JULIANA RUBIRA GEREZ

FUSARIOTOXINAS EM SUÍNOS: EFEITOS TÓXICOS *IN VIVO* E *EX VIVO*

Londrina
2014

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Tese apresentada ao Programa de Pós-Graduação em Ciência Animal, área de concentração Sanidade Animal, da Universidade Estadual de Londrina, como requisito à obtenção do título de Doutor.

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

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Tese apresentada ao Programa de Pós-Graduação em Ciência Animal, área de concentração Sanidade Animal, da Universidade Estadual de Londrina, como requisito à obtenção do título de Doutor.

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Dedico este trabalho a Darci Rubira Redondo, minha amiga e mãe,
cuja força e coragem faz sonhos se tornarem realidade.

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“Se vi mais longe... é porque me apoiei nos ombros de gigantes.”

Isaac Newton

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RESUMO

Com objetivo de avaliar os efeitos sistêmicos da contaminação alimentar por micotoxinas, enfatizando aspectos morfológicos e imunohistoquímicos, dois experimentos foram realizados. No primeiro experimento, 20 leitões de cinco semanas de idade foram divididos em quatro grupos experimentais. Os animais receberam durante 28 dias os seguintes tratamentos: dieta controle, dieta contaminada com desoxinivalenol (DON) (1,5 mg/kg), dieta contaminada com DON (2 mg/kg) + nivalenol (NIV) (1,3 mg/kg) + zearalenona (ZEA) (1,5 mg/kg) ou dieta contaminada com DON (3 mg/kg) + NIV (1,3 mg/kg) + ZEA (1,5 mg/kg). A dieta mono-contaminada não alterou o ganho de peso, entretanto os animais expostos à dieta multi-contaminada apresentaram uma diminuição significativa no ganho de peso final. A ingestão crônica das dietas contaminadas induziu alterações histológicas no intestino, como atrofia e fusão de vilosidades, diminuição na altura de vilosidades e na profundidade de criptas, redução no número de células caliciformes e linfócitos. O fígado, os linfonodos mesentéricos e o baço dos animais expostos às dietas contaminadas apresentaram um aumento significativo de lesões. Um aumento significativo na expressão de caspase-3 nos órgãos linfoides foi observado nos animais que receberam as dietas contaminadas. No segundo experimento, 12 leitões de 4 a 5 semanas de idade foram eutanasiados para obtenção de explantes jejunais, os quais foram expostos a cinco tratamentos por 4 horas, sob agitação constante a 37ºC em atmosfera umidificada a 5% de CO_2. No tratamento controle foi utilizado meio de cultura William E sem ou com o diluente DMSO a 0,1%. Nos demais tratamentos os explantes foram expostos a DON, NIV (1, 3, 10 µM) e fusarenona X (FX) (0,3, 1 e 3 µM). Após incubação, as amostras de tecidos foram processadas histologicamente e analisadas utilizando-se escore histológico. Não foi observada diferença significativa no escore histológico entre amostras não incubadas e incubadas com meio de cultura na ausência ou presença de DMSO. As principais alterações observadas nos explantes expostos às micotoxinas foram atrofia de vilosidades, células epiteliais cúbicas e pavimentosas, edema na lâmina própria e desnudamento apical com perda de enterócitos. Os tratamentos individuais com DON, NIV e FX induziram uma diminuição do escore histológico a partir das doses de 3µM, 1µM e 0,3 µM, respectivamente. Em conclusão, os dados obtidos fornecem uma melhor compreensão dos efeitos das fusariotoxinas isoladas ou em combinação sobre a morfologia intestinal e de órgãos linfoides, as quais podem predispor os animais a infecções secundárias.

Palavras-chaves: Micotoxinas. Fusariotoxinas. Morfologia. Órgãos linfoides. Explantes jejunais.

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ABSTRACT

In order to evaluate the systemic effects of food contamination by mycotoxins, emphasizing morphological and immunohistochemical aspects, two experiments were conducted. In the first experiment, 20 5-week-old piglets were randomly assigned to four groups. The animals received for 28 days the following treatments: control diet, a diet contaminated with deoxynivalenol DON (1.5 mg/kg), a diet contaminated with DON (2.0 mg/kg) + nivalenol (NIV) (1.3 mg/kg) + zearalenone (ZEA) (1.5 mg/kg) or a diet contaminated with DON (3.0 mg/kg) + NIV (1.3 mg/kg) + ZEA (1.5 mg/kg). The mono-contaminated diet showed no difference in weight gain, however the animals fed the multi–contaminated diets presented a significant decrease in final weight gain. The chronic ingestion of these contaminated diets induced histological changes in the intestine as show by atrophy and fusion of villi, decreased villi height and crypth depth, and reduced number of goblet cells and lymphocytes. The liver, mesenteric lymph nodes and spleen of animals exposed to contaminanted diets showed a significant increase of lesions. A significant increase in caspase-3 expression in lymph nodes and spleen was observed in animals receiving the contaminated diets. In the second experiment, 12 4 to 5-week-old piglets were euthanized to obtain jejunal explants, which were exposed to 5 treatments for 4 hours, under constant stiring at 37ºC and 5% CO_2 humidified atmosphere. In the control treatment were used the William's medium E without or with the diluent DMSO to 0.1%. In the other treatments the explants were exposed to DON, NIV (1, 3, 10 µM) and fusarenone X (FX) (0.3, 1 and 3 µM). After incubation, the tissue samples were histologically processed and analyzed using histological score. The analyse of samples non-incubated and incubated with culture medium in the absence or presence of DMSO showed no significant different of histopathological score. The main lesions observed in the explants exposed to mycotoxins were villi atrophy, cuboidal and squamous epithelial cells, areas of oedema in the lamina propria and apical denudation with loss of enterocytes. The individual treatment with DON, NIV and FX resulted in a significant decrease of the histopathologic score from doses of 3µM, 1µM and 0.3 µM, respectively. In conclusion, the data obtained provide a better understanding of the possible effects of *Fusarium* toxins, alone or in combination on the morphology of the intestine and lymphoid organs, which would may predispose animals to secondary infections.

Keywords: Mycotoxins. *Fusarium* toxins. Morphology. Lymphoid organs. Jejunal explants.

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Caco-2	Células de Adenocarcinoma de cólon humano
DMSO	Dimetilsulfóxido
DON	Desoxinivalenol
EDTA	*Ethylenediamine Tetraacetic Acid* (Ácido Tetracético Etilenodiamino)
ENVT	*École Nationale Vétérinaire de Toulouse*
ERK	*Extracellular Signal-regulated Kinases*
FAO	*Food and Agricultural Organization*
FAOSTAT	*Food and Agriculture Organization of the United Nation*
FB_1	Fumonisina B_1
FX	Fusarenona X
HE	Hematoxilina-eosina
IC_{50}	Concentração inibitória de 50% de viabilidade celular
IARC	*International Agency for Research on Cancer*
IEC-6	*Intestinal epithelial cells*
INRA	*Institut National de la Recherche Agronomique*
IPEC	*Intestinal Porcine Epithelial Cells*
JNK	*c Jun N-terminal Kinases*
LOAEL	*Lowest-observed-adverse-effect level*
MAPK	*Mitogen-activated Protein Kinase*
NIV	Nivalenol
NOEL	*No observable effect level*
SCF	*Scientific Committee on Food*
SDM	*Mean Standard Deviation*
SD	*Standard Deviation*
TCA	*Trichloroacetic Acid*
ZEA	Zearalenona

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1 INTRODUÇÃO

A carne suína é a fonte de proteína animal mais consumida no mundo. De acordo com o *United States Departament of Agriculture* (USDA), no ano de 2012 foram produzidas 104 milhões de toneladas de carne suína, sendo que aproximadamente 50% deste total foram produzidos na China. O bloco da União Europeia, composto por 27 países, é o segundo maior produtor, com uma produção de 23 milhões de toneladas. O terceiro maior produtor são os Estados Unidos, com 10 milhões de toneladas. O Brasil representa 3,1% da produção mundial (GERVÁSIO, 2013).

Com um sistema produtivo baseado na integração, disponibilidade de insumos básicos para a produção, principalmente de grãos como soja e milho, e investimentos em tecnologia, a suinocultura no Brasil apresenta custos inferiores aos principais competidores mundiais (FISCHER, 2011). Além disso, o Brasil é considerado o segundo e o terceiro maior produtor mundial de soja e milho, respectivamente (FAOST, 2012).

O desenvolvimento crescente da cadeia suinícola mundial e brasileira tem levado também a preocupação em relação à qualidade sanitária das matérias primas destinadas à alimentação humana e animal (GONZALES-MARTINEZ; CORRADINI, PELEG, 2003). A busca por qualidade está relacionada ao controle de fitopatógenos, como fungos toxigênicos, visando minimizar a produção de toxinas naturais nas etapas de pré, pós-colheita e armazenamento (FRANCESCHINI et al., 2001). Sendo assim, é necessário preocupar-se com a possível contaminação por fungos nos alimentos destinados ao homem e aos animais e o risco associado à presença de micotoxinas.

Efeitos adversos na saúde e prejuízos na produção animal têm sido reconhecidos em rebanhos suínos e de aves que ingerem alimentos contaminados com micotxinas. Estas espécies são particularmente suscetíveis devido a consumirem altas concentrações de cereais em suas dietas (SMITH, 1994). De acordo com a *Food and Agricultural Organization* (FAO), estima-se que as perdas globais de alimentos pela contaminação com micotoxinas estejam em torno de um milhão de toneladas ao ano (FAO, 2004).

Os cereais que constituem a dieta dos suínos certamente são a principal fonte dessas toxinas, uma vez que os mesmos servem de substrato para o crescimento dos fungos e para a consequente produção de micotoxinas. Porém, nem todo o cereal que apresenta fungos está necessariamente contaminado por micotoxinas, devido ao fato que a produção e concentração dessas substâncias são determinadas por diversos efeitos

combinados, como espécies de fungos presentes, temperatura e umidade do grão (LANCOVA et al., 2008). Quando as micotoxinas estão presentes na dieta, vários outros fatores como espécie animal afetada, concentração e natureza da toxina vão determinar o efeito no organismo exposto a essas substâncias.

Estudos revelam que aproximadamente 90% das intoxicações por micotoxinas são crônicas e não apresentam sinais clínicos específicos, podendo ser facilmente confundidos com desnutrição, deficiência de manejo ou outras doenças crônicas que implicam na diminuição da produtividade animal (DILKIN, 2002). Poucas vezes as micotoxicoses se manifestam como doença aguda, culminando com a morte dos animais.

O objetivo desta tese foi avaliar os efeitos isolados de fusariotoxinas utilizando o modelo do explante intestinal, bem como avaliar os efeitos sistêmicos da mono e multi-contaminação micotóxica em suínos, com ênfase sobre os órgãos linfoides e morfologia intestinal. Suspeita-se dos efeitos imunossupressores das micotoxinas, todavia, seu estudo sistemático é relativamente recente e parcial. No presente trabalho postulamos que, mesmo em pequenas doses, as micotoxinas podem agir sobre a morfologia intestinal e órgãos linfoides, aumentando a sensibilidade do ser humano e dos animais às infecções.

2 REVISÃO DE LITERATURA

2.1 MICOTOXINAS

As micotoxinas são metabólitos secundários orgânicos de baixo peso molecular produzidas em sua grande maioria por fungos filamentosos do gênero *Aspergillus* ssp., *Penicillium* ssp. e *Fusarium* ssp. (OKOLI et al., 2007). As micotoxicoses, doenças causadas pela ingestão de micotoxinas, não envolvem necessariamente a presença do fungo produtor da toxina. Outra consideração relevante é que o fungo pode produzir mais de um tipo de micotoxina, e que estas toxinas, por sua vez, podem ser produzidas por diferentes espécies de fungos (FINK-GREMMELS, 1999). As características clínicas das micotoxicoses são as seguintes: doença não transmissível; tratamentos com drogas e antibióticos têm pouco ou nenhum efeito; os surtos são muitas vezes sazonais; os surtos são normalmente associados com um determinado gênero alimentício e o exame do alimento suspeito muitas vezes revela a presença de micotoxinas (MARIN et al., 2013).

As micotoxinas são metabólitos secundários não essenciais ao crescimento fúngico, produzidas eventualmente sob condição de estresse. Micotoxinas podem ser produzidas durante o cultivo, processamento e estocagem de cereais. Por isso, prevenir a ocorrência destas toxinas pode ser uma difícil tarefa. A produção das micotoxinas bem como o grau de contaminação nos alimentos é regulada por fatores como umidade do substrato (10 a 20%), umidade relativa do ambiente (≥70%), temperatura (0 a 50°C, dependendo da espécie de fungo) e disponibilidade de oxigênio (KANORA; MAES, 2009).

As micotoxinas podem contaminar uma variedade de alimentos consumidos por animais e humanos, como cereais, frutas, grãos e produtos manufaturados (BOUHET; OSWALD, 2005). De acordo com a *Food and Agriculture Organization* das Nações Unidas estima-se que aproximadamente 25% dos cereais produzidos em todo o mundo pode estar contaminado por micotoxinas (FAO, 2004). Devido às suas propriedades tóxicas e sua alta estabilidade a tratamentos tecnológicos usuais, permanecem mesmo após a eliminação do fungo no alimento. A presença das micotoxinas na cadeia alimentar é um risco potencial à saúde de humanos e animais (BOUHET; OSWALD, 2005).

Dentre as espécies animais, os suínos exibem grande sensibilidade às micotoxinas, e considerando que sua dieta é rica em cereais, pode ser exposto a estas toxinas com frequência. Além disso, o suíno pode ser considerado um bom modelo a ser extrapolado

para o homem, devido às similaridades relacionadas ao sistema digestório e imune (ROTHKÖTTER et al. 2002).

As diferentes espécies de *Fusarium* ssp. podem produzir mais de 180 metabólitos secundários (OSWALD et al., 2005). Entre as fusariotoxinas, destacam-se tricotecenos, fumonisinas e zearalenona, mas também as eniatinas, beauvericina, moniliformina e fusarioproliferina, as quais são frequentemente referidas como micotoxinas emergentes (JESTOI, 2008).

Através da resolução RDC nº 7 de 18 de fevereiro de 2011, a Agência Nacional de Vigilância Sanitária (ANVISA) estabeleceu limites máximos toleráveis de micotoxinas em diferentes classes de alimentos no Brasil. Em janeiro de 2012, os limites de desoxinivalenol, fumonisina (B1 e B2) e zearalenona foram estendidos para o restante das categorias de alimentos de interesse. No início de 2014 a resolução entrou em vigor para as matérias-primas. Para 2016, a resolução prevê uma redução de limites em relação àqueles estabelecidos em 2012, de forma a aproximá-los do que hoje está definido em outros países. A resolução RDC 07/2011 publicada no Diário Oficial em nove de março de 2011 se encontra nos anexos.

No presente trabalho, discutiremos a respeito de quatro fusariotoxinas presentes em cereais: desoxinivalenol (DON), nivalenol (NIV), fusarenona-X (FX) e zearalenona (ZEA).

2.1.1 Tricotecenos

Tricotecenos são um grande grupo de micotoxinas sesquiterpenóides produzidas principalmente por várias espécies de *Fusarium* (*F. sporotrichioides*, *F. graminearum*, *F. poae*, e *F. culmorum*), podendo ser também produzidos por membros de outros gêneros, como *Myrothecium* ssp., *Trichoderma* ssp., *Trichothecium* ssp., *Cephalosporium* ssp., *Verticimonosporiu* ssp. e *Stachybotrys* ssp. (HE et al., 2010). O nome tricoteceno advém do primeiro isolamento ter ocorrido na espécie *Trichothecium roseum* por Freeman em 1948.

Todas as micotoxinas deste grupo possuem em comum um núcleo tetracíclico, com uma ligação dupla entre os carbonos 9 e 10 e um grupamento epóxido nas posições 12 e 13, o que lhes confere grande reatividade e toxicidade (Figura 1). De acordo com o substituinte encontrado no C-8, os tricotecenos produzidos pelo gênero *Fusarium* ssp.

são classificados como tipo A (C-8 grupo isovaleril; toxina T2 e HT-2) e o tipo B (C-8 grupo cetona; DON e NIV) (Quadro 1).

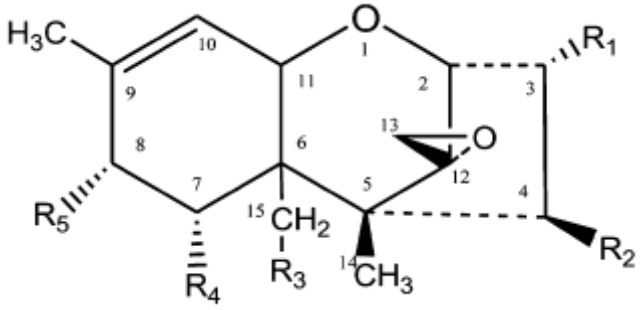

Figura 1. Estrutura básica dos tricotecenos. Fonte: HE et al., 2010

Quadro 1 – Estruturas químicas dos substituintes R1 – R4 dos Tricotecenos dos Tipos A e B.

Toxina	Abreviação	R1	R2	R3	R4
Tipo A					
Toxina T-2	T-2	OH	OCOCH	OCOCH	$OCOCH_2CH(CH_3)_2$
Toxina HT-2	HT-2	OH	OH	OCOCH	$OCOCH_2CH(CH_3)_2$
Diacetoxiscirpenol	DAS	OH	OCOCH	OCOCH	H
Tipo B					
Nivalenol	NIV	OH	OH	OH	-
Desoxinivalenol	DON	OH	H	OH	-
3-acetil-Desoxinivalenol	3aDON	OCOCH	H	OH	-
15-acetil-Desoxinivalenol	15aDON	OH	H	OCOCH	-
Fusarenona X	FX	OH	OCOCH	OH	-

Fonte: Goyarts (2006) (modificado)

As duas outras categorias de tricotecenos, tipo C e D, são produzidas por fungos do gênero *Myrothecium* spp., e são caracterizadas por um segundo grupo epóxi em C-7 ou C-9, 10 (tipo C) ou um anel macrocíclico entre C-4 e C-15 com dois ésteres (tipo C) (UENO, 1985).

Tricotecenos são componentes com peso molecular entre 250 e 550 Da, a maioria é insolúvel em água, mas altamente solúvel em solventes orgânicos como acetona, etilacetato, clorofórmio e dimetilsufóxido. Estas micotoxinas sãos estáveis quando expostas ao ar e/luz, mas podem ser degradados por bactérias e fungos. Os tricotecenos não são inativados em condições de 121ºC/ 15 min/ 1 atm, mas podem ser inativados em meios fortemente básicos ou ácidos e por aquecimento com temperatura de 482ºC por 10 minutos ou 260ºC por 30 minutos (SUDAKIN, 2003). Assim, não são degradados durante o processo de industrialização dos alimentos e não são hidrolisados pelo processo de digestão no sistema gastrointestinal (LAUREN; SMITH, 2001).

As principais fontes de tricotecenos na alimentação humana e animal são o trigo, a cevada e o milho. Estas micotoxinas podem entrar na cadeia alimentar humana e animal através de cereais matinais, produtos de panificação, cerveja, e componentes alimentares derivados de grãos (LANCOVA et al., 2008). Além disso, os humanos podem ingerir os tricotecenos através do consumo de ovos, leite e carne oriundos de animais alimentados com ração contaminada por estas micotoxinas. Embora a exposição de risco a humanos pelo consumo de tecidos comestíveis contaminados exista, esta é menor quando comparado ao consumo direto e indireto de grãos (RATCLIFF, 2002).

Os principais efeitos dos tricotecenos são decorrentes da acentuada inibição da síntese proteica (CUNDLIFFE; CANNON; DAVIES, 1974). Tricotecenos são moléculas hidrofóbicas que podem atravessar as membranas celulares e interagir com vários alvos, incluindo ribossomos e mitocôndrias (BIN-UMER et al., 2011). Dependendo dos substituintes, os tricotecenos atuam inibindo tanto a iniciação quanto o alongamento e terminação da síntese proteica (EHRLICH; DAIGLE, 1987). Tricotecenos que inibem a iniciação da cadeia peptídica são inúmeras vezes mais potentes do que aqueles que afetam o alongamento e terminação das cadeias peptídicas (EHRLICH; DAIGLE, 1985).

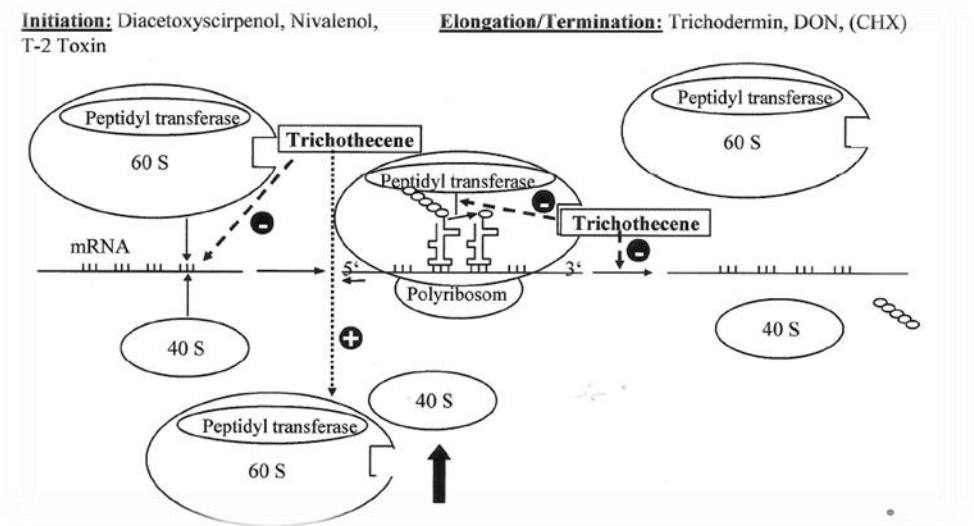

Figura 2. Mecanismo ação de inibição protéica dos tricotecenos. Inibidores de iniciação da cadeia polipeptídica irão acumular ribossomos livres (40S+60S), estes não são capazes de se ligar ao mRNA. Inibidores de elongação e terminação vão aumentar a quantidade de poliribossomos (80S) bem como o desacoplamento de mRNA e a liberação da cadeia peptídica é inibida por efeitos inibitórios ou de ativação. Fonte: Goyarts, 2006.

Inibidores da peptidiltransferase podem desencadear uma reação denominada como resposta ao estresse ribotóxico, o que ativa proteínas quinases ativadas por

mitógeno (MAPK), consideradas componentes de uma cascata de sinalização que induz apoptose e regula a sobrevivência celular em resposta ao estrese (SHIFRIN; ANDRESON, 1999). Além da inibição da síntese proteica, os tricotecenos apresentam vários outros efeitos inibitórios sobre as células eucariontes, como a inibição da síntese de DNA e RNA, bem como efeitos adversos na função mitocondrial (CHAROENPORNSOOK et al. 1998, MEKHANCHA-DAHEL et al., 1990; MINERVINI et al., 2004). No entanto, esses efeitos parecem ser secundários a inibição da síntese proteica (THOMPSON; WANNEMACHER, 1990).

2.1.1.1 Desoxinivalenol

O DON foi primeiramente caracterizado e isolado de cevada contaminada no Japão. DON é produzido principalmente por *F. graminearum* e *F. culmorum* (MOSS, 2002). Ambas as espécies têm diferentes temperaturas ótimas de crescimento e isso provavelmente afeta sua distribuição geográfica (MOSS, 2002). Em países desenvolvidos, onde os grãos são secos obtendo um teor de umidade ≤13%, DON é uma importante micotoxina encontrada na fase de pré-colheita. Entretanto, em países onde o teor de umidade dos grãos é menos rigorosamente controlado, DON pode também ser produzido durante a estocagem. Infecções fúngicas que resultam na produção de DON no campo são principalmente dependentes de condições climáticas e são favorecidas por baixas temperaturas e alta umidade (EFSA, 2013).

Vários reagentes químicos tais como amônia, hidróxido de cálcio, cloro, ácido clorídrico, ozônio, bissulfato de sódio e hidróxido de sódio podem degradar DON. Entretanto, nenhum destes produtos pode ser aplicado, uma vez que interferem com o processamento de grãos e representam riscos para à saúde humana e animal (EFSA, 2013). DON é estável sob condição fracamente ácida, mas é instável sob condições alcalinas (LARSEN et al., 2004).

DON é uma micotoxina que comumente contamina os alimentos a base de cereais. É detectada frequentemente em nível de mg/Kg (PESTKA, 2007). A contaminação mundial de DON é observada com maior frequência em grãos de cereais como trigo, milho ou cevada (EFSA, 2013). Na década de 1990, constatou-se que 80% das lavouras de trigo e cevada nos Estados Unidos e Canadá estavam contaminadas com *F. graminearum*, sendo que apenas 19% dos grãos produzidos apresentavam níveis abaixo de 0,5 mg/kg (WINDELS,

2000). Um estudo realizado com 11.022 amostras de cereais de 12 países europeus mostrou que 57% foram positivas para DON e que em 7% dessas a concentração de DON era igual ou superior a 0,00075 mg/kg (SCOOP, 2003).

No sul do Brasil, 24,9% de 297 amostras de trigo utilizadas na alimentação humana apresentaram contaminação por DON, com níveis variando entre 0,0006 mg/kg a 8,5 mg/kg (MALMANN et al., 2003). Em outro estudo, realizado no Estado de São Paulo, constatou-se a contaminação por DON em 45% das 42 amostras de trigo analisadas em níveis que variaram de 0,8 a 1,5 mg/kg (LAMARDO et al., 2006). Do total de 50 amostras de trigo provenientes dos Estados de São Paulo (SP), Paraná (PR) e Rio Grande do Sul (RS) e 50 amostras de trigo importado provenientes da Argentina e Paraguai, 94% do trigo nacional e 88% do trigo importado apresentaram-se contaminados com DON em níveis médios de 0,332 mg/kg (nacional) e 0,00009 mg/kg (importado) (CALORI-DOMINGUES et al., 2007). Através da análise de 38 amostras de trigo provenientes de diferentes cultivares e localidades do PR e RS, Santos et al. (2011) detectaram a presença de DON em 29 amostras (76,3%) através do teste ic-ELISA (0,282-12,291 mg/kg) e em 22 amostras (57,9%) através da técnica LC-MS (0,155-9,907 mg/kg). Savi et al. (2014) analisando amostras de grãos de trigo colhido no sul do Brasil na safra de 2012, detectaram a presença de DON em 47,2% (25 de 53 das amostras analisadas), com níveis entre 0,2437 mg/kg a 2,2813 mg/kg. Segundo a ANVISA (2011), a partir de janeiro de 2014 o limite máximo tolerável de DON na farinha de trigo é 1,25 mg/kg (Anexo B), enquanto o limite máximo na União Europeia é 0,5 mg/kg (para produtos de cereais) e 0,75 mg/kg para farinha usada como matéria prima (SCOOP, 2003).

Na célula, DON interage com a peptidiltransferase na subunidade ribossômica 60S, causando um bloqueio na tradução proteica (UENO, 1985; PESTKA, 2004). Como resultado, a síntese proteica é afetada, ocorrendo a chamada “síndrome do stress ribotóxico” (PESTKA et al., 2004; YANG et al., 2000), resultando na ativação das MAPKs e de seus mecanismos aplicados na apoptose e inflamação (SHIFRIN; ANDERSON, 1999; LASKIN et al., 2002).

Os estudos sobre toxicidade aguda em animais sensíveis incluem desconforto abdominal, aumento da salivação, mal-estar, diarreia e anorexia. Os efeitos da toxidade crônica em estudos animais são diminuição do desempenho zootécnico gerando uma grande perda econômica, especialmente na produção suína (GOYARTS, 2006). Os efeitos anoréxicos e eméticos provocados por desoxinivalenol são, supostamente, mediados pelo sistema serotoninérgico, conforme já descrito por Rotter et al. (1996), onde DON diminui as concentrações de serotonina e seus metabólitos no fluido cerebroespinhal de suínos

(PRELUSKY et al., 1993). Segundo estudos realizados por Pestka et al. (2004), DON é menos tóxico do que outros tricotecenos, como a toxina T-2, mas a exposição aguda a altas doses de desoxinivalenol pode levar o animal à morte por choque.

Estudos *in vivo* e *in vitro* demonstram que o sistema imune inato é o principal alvo de DON, sendo que os tricotecenos podem afetar leucócitos pela superexpressão na produção de citocinas e pela indução da apoptose (ZHOU et al., 1998, 2003). Dependendo da dose e frequência de exposição, DON pode ter ação imunossuspressora ou imunoestimulatória (PESTKA; SMOLINSKI, 2005; PINTON et al., 2008). A exposição crônica a baixas doses de tricotecenos é responsável pela redução na produtividade e proliferação linfocitária, resistência do hospedeiro, função imune humoral e celular e pelo aumento da suscetibilidade a doenças infecciosas (BONDY; PESTKA, 2000; PESTKA et al., 2004).

A Agência Internacional de Pesquisa do Câncer (IARC) concluiu em 1993 que «não há evidências em animais experimentais para a carcinogenicidade do desoxinivalenol». Na interpretação dos resultados obtidos em literatura, a IARC (1993) concluiu que DON induz à transformação celular, aberrações cromossômicas e inibe a comunicação entre as junções intercelulares em culturas de células de mamíferos. DON está classificado no Grupo 3, «não classificados quanto a sua carcinogenicidade em seres humanos».

2.1.1.2 Nivalenol

NIV é produzido principalmente por *Fusarium cerealis* e *F. poae*, no entanto *F. culmorum* e *F. graminearum* também podem produzi-lo. *F. poae* é mais difundido na Europa e um importante produtor de NIV (LARSEN 2004). No Japão, amostras analisadas, apresentaram tricotecenos e especialmente NIV em grãos de trigo e cevada (YOSHIZAWA; JIN, 1995).

NIV ocorre em várias colheitas de cereais tais como trigo, milho, cevada, aveia e centeio (SCF, 2000). Esta micotoxina ocorre mais frequentemente em safras com épocas de cultivo quentes e secas. NIV é mais comumente identificado na Europa, Austrália e Ásia do que na América. Ambos os níveis médios e incidência de amostras positivas são mais baixos do que para DON, mesmo nos países nórdicos e na Europa (SCF, 2000).

Rodrígues-Carrasco et al. (2013), identificaram que 13,4% das 119 amostras de trigo coletadas na região de Valencia em 2012 estavam contaminadas com NIV em

concentrações permitidas pela legislação europeia. Em estudo realizado com 19 amostras de trigo coletadas das principais regiões produtoras da Argentina entre 2000 e 2002, apenas duas amostras apresentavam contaminação por NIV (0,05 mg/kg e 0,1 mg/kg) com a presença de DON (7,5 mg/kg e 6,7 mg/kg) (PINTO et al., 2008). No Brasil, 20% das 80 amostras de milho analisadas produzidas no Estado de São Paulo entre 2001 e 2002 apresentaram níveis de contaminação por NIV entre o limite de detecção e o limite de quantificação.

NIV é um potente inibidor da síntese de proteína, RNA e DNA em células de mamíferos, causando morte celular em células com alta taxa proliferativa (SCHLATTER, 2004). Assim, tecidos com alta taxa de divisão celular, tais como órgãos linfoides e mucosa intestinal, são altamente sensíveis a esta micotoxina (UENO et al., 1985).

Suínos expostos a dieta contendo 2,5 ou 5 mg de NIV purificado/kg de alimento por três semanas não apresentaram nenhum sinal clínico. Entretanto, no exame macroscópico, a maioria dos animais expostos à dieta contaminada revelou erosões gastrointestinais e sinais de nefropatia. Uma diminuição no número de linfócitos do baço foi observada, bem como aumento nos níveis de IgA plasmática (HEDMAN et al., 1997).

Para ratos machos e fêmeas F344 expostos a NIV via oral, uma (Dose Letal 50 (DL_{50}) de 19,5 mg/kg de peso corpóreo foi relatada (KAWASAKI et al., 1990). Os efeitos observados foram sedação, fechamento de pálpebra, diarreia e congestão nos pulmões e trato digestório. Em outro trabalho com ratos machos e fêmeas F344 submetidos a uma dieta contendo 6,25; 25 ou 100 mg/kg de NIV por 90 dias observou-se redução do peso corpóreo e fezes amolecidas. Análise hematológica revelou uma diminuição de leucócitos e eritrócitos, como também diminuição na concentração de hemoglobina e plaquetas. As principais alterações histológicas observadas foram atrofia tímica, hipocelularidade na medula óssea e aumento de folículos ovarianos atrésicos. Baseados nos dados hematológicos, o NOEL (Nível de efeito adverso não observado) de NIV foi de 0,4 mg/kg de peso corpóreo para os animais machos e fêmeas (TAKAHASHI et al., 2008).

Em camundongo machos ddY, NIV parece ser menos tóxico após a administração oral (DL_{50} de 38,9 mg/kg de peso corpóreo) em relação a administrações intraperitoneal, intravenosa ou subcutânea (DL_{50} de 7,4; 7,3 e 7,2 mg/kg de peso corpóreo, respectivamente). Independente da via de administração, a maioria das mortes aconteceu dentro de três dias após exposição a esta micotoxina. A análise histopatológica revelou intensa congestão e hemorragia nos intestinos (RYU et al., 1988). A administração oral de 15 mg NIV/kg de peso corpóreo em camundongos fêmeas BALB/c induziu apoptose em placas de Peyer, nódulos linfáticos mesentéricos e timo (POAPOLATHEP et al., 2002).

Em camundongos, NIV é embriotóxico e fetotóxico, mas não teratogênico (PRONK; SCHOTHORS; EGMOND, 2002). NIV aumenta ligeiramente as frequências de aberrações cromossomais e alterações em cromátides irmãs em células de hamster chinês (SCF, 2000). NIV apresenta-se como um fraco indutor de aberrações cromossômicas em estudo *in vitro* com células de mamíferos, mas é capaz de induzir dano na molécula de DNA. Entretanto as informações disponíveis são limitadas para avaliar o real potencial genotóxico (PRONK; SCHOTHORS; EGMOND, 2002) e carcinogênico de NIV (SCF, 2000).

2.1.1.3 Fusarenona-X

FX foi isolada inicialmente em 1968 e caracterizada em 1969. FX é produzida por diferentes espécies do gênero *Fusarium*, como *F. nivale*, *F. graminearum*, *F. oxysporum*, *F. semitectum*, *F. sporotrichioides*, *F. sambucinum* (WEIDENBÖRNER, 2000).

Esta micotoxina pode ser encontrada no alho, milho, aveia e trigo (WEIDENBÖRNER, 2000). Embora NIV e FX possam ocorrer simultaneamente com DON nos grãos de cereais, essas toxinas não têm sido estudadas tão extensivamente quanto DON (IARC, 1993). Frequentemente a ocorrência de FX é detectada em concentrações correspondentes a 10% a 20% do valor de nivalenol presente na amostra (TRITSCHER; PAGE, 2004).

Como membro do grupo de tricotecenos tipo-B, FX é um potente inibidor da síntese proteica em células eucariontes (CARTER; CANNON, 1978). FX liga-se ao sítio ativo da enzima peptidiltransferase localizada nos ribossomos e assim bloqueia a extensão da cadeia peptídica (CARTER; CANNON, 1978). Esta micotoxina, além de inibir a síntese de proteinas, tem mostrado efeito inibidor sobre a síntese de DNA, enquanto que a síntese de RNA apresenta-se menos sensível a esta toxina (PRONK; SCHOTHORST; EGMOND, 2002). Devido a sua potente inibição da síntese protéica, interfere principalmente com tecidos com elevada taxa de divisão, tais como baço, medula óssea, timo e mucosa intestinal (BONY, 2007).

FX é imunossupressor, citotóxico, emético, causando diarreia, hipotermia e diminuição da taxa respiratória em animais experimentais (COUNCIL FOR AGRICULTURAL SCIENCE AND TECHNOLOGY, 2003). A DL_{50} oral em camundongo de NIV é 38,9 mg/kg (RYU et al., 1988), enquanto que para FX é de 4,5mg/kg na mesma espécie animal (POAPOLATHEP et al., 2003). Poapolathep et al. (2003), demostraram que

FX em camundongos é absorvida a partir do trato gastrointestinal de forma mais eficiente que NIV, e que após ser absorvida é rapidamente metabolizada a NIV pelo fígado e rins.

Estudos prévios demonstram que FX induz apoptose em timócitos e linfócitos murinos, bem como em células de hepatoblastoma de origem humana (WIJNANDS; LEUSDEN, 2000; MIURA; AMINOVA; MURAYAMA, 2002; MIURA, AMINOVA, MURAYAMA, 2002). Também induz genotoxicidade sobre células intestinais humanas (BONY et al., 2007). Embora exista uma exposição de risco a FX pelo consumo de alimento contaminado, não existem dados que avaliem o potencial teratogênico ou efeitos cromossômicos de FX em humanos (WIJNANDS; LEUSDEN, 2000). A IARC, em 1993, concluiu que os trabalhos com animais experimentais são inconclusivos, classificando esta micotoxina no grupo 3 «não classificados quanto a sua carcinogenicidade em seres humanos».

2.1.2 Zearalenona

ZEA foi descoberta como a causa de uma desordem reprodutiva em suínos conhecida como vulvovaginite (MOSS, 2002). ZEA (previamente conhecida como toxina F-2) é uma micotoxina estrogênica não-esteroidal biossintetizada por uma variedade de fungos *Fusarium*, incluindo *F. graminearum* (*Gibberella zeae*), *F. culmorum*, *F. cerealis*, *F. equiseti*, *F. crookwellense* e *F. semitectum*. Estas espécies de fungos são comumente encontradas no solo de países temperados e quentes e frequentemente contaminam cereais em todo o mundo (BENNETT; KLICH, 2003).

Essa micotoxina é um ácido resorcíclico lactona, estável durante o armazenamento, a moagem, o processamento e o cozimento (EFSA, 2004). Embora cereais infectados por *Fusarium* no campo possam acumular ZEA antes da colheita, numerosos experimentos indicam que altos níveis de ZEA reportados no cultivo de milho destinado a alimentação animal resultam principalmente de estocagem inadequada (RESNIK et al., 1996).

ZEA é encontrada, especialmente, como um contaminante do milho, porém pode também ocorrer em cereais como aveia, cevada, trigo e sorgo (WODD, 1992). A característica predominante na distribuição de ZEA nos grãos de cereais e de alimentos destinados a humanos e animais é a sua ocorrência com outras fusariotoxinas, incluindo tricotecenos e fumonisinas (D'MELLO et al., 1997).

No Brasil, a ocorrência de ZEA foi relatada em milho em diversos estudos (SABINO et al., 1989, SILVA; VARGAS, 2001, VARGAS et al., 2001, QUEIROZ et al., 2012). A contaminação de trigo foi também relatada (FURLONG et al., 1995). No Uruguai,

um estudo com objetivo de monitorar a contaminação de alimentos destinados a humanos e animais mostrou a presença desta toxina no milho, cevada, malte, bem como em frutos e legumes secos (PINEIRO et al., 1996a,b). Na Argentina, ZEA foi identificada em grãos (LOPEZ; TAPIA, 1980), trigo (QUIROGA et al., 1995), alimentos a base de milho (RESNIK et al., 1996) e em rações destinadas a aves (DALCERO et al., 1998).

Para ZEA, vários países tem estabelecido o limite máximo entre 0,02 e 1 mg/kg tanto para matéria-prima como para alimentos processados (FAO, 2004). Na China, um limite máximo de 0,06 mg/kg foi estabelecido para o milho e trigo (MINISTRY OF HEALTH OF REPUBLIC OF CHINA, 2005). Na Europa, o limite máximo para esta toxina é de 0,05 mg/kg (FAO, 2004), sendo que no Brasil o limite máximo para milho e trigo é 0,4 mg/kg (Anexo B).

ZEA tem um forte efeito estrogênico devido sua competição com o 17-β-estradiol em ligar-se aos receptores estrogênicos citoplasmáticos presentes no útero, glândulas mamárias, hipófise e hipotálamo (KUIPER-GOODMAN et al., 1987), como também em células do sistema imunológico (IGARASHI et al., 2001).

ZEA e alguns de seus metabólitos, competitivamente ligam-se aos receptores de estrógeno. Desse modo, a toxicidade desta micotoxina está associada principalmente com problemas reprodutivos em animais e humanos (MINERVINI et a., 2005). ZEA foi avaliada pela IARC em 1993, baseada em dados humanos incompletos e evidência limitada obtida a partir de estudos experimentais com animais. Esta toxina foi classificada juntamente com as demais fusariotoxinas no grupo 3 «não classificados quanto a sua carcinogenicidade em seres humanos». Entretanto, tem sido demostrado que ZEA pode potencialmente estimular o crescimento de células que apresentam receptores estrogênicos em glândulas mamárias (MINERVINI et al., 2005). Este resultado juntamente com outros estudos epidemiológicos, suporta a hipótese que ZEA pode participar na etiologia do câncer de mama em humanos (YU et al., 2005). Além disso, ZEA pode ser identificada no tecido endometrial de mulheres portadoras de adenocarcinoma ou de hiperplasia endometrial (TOMASZEWSKI et al., 1998). Em adição, entre 1978 e 1981 foi suspeitado que ZEA e seu metabólito zearalenol foram os agentes causais do episódio de puberdade precoce que afetou várias crianças em Puerto Rico (RODRÍGUEZ et al., 1985).

Trabalhos sobre a toxicidade aguda de ZEA mostram que o valor de DL_{50} desta toxina varia entre 2 a 20 mg/kg, dependendo da espécie animal analisada (FLANNIGAN, 1991). Altas concentrações de ZEA em suínos expostos a uma alimentação contaminada causou distúrbios relacionados à concepção e aborto, entre outros (KURTZ;

MIROCHA, 1978). Em animais experimentais, efeitos tóxicos devido ao consumo prolongado de dieta contaminada por ZEA incluem carcinogenicidade, genotoxicidade, distúrbios reprodutivos e endócrinos, além de imunotoxicidade. Suínos são uma das espécies animais mais susceptíveis, com NOEL de 40 mg/kg/dia comparado ao NOEL de 100 mg/kg/dia em ratos. Esta suscetibilidade tem efeitos severos sobre a produção animal, pois ZEA reduz a sobrevivência de embriões e o peso fetal. Adicionalmente, ZEA produz dilatação vulvar, vulvovaginites, retenção ou ausência de leite e prolapso retal. Em machos, ZEA pode induzir diminuição nos níveis de testosterona, no peso de testículos e na espermatogênese, induzindo feminização e redução de libido (ZINEDINE et al., 2007).

2.2 Efeitos das Micotoxinas no Epitélio Intestinal e no Sistema Imune

Embora a literatura sobre micotoxinas seja rica em relação ao número de trabalhos sobre mecanismo e toxicidade celular associada à patologia e desempenho animal, estudos que avaliem os efeitos destes componentes sobre o trato gastrointestinal são limitados (BOUHET; OSWALD 2005).

Considerando que a interação inicial das micotoxinas é com o epitélio intestinal, esta tem sido objeto de interesse na última década, devido a várias razões. Primeiro, a saúde do trato intestinal está fortemente relacionada com a saúde animal e humana. Segundo, células com alta taxa de divisão celular e síntese proteica são predominantes no epitélio intestinal, assim células intestinais podem ser consideradas alvo das micotoxinas uma vez que muitos destes metabólitos são inibidores da síntese proteica. Além disso, devido às micotoxinas serem absorvidas ao longo do trato gastrointestinal, o epitélio intestinal é repetidamente exposto a estas toxinas em concentrações mais altas que outros tecidos (GRENIER; APPLEGATE, 2013).

A manutenção morfológica do trato gastrointestinal é crucial, uma vez que assegura que os nutrientes sejam absorvidos e fornece uma proteção eficaz contra patógenos. Vilosidades intestinais aumentam a área da superfície interna das paredes intestinais, permitindo um aumento da superfície disponível para a absorção de nutrientes. Logo, se a integridade da parede intestinal é comprometida, a efetividade da absorção de nutrientes pode ser afetada (LORENZONI, 2010).

Embora trabalhos relatem efeitos adversos das micotoxinas sobre a morfologia das vilosidades intestinais, até o momento os efeitos destes metabólitos fúngicos sobre a diferenciação ou migração dos enterócitos ao longo da superfície das vilosidades

intestinais não foi elucidado (GRENIER; APPLEGATE, 2013). Em suínos, a exposição do epitélio intestinal a baixas doses de DON reduziu a altura das vilosidades intestinais, causando atrofia e fusão das vilosidades do jejuno (KOLF-CLAUW et al., 2009; BRACARENSE et al., 2012, BASSO et al., 2013).

Da mesma forma, em aves, tanto níveis baixos ou moderados de DON nos alimentos, assim como a sua combinação com outras toxinas de *Fusarium* foram capazes de reduzir a área de superfície de absorção através de uma diminuição da altura das vilosidades no duodeno e jejuno (AWAD et al., 2011; YUNUS et al., 2012).

Em relação à micotoxina NIV, poucos são os trabalhos que têm analisado a ação deste metabólito fúngico sobre a morfologia intestinal (HEDMAN et al., 1997; OHTSUBO et al., 1989), enquanto nenhum estudo até o momento tem analisado a toxicidade de FX sobre a morfologia intestinal. Deste modo, estudos que avaliem a ação de micotoxinas sobre a morfologia intestinal são de extrema importância, uma vez que este critério pode ser considerado como um indicador de saúde animal.

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OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar os efeitos tóxicos das fusariotoxinas (DON, NIV, FX e ZEA) em suínos utilizando modelos *in vivo* e *ex vivo.*

3.2 OBJETIVOS ESPECÍFICOS

Avaliar o efeito tóxico individual e combinado de DON associado a NIV e ZEA em suínos com relação ao:

i. Ganho de peso
ii. Morfologia do intestino, fígado e órgãos linfóides
iii. Apoptose de órgãos linfóides

Avaliar os efeitos isolados de DON, NIV e FX sobre a morfologia intestinal de suínos utilizando o modelo *ex vivo* (explantes de jejuno).

4 ARTIGOS PARA PUBLICAÇÃO

Artigo 1. Deoxynivalenol alone or in combination with nivalenol and zearalenone induce systemic histological changes in pigs.

Artigo 2. Histopathological changes induced by deoxynivalenol, nivalenol and fusarenone X in pig jejunal explants.

Artigo 1

Deoxynivalenol alone or in combination with nivalenol and zearalenone induce systemic histological changes in pigs

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Artigo editado de acordo com as normas de publicação da *British Journal of Nutrition*

Deoxynivalenol alone or in combination with nivalenol and zearalenone induce systemic histological changes in pigs

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Abstract

Deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) are mycotoxins produced by *Fusarium* species, which naturally co-occur in animal diets. The purpose of the present study was to investigate the effects of DON alone and in combination with NIV and ZEA, on several parameters including animal performance, morphology and histology of intestine, liver and lymphoid organs of pigs submitted to chronic intoxication. A total of twenty, 5-week-old piglets were randomly assigned into four different groups, receiving separate diets for 28 days: a control diet, a diet mono-contaminated with DON (1.5 mg/kg), a diet multi-contaminated with DON (2 mg/kg) + NIV (1.3mg/kg) + ZEA (1.5 mg/kg) or a diet contaminated with DON (3 mg/kg) + NIV (1.3mg/kg) + ZEA (1.5 mg/kg). The control and mono-contaminated diet showed no difference in weight gain, whereas animals fed the multi–contaminated diets presented a significant decrease in weight gain over the total period. The chronic ingestion of these contaminated diets induced histological changes in the intestine such as atrophy and fusion of villi, reduction of villi height and crypth depth, and low number of goblet cells and lymphocytes. In addition, contaminated diets induced a significant increase of histological changes in liver and lymphoid organs. A significant increase on the caspase-3 expression in the lymph nodes and spleen was observed in the animals receiving contaminated diets too. These data provide a better understanding of the possible effects of *Fusarium* toxins, alone or in combinations on the morphology of the intestine and lymphoid organs, which would contribute to the risk assessment of these toxins.

Key-words: **Multi-contamination: Mycotoxins: Deoxynivalenol: Nivalenol: Zearalenone: Intestinal morphology**

Introduction

Mycotoxins are secondary metabolites produced by a wide variety of fungal species that cause nutritional losses and represent a significant hazard to the food chain. According to the Food and Agriculture Organization (FAO) of the United Nations, it is estimated that approximately 25% of the cereals produced in the world are contaminated by mycotoxins[1].

Zearalenone (ZEA), fumonisin B1 (FB1) and trichothecenes, in particular, deoxynivalenol (DON) and nivalenol (NIV), are amongst the most toxicologically important *Fusarium* toxins that occur frequently in combination in cereal grains. In Europe, DON is the major contaminant, often in co-occurrence with ZEA[2]. However, to date, very little is known about the potential interactive toxic effects among fusariotoxins[3,4].

It is well-known that trichothecenes induce various toxic effects in animals such as suppression of body growth and immune function, diarrhea, and general loss of condition. Trichothecenes exhibit inhibitory influence on protein synthesis by binding to ribosomes, and inhibition of DNA and RNA synthesis has also been reported[5,6]. Therefore, organs/tissues showing high rate of cell turnover are regarded as particularly susceptible to trichothecenes, such as the lymphoid and hematopoietic tissues, and the gastrointestinal tract[5-7]. In addition, trichothecenes have been shown to affect immunological functions by deregulating production of cytokines and immunoglobulins and by inducing apoptosis[8,9].

DON causes toxic and immunotoxic effects in a variety of cell systems and animal species[5,10]. Swine are more sensitive to DON than other species, in part because of differences in the metabolism of DON. Chronic low dietary concentrations (0.28; 0.56; 0.84 mg/kg of feed) induced no changes on animal performance, biochemical and hematological variables and immunological parameters[11], while acute higher doses induce vomiting, haemorrhagic diarrhea and circulatory shock[10].

Long-term NIV chronic exposure in mice induced a reduced body gain and feed efficiency, and an increase in relative organ weight or severe leucopenia[12]. However, young pigs exposed to NIV by three weeks showed no changes in body or organ weight, but in the macroscopic examination revealed lesions in the kidneys and gastrointestinal tract and reduction in the number of splenocytes[13]. Associated with other trichothecenes, NIV has been shown to correlate with the high incidence of oesophageal cancer in China[14].

ZEA and its metabolites exhibit potent estrogenic activity; hence it is often referred as a mycoestrogen. This non-steroidal mycostrogen binds to oestrogen receptors leading to hyperestrogenicity in several animal species, especially pigs[15]. *In vivo* studies showed that rats fed with ZEA developed liver lesions and hepatocarcinomas[16]. Prepuberal gilts fed diets

contaminated with DON (2.1 to 9.57 mg/kg) and ZEA (0.004 to 0.358 mg/kg) showed hepatocyte glycogen depletion and expansion of hepatic interlobular connective tissue [17] and hemossiderosis in spleen[18]. ZEA induced intracellular oxidative stress that results in induction of oxidative DNA damage[19] and apoptosis[20]. The immune system is a potent target for estrogenic endocrine disruptors considering that its cells express estrogenic receptors[21]. In spite of that, only few studies have been carried out on the immune effects of ZEA and its metabolites. In particular, reduction of mitotic index and cell survival of porcine lymphocytes was reported when high concentrations of ZEA were used in *in vitro*[22]. ZEA and its derivatives showed toxic effects on porcine neutrophils and decreased IgG, IgA and IgM levels as well as TNF-α synthesis in an *in vitro* model[23,24].

Considering that food and feed commodities are often contaminated by more than one mycotoxin[25], studying the interactions between different mycotoxins can be useful. It is known that *Fusarium* toxins can exert additive and synergistic effects[26]; but mycotoxins may also act as antagonists. The data on combined toxic effects of mycotoxins are limited and, therefore, the actual combined health risk from exposure to mycotoxins is unknown[27]. Assessment of the interaction of *Fusarium* mycotoxins has been investigated *in vitro* on porcine immune cells, swine and human intestinal epithelial cells[3,22,28,29]. *In vivo* experiments have also been performed on mice, pigs and poultry using high doses of toxins in which the authors mainly looked for differences in animal performance[27]. Among them, few studies were concerned with the interaction between the *Fusarium* toxins DON, NIV and ZEA[22].

The purpose of this study was to evaluate the weight gain and to determine the extent of histological lesions in selected organs of piglets submitted to chronic intoxication by ingestion of feed naturally contaminated with DON or multi-contaminated with DON, NIV and ZEA.

Material and methods

Animals and diets

The experiment was conducted in Arvalis – Institut du vegetal (Villerable, France) facilities. Twenty castrated male piglets (Pietrain), 5-week-old were divided in four groups of homogeneous weight. The animals were subjected for 28 days to control diet or one of three diets naturally contaminated with *Fusarium* toxins. Two lots of wheat (healthy or contaminated with *Fusarium* toxin) and a batch of corn (*Fusarium* toxin-contaminated) were selected and used for the manufacture of four experimental diets. Table 1 shows the ingredients that differ depending on the regime and the level of contamination by mycotoxins. The ingredients common to the four diets (isoenergetic and isoproteic diets) are listed below

and expressed in percentage: L-Lysine HCL (0.60), L-Threonine (0.25), DL-Methionine (0.20), L-Tryptophane (0.04), vitamins and mineral premix (4.0). The concentrations of *Fusarium* toxins were determined by High Performance Liquid Chromatography by laboratories CAPINOV (Landerneau, France) and LDA22 (Ploufragan, France).

During the experiment, piglets were weighed on the 14^{th} and 28^{th} and body weight gain was calculated for the first 14 days and for the last 14 days. At the end of the experimental period, pigs were fasted overnight before being subjected to electrical stunning and euthanized by exsanguination. Samples from the medium jejunum, proximal ileum, liver, spleen and mesenteric lymph nodes were collected and fixed in 10% buffered formalin solution for histological analysis. The experimental design used in the histopathological analysis was entirely randomized with five replicates (each animal representing one replicate). The institutional Ethics Committee for Animal Experimentation approved the study.

Table 1. Composition of the experimental diet

Igredients (%)	Feed 1	Feed 2	Feed 3	Feed 4
Wheat	75.41	5.37	52.43	0
Contaminated Wheat	0	70.00	70.00	53.77
Contaminated maize Andoins	0	0	13.00	13.00
Contaminated maize Cribs	0	0	8.50	8.00
Soybean	19.5	18.5	21.00	19.6
Lysine HCL	0.61	0.64	0.58	0.63
Threonine	0.25	0.25	0.25	0.25
Methionine	0.20	0.20	0.20	0.20
Tryptophan	0.03	0.04	0.04	0.05
Vitamin and mineral premix	4.00	4.00	4.00	4.00
Vegetable oil	0	1.00	0	0.50
Composition (%)				
Crude protein	20.43	20.48	20.33	20.20
Crude fibre	2.75	3.08	2.77	3.02
Ethereus extract	1.29	2.70	1.83	2.61
Digestble energy	3866	3851	3861	3833
Net energy	2770	2771	2775	2764
Lysine D	1.34	1.35	1.33	1.34
Ca	1.46	1.46	1.53	1.53
P	0.80	0.79	0.79	0.69
Mycotoxin (mg/kg)				
DON	0.12	1.5	2.08	3.0
NIV	<LQ (0.2)	<LQ (0.2)	1.30	1.30
ZEA	0.02	0.18	1.50	1.50
T-2 toxin	<LQ (0.01)	<LQ (0.01)	0.01	0.012
HT-2 toxin	<LQ (0.01)	<LQ (0.01)	0.02	0.02
Diacetoxyscispernol	<LQ (0.05)	<LQ (0.05)	<LQ (0.05)	<LQ (0.05)
3 + 15 acetyl DON	<LQ (0.01)	<LQ (0.01)	<LQ (0.01)	<LQ (0.01)
Fusarenon-X	<LQ (0.01)	<LQ (0.01)	<LQ (0.01)	<LQ (0.01)
Fumonisin B1	<LQ (0.01)	<LQ (0.01)	0.21	0.20
Fumonisin B2	<LQ (0.01)	<LQ (0.01)	<LQ (0.01)	<LQ (0.01)

LQ = Limit of quantification

Histological assessment

Tissue fragments were dehydrated through graded alcohols and embedded in paraffin wax. Sections of 3 µm were stained with hematoxylin–eosin (HE) for histopathological evaluation. For each organ, three slides per animal were prepared for analysis, and an area of 2000–2500 mm^2 per slide was observed. As displayed in Table 2, microscopic observations led to the identification of different lesions and severity in the organs, and allowed for establishing a lesion score per animal. For each tissue, the minimal score was 0 and the maximal lesional score was 36 for intestine, 27 for liver, 12 for lymph nodes and 18 for spleen (Table 2). The morphometric analysis of the number of goblet cells, inflammatory infiltrate and mitosis in the intestinal epithelium as well as the villus height and crypt depth were assessed as described by Bracarense et al.[30]

Table 2. Establishment of a lesion score – endpoints used to assess histological lesions[a]

Tissue	Type of lesions (severity factor)	Maximal score
Intestine	Lymphatic vessel dilation (1)	36
	Cubic enterocytes (2)	
	Villus flattening (2)	
	Villus fusion (2)	
	Interstitial oedema (2)	
	Villi apical necrosis (3)	
Liver	Disorganization of hepatic cords (1)	24
	Hepatic cell vacuolization (1)	
	Apoptosis (2)	
	Nuclear vacuolization (1)	
	Necrosis (3)	
Lymph organs	Depletion (1)	12
	Germinal centre (1)	21
	Apoptosis (2)	
	Necrosis (3)*	

a) The score for each lesion was obtained by multiplying the severity factor with the extent of the lesion. The organ score was then obtained by the sum of each lesion score. Severity factor (or degree of severity), 1= mild lesions, 2= moderate lesion, 3=severe lesion; the extent of each lesion (intensity or observed frequency) was evaluated and scored as 0= no lesion, 1= low extent, 2= intermediate extent, 3= large extent. *Lesion analysed only in the spleen.

Assessment of apoptosis

Evaluation of apoptosis was performed on lymphoid tissues using antibody anti-cleaved caspase-3 (CCasp3) (clone Asp 175, 1:200 dilution, Cell Signaling Technology, Beverly, MA). The immunohistochemical procedures were described elsewhere [30].

The immunoexpression of CCasp3 in the lymph nodes and spleen was estimated by counting strongly positive immunostaining of cell cytoplasm in twelve random lymphoid follicles at 400x magnification.

Statistical analysis

Data are presented as means with SED. They were statistically analyzed using normality (Shapiro-Wilk) and homogeneity (Bartlett) tests. When these two assumptions were met, the analysis of variance (ANOVA) was applied, followed by Duncan's test at 0.05 significance.

Results

Individual effect of DON or combined effects of DON, NIV and ZEA on weight gain

During the experiment, piglets were weighed individually on the 14th and 28th day. Animals fed a mono-contaminated diet with DON showed no difference in weight gain when compared with animals fed the control diet; however animals fed the combined DON (2 or 3 mg/kg), NIV (1.3 mg/kg) and ZEA (1.5 mg/kg) – contaminated diets presented a significant decrease in weight gain only during the last 14 days (Table 3).

Table 3. Individual or combined effects of DON with NIV+ZEA on weight gain.

Body weight gain/day (kg)	Animals diets			
	Control	DON	DON+NIV+ZEA[1]	DON+NIV+ZEA[2]
Days 1-14	0.46±0.04^{ab}	0.40±0.06^{a}	0.42±0.02^{ab}	0.48±0.06^{b}
Days 14-28	0.75±0.05^{a}	0.71±0.05^{ab}	0.64±0.06^{b}	0.68±0.04^{b}

Results are expressed as mean ± SEM for five animals. [1]DON (2.0mg/kg)+NIV (1.3 mg/kg)+ZEA (1.5 mg/kg); [2] DON (3.0 mg/kg)+NIV (1.3 mg/kg)+ZEA (1.5 mg/kg)

Individual effect of DON or combined effects of DON, NIV and ZEA on the histology and morphometry of the intestine

Samples of the jejunum and ileum were collected for histomorphometrical analysis. In the jejunum, a significant decrease in villi height was observed in the animals receiving DON or DON+NIV+ZEA-contaminated diets (Fig. 1). In the ileum, villi height decreased only in the animals treated with the multi-contaminated diet when compared with control piglets. A significant reduction in crypt depth was observed in both regions of the intestine in the animals fed the DON mono-contaminated diet.

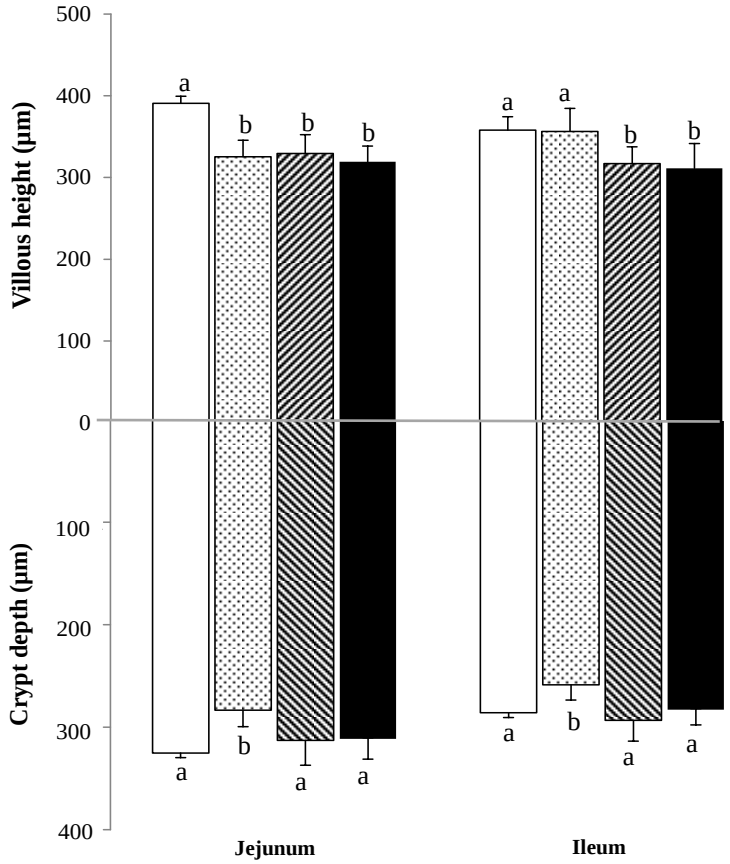

Figure 1. Individual and combined effects of DON with NIV+ZEA on pigs jejunum and ileum villi height and crypt depth fed with a control diet (□), diet contaminated with DON (1.5 mg/kg) (▨), DON (2.0mg/kg)+NIV (1.3 mg/kg)+ZEA (1.5 mg/kg) (▨) or DON (3.0 mg/kg)+NIV (1.3 mg/kg)+ZEA (1.5 mg/kg) (■) for 28 days. Values are mean ± SEM represented by vertical bars (*n* 5 animals). [a,b] Mean values with unlike letters were significantly different ($P < 0.05$).

Piglets fed the diets contaminated with mycotoxins showed mild to moderate intestinal lesions. The main histological changes observed were multifocal atrophy, villi fusion and oedema of lamina propria (Fig. 2). Lymphatic vessel dilation and prominent lymphoid

follicles were also observed. As indicated by the lesional scores, piglets fed mycotoxin-contaminated diets (DON or DON + NIV+ ZEA) displayed significant jejunal and ileal lesions when compared with animals fed the control diet (Fig. 2). In the jejunum, the histological changes were significantly more severe in the group fed the co-contaminated diet with the higher dose of DON when compared with the DON group, mainly due to apical villi necrosis (Fig. 2).

Goblet cells synthesize and secrete mucin, which is involved in gut barrier function. The number of goblet cells decreased significantly in the jejunum and the ileum of piglets fed with DON or DON+NIV+ZEA-contaminated diets (Fig. 3A). In the groups receiving a mycotoxin-contaminated diet, a reduction in lymphocytic infiltration was observed in both regions of the intestine. However, this decrease was only significant in the jejunum of DON-treated animals and in the ileum of DON (3 mg/kg)+NIV (1.3 mg/kg)+ZEA (1.5 mg/kg)-treated piglets (Fig. 3B). In relation to the number of plasma cells, the animals fed the contaminated diets showed no difference compared to the control group, but there was a significant reduction in the number of plasma cells in the intestine of animals receiving the DON-contaminated diet compared to the multi-contaminated diets (Fig. 3C).

Individual effect of DON or combined effects of DON, NIV and ZEA on the intestinal cell proliferation

Epithelial cell proliferation was estimated by counting mitotic figures in intestinal crypts on hematoxylin–eosin stained slides. The mean number of mitosis per microscopic field in the jejunum was 1.99±0.21 in the control group, 0.84±0.17 in the DON-treated group, 1.81±0.17 in the DON (2 mg/kg)+NIV (1.3 mg/kg)+ZEA-treated group (1.5 mg/kg) and 1.33±0.26 in the DON (3 mg/kg)+NIV (1.3 mg/kg)+ZEA (1.5 mg/kg)-treated group. In the ileum, the mean number of mitosis per microscopic field (1.5mm^2) was 2.14±0.53, 0.99±0.17, 2.25±0.18, 1.25±0.23 for the control group, DON-treated group and DON+NIV+ZEA-treated groups with the lower and higher doses, respectively. A significant decrease ($P<0.05$) was observed in the jejunum and ileum of the groups fed DON mono-contaminated diet compared with the control group.

Individual effect of DON or combined effects of DON, NIV and ZEA on the liver

Piglets fed either DON or DON+NIV+ZEA-contaminated diets displayed significant liver lesions when compared to animals fed the control diet. The higher lesional score was observed in animals fed the diet contaminated with DON (3 mg/kg)+NIV (1.3 mg/kg)+ZEA (1.5 mg/kg) (Fig. 2B). The main histological lesions observed in the liver were

disorganization of hepatic cords, cytoplasmatic vacuolization of hepatocytes and megalocytosis. Focal necrosis was observed in piglets fed the co-contaminated diet with the higher dose of DON (Fig. 2C).

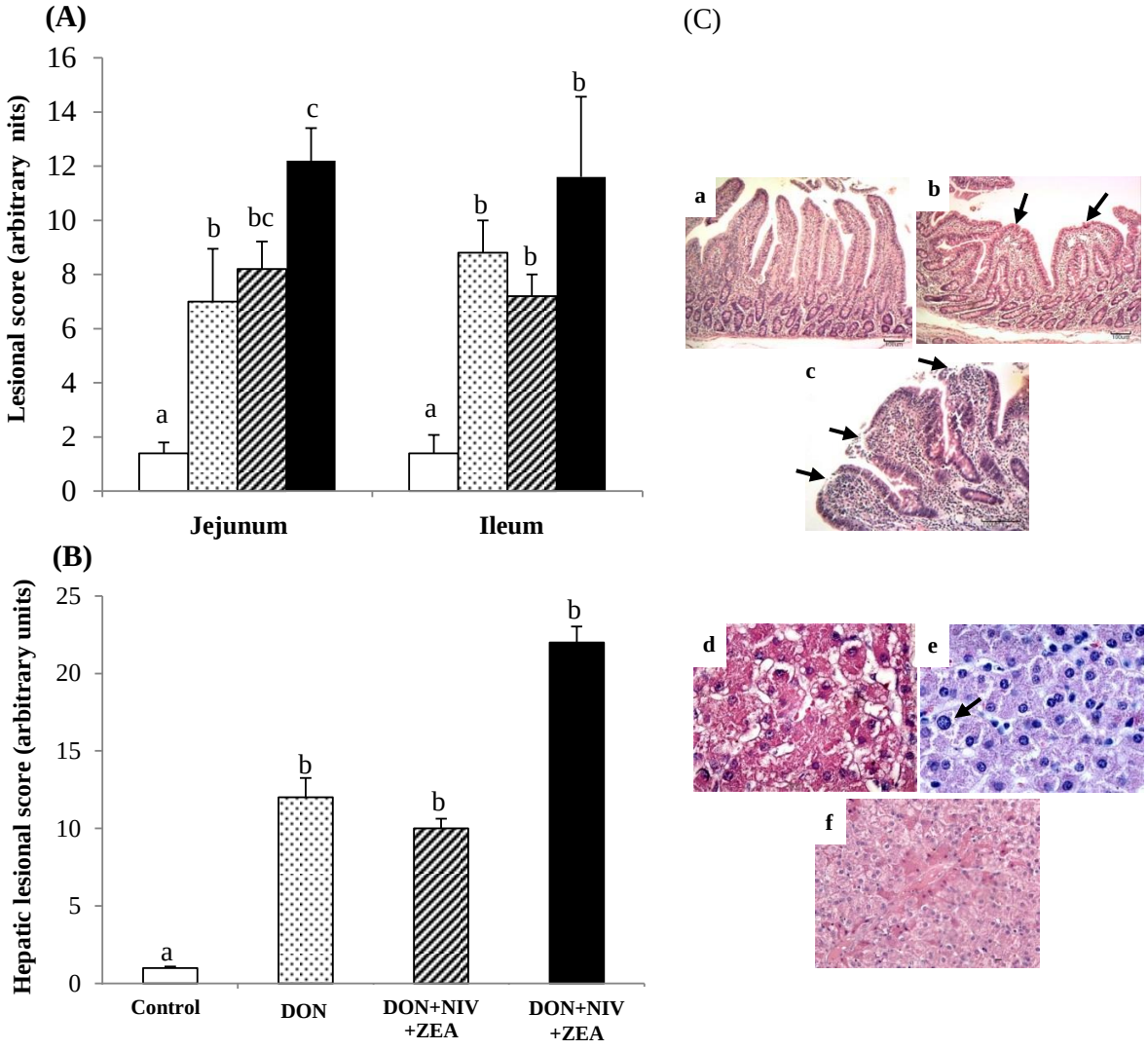

Figure 2. Individual and combined effects of DON with NIV+ZEA on pigs intestine (A) and liver (B) fed with: a control diet (□),diet contaminated with DON (1.5 mg/kg) (▨), DON (2.0mg/kg)+NIV (1.3 mg/kg)+ZEA (1.5 mg/kg) (▩) or DON (3.0 mg/kg)+NIV (1.3 mg/kg)+ZEA (1.5 mg/kg) (■) for 28 days. Histological aspects of intestine and liver (C). Jejunum of a control piglet with normal villi (a). HE, 10x; jejunum of a 1.5 mg/kg DON-treated piglet with villi flattening and fusion (arrow) (b). HE, 10x; jejunum of a 3 mg/kg DON plus NIV and ZEA-treated piglet with villi apical necrosis (c). HE, 20x. Liver of a piglet treated with a mono-contaminated diet. Hepatocyte cytoplasmatic vacuolization (d), HE, 40x. Liver of a piglet treated with a co-contaminated diet with 2 mg/kg of DON. Hepatocyte megalocytosis (arrow) (e). HE, 20x. Liver of a piglet treated with a co-contaminated diet with 3 mg/kg of DON. Focal hepatocyte necrosis. (f). HE, 40x. Values are mean ± SEM for five animals. [a,b] Mean values with unlike letters were significantly different ($P < 0.05$).

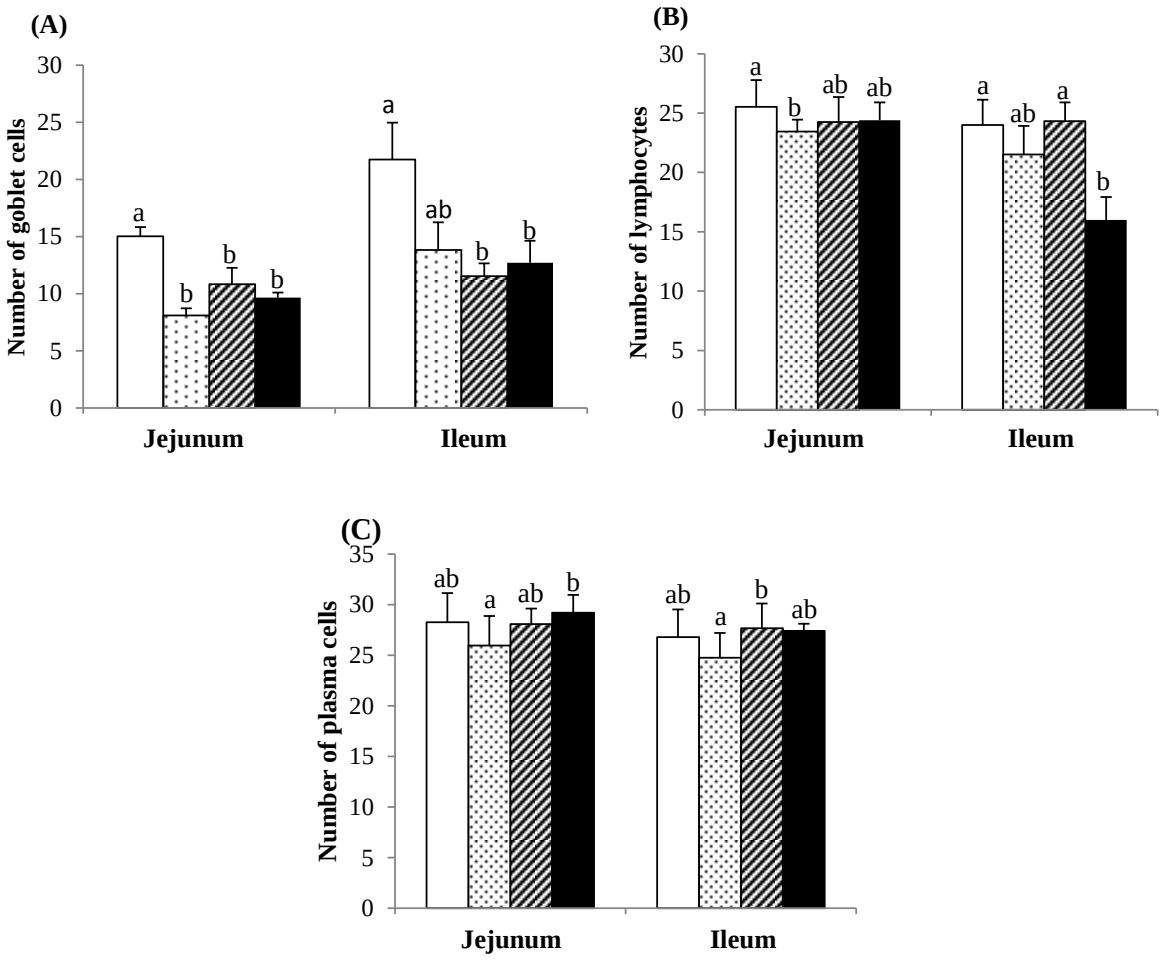

Figure 3. Effect of individual DON and combined with NIV and ZEA exposure on the number of goblet cells (A), lymphocytes (B) and plasma cells (C) in the jejunum and ileum of pigs fed with a control diet (□), diet contaminated with DON (1.5 mg/kg), (▨) DON (2.0mg/kg)+NIV (1.3 mg/kg)+ZEA (1.5 mg/kg) (▩) or DON (3.0 mg/kg)+NIV (1.3 mg/kg)+ZEA (1.5 mg/kg) (■) for 28 days. Values are mean numbers of inflammatory and goblet cells per field (1.5mm^2; *n* 5 animals), with standard errors of the mean represented by vertical bars. ^{a,b} Mean values with unlike letters were significantly different (*P*<0.05).

Individual effect of DON or combined effects of DON, NIV and ZEA on the lymphoid organs

Changes in the mesenteric lymph nodes and spleen were mild and characterized by lymphocyte apoptosis and follicular depletion. A significant increase in the spleen lesional score was observed in pigs fed the diet contaminated with DON or DON+NIV+ZEA. Pigs submitted to the co-contaminated diets showed no effect on the lesional score of lymph nodes while the mono-contaminated diet induced a significant increase in the lymph nodes (Fig. 4A).

Trichotecenes can modulate immune function by inducing apoptosis or cell proliferation[31]. We are interested to investigate in the tissue sections the effects of mycotoxins in apoptosis, so an immunohistochemical assay was performed using CCasp3 antibody. A significant increase in cell apoptosis was observed in the spleen of both groups receiving the contaminated diets with mycotoxins compared to control, however in lymph nodes the increase occurred only in the DON-treated group (Fig. 4B and C).

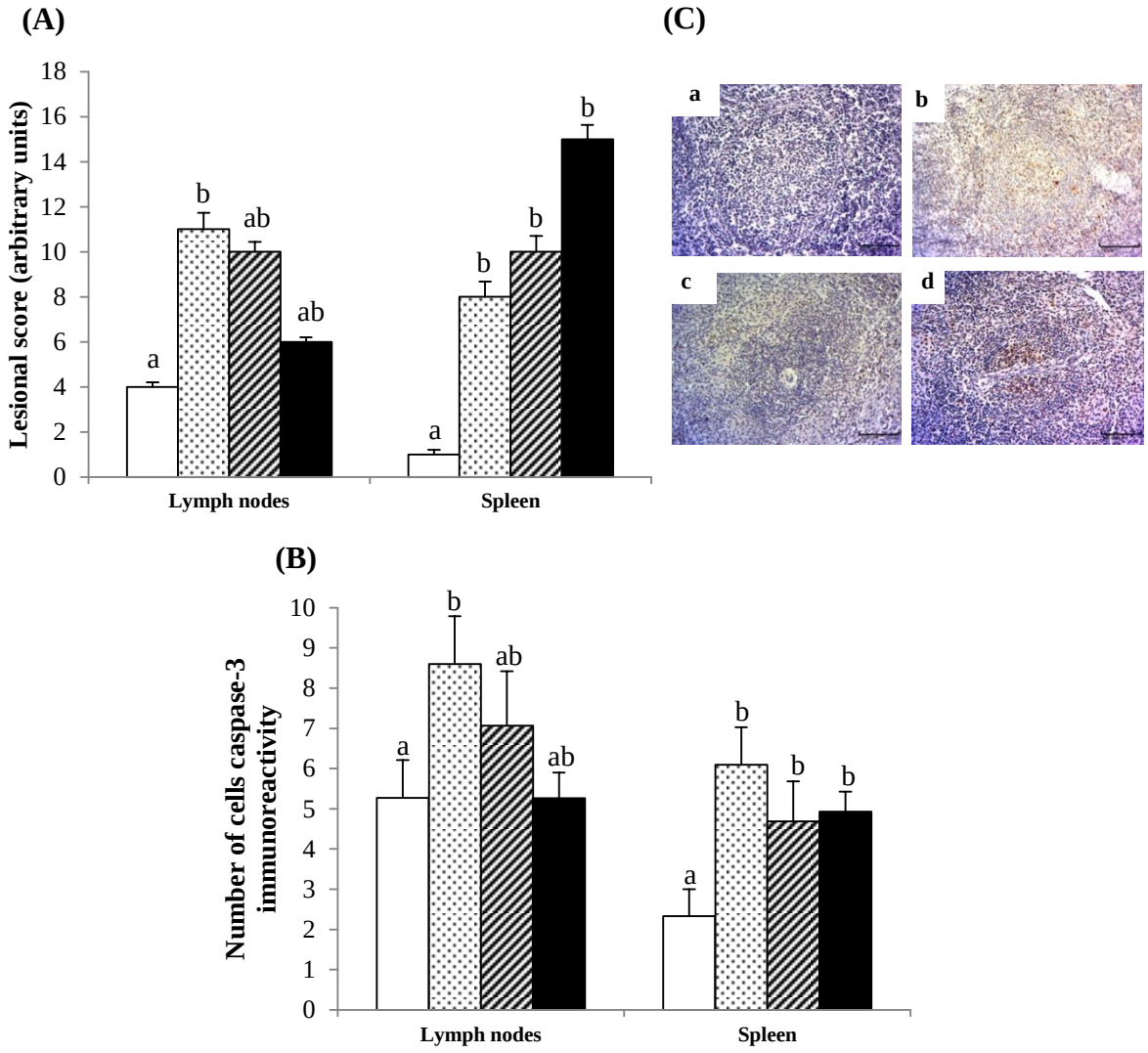

Figure 4. Effect of mycotoxins exposure on morphology (A) and caspase-3 cell immunoreactivity of lymphoid organs of pigs fed with a control diet (□), diet contaminated with DON (1.5 mg/kg), (▨) DON (2.0 mg/kg)+NIV (1.3 mg/kg)+ZEA (1.5 mg/kg) (▩) or DON (3.0 mg/kg)+NIV (1.3 mg/kg)+ZEA (1.5 mg/kg) (■) for 28 days. Values are mean ± SEM for five animals. [a,b] Mean values with unlike letters were significantly different ($P<0.05$). (B) Immunohistochemistry to caspase-3 on lymph nodes and spleen. (C) Control piglets with mild immunostaining to caspase-3 (a, c). Increase in the number of cells immunostained to caspase-3 in lymph nodes (b) and spleen (d). Immunoperoxidase, bar 100 µm.

Discussion

In the present study, piglets were exposed to low doses of three major *Fusarium* mycotoxins (DON, NIV and ZEA) at levels commonly found in crops. Most of the effects of the three toxins in animals, including rodents and gilts, have been evaluated using mono-contaminated feed[13,32-34]. Thus it was of interest to determine the effects of ingestion of feeds contaminated with low levels of these toxins in combination, on some zootechnical and histopatological parameters of piglets.

The body weight gain showed no changes in control animals and in animals fed the mono-contaminated diet with DON. This result was similar to that reported a decreased body weight gain in pigs fed a diet naturally contaminated with 3.5 mg/kg of DON, whereas a dose of 1.7 mg/kg showed no changes[35] .

On the other hand, a weight gain reduction was observed when DON, NIV and ZEA were given together. This result agrees with the previous data reported for gilts fed with different doses of DON and ZEA[36]. In contrast, pigs fed with varying amounts of purified ZEA (0-6 mg ZEA/kg/feed) added to naturally contaminated wheat with 0-5 mg DON/kg feed showed no significant interaction between both toxins. ZEA did not affect DON-toxicity measured by the same parameters in mice[37]. We can suggest from our data that the weight gain reduction observed when DON, NIV and ZEA were given together may be attributable to presence of the trichothecene NIV that has a higher toxicity than DON[38,39]. Moreover, subchronic and chronic ingestion of NIV alone induced a significant reduction in weight gain in mice and rats, respectively[40,41]. Another hypothesis is that the greater doses of DON in the co-contaminated diet contributed to the reduced weight gain.

The main histological intestinal findings were multifocal villi atrophy, villi fusion and reduction in the number of goblet cells. Similar histopathological changes were observed during *in vivo* and *ex vivo* exposure of the intestine to DON[30,42].The lesional score showed a 7, 8.2 and 12.2 fold increase for the DON, co-contaminated diet with lower dose of DON and co-contaminated diet with higher dose of DON, respectively. Pigs fed the co-contaminated diet with 3 mg/kg of DON presented a significant increase on jejunum lesional score when compared to animals receiving the mono-contaminated diet. This result suggests that the toxic effects on the intestine were due to a dose effect and not an interaction between mycotoxins. However, synergistic effects were reported for DON+NIV+ZEA inducing a decrease in cell viability and proliferation on *in vitro* models[3,43]. Also, an additive effect was reported for DON plus NIV promoting a reduction on lymphocyte proliferation[44]. Thereby, for interaction where trichothecenes are involved, this way reflects the effects on protein synthesis which can increase the susceptibility to other *Fusarium* toxins.

We observed a significant decrease in villi height in the jejunum of piglets fed the diet contaminated with DON and in the jejunum and ileum of animals fed the co-contaminated diet. Villi flattening in the jejunum are probably due to the impairment of cell proliferation, as could be observed by the decrease in the number of mitotic figures in the same region. This data agrees with previous studies in piglets and broiler chickens that showed a significant decreased in villi height after ingestion of a diet contaminated with DON in duodenum and jejunum[30,45]. We speculate that this may be explained by the fact that under normal circumstances the major absorption of nutrients occurs in the duodenum and proximal jejunum[46] that can result in a higher exposure of the jejunum to mycotoxins. The co-contaminated diet caused a villi flattening in both intestinal regions analyzed, suggesting a possible interaction of ZEA on the toxicity of the trichothecenes DON and NIV. This result is expected since previous studies have shown that the ZEA induced apoptosis in *in vitro* models[47,48].

In both regions crypt depth showed a significant reduction in piglets fed the DON-contaminated diet. These results differ from those previously reported[30] where no difference in animals receiving a DON-diet was observed. In the current study, probably the reduction in crypt depth is related to the decrease in the number of mitotic figures that occurred in the same regions.

The toxic effects of the trichothecenes DON and NIV is associated with inhibition of protein synthesis, affecting mainly tissues with a high rate of cell turnover, as lymphoid, hematopoietic and gastrointestinal tissues[5,6,49]. In relation to toxic mechanisms of ZEA in addition to their binding affinity to estrogen receptors, studies *in vitro* show that this mycotoxin induces changes in cell cycle, inhibition of protein and DNA synthesis and oxidative stress in Caco-2 cells[20,47].

The number of goblet cells in the intestinal wall reflects the intestinal potential of mucin production. The large protein synthesis load of these secretory cells renders them susceptible to endoplasmatic reticulum stress[50]. In this study, a decrease in the number of goblet cells was verified in piglets fed the contaminated diets. Our hypothesis is that DON in mucus-producing cell lines induces endoplasmic reticulum stress, leading to changes in intestinal cell density. A reduction in the number of goblets cells in jejunum was also observed in piglets fed DON-contaminated diet[30]. No data were reported about the effects of NIV and ZEA on goblet cell density. Intestinal mucus protects the epithelium against adhesion and invasion by pathogens[51]; therefore, a reduction in the number of goblet cells can affect the intestinal barrier function. The mechanisms involved in the alterations on the

423 production and composition of the intestinal mucus layer by mycotoxins are still unknown
424 and further studies are required.

425 We have shown that DON and DON+NIV+ZEA induced a significant decrease in the
426 number of lymphocytes in the jejunum of the animals fed the mono-contaminated diet and in
427 the ileum of swine fed the higher DON dose of co-contaminated diet. The present data agrees
428 with the study of Bracarense et al.[30] that showed a significant decrease of lymphocytes only
429 in the jejunum of piglets fed with diet containing 2.8 mg DON/kg feed. DON[9], NIV[52] and
430 ZEA[24] directly induce apoptosis on lymphocytes *in vitro*, which might suppress immune
431 function. This immunosuppressive effect was associated with the induction of apoptosis by
432 the activation of c-Jun terminal kinase, p38 mitogen-activated protein kinase and caspases[53].
433 Because lymphoid cells are constantly renewing, lymphocytes could be particularly sensitive
434 to DON.

435 Hepatocyte vacuolization was the main lesion observed in the liver of pigs fed
436 contaminated diets. Ingestion of both contaminated diets induced a significant increase in
437 liver histological changes (12, 10 and 22 fold increase for mono, co-contaminated diet with
438 2mg/kg of DON and 3 mg/kg of DON, respectively) compared to control group. Hepatic
439 histological changes were also reported in piglets submitted to a chronic[30] and acute[54]
440 exposition to DON. Also in *in vitro* studies with porcine and human hepatocytes treated with
441 different doses of DON (0.1 µM to 100 µM/ml) reduced the cell viability[55,56]. In this study,
442 the higher dose of DON co-contaminated diet induced more lesions than the mono-
443 contaminated diet. Ingestion of low doses of DON (1mg/kg) plus ZEA (250 µg/kg) also
444 induced histological changes in the liver of pigs[57]. Glycogen depletion, hemosiderin and
445 lobular connective tissue increase were reported[17,18] in gilts fed a DON plus ZEA diet.

446 The sensitivity of the immune system to mycotoxins arises from the vulnerability of
447 the continually proliferating and differentiating cells that participate in immune mediated
448 activities[58]. In this study, the main histological changes observed in lymphoid organs were
449 marked cell death and mild follicle depletion. We verified on the spleen an 8, 10 and 15 fold
450 increase on the lesional score for the mono-contaminated diet, the co-contaminated with 2
451 mg/kg of DON and co-contaminated diet with 3 mg/kg of DON, respectively. The increase in
452 cell death in lymphoid organs in pigs fed the contaminated diets was confirmed by
453 immunostaining to caspase-3. Induction of lymphocytic apoptosis by DON has been
454 previously reported in murine lymphoid organs[59]. We confirmed that DON induced
455 apoptosis via caspase-3 in both lymphoid organs in pigs chronically fed a DON diet. Mild
456 lympholysis in mesenteric lymph nodes of piglets was reported after intravenously DON
457 administration (0.5 mg DON kg^{-1} body weight)[60], as well as apoptotic changes and caspase-3

activation in lymphoid tissues when DON was intravenously injected (1 mg/kg) in piglets[54]. Data about the effects of NIV and ZEA on *in vivo* models on lymphoid tissue are scarce[13] and to the best of our knowledge no study was the first done with co-contamination.

In conclusion, we observed that mono and multi-contaminated diets with DON induced toxic effects on pig tissues and weight gain. Our results suggest that no interaction occurred when animals are exposed to co-contamination. However, more studies are necessary to evaluate the interaction between the three toxins. It is important to determine or revalidate the toxic effects of combinative mycotoxins in animal models in order to elucidate the possible interactions between then.

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Conflict of interest

The authors state that there is no conflict of interest.

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Artigo 2

Histopathological changes induced by deoxynivalenol, nivalenol and fusarenone X in pig jejunal explants

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Histopathological changes induced by deoxynivalenol, nivalenol and fusarenone X in pig jejunal explants

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Abstract

Deoxynivalenol (DON), nivalenol (NIV) and fusarenon-X (FX) cause deleterious effects on pigs intestine. The purpose of the present study was to investigate the effects of the three toxins alone on the intestinal morphology. Crossbreed weanling piglets of 4–5 week-old (n = 12) were used for explanting jejunal tissue. The pigs jejunal explants were subjected to four treatments during 4 hours under 5% CO_2 humidified atmosphere. In the control treatment William's medium E without or with 0.1% of DMSO was used. Explants were incubated in culture medium containing DON, NIV (1, 3, 10 µM) and FX (0.3, 1 and 3 µM). The main lesions observed were cubic epithelial cells, areas of oedema in the lamina propria, villi atrophy and denudation of villi with apical loss of enterocytes. The individual treatment with DON, NIV or FX resulted in a significant decrease of the histological score from doses of 3µM, 1µM and 0.3 µM, respectively. The present data indicate that pig intestinal explants are adequate for assessing intestinal toxicity induced by exposure to DON, NIV or FX, where the comparison of the toxic effects of the three mycotoxins tested allows the following range: DON<NIV< FX.

Keywords: *Fusarium* spp.; mycotoxins; toxicity; Intestinal explant culture; histopathology; food contaminants.

1. Introduction

Fungi of the *Fusarium* genus commonly contaminate cereals in the temperate climatic zones of the world and contribute to undervalue grains entering the food and feed chain. Among the mycotoxins produced by this genus, the wide group of trichothecenes is extremely prevalent, particularly through the deoxynivalenol (DON) for which many exposure and toxicological surveys have been realized or reviewed recently (Pestka, 2010; Smith et al., 2012; Dänicke et al., 2013). Nivalenol (NIV) and the 4-acetylnivalenol or Fusarenon X (FX) both classified with DON as type B trichothecenes, are known as two other biologically active metabolites of DON, which have been detected in agricultural commodities (Tanaka et al., 2004; Schollenberger et al., 2005). A large scale data survey indicates that DON, NIV and FX are present in 57%, 16% and 10%, respectively, of food samples collected in the European Union (Schothorst and van Egmond, 2004).

From their first discovery, there has been concern about the relationship between trichothecenes exposure and health damage based on both experimental toxicity and epidemiological data. Studies have shown that mycotoxins cause toxic effects in humans as well as in all animal species so far investigated (Pestka, Smolinski, 2005; Zain, 2011). Studies in laboratory and food animals surveys have revealed a complex spectrum of toxic effects. Experimentally, low to moderate dose acute oral exposure to trichothecenes cause vomiting, diarrhea and gastroenteritis, whereas higher doses cause severe damage to the lymphoid and epithelial cells of the gastrointestinal mucosa resulting in hemorrhage, endotoxemia and shock (Ueno, 1984). Chronic exposure to trichothecenes can cause anorexia, reduced weight gain, diminished nutricional efficiency, neuroendocrine changes, and immune modulation (Rotter et al., 1996).

Although not as prevalent as DON (Leblanc et al., 2005), NIV and FX show higher acute toxicity than deoxynivalenol in animal studies, with oral LD_{50} values in mice of 78, 39 and 4.5 mg/kg for DON, NIV and FX, respectively (IARC, 1993). These last two toxins are of added concern for food safety but information for assessing the health risk still scarce (SCF, 2002; Pronk et al., 2002). At the molecular level, as other trichothecenes, DON, NIV and FX display multiple inhibitory effects on the primary metabolism of eukaryotic cells including the inhibition of proteins, DNA and RNA synthesis

(Rocha et al., 2005). This impairment leads to the alteration in cell proliferation in tissue with high rates of cell turnover such as spleen, bone marrow, thymus and intestinal mucosa (De Walle et al., 2010).

Following ingestion of contaminated food or feed, the intestine and the intestinal epithelial layer cell can be exposed to a high concentration of food contaminant such as mycotoxins (Maresca et al., 2008). The intestinal layer is the first barrier preventing the entry of foreign antigens, including food proteins, natural toxins, commensal gut flora and pathogens, into the underlying tissues (Bouhet, Oswald, 2005). Studies focusing on the influence of food antigens in intestinal morphology as an indicator of animal health have been conducted, however, there are few studies investigating the effects of mycotoxins on the morphology of the intestinal epithelium, whereas increasing evidence of its repeated exposure to mycotoxins at a higher concentration than other tissues (Grenier and Applegate, 2013). Pigs treated with 3mg/kg feed of DON for 5 weeks showed significant histopathological changes compared to control animals, as atrophy and fusion of villi and reduction in the number of goblet and inflammatory cells (Bracarense et al., 2012).

Little is known about the effects of NIV and FX on intestinal tract of pigs. Pigs treated with 2.5 or 5 mg NIV/kg of feed for 3 weeks, revealed gastrointestinal erosions (Hedman et al., 1997) and reduced enzymatic ability of intestinal epithelium to utilize alpha-ketoglutarate in the tricarboxylic acid cycle (TCA-cycle) (Madej et al., 1999). Bony et al. (2007) demonstrated in Caco-2 a genotoxic potential for NIV and FX at low exposure levels, whereas FX was 10-fold more cytotoxic than NIV.

In vitro models of intestinal mucosa have been developed for studying enteric diseases (Girard et al., 2007). In the context of reducing the number of experimental animals (3Rs principles, Russel and Burch, 1959), intestinal explants represent a powerful model. Organ culture of intestinal explants allows preserving normal histological structure *in vitro* (Nietfeld et al., 1991). Large numbers of explants can be prepared from a single animal, thus reducing the number of animals required for a given study. The pig jejunal explant model has been used for studying the digestive effects of the mycotoxin DON, both at histopathological level and at biochemical level (Kolf-Clauw et al., 2009; Lucioli et al., 2013; Basso et al., 2013).

Therefore considering pig jejunal explant culture as an efficient methodology of study, this work estimated the impact of the diluent DMSO in this model, as well compared the histopathological changes induced by the mycotoxins DON, NIV and FX on the pigs digestive barrier.

2. Material and methods

2.1. Animals

Twelve crossbred piglets of 4–5 week-old were kept in the animal facility of the INRA ToxAlim Laboratory (Toulouse, France). The experimental procedures were carried out in accordance with European Guidelines for the Care and Use of Animals for Research Purposes. The animals were fed *ad libitum* with free access to water.

2.2. Toxins

DON was acquired from Sigma (St Quentin Fallavier, France); NIV, FX from Waco Pure Chemical Industries LTD (Osaka, Japan). Stock solutions of mycotoxins were dissolved in Dimethyl sulfoxide (DMSO) to the following concentrations: 15 mM DON and 10 mM NIV and FX. Stock solutions were stored at −20 °C and working dilutions were prepared in cell culture medium, William's medium E (WME-Sigma, Saint-Quentin Fallavier, France).

2.3. Jejunum explants culture

The animals were used to obtain explants of jejunal tissue and the procedures for the culture of the explants were as previously described (Kolf-Clauw et al., 2009) with minor modifications. Explants were incubated for 4 h with Williams E Medium (WME) (n=42) containing 100 U/mL penicillin, 100 µg/mL streptomycin and 50 µg/mL gentamicin and supplemented with D-glucose (2.5 g/L) and 30 mM Alanine-Glutamine (Sigma) at 37 °C under CO_2-controlled atmosphere with orbital shaking.

Uncultured control tissue was placed into fixative at the end of dissection time, as time 0 controls (T0, n=12). Considering the possible effects of DMSO on intestinal morphology, the final concentration of 0.1% DMSO corresponding to the highest DMSO concentration of working dilutions was tested in 42 explants.

Twelve explants were exposed to purified DON and NIV at 1, 3 and 10 µM, or purified FX at 0.3, 1 and 3 µM, for 4 h, respectively. These concentrations were chosen after preliminary explants cultures with 0.1 to 30 µM of each toxin.

2.4. Histological and Morphometric Analysis

For histological analysis, the explants fixed in 10% formalin were embedded in paraffin and sectioned at 5 µm slides parallel to the villi axis and stained by haematoxylin and eosin (HE) using standard procedures. The resulting slides were analyzed independently by two observers, at a magnification of 100x. Mean villous height was assessed by histomorphometrical analysis of a number of at least ten villi per explant (Motic Image Plus 2.0 software using a 10 × magnification).

The histological changes were evaluated using a tissue scoring system (Kolf-Clauw et al., 2009) with minor modifications. The scoring system includes both morphological and lesional criteria as shown in Table 1. The maximal score was attributed to the T0 tissue, before incubation, for each criterion. The morphological score includes the number of villi per explant and the fusion of villi. This latter was expressed as the 97.5^{th} percentile of the percentage of fused villi (number of villi fused/non fused villix100). A number of at least 25 villi per explant allow the score of 3. The score 3 for the villi fusion corresponded to a maximum of 11% of fused villi.

The lesional score included morphology of enterocytes (score 3 for columnar epithelium), the degrees of edema and apoptotis in the lamina propria (score 2 for slight flattening of villi), and the extent of discontinued epithelium qualified as apical denudation of villi. This endpoint was quantified by the 97.5^{th} percentile of the percentage of the villi showing apical denudation (score 3 for T0 explants). For explants lesions, the score 3 corresponded to a maximum of 10% apical denudation and the scores of 2, 1, 0, to the extent of 11-40%, 41-70%, and 71-100% respectively. For the lesion of the

193 lamina propria, localized edema was scored 1 whereas multifocal edema and apoptosis accounted for
194 0.

195 The total score was calculated by taking into account the degree of severity for the lesions
196 (severity factor). For each lesion, the score (according to intensity or observed frequency, scored from
197 0 to 3 or 0 to 2) was multiplied by the severity factor of 2. Then through the sum of each criterion was
198 obtained the value of the total score for each sample. Each score value was the result of 2 explants
199 from the same pig submitted to different treatments. The maximum score (22 points) indicates the
200 overall integrity of the intestine.

201 The histopathological scoring system was applied for comparing the morphological and lesional
202 changes after explants exposure to DON, NIV and FX, respectively.

203

204

Table 1. Histopathological score – endpoints used and severity factor

	Criteria (severity factor)	End-point	Score
Lesional Score	Enterocytes morfology (2)	Columnar epithelium	3
		<50% cuboid epithelium	2
		>50% cuboid epithelium	1
		Flattened epithelium	0
	Apical denudation of villi (2)	0 -10%	3
		11-40%	2
		41-70%	1
		71-100%	0
	Lesions of lamina propria(2)	No lesions, slight flattening of villi	2
		Localized edema and apoptosis	1
		Multifocal edema and apoptosis	0
Morphological Score	Villi fusion (1)	0-11%	3
		12-40%	2
		41-70%	1
		71-100%	0
	Number of villi (1)	≥25	3
		16 a 24	2
		5 a 15	1
		≤4	0

205

206 *2.5. Statistical analysis*

207

208 The values of scores are presented as means ± SD of independent experiments with 12 different
209 animals. The scores were statistically analyzed using normality (Shapiro-Wilk) and homogeneity
210 (Bartlett) tests. When these two assumptions were met, the analysis of variance (ANOVA) was
211 applied, followed by Tukey's test at 0.05 significance.

212

213 **3. Results**

214

215 *3.1. Histological and morphometric analysis before and after incubation and effect of DMSO*

216

217 We first investigated the effects of the culture and of DMSO on the histology and morphometry of
218 the jejunal explants. The explants were observed microscopically and scored from 22 (no lesion) to 0
219 (destroyed tissues).

220 Before incubation (T0), scores values were between 16 to 21 for all the explants T0 (Fig. 1/I). The
221 histological lesions observed were mild oedema in the lamina propria and light dilatation of the
222 lymphatic vessels, resulting in an average score of 18.09±1.98 and a mean villi height of 165.03±18.00
223 µm (Fig 1/I and IIa).

224 After 4-hours incubation with WME, the scores did not differ significantly in the presence or
225 absence of DMSO, with a mean value of 16.75 ± 2.20, which represented 92.59% of the value of the
226 non-incubated T0 explants (non-significant decrease) (Fig. 1/I). The pattern of lesions observed were
227 the same as described in the non-incubated explants; however after 4 hours of incubation the flattening
228 of the villi was evidenced (Fig. 1/IIb). Thus, villous height was analyzed by histomorphometrical
229 analysis. The mean villous height was 141.53±29.72 µm in the explants incubated with WME and
230 146.90±40.79 µm in the explants incubated in the presence of DMSO and did not differ significantly
231 from the T0 results. No statistically significant difference was observed between the different
232 incubation groups, with 0.1% DMSO, or without DMSO (Fig 1/I). The scores of treated explants in

the presence and absence of DMSO were grouped into a single 4-h culture control group for subsequent analyzes (n =84).

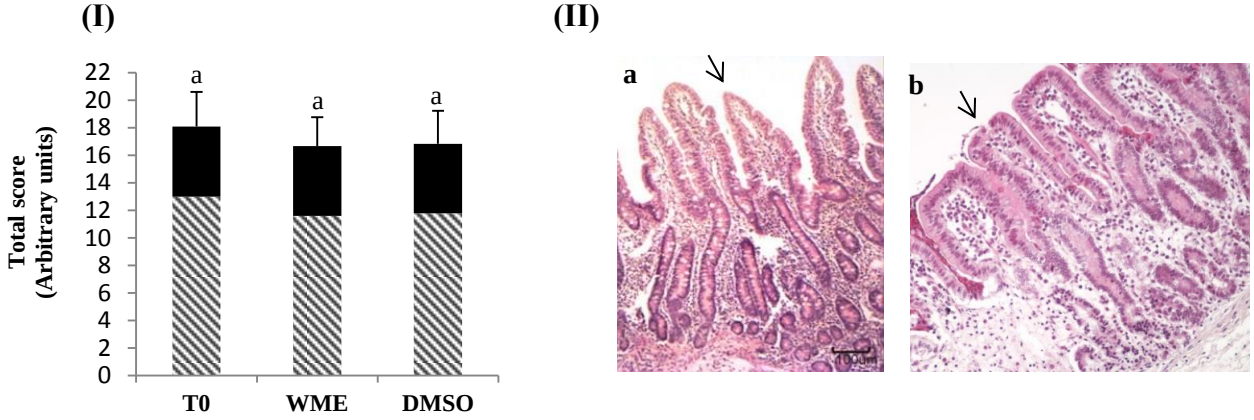

Figure 1. Morphology of jejunal explants of pigles. (I) Effect of the treatments (T0, MWE and DMSO) on morphological score (▨) and lesional score (■) after 4 hours of exposition. Values are mean ± SD.[a,b] Mean values with unlike letters were significantly different (P<0.05). (II) a) Jejunal explant uncultured (T0; n = 12). Light dilatation of the lymphatic vessels (arrow), HE, 10x; b) explants exposed to MWE with 0.1% of DMSO (DMSO n=42). Edema of the lamina propria and mild villi atrophy (arrow), HE 20x.

3.2. Effect of mycotoxins on the histological and morphometric analysis

Each treatment, DON (1-10 µM), NIV (1-10 µM) and FX (0.3-3 µM) induced a dose-dependent decrease in the histopathological scores of the jejunal explants after 4 hours of exposure (*P<0.01*) (Fig. 2/I). In the explants exposed to DON, the main morphological change was the coalescent villi with moderate fusion. Lesions included cubic epithelial cells instead of the cylindric epithelial cells seen in the control, areas of oedema in the lamina propria, villi atrophy and apical denudation of villi with focal loss of apical enterocytes (Fig. 2/IIb). In the group treated with NIV, the changes were similar to the DON group; however the flattening of the epithelial cells and apical denudation of villi were more severe (Fig 2/IIc). The individual treatment with the mycotoxins DON and NIV resulted in a significant decrease of the histopathologic score from doses of 3µM and 1µM, respectively. The corresponding scores were reduced to about 70% of the control explants at 3 µM and 10 µM DON or 1 µM NIV, to almost half the mean score (59.01%±6.08) of control explants at the highest concentration of NIV (Fig. 2/I).

A significant decrease in histological score was observed in all explants treated with the mycotoxin FX when compared to the control group, from the lowest concentration of 0.3 µM. The reduction was 81.41%, 74.69% and 55.21% for the respective doses of 0.3, 1 and 3µM (Fig. 2/I). Histopathological analysis showed that explants exposed to mycotoxin FX presented similar lesions compared to other treatments, however villi with absence of epithelia, severe atrophy, diffuse cellular debris along the surface of the explants and a reduction in the villi number were observed at the highest dose, as shown in Fig 2/IId.

The comparison of the toxic effects from the less toxic to the most highly toxic of the three mycotoxins tested allows the following range: DON<NIV< FX.

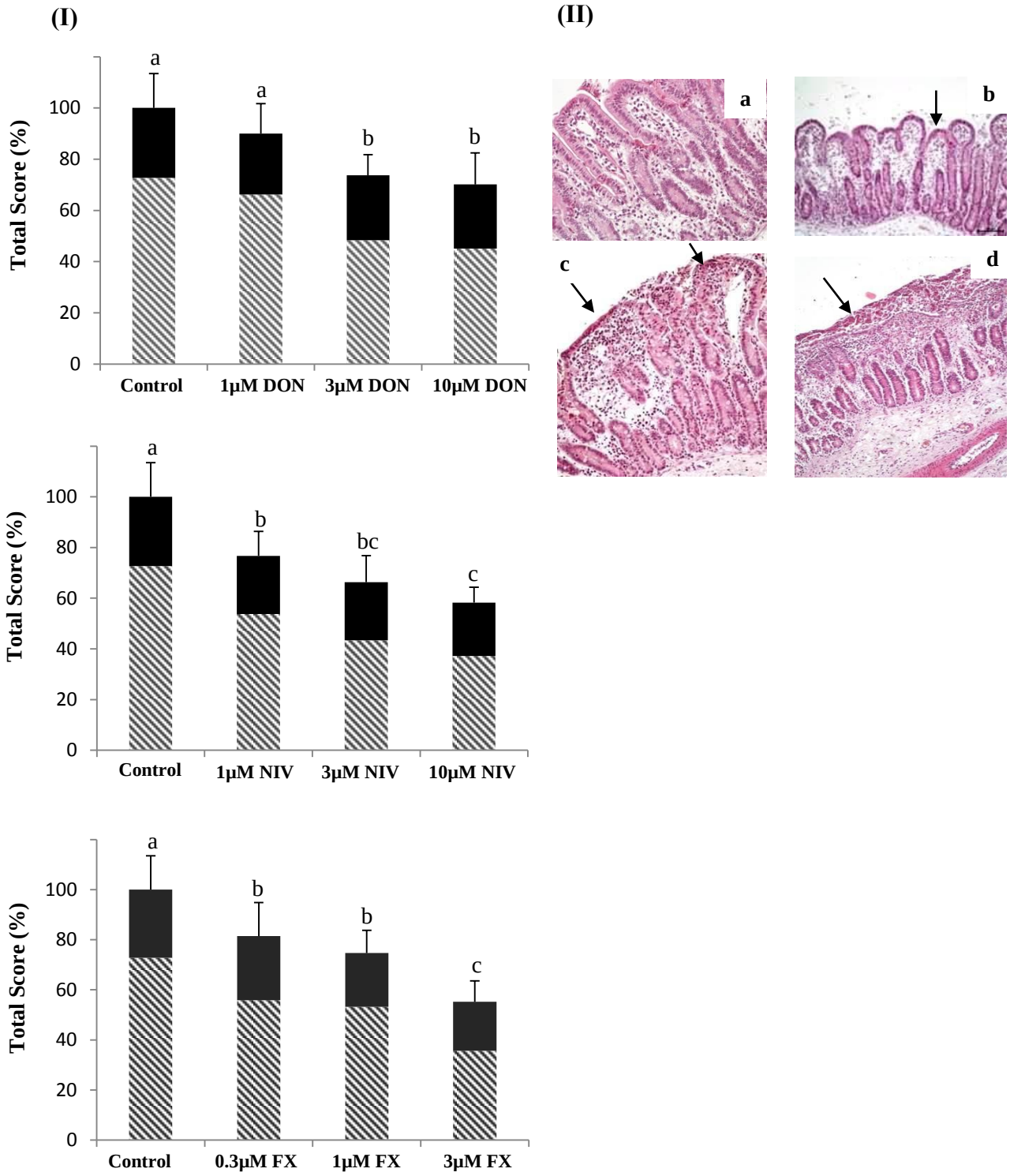

Figure 2. Effect of individual exposition of jejunal explants to DON, NIV and FX. (I) Effect of the treatments on morphological score (▨) and lesional score (■) after 4 hours of exposition. Values are mean ± SD. [a,b] Mean values with unlike letters were significantly different ($P<0.05$). (II) a) Control explants (H.E., 20x); b) 3µM DON-exposed explant. Moderate fusion and cubic epithelial cells (arrow) (H.E., 10x); c) 10µM NIV-exposed explant. Fusion and atrophy of villi with severe flattening epitelial (arrow). d) 3µM FX-exposed explant (H.E., 20x). Villi with absence of epithelia, severe atrophy and diffuse cellular debris (H.E., 10x).

4. Discussion

The intestinal epithelium is a major target for xenobiotics (Randall et al., 2011). The data obtained in the current study confirm that pig jejunal explants represent a sensitive model to assess intestinal epithelium toxicity, was not affected by DMSO, and allowed to compare the effects of the three mycotoxins. In their pioneering work on the application of jejunal explants from pigs for toxicological studies, Kolf-Clauw et al (2009), showed that the culture of pig intestinal tissue represent a relevant model to investigate the effects of food and feed contaminants, especially regarding both the relevance of the pig model-species compared to humans, and its high sensitivity to mycotoxins. In the current study, we refined the histopathological scoring of this *ex vivo* model and we analyzed and compared the toxicity of the mycotoxins DON, NIV and FX.

The tissue explanted and incubated for 4h was not affected by 0.1% DMSO, the highest concentration in our cultures. DMSO is used as a reagent in the field of bioscience, as an universal polar solvent of insoluble compounds or as a protectant in the freezing process involved in the cryopreservation of living cells. DMSO exerts a number of biological effects *in vitro* including acting as an inducer for cell differentiation (Santos-Beneit and Mollinedo, 2000), or as an apoptosis regulator (Fiore and Degrassi, 1999; Liu et al., 2001). Thus the samples incubated with WME in the presence or absence of DMSO were evaluated regarding morphological preservation once the toxicity of DMSO for cultured cells or animals is well known (Worthley and Schott, 1969; Cavaletti et al., 2000). The relative degree of hypoxia and the solvent DMSO resulted in no significant effect on intestinal morphology. This result agrees with that reported by Kolf-Clauw et al. (2009) that despite verify shorter and wider villi, observed no significant differences in intestinal histology between samples not incubated and incubated for 4h.

Our study showed dose-related toxicity of DON and related mycotoxins in the explants model from weanling pigs after 4 h of incubation. To the best of our knowledge, the present study is the first to analyze the action of FX on intestinal morphology of swine. In this work, the mycotoxin FX was responsible for inducing the most severe changes observed. In all the concentrations analyzed (0.3-3µM) the morphological and lesional scores were significantly decreased compared to the control

345 explants. In previous acute, subacute toxicity studies, FX induced extensive hemorrhage in the
346 intestine with cellular destruction and karyorrhexis in the intestinal mucous of mice (Ueno, 1973;
347 Ueno et al., 1984). Mice chronically treated with FX showed atypical hyperplasia in the gastric and
348 intestinal mucosa (Saito and Ohtsubo, 1974). FX can induce apoptotic cell death in rat gastric mucosa
349 and thymus of mice (Li and Shimizu, 1997; Miura et al., 1998) and HL-60 cells (Miura et al., 2002).
350 Changes were significant in explants exposed from dose of 3 µM DON. The main histological
351 changes were focal lysis of enterocytes, moderate atrophy and fusion of villi. The histopathological
352 lesions observed in the present work, following short-term exposure to DON, are in accordance with
353 the morphological alterations showed previously in *ex vivo* studies (Kolf-Clauw et al., 2009; Pinton et
354 al. 2012; Basso et al., 2013). The reduction of 30% in the histopathological score observed in the
355 samples treated with the dose of 10µM DON were similar to the results presented by Basso et al
356 (2013) that obtained a decrease of the histopathological score around 37%.
357 Few studies have analyzed the action of NIV on intestinal morphology. In this experiment, all
358 doses of NIV caused significant decrease of the histopathologic score in the intestinal explants.
359 Chronic ingestion of NIV induced gastrointestinal erosions in young pigs (2.5 or 5mg/kg) (Hedman et
360 al., 1997), whereas in C57BL/6 mice, subjected to acute, subchronic and chronic feeding study with
361 NIV showed no alterations in the histological architecture of small intestine (Yamamura et al., 1989;
362 Ryu et al., 1988) . This differences is probably related to fact of swine are among the most sensitive
363 species to mycotoxins (Kararli, 1995). The toxicity of NIV on intestine cells has already been reported
364 in *in vitro* studies. In accordance with the results of Alassane-Kpembi et al. (2013) and Wan et al.
365 (2013) NIV has dose-dependent cytotoxic effect on cell viability of Caco-2 and IPEC-J2 cells,
366 respectively. In previous study, the reduction of IEC-6 viability due to treatment with NIV was related
367 to apoptosis induction (Bianco et al., 2012).
368 Interestingly the individual treatment with the mycotoxins DON, NIV and FX resulted in a
369 significant decrease of the histopathologic score from doses of 3µM, 1µM and 0.3 µM, respectively.
370 Our results suggest that NIV was less toxic to intestinal explants than its acetyl derivative FX. Similar
371 results were reported in Caco-2 cells where NIV was 10-20 times or 20-35 less toxic than FX (Bony et
372 al., 2007; Allassane-Kpembi et al., 2013). In other cell lines, FX has been found to be more toxic than

NIV in unsettled proportions (Forsell and Pestka, 1985; Thompson and Wannemacher, 1986; Eriksen et al., 2004). The less severe toxic effect of DON on intestinal explants in relation to NIV is also in accordance with previous studies, which demonstrated that NIV exerted a stronger effect compared to DON on intestinal and not- intestinal cells lines (Bianco et al., 2012; Alassane-Kpembi et al., 2013; Marzocco et al., 2009; Luongo et al., 2008; Minervini et al., 2004). The higher oral toxicity of FX than NIV can be due to the FX be absorbed from the gastrointestinal tract more rapidly and efficiently than NIV, as showed by Poapolathep et al. (2003) in mice and rats.

The present study compares the effects of mycotoxins at realistic concentrations, considering the concentration of mycotoxins to which the consumer can be exposed via the food. So, the present results are of high biological relevance. DON concentrations of 0.16–2 μg/mL (0.5–7 μM) can be considered as realistic in human gut (Sergent et al., 2006). The lower concentration value corresponds to the mean estimated daily intake of French adult consumers on a chronic basis (Sirot et al., 2013). The higher concentration value simulates levels that can be reached after the consumption of heavily contaminated food, as can be occasionally encountered. The amount of NIV in cereal products varies considerably among world regions (from 20–60 μg/kg in France, to 584–1780 μg/kg in China) (Hsia et al., 2004), depending on weather and culture conditions (Edwards, 2004). The significant morphological and lesional alterations were observed from doses of 1μM NIV in this study (corresponding to about to 312 μg/Kg) and is consistent with the levels plausibly encountered in the gastrointestinal tract after consumption of heavily contaminated food. This may occur particularly in the case of unfavorable weather conditions or in eastern regions of Asia (Tanaka et al., 1985; Sugiura et al., 1993; Hsia et al., 2004), where particularly *Fusarium* species (e.g. *F. poae* and *F. crookwellense*) seems to be responsible for heavy contamination of cereals by NIV.

In European countries, the European Food Safety Authority Panel on Contaminants in the Food Chain (CONTAM) establishes the tolerable daily intake (TDI) of NIV as 1.2 μg/kg bw/day based on a lowest-observed-adverse-effect (LOAEL) of 0.7 mg/kg bw/day found in long-term dietary studies in mice (EFSA, 2013; SCF, 2000, 2002; Gareis et al., 2003; Schlatter, 2004), while to DON and its acetylated derivates the TDI established was of 1 μg/kg bw/day (SCF, 2002). On the other hand, the TDI for FX was considered difficult, due to the limited number of studies and varied test results on the

combined effects and the fact that the mechanism of action of toxin has not been fully clarified (Saengtienchai et al., 2014).

To conclude, the present study demonstrates that pig intestinal explants represent a sensitive model to investigate the digestive effects of DON, NIV and FX. Intestinal explants can also contribute to improve our knowledge on plausible interactions of contaminants present simultaneously at the intestinal level. For this purpose, in parallel to histological analysis, specific cellular responses of toxic insult such as, for example, the expression of inflammatory cytokines will need to be investigated.

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Conflict of interest

The authors state that there is no conflict of interest.

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5 **CONCLUSÃO**

i. A ingestão crônica de dieta contaminada com DON+ NIV + ZEA promoveu a redução do ganho de peso.

ii. A ingestão crônica das dietas contaminadas induziu alterações morfológicas intestinais, hepáticas e nos órgãos linfoides.

iii. A ingestão crônica das dietas contaminadas induziu apoptose nos órgãos linfoides.

iv. As micotoxinas DON, NIV e FX apresentam um efeito dose-dependente sobre a morfologia intestinal e podem ser classificadas na seguinte ordem de toxicidade: DON<NIV< FX.

ANEXOS

ANEXO A

Artigo publicado em colaboração *Archives of Toxicology*

The emerging mycotoxin, enniatin B1, down‑modulates the gastrointestinal toxicity of T‑2 toxin in vitro on intestinal epithelial cells and ex vivo on intestinal explants

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ORGAN TOXICITY AND MECHANISMS

The emerging mycotoxin, enniatin B1, down-modulates the gastrointestinal toxicity of T-2 toxin in vitro on intestinal epithelial cells and ex vivo on intestinal explants

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Abstract Enniatins, the most prevalent emerging mycotoxins, represent an emerging food safety issue, because of their common co-occurrence with other fusariotoxins such as trichothecenes co-produced by *Fusarium spp* on field grains and because of their extensive prevalence in grains. In this study, the intestinal toxicity of enniatin B1 (ENN) alone and mixed with the most toxic trichothecene T-2 toxin (T2) was characterized by using two biological models from pig, the most sensitive species: the intestinal cell line IPEC1 (in vitro exposure) and jejunal explants (ex vivo exposure). Dose-dependent decreases in cell proliferation in IPEC1 and in the histopathological scores of explants were observed for ENN at μM-levels and for T2 at nM-levels, with IC50 values for ENN of 15.8 and 29.7 μM, and for T2 of 9.3 and 15.1 nM in vitro and ex vivo, respectively. Interaction analysis by probabilistic and by determinist approaches showed a less than additive effect both in vitro and ex vivo, at IC50 values, with increasing antagonism with decreasing concentrations of toxins. The results obtained by the determinist median-effect dose analysis and by the nonlinear regression analysis were concordant. All the median-effect doses estimated for IPEC cells were included in the IC50 confidence intervals of the nonlinear regression fitting. Given the occurrence of enniatins, potential synergy following the co-occurrence of enniatins and the major fusariotoxins, especially trichothecene B deoxynivalenol should be investigated.

Keywords Emerging mycotoxins · Enniatins · Gastrointestinal toxicity · Jejunal explants · Interactions · Combination index

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Introduction

The increasing world population requires more and safe food in the future, but the worldwide contamination of cereals and cereal products by mycotoxins, secondary metabolites produced by filamentous fungi, is of potential concern for human and animal health. In Europe, the contamination of cereals by *Fusarium* mycotoxins raises a worrisome problem linked to climatic changes and to co-contaminations (Van Der Fels-Klerx et al. 2012). Fusariotoxins, produced by various *Fusarium spp*, are most frequently present as mixtures (Rodrigues and Nahrer 2012). They include the well-known trichothecenes, fumonisins and zearalenone chemical groups, but also the enniatins, beauvericin, moniliformin and fusaproliferin, which are frequently referred as emerging mycotoxins (Jestoi 2008). These emerging mycotoxins are usually co-produced with the well-known fusariotoxins, but have very rarely been

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studied (review in Jestoi 2008; Santini et al. 2012). The natural co-occurrence of mycotoxins means that a better knowledge of their potential interactions must be considered in the risk assessment for human health. Both enniatin B1 and T-2 toxin can be produced by the same strains of *Fusarium* species, and their co-ocurrence has been observed in wheat, barley, oats, rye and grain-based products from Finland and Italy (Jestoi 2008). However, studies on the possible interaction between enniatins and co-occurring trichothecenes are lacking, that is, additive, synergic or antagonist toxic effects, and to date, the health risk of these combined exposures is unknown.

Enniatins represent an emerging food safety issue and possess a wide range of biological properties, but their toxicity in vivo has been little studied formerly. Oral doses of 0.5–1 g/kg body weight (bw) per day over 6 days to mice and single oral doses of up to 50 mg/kg bw in rats did not produce toxic effects (Gaümann et al. 1950; Bosch et al. 1989). Chronic exposure by feeding experiments may induce feed refusal, weight loss and reduced productivity (Jestoi 2008). In vitro, general cytotoxicity was described at low micromolar concentrations (Behm et al. 2009; Ivanova et al. 2006; Jestoi 2008). Enniatins derive structurally from amino-acids, naturally present as mixtures of cyclic depsipeptides, acting as ionophores with antibacterial activities. Among the enniatins, enniatin B1 (ENN) is a frequent contaminant in cereals, but despite this, very limited data are available on its toxicity.

The trichothecene type A T-2 toxin (T2) is recognized as the most acutely toxic trichothecene (for review, see EFSA 2011) showing high gastrointestinal sensitivity (Pinton et al. 2012a). The effects observed in various species after acute oral T2 exposure to doses ranging from 0.06 to 10 mg/kg bw include nonspecific symptoms like weight loss, feed refusal, dermatitis, vomiting, diarrhoea, haemorrhages and histopathological signs of necrosis of the gastrointestinal epithelium and of other target tissues (necrotoxin). T2 acute toxicity on actively dividing tissues, including intestinal mucosa, makes it a potential candidate as a biological warfare agent. Apoptosis has been demonstrated in vitro and in vivo in gastrointestinal, haematopoietic and lymphoid tissues (Doi et al. 2006), but the mechanisms involved are still controversial. The most sensitive species is the pig, and the risk assessment for consumers is based on a feeding study in this species (EFSA 2011).

The aim of the present study was to investigate how ENN modulates T2 toxicity in the situation of co-contamination, by analysing the acute toxicity of T2 on the digestive target. Two relevant models from pig were used: the IPEC cell line in culture (Bouhet et al. 2006) and pig jejunal explants (Kolf-Clauw et al. 2009). Two analytical methodologies were used and compared to study the interactions, one based on a statistical approach and the other based on a determinist approach.

Materials and methods

Toxins

Purified T2 from Sigma-Aldrich (Saint-Quentin Fallavier, France) and ENN from BioAustralis (Le Perray en Yvelines, France), were dissolved in DMSO and stored at −20 °C before dilution in cell culture media. The range of concentration of toxins in single exposure (0.3–100 µmol/L and 0.3–100 nmol/L for ENN and T2, respectively) was chosen according to previously described cytotoxic ranges and to preliminary cell assays.

In vitro assay: cell culture

IPEC-1 cells, derived from the small intestine of a newborn unsuckled piglet were maintained as previously described (Bouhet et al. 2006). Cell proliferation was used as the endpoint for a cytotoxic effect. The cytotoxicity of each toxin alone and in combination was evaluated by determining the molecular concentrations giving 50 % inhibition of cell proliferation (IC_{50}). The IC_{50} values for cells were determined for T2, ENN and the association T2 + ENN, with a constant T2:ENN ratio of 1:1,000, by using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Charbonnières-les-Bains, France) according to manufacturer instructions. The mono-oxygenation of luciferin is catalysed by luciferase and depends on the presence of Mg^{2+}, ATP and molecular oxygen. The quantity of ATP is directly proportional to the number of cells. IPEC-1 cells were seeded at 4×10^3 cells/well in 100 µl of complete proliferation medium in flat-bottomed white chimney 96-well plates (Greiner, Courtaboeuf, France). After 48 h of culture, concentrations ranging from 0 to 30 µmol/L of ENN and 0–30 nmol/L of T2 were added to the cells, either separately or concomitantly (co-contamination exposure). After 48 h of treatment, 100 µl of CellTiter-Glo® Reagent were added per well and mixed for 2 min in an orbital shaker to induce cell lysis. Data were recorded 10 min after with a microplate luminometer reader (Tecan, Lyon, France) and corrected for the background signal resulting from reagent-treated medium without cells. The effect of the mycotoxins on cell proliferation was calculated as a percentage of the proliferation of the control cells without mycotoxins (relative proliferation).

Ex vivo assay: intestinal explant culture

Six crossbreed 4–5 week-old weaning piglets were used to obtain explants of jejunal tissue and the procedures for the culture of the explants were as previously described (Kolf-Clauw et al. 2009) with minor modifications. Explants were incubated for 4 h in William's Medium E (WME)

containing 100 U/mL penicillin, 100 μg/mL streptomycin and 50 μg/mL gentamicin and supplemented with D glucose (2.5 g/L) and 30 mM Alanine-Glutamine (Sigma) at 37 °C under CO_2-controlled atmosphere with orbital shaking. Uncultured control tissue was placed into fixative at the end of dissection time (0 h). Explants were exposed to purified T2 at 0, 0.3, 1, 3, 10 and 30 nM, or purified ENN at 0, 0.3, 1, 3, 10 and 30 μM, or to the mixture of T2:ENN at the same ratio as for cell culture (1:1,000) for 4 h. For histological analysis, jejunum explants were fixed in 10 % buffered formalin for 24 h. Histopathological and morphological assessment of the explants were carried out as previously described (Kolf-Clauw et al. 2009).

Data analysis: probabilistic and determinist approaches

For data analysis, two toxin-based approaches by response-additivity modelling (Berenbaum 1985) were used and compared: one statistically based analysis, with regression curve fitting and *P* value determination and the other based on determinist analysis.

Statistical analysis

Firstly, the individual dose–effect data were analysed to verify that both drugs produced effects that increased with dose. For each dose level, the percentage of proliferative cells compared to controls, and the explant scores are presented, as mean ± SD of 5–6 independent experiments (from different animals for the intestinal explants). To test the individual and combined effect of the toxins according to the concentration, ANOVA analysis was used (Systat Software Inc, version 10, San Jose, USA) followed by Tukey and Dunnet tests if significantly different (*P* values ≤ 0.05). The IC_{50} values of each mycotoxin alone and of the mixture were calculated by extrapolating results from the concentration–response curves from each experiment, by nonlinear regression Hillslope modelling using the raw data (GraphPad Prism5 software Inc, San Diego, California, USA). To test the interactive effect, an isobologram approach was used, with the assumption of a constant potency ratio of ENN/T2, employing the concept of dose equivalence (Tallarida 2006). A constant potency ratio of 1,000:1 (ENN:T2) was used, and additive isobolograms represented by a line joining equally effective doses were constructed. The cytotoxic effect was identified as synergist, additive or antagonist according to the position of the combined effect related to the line of additivity (Borgert et al. 2001).

Median-effect doses and combination index

The second approach was based on a determinist analysis with the determination of combination index values (CI) by the Chou-Talalay median-effect equation (Chou and Talalay 1984). The median-effect plot of Chou was applied to the individual toxins, and a median-proliferation dose (Dm) was calculated for each toxin (CompuSyn Software 2007) and compared with the IC_{50} values calculated by the regression model. For the mixture of T2 and ENN, the Dm for the mixture was calculated. The combination index (CI) was calculated for a 10, 20 and 50 % inhibition of cell proliferation. CI is a quantitative measure of the degree of drug interaction of synergism and antagonism for a given endpoint of the effect measurement with $CI < 1$ indicating synergism, that is, a greater than expected additive effect and $CI > 1$ indicating antagonism, a smaller than expected additive effect.

Results

Individual and combined effects of the ENN and T2 on intestinal epithelial cell proliferation

Each treatment, ENN (0.3–100 μM), T2 (0.3–100 nM) and ENN+ T-2 induced a dose-dependent decrease in intestinal epithelial cell proliferation ($P < 0.001$). ENN significantly decreased cell proliferation at all the concentrations tested (0.3–100 μM), with values ranging from 96.2 to 13.6 % proliferation when compared to control cells. T2 also decreased cell proliferation, from concentration of 3 nM and above, with relative cell proliferation of 80 % decreasing to 11.6 % at the highest concentration (Fig. 1). The IC_{50} values were 15.80 μM (confidence interval of 13.02–19.17 μM) and 9.35 nM (confidence interval of 6.94–12.6 nM) for ENN and T2, respectively (Table 1). The binary mixture of T2 and ENN at the 1:1,000 ratio showed a dose-dependent effect on cell proliferation, ranging from 96.3 to 3.1 % compared to the control cells (Fig. 1), and an IC_{50} of 14.41 μM (confidence interval of 10.55–19.67). The determination coefficients of the nonlinear regression Hillslope showed a better goodness of fit for isolated toxins than for the mixture, with values of 0.995 for enniatin, 0.989 for T2-toxin and 0.840 for the mixture (Table 1). The median-effect doses (Dm) calculated according to the determinist method were highly correlated to the IC_{50} values, and the coefficients of correlation of the median-effect plot were between 0.925 and 0.997 (Table 1).

Individual and combined effects of the toxins on jejunal explant histopathology

Each treatment, ENN (0.3–30 μM), T2 (0.3–30 nM) and ENN + T2 at similar concentrations as the toxins alone induced a dose-dependent decrease in the histopathological scores of the jejunal explants after 4 h of exposure

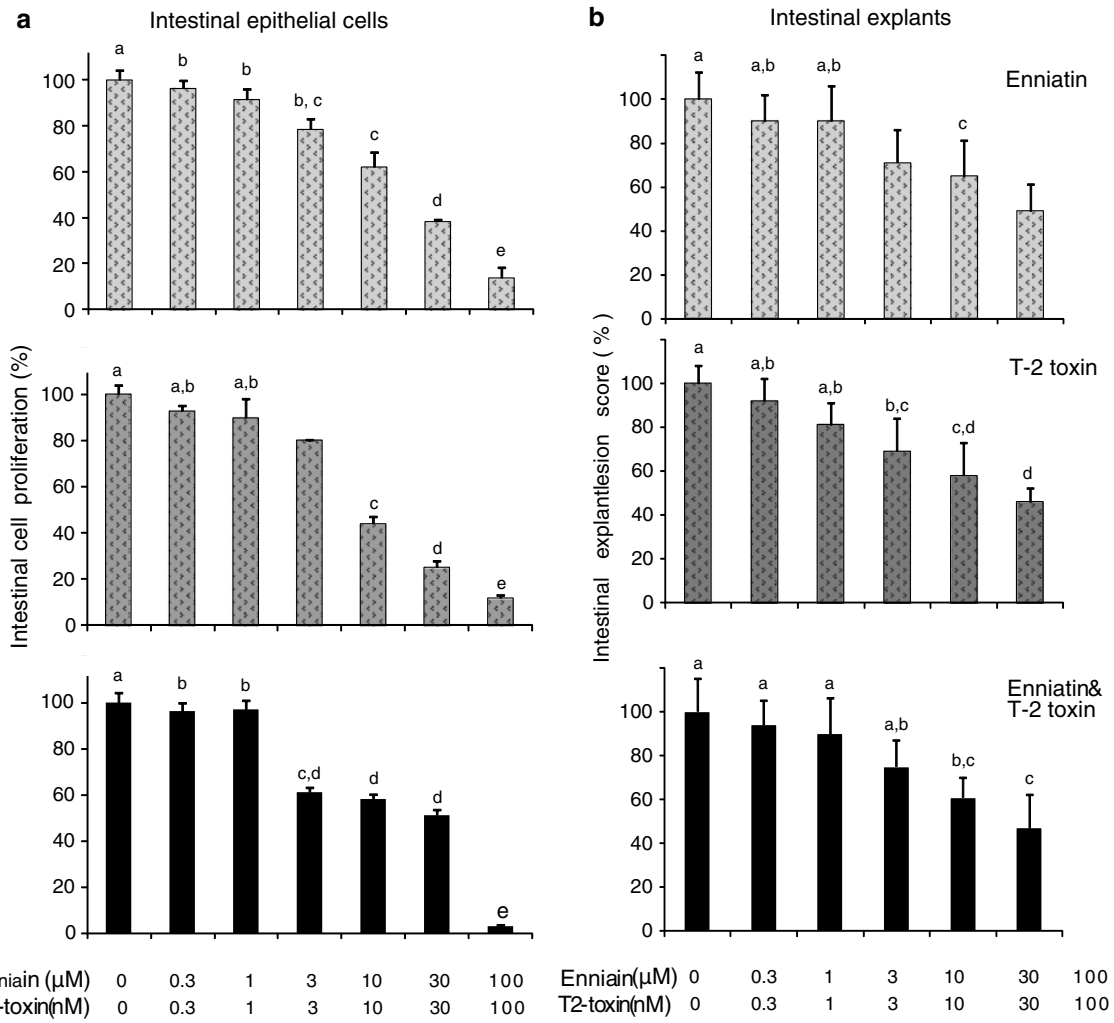

Fig. 1 **a** Effects of mycotoxins on the proliferation of IPEC cell cultures after 48-h exposure to different concentrations of enniatin B1 (ENN), T-2 toxin (T2) and their combination (ENN/T2-toxin 1,000:1). Mean relative proliferation compared to control cells (expressed as %) and the standard deviation (SD) of the mean. ANOVA analysis, *bars* without a common *letters* differ ($P < 0.05$). **b** Jejunal explants from 4 to 5 week-old piglets were exposed in vitro for 4 h to different concentrations of enniatin B1 (ENN), T-2 toxin (T2) and their combination (ENN/T2 1,000:1) before histopathological examination and scoring assessment. For each concentration, 2–4 explants from the same animal were scored. Data are mean relative scores ± SD from 6 different animals (expressed as %). ANOVA analysis, *bars* without a common *letters* differ ($P < 0.05$)

Table 1 IC50 and median-effect dose (Dm) in IPEC cell culture (Intestinal Porcine Epithelial Cell) and in porcine jejunal explants culture, following exposure to mycotoxins alone or in combination

	Enniatin B1 µM		T-2 toxin nM		T-2 toxin + Enniatin B1 (1:1,000) µM	
	IC50	Dm	IC50	Dm	IC50	Dm
IPEC	15.80 (13.02–19.17) $R^2 = 0.995$	14.97 ($r = 0.997$)	9.35 (6.94–12.60) $R^2 = 0.989$	9.34 ($r = 0.985$)	14.41 µM (10.55–19.67) $R^2 = 0.840$	10.5 ($r = 0.925$)
Jejunum explants	29.71 $R^2 = 0.60$	28.32 ($r = 0.959$)	15.11 $R^2 = 0.60$	18.08 ($r = 0.987$)	18.58 $R^2 = 0.787$	18.76 ($r = 0.993$)

IC50 values obtained using the Hillslope model (confidence interval of IC50)

Dm, Median-effect dose calculated by the Chou-Talalay median-effect equation: determinist approach; *r* coefficient of correlation; R^2 determination coefficient of the nonlinear regression model

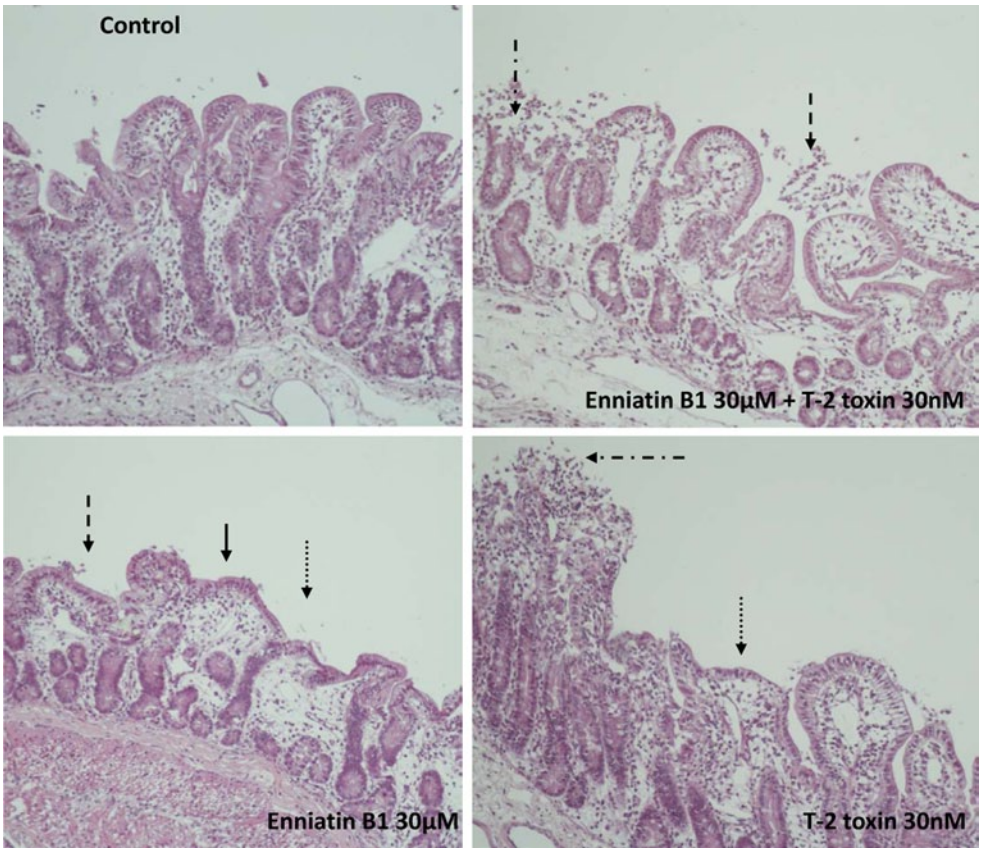

Fig. 2 Morphology of 4–5 week-old piglet jejunal explants incubated for 4 h with T-2 toxin, Enniatin B1 and the binary mixture of Enniatin B1 and T-2 toxin (1,000:1). Negative control (*upper left*) and treated samples with indication of lesions observed for the scoring system analysis. Obj 10x, H&E staining. ——▶ Coalescence, - - - - -▶ Cellular debris, ...▶ Cuboïd cells, -·-·-·▶ Villi lysis

($P < 0.001$). The morphological changes consisted of coalescent villi with cubic epithelial cells instead of the cylindric epithelial cells seen in the control. Lesions included lysis of villi, areas of oedema in the lamina propria and cellular debris (Fig. 2). The morphological and lesional scores were significantly decreased compared to the control explants from the concentrations of 3 µM ENN, 3 nM T2 and the combination of both toxins at 10 µM/10 nM (ENN/T2, 1,000:1). The corresponding scores were reduced to about 70 % of the control explants at 3 nM T2 or 3 µM ENN (and to almost three-quarters of the control values for the similar points in the mixture) to less than half the mean score (45.7–48.9 %) of control explants at the highest concentrations (Fig. 1). The IC_{50} values could not be precisely estimated in the case of explants as the 95 % confidence intervals were too large to test the interaction by the classical isobologram approach. However, the nonlinear fitting of the raw data enabled the $IC_{50.}$ values to be obtained: 29.71 µM for enniatin, 15.11 nM for T2-toxin and 18.58 µM for the mixture (Table 1). These values are very similar to and concordant with those obtained by the determinist approach with Dm of 28.32 M, 18.08nM and 18.76 µM for ENN, T2 and the mixture, respectively. The correlation coefficients of the median-effect plot were between 0.959 and 0.993 for the explants model.

Determination of the type of interaction between T2 and ENN at the intestinal level

A less than additive effect was observed in the two biological models by both methodologies for the mixture ENN/T2 (1,000:1). The two mathematical approaches resulted in similar results. In the determinist approach, the goodness of fit was attested by coefficients of correlation higher than 0.925 for the modelization using the two biological models (Table 1). The comparison of Dm and IC_{50} shows highly correlated values (Table 1). The Chou and Talalay method does not allow a confidence interval to be calculated with all the raw data, but we show here that all the calculated Dm for the IPEC cell culture model were included in the IC_{50} Hillslope confidence intervals. At the Dm, that is, 50 % effect level, the CI were 1.81 and 1.69 for IPEC and explants, respectively, indicating antagonism. CI values at doses lower than the Dm, more relevant for risk assessment purposes, were estimated for the 10, 20 and 30 % effect levels. Antagonism, that is, a less than additive effect, was also observed for these effect levels (Fig. 3).

The less than additive effect was demonstrated in both biological models, with the CI index calculated in both models, IPEC cells or jejunal explants giving very similar

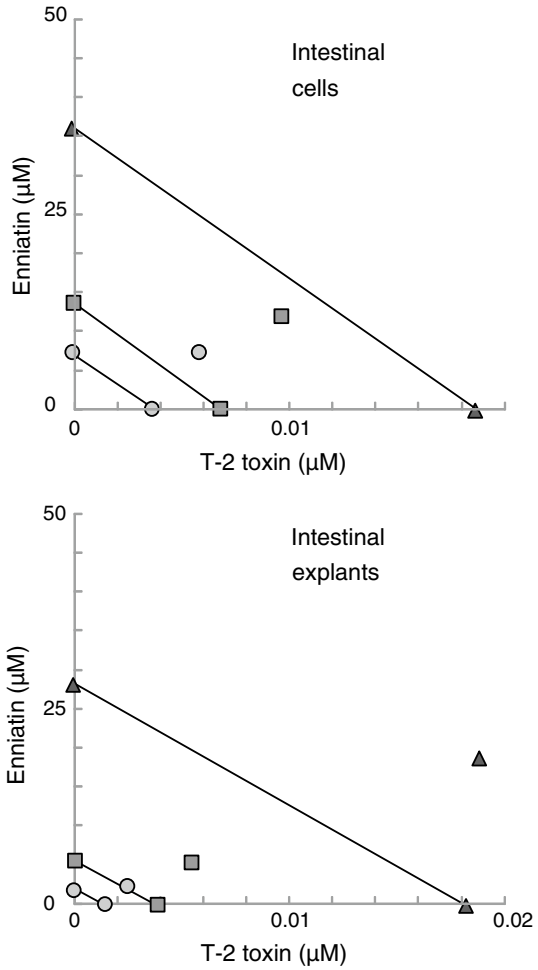

Fig. 3 Isobolograms illustrating the combined effect of the mixture of Enniatin B1 and T-2 toxin (1,000:1) for reaching 20 % (Fa = 0.2, *filled circle*), 30 % (Fa = 0.3, *filled square*), or 50 % inhibition (Fa = 0.5, *filled triangle*) of IPEC cell proliferation or of the scores of explants. The points on each axis are mean concentrations of dose–response curves of each toxin alone (*Compusyn*® software analysis)

values and well correlated: $r = 0.98$ (Table 2). In both models, the binary mixture of the toxins produced antagonism, tending towards strong antagonism with decreasing the effect (strong antagonism according to Chou 2006, means a CI between 3.3 and 10). The two alternative models gave similar and highly correlated results for moderate effects, that is, less than the median-effect that is biologically relevant. Above the effect dose of 95 % inhibition for cell relative proliferation and for the relative score of explants, CI values were lower than 0.90, indicating synergy. However, from a biological and toxicological point of view, this result should not be considered as relevant, because mixture interaction should be identified at non-toxic levels that are relevant and realistic in terms of the actual exposure of consumers.

Table 2 Combination index (CI) values in the two models

Effect level	10 %	20 %	30 %	50 %
IPEC cells	3.26	2.63	2.27	1.81
Jejunum explants	4.10	2.96	2.38	1.69

Combination index (CI) calculated by the Chou-Talalay median-effect equation (1984) by using CompuSyn software (3.0.1, 2010) in the two pig models: intestinal IPEC-1 cells (in vitro model) and jejunal explants (ex vivo model). Effect levels for 10, 20, 30 and 50 % of the relative proliferation for treated cells compared to control cells, or the relative scores for explants. CI is a quantitative measure of toxin interaction in terms of synergism and antagonism for a given endpoint of the effect measurement. CI = 1 means additive effect, combined effect predicted by the mass-action law principle in the absence of synergism (CI < 1: greater than expected additive effect) or antagonism (CI > 1: smaller than expected additive effect)

Discussion

Both fusariotoxins, T2 and ENN individually induced dose-dependent gastrointestinal toxicity with a one-thousand-fold difference in potency, when assessed on an intestinal cell line (IPEC) and on intestinal explants. The ratio in the cytotoxic effect between ENN and T2 (1,000:1) was similar to other studies (Behm et al. 2012). In combination, these toxins showed a less than additive effect in the two biological models. To our knowledge, no previous interaction study has used two different biological models of different complexity, at the cellular level for the IPEC cell line and at the tissue level for jejunal explants. These two intestinal models were from pig, considered the most relevant animal species for studying fusariotoxin toxicity (Pinton et al. 2012a). The mycotoxins T2 and ENN were evaluated because of their special interest and natural co-occurrence. T2 is of concern as the most acutely toxic member of the trichothecenes. Our results confirm the high toxicity of T2 towards rapidly dividing cells such as mammalian kidney epithelium (Ruiz et al. 2011b) or in human monocytes (Hymery et al. 2006). The digestive tract as a target is well-known for T2, with the induction of necrotic lesions of the gastrointestinal tract following high doses in several species (Pinton et al. 2012a). In the present study, the co-contamination with ENN down-modulated this toxicity. Identifying intestinal target is of major importance because the intestinal epithelium represents the first barrier to the access of food contaminants or pathogens to the whole body. Its integrity is also necessary to avoid any indirect effects of food contaminants. More recently, another fusariotoxin, deoxynivalenol, has been shown to alter the intestinal epithelium in vitro, ex vivo and in vivo (Bracarense et al. 2011; Kolf-Clauw et al. 2009; Pinton et al. 2009). The morphological changes observed in jejunal explants with T2 or ENN in the current study were similar to those

described with deoxynivalenol (Kolf-Clauw et al. 2009; Pinton et al. 2012b). Studying the toxicity of the enniatins alone and in combination, in situ on the digestive target is especially relevant.

ENN is an emerging fusariotoxin, which has led to interest concerning its toxicity over the last few years. Even though enniatins apparently are of low acute toxicity in vivo, their effects in combination with other mycotoxins remain unknown. They represent an emerging food safety issue because of their extensive incidence, documented in recent decades, in various grain cereals. In a Spanish survey, the simultaneous presence of two or more mycotoxins was observed in a high percentage of the samples, and the prevalence of the emerging mycotoxins in cereal products was considered likely to pose a health risk to general population (Serrano et al. 2013). Our cytotoxicity data for ENN are in accordance with literature data reporting enniatin cytotoxicity (Jestoi 2008). The measured IC50 values were similar to those reported in Caco-2 cells (Meca et al. 2012) and were in agreement with other studies reporting IC50 values in the lower μM-range (Föllmann et al. 2009; Ivanova et al. 2006), with a higher sensitivity for cancer cells, possibly making enniatins interesting as new anticancer drugs (Wätjen et al. 2009). Recently, the cytotoxicity of enniatins was shown to be due to lysosomal destabilization and mitochondrial permeabilization, possibly related to their ionophoric properties, finally resulting in apoptotic cell death (Gammelsrud et al. 2012; Ivanova et al. 2012).

Co-contaminations are frequent in cereals in Europe and worldwide (Rodrigues and Naehrer 2012) and studying combined toxicity of mycotoxins should help to protect consumers. The simultaneous appearance of T-2 toxin and enniatin B1 in the same samples can be inferred by the recent multi-mycotoxin analysis study of 83 feed samples (Streit et al. 2013). The chosen combinations were realistic, corresponding to some actual situations in food or feed, as T2 and ENN concentrations have been reported in the μg/kg (Van der Fels-Klerx and Stratakou 2010) and in the mg/kg (Santini et al. 2012; Uhlig et al. 2006) ranges, respectively. In our study, two different analytical models were used that gave concordant results for the interaction. The T2 and ENN interaction was analysed according to a classical probabilistic approach, and also with a determinist method (Chou and Talalay 1984). This latter method provides a fundamental basis for assessing whether a combined effect is greater, equal or smaller than expected effect. This method was initially described for optimizing drug associations for pharmacologists (Chou 2006) and has subsequently been applied to toxicology studies of mycotoxins (Ruiz et al. 2011a, b). The present results from determinist analysis were correlated with the probabilistic results in the two biological models. For cell cytotoxicity, all the median-doses estimated by the determinist method were included in the confidence intervals of Hillslope model, demonstrating a good concordance with the probabilistic approach. The Chou and Talalay method enables the nature of an interaction at various dose levels to be easily predicted. Interactions previously described between co-occurring mycotoxins showed either additive affects, antagonism or synergism, according to the biological model, the toxins, and the level of the effect. The combination of the two major trichothecenes T2 and DON was previously reported as antagonist in kidney cells, and similarly to our results, the strongest antagonism on cytotoxicity was observed at lower doses (Ruiz et al. 2011b). These latter authors used Chou and Talalay's method to analyse the fractional inhibition of proliferation by beauvericin, DON and T-2 toxin in combination. Strong antagonism was also observed with T-2 toxin and the emerging mycotoxin beauvericin, as for the IPEC cells and jejunal explants with ENN/T2 in our study. We observed stronger antagonism below 50 % relative proliferation inhibition in the two biological models, but interactions tending towards synergism at higher levels of the effects. Testing a combination at low concentrations appears highly relevant biologically compared to testing at high concentrations, such as above 75 % inhibition for cell relative proliferation as described previously (Ruiz et al. 2011b). We have to wonder about the relevance of the mechanisms underlying these interactions observed at high toxic concentration, whereas interactions at very low levels of effects are very relevant, because based on healthy cell metabolism and reactivity, allowing hypothesis relative to the mechanisms of these interactions.

Biologically, several hypotheses could be proposed to explain the less than additive effect of the two toxins observed in vitro and ex vivo on the digestive tract. First, antagonism may be explained by the interactions at membrane level, with ENN potentially lowering the cell bioavailability of T2. Indeed, the ionophoric properties of ENN might interact with the cell membrane, preventing T2 from reaching its cell target. Another hypothesis at membrane level is an interaction involving an efflux pump and preventing the molecular target to be reached, as enniatins interact with ATP-binding cassette transporters below toxic concentrations (Ivanova et al. 2010; Tedjiotsop Feudjio et al. 2010). Enzymatic inhibition of HT2 production is another hypothesis, as well as an antagonism at the intracellular target level. T-2 toxin was shown to induce apoptosis in the intestinal crypt epithelial cells in mice (Li et al. 1997) that correlated in vitro with MAPK activation (Yang et al. 2000). A target antagonism could explain, at least partly, our results at the level of MAP-Kinases, as T2 activates extracellular regulated protein kinases ERK (ERK1/2, Yang et al. 2000), whereas enniatin B1 was recently shown to decrease the activation of ERK

(Wätjen et al. 2009). These different hypotheses should be investigated to explain the observed interactions between ENN and T2.

To conclude, the present study demonstrates the down-modulation of the gastrointestinal toxicity of T-2 toxin by the emerging enniatin B1 below toxic concentrations and confirms the relevance of the determinist approach for the analysis of toxin interactions, giving concordant results with the probabilistic approach in two different pig intestinal models. Further toxicity studies are needed with the emerging mycotoxins, notably investigations into the interactions following co-occurrence of enniatins A1 and B1 and major fusariotoxins, especially the trichothecene B deoxynivalenol. In addition, the potential bioaccumulation in animal and human tissues of the enniatins due to their lipophilicity is a matter of concern.

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ANEXO B

Resolução – RDC Nº 7, de feveireiro de 2011 – ANVISA

Art. 1º Deferir registro de medicamento, cancelamento do registro de medicamento, renovação de registro de medicamento, retificação de publicação e inclusão de novo acondicionamento, conforme relação anexa.

Art. 2º Esta Resolução entra em vigor na data de sua publicação.

DIRCEU BRÁS APARECIDO BARBANO

(*) Esta Resolução e o anexo a que se refere serão publicados em suplemento à presente edição

RESOLUÇÃO - RE Nº 990, DE 4 DE MARÇO DE 2011(*)

O Diretor-Presidente Substituto da Agência Nacional de Vigilância Sanitária, no uso das atribuições que lhe conferem o Decreto de nomeação de 10 de outubro de 2008 do Presidente da República, publicado no DOU de 13 de outubro de 2008, e a Portaria GM/MS n° 3.177, de 29 de dezembro de 2008, tendo em vista o disposto no inciso X, do art.13 do Regulamento da ANVISA, aprovado pelo Decreto n° 3.029, de 16 de abril de 1999, no inciso VIII do art. 16, e no inciso I, § 1º do art. 55 do Regimento Interno aprovado nos termos do Anexo I da Portaria n.º 354 da ANVISA, de 11 de agosto de 2006, republicada no DOU de 21 de agosto de 2006,

considerando o art. 12, 15 e o art. 33 e seguintes da Lei n.º 6.360, de 23 de setembro de 1976, bem como o inciso IX, do art. 7º da Lei n.º 9.782, de 26 de janeiro de 1999, resolve:

Art. 1º Indeferir as petições dos produtos Saneantes Domissanitários, conforme relação anexa.

Art. 2º Esta Resolução entra em vigor na data de sua publicação

DIRCEU BRÁS APARECIDO BARBANO

(*) Esta Resolução e o anexo a que se refere serão publicados em suplemento à presente edição.

RESOLUÇÃO - RE Nº 991, DE 4 DE MARÇO DE 2011(*)

O Diretor-Presidente Substituto da Agência Nacional de Vigilância Sanitária, no uso das atribuições que lhe conferem o Decreto de nomeação de 10 de outubro de 2008 do Presidente da República, publicado no DOU de 13 de outubro de 2008, e a Portaria GM/MS n° 3.177, de 29 de dezembro de 2008, tendo em vista o disposto no inciso X, do art.13 do Regulamento da ANVISA, aprovado pelo Decreto n° 3.029, de 16 de abril de 1999, no inciso VIII do art. 16, e no inciso I, § 1º do art. 55 do Regimento Interno aprovado nos termos do Anexo I da Portaria n.º 354 da ANVISA, de 11 de agosto de 2006, republicada no DOU de 21 de agosto de 2006,

considerando o art. 12 e o art. 33 e seguintes da Lei n.º 6.360, de 23 de setembro de 1976, bem como o inciso IX, do art. 7º da Lei n.º 9.782, de 26 de janeiro de 1999, resolve:

Art. 1º Deferir as petições dos produtos Saneantes Domissanitários, conforme relação anexa.

Art. 2º Esta Resolução entra em vigor na data de sua publicação.

DIRCEU BRÁS APARECIDO BARBANO

(*) Esta Resolução e o anexo a que se refere serão publicados em suplemento à presente edição.

RESOLUÇÃO - RE Nº 992, DE 4 DE MARÇO DE 2011(*)

O Diretor-Presidente Substituto da Agência Nacional de Vigilância Sanitária, no uso das atribuições que lhe conferem o Decreto de nomeação de 10 de outubro de 2008 do Presidente da República, publicado no DOU de 13 de outubro de 2008, e a Portaria GM/MS n° 3.177, de 29 de dezembro de 2008, tendo em vista o disposto no inciso X, do art.13 do Regulamento da ANVISA, aprovado pelo Decreto n° 3.029, de 16 de abril de 1999, no inciso VIII do art. 16, e no inciso I, § 1º do art. 55 do Regimento Interno aprovado nos termos do Anexo I da Portaria n.º 354 da ANVISA, de 11 de agosto de 2006, republicada no DOU de 21 de agosto de 2006,

considerando o art. 12 e o art. 33 e seguintes da Lei n.º 6.360, de 23 de setembro de 1976, bem como o inciso IX, do art. 7º da Lei n.º 9.782, de 26 de janeiro de 1999, resolve:

Art. 1º Deferir as petições dos produtos Saneantes Domissanitários, conforme relação anexa.

Art. 2º Esta Resolução entra em vigor na data de sua publicação.

DIRCEU BRÁS APARECIDO BARBANO

(*) Esta Resolução e o anexo a que se refere serão publicados em suplemento à presente edição.

RETIFICAÇÃO

No Diário Oficial da União nº 181, de 21 de setembro de 2010, Seção 1, pág. 54 e no Suplemento, pág. 78,

Onde se lê:

"RESOLUÇÃO - RE nº 4.345, de 17 de setembro de 2010"

Leia-se:

"RESOLUÇÃO - RE nº 4.340, de 17 de setembro de 2010"

DIRETORIA COLEGIADA

RESOLUÇÃO - RDC Nº 7, DE 18 DE FEVEREIRO DE 2011(*)

Dispõe sobre limites máximos tolerados (LMT) para micotoxinas em alimentos.

A Diretoria Colegiada da Agência Nacional de Vigilância Sanitária, no uso da atribuição que lhe confere o inciso IV do art. 11 do Regulamento aprovado pelo Decreto nº. 3.029, de 16 de abril de 1999, e tendo em vista o disposto no inciso II e nos §§ 1º e 3º do art. 54 do Regimento Interno aprovado nos termos do Anexo I da Portaria nº. 354 da Anvisa, de 11 de agosto de 2006, republicada no DOU de 21 de agosto de 2006, em reunião realizada em 15 de fevereiro de 2011,

adota a seguinte Resolução da Diretoria Colegiada e eu, Diretor-Presidente Substituto, determino a sua publicação:

Art. 1º Fica aprovado o Regulamento Técnico sobre limites máximos tolerados (LMT) para micotoxinas em alimentos, nos termos desta Resolução.

Art. 2º Este Regulamento possui o objetivo de estabelecer os limites máximos para aflatoxinas (AFB1+AFB2+AFG1+AFG2 e AFM1), ocratoxina A (OTA), desoxinivalenol (DON), fumonisinas (FB1 + FB2), patulina (PAT) e zearalenona (ZON) admissíveis em alimentos prontos para oferta ao consumidor e em matérias- primas, conforme os Anexos I, II, III e IV desta Resolução.

Parágrafo único. Os limites máximos tolerados referem-se aos resultados obtidos por metodologias que atendam aos critérios de desempenho estabelecidos pelo Codex Alimentarius.

Art. 3º Este Regulamento aplica-se às empresas que importem, produzam, distribuam e comercializem as seguintes categorias de bebidas, alimentos e matérias primas:

I - amendoim e seus derivados;

II - alimentos à base de cereais para alimentação infantil (lactentes e crianças de primeira infância);

III - café torrado (moído ou em grão) e solúvel;

IV - cereais e produtos de cereais;

V - especiarias;

VI - frutas secas e desidratadas;

VII - nozes e castanhas;

VIII - amêndoas de cacau e seus derivados;

IX - suco de maçã e polpa de maçã;

X - suco de uva e polpa de uva;

XI - vinho e seus derivados;

XII - fórmulas infantis para lactentes e fórmulas infantis de seguimento para lactentes e crianças de primeira infância;

XIII - leite e produtos lácteos, e

XIV - leguminosas e seus derivados.

Art. 4º Os níveis de micotoxinas deverão ser tão baixos quanto razoavelmente possível, devendo ser aplicadas as melhores práticas e tecnologias na produção, manipulação, armazenamento, processamento e embalagem, de forma a evitar que um alimento contaminado seja comercializado ou consumido.

Art. 5º No caso de produtos não previstos no art. 3º desta Resolução e que sejam produzidos a partir de ingredientes com limites estabelecidos na forma dos Anexos deste Regulamento, que forem desidratados ou secos, diluídos, transformados e compostos, os limites máximos tolerados devem considerar as proporções relativas dos ingredientes no produto, concentração e diluição em relação aos limites estabelecidos para os ingredientes.

§ 1º Na hipótese do "caput" deste artigo, o interessado será notificado para fornecer informações relativas à proporção dos ingredientes no produto, bem como aos fatores específicos de concentração e diluição, caso seja necessário.

§ 2º A não apresentação das informações mencionadas no § 1º no prazo de 10 (dez) dias, ou sua inadequação, ensejará conclusão com base nos dados disponíveis.

Art. 6º Os limites máximos tolerados se aplicam à parte comestível dos produtos alimentícios em questão, salvo especificação em contrário.

Art. 7º O descumprimento das disposições contidas nesta Resolução constitui infração sanitária, nos termos da Lei nº. 6.437, de 20 de agosto de 1977, sem prejuízo das responsabilidades civil, administrativa e penal cabíveis.

Art. 8º Ficam revogadas a Resolução CNNPA nº 34, de 1976, publicada no D.O.U. de 19/01/1977, e a Resolução RDC nº 274, de 15 de outubro de 2002.

Art. 9º São concedidos prazos para aplicação dos limites máximos tolerados estabelecidos nos anexos desta Resolução, tendo em vista a necessidade de adequação do setor produtivo, com exceção dos limites estabelecidos no Anexo I.

Art. 10. Os Limites Máximos Tolerados (LMT) estabelecidos para as Micotoxinas e as respectivas categorias de alimentos especificadas no Anexo II entrarão em vigor em 1º de janeiro de 2012.

Art. 11. Os Limites Máximos Tolerados (LMT) estabelecidos para as Micotoxinas e as respectivas categorias de alimentos especificadas no Anexo III entrarão em vigor em 1º de janeiro de 2014.

Art. 12. Os Limites Máximos Tolerados (LMT) estabelecidos para as Micotoxinas e as respectivas categorias de alimentos especificadas no Anexo IV entrarão em vigor em 1º de janeiro de 2016.

Art. 13. Esta Resolução e seu Anexo I entram em vigor na data de sua publicação.

DIRCEU BRÁS APARECIDO BARBANO

ANEXO I - Aplicação Imediata

LIMITES MÁXIMOS TOLERADOS (LMT) PARA MICOTOXINAS

Micotoxinas	Alimento	LMT (µg/kg)
Aflatoxina M1	Leite fluído	**0,5**
	Leite em pó	**5**
	Queijos	**2,5**
Aflatoxinas B1, B2, G1, G2	Cereais e produtos de cereais, exceto milho e derivados, incluindo cevada malteada	**5**
	Feijão	**5**
	Castanhas exceto Castanha-do-Brasil, incluindo nozes, pistachios, avelãs e amêndoas	**10**
	Frutas desidratadas e secas	**10**

	Castanha-do-Brasil com casca para consumo direto	**20**
	Castanha-do-Brasil sem casca para consumo direto	**10**
	Castanha-do-Brasil sem casca para processamento posterior	**15**
	Alimentos à base de cereais para alimentação infantil (lactentes e crianças de primeira infância)	**1**
	Fórmulas infantis para lactentes e fórmulas infantis de seguimento para lactentes e crianças de primeira infância	**1**
	Amêndoas de cacau	**10**
	Produtos de cacau e chocolate	**5**
	Especiarias: Capsicum spp. (o fruto seco, inteiro ou triturado, incluindo pimentas, pimenta em pó, pimenta de caiena e pimentão-doce); Piper spp. (o fruto, incluindo a pimenta branca e a pimenta preta) Myristica fragrans (noz-moscada) Zingiber officinale (gengibre) Curcuma longa (curcuma). Misturas de especiarias que contenham uma ou mais das especiarias acima indicadas	**20**
	Amendoim (com casca), (descascado, cru ou tostado), pasta de amendoim ou manteiga de amendoim	**20**
	Milho, milho em grão (inteiro, partido, amassado, moído), farinhas ou sêmolas de milho	**20**
Ocratoxina A	Cereais e produtos de cereais, incluindo cevada malteada	**10**
	Feijão	**10**
	Café torrado (moído ou em grão) e café solúvel	**10**
	Vinho e seus derivados	**2**
	Suco de uva e polpa de uva	**2**
	Especiarias: Capsicum spp. (o fruto seco, inteiro ou triturado, incluindo pimentas, pimenta em pó, pimenta de caiena e pimentão-doce) Piper spp. (o fruto, incluindo a pimenta branca e a pimenta preta) Myristica fragrans (noz-moscada) Zingiber officinale (gengibre) Curcuma longa (curcuma) Misturas de especiarias que contenham uma ou mais das especiarias acima indicadas	**30**
	Alimentos a base de cereais para alimentação infantil (lactentes e crianças de primeira infância)	**2**
	Produtos de cacau e chocolate	**5,0**
	Amêndoa de cacau	**10**
	Frutas secas e desidratadas	**10**
Desoxinivalenol (DON)	Arroz beneficiado e derivados	**750**
	Alimentos a base de cereais para alimentação infantil (lactentes e crianças de primeira infância)	**200**
Fumonisinas (B1 + B2)	Milho de pipoca	**2000**
	Alimentos a base de milho para alimentação infantil (lactentes e crianças de primeira infância)	**200**
Zearalenona	Alimentos a base de cereais para alimentação infantil (lactentes e crianças de primeira infância)	**20**
Patulina	Suco de maçã e polpa de maçã	**50**

ANEXO II - Aplicação em janeiro de 2012

LIMITES MÁXIMOS TOLERADOS (LMT) PARA MICOTOXINAS

Micotoxinas	Alimento	LMT (µg/kg)
Desoxinivalenol (DON)	Trigo integral, trigo para quibe, farinha de trigo integral, farelo de trigo, farelo de arroz, grão de cevada	**2000**
	Farinha de trigo, massas, crackers, biscoitos de água e sal, e produtos de panificação, cereais e produtos de cereais exceto trigo e incluindo cevada malteada	**1750**
Fumonisinas (B1 + B2)	Farinha de milho, creme de milho, fubá, flocos, canjica, canjiquinha	**2500**
	Amido de milho e outros produtos à base de milho	**2000**
Zearalenona	Farinha de trigo, massas, crackers e produtos de panificação, cereais e produtos de cereais exceto trigo e incluindo cevada malteada	**200**
	Arroz beneficiado e derivados	**200**
	Arroz integral	**800**
	Farelo de arroz	**1000**
	Milho de pipoca, canjiquinha, canjica, produtos e subprodutos à base de milho	**300**
	Trigo integral, farinha de trigo integral, farelo de trigo	**400**

ANEXO III- Aplicação em janeiro de 2014

LIMITES MÁXIMOS TOLERADOS (LMT) PARA MICOTOXINAS

Micotoxinas	Alimento	LMT (µg/kg)
Ocratoxina A	Cereais para posterior processamento, incluindo grão de cevada	**20**
Desoxinivalenol (DON)	Trigo e milho em grãos para posterior processamento	**3000**
	Trigo integral, trigo para quibe, farinha de trigo integral, farelo de trigo, farelo de arroz, grão de cevada	**1500**
	Farinha de trigo, massas, crackers, biscoitos de água e sal, e produtos de panificação, cereais e produtos de cereais exceto trigo e incluindo cevada malteada.	**1250**
Fumonisinas (B1 + B2)	Milho em grão para posterior processamento	**5000**
Zearalenona	Milho em grão e trigo para posterior processamento	**400**

ANEXO IV - Aplicação em janeiro de 2016

LIMITES MÁXIMOS TOLERADOS (LMT) PARA MICOTOXINAS

Micotoxinas	Alimento	LMT (µg/kg)
Desoxinivalenol (DON)	Trigo integral, trigo para quibe, farinha de trigo integral, farelo de trigo, farelo de arroz, grão de cevada	**1000**
	Farinha de trigo, massas, crackers, biscoitos de água e sal, e produtos de panificação, cereais e produtos de cereais exceto trigo e incluindo cevada malteada.	**750**
Fumonisinas (B1 + B2)	Farinha de milho, creme de milho, fubá, flocos, canjica, canjiquinha	**1500**
	Amido de milho e outros produtos a base de milho	**1000**
Zearalenona	Farinha de trigo, massas, crackers e produtos de panificação, cereais e produtos de cereais exceto trigo e incluindo cevada malteada.	**100**
	Arroz beneficiado e derivados	**100**
	Arroz integral	**400**
	Farelo de arroz	**600**
	Milho de pipoca, canjiquinha, canjica, produtos e sub-produtos à base de milho	**150**
	Trigo integral, farinha de trigo integral, farelo de trigo	**200**

(*) Republicada por ter saído, no DOU nº 37, de 22-2-2011, Seção 1, pág. 72, com incorreção no original.

RESOLUÇÃO-RE Nº 960, DE 2 DE MARÇO DE 2011(*)

O Diretor da Diretoria Colegiada da Agência Nacional de Vigilância Sanitária, no uso das atribuições que lhe conferem o Decreto de nomeação de 26 de agosto de 2010 do Presidente da República, publicado no DOU de 27 de agosto de 2010, o inciso VIII do art. 15, e o inciso I e o § 1º do art. 55 do Regimento Interno aprovado nos termos do Anexo I da Portaria n.º 354 da ANVISA, de 11 de agosto de 2006, republicada no DOU de 21 de agosto de 2006, e a Portaria nº 29 da ANVISA, de 11 de janeiro de 2011, e ainda amparado pela Resolução RDC n° 345, de 16 de dezembro de 2002, resolve:

Art. 1º Conceder Renovação da Autorização de Funcionamento de Empresas em conformidade com o disposto no anexo.

Art. 2º Esta Resolução entra em vigor na data de sua publicação.

JOSÉ AGENOR ÁLVARES DA SILVA

(*) Esta Resolução e o anexo a que se refere serão publicados em suplemento à presente edição

RESOLUÇÃO-RE Nº 961, DE 2 DE MARÇO DE 2011(*)

O Diretor da Diretoria Colegiada da Agência Nacional de Vigilância Sanitária, no uso das atribuições que lhe conferem o Decreto de nomeação de 26 de agosto de 2010 do Presidente da República, publicado no DOU de 27 de agosto de 2010, o inciso VIII do art. 15, e o inciso I e o § 1º do art. 55 do Regimento Interno aprovado nos termos do Anexo I da Portaria n.º 354 da ANVISA, de 11 de agosto de 2006, republicada no DOU de 21 de agosto de 2006, e a Portaria nº 29 da ANVISA, de 11 de janeiro de 2011, e ainda amparado pela Resolução RDC nº 345, de 16 de dezembro de 2002, resolve:

Art. 1º Conceder Renovação da Autorização de Funcionamento de Empresas em conformidade com o disposto no anexo.

Art. 2º Esta Resolução entra em vigor na data de sua publicação.

JOSÉ AGENOR ÁLVARES DA SILVA

(*) Esta Resolução e o anexo a que se refere serão publicados em suplemento à presente edição

RESOLUÇÃO-RE Nº 962, DE 2 DE MARÇO DE 2011(*)

O Diretor da Diretoria Colegiada da Agência Nacional de Vigilância Sanitária, no uso das atribuições que lhe conferem o Decreto de nomeação de 26 de agosto de 2010 do Presidente da República, publicado no DOU de 27 de agosto de 2010, o inciso VIII do art. 15, e o inciso I e o § 1º do art. 55 do Regimento Interno aprovado nos termos do Anexo I da Portaria nº. 354 da ANVISA, de 11 de agosto de 2006, republicada no DOU de 21 de agosto de 2006, e a Portaria nº. 29, de 11 de janeiro de 2011 e ainda amparado pela Resolução RDC n° 345, de 16 de dezembro de 2002, resolve:

Art. 1º Conceder Renovação de Autorização de Funcionamento de Empresas em conformidade com o disposto no anexo.

Art. 2º Esta Resolução entra em vigor na data de sua publicação.

JOSÉ AGENOR ÁLVARES DA SILVA

(*) Esta Resolução e o anexo a que se refere serão publicados em suplemento à presente edição

ANEXO C

Normas para publicação *British Journal of Nutrition*

Directions to Contributors

British Journal of Nutrition

(Revised November 2013)

The *British Journal of Nutrition* is an international peer-reviewed journal that publishes original papers and review articles in all branches of nutritional science. The underlying aim of all work should be to develop nutritional concepts.

[Please note that URLs cited in this document may not work with all internet browsers. It is recommended that URLs are copied and pasted into the browser address bar]

The publication remit of the journal. The *British Journal of Nutrition* encompasses the full spectrum of nutritional science and reports of studies in the following areas will be considered for publication: Epidemiology, dietary surveys, nutritional requirements and behaviour, metabolic studies, body composition, energetics, appetite, obesity, ageing, endocrinology, immunology, neuroscience, microbiology, genetics and molecular and cell biology.

The journal does not publish papers on the following topics: Case studies; papers on food technology, food science or food chemistry; studies of primarily local interest; papers on pharmaceutical agents or substances that are considered primarily as medicinal agents; studies in which a nutrient or extract is administered by a route other than orally (unless the specific aim of the study is to investigate parenteral nutrition) nor studies using supra-physiological amounts of nutrients (unless the specific aim of the study is to investigate toxic effects).

Guidelines on studies reporting *in vivo* or *in vitro* models. Studies involving animal models of human nutrition and health or disease will be considered for publication provided that the amount of a nutrient or combination of nutrients used could reasonably be expected to be achieved in humans.

Studies involving *in vitro* models will be considered for publication provided that the amount of a nutrient or combination of nutrients is within the range that could reasonably be expected to be encountered *in vivo* and that the molecular form of the nutrient or nutrients is the same as what the cell type used in the model would encounter *in vivo*.

Guidelines on studies reporting the effects of extracts. Studies involving extracts will be considered for publication provided that the source of starting material is readily accessible to other researchers and that there are appropriate measures for quality control, that the method of extraction is described in sufficient detail with appropriate quality control measures, that the nutrient composition of the extract is characterised in detail and that there are measures to control the quality of the composition of the extract between preparations, and that the amount of extract used could reasonably be expected to be achieved in humans (or in animals if they are the specific target of an intervention).

Studies involving extracts in *in vitro* models will be considered for publication provided that the above guidelines for studies involving extracts are followed and that the amount and molecular form of the extract is the same as that which would be encountered by the cell type used in the model *in vivo*.

Ethical standards for studies involving humans or other vertebrate animals

Experiments involving human subjects. The notice of contributors is drawn to the guidelines in the World Medical Association (2000) Declaration of Helsinki: ethical principles for medical research involving human subjects, with notes of clarification of 2002 and 2004 (http://www.wma.net/en/30publications/10policies/b3/), the *Guidelines on the Practice of Ethics Committees Involved in Medical Research Involving Human Subjects* (3rd ed., 1996; London: The Royal College of Physicians) and the Guidelines for the ethical conduct of medical research involving children, revised in 2000 by the Royal College of Paediatrics and Child Health: Ethics Advisory Committee (*Arch Dis Child* (2000) **82**, 177–182). A paper describing any experimental work on human subjects must include the following statement in the materials/methods section: "This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the [insert name of the ethics committee; a specific ethics number may be inserted if you wish]. Written [or Verbal] informed consent was obtained from all subjects/patients. [Where verbal consent was obtained this must be followed by a statement such as: Verbal consent was witnessed and formally recorded]."

Experiments involving the use of other vertebrate animals. Papers that report studies involving vertebrate animals must conform to the 'ARRIVE Guidelines for Reporting Animal Research' detailed in Kilkenny *et al***. (***J Pharmacol Pharmacother* (2010) **1**,94-99) and summarised at www.nc3rs.org.uk . Authors must ensure that their manuscript conforms to the checklist that is available from the nc3Rs website. The attention of authors is drawn particularly to the ARRIVE guidelines point 3b ('Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology', point 9c ('Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment') and point 17a ('Give details of all important adverse events in each experimental group'). The Editors will not accept papers reporting work carried out using inhumane procedures, or experiments that have not been reviewed and approved by an animal experimentation ethics committee. When reporting on experiments involving the use of vertebrate animals, authors must state the institutional and national guidelines for the care and use of animals that were followed and that all experimental procedures

involving animals were approved by the [insert name of the ethics committee or other approving body; wherever possible authors should also insert a specific ethics/approval number].

General guidelines

As a contributor you must follow the guidelines set out below. Prospective authors may contact the Publications Office directly on +44 (0)1223 347954 (telephone) or bjn.edoffice@cambridge.org (email).

Papers submitted for publication must be written in English and be as concise as possible. If English is not the first language of the authors, then the paper should be checked by an English speaker. It is the responsibility of the author to ensure that the use of English in a manuscript meets the required standard for publication. **The *British Journal of Nutrition* operates an on-line submission and reviewing system (eJournalPress). Authors should submit to the following address:** http://bjn.msubmit.net/ Receipt of papers will be acknowledged immediately.

Papers should be accompanied by a statement of acceptance of the conditions laid down in the Directions to Contributors. The statement must affirm that the submission represents original work that has not been published previously, that it is not currently being considered by another journal, and that if accepted for the *British Journal of Nutrition* it will not be published elsewhere in the same form, in English or in any other language, without the written consent of the Nutrition Society. It should also confirm that each author has seen and approved the contents of the submitted manuscript. **At the time of acceptance the authors should provide a completed copy of the 'Licence to Publish' (in lieu of copyright transfer), which is available on the Nutrition Society's web pages** (http://www.nutritionsociety.org/publications/nutrition-society-journals/british-journal-of-nutrition); the Society no longer requires copyright of the material published in the journal, only a 'Licence to Publish.' **The authors or their institutions retain the copyright.**

Conflicts of interest and contributions of funding organisations

The manuscript must include statements reporting any conflicts of interest, all sources of funding and the contribution of each author to the manuscript. These statements should be placed at the end of the text of the manuscript before the references are listed, as instructed below.

This journal adheres to the Committee on Publication Ethics (COPE) guidelines on research and publications ethics http://publicationethics.org/resources/guidelines

Types of papers published by the *British Journal of Nutrition*

The *British Journal of Nutrition* publishes the following: Full Papers, Review Articles, Systematic Reviews, Horizons in Nutritional Science, Workshop Reports, Invited Commentaries, Letters to the Editor/Nutrition Discussion Forums, Obituaries, and Editorials.

Full Papers, Reviews, Systematic Reviews, Horizons Articles and Workshop Reports should be submitted to: http://bjn.msubmit.net/ Please contact the Publications Office on edoffice@nutsoc.org.uk regarding any other types of article. Articles reporting randomised trials must conform to the standards set by the Consolidated Standards of Reporting Trials consortium http://www.consort-statement.org/ . Studies reporting the findings of microarray analyses must comply with the "Minimum Information about a Microarray Experiment" (MIAME) guidelines (http://www.mged.org/Workgroups/MIAME/miame.html) and the data must be deposited in a publically accessible repository.

Review Articles The BJN is willing to accept critical reviews that are designed to advance knowledge, policy and practice in nutritional science. Current knowledge should be appropriately contextualised and presented such that knowledge gaps and research needs can be characterised and prioritised, or so that changes in policy and practice can be proposed along with suggestions as to how any changes can be monitored. The purpose or objective of a review should be clearly expressed, perhaps as question in the Introduction, and the review's conclusions should be congruent with the initial objective or question. Reviews will be handled by specialist Reviews Editors. Please contact the Publications Office with any queries regarding the submission of potential review articles. All reviews, including systematic reviews and meta-analyses should present the uncertainties and variabilities associated with the papers and data being reviewed; in particular the BJN cautions against uncritical acceptance of definitions and non-specific global terminology, the advice of advisory bodies, and reference ranges for example.

Reviews. These articles are written in a narrative style which aim to critically evaluate a specific topic in nutritional science.

*Horizons in Nutritional Science***.** These are shorter than Review articles and aim to critically evaluate recent novel developments which are likely to produce substantial advances in nutritional science. These articles should be thought-provoking and possibly controversial.

Systematic Reviews and meta-analyses. The journal endorses the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Statement, a guideline to help authors report a systematic review and meta-analysis http:/prisma-statement.org (see *British Medical Journal* (2009) **339**, b2535). A systematic review or meta-analysis of randomised trials and other evaluation studies should follow the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (http:/prisma-statement.org).

Letters to the Editor/Nutrition Discussion Forum. Letters are invited that discuss, criticise or develop themes put forward in papers published in the *British Journal of Nutrition* or that deal with matters relevant to it. They should not, however, be used as a means of publishing new work. Acceptance will be at the discretion of the Editorial Board, and editorial changes may be required. Wherever possible, letters from responding authors will be included in the same issue.

Revision of manuscripts following review. When substantial revisions are required to manuscripts, authors are normally given the opportunity to do this once only; the need for any further changes should at most reflect only minor issues. If a paper requiring revision is not resubmitted within 2 months, it may, on resubmission, be deemed a new paper and the date of receipt altered accordingly.

Form of full papers submitted for publication. The onus of preparing a paper in a form suitable for sending to press lies with the author. Authors are advised to consult a current issue in order to make themselves familiar with the *British Journal of Nutrition* as to typographical and other conventions, layout of tables etc. Sufficient information should be given to permit repetition of the published work by any competent reader of the *British Journal of Nutrition*. The requirements of *British Journal of Nutrition* are in accordance with the Uniform Requirements for Manuscripts Submitted to Biomedical Journals produced by the International Committee of Medical Journal Editors (ICMJE), and authors are encouraged to consult the latest guidelines, which contain a lot of useful generic information about preparing scientific papers http://www.icmje.org/ .

Plagiarism: Text taken directly or closely paraphrased from earlier published work that has not been acknowledged or referenced will be considered plagiarism. Submitted manuscripts in which such text is identified will be withdrawn from the editorial process. If a concern is raised about possible plagiarism in an article published in the *British Journal of Nutrition*, this will be investigated fully and dealt with in accordance with the Committee on Publication Ethics guidelines (www. http://publicationethics.org/).

Authors are invited to nominate up to four potential referees who may then be asked by the Editorial Board to help review the work.

Typescripts should be prepared with 1·5 line spacing and wide margins (2 cm), the preferred font being Times New Roman size 12. At the ends of lines words should not be hyphenated unless hyphens are to be printed. Line numbering and page numbering is required.

Spelling should generally be that of the *Concise Oxford Dictionary* (1995), 9th ed. Oxford: Clarendon Press. Papers should normally be divided into the following parts:

(a) *Title page:* authors' names should be given without titles or degrees and one forename may be given in full. The name and address of the institution where the work was performed should be given, as well as the main address for each author. The name and address of the author to whom correspondence should be sent should be clearly stated, together with telephone and fax numbers and email address. Other authors should be linked to their address using superscript Arabic numerals. Any necessary descriptive material about the authors, e.g. Beit Memorial Fellow, should appear at the end of the paper in the Acknowledgments.

Authorship. Although discretion is allowed, authors must fulfill the criteria set out by the International Committee of Medical Journal Editors (http://www.icmje.org) and described by as follows by Calder (*Br J Nutr* (2009) **101**, 775). Authors should meet each of the following requirements by substantial contributions to '(a) the conception and design, acquisition of data, or analysis and interpretation of data; (b) the drafting the article or revising it critically for important intellectual content; (c) final approval of the version to be published'. The contribution of individuals who were involved in the study, but do not meet these criteria should be acknowledged in the Acknowledgments section.

If the paper is one of a series of papers that have a common main title followed by a subtitle specific to the individual paper, numbering should not be used to indicate the sequence of papers. The format should be 'common title: specific subtitle', with a short common title, e.g. Partitioning of limiting protein and energy in the growing pig: testing quantitative rules against experimental data.

The title page should also contain a shortened version of the paper's title, not exceeding forty-five letters and spaces in length, suitable for use as a running title in the published paper.

Authors are asked to supply three or four key words or phrases (each containing up to three words) on the title page of the typescript.

(b) *Abstract*: each paper must open with an abstract of **not more than 250 words**. The abstract should be a single paragraph of continuous text outlining the aims of the work, the experimental approach taken, the principal results and the conclusions and their relevance to nutritional science.

(c) *Introduction*: it is not necessary to introduce a paper with a full account of the relevant literature, but the introduction should indicate briefly the nature of the question asked and the reasons for asking it. It should be **no longer than two pages**.

(d) *Experimental methods*: methods should appear after the introduction. The methods section must include a subsection that describes the methods used for statistical analysis (see the section on statistical analysis below) and the sample size must be justified by the results of appropriate calculations and related to the study outcomes.

All analytical procedures must be accompanied by a statement of within and between assay precision.

(e) *Results*: these should be given as concisely as possible, using figures or tables as appropriate. Data must not be duplicated in tables and figures.

(f) *Discussion*: while it is generally desirable that the presentation of the results and the discussion of their significance should be presented separately, there may be occasions when combining these sections may be beneficial. Authors may also find that additional or alternative sections such as 'conclusions' may be useful. The discussion should be **no longer than five pages**.

(g) *Acknowledgments*: here you may acknowledge individuals or organizations that provide advice and/or support (non-financial). Formal financial support and funding should be listed in the following section.

(h) *Financial Support:* please provide details of the sources of financial support for all authors, including grant numbers. This is particularly important in the case of research that is supported by industry. Support from industry not only includes direct financial support for the study but also support in kind such as provision of medications, equipment, kits or reagents without charge or at reduced cost and provision of services such as statistical analysis; all such support must be disclosed here and if no

such support was received this must be stated. For example, “This work was supported by the Medical research Council (grant number XXXXXXX)”. Multiple grant numbers should be separated by a comma and space, and where research was funded by more than one agency the different agencies should be separated by a semi-colon, with “and” before the final funder. Grants held by different authors should be identified as belonging to individual authors by the authors’ initials. For example, “This work was supported by the Wellcome Trust (A.B., grant numbers XXXX, YYYY), (C.D., grant number ZZZZ); the Natural Environment Research Council (E.F., grant number FFFF); and the National Institutes of Health (A.B., grant number GGGG), (E.F., grant number HHHH)”. Where no specific funding has been provided for research, please provide the following statement: “This research received no specific grant from any funding agency, commercial or not-for-profit sectors.” In addition to the source of financial support, please state whether the funder contributed to the study design, conduct of the study, analysis of samples or data, interpretation of findings or the preparation of the manuscript. If the funder made no such contribution, please provide the following statement: "[Funder's name] had no role in the design, analysis or writing of this article.”

(i) *Conflict of Interest:* Conflict of interest exists when an author has interests that might inappropriately influence his or her judgement, even if that judgement is not influenced; for further detail, see http://www.icmje.org/ethical_4conflicts.html. Because of this, authors must disclose potentially conflicting interests so that others can make judgements about such effects. At the time of submission authors should disclose any financial arrangements or connections they may have that are pertinent to the submitted manuscript and that may be perceived as potentially biasing their paper. Non-financial interests that could be relevant in this context should also be disclosed. If there are no interests to declare, the title should be inserted followed by “None”.

(j) *Authorship:* Please provide a very brief description of the contribution of each author to the research. Their roles in formulating the research question(s), designing the study, carrying it out, analysing the data and writing the article should be made plain.

(k) *References*: these should be given in the text using the Vancouver system. They should be numbered consecutively in the order in which they first appear in the text using superscript Arabic numerals in parentheses, e.g. ‘The conceptual difficulty of this approach has recently been highlighted[1,2–4]’. If a reference is cited more than once the same number should be used each time. References cited only in tables and figure legends and not in the text should be numbered in sequence from the last number used in the text and in the order of mention of the individual tables and figures in the text. At the end of the paper, on a page(s) separate from the text, references should be listed in numerical order. When an article has more than three authors only the names of the first three authors should be given followed by ‘*et al*.’ The issue number should be omitted if there is continuous pagination throughout a volume. Names and initials of authors of unpublished work should be given in the text as ‘unpublished results’ and not included in the References. Titles of journals should appear in their abbreviated form using the NCBI LinkOut page http://www.ncbi.nlm.nih.gov/projects/linkout/journals/jourlists.fcgi?typeid=1&type=journals&operation=Show References to books and monographs should include the town of publication and the number of the edition to which reference is made. Thus:

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19. Keiding L (1997) *Astma, Allergi og Anden Overfølsomhed i Danmark – Og Udviklingen 1987–199I* (*Asthma, Allergy and Other Hypersensitivities in Denmark, 1987–1991*). Copenhagen, Denmark: Dansk Institut for Klinisk Epidemiologi.

References to material available on websites should include the full Internet address, and the date of the version cited. Thus:

20. Department of Health (1997) Committee on Toxicity of Chemicals in Food Consumer Products and the Environment. Statement on vitamin B_6 (pyridoxine) toxicity. http://www.open.gov.uk/doh/hef/B6.htm
21. Kramer MS & Kakuma R (2002) *The Optimal Duration of Exclusive Breastfeeding: A Systematic Review*. Rome: WHO; available at http://www.who.int/nut/documents/optimal_duration_of_exc_bfeeding_review_eng.pd
22. Hooper L, Thompson RL, Harrison RA *et al*. (2004) Omega 3 fatty acids for prevention and treatment of cardiovascular disease. *Cochrane Database of Systematic Reviews*, issue 4, CD003177. http://www.mrw.interscience.wiley.com/cochrane/clsysrev/articles/CD003177/frame.html
23. Nationmaster (2005) HIV AIDS – Adult prevalence rate. http://www.nationmaster.com/graph-T/hea_hiv_aid_adu_pre_rat (accessed June 2005).

(l) *Supplementary data:* Additional data (e.g. data files, large tables) relevant to the paper can be submitted for publication online only, where they are made available via a link from the abstract and the paper. The paper should stand alone without these data. Supplementary data should be supplied as a PDF for the review process and must be cited in a relevant place in the text of the paper.

Mathematical modelling of nutritional processes. Papers in which mathematical modelling of nutritional processes forms the principal element will be considered for publication provided: (a) they are based on sound biological and mathematical principles; (b) they advance nutritional concepts or identify new avenues likely to lead to such advances; (c) assumptions used in their construction are fully described and supported by appropriate argument; (d) they are described in such a way that the nutritional purpose is clearly apparent; (e) the contribution of the model to the design of future experimentation is clearly defined.

Units. Results should be presented in metric units according to the International System of Units (see Quantities, Units and Symbols in Physical Chemistry, 3rd ed. (2007) Cambridge: RSC Publishing), and Metric Units, Conversion Factors and Nomenclature in Nutritional and Food Sciences (1972) London: The Royal Society – as reproduced in *Proceedings of the Nutrition Society* (1972) **31**, 239–247). SI units should be used throughout the paper. The author will be asked to convert any values that are given in any other form. The only exception is where there is a unique way of expressing a particular variable that is in widespread use. Energy values must be given in Joules (MJ or kJ) using the conversion factor 1 kcal = 4·184 kJ. If required by the author, the value in kcal can be given afterwards in parentheses. Temperature is given in degrees Celsius (ºC). Vitamins should be given as mg or µg, not as IU.

For substances of known molecular mass (Da) or relative molecular mass, e.g. glucose, urea, Ca, Na, Fe, K, P, values should be expressed as mol/l; for substances of indeterminate molecular mass (Da) or relative molecular mass, e.g. phospholipids, proteins, and for trace elements, e.g. Cu, Zn, then g/l should be used.

Time. The 24 h clock should be used, e.g. 15.00 hours.

Units are: year, month, week, d, h, min, s, kg, g, mg, µg, litre, ml, µl, fl. To avoid misunderstandings, the word litre should be used in full, except in terms like g/l. Radioactivity should be given in becquerels (Bq or GBq) not in Ci. 1 MBq = 27·03 µCi (1Bq = 1 disintegration/s).

Statistical treatment of results. Data from individual replicates should not be given for large experiments, but may be given for small studies. The methods of statistical analysis used should be described, and references to statistical analysis packages included in the text, for example: Statistical Analysis Systems statistical software package version 6.11 (SAS Institute, Cary, NC, USA). The description should provide enough information for a statistician with access to the data to reproduce the results presented. Information such as analysis of variance tables should be given in the paper only if they are relevant to the discussion. A statement of the number of replicates, their average value and some appropriate measure of variability is usually sufficient. Authors must state whether their data follow a Gaussian distribution or not, and the choice of statistical tests must be consistent with the distribution of the data.

Justification for the sample size must be given. If the study is based on a power calculation, details of this should be provided including the desired effect size and power as well as the estimate of variability that was used.

Comparisons between means can be made by using either confidence intervals (CI) or significance tests. The most appropriate of such measures is usually the standard error of a difference between means (SED) or the standard errors of the means (SEM). The SEM represents the uncertainty associated with the estimation of a given mean and is not directly related to the SED or comparisons among means in mixed models as it is in fixed effects models. The SED estimates the uncertainty associated with the difference between two means; because it is used in various mean comparisons tests, SED can be implied within the tests *per se*. The standard deviation (SD) is more useful only when there is specific interest in the variability of individual values and no treatment means are being compared. The sample size (n per treatment) should also be stated in text or in the table. Standard analysis of variance assumes homogeneous variance. Unless there is heterogeneous variance, as tested by an appropriate statistic, or there is unequal n, a pooled SEM or SED simplifies tables and is preferred. The number of decimal places quoted should be sufficient but not excessive. If data transformations are being used, text should clearly state which variables have been transformed in which way and how that was decision was reached (e.g., tests for normality, diagnostic plots).

Authors should consider whether their study is rather of explorative (hypothesis-generating) or confirmative (hypothesis-testing) nature. This is particularly important when results from multiple tests are being presented, which can be the case when various

treatments are being compared, multiple endpoints are considered, or different subgroups are being analysed. Such multiple testing issues occur often in exploratory studies, and authors should take care not to overstate findings in these situations. At least the number of significant results should be compared to the number of tests compared, where 1 in 20 findings would be expected by chance alone. Methods that control certain error rates (experiment-wise error rate, false discovery rate, etc…) such as post-hoc tests can be used in this context, but are not obligatory, as long as the exploratory nature of the results is made clear. In confirmative studies, pre-planned comparisons or primary endpoints should be stated upfront and analysed by appropriate tools such as contrast testing for pre-planned comparisons. Unnecessary multiple testing corrections with respect to secondary comparisons or endpoints should be avoided to not compromise the power of the study.

Measurements on the same experimental unit over time or in different sections of tissue generally are not independent. If the repeated measures are taken from the same animal or human subject, which are expected to be randomly chosen to represent a population, an appropriate mixed model should be fitted while investigating the best covariance of error structures. All major statistical software packages offer a wide variety of structures; the one chosen should be stated.

If comparisons between means are made using CI, the format for presentation is, e.g. 'difference between means 0·73 (95 % CI 0·314, 1·36) g'. If significance tests are used, a statement that the difference between the means for two groups of values is (or is not) statistically significant should include the level of significance attained, preferably as an explicit *P* value (e.g. *P*=0·016 or *P*=0·32) rather than as a range (e.g. *P*<0·05 or *P*>0·05). It should be stated whether the significance levels quoted are one-sided or two-sided (when relevant). Where a multiple comparison procedure is used, a description or explicit reference should be given. Where appropriate, a superscript notation may be used in tables to denote levels of significance; similar superscripts should denote lack of a significant difference.

When the method of analysis is unusual, or if the experimental design is at all complex, further details (e.g., experimental plan, raw data, confirmation of assumptions, analysis of variance tables, etc.) should be included. Adequate detail should be provided for a subsequent reader to interpret and potentially repeat the approach used. For example, the statistical model should be provided or described in adequate detail, and all blocking factors and criteria should be provided. Regressions should provide appropriate estimates of parameter uncertainty (not necessarily provided by graphing software).

Figures. Figures should not be incorporated into the article file and should be supplied as separate electronic files. Figure legends should be grouped in a section at the end of the text. Each figure should be clearly marked with its number and separate panels within figures should be clearly marked (a), (b), (c) etc. so that they are easily identifiable when the article and figure files are merged for review.

In curves presenting experimental results the determined points should be clearly shown, the symbols used being, in order of preference, ○, ●, Δ, ▲, □, ■, ×, +. Curves and symbols should not extend beyond the experimental points. Scale-marks on the axes should be on the inner side of each axis and should extend beyond the last experimental point. Ensure that lines and symbols used in graphs and shading used in histograms are large enough to be easily identified when the figure is reduced to fit the printed page.

Figures and diagrams can be prepared using most applications but please do not use the following: cdx, chm, jnb or PDF. All figures should be numbered and legends should be provided. Each figure, with its legend, should be comprehensible without reference to the text and should include definitions of abbreviations. Latin names for unusual species should be included unless they have already been specified in the text. Each figure will be positioned near the point in the text at which it is first introduced unless instructed otherwise.

Note that authors will be charged 350 GBP for the publication of colour figures. Authors from countries entitled to free journal access through Research4Life will be exempt from these charges: http://www.research4life.org/

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Image integrity. Images submitted with a manuscript should be minimally processed (e.g. the addition of labelling). Authors should retain their original data, as Editors may request them for comparison during manuscript review. If such data are unavailable the manuscript may be withdrawn from the review process. Submitted manuscripts in which there appear to be manipulation of images will be withdrawn from the editorial process.

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Footnotes are given in the following order: (1) abbreviations, (2) superscript letters, (3) symbols. Abbreviations are given in the format: RS, resistant starch. Abbreviations appear in the footnote in the order that they appear in the table (reading from left to right across the table, then down each column). Abbreviations in tables must be defined in footnotes. Symbols for footnotes should be used in the sequence: *†‡§‖¶, then ** etc. (omit * or †, or both, from the sequence if they are used to indicate levels of significance).

For indicating statistical significance, superscript letters or symbols may be used. Superscript letters are useful where comparisons are within a row or column and the level of significance is uniform, e.g. '^{a,b,c}Mean values within a column with unlike superscript letters were significantly different (P<0·05)'. Symbols are useful for indicating significant differences between rows or columns, especially where different levels of significance are found, e.g. 'Mean values were significantly different from those of the control group: *P<0·05, **P<0·01, ***P<0·001'. The symbols used for P values in the tables must be consistent.

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Please refer to a recent copy of the journal for examples of tables.

Chemical formulas. These should be written as far as possible on a single horizontal line. With inorganic substances, formulas may be used from first mention. With salts, it must be stated whether or not the anhydrous material is used, e.g. anhydrous $CuSO_4$, or which of the different crystalline forms is meant, e.g. $CuSO_4.5H_2O$, $CuSO_4.H_2O$.

Descriptions of solutions, compositions and concentrations. Solutions of common acids, bases and salts should be defined in terms of molarity (M), e.g. 0·1 M-NaH_2PO_4. Compositions expressed as mass per unit mass (w/w) should have values expressed as ng, µg, mg or g per kg; similarly for concentrations expressed as mass per unit volume (w/v), the denominator being the litre. If concentrations or compositions are expressed as a percentage, the basis for the composition should be specified (e.g. % (w/w) or % (w/v) etc.). The common measurements used in nutritional studies, e.g. digestibility, biological value and net protein utilization, should be expressed as decimals rather than as percentages, so that amounts of available nutrients can be obtained from analytical results by direct multiplication. See *Metric Units, Conversion Factors and Nomenclature in Nutritional and Food Sciences*. London: The Royal Society, 1972 (para. 8).

Cell lines. The Journal expects authors to deposit cell lines (including microbial strains) used in any study to be published in publicly accessible culture collections, for example, the European Collection of Cell Cultures (ECACC) or the American Type Culture Collection (ATCC) and to refer to the collection and line or strain numbers in the text (e.g. ATCC 53103). Since the authenticity of subcultures of culture collection specimens that are distributed by individuals cannot be ensured, authors should indicate laboratory line or strain designations and donor sources as well as original culture collection identification numbers.

Gene nomenclature and symbols. The use of symbols and nomenclature recommended by the HUGO Gene Nomenclature Committee (http://www.genenames.org/) is encouraged. Information on human genes is also available from Entrez Gene (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene), on mouse genes from the Mouse Genome Database (http://www.informatics.jax.org/) and on rat genes from the Rat Genome Database (http://rgd.mcw.edu/).

Nomenclature of vitamins. Most of the names for vitamins and related compounds that are accepted by the Editors are those recommended by the IUNS Committee on Nomenclature. See *Nutrition Abstracts and Reviews* (1978) **48**A, 831–835.

Acceptable name	*Other names**
Vitamin A	
Retinol	Vitamin A_1
Retinaldehyde, retinal	Retinene
Retinoic acid (all-*trans* or 13-*cis*)	Vitamin A_1 acid
3-Dehydroretinol	Vitamin A_2
Vitamin D	
Ergocalciferol, ercalciol	Vitamin D_2 calciferol
Cholecalciferol, calciol	Vitamin D_3
Vitamin E	
α-, β- and γ-tocopherols plus tocotrienols	
Vitamin K	
Phylloquinone	Vitamin K_1
Menaquinone-n (MK-n)†	Vitamin K_2
Menadione	Vitamin K_3, menaquinone, menaphthone
Vitamin B_1	
Thiamin	Aneurin(e), thiamine
Vitamin B_2	
Riboflavin	Vitamin G, riboflavine, lactoflavin

Niacin	
Nicotinamide	Vitamin PP
Nicotinic acid	
Folic Acid	
Pteroyl(mono)glutamic acid	Folacin, vitamin B_c or M
Vitamin B_6	
Pyridoxine	Pyridoxol
Pyridoxal	
Pyridoxamine	
Vitamin B_{12}	
Cyanocobalamin	
Hydroxocobalamin	Vitamin B_{12a} or B_{12b}
Aquocobalamin	
Methylcobalamin	
Adenosylcobalamin	
Inositol	
Myo-inositol	*Meso*-inositol
Choline	
Pantothenic acid	
Biotin	Vitamin H
Vitamin C	
Ascorbic acid	
Dehydroascorbic acid	

*Including some names that are still in use elsewhere, but are not used by the *British Journal of Nutrition*.

†Details of the nomenclature for these and other naturally-occurring quinones should follow the Tentative Rules of the IUPAC-IUB Commission on Biochemical Nomenclature (see *European Journal of Biochemistry* (1975) **53**, 15–18)

Generic descriptors. The terms **vitamin A**, **vitamin C** and **vitamin D** may still be used where appropriate, for example in phrases such as 'vitamin A deficiency', 'vitamin D activity'.

Vitamin E. The term **vitamin E** should be used as the descriptor for all tocol and tocotrienol derivatives exhibiting qualitatively the biological activity of α-tocopherol. The term **tocopherols** should be used as the generic descriptor for all methyl tocols. Thus, the term **tocopherol** is not synonymous with the term **vitamin E**.

Vitamin K. The term **vitamin K** should be used as the generic descriptor for 2-methyl-1,4-naphthoquinone (menaphthone) and all derivatives exhibiting qualitatively the biological activity of phylloquinone (phytylmenaquinone).

Niacin. The term **niacin** should be used as the generic descriptor for pyridine 3-carboxylic acid and derivatives exhibiting qualitatively the biological activity of nicotinamide.

Vitamin B_6. The term **vitamin B_6** should be used as the generic descriptor for all 2-methylpyridine derivatives exhibiting qualitatively the biological activity of pyridoxine.

Folate. Due to the wide range of C-substituted, unsubstituted, oxidized, reduced and mono- or polyglutamyl side-chain derivatives of pteroylmonoglutamic acid that exist in nature, it is not possible to provide a complete list. Authors are encouraged to use either the generic name or the correct scientific name(s) of the derivative(s), as appropriate for each circumstance.

Vitamin B_{12}. The term **vitamin B_{12}** should be used as the generic descriptor for all corrinoids exhibiting qualitatively the biological activity of cyanocobalamin. The term **corrinoids** should be used as the generic descriptor for all compounds containing the corrin nucleus and thus chemically related to cyanocobalamin. The term **corrinoid** is not synonymous with the term **vitamin B_{12}**.

Vitamin C. The terms **ascorbic acid** and **dehydroascorbic acid** will normally be taken as referring to the naturally-occurring L-forms. If the subject matter includes other optical isomers, authors are encouraged to include the L- or D- prefixes, as appropriate. The same is true for all those vitamins which can exist in both natural and alternative isomeric forms.

Amounts of vitamins and summation. Weight units are acceptable for the amounts of vitamins in foods and diets. For concentrations in biological tissues, SI units should be used; however, the authors may, if they wish, also include other units, such as weights or international units, in parentheses.

See *Metric Units, Conversion Factors and Nomenclature in Nutritional and Food Sciences* (1972) paras 8 and 14–20. London: The Royal Society.

Nomenclature of fatty acids and lipids. In the description of results obtained for the analysis of fatty acids by conventional GLC, the shorthand designation proposed by Farquhar JW, Insull W, Rosen P, Stoffel W & Ahrens EH (*Nutrition Reviews* (1959), **17**, Suppl.) for individual fatty acids should be used in the text, tables and figures. Thus, 18 : 1 should be used to represent a fatty acid with eighteen carbon atoms and one double bond; if the position and configuration of the double bond is unknown. The shorthand designation should also be used in the abstract. If the positions and configurations of the double bonds are known, and these are important to the discussion, then a fatty acid such as linoleic acid may be referred to as *cis*-9,*cis*-12-18 : 2 (positions of double bonds related to the carboxyl carbon atom 1). However, to illustrate the metabolic relationship between different unsaturated fatty acid families, it is sometimes more helpful to number the double bonds in relation to the terminal methyl carbon atom, *n*. The preferred nomenclature is then: 18 : 3*n*-3 and 18 : 3*n*-6 for α-linolenic and γ-linolenic acids respectively; 18 : 2*n*-6 and 20 : 4*n*-6 for linoleic and arachidonic acids respectively and 18 : 1*n*-9 for oleic acid. Positional isomers such as α- and γ-linolenic acid should always be clearly distinguished. It is assumed that the double bonds are methylene-interrupted and are of the *cis*-configuration (see

Holman RT in *Progress in the Chemistry of Fats and Other Lipids* (1966) vol. 9, part 1, p. 3. Oxford: Pergamon Press). Groups of fatty acids that have a common chain length but vary in their double bond content or double bond position should be referred to, for example, as C_{20} fatty acids or C_{20} PUFA. The modern nomenclature for glycerol esters should be used, i.e. triacylglycerol, diacylglycerol, monoacylglycerol *not* triglyceride, diglyceride, monoglyceride. The form of fatty acids used in diets should be clearly stated, i.e. whether ethyl esters, natural or refined fats or oils. The composition of the fatty acids in the dietary fat and tissue fats should be stated clearly, expressed as mol/100 mol or g/100 g total fatty acids.

Nomenclature of micro-organisms. The correct name of the organism, conforming with international rules of nomenclature, should be used: if desired, synonyms may be added in parentheses when the name is first mentioned. Names of bacteria should conform to the current Bacteriological Code and the opinions issued by the International Committee on Systematic Bacteriology. Names of algae and fungi must conform to the current International Code of Botanical Nomenclature. Names of protozoa should conform to the current International Code of Zoological Nomenclature.

Nomenclature of plants. For plant species where a common name is used that may not be universally intelligible, the Latin name in italics should follow the first mention of the common name. The cultivar should be given where appropriate.

Other nomenclature, symbols and abbreviations. Authors should consult recent issues of the *British Journal of Nutrition* for guidance. The IUPAC rules on chemical nomenclature should be followed, and the recommendations of the Nomenclature Committee of IUBMB and the IUPAC-IUBMB Joint Commission on Biochemical Nomenclature and Nomenclature Commission of IUBMB in *Biochemical Nomenclature and Related Documents* (1992), 2nd ed., London: Portland Press (http://www.chem.qmul.ac.uk/iupac/bibliog/white.html). The symbols and abbreviations, other than units, are essentially those listed in *British Standard* 5775 (1979–1982), *Specifications for Quantities, Units and Symbols*, parts 0–13. Day should be abbreviated to d, for example 7 d, except for 'each day', '7th day' and 'day 1'.

Elements and simple chemicals (e.g. Fe and CO_2) can be referred to by their chemical symbol (with the exception of arsenic and iodine, which should be written in full) or formula from the first mention in the text; the title, text and table headings, and figure legends can be taken as exceptions,. Well-known abbreviations for chemical substances may be used without explanation, thus: RNA for ribonucleic acid and DNA for deoxyribonucleic acid. Other substances that are mentioned frequently (five or more times) may also be abbreviated, the abbreviation being placed in parentheses at the first mention, thus: lipoprotein lipase (LPL), after that, LPL, and an alphabetical list of abbreviations used should be included. Only accepted abbreviations may be used in the title and text headings. If an author's initials are mentioned in the text, they should be distinguished from other abbreviations by the use of stops, e.g. 'one of us (P. J. H.)…'. For UK counties the official names given in the *Concise Oxford Dictionary* (1995) should be used and for states of the USA two-letter abbreviations should be used, e.g. MA (not Mass.) and IL (not Ill.). Terms such as 'bioavailability' or 'available' may be used providing that the use of the term is adequately defined.

Spectrophotometric terms and symbols are those proposed in *IUPAC Manual of Symbols and Terminology for Physicochemical Quantities and Units* (1979) London: Butterworths. The attention of authors is particularly drawn to the following symbols: m (milli, 10^{-3}), μ (micro, 10^{-6}), n (nano, 10^{-9}) and p (pico, 10^{-12}). Note also that ml (millilitre) should be used instead of cc, μm (micrometre) instead of μ (micron) and μg (microgram) instead of γ.

Numbers. Numerals should be used with units, for example, 10 g, 7 d, 4 years (except when beginning a sentence, thus: 'Four years ago...'); otherwise, words (except when 100 or more), thus: one man, ten ewes, ninety-nine flasks, three times (but with decimal, 2·5 times), 100 patients, 120 cows, 136 samples.

Abbreviations. The following abbreviations are accepted without definition by the *British Journal of Nutrition:*

ADP (GDP)	adenosine (guanosine) 5'-disphosphate
AIDS	acquired immune deficiency syndrome
AMP (GMP)	adenosine (guanosine) 5'-monophosphate
ANCOVA	analysis of covariance
ANOVA	analysis of variance
apo	apolipoprotein
ATP (GTP)	adenosine (guanosine) 5'-triphosphate
AUC	area under the curve
BMI	body mass index
BMR	basal metabolic rate
bp	base pair
BSE	bovine spongiform encephalopathy
CHD	coronary heart disease
CI	confidence interval
CJD	Creutzfeldt-Jacob disease
CoA and acyl-CoA	co-enzyme A and its acyl derivatives
CV	coefficient of variation
CVD	cardiovascular disease
Df	degrees of freedom
DHA	docosahexaenoic acid
DM	dry matter
DNA	deoxyribonucleic acid

dpm	disintegrations per minute
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid
Expt	experiment (for specified experiment, e.g. Expt 1)
FAD	flavin-adenine dinucleotide
FAO	Food and Agriculture Organization (except when used as an author)
FFQ	food-frequency questionnaire
FMN	flavin mononucleotide
GC	gas chromatography
GLC	gas–liquid chromatography
GLUT	glucose transporter
GM	genetically modified
Hb	haemoglobin
HDL	high-density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
Ig	immunoglobulin
IHD	ischaemic heart disease
IL	interleukin
IR	infra red
kb	kilobases
K_m	Michaelis constant
LDL	low-density lipoprotein
MHC	major histocompatibility complex
MRI	magnetic resonance imaging
MS	mass spectrometry
MUFA	monounsaturated fatty acids
NAD+, NADH	oxidized and reduced nicotinamide-adenine dinucleotide
NADP+, NADPH	oxidized and reduced nicotinamide-adenine dinucleotide phosphate
NEFA	non-esterified fatty acids
NF-κB	nuclear factor kappa B
NMR	nuclear magnetic resonance
NS	not significant
NSP	non-starch polysaccharide
OR	odds ratio
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PG	prostaglandin
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acids
RDA	recommended dietary allowance
RER	respiratory exchange ratio
RIA	radioimmunoassay
RMR	resting metabolic rate
RNA, mRNA etc.	ribonucleic acid, messenger RNA etc.
rpm	revolutions per minute
RT	reverse transcriptase
SCFA	short-chain fatty acids
SDS	sodium dodecyl sulphate
SED	standard error of the difference between means
SFA	saturated fatty acids
SNP	single nucleotide polymorphism
TAG	triacylglycerol
TCA	trichloroacetic acid
TLC	thin-layer chromatography
TNF	tumour necrosis factor
UN	United Nations (except when used as an author)
UNICEF	United Nations International Children's Emergency Fund
UV	ultra violet
VLDL	very-low-density lipoprotein
V_{O2}	O_2 consumption
V_{O2max}	maximum O_2 consumption

WHO World Health Organization (except when used as an author)

Use of three-letter versions of amino acids in tables: Leu, His, etc.
CTP, UTP, GTP, ITP, as we already use ATP, AMP etc.

Disallowed words and phrases. The following are disallowed by the *British Journal of Nutrition*:

deuterium or tritium (use ^{2}H and ^{3}H)
c.a. or around (use approximately or about)
canola (use rapeseed)
ether (use diethyl ether)
free fatty acids (use NEFA)
isocalorific/calorie (use isoenergetic/energy)
quantitate (use quantify)
unpublished data or observations (use unpublished results)

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ANEXO D

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AUTHOR INFORMATION PACK

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