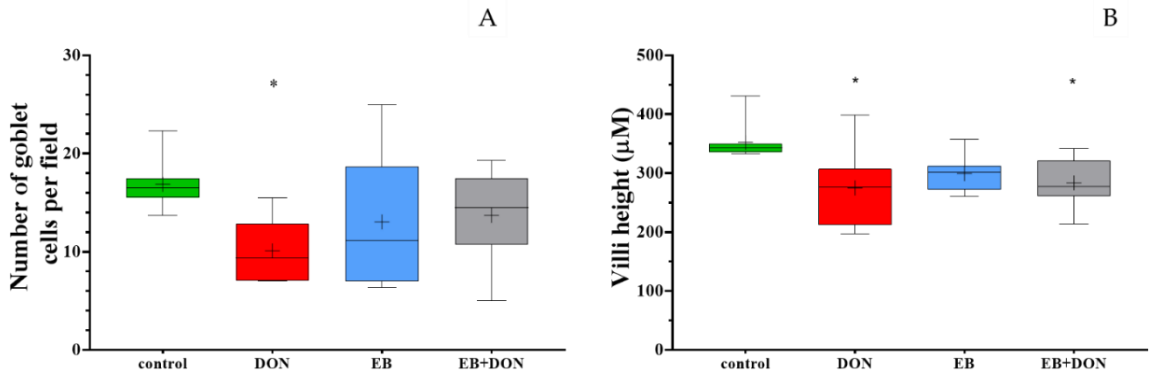


Figure 8. Number of goblet cells per field (**A**) and villi height (**B**) in the jejunum (n = 8). Significant differences are indicated with * $p \leq 0.05$.

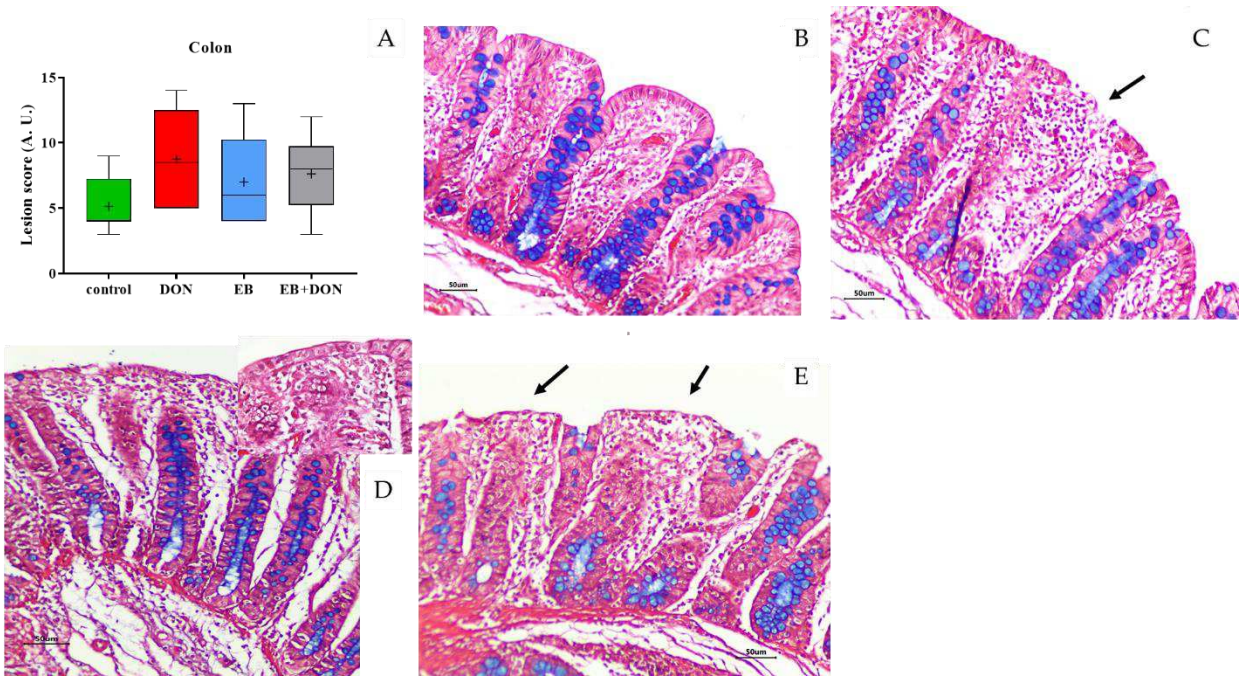


Figure 9. Lesion score of colon tissue (**A**). Control: Normal aspect of colon (**B**). DON: Edema of the lamina propria, focal necrosis of enterocytes (arrow) (**C**). EB: Edema of lamina propria, flattening of enterocytes. Insert: Flattening of enterocytes (**D**). EB+DON: Multifocal flattening of enterocytes and necrosis (arrows) (**E**). Alcian blue staining. Scale bar 50 µm.

In the liver, animals fed EB and EB+DON diets showed a significant increase ($p = 0.043$ and $p = 0.011$, respectively) in histological changes when compared to the control (Figure 10A). Control animals showed well-organized hepatocytes forming trabeculae, while mild cytoplasmic vacuolation of hepatocytes was frequently detected in this group (Figure 10B). Animals receiving the contaminated diets showed mainly moderate to severe vacuolation of hepatocytes and megalocytosis (Figure 10C-F). Apoptosis of hepatocytes and focal necrosis were also detected in DON and EB+DON groups (Figure 10C+E).

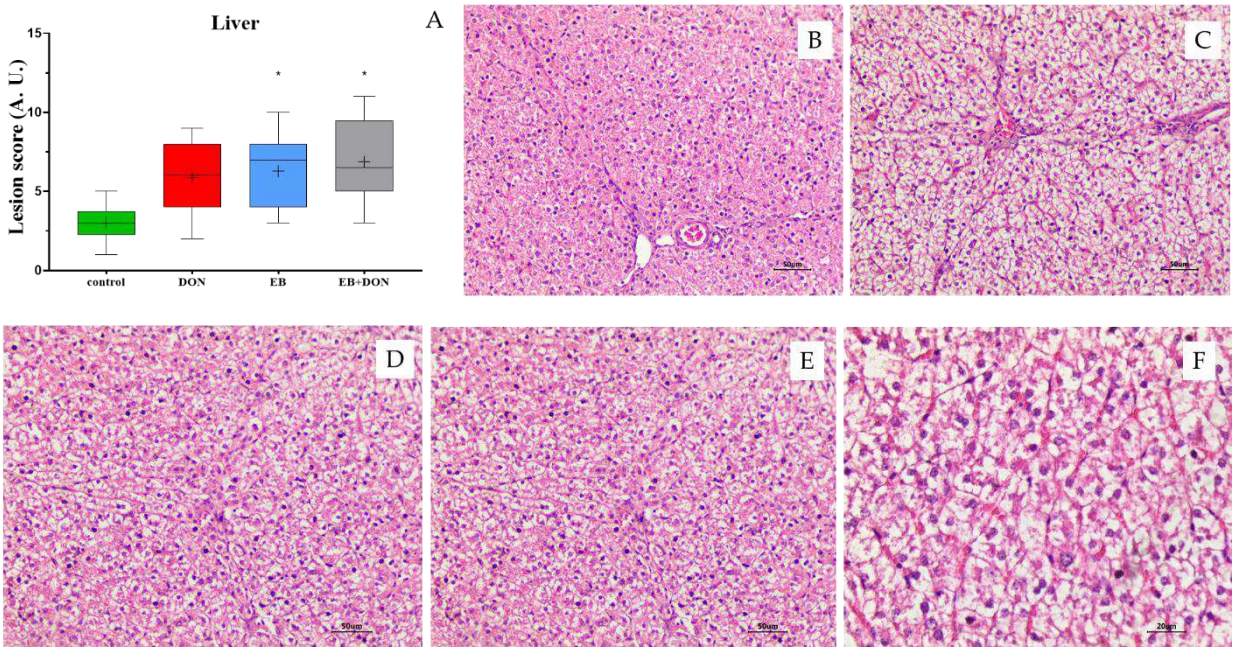


Figure 10. Lesion score of liver tissue (**A**) and histological images of control (**B**) showing a normal aspect of liver; DON (**C**) showing diffuse and severe vacuolar degeneration of hepatocytes; EB (**D**) showing moderate vacuolar degeneration of hepatocytes; EB+DON showing moderate vacuolar degeneration of hepatocytes (**E**) and megalocytosis (**F**). Hematoxylin and Eosin (H&E) staining. Scale bar 100 µm (**B-E**), 20 µm (**F**). Significant differences are indicated with * $p \leq 0.05$.

A significant increase in histological changes ($p = 0.022$) was detected in lymph nodes of animals exposed to DON in comparison to control. Lymph nodes of control animals presented a histological aspect within normal limits. In pigs fed the DON or EB+DON contaminated diets, the main findings were lymphoid depletion and apoptosis, while in the EB group lymphoid hyperplasia was the most frequent change (Figure 11).

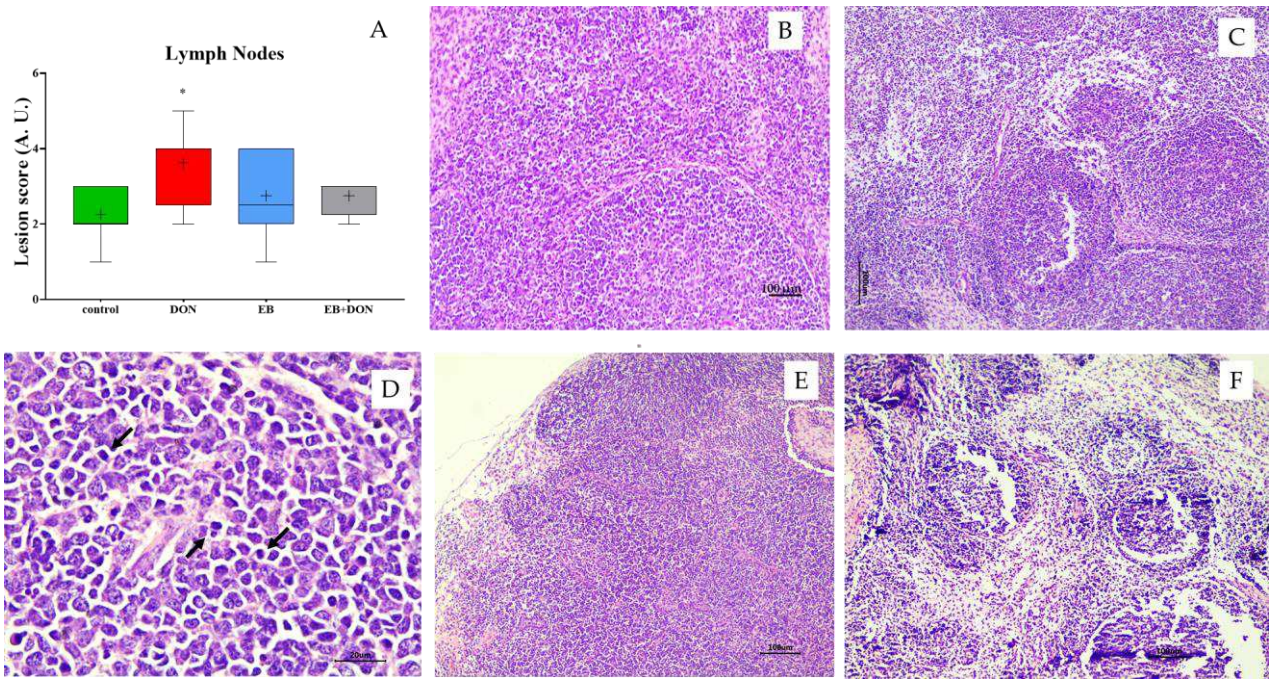


Figure 11. Lesion score of lymph nodes tissue (**A**) and histological images of control (**B**) showing a normal aspect of lymph node; DON showing lymphoid depletion (**C**) and lymphocyte apoptosis (**D**); EB (**E**) showing lymphoid hyperplasia; EB+DON (**F**) showing severe lymphoid depletion. Hematoxylin and Eosin (H&E) staining. Scale bar 100 µm (**B, C, E** and **F**), 20 µm (**D**). Significant differences are indicated with *p ≤ 0.05.

3.5. *Metagenomic data of the fecal microbiome*

Mycotoxin applications substantially impacted the diversity and structure of fecal microbiota in weaned piglets. Shotgun metagenomics of 32 collected fecal samples using the MK1C platform generated over 11 million 1D reads with the quality score > 8 with a mean number of about 300 k reads per sample. Taxonomic profiling of the fecal samples in terms of the most abundant microbial phyla and species is shown in Figure 12. In total, 1,945 different microbial species were observed, of which Firmicutes, followed by Ascomycota and Proteobacteria, were the most dominant members of the piglet's fecal microbiota. Interestingly, the phyla Actinobacteria was more dominant in the EB group which goes along with a reduction in Ascomycota (Figure 12A). In regard to the most abundant species, *Aureobasidium namibiae* completely disappeared in the EB and EB+DON groups, while the *Mycobacterium branderi* is found in a remarkable amount in the EB group, but not in the others (Figure 12B).

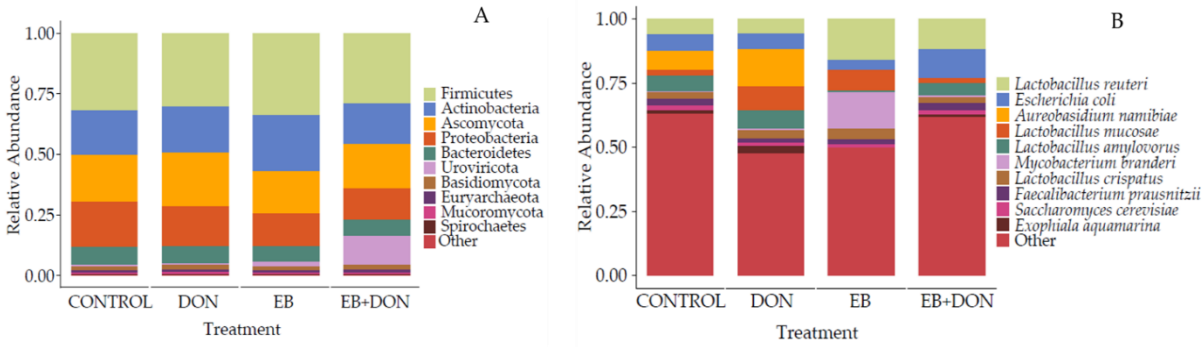


Figure 12. Relative abundance of 10 most abundant microbial phyla (**A**) and microbial species (**B**) found in the fecal microbiome of pigs fed with the different diets.

Permutational Multivariate Analysis of Variance (PERMANOVA) statistical analysis calculated on Bray–Curtis dissimilarity matrices from Kraken2 and Bracken data showed that the difference in the treatment groups of the piglets explained 14% of the variation in fecal microbiota profiles of the piglets in the current study (R2 = 0.13351, p = 0.005), showing a significant impact of the applied mycotoxins on the structure and community membership of the piglet's fecal microbiota. Furthermore, pairwise post-hoc comparisons (https://rdrr.io/github/GuillemSalazar/-EcolUtils/man/adonis.pair.html) showed that among different groups, fecal microbiota of the piglets in the EB group showed the most significant difference in the profiles of the relative abundance of taxa compared to the animals in the control group (control vs EB, R2= 0.10, FDR-corrected p = 0.005), followed by DON (control vs DON, R2= 0.072, FDR-corrected p = 0.02) and EB+DON (control vs EBD, R2= 0.068, FDR-corrected p = 0.02). In line with the observation from the beta diversity analysis, a significant effect of mycotoxins contamination on the alpha diversity indices was observed, with the EB+DON resulted in a significantly lower microbial diversity (measured by Shannon index, p = 0.004 and 0.088, respectively) and richness (measured by Observed index, p = 0.035 for EB) in the fecal microbiota of the piglets (Figure 13).

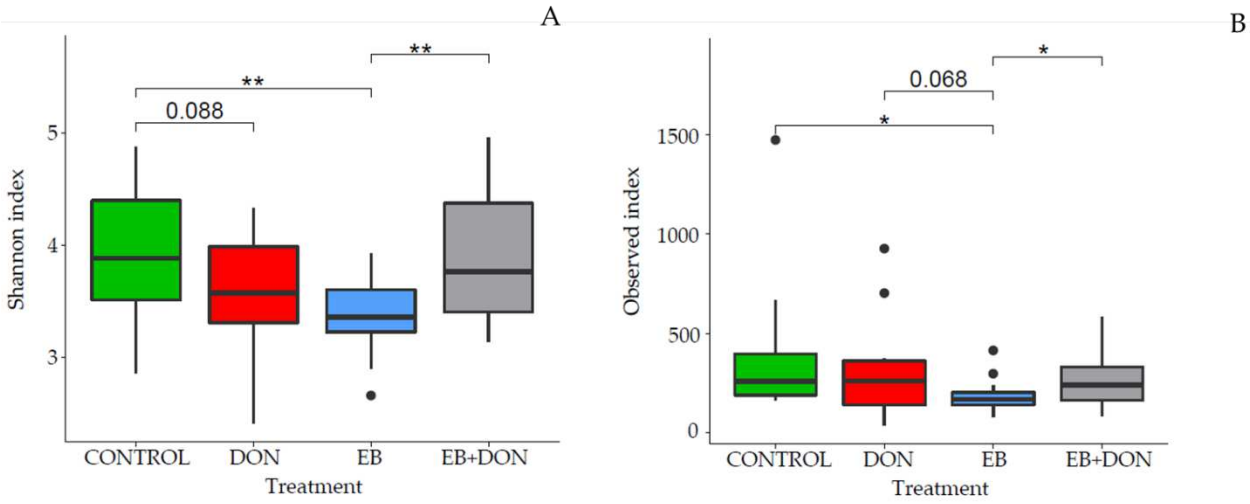


Figure 13 Alpha diversity analysis of the taxa in pig fecal microbiome (**A**: Shannon index, **B**: observed index); n= = 8; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

4. Discussion

In this study, we investigated, for the first time the effects of a diet contaminated with the emerging mycotoxins enniatin B, enniatin B1 and beauvericin (EB) on piglets. As enniatins (ENNs) and BEA frequently co-occur with the regulated *Fusarium* mycotoxin DON (Kovalsky et al., 2016; Lindblad et al., 2013), we included a combinatory group to determine possible combined effects. We used a BEA concentration of 2570 µg/kg and 3578 µg/kg, as well as a joint ENN B and ENN B1 concentration of 1345 µg/kg and 1830 µg/kg in the EB and EB+DON group, respectively. In the field, BEA is found in rather low concentrations (~100 µg/kg), however, also highly contamination up to 26300 µg/kg were already determined in specific feed stuff and countries. Regarding ENN B and B1, the usual contamination is higher with detected maximum levels of 81100 µg/kg and 795000 µg/kg, respectively (Fraeyman et al., 2017). However, concentrations are difficult to compare as many variables have to be considered such as feed stuff, country region, detection analytical method, sampling as well as weather and storage conditions.

As a first indicator, we determined weight gain and feed consumption of the animals. In the 14 experimental days, the weight gain of 2.64 ± 0.56 kg in the control group was significantly decreased to only 1.99 ± 0.60 kg in the EB+DON group but remained constant in the EB group with 2.68 ± 0.34 kg. As with 2.03 ± 0.66 kg the weight gain in the DON group was also low (by trend, Figure 1A), the observed negative effect of EB+DON derived due to an enhanced intake of DON through the gut barrier,

377 triggered by the ionophoric properties of the emerging mycotoxins (Tonshin et al., 2010). Also *in vitro*
378 experiments showed synergistic effects of ENNs with DON on the gut integrity in intestinal porcine
379 epithelial cells (Springler et al., 2016). The direct reason might be the reduced feed consumption in both
380 DON groups. Animals which received the DON-contaminated diet ate 6763 ± 404 g of feed on average;
381 EB+DON-fed animals only 6943 ± 832 g (Figure 1B). A significant difference was detected only for
382 the DON group compared to the control (7839 ± 135 g). A lower consumption of DON-contaminated
383 feed has been described by several other scientists (FAO/WHO, 2001; Prelusky, 1997; Rotter et al.,
384 1996; Trenholm et al., 1994) and is believed to be a consequence of the impact of DON on satiety
385 hormones, such as peptide YY (PYY) and cholecystokinin (CCK) (Flannery et al., 2012; Knutsen et al.,
386 2017). However, only a slight, but not statistically significant up-regulation of the expression of genes
387 coding for satiety and growth hormones was observed (Figure 6), which could be a matter of sampling
388 time point (Taylor et al., 2019). One of the most remarkable effects of DON is the anorexia and also
389 emesis that are elicited by the direct action on the central nervous system and by the indirect action of
390 enteroendocrine cells that secretes several gut hormones (Terciolo et al., 2018).

391 In general, due to the inter-individual variability and the limited number of animals in this trial, the
392 number of genes whose expression was significantly regulated by the ingestion of a mycotoxin-
393 contaminated diet was limited. However, the tendency of some genes to be upregulated was consistent
394 with previously described effects of DON. Increased production of cytokines upon DON exposure has
395 already been demonstrated in human cells (Maresca et al., 2008), in porcine intestinal cells (Cano et al.,
396 2013), in jejunal explants from pigs (Pierron et al., 2016b) as well as *in vivo* in pigs and mice (Azcona-
397 Olivera et al., 1995; Bracarense et al., 2012, 2020; Pierron et al., 2018). In the present study, a 2-weeks-
398 week exposure to ~2 ppm DON showed a tendency to elicit a pro-inflammatory response. As
399 zearalenone (ZEN) concentration with 342 and 263 µg/kg, respectively, in both DON groups was quite
400 high, an impact of ZEN on inflammation mediators could be possible (Pistol et al., 2015). However,
401 clinical effects on estrogenic parameters such as an estrogenic effect increased, swollen vulva in female
402 pigs as described for ZEN were not detected (Gajecki, 2002). Since ZEN is not known to elicit any
403 effect on our analyzed parameters, we assume that our findings are mainly caused by EB and/or DON.
404 Most interestingly, co-exposure to EB seemed to reduce the inflammatory potential of DON and led to

405 a down-regulation of cytokines (Figure 6), possibly due to their ionophoric properties disrupting several
406 functions of cells (EFSA, 2014). Antagonistic cytotoxic effects of DON and EB have already been
407 described in IPEC-1 cells (Khoshal et al., 2019). Our results, both on feed consumption and growth and
408 on mRNA expression, corroborate the indirect action of hormones on the anorexic mechanism of DON
409 and, again, the association with EB partially antagonized this effect. Recent studies have highlighted the
410 involvement of mitochondrial genes in the acute toxicity of DON (Wang et al., 2021), BEA and ENNs
411 (Alonso-Garrido et al., 2018; Escrivá et al., 2018, 2019) in cultured cells. Our experiment indicates that
412 the expression of these genes was also slightly exacerbated upon *in vivo* chronic exposure to the toxins
413 (Figure 6).
414 Besides tissue samples from different organs, we also took plasma samples at the middle and at the end
415 of the trial. Increased concentrations of albumin and total proteins were observed in pigs exposed to EB
416 at D7 (Table 3). Co-exposure to EB and DON showed a similar effect on albumin but not on total
417 proteins (Table 3). Albumin has a turnover of about 25 days and there is a relative consensus in the
418 literature that hyperalbuminemia, an increase of in the albumin concentration in serum is mainly due to
419 dehydration (Busher, 1990; Levitt and Levitt, 2016). Increased serum albumin levels in our trial could
420 be due to the ionophoric effect of ENNs and BEA on cell membranes leading to a disturbance of osmosis.
421 The lack of increase of in total protein concentration in the EB+DON group could be related to the well-
422 characterized inhibitory effect of DON on protein synthesis after its binding to the ribosome (Payros et
423 al., 2016). The increase in albumin level in the EB+DON group accompanied an increase of in
424 circulating calcium (Table 3). The lack of an increase in calcium in the EB group could be due to the
425 fact that BEA increases cytoplasmic calcium concentration, thereby reducing the amount of circulating
426 calcium (Chen et al., 2006). These effects seem to be temporary since no differences between the groups
427 were seen at D14.
428 At D14, the ALP concentration was decreased in DON-receiving groups (Table 3), although usually
429 an increase in liver enzyme activity is associated with liver damage(Giannini et al., 2005). An
430 explanation could be that enzymes increase rapidly after the first days of exposure and on D14, the
431 activity has already dropped to a lower level. A positive correlation between food consumption and
432 serum ALP was reported previously (Amacher, 2002). So, the decreased feed consumption may have

led to a reduction in serum ALP in this study. Another reason could be a zinc deficiency elicited by an inadequate absorption and which correlates with a low ALP (Cho et al., 2007). ALP activity is a good marker of intestinal function. Indeed, it was shown to be higher in rat intestinal mucosa than in liver (Ramaiah, 2007). ALP participates in the metabolism of vitamin B6 which is a co-factor for both ALT and AST (Ono et al., 1995). In fact, both groups exhibiting ALP reduction (DON, EB-DON) also presented reductions in ALT and AST activity (Table 3). (EB-DON). Although an increase in liver enzyme activity is associated with liver injury, also reductions on ALT and AST activities have been seen in cases of chronic liver diseases usually associated with other pre-existent condition such as kidney disease (Ono et al., 1995).

We additionally analyzed the effects of mycotoxin-contaminated diets on proteins in porcine plasma associated with growth or gut barrier functionality, i.e. i-FABP, IGF-1 and ZON, in the porcine plasma. IGF-1 is known to correlate with growth performance and a reduction of this biomarker has been already described upon DON exposure (Pestka, 2010; Voss, 2010). However, in our study, no significant impact of mycotoxin-contaminated diets on IGF-1 in serum was seen (Figure 4), although trends were determined in the DON group on both sampling days as well as in the EB+DON group on D14. Additionally, the IGF-1 mRNA expression in jejunum was slightly increased (Figure 5), which is in accordance with a previous study in mice, in which a higher gene expression in the liver was observed, but circulating IGF-1 was decreased (Amuzie and Pestka, 2009).

ZON, a widely-discussed biomarker for mucosal barrier integrity (Fasano, 2020), has been described in humans suffering from inflammatory bowel disease (Caviglia et al., 2018). In DON-fed piglets a significant increase in ZON was associated with the damaging effect of this mycotoxin on the gut barrier (Wang et al., 2018). In contrast to our expectations, we observed a slightly lower ZON level in the DON group (318.7 ng/mL) compared to the control group (363.6 ng/mL). This has also been shown in another study in patients with chronic kidney diseases and the authors of this study hypothesized that low ZON concentration is caused by impaired defensive mechanisms due to a suppressed immune system (Lukaszyk et al., 2018).

As another biomarker for intestinal permeability, we analyzed i-FABP, which is considered to be released into the blood stream from damaged mucosal tissue (Funaoka et al., 2010). Again, we detected

461 a significant decrease of in i-FABP in both EB-containing diets on D14, instead of the expected increase
462 (Figure 2). However, Lau *et al.* also reported an unexpected decrease inof i-FABP plasma levels with a
463 parallel increased gene expression in the jejunum (Lau et al., 2016). They hypothesized that increased
464 i-FABP levels could be a result of an increased production by enterocytes rather than tissue damage.
465 Furthermore, EB inhibit protein biosynthesis (Olleik et al., 2019) and thereby, might lead to a reduced
466 i-FABP plasma concentration.

467 As blood parameters alone can be difficult to interpret due to limitations in methods, dependence
468 on sampling time point and choosing the right parameter (Celi et al., 2019), we additionally investigated
469 the extent of histological lesions in selected organs. As the intestine and the liver are the main sites of
470 drug metabolism (Chhabra, 1979), we focused on jejunum, colon, liver and additionally on lymph nodes.
471 DON-containing diets led to significant changes in the jejunum seen as villi atrophy, flattening and
472 cytoplasmic vacuolation of enterocytes, reduced number of goblet cells and shorter villi (Figures 8A+B).
473 This is in accordance with several other studies (Bracarense et al., 2012; Pinton and Oswald, 2014;
474 Gerez et al., 2015; Przybylska-Gornowicz et al., 2018). The most frequent change observed in EB-fed
475 animals was vacuolation of enterocytes. This change may be associated with the ionophoric properties
476 of ENNs and BEA resulting in an imbalance of cell membrane permeability. Enniatins have the ability
477 to incorporate into cell membranes forming cation selective pores with high affinity to Na+ (Kouri et
478 al., 2003). Increases in cytosolic Na+ is followed by intracellular water rise and cell swelling (Myers et
479 al., 2012). No significant effects were seen in the histological part of colon tissue between the different
480 groups. Most likely, the parent fungal compounds were already metabolized into less-toxic substances
481 as known for DON and its metabolite deepoxy-DON (DOM-1) (Bracarense et al., 2020). In general, the
482 effects of EB were more significant on liver tissue than in the intestine (Figures 7 – 10), which might be
483 due to rapid absorption known for ENN B1, and its fast metabolism in the liver (Devreese et al., 2014).
484 Piglets receiving the EB-diets showed moderate to severe vacuolation of hepatocytes and megalocytosis
485 (Figure 10). This could be due to a possible bioaccumulation of lipophilic ENNs and BEA in fat-rich
486 tissue as seen in mice (Rodríguez-Carrasco et al., 2016) In addition, the administration of 50, 100 or 200
487 mg/kg b.w. of BEA or ENN B by oral gavage in mice induced mild toxic effects in the liver (Maranghi
488 et al., 2018). However, those doses are very high and perhaps mice may be more tolerant to these

mycotoxins than pigs, but it is hard to make this conclusion confirm due to the lack of *in vivo* studies with emerging mycotoxins. In total, apoptosis and necrosis were detected only in animals fed the DON-diets. An increase in the lesion score of piglets fed with DON-diets was reported previously (Mikami et al., 2010; Pierron et al., 2018; Bracarense et al., 2020), but apoptosis was not present in the liver of rats despite the increased lesion score in this organ (Bracarense et al., 2017). In lymph nodes, a significant increase in the histological score was detected in DON-exposed animals showing lymphoid depletion and apoptosis (Figure 11). This is in accordance with several studies performed by our group, which found that DON increased the lesion score in the lymph nodes in both, piglets and rats (Bracarense et al., 2017, 2020; Gerez et al., 2015; Mikami et al., 2010; Pierron et al., 2016). In this study, lymphoid hyperplasia was frequent in the EB group, but the lesion score did not differ from the control group (Figure 11).

The antibiotic activity of ENNs and BEA against mostly several gram-positive bacteria and fungi due to their ionophoric properties has already been studied (Olleik et al., 2019; Sy-Cordero et al., 2012), and could explain differences in fecal microbiome between groups in our study. The fecal microbiome of animals receiving the EB diet showed a lower diversity compared to the control group, but also compared to the EB+DON group. Therefore, it is tempting to speculate that DON antagonized the antibiotic effect of EB. Furthermore, a change in the microbial pattern was seen in all mycotoxin-containing groups (Figure 12). This life stage of pigs, only two weeks after weaning, is crucial as the microbial flora is not fully developed which can lead to higher susceptibility toto diarrhea, weight loss and mortality (Rhouma et al., 2017). The importance of the intestinal gut microbiome has been studied more extensively in the last decade due to development of the ”omics” methodologies. Therefore, we already know the strong correlation between a high diverse microbiota and appropriate functions such as enhanced feed efficiency, production of volatile fatty acids, resistance against pathogenic bacteria and improved immune system (Ghanbari et al., 2019; Guevarra et al., 2019; Kraler et al., 2016; Stokes, 2017; Yang et al., 2017). One study showed that DON, applied in a concentration of 2.8 mg/kg, changed the microflora balance and modified the richness index in 9-week-old pigs, but did not influence the diversity (Waché et al., 2009). In our study, DON alone also led to a slight decrease of in the diversity of taxa, but not significantly ($p = 0.088$) and in the EB+DON group no effect was found (Figure 13). A

517 higher translocation of a pathogenic *Escherichia coli* strain was seen *in vitro* using intestinal porcine
518 cells (IPEC-1), when treated with DON (Pinton et al., 2009). The relative abundance of *E. coli* in the
519 DON group was not increased (Figure 12). However, in animals that received DON+EB, an increase in
520 *E. coli* abundance was seen. Another remarkable alteration in EB-fed piglets was the increase of in
521 *Actinobacteria* and *Mycobacterium branderi* with a parallel decrease in *Ascomycota* and the species
522 *Aureobasidium namibiae*. An alteration of the mycobiome (the fungal part of the microbiome) has been
523 associated with several diseases in humans (Chin et al., 2020). There is not much literature about the
524 species *M. branderi*, belonging to the phylum *Actinobacteria*, except that it is a potential pathogen first
525 isolated from human respiratory tract (Koukila-Kahkola et al., 1995). Also, the abundant *Lactobacillus*
526 species *L. amylovorus* known to exhibit positive probiotic activities in weaning piglets (Konstantinov et
527 al., 2006) was reduced only in the EB-fed group, but not in the EB+DON group. On the other hand, the
528 relative abundance of *L. reuteri* was increased in the EB group and also, to a lesser extent, in the
529 EB+DON group compared to the control and the DON group. *L. reuteri* is a lactic acid bacterium and
530 proven to reduce *Cryptosporidium parvum*-induced diarrhea in piglets (Casas and Dobrogosz, 2000).
531 This is in contrast to a recent publication (Reddy et al., 2018), in which *Lactobacillus* was significantly
532 more abundant in piglets fed a DON-diet (7.38 mg/kg) compared to the control. However, in this study
533 8-week-old piglets were used for a 4-week trial with a quite high DON contamination, which is in
534 contrast to our approach.

535 ***5. Conclusion***

536 All together it could be seen that a contaminated diet including EB led to a decrease in the diversity
537 of the gut microbiome as well as to a shift in certain fungal, viral, and bacterial genera in weaning piglets.
538 Interestingly, these effects were not so evident when DON was additionally included in the diet, which
539 indicates a combinatory effect. The EB+DON combinatory diet of EB+DON significantly decreased the
540 weight gain within the short period of 14 days, which was not seen in the EB or DON groups.
541 Furthermore, all mycotoxins-receiving animals showed moderate to severe histological changes in the
542 intestine, the liver, and the lymph nodes to different extents, although plasma biomarkers were not as
543 clearly affected. Our study shows that the emerging mycotoxins alone, but also with the frequently co-

occurring metabolite DON, impact the growth performance, lead to organ lesions and to an alteration of microbial community composition for which later consequences have to be in the focus of further studies. Therefore, regarding the high prevalence and co-occurrence of EB with regulated mycotoxins in different feed commodities, more *in vivo* results must be generated also in other animal species to provide sufficient data for a reliable risk assessment.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, B.N., A.L., P.P. and D.S.; methodology, B.N., A.L., M.N. and C.E.; validation, B.N. and A.L.; formal analysis, B.N., A.L., M.G., A.B., V.M. and P.P.; data curation, B.N., A.L., M.G., A.B., V.M. and P.P.; writing—original draft preparation, B.N. and A.L.; writing—review and editing, B.N., A.L., A.B., S.W., P.P. and IPO.; visualization, B.N. and A.L.; supervision, IPO. and D.S.; project administration, B.N. and A.L.; funding acquisition, B.N., A.L., IPO and P.P. All authors have read and agreed to the published version of the manuscript.

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7 ARTIGO B – DEOXYNIVALENOL INDUCES APOPTOSIS AND INFLAMMATION IN THE LIVER: ANALYSIS USING PRECISION-CUT LIVER SLICES

Deoxynivalenol induces apoptosis and inflammation in the liver: Analysis using precision-cut liver slices

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ABSTRACT: Deoxynivalenol (DON) is one of the most common mycotoxins in cereals and their by-products. Its adverse effects on animal and human health have been extensively studied in the intestine, but little attention has been paid to another target organ for mycotoxins, the liver, which is potentially exposed after intestinal absorption and enterohepatic circulation. To assess DON's toxicity in an ex vivo model structurally and physiologically closer to the whole liver, we developed a pig precision-cut liver slices (PCLS) model. PCLS contain all cell types and maintain intercellular and cell-matrix interactions, among other architectural features of the liver. The human HepG2 cell line was used for comparison. We observed that after a short exposure, DON reduced the cell viability of HepG2 and induced the expression of genes involved in apoptosis, inflammation, and oxidative stress. When PCLS were exposed to DON, damage to the tissues was observed, with no changes in markers of liver function or injury. Exposure to the toxin also triggered liver inflammation and apoptosis, effects already observed in pigs fed a DON-contaminated diet. Overall, these data demonstrate that DON had toxic effects on a liver cell line and on whole liver tissue, consistent with the effect observed during in vivo exposure. They also indicate that pig PCLS is a relevant and sensitive model to investigate the liver toxicity of food contaminants.

Keywords: cell death, liver, explants, histology, inflammation, apoptosis, mycotoxins

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1. *Introduction*

Mycotoxins are toxic secondary metabolites produced by fungi, mainly by Aspergillus, Penicillium, Alternaria, Fusarium, and Claviceps. They are the most frequently occurring natural contaminants in human food and animal feed. They are highly resistant to various food processes

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including cooking, and therefore end up on our plates (Payros et al., 2021a). Estimates show that 60-80% of food crops worldwide are contaminated with mycotoxins (Eskola et al., 2020).

Among mycotoxins, deoxynivalenol (DON) is the most prevalent member of the trichothecene family. It is mainly produced by Fusarium graminearum and F. culmorum (Knutsen et al., 2017). In Europe, DON was detected in almost half of 26,613 cereal samples collected from 21 countries, with the highest levels observed in wheat, maize, and oat grains (Knutsen et al., 2017). Similarly, in the southern region of Brazil, one of the largest crops producing regions, 48% of the maize samples analyzed were contaminated with this toxin (Oliveira et al., 2017).

In 2017, EFSA set a tolerable daily intake (TDI) of 1 μg/kg of body weight/day for the sum of DON and its modified forms (Knutsen et al., 2017). Data obtained at the European and national levels indicate that this TDI may be exceeded in some parts of the population, particularly children (Knutsen et al., 2017; Narváez et al., 2022; Vin et al., 2020); indicating that exposure to DON is of concern for humans.

At the molecular level, DON binds to the 60S ribosomal subunit, inducing ribotoxic stress, inhibiting protein synthesis, and activating mitogen-activated protein kinases and their downstream pathways (Pestka, 2010a). Vomiting and bloody diarrhea have been reported following acute exposure to DON (Pestka, 2010b; Ruan et al., 2020). Chronic exposure to the toxin reduces food consumption and weight gain and induces neuroendocrine changes (Payros et al., 2016; Terciolo et al., 2018), alteration of the immune system (Pinton et al., 2008), and disturbance of intestinal morphology and functions (Pinton and Oswald, 2014; Robert et al., 2017).

The effect of DON on the liver is poorly described even though it can be a target for mycotoxins. The functional capacities of this organ are mainly related to anabolic and catabolic processes including energy metabolism and xenobiotics detoxification, storage and circulation of nutrients, as well as protein synthesis and bile production. In vitro studies with hepatic human cells lines have focused on cytotoxicity, apoptotic, and transcription factors (Fernández-Blanco et al., 2018; Mayer et al., 2017; Smith et al., 2017a, 2017b; Zhang et al., 2009). The only study on human primary hepatocytes demonstrated cytotoxicity, decreased albumin secretion, and increased apoptosis (Königs et al., 2008). *In vivo* animal trials showed that ingestion of DON targets the liver; oral exposure to the toxin alters the liver morphology, induces an inflammatory response, increases apoptosis and oxidative stress

(Bracarense et al., 2017; Gerez et al., 2015; Grenier et al., 2011; Souza et al., 2020; Sun et al., 2014).

In the context of reducing the number of experimental animals (3Rs principles), *ex vivo* organ culture is a powerful model that preserves normal histological structure (Maresca et al., 2018). Indeed, large numbers of explants can be prepared from a single animal, thus reducing the number of animals, and allowing multiple tests. The advantage of *ex vivo* approaches for the intestine has been demonstrated through observation of reproducible toxic effects of mycotoxins, including DON (Alassane-Kpembi et al., 2017a, 2017b; Gerez et al., 2021; Pierron et al., 2022; Silva et al., 2019, 2014). Precision-cut liver slices (PCLS) are a physiologically relevant *ex vivo* model for hepatic studies (Palma et al., 2019). This model has features close to the *in vivo* situation, including the conservation of the complex 3D liver structure, as well as cell–cell and cell–matrix interactions. Moreover, PCLS provides the possibility to work with a complete multicellular liver model including hepatocytes, Kupffer cells, stellate cells, cholangiocytes, and liver sinusoidal endothelial cells. As normal metabolism is necessary for morphological preservation, structural integrity is a valid criterion to demonstrate a successful culture (Starokozhko et al., 2015). Methodologies and parameters to produce and culture PCLS have been optimized over the past decades (de Graaf et al., 2010; Dewyse et al., 2021). It is now considered that PCLS provides a useful *ex vivo* tool to obtain multiple read-outs and that it is relevant for studies investigating liver functionality, toxicity, chronic liver diseases, and potential adverse effects on the immune system (Dewyse et al., 2021; Kasper et al., 2005; Wu et al., 2018). However, studies using *ex vivo* models from the liver are scarce and their advantage in analyzing the effects of mycotoxins remains to be demonstrated.

The objective of this work was thus to study the impact of DON on the liver using a liver slice model. In this model, histological damages, hepatic biomarkers, and changes in gene expression were evaluated. HepG2 cells, which are widely used for hepatotoxicity studies, were used for comparison.

2. *Materials and methods*

2.1 Mycotoxin

DON was acquired from Sigma-Aldrich (Saint Quentin Fallavier, France), dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich), and stored at -20°C until use. Except for cytotoxicity assays, the

DON working concentration was 10 µM, in accordance with our previous studies performed in different experimental models and tissues (Bracarense et al., 2020; Gerez et al., 2021, 2017).

2.2 In vitro assay

To evaluate DON hepatotoxicity and gene expression, in vitro assays were performed using HepG2, a human liver cancer cell line (Sigma, 85011430). Cells were maintained as previously described (Luo et al., 2019) for gene expression analysis. To assess the cytotoxicity, HepG2 cells were seeded in 96-white-well flat-bottom cell culture plates (Greiner, Courtaboeuf, France) (10^3 cells/well in 100 µL culture medium). After 24 h, the medium was replaced by a complete medium (without fetal calf serum) containing DON or vehicle (DMSO) and incubated for 4, 24, and 48 h. The cytotoxic effects of DON were determined by measuring the reducing potential of living cells, and hence their metabolism, using the RealTime-Glo™ MT Cell Viability Assay (Promega, Charbonnières-les-Bains, France). Six biological replicates were performed.

For gene expression analysis, cells were incubated for 4 h with the mycotoxin or vehicle, lysed with Extract All (Eurobio, Les Ulis, France), and stored at -80°C prior to mRNA extraction. Six biological replicates were performed.

2.3 Preparation of liver slices

Animal experimentation procedures were approved by the Ethics Committee of Pharmacology-Toxicology of Toulouse-Midi-Pyrénées in animal experimentation (Toxcométhique) (APAFIS#N2016080314392462), in accordance with the European Directive on the protection of animals used for scientific purposes.

Liver slices (PCLS) were prepared from liver lateral lobe resected from nine euthanized male 4–5-weeks-old piglets. The lobe was immediately flushed with an ice-cold sodium chloride solution (0.9% NaCl) to limit ischemia and remove hemoglobin.

To improve their viability during coring, slicing, and storage procedures, the liver slices were maintained in Krebs-Henseleit ice-cold buffer supplemented with NaHCO3 (2.1 g/L) and CaCl2, 2H2O (0.373 g/L), previously bubbled with carbogen for 1 h. Eight-millimeter diameter cores were cut out and transferred to the Krumdieck tissue slicer (Alabama Research and Development, AL, USA) where 250

µm thick slices were cut (de Graaf et al., 2010). Damaged slices were discarded, and perfect slices underwent homeostasis recovery during a 1 h regeneration step in ice-cold William's E Medium supplemented with 1% glutamine and 0.5% gentamycin (Eurobio) bubbled in carbogen. The slices were deposited in 12-wells culture plates (1 slice per well) for 4 h in 2 mL of complete William's E Medium previously bubbled in carbogen and containing 10 µM DON or vehicle. Incubation took place at 37°C under a 95% O2 and 5% CO2-controlled atmosphere.

After the incubation period, the cell culture medium was collected and frozen to detect hepatic injury markers. Some liver slices were snap-frozen and stored at -80°C until transcriptomic analyses. Others were fixed for 24 h in 4% buffered formaldehyde (VWR, Rosny-sous-Bois, France) and stored in 70% ethanol for histology and immunohistochemical experiments.

2.3.1 Histological evaluation

PCLS incubated for four hours were dehydrated in a series of alcohol solutions of increasing concentration and embedded in paraffin for histological examination. Sections of 5 µm were stained with hematoxylin and eosin (HE) and mounted with coverslips for histological analysis. A morphological lesion score as described by Bracarense et al. (2012) and Terciolo et al. (2019) was used to evaluate histological changes in the liver. The extent of each lesion (according to the intensity or the frequency observed, scored from 0 to 3) and the severity factor were used to establish the lesion score scale (Table 1).

Table 1. Histological criteria used to establish liver lesion score.

Type of lesion	Severity factor	Maximal score
Disorganization of hepatic cords	1	33
Inflammatory infiltrate	1	
Cytoplasmic vacuolation	1	
Nuclear vacuolation	1	
Megalocytosis	2	
Apoptosis	2	
Necrosis	3	

The maximum lesion score was obtained by multiplying the lesion's maximum extent level by the organ's maximum severity factor. The lesion score was obtained by multiplying the severity factor by the extent of the lesion.

2.3.2 Hepatic biomarkers

To assess liver damage and function, hepatic enzyme activity and protein concentration were evaluated in the medium after a 4 h incubation period. Alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) activities, and total protein (TP) concentrations were determined on a Pentra 400 chemistry analyzer (Horiba, Les Ulis, France) at the Anexplo Platform in Toulouse (France).

2.3.3 Immunohistochemical assessment of apoptosis

Apoptosis was assessed on liver slices by immunohistochemical analysis using the antibody anti-cleaved caspase-3 (CCasp3) (clone Asp 175, 1:200 dilution, Cell Signaling Technology, Beverly, MA) and the procedures previously described (Bracarense et al., 2017). Cells with positive immunostaining were counted in five random fields per section at 400x magnification, as described previously (Gerez et al., 2015).

2.4 Expression of mRNA encoding genes by real-time qPCR

The RNA from tissue and cells was extracted as already described (Grenier et al., 2012). The concentration and quality of the samples were analyzed, and reverse transcription and real-time qPCR were performed as previously described (Maruo et al., 2018) using specific primers sequences purchased from Sigma (Tables 2 and 3). Most of the primers were specifically designed for this experiment.

2.4.1 Total RNA Extraction and Reverse Transcription

PCLS were lysed using 1 mL of Extract-All with ceramic beads (MP Biomedicals, Illkirch, France). Total RNA from PCLS and HepG2 assays were purified, and their concentration and integrity were determined as previously described (Maruo *et al.*, 2018). For each sample, 2 µg of total RNA was reverse transcribed into cDNA and diluted 1:20 with nuclease-free/injection water (Ambion, Austin, TX) and stored at −20°C until use.

2.4.2 Quantitative Real-time PCR (qPCR) Analysis

To quantify mRNA expression levels, RT-qPCR was performed using a Viia™ 7 thermocycler (Applied Biosystems, California). The specificity of the qPCR products was assessed at the end of the

reactions by analyzing the dissociation curves. TOP2B and ACTB for PCLS samples, and GAPDH and PPIA for HepG2 cells were selected for their stable expression upon treatments, assessed with NormFinder software (Andersen et al., 2004), and used as reference genes. Non-reverse transcribed RNA was used as non-template control (NTC) for verification of the genomic DNA amplification signal. Data from qPCR were analyzed with the LinRegPCR 2016 program enabling the determination of the starting concentrations (N0) based on the observed PCR efficiency values. mRNA expression levels in samples exposed to DON were expressed relative to the mean of the control (Maruo et al., 2018).

Table 2: Table of human primer sequences used for RT-qPCR analysis (F: Forward; R: Reverse)

Gene symbol	Gene name	Primer sequence	mRNA	References
FOS	AP-1 Transcription Factor Subunit	F: ACGCGCAGGACTTCTGCAC R: GAATGAAGTTGGCACTGGAGAC	AH003773.2	Present study
ATF3	Activating Transcription Factor 3	F: GGCGACGAGAAAGAAATAAG R: CAGCTTCTCCGACTCTTT	NM_001674.4	Present study
HNF4	Hepatocyte Nuclear Factor 4 Alpha	F: CAGATGATCGAGCAGATCCA R: CGTTGGTTCCCATATGTTCC	X76930.1	Present study
CASP3	Caspase 3	F: GAGGCCGACTTCTTGTAT R: CAAAGCGACTGGATGAAC	NM_004346.4	Present study
CASP 9	Caspase 9	F: CACACCCAGTGACATCTTT R: AGGGTCTCAACGTACCA	AB020979.1	Present study
CAT	Catalase	F: CTTCGACCCAAGCAACAT R: GGGACAGTTCACAGGTATATG	NM_001752.4	Present study
SOD2	Superoxide Dismutase 2	F: GGTTGGCTTGGTTTCAATAAG R: GTGCTCCCACACATCAAT	NM_000636.4	Present study
HMOX1	Heme Oxygenase 1	F: CAGGCAGAGGGTGATAGA R: CTCCTGCAACTCCTCAAAG	NM_002133	Present study
IL8	Interleukin 8	F: GATTTCTGCAGCTCTGTG R: GTGGAAAGGTTTGGAGTATG	NM_000584	Present study
TNFα	Tumor Necrosis Factor Alpha	F: CCTGTAGCCCATGTTGTA R: CCAGCTGGTTATCTCTCA	NM_000594	Present study
NF-κβ	Nuclear Factor Kappa B	F: GAGCAACCTAAACAGAGAGG R: TTGACCTGAGGGTAAGACT	NM_003998.4	Present study
CCL20	C-C Motif Chemokine Ligand 20	F: GGCTGCTTTGATGTCAGT R: GAAGAATACGGTCTGTGTATCC	NM_001130046.2	Present study
PPIA	Peptidylprolyl Isomerase A	F: GTCAACCCCACCGTGTTCTTC R:TTTCTGCTGTCTTTGGGACCTTG	ENST00000355968	Present study

GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	F: TCAAGGCTGAGAACGGGAAG R: CCACTTGATTTTGGAGGGATCTC	NM_002046	Luo et al., 2021

Table 3: Table of pig primer sequences used for RT-qPCR analysis (F: Forward; R: Reverse)

Gene symbol	Gene name	Primer sequence	mRNA	References
FOS	AP-1 Transcription Factor Subunit	F: CACTGTGAACAGATCAGC R: GAAGTCGGTCAGTTCTTTC	ENSSSCT00000014174.4	Present study
ATF3	Activating Transcription Factor 3	F: TGAGGTTCGCCATCCA R: TACCTCGGCTTTCGTGA	ENSSSCT00000033171.2	Present study
HNF4	Hepatocyte Nuclear Factor 4 Alpha	F: TACTGCAGGCTCAAGAAGTG R: CTGCTATCCTCGTAGCTTGAC	NM_001044571.1	Present study
IL1α	Interleukin 1 Alpha	F: GCCAATGACACAGAAGAAGA R: ATGCACTGGTGGTTGATG	NM_214029	Pierron et al., 2022
IL8	Interleukin 8	F: GCTCTCTGTGAGGCTGCAGTTC R:AAGGTGTGGAATGCGTATTTATGC	NM_213867	Grenier et al., 2011
TNFα	Tumor necrosis factor alpha	F: ACTGCACTTCGAGGTTATCGG R: GGCGACGGGCTTATCTGA	NM_214022	Cano et al. 2013
NF-κβ	Nuclear Factor Kappa β	F: CCTCCACAAGGCAGCAAATAG R: TCCACACCGCTGTCACAGA	ENSSSCT00000033438	Alassane-Kpembi et al, 2017a
CCL20	C-C Motif Chemokine Ligand 20	F: GCTCCTGGCTGCTTTGATGTC R: CATTGGCGAGCTGCTGTGTG	NM_001024589	Meurens et al., 2009
ACTB	Actin β	F: GCACCACACCTTCTACA R: ATCTGGGTCATCTTCTCAC	ENSSSCT00000008324.1	Present study
TOP2B	DNA Topoisomerase II Beta	F: AAGGGCGAGAGGTCAATGAT R: ACATCTTCTCGTTCTTGCGC	ENSSSCT00015080370.1	Park et al., 2015

2.5 Statistical analysis

All statistical analyses were performed using GraphPad Prism 9.0.2 software (GraphPad Software Inc., La Jolla, USA). Data are expressed as mean ± SEM (standard error of the mean). Significant differences were assessed by one-way ANOVA followed by Tukey's test for cytotoxicity and by the unpaired Student's t-test for parametric data and Mann Whitney test for non-parametric data for all the other analyses. p-value≤0.05 was considered statistically significant.

3. *Results*

3.1 Effects of DON on HepG2 cells

DON cytotoxicity was first evaluated on human liver cells (Figure 1). After 4 h exposure to DON, the viability of HepG2 cells decreased significantly at concentrations of 6.25 µM (12% reduction) to 100 µM (17% reduction). After 24 h, viability decreased after DON exposure from 0.8 (9% reduction) to 100 µM (40% reduction). DON showed higher cytotoxic effects after 48 h exposure from 1.6 to 100 µM (15% and 50% inhibition, respectively).

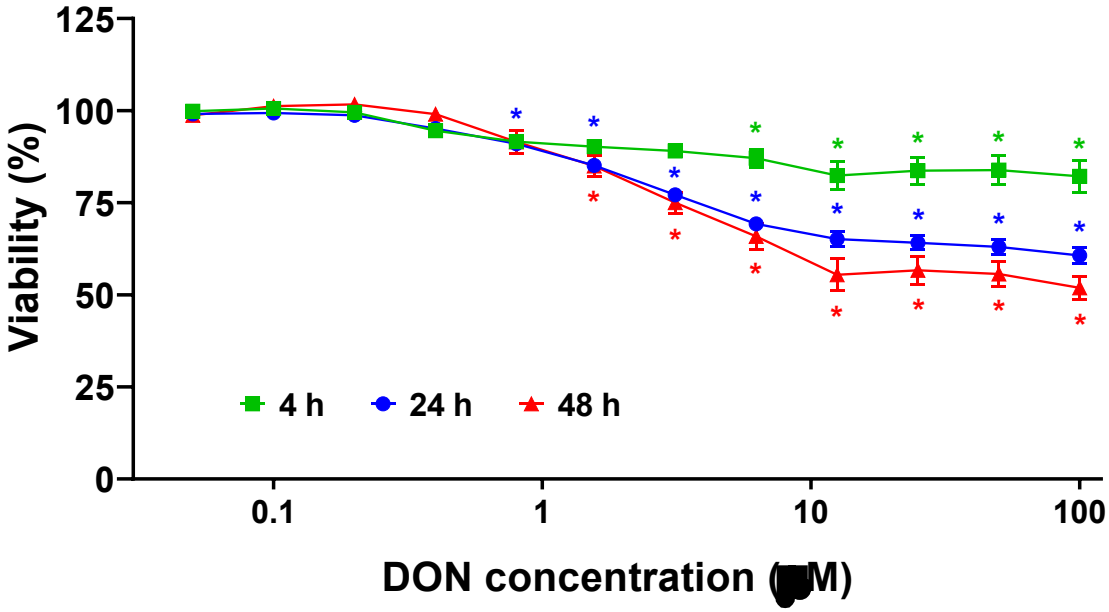


Figure 1. Toxicity of DON in HepG2 cells evaluated by measurement of the reducing potential of cells (RealTime-Glo™ MT Cell Viability Assay) after 4, 24, and 48 h. Cell viability is expressed as % of control cells. Data are presented as mean ± SEM of six replicates, *p ≤ 0.05.

To complete the analysis of the hepatic toxicity of DON, the abundance of genes involved in apoptosis, inflammation, and oxidative stress were evaluated by RT-qPCR. The liver is exposed to a multitude of xenobiotics, including mycotoxins. Being the primary target tissue and main metabolizer of many xenobiotics, it is very sensitive to tissue injury leading to cell apoptosis. We thus evaluated the expression of genes involved in apoptosis. We observed that the expression of caspase-3 (CASP3) and caspase-9 (CASP9) increased in HepG2 exposed to DON (2.7- and 1.4-fold, respectively) (Figure 2).

Besides its metabolic role, the liver is also a key tissue involved in the immune system (Kubes and Jenne, 2018). For this reason, we also assessed the expression of cytokine genes. Upon exposure to DON, increased expression of interleukin 8 (IL8) (16.6-fold), tumor necrosis factor α (TNFα) (17.6-fold), and the C-C motif chemokine ligand 20 (CCL20) (8.8-fold) was observed, but no changes in

interleukin 1α (IL1α) (Figure 2). The expression of the transcription factor nuclear factor-kappa B (NF-κB) (4.7-fold), which controls the transcription of many inflammatory cytokines, was also found to be up-regulated upon exposure to DON (Figure 2). Similarly, the Fos proto-oncogene (FOS) (66.8-fold), the expression of activating transcription factor 3 (ATF3) (28.7-fold), and of hepatocyte nuclear factor 4 α (HNF4) (1.3-fold) were increased upon exposure to DON (Figure 2).

DON, like other mycotoxins, can induce oxidative stress in the liver. Thus, the last set of genes tested concerned enzymes involved in oxidative stress. In HepG2 cells, a significant increase (2.2-fold) only in the catalase (CAT) and superoxide dismutase 2 (SOD2) (2.5-fold) genes, and a (0.7-fold) decrease in heme oxygenase 1 (HMOX1) were observed (Figure 2).

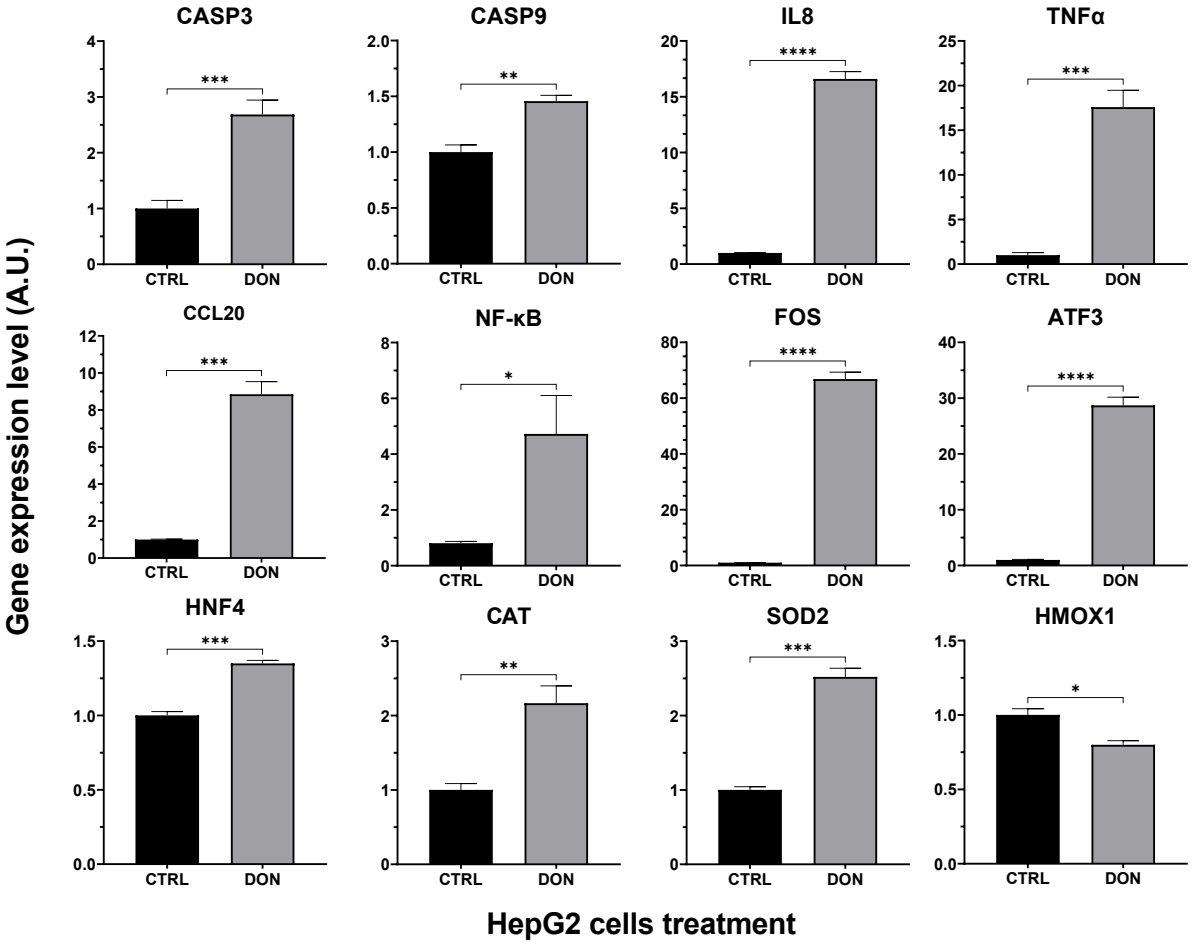


Figure 2. Apoptosis, inflammatory, transcription factors, and oxidative stress mRNA levels measured by RT-PCR in HepG2 cells exposed or not to 10 µM DON for 4 h. Data are presented as mean ± SEM of six replicates, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, and ****p ≤ 0.0001.

3.2 Effects of DON on PCLS

To not limit our observations to a hepatic cell line, experiments were also performed on porcine PCLS. This later model has the advantage to include all liver cell types within the context of a preserved hepatic architecture, including the conservation of intercellular and cell-matrix interactions (de Graaf et al., 2010).

The hepatic effects of DON were first assessed histologically. A significant increase in the lesion score was observed in DON-treated PCLS ($p \leq 0.001$). The main histological changes in PCLS were disorganization of hepatic cords, apoptosis, and cytoplasmic vacuolation of hepatocytes (Figure 3). Despite damages to the tissue, we observed no significant increase in biomarkers of liver injury (alanine transaminase - ALT, aspartate aminotransferase – AST, and lactate dehydrogenase - LDH), cholestasis (alkaline phosphatase - ALP), or hepatic function (total proteins) in the PCLS supernatant upon DON treatment (Supplementary data - Table S1).

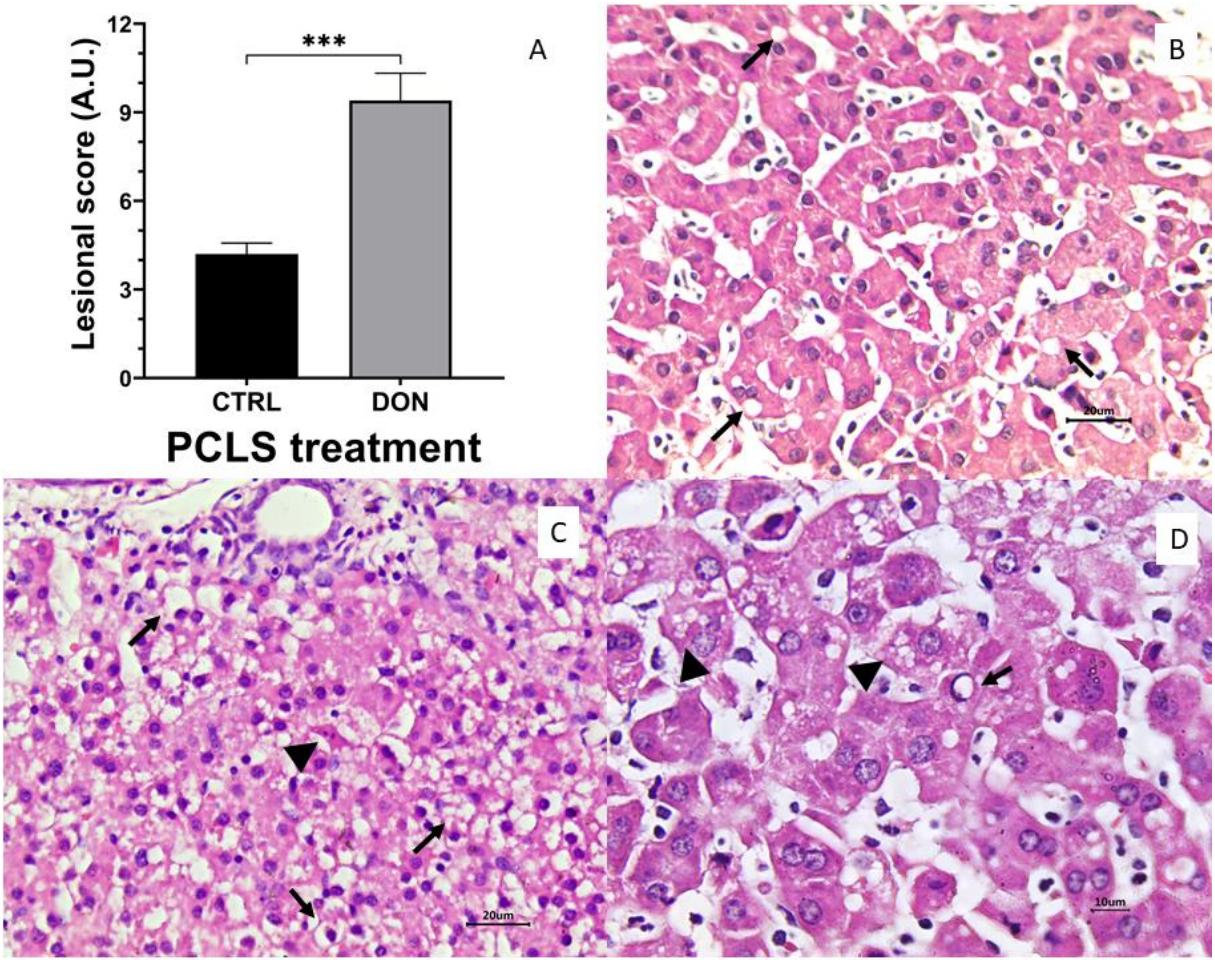


Figure 3. Effects of 4 h DON exposure on PCLS. A- Lesion score (A.U. – arbitrary units). Data are presented as mean ± SEM of five different animals, ***$p \leq 0.001$. B- control group. Mild disorganization of hepatocytes trabeculae and cytoplasmic vacuolation (arrows). HE. Scale bar 20 µm. C – DON group.

Moderate cytoplasmic vacuolation of hepatocytes (arrows) and apoptosis (arrowhead). HE. Scale bar 20 µm. D- DON group. Cytoplasmic vacuolation (arrowheads) and nuclear vacuolation (arrows) of hepatocytes. HE. Scale bar 10 µm.

Because apoptosis was an important histological finding in DON-exposed PCLS, we sought to confirm activation of a cysteine protease, caspase-3 (CASP3), a key effector in cell apoptosis. A significant increase in CASP3 immunostaining was observed in the treated group ($p \leq 0.01$) compared to the control (Figure 4). The brownish immunostaining was randomly distributed in hepatocytes and Kupffer cells.

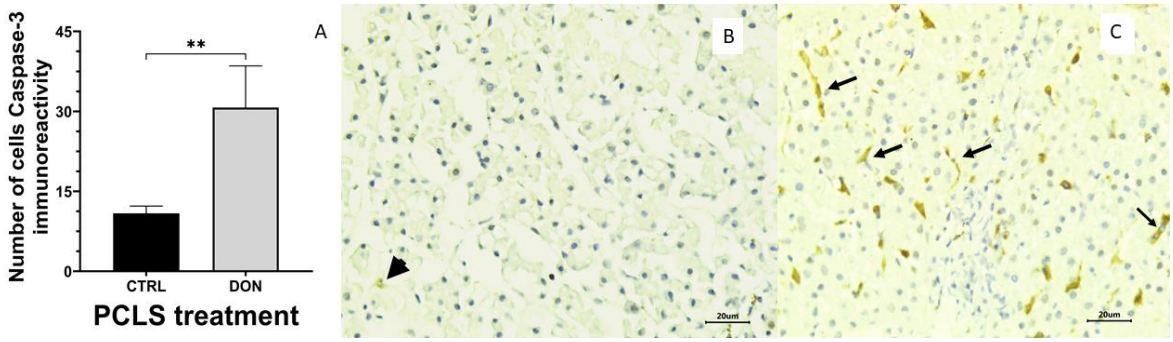
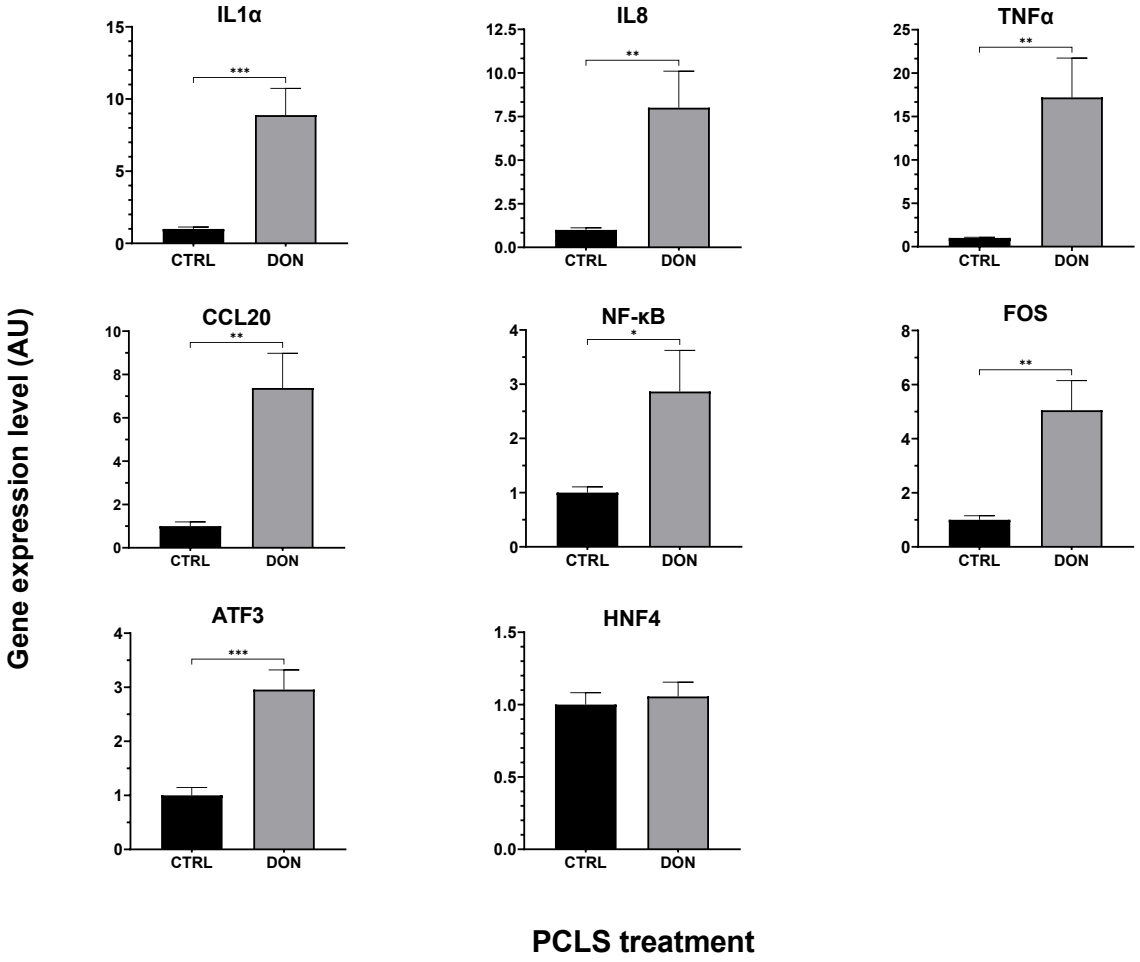


Figure 4. Analysis of apoptosis in PCLS. A- Mean number of immunoreactive cells for CASP3 in PCLS/field. Data are presented as mean ± SEM of five different animals), **p ≤ 0.01. B–C- Immunoperoxidase staining. B- control group. Mild positive CASP3 staining (arrowhead). Scale bar 20 µm. C- DON group. Moderate CASP3 positive staining in hepatocytes and Kupffer cells (arrows). Scale bar 20 µm.

Next, we investigated the effect of DON on gene expression. As already observed in HepG2 cells, exposure of PCLS to 10 µM DON stimulates the expression of IL8 (8-fold), TNFα (17.2-fold), and CCL20 (7.4-fold) (Figure 5). In these samples, DON also induced the expression of IL1α (8.9-fold). Like HepG2, DON upregulated in PCLS the transcription factors NF-κB, FOS, and ATF3 (2.9-, 5-, and 3-fold, respectively) (Figure 5).



219

220 **Figure 5.** Inflammatory and transcription factors mRNA levels measured by RT-PCR in PCLS exposed
221 or not to DON for 4 h. Data are mean ± SEM from 9 different animals. *p ≤ 0.05, **p ≤ 0.01, and ***p
222 ≤ 0.001.

223 By contrast in PCLS, no significant differences were observed in the expression of any of the
224 oxidative stress genes analyzed (data not shown).

225 **4. *Discussion***

226 Many studies have reported an effect of DON on the intestine and the lymphoid organs (Luo et al.,
227 2019; Novak et al., 2021; Pinton and Oswald, 2014), but investigations on the impact of DON on the
228 liver are scarce. The present study evaluated the effects of DON on liver tissue, particularly PCLS. The
229 human hepatocyte cell line, HepG2, was used for comparison.

230 Due to the difficulties in accessing human liver samples, the study was performed on porcine PCLS.
231 Pig is considered a good model for extrapolation to humans (Helke and Swindle, 2013). PCLS retain

the original three-dimensional structure of the liver, preserve hepatocytes and Kupffer cells as well as cellular crosstalk and cell-matrix interactions (de Graaf et al., 2010). PCLS thus appear to be highly suitable for studies of the toxic effects of food contaminants. This model has two main advantages, (i) cultured explants are in line with 3R principles, as one animal allows the assessment of multiple doses and (ii) it gives access to a fully differentiated three-dimensional model for histopathology assessment. A few studies using rat PCLS have addressed the effects of mycotoxins, one assessed the effect of fumonisin B1 on sphingoid base (Norred et al., 1996), another one the impact of aflatoxins on RNA synthesis (Friedman et al., 1997), and a third one described the metabolization of Alternaria toxins into potentially toxic compounds (Burkhardt et al., 2011).

A cytotoxic effect of DON was observed in HepG2 cells at low concentrations (0.8-1.6 µM), regardless of the duration of exposure (4, 24, and 48 h). The decrease observed at these low concentrations after 24 h is in agreement with the results of previous studies (Darwish et al., 2020; Mayer et al., 2017) indicating that short exposure to realistic doses of DON alters hepatic cells.

Damages to the tissue were observed in PCLS exposed to DON, despite the short time of exposure (4 h), but no changes in markers of liver function or injury. Thus, histology can be considered a sensitive endpoint for liver damage. In PCLS, disorganization of hepatic cords, apoptosis, and cytoplasmic vacuolation of hepatocytes were observed after 4 h of exposure to 10 µM DON. These histological changes are similar to those obtained in subchronic *in vivo* trials. Indeed, disorganization of hepatic cords, apoptosis, cytoplasmic and nuclear vacuolation of hepatocytes, megalocytosis, and focal necrosis were observed in piglets or gilts fed diets contaminated with 0.012 to 3 mg/kg DON for one to six weeks (Bracarense et al., 2020; Gerez et al., 2015; Grenier et al., 2011; Pierron et al., 2018; Skiepko et al., 2020).

The cytoplasmic vacuolation observed in PCLS may be related to the DON-induced impairment of mitochondrial function (Springler et al., 2017). Indeed, mitochondrial dysfunction leads to failure of the Na^+/K^+-ATPase pump causing an influx of Na^+, Ca^{+2}, and water into the cytosol, inducing, cytoplasmic vacuolation (Miller and Zachary, 2017). Moreover, an increased number of apoptotic hepatocytes and cells in hepatic sinusoids was observed in gilts exposed to a fusariotoxins-contaminated diet (Dolenšek et al., 2021).

Apoptosis observed in PCLS was confirmed by immunostaining of CASP3. Liver apoptosis has already been reported in piglets injected intra-venously with DON (Mikami et al., 2010) as well as in mice and pigs fed a DON-contaminated diet (Bracarense et al., 2017; Gerez et al., 2015; Sun et al., 2014). Apoptosis can occur via different pathways (Elmore, 2007). The extrinsic pathway induced by transmembrane death-receptor interactions activates CASP8 and subsequently CASP3. The intrinsic or mitochondrial pathway involves intracellular signals such as p53, resulting in the activation of CASP9 and subsequently of CASP3 (van Cruchten and van den Broeck, 2002). In the present study, we observed upregulation of both CASP3 and CAPS9. CASP9 activation suggests that in the liver the intrinsic pathway is involved in DON-induced apoptosis, as already demonstrated in the intestine (Payros et al., 2021b). In the same way as observed for the histological evaluation of PCLS exposed to DON, it should be noted that the evaluation of apoptosis has revealed similar effects to those observed *in vivo* in different species.

DON is known to activate the inflammatory response. Indeed, DON-induced ribotoxic stress leads to the activation of the MAPK1/2 pathway, which in turn activates several immediate-early genes and induces the production of cytokines, the expression of cyclooxygenase-2 (COX2), and apoptosis (Pestka, 2010a; Pierron et al., 2016) in different target organs (Payros et al., 2016; Pestka, 2010a). In the present study, we observed overexpression of various cytokines in both HepG2 and PCLS. Such pro-inflammatory expression patterns have already been observed in the liver of pigs and rats fed a DON-contaminated diet (Abdel-Wahhab et al., 2015; Chen et al., 2008). In our study, IL1α gene expression was only upregulated in PCLS. This difference might be due to the presence of Kupffer cells in hepatic tissue that are known to produce high levels of this cytokine (Bilzer et al., 2006) in the liver (Bai et al., 2021). The IL1α was also shown to be expressed in the intestine after in vivo exposure to DON (Pestka, 2010b). Taken together, these results show that pro-inflammatory cytokines are sensitive markers when the liver is exposed to DON and that such inflammation can be observed *in vitro* (hepatocytes), *ex vivo* (PCLS), and *in vivo*.

Concerning transcription factors, DON-activation of MAPK pathway activates immediate-early genes, such as FOS and ATF3, in turn promoting the expression of COX2, cytokines, and apoptosis (Nielsen et al., 2009; Smith et al., 2017b; Sun et al., 2015; Yuan et al., 2018). In the present study, we

288 demonstrated an increase in the expression of FOS, ATF3 in PCLS, and of HNF4 only, in the cells.
289 ATF3 reveals endoplasmic reticulum (ER) stress. In absence of relief of ER, cells can go into apoptosis
290 (Holtz et al., 2006; Li et al., 2006). FOS is highly induced in HepG2 cells, probably due to its
291 stabilization by sustained extracellular signal-regulated kinase (ERK) signaling (Murphy et al., 2002).
292 In HepG2 cells, but not in PCLS, we observed increased expression of HNF4, a transcription factor that
293 controls the expression of several hepatic-specific genes involved in hepatic metabolism (Madec et al.,
294 2011; Petrescu et al., 2002). The cancerous origin of HepG2 cells, which involves different demands on
295 metabolic processes (Coller, 2014), could explain the high induction of these transcription factors.

296 **5. *Conclusions***

297 We observed that short exposure to DON reduces HepG2 cells viability and induces toxic effects
298 on hepatic cells and liver tissue. In particular, we demonstrated that in accordance with the effects
299 observed in pigs fed a DON-contaminated diet, this toxin induces the expression of genes involved in
300 liver apoptosis and inflammation and histological damages.

301 To the best of our knowledge, this is the first study to use PCLS for the identification of DON toxic
302 effects on liver structure and functions.

303 Pig PCLS is a promising model to study the effects of DON, and potentially other mycotoxins on
304 the liver. It enables the demonstration of histomorphological and molecular effects at realistic dietary
305 levels, in very good accordance with data obtained in in vivo trials. As pig is a good model for
306 extrapolation to humans and is very sensitive to mycotoxins, particularly to DON, our results are
307 relevant for progressing towards a better assessment of human consumers' risk related to mycotoxins.

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310 investigation, A.L.H., A.P.B, P.P., A.K., S.P., S.B. and E.P.; resources, A.P.B. and P.P.; data curation,
311 A.L.H. and P.P.; writing—original draft preparation, A.L.H.; writing—review and editing, A.L.H,
312 A.P.B., P.P., and I.P.O.; visualization, A.L.H. and P.P.; supervision, A.P.B and I.P.O.; project
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Appendix A: Supplementary data to this article can be found online at: https://doi.org/10.1016/j.fct.2022.112930.

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8 ARTIGO C – EMERGING MYCOTOXINS INDUCE EX VIVO AND IN VITRO HEPATOTOXICITY IN PIGS PRECISION-CUT LIVER SLICES AND HEPG2 CELLS

Emerging mycotoxins induce *ex vivo* and *in vitro* hepatotoxicity in pigs precision-cut liver slices and HepG2 cells

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ABSTRACT: Emerging mycotoxins are currently gaining more attention due to their high frequency of contamination in foods and grains. However, most data available in the literature are in vitro, with few in vivo results that do not allow establishing their regulation. Beauvericin (BEA), eniatins (ENNs), emodin (EMO), apicidin (API) and aurofusarin (AFN) are emerging mycotoxins frequently found contaminating food and there is growing interest in studying their impact on the liver, a key organ in the metabolization of these components. We used an ex vivo model of precision-cut liver slices (PCLS) to verify morphological and transcriptional changes after acute exposure (4 h) to these mycotoxins. The human liver cell line HepG2 was used for comparison purposes. Most of the emerging mycotoxins were cytotoxic to the cells, except for AFN. In cells, BEA and ENNs were able to increase the expression of genes related to transcription factors, inflammation, and hepatic metabolism. In the explants, only ENN B1 led to significant changes in the morphology and expression of few genes. Overall, our results demonstrate that BEA, ENNs and API have the potential to be hepatotoxic.

Keywords: cell death, histology, liver explants, non-regulated mycotoxins, swine.

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1. Introduction

Toxic secondary fungal metabolites, as mycotoxins, can contaminate food and feed commodities (Marin et al., 2013). Grain contamination by mycotoxins can occur throughout the entire food chain process, including food processes such as cooking, as they are highly resistant to high temperatures (Payros et al., 2021).

As some of these mycotoxins, such as deoxynivalenol (DON), zearalenone (ZEN), fumonisins, T-2/HT-2, aflatoxins, ochratoxin A (OTA), and patulin have toxic effects in the organism and occur with high frequency in the food and feed grains, they are regulated in Europe (European Commission, 2006). However, some mycotoxins have recently "emerged", and they were not routinely determined, nor regulated. Therefore, they are called emerging mycotoxins (Vaclavikova et al., 2013). A recent study estimates that 60-80% of crops worldwide are contaminated by mycotoxins (Eskola et al., 2020), and emerging mycotoxins are frequently associated with some other mycotoxins in contaminated crops (Fraeyman et al., 2017).

The most frequent emerging mycotoxins found in grains are the *Fusarium*-toxins – beauvericin (BEA – 12-100%), enniatins (ENNs – 96-100%), apicidin (API – 29-55%), aurofusarin (AFN – 62-79%), moniliformin, fusaproliferin, culmorin, butenolide, and fusaric acid (Drakopoulos et al., 2021; Gruber-Dorninger et al., 2017; Jestoi, 2008). Moreover, emodin (EMO – 63-74%), a mycotoxin produced by many plants and *Aspergillus* species, is important because often contaminates pig feed and grains, and it is probably the most ubiquitous anthraquinone found worldwide (Gruber-Dorninger et al., 2017; Novak et al., 2019; Streit et al., 2013).

BEA and ENNs are cyclic hexadepsipeptides and their toxicity are related to their ionophore properties by inserting into the cell membrane and forming cation-selective pores and consequently interfering with cell homeostasis (Fraeyman et al., 2017). The mechanism and toxic effects of AFN and API are poorly studied and understood. AFN belongs to the naphthoquinone group and had only moderate toxicity to intestinal swine and human cells (Frandsen et al., 2006; Khoshal et al., 2019; Vejdovszky et al., 2016). API is a cyclic tetrapeptide and a high toxicity for intestinal swine cells was reported (Niehaus et al., 2014; Novak et al., 2019). EMO is a compound known to have beneficial effects on cancer cells and is widely used in Chinese medicine for decades (Tuli et al., 2021).

In vitro studies in various cell types exposed to some of these mycotoxins were already published, showing the cytotoxicity and genotoxicity, oxidative and inflammatory effects in intestinal, hepatic, reproductive, neurological, and lymphocytic cells (Escrivá et al., 2018; Juan-García et al., 2019; Krug et al., 2018; Liu et al., 2009; Prosperini et al., 2013a; Schoevers et al., 2016). Few *in vivo* studies about the toxic and immune effects of some of these emerging mycotoxins were performed to date, mainly in

chicken and mice (Fraeyman et al., 2018a; Liu et al., 2009; Maranghi et al., 2018; Wu et al., 2013), and recent studies in piglets and rats (Bhateria et al., 2022; Novak et al., 2021). The European Food Safety Authority (EFSA) publish a scientific opinion in 2014 about the risk of BEA and ENNs to human and animal health, but due to the shortage of *in vivo* data, no regulations were yet established (EFSA, 2014).

The lack of *in vivo* information needs to be fulfilled. Thinking of that and valuing the 3Rs principle, *ex vivo* models are proven to be good substitutes for the use of several animals to assess mycotoxin toxicity (Gerez et al., 2021; Pierron et al., 2022). The liver is a major organ that promotes the detoxification of several toxic substances and the effects of mycotoxins in this organ are poorly described. A recent study using a promising *ex vivo* model of precision-cut liver slices (PCLS) in the toxic effects of DON showed that liver explants have good results that agree with *in vivo* experiments (Hasuda et al., 2022).

This study aimed to investigate the effects of the emerging mycotoxins (BEA, ENN A1, ENN B, ENN B1, EMO, AFN, and API) short exposure in PCLS explants. Histological and biochemical changes, cytotoxicity, and gene expression were evaluated in these explants. The HepG2 cell, a hepatic cell type that is widely used in toxicological studies, was used for comparison.

2. Material and methods

2.1. Mycotoxin

The mycotoxins BEA, ENN A1, ENN B, ENN B1, EMO, AFN, and API were acquired from Sigma-Aldrich (Saint Quentin Fallavier, France), dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich), and stored at -20 °C until use. Except for cytotoxicity assays, the mycotoxins' working concentration was 10 µM, a dose that is cytotoxic according to a previous study performed by our research team in intestinal cells (Khoshal et al., 2019).

2.2. In vitro assay

To evaluate the effects of emerging mycotoxins on hepatic toxicity and gene expression, *in vitro* assays were performed using HepG2 cells, a human liver cancer cell line (Sigma, 85011430). Cells were maintained as previously described (Luo et al., 2019) for gene expression analysis. To assess

the cytotoxicity, HepG2 cells were seeded in 96-white-well flat-bottom cell culture plates (Greiner, Courtaboeuf, France) (10^3 cells/well in 100 µL culture medium). After 24 h, the medium was replaced by a complete medium (without fetal calf serum) containing crescent concentrations (0.1-100 µM) of BEA, ENN A1, ENN B, ENN B1, EMO, AFN, API, or vehicle (DMSO) and incubated for 24 h. The cytotoxic effects of the emerging mycotoxins were determined by measuring the ATP production, thus their metabolism, using the CellTiter-Glo® Luminiscent Cell Viability Assay (Promega, Charbonnières-les-Bains, France). Six biological replicates were performed.

For gene expression analysis, cells were incubated for 4 h with the mycotoxins (BEA, ENN A1, ENN B, ENN B1, EMO, AFN, and API) or vehicle, lysed with Extract All (Eurobio, Les Ulis, France), and stored at -80 °C before mRNA extraction. Six biological replicates were performed.

2.3. Preparation of liver explants

Animal experimentation procedures were approved by the Ethics Committee of Pharmacology-Toxicology of Toulouse-Midi-Pyrénées in animal experimentation (Toxcométhique) (APAFIS#N2016080314392462), following the European Directive on the protection of animals used for scientific purposes.

For liver explants sampling, six male 4–5-week-old crossbred piglets were euthanized to obtain precision-cut liver slices (PCLS) that were prepared from the resected liver lateral lobe. The lobe was immediately flushed with an ice-cold sodium chloride solution (0.9% NaCl) to limit ischemia and remove hemoglobin.

All the procedures to prepare liver slices were made as described (Hasuda et al., 2022). The slices were deposited in 12-well culture plates (1 slice per well) for 4 h in 2 mL of complete William's E Medium previously bubbled in carbogen and containing 10 µM of BEA, ENN A1, ENN B, ENN B1, EMO, AFN, API, or vehicle. Incubation took place at 37 °C under a 95% O_2 and 5% CO_2-controlled atmosphere.

After the incubation period, the culture medium was collected and frozen to measure liver injury enzymes. Some liver slices were snap-frozen and stored at -80 °C until transcriptomic analyses. Others were fixed for 24 h in 4% buffered formaldehyde (VWR, Rosny-sous-Bois, France) and

stored in 70% ethanol for histology analysis.

2.3.1. Histological evaluation

After four hours of incubation, the PCLS were dehydrated in a series of alcohol solutions of increasing concentration and embedded in paraffin for histological examination. Sections of 5 µm were stained with hematoxylin and eosin (HE) and mounted with coverslips for histological analysis. A morphological lesion score as described by Bracarense et al. (2012) was used to evaluate histological changes in the liver. The extent of each lesion (according to the intensity or the observed frequency, scored from 0 to 3) and the severity factor were used to establish the lesion score scale (Table 1).

Table 1. Histological criteria used to establish liver and intestinal lesion scores.

Organ	Type of lesion	Severity factor	Maximal score
Liver	Disorganization of hepatic cords	1	33
	Inflammatory infiltrate	1	
	Cytoplasmic vacuolation	1	
	Nuclear vacuolation	1	
	Megalocytosis	2	
	Apoptosis	2	
	Necrosis	3	

The maximum lesion score was obtained by multiplying the lesion's maximum extent level by the organ's maximum severity factor. The lesion score was obtained by multiplying the severity factor by the extent of the lesion.

2.3.2. Liver biochemicals

To assess liver damage and function, hepatic enzyme activity and protein concentration were evaluated in the culture medium after a 4 h incubation period. Alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) activities, and total protein (TP) concentration were determined on a Pentra 400 chemistry analyzer (Horiba, Les Ulis, France) at the Anexplo Platform in Toulouse (France).

2.4. Expression of mRNA encoding genes by real-time qPCR

Expression of mRNA encoding genes by real-time qPCR The RNA from tissue and cells was extracted as already described in (Grenier et al., 2012). The concentration and quality of the samples

were analyzed, and reverse transcription and real-time qPCR were performed as previously described in (Maruo et al., 2018) using specific primers sequences purchased from Sigma (Tables 2 and 3).

2.5. Total RNA extraction and reverse transcription

Total RNA extraction and reverse transcription PCLS were lysed using 1 mL of Extract-All with ceramic beads (MP Biomedicals, Illkirch, France). Total RNA from PCLS and HepG2 cells assays were purified, and their concentration and integrity were determined as previously described (Maruo et al., 2018). For each sample, 2 µg of total RNA was reverse transcribed into cDNA and diluted 1:20 with nuclease-free/injection water (Ambion, Austin, TX) and stored at -20 °C until use.

Table 2: Table of human primer sequences used for RT-qPCR analysis (F: Forward; R: Reverse).

Gene symbol	Gene name	Primer sequence	mRNA	References
ATF3	Activating Transcription Factor 3	F: GGCGACGAGAAAGAAATAAG R: CAGCTTCTCCGACTCTTT	NM_001674.4	(Hasuda et al., 2022)
CYP1A1	Cytochrome p450 1A1	F: GGTGTTAAGTGAGAAGGTGATTATC R: AGCAGGATAGCCAGGAAGAG	NM_000499.5	(Budin et al., 2021)
CYP3A4	Cytochrome p450 3A4	F: GGATCCATTCTTTCTCTCAATAA R: AATTTGTAACTTCTCTTGGAAAC	ENST00000651514.1	(Budin et al., 2021)
P53	Tumor protein 53	F: CGTGTGGAGTATTTGGATGAC R: TGTAGTGGATGGTGGTACAG	ENST00000617185.4	Present study
PPARa	Peroxisome proliferator-activated receptor alpha	F: AGCTTTGGCTTTACGGAATA R: AGGATAAGTCACCGAGGAG	NM_001001928.3	Present study
IL8	Interleukin 8	F: GATTTCTGCAGCTCTGTG R: GTGGAAAGGTTTGGAGTATG	NM_000584	(Hasuda et al., 2022)
TNFα	Tumor Necrosis Factor Alpha	F: CCTGTAGCCCATGTTGTA R: CCAGCTGGTTATCTCTCA	NM_000594	(Hasuda et al., 2022)
NF-κB	Nuclear Factor Kappa B	F: GAGCAACCTAAACAGAGAGG R: TTGACCTGAGGGTAAGACT R: GAAGAATACGGTCTGTGTATCC	NM_003998.4	(Hasuda et al., 2022)
PPIA	Peptidylprolyl Isomerase A	F: GTCAACCCCACCGTGTTCTTC R: TTTCTGCTGTCTTTGGGACCTTG	ENST00000355968	(Hasuda et al., 2022)

GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	F: TCAAGGCTGAGAACGGGAAG R: CCACTTGATTTTGGAGGGATCTC	NM_002046	(Luo et al., 2021)

Table 3: Table of pig primer sequences used for RT-qPCR analysis (F: Forward; R: Reverse).

Gene symbol	Gene name	Primer sequence	mRNA	References
ATF3	Activating Transcription Factor 3	F: TGAGGTTCGCCATCCA R: TACCTCGGCTTTCGTGA	ENSSSCT00000033171.2	(Hasuda et al., 2022)
CYP7A1	Cytochrome p450 7A1	F: GGATCCATTCTTTCTCTCAATAA R: AATTTGTAACTTCTCTTGGAAAC	ENST00000651500.1	Present study
CYP1A1	Cytochrome p450 1A1	F: GGATCCATTCTTTCTCTCAATAA R: AATTTGTAACTTCTCTTGGAAAC	ENST00000651602.1	Present study
IL1α	Interleukin 1 Alpha	F: GCCAATGACACAGAAGAAGA R: ATGCACTGGTGGTTGATG	NM_214029	(Pierron et al., 2022)
IL8	Interleukin 8	F: GCTCTCTGTGAGGCTGCAGTTC R: AAGGTGTGGAATGCGTATTTATGC	NM_213867	(Grenier et al., 2011)
TNFα	Tumor necrosis factor alpha	F: ACTGCACTTCGAGGTTATCGG R: GGCGACGGGCTTATCTGA	NM_214022	(Cano et al., 2013)
NF-κB	Nuclear Factor Kappa B	F: CCTCCACAAGGCAGCAAATAG R: TCCACACCGCTGTCACAGA	ENSSSCT00000033438	(Alassane-Kpembi et al., 2017a)
CCL20	C-C Motif Chemokine Ligand 20	F: GCTCCTGGCTGCTTTGATGTC R: CATTGGCGAGCTGCTGTGTG	NM_001024589	(Meurens et al., 2009)
ACTB	Actin β	F: GCACCACACCTTCTACA R: ATCTGGGTCATCTTCTCAC	ENSSSCT00000008324.1	(Hasuda et al., 2022)
TOP2B	DNA Topoisomerase II Beta	F: AAGGGCGAGAGGTCAATGAT R: ACATCTTCTCGTTCTTGCGC	ENSSSCT00015080370.1	(Park et al., 2015)

2.6. Quantitative Real-time PCR (qPCR) Analysis

To quantify mRNA expression levels, RT-qPCR was performed using a Viia™ 7 thermocycler (Applied Biosystems, California). The specificity of the qPCR products was assessed at the end of the reactions by analyzing the dissociation curves. TOP2B and ACTB for PCLS samples, and GAPDH and PPIA for HepG2 cells were selected for their stable expression upon treatments, assessed with NormFinder software (Andersen et al., 2004), and used as reference genes. Non-reverse transcribed RNA was used as non-template control (NTC) for verification of the genomic

DNA amplification signal. Data from qPCR were analyzed with the LinRegPCR 2016 program enabling the determination of the starting concentrations (N0) based on the observed PCR efficiency values. mRNA expression levels in samples exposed to the micotoxins were expressed relative to the mean of the control (Maruo et al., 2018).

2.7. Statistical analysis

All statistical analyses were performed using GraphPad Prism 9.0.2 software (GraphPad Software Inc., La Jolla, USA). Data were expressed as mean ± SEM (standard error of the mean) for normal distribution or median and interquartile range when data were not normally distributed. They were submitted to statistical analysis, using normality (Shapiro–Wilk) and homogeneity (Bartlett) tests. Significant differences were assessed by one-way ANOVA. Multiple comparisons were performed by Bonferroni's test for parametric data and by Kruskal-Wallis' test followed by Dunn's test for nonparametric data. p-value ≤0.05 was considered statistically significant.

3. Results

3.1. In vitro analysis

3.1.1. Cytotoxicity

After 24 h of exposure all mycotoxins, except AFN, decreased the cell viability (Fig. 1). ENN A1 and BEA showed the highest cytotoxicity, with a decrease in the viability from low concentrations (0.1 and 1 μM, respectively). AFN was not toxic for HepG2 cells in any concentration. High concentrations of ENN B (100 μM), AFN (100 μM) and ENN B1 (30 μM) stimulated the production of ATP in HepG2 cells.

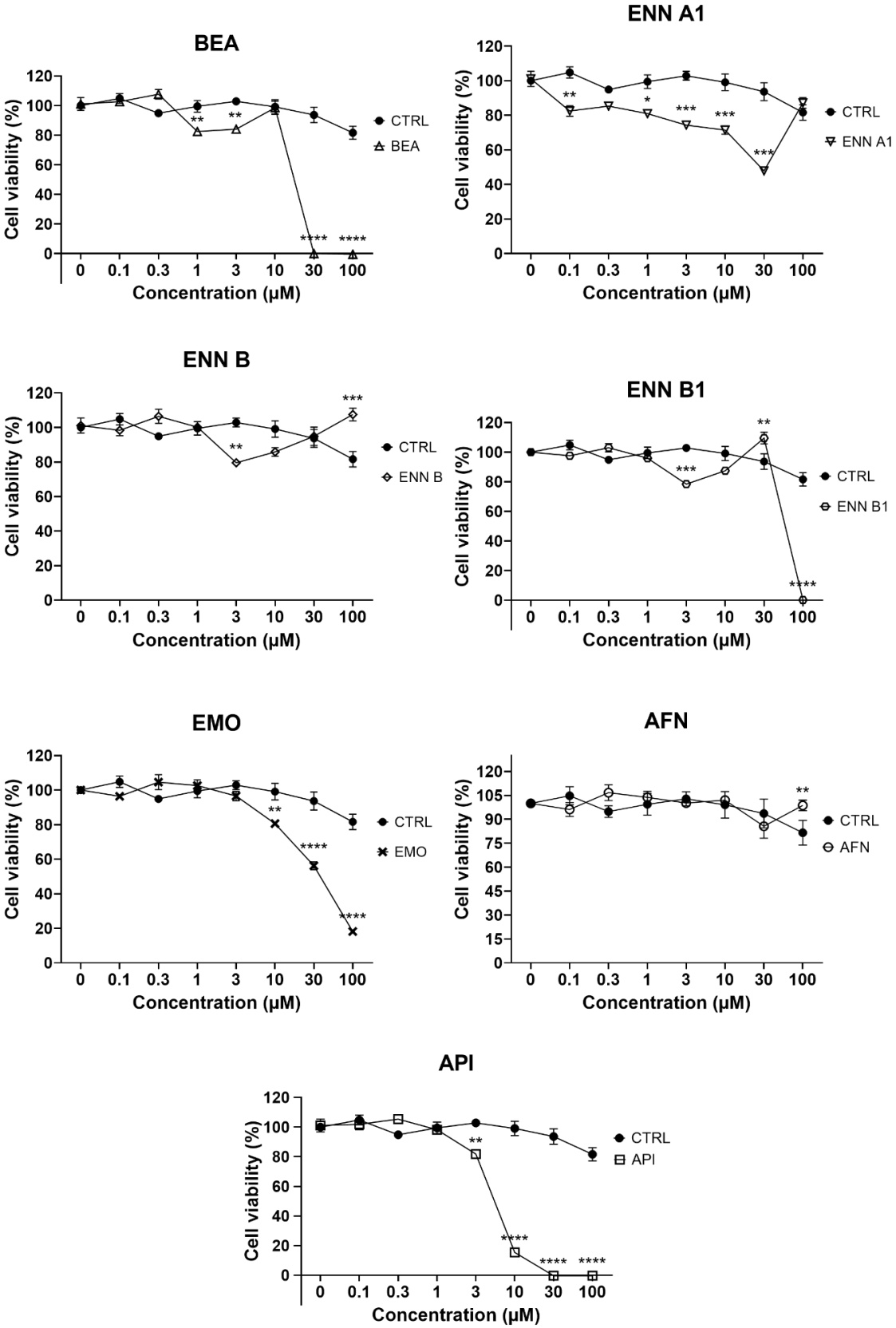

Figure 1. Toxicity of DON in HepG2 cells by CellTiter-Glo® Assay after 24h. Cell viability was evaluated by measurement of ATP and was expressed as % of control cells. Data are expressed as mean ± SEM of six replicates. *p≤0.05, **p≤0.01, ***p≤0.001, and ****p≤0.0001.

3.1.2. Gene expression

After 4 h incubation with a 10 µM concentration, some of the tested emerging mycotoxins were able to change the expression of genes in HepG2 cells and PCLS.

In the cells, all the ENNs stimulated the expression of interleukin-8 (IL8) and tumor necrosis factor-alpha (TNFα), both inflammatory proteins. The overexpression of the last one was induced by BEA and API. BEA also increased the expression of the transcription nuclear factor kappa B (NF-κB). In addition, BEA, ENNs, and EMO increased the expression of the activating transcription factor 3 (ATF3). BEA and the enniatins also increased a gene involved in hepatic metabolism, the CYP1A1, while AFN and EMO decreased the expression of this gene (Fig. 2).

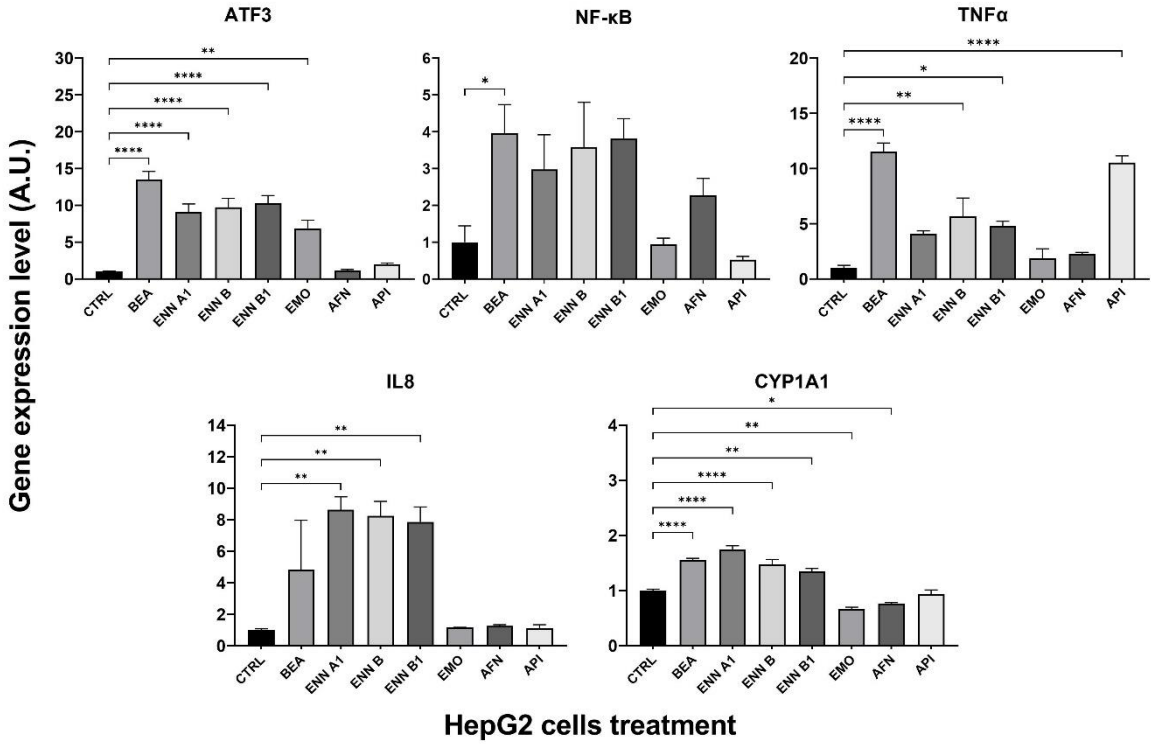

Figure 2. Inflammatory, transcription factors, and liver metabolism mRNA levels measured by RT-qPCR in HepG2 cells exposed or not to 10 µM emerging mycotoxins for 4 h. Data are presented as mean ± SEM of six replicates, **p ≤0.01, **p ≤0.01, ***p ≤0.001 and ****p ≤0.0001.

3.2. Ex vivo analysis

3.2.1. Histology

Among all the tested mycotoxins, only ENN B1 increased the liver lesion score compared to

the control after 4 h incubation (Fig. 3). The main histological changes found in the liver were disorganization of hepatic cords, cytoplasmic vacuolation of hepatocytes, apoptosis, megalocytosis, nuclear vacuolation of hepatocytes, and inflammatory infiltrate.

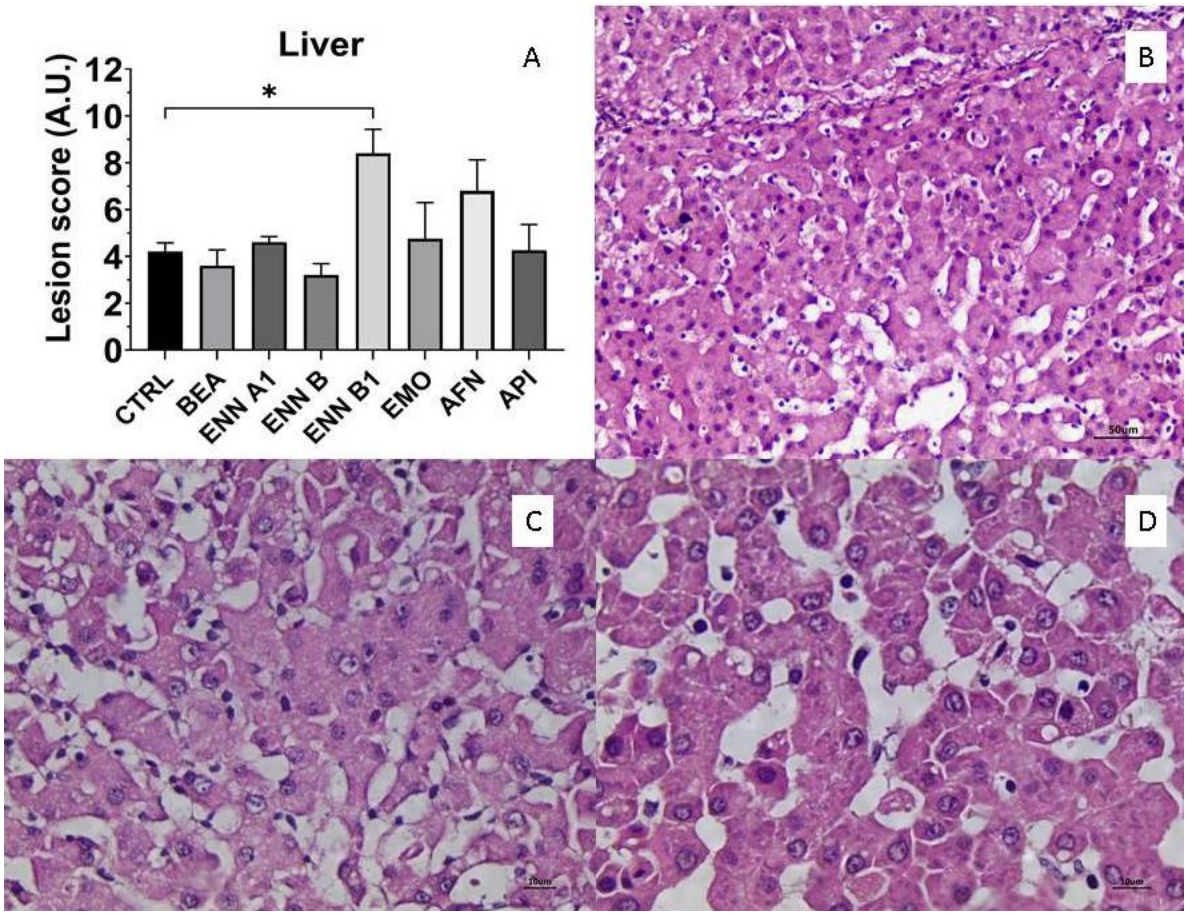

Figure 3. Effects of emerging mycotoxins exposure on liver PCLS. A) Lesion score (A.U. – arbitrary units), data are presented as mean ± SEM, n=6. B) control, mild disorganization of hepatic cords, HE, 50 µm bar. C) ENN B1, megalocytosis, HE, 10 µm bar. D) ENN B1, nuclear vacuolation of hepatocytes, HE, 10 µm bar.

3.2.2. Liver biochemicals

The liver enzyme activity and total protein content had no significant changes among the groups (Supplementary material - Table S1).

3.2.3. Gene expression

In the PCLS, we decided to test only ENN B1 and AFN, which were the mycotoxins with the higher lesion score in histology for qPCR analysis. The expression of most genes was not changed by any mycotoxin. Only superoxide dismutase 2 (SOD2) and Chemokine (C-C motif)

ligand 20 (CCL20) were downregulated and E-cadherin (Ecad) was upregulated with the ENN B1 treatment (Fig 4). The IL10 cytokine tended to increase with mycotoxin treatment (Fig 5), but with no significative changes as well as the other inflammatory genes that were not changed.

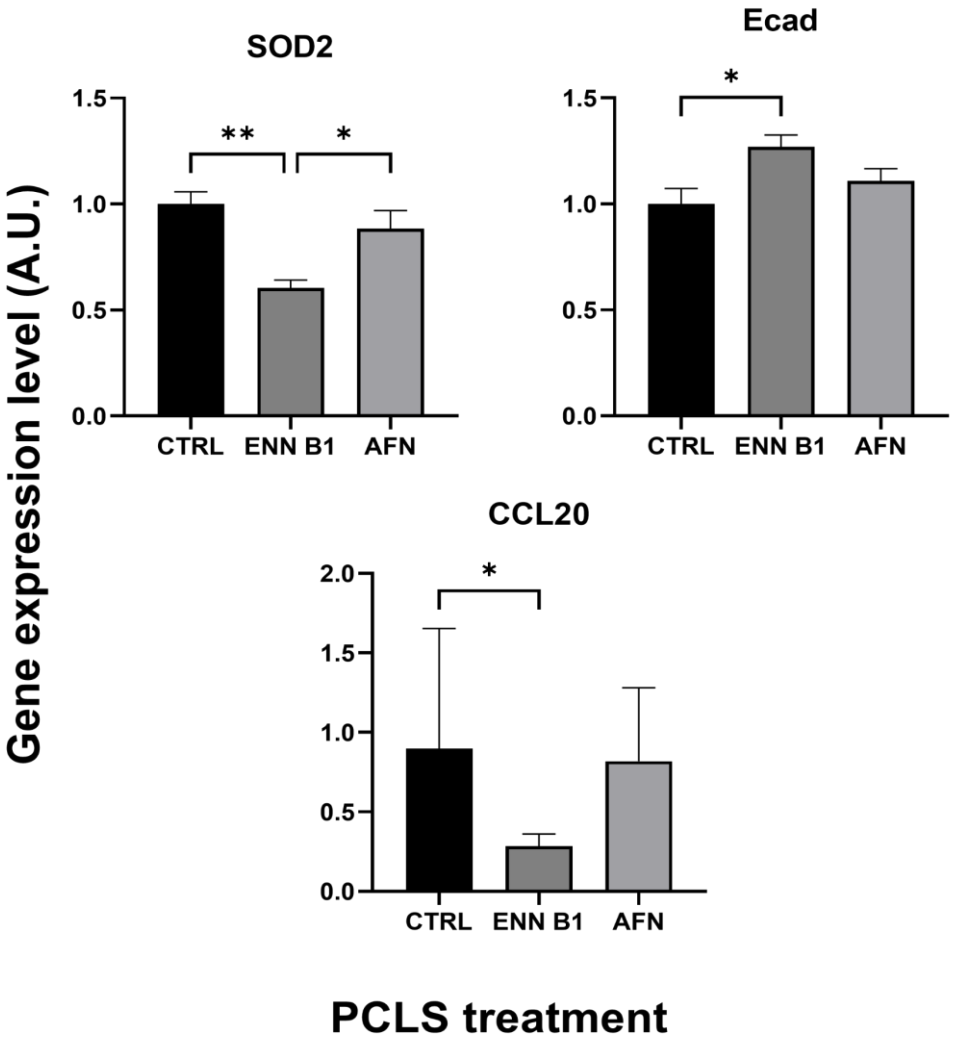

Figure 4. Significative changes in mRNA levels measured by RT-qPCR in PCLS exposed or not to 10 µM of ENN B1 and AFN for 4 h. Data are presented as mean ± SEM (SOD2 and Ecad) or median ± interquartile (CCL20), n=6, **p ≤0.01 and **p ≤0.01.

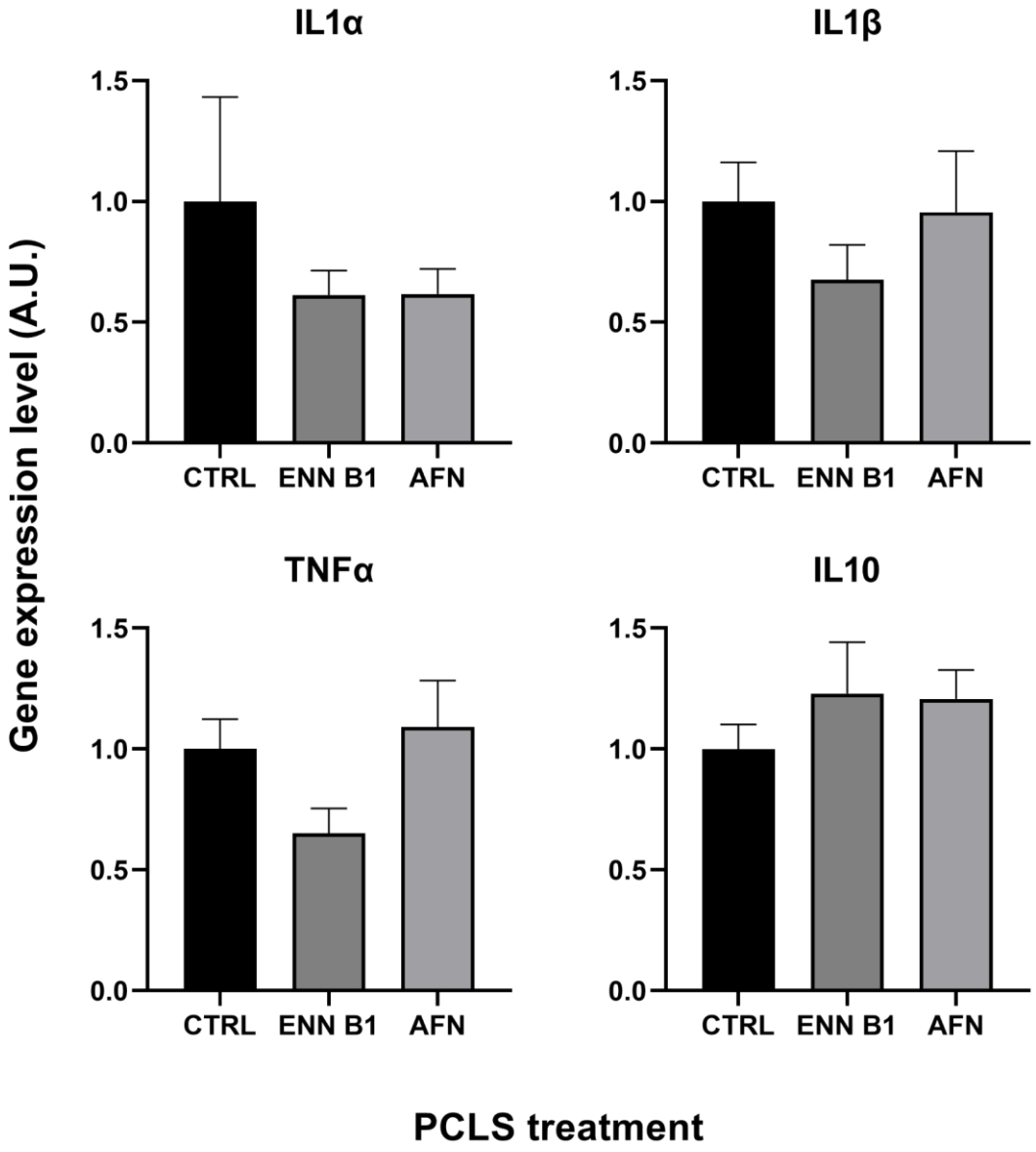

Figure 5. Cytokines mRNA levels measured by RT-qPCR in PCLS exposed or not to 10 µM of ENN B1 and AFN for 4 h. Data are presented as mean ± SEM, n=6.

4. Discussion

Cell viability is an important parameter to evaluate the toxicity of mycotoxins in *in vitro* studies. In the present study, we have verified hepatocyte viability by a test (CellTiter-Glo®) that measures ATP production by cells. The cytotoxicity observed after exposure to BEA and ENNs (A1, B, and B1) agrees with what has already been described in previous studies with similar concentrations in intestinal (Caco-2), liver (HepG2) and ovarian (CHO -K1) cells (Fraeyman et al., 2018b; Juan-García et al., 2019; Zouaoui et al., 2016). Furthermore, BEA and ENNs (A, A1, B, and B1) can promote cell cycle arrest in

different phases, thus inhibiting the proliferation of intestinal cells (Prosperini et al., 2013a, 2013b).

The cytotoxicity presented by API in HepG2 cells has also been observed in intestinal cells (Khoshal et al., 2019; Novak et al., 2019). The API toxicity mechanism is still not well elucidated, but it is known that it has antiparasitic properties through the inhibition of histone deacetylase and antiproliferative activity against various cancer cell lines (Han et al., 2000).

On the other hand, treatments with high concentrations of ENN B (100 μM), ENN B1 (30 μM), and AFN (100 μM), induced an increase in cell viability, indicating greater ATP production. It is known that ENNs have ionophore properties, which can form pores in the mitochondrial membrane, and have a greater affinity for the potassium ion (K^+), allowing the passage of this ion through the mitochondrial membrane (Tonshin et al., 2010). Therefore, it is possible that there was an influx of K^+ into the mitochondria, so the Na^+/K^+ ATPase pump needs more ATP to carry out the active transport of K^+ ions from the extracellular environment to the cytoplasm in order to reestablish intracellular homeostasis (Miller and Zachary, 2017).

Regarding AFN, previous results in IPEC-J2 cells exposed to 10 μM of AFN show that cell viability was not affected, even after 72 h (Springler et al., 2016). However, in Caco-2 cells, the same concentration of AFN for 24 h was cytotoxic, decreasing cell viability measured by mitochondrial activity by 51% (Vejdovszky et al., 2016). The present study appears to be one of the first to evaluate the cytotoxicity of AFN in liver cells, and the differences in results between cell types suggest that human liver cells may be more resistant than human intestinal cells.

In addition to cell viability, we also tested gene expression, but in a shorter exposure time (4h) which was the same period that PCLS explants were exposed to treatments for comparison purposes. The transcription factors-related genes (ATF3 and NF-κB) were increased after exposure to BEA, ENNs (A1, B, and B1), and EMO. ATF3 is related to endoplasmic reticulum (ER) stress. When there is no ER relief, cells may undergo apoptosis (Li et al., 2006), which is an already described effect that BEA and ENNs (A1 and B1) cause in HepG2 cells (Juan-García et al., 2015). Therefore, the higher rate of apoptosis and overexpression of ATF3 may be related.

Furthermore, inflammatory genes (IL8 and TNFα) were tested and had an increase in their expression with BEA, ENNs (A1, B, and B1), and API treatments. But in contradiction with our results,

in immune cells, BEA and ENNs stimulate IL10 secretion in macrophages and impair their phagocytic capacity (Ficheux et al., 2013) and even lower concentrations of BEA (0.1-4.5 μM) inhibited the NF-κB transcription factor pathway in macrophages (Yoo et al., 2017). It is possible that acute exposure (4h) to BEA and ENNs exerts different effects since our results show an increase in the expression of pro-inflammatory cytokines and transcription factors. While EMO and AFN did not increase the expression of cytokines, it is likely that these mycotoxins are not hepatotoxic after a short exposure, as they also did not cause a change in the morphology of the explants, nor the activity of liver enzymes.

The liver is the main organ to metabolize toxic compounds, including mycotoxins. In the liver, there are several enzymes involved in the metabolism of these compounds, including cytochrome p450 (CYPs). Therefore, we investigated the effects of metabolic enzymes on emerging mycotoxins metabolism in HepG2 cells. Even a short exposure (4h) to BEA and ENNs (A1, B, and B1) increased CYP1A1 expression. In other kinds of liver and endocrine cells, ENN B was able to promote the same effect. CYP1A1 is involved in an NADPH-dependent electron transport pathway and the metabolism of ENN B in human, canine, and rat liver microsomes (Fæste et al., 2011; Kalayou et al., 2015).

In contrast, a decrease in the expression of CYP1A1 by EMO and AFN was observed. EMO exerts a hepatoprotective effect in HepG2 cells against alcohol-induced damage by decreasing the expression of CYP2E1 (Qian et al., 2011). Moreover, CYP1A2 is also involved in the metabolism of EMO in the liver of rats (Zhou et al., 2023). Contrary to previous results, in which EMO exerted a hepatotoxic effect (Wang et al., 2022), in the present study we demonstrated that CYP1A1 is also affected by EMO in liver cells, but with a decrease in the expression of this gene. The difference can be explained due to the short exposure time and the lower concentration used in the explants.

Studies regarding gene expression with AFN and API have practically not been carried out to date. It is possible that AFN has also the same effect as EMO regarding CYP1A1 and that API can produce inflammation in the liver by increasing TNFα expression based on the results found. Therefore, the small amount of information available in the literature makes it even more important to further study the effects of these mycotoxins.

Although *in vitro* studies are of great importance to assess the toxic effects of mycotoxins, they do not fully replace studies in a living organism, where there are greater complexity and system

interactions. Therefore, we decided to use the *ex vivo* technique, in which there are fewer animals used in experimentation, with good results like those found *in vivo* (Alassane-Kpembi et al., 2017b; Gerez et al., 2021; Pierron et al., 2022).

Tissue damage was observed in PCLS exposed to ENN B1, despite the short time of exposure (4 h), but no changes in the injury liver enzymes were verified. The PCLS model in the study of mycotoxin toxicity has already proven effective in detecting the same histological changes found *in vivo* (Hasuda et al., 2022). Increased lesion score and histological changes caused by ENNs and BEA have already been reported *in vivo* in piglets (Novak et al., 2021). The same histological pattern was observed in the present *ex vivo* study for ENN B1, thus corroborating previous results. ENN B1 is rapidly absorbed orally, bioaccumulates, and is metabolized in the liver to be excreted (Devreese et al., 2014; Rodríguez-Carrasco et al., 2016).

As only ENN B1 had a significant increase in lesion score and AFN was the other mycotoxin with the highest lesion score, but without a significant increase, we decided to test the gene expression only with these two mycotoxins to see if there was any change in the expression of genes related to liver metabolism and health, inflammation, oxidative stress, and apoptosis. Despite the variety of genes tested, only SOD2, CCL20, and Ecad changed with ENN B1 treatment.

SOD2 is an important antioxidant enzyme that has manganese as a cofactor and is present in the mitochondria of aerobic cells (Zelko et al., 2002). SOD2 is a key molecule in cells because it provides resistance against oxidative damage (Zou et al., 2017). In the intestine of Wistar rats, acute treatment (8 h) of ENNs led to a decrease in the expression of genes associated with the enzymatic antioxidant defense system (SOD1 and GPx1) (Cimbalo et al., 2021). In PCLS explants, ENN B1 treatment also impairs the enzymatic antioxidant system, with a decrease in SOD2.

CCL20 is an inflammatory chemokine related to lymphocyte attraction and it is negatively regulated by IL10 secretion (Hieshima et al., 1997). In the present study, we found a downregulation of CLL20, and a tendency to increase the IL10 expression with ENN B1 treatment, which could explain the decrease in CCL20 expression.

Ecad is an important cell junction protein. In general, it decreases in the intestine of in vivo and ex vivo studies with mycotoxins such as deoxynivalenol and fumonisin B1 (Basso et al., 2013; Lucioli

et al., 2013; Silva et al., 2019). However, in another study with pigs, acute mycotoxin exposure led to an increase in Ecad expression (Bracarense et al., 2020). It is possible that an acute exposure leads to an increase in Ecad as a way of trying to protect the organ, but in more chronic exposures, such as *in vivo* studies, this expression decreases, as mycotoxins impair the structural integrity of the organ (Pinton et al., 2009).

The lack of changes in the liver biochemicals and gene expression could be due to the short exposure time in which the explants were submitted. Also, it is possible that an interindividual variability could cause this lack of significance. More studies for a longer exposure time should be performed to better understand the possible hepatotoxic effects that emerging mycotoxins, especially the poorly studied ones like AFN and API, can develop. In addition, our results can contribute to *ex vivo* data to aid in the establishment of regulation of emerging mycotoxins like BEA and ENNs.

5. Conclusion

. Taken together, our results indicate that emerging mycotoxins, particularly beauvericin, enniatins, and apicidin have the potential to be hepatotoxic. These mycotoxins were able to decrease cell viability and changed the expression of genes related to inflammation and hepatic metabolism in human liver cells. In PCLS explants, ENN B1 in particular changed the liver morphology and the expression of some genes after acute exposure. Although changes indicating loss of function or liver damage were not identified in the supernatant of the medium in which the explants were cultured.

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9 CONSIDERAÇÕES FINAIS

No geral, os resultados desse estudo forneceram novos *insights* sobre o impacto negativo da ingestão de micotoxinas emergentes sozinhas ou associadas com DON no desempenho, saúde intestinal e parâmetros imunológicos em suínos. Mostrando que essas micotoxinas causaram alterações histológicas em fígado, intestino delgado e linfonodos dos suínos. Também impactaram negativamente na microbiota fecal, com diminuição da diversidade, indicando uma piora na saúde intestinal, podendo afetar também a imunidade desses animais.

Além disso, os resultados encontrados forneceram dados adicionais sobre os efeitos tóxicos do DON e outras micotoxinas emergentes, principalmente BEA e ENNs no fígado, evidenciando que explantes de fígado cortados com precisão (PCLS) são uma ferramenta relevante para avaliar a toxicologia de contaminantes alimentares como as micotoxinas.

O modelo *ex vivo* de PCLS demonstrou ser uma alternativa melhor ao modelo *in vitro*, pois no modelo de explantes há conservação da estrutura 3D, bem como interações célula-célula e célula-matriz que não ocorrem no cultivo celular. Também é um bom substituto ao modelo *in* vivo, já que mantém características próximas ao *in vivo* e menos animais são utilizados na experimentação.

Além das vantagens fornecidas pelo modelo *ex vivo*, mais estudos devem ser realizados para avaliar se uma exposição longa (superior a quatro horas) às micotoxinas emergentes também podem causar alterações morfológicas e transcricionais significativas tardias em explantes de fígado cortados com precisão.

APÊNDICES

APÊNDICE A

Tabela complementar ao artigo B “Deoxynivalenol induces apoptosis and inflammation in the liver: Analysis using precision-cut liver slices”

Table S1. Effects of DON exposure (10 μM) on hepatic biomarkers in PCLS supernatant (mean ± SEM).

Hepatic biomarkers	**Control**	**DON**
Alkaline phosphatase (U/L)	4.393±1.599	3.687±1.892
Alanine transaminase (U/L)	6.333±4.885	6.500±2.811
Aspartate aminotransferase (U/L)	260.8±153.6	278.2±99.23
Lactate dehydrogenase (U/L)	83.02±40.27	100.3±47.71
Total proteins (g/L)	5.707±0.573	6.311±0.452

APÊNDICE B

Tabela complementar ao artigo C “Emerging mycotoxins induce ex vivo and in vitro hepatotoxicity in pigs precision-cut liver slices and HepG2 cells”

Table S1: Effects of emerging mycotoxins exposure (10μM) on liver enzymes and protein content in PCLS supernatant (mean ± SEM).

Liver biochemicals	**ALP (U/L)**	**ALT (U/L)**	**AST (U/L)**	**LDH (U/L)**	**TP (g/L)**
Control	4.39±1.31a	6.33±4.46a	260.83±140.19a	83.02±36.76a	5.71±0.52a
BEA	3.70±1.18a	6.17±2.54a	238.00±71.16a	84.48±32.87a	6.28±0.55a
ENN A1	3.00±1.63a	6.33±3.77a	238.00±100.34a	83.02±43.89a	5.88±0.83a
ENN B	3.67±1.77a	6.17±2.61a	259.67±92.58a	94.10±39.19a	6.31±0.66a
ENN B1	3.65±1.17a	6.33±2.92a	242.17±91.80a	81.28±36.67a	6.21±0.56a
AFN	4.06±2.02a	8.00±3.37a	249.00±22.83a	104.88±33.38a	5.81±0.69a
API	3.18±1.24a	5.33±3.09a	221.33±122.22a	90.07±48.27a	5.90±0.99a