JOELMA LUCIOLI

EFEITOS SISTÊMICOS DA CONTAMINAÇÃO POR DESOXINIVALENOL, FUMONISINA B E SUA ASSOCIAÇÃO EM SUÍNOS

Londrina
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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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BANCA EXAMINADORA

_____
Prof. Dra. Ana Paula F. R. L. Bracarense
UEL – Londrina – PR

_____
Profa. Dra. Elizabeth Santin
UFPR – Curitiba – PR

_____
Prof. Dr. Geraldo Camilo Alberton
UFPR – Curitiba – PR

_____
Profa. Dra. Joice Sifuentes dos Santos
UEL – Londrina – PR

_____
Prof. Dr. Mario Augusto Ono
UEL – Londrina – PR

Londrina, 04 de novembro de 2011.

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“A imaginação é mais importante que conhecimento”.

Albert Einstein

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RESUMO

Com o objetivo de avaliar os efeitos sistêmicos da contaminação por micotoxinas, enfatizando aspectos imunológicos e morfológicos, dois experimentos foram realizados. No primeiro experimento, 24 leitões de 5 semanas de idade foram divididos em 4 grupos, cada um recebendo dieta controle, dieta contaminada com 2,8 mg/Kg DON ou 5,9 mg/Kg FB ou 6,5 mg/Kg DON+FB. Os animais foram imunizados com ovalbumina no 4º e 16º dia e amostras de sangue foram coletadas. Após 35 dias, os animais foram eutanasiados. Amostras de tecidos foram coletadas e fixadas em formol 10% e em nitrogênio líquido a -80ºC. Com as amostras de sangue, realizaram-se exames hematológicos, bioquímicos e imunológicos. As amostras de tecidos fixadas em formol 10% foram submetidas a processamento histológico e imunoistoquímico, enquanto que as amostras congeladas foram submetidas a Western Blot e PCR para avaliação da expressão de proteínas de junção e citocinas. No segundo experimento foram utilizados dois modelos experimentais. No modelo *in vivo*, 24 leitões de 4 semanas de idade foram distribuídos em 2 grupos. Durante 28 dias, um grupo recebeu dieta controle e o outro dieta contaminada com 2,3 mg/Kg DON. Ao término do experimento, seis animais de cada grupo foram eutanasiados e amostras de jejuno e íleo foram coletadas e fixadas em formol a 10% e em nitrogênio líquido a -80ºC. Para realização do modelo *ex vivo* foram eutanasiados seis animais de 4 semanas de idade para a obtenção dos explantes jejunais, os quais foram expostos a 5 e 10µmol/L de DON durante 4 horas, sob agitação constante a 39ºC. Após a incubação, estes foram fixados em formol 10% ou nitrogênio líquido a -80ºC. Com as amostras obtidas nos 2 modelos experimentais foram realizadas as técnicas de Western Blot e histopatologia, para avaliação da expressão de MAPK's e morfologia intestinal. A ingestão de dietas contaminadas com 2,8 mg/Kg de DON induziu ao aumento da expressão de citocinas de jejuno e íleo, enquanto que a ingestão de dietas contaminadas com 5,9 mg/Kg de FB aumentou a expressão de citocinas no íleo. Nos animais que ingeriram dietas contaminadas com 6,5 mg/Kg DON+FB houve uma diminuição na resposta imune sistêmica. Alterações morfológicas em fígado, pulmão, rins e intestinos foram observadas em animais que ingeriram as micotoxinas de forma isolada ou em associação. A expressão das proteínas de junção E-caderina e Ocludina diminuiu significativamente nos animais que ingeriram dietas contaminadas com 2,8 mg/Kg DON e 6,5 mg/Kg DON+FB. No segundo experimento, os dados obtidos no modelo *ex vivo* demonstraram a capacidade de DON, nas doses de 5 e 10 µmol/L, de provocar lise de enterócitos, edema intersticial e fusão de vilosidades. Em ambos os modelos experimentais, verificou-se que DON ativou a expressão das MAPK's p44/42 ERK ½ e phospho p38, não sendo observada a ativação de SAPK/JNK. Em conclusão, os dados obtidos indicam que a ingestão de dietas mono ou co-contaminadas induziram alterações morfológicas e imunológicas que podem predispor os animais a infecções secundárias.

Palavras-chaves: Co-contaminação. Resposta Imune. MAPK's. Proteínas de junção.

LUCIOLI, Joelma. **Systemic effects of contamination by Deoxynivalenol, Fumonisin B and their association in swines**. 2011. 177p. Thesis (Doctorate Degree in Animal Science) Londrina State University, Londrina, 2010.

ABSTRACT

Two experiments were conducted in order to evaluate the systemic effects of mycotoxin contamination, emphasizing morphological and immunological aspects. In the first study, 24 5-week-old piglets were divided into four groups, each group receiving a diet: one diet negative control and 3 diets contaminated with 2.8 mg/Kg DON, 5.9 mg/Kg FB and 6.5 mg/Kg DON + FB respectively. The animals were immunized with ovalbumin on the 4th and 16th days and blood samples were collected. After 35 days, the animals were euthanized. Tissue samples were collected and fixed in 10% formalin and in liquid nitrogen at -80 °C. The blood samples were used in hematological, biochemical and immunological tests. The tissue samples fixed in formaldehyde 10% were subjected to histological and immunohistochemical processing while the frozen samples were subjected to Western Blot and PCR to evaluate the expression of junction proteins and cytokines. In the second experiment, an *in vivo* study, twenty four 4-weeks-old piglets were distributed into two groups. For 28 days, one group received a control diet and the other a diet contaminated with 2.3 mg/Kg DON. At the end of the experiment, six animals from each group were euthanized. Samples of jejunum and ileum were collected and fixed in 10% formalin and in liquid nitrogen at -80°C. Using the *ex vivo* model , six 4-week-old animals were euthanized to obtain jejunal explants, which were exposed to 5 and 10μmol/L of DON for 4 hours, under constant stirring at 39°C. After incubation, these were fixed in 10% formalin and in liquid nitrogen at -80°C. The samples obtained from the two experimental models were analyzed with western blot techniques and histopathology, to evaluate the expression of MAPK's and intestinal morphology. Ingestion of diets contaminated with 2.8 mg /Kg DON induced increased expression of cytokines in the jejunum and ileum, whereas the intake of diets contaminated with 5.9 mg/Kg FB increased the expression of cytokines in the ileum. In animals fed diets contaminated with 6.5 mg/Kg DON + FB there was a decrease in systemic immune response. Morphological changes in liver, lung, kidneys and intestines were observed in animals fed mycotoxins in isolation or in combination. The expression of junction protein e-cadherin and ocludin decreased significantly in animals fed diets contaminated with 2.8 mg/Kg DON and 6.5 mg/Kg DON + FB. In the second experiment, the data obtained in the *ex vivo* model demonstrated the ability of DON, at doses of 5 and 10 μmol/L, to cause lysis of enterocytes, interstitial edema and fusion of villi. In both experimental models, it was observed that DON activated the expression of MAPK's p44/42 ERK ½ and phospho p38, with no observed activation of SAPK/JNK. In conclusion, these data indicate that intake of mono or co-infected diets induced morphological and immunological changes that may predispose animals to secondary infections.

Keywords: Co-contamination. Immune response. MAPK's. Junction proteins.

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LISTA DE ABREVIATURAS E SIGLAS

µM	Micromolar
µl	Microlitro
µg/ml	Micrograma por Mililitro
µmol/L	Micromol por Litro
15aDON	15-acetil-Desoxinivalenol
3aDON	3-acetil-Desoxinivalenol
ABIPESC	Associação Brasileira da Indústria Produtora e Exportadora de Carne Suína
AEBSF	*4,2 Aminoethyl Benzenesulfonyl Fluoride Hydrochloride*
ANRT	*Association Nationale de la Recherche Technique*
ANVISA	Agência Nacional de Vigilância Sanitária
APC	*Antigen-presenting Cells* (Células Apresentadoras de Antígeno)
BALT	*Bronchus-associated Lymphatic Tissue* (Tecido Linfóide associado à Mucosa Brônquica)
CACO-2	Células de Adenocarcinoma Humano
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CMAADLV	Comitê de Micotoxinas da Associação Americana de Diagnóstico Laboratorial Veterinário
COFECUB	Comitê Francês de Avaliação da Cooperação Universitária e Científica com o Brasil
DART PCR	*Differential amplifying RT-PCR* (Amplificação Diferencial da Reação em Cadeia de Polimerase Real Time)
DAS	Diacetoxiscirpenol
DL50	Dose Letal 50%
DMSO	Dimetilsulfóxido
DNA	*Deoxyribonucleic Acid* (Ácido Desoxirribonucléico)
DON	Desoxinivalenol
EDTA	*Ethylenediamine Tetraacetic Acid* (Ácido Tetracético Etilenodiamino)
ELISA	*Enzyme Linked Immunosorbent Assay*
ENVT	*École Nationale Vétérinaire de Toulouse*
ERK	*Extracellular Signal-regulated Kinases*
FAO	*Food and Agricultural Organisation*
FB_1	Fumonisina B_1

FB_2	Fumonisina B_2
FX	Fusarenona X
GLDH	Glutamato Desidrogenase
g	Grama
g/L	Grama por Litro
GGT	*Gamma Glutamyl Transferase* (Gama glutamil transferase)
HCl	Ácido Clorídrico
HE	Hematoxilina-eosina
i.p.	Intraperitoneal
i.v.	Intravenosa
IARC	*International Agency for Research on Cancer*
IgA	Imunoglobulina A
IgG	Imonoglobulina G
IHQ	Imunoistoquímica
IL-1β	Interleucina 1β
IL-6	Interleucina 6
IL-8	Interleucina 8
INRA	*Institut National de la Recherche Agronomique*
IPEC	*Intestinal Porcine Epithelial Cells*
JNK	*c Jun N-terminal Kinases*
LMT	Limite Máximo Tolerável
mA	Miliampére
MAPK	*Mitogen-activated Protein Kinase*
mg	Miligrama
mg/Kg	Miligrama por Quilo
MIP-1β	Proteína inflamatório do macrófago 1β
M-MLV	*Moloney Murine Leukemia Virus*
mmol/L	Milimol por Litro
mRNA	*Ribonucleic acid Messenger* (Ácido Ribonucléico Mensageiro)
nm	Nanometro
NaOH	Hidróxido de Sódio
NaN_3	Azida Sódica
NBB	*Naphtol Blue Black*
NIV	Nivalenol

NTC	*Non template control*
OVA	Ovalbumina
PBS	*Phosphate Buffered Saline*
PCR	*Polymerase Chain Reaction* (Reaçao em Cadeia da Polimerase)
pH	Potencial Hidrogeniônico
PV	Peso Vivo
RT-PCR	*Transcriptave Reverse Polymerase Chain Reaction*
Sa	Esfinganina
SCF	*Scientific Committee on Food*
SD	*Standard Deviation*
SDS	*Sodium Dodecyl Sulfate*
SDS-PAGE	*Sodium dodecyl Sulfate Polyacrilamide Gel Electrophoresis*
SEM	*Standard Error of the Mean*
So	Esgingosina
TCA	*Trichloroacetic Acid*
TEER	*Transepithelial Electrical Resistance* (Resistência Elétrica Trans-epitelial)
TRIS	*Tris(hydroximethil) Aminomethane*
UEL	Universidade Estadual de Londrina
UI	Unidade internacional
V	Volts
v.o.	Via oral
WB	*Western Blot*
WHO	*World Health Organisation*

SUMARIO

1 INTRODUÇÃO

O Brasil é o quarto maior produtor mundial de carne suína, tendo produzido, em 2010, um total de 3,24 milhões de toneladas de carne suína. O país é também o quarto maior exportador deste produto, sendo o responsável pela exportação de 625 mil toneladas de carne neste mesmo período (ABIPESC, 2010). A produção de carne suína concentra-se na região sul do país, representante da maior parte da produção brasileira. Santa Catarina lidera o ranking com 25,6% do total da carne produzida, seguida pelo Rio Grande do Sul com 16,3% e Paraná com 14,8%. Além da concentração de produção suína existente no sul do país, observa-se a difusão da produção para outras regiões do país, como o centro-oeste (SAAB; CLAUDIO, 2010).

Com um sistema produtivo baseado na integração vertical, disponibilidade de insumos básicos para a produção, principalmente de grãos como soja e milho, e investimentos em tecnologia, a produção de suínos no Brasil apresenta custos inferiores aos principais competidores mundiais. Além disso, o Brasil é o terceiro maior produtor mundial de milho, totalizando 53,2 milhões de toneladas na safra 2009/2010 (Ministério da Agricultura. 2011). Diante de tamanha produção instala-se, não apenas no Brasil, mas também em outras partes do mundo, uma preocupação crescente em relação à qualidade sanitária das matérias primas destinada à alimentação humana e animal. A detecção sistemática de micotoxinas nos produtos agrícolas é cada vez mais discutida visando estabelecer níveis de recomendação e/ou regulamentação para o seu uso. Sendo assim, é necessário preocupar-se sobre a possível contaminação por fungos dos alimentos destinados ao homem e aos animais e o risco associado à presença de micotoxinas.

Nenhuma região do mundo encontra-se livre dos problemas causados pelas micotoxinas. Sabe-se que 25% das plantações mundiais são afetadas e em certas áreas geográficas do mundo algumas micotoxinas são produzidas mais prontamente que outras (LAWLOY; LYNCH, 2005). No Brasil, embora as micotoxinas sejam responsáveis por expressivos prejuízos na produção de grãos, praticamente não existem estimativas das perdas econômicas associadas a elas.

Efeitos adversos na saúde e prejuízos na produção animal têm sido reconhecidos em rebanhos suínos, bovinos e de aves, como consequência do consumo de altas concentrações de cereais em sua dieta (SMITH, 1994). De acordo com a *Food and Agricultural Organisation* (FAO), estima-se que as perdas globais de alimentos pela

contaminação com micotoxinas estejam em torno de um milhão de toneladas ao ano (IHESHIULOR et al., 2011).

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, uma vez que a produção e concentração dessas substâncias são determinadas por diversos efeitos combinados, como espécies de fungos presentes, temperatura e umidade do grão (RAMAKRISHNA et al., 1996). Quando as micotoxinas estão presentes na dieta, vários outros fatores como espécie animal, 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 dos animais (DILKIN, 2002). Poucas vezes as micotoxicoses se manifestam como doença aguda, culminando com a morte dos animais.

O objetivo desta tese foi o de avaliar os efeitos sistêmicos da multi-contaminação por micotoxinas em suínos, com ênfase sobre a resposta imunitária e morfologia intestinal. Suspeita-se dos efeitos imunossupressores das micotoxinas há bastante tempo, todavia, seu estudo sistemático é relativamente recente e parcial. Poucos relatos existem sobre os mecanismos que modulam os efeitos das micotoxinas sobre as células envolvidas na resposta imunitária e na proliferação e adesão celular. No presente trabalho postulamos que, mesmo em pequenas doses, as micotoxinas podem agir sobre a resposta imune e a barreira intestinal, aumentando a sensibilidade do ser humano e dos animais às infecções.

2 REVISÃO DE LITERATURA

2.1 MICOTOXINAS

Micotoxina é um termo derivado do grego (*Mikes* = fungo e *Toxicum* = veneno), utilizado para descrever substâncias tóxicas produzidas por fungos em diferentes etapas do desenvolvimento micelial (D'MELLO; MacDONALD, 1997). São metabólitos secundários de baixa massa molecular, considerados como contaminantes de alimentos de consumo humano e animal, produzidos por certas cepas de fungos filamentosos como *Aspergillus* spp., *Penicilium* spp. e *Fusarium* spp. (OKOLI et al., 2007).

A presença de fungos toxigênicos nos cereais ou alimentos indica risco potencial, mas somente a detecção de toxinas específicas determina a toxicidade dos mesmos. Da mesma forma, a ausência de fungos não indica ausência de toxinas, sendo que esta pode persistir após a eliminação do agente (SANTIN, 2000). Outra consideração importante a ser feita, é a de que o fungo pode produzir mais de uma micotoxina e que a micotoxina, por sua vez, pode ser produzida por diferentes tipos de fungos (FINK-GREMMELS, 1999). Quando em associação, fungos e micotoxinas representam um impacto negativo na saúde e na produtividade animal, em comparação aos seus efeitos individuais (SMITH; SEDDON, 1998).

A produção de toxinas e o grau de contaminação dos alimentos são regulados por fatores ambientais, composição e textura do substrato, umidade e temperatura. As plantas são mais sensíveis à invasão fúngica sob condições de estresse, como seca, excesso de irrigação ou chuva, ataques constantes de insetos ou exposição excessiva a inseticidas (FINK-GREMMELS, 1999).

Os efeitos biológicos das micotoxinas dependem da quantidade ingerida, duração da exposição e sensibilidade animal (AKANDE et al., 2006). Micotoxinas podem afetar o *status* imune do animal favorecendo diversas infecções, sendo essa a principal razão da dificuldade de diagnóstico das micotoxicoses. Também podem induzir problemas de saúde que são específicos para cada toxina (IHESHIULOR et al., 2011).

Entre os diversos fungos micotoxigênicos, o gênero *Fusarium* spp. está presente nas culturas de cereais no Brasil e em todo o mundo. As diferentes espécies de *Fusarium* spp. podem produzir mais de 180 metabólitos secundários, dentre os quais alguns afetam a saúde humana e animal (OSWALD et al. 2005). As fusariotoxinas que podem ser encontradas em concentrações significativas nos cereais são as fumonisinas, a zearalenona e

os tricotecenos dos grupos A (toxina T_2 e HT_2) e B (nivalenol e desoxinivalenol). Estas micotoxinas podem provocar perdas econômicas em todas as etapas da cadeia alimentar, além de afetar a saúde humana e animal.

Recentemente, a ANVISA – Agência Nacional de Vigilância Sanitária, através da resolução RDC nº 7, de 18 de fevereiro de 2011, estabeleceu limites máximos toleráveis de micotoxinas para diferentes classes de alimentos. Os limites máximos admissíveis em alimentos prontos para consumo e em matérias-primas foram estabelecidos de acordo com a categoria. A resolução começou a ser aplicada em 18 de fevereiro do corrente ano. Os níveis máximos estabelecidos para as diferentes micotoxinas estão dispostos em tabelas que se encontram nos Anexos.

No presente trabalho, discutiremos a respeito de duas fusariotoxinas presentes em cereais: a Fumonisina B_1 (grupo das fumonisinas) e o Desoxinivalenol (grupo B dos tricotecenos).

2.1.1 Fumonisinas

As fumonisinas são metabólitos secundários produzidos por *Fusarium verticillioides* (anteriormente *Fusarium moniliforme* - GELDERBLOM et al., 1988), primeiramente isolados e identificados em 1988 (BEZUIDENHOUT et al., 1988). Até o momento, 28 fumonisinas têm sido isoladas e podem ser divididos em quatro grupos, conhecidos como A, B, C e P (SORIANO et al., 2005; WANG et al., 2008). A Fumonisina B_1 (FB_1) é a mais importante, e além dos 28 análogos, existem outros metabólitos de menor importância não detectados como contaminantes naturais (WHO, 2000).

A primeira descrição sobre a ocorrência natural de FB_1 foi em 1990 a partir de milho mofado coletado em Transkei, África do Sul, região que apresentava alta incidência de câncer de esôfago em humanos (SYDENHAUM et al., 1990).

A FB_1 é mundialmente encontrada em grãos, principalmente no milho. A toxina é estável ao calor e só é reduzida significativamente durante processos nos quais a temperatura excede os 150°C. Ocorre pouca degradação da FB_1 durante a fermentação ou manufatura por trituração úmida do amido de milho, uma vez que esta é solúvel em água (SCOTT, 1993; KAWASHIMA; VALENTE-SOARES, 2006; CALDAS; SILVA, 2007).

As fumonisinas foram detectadas em vários tipos de alimentos em diferentes países, como Canadá, Egito, Peru, África do Sul e Estados Unidos. No Brasil, Yamaguchi et al (1992) ao analisar diferentes lotes de milho provenientes da safra de quatro regiões do

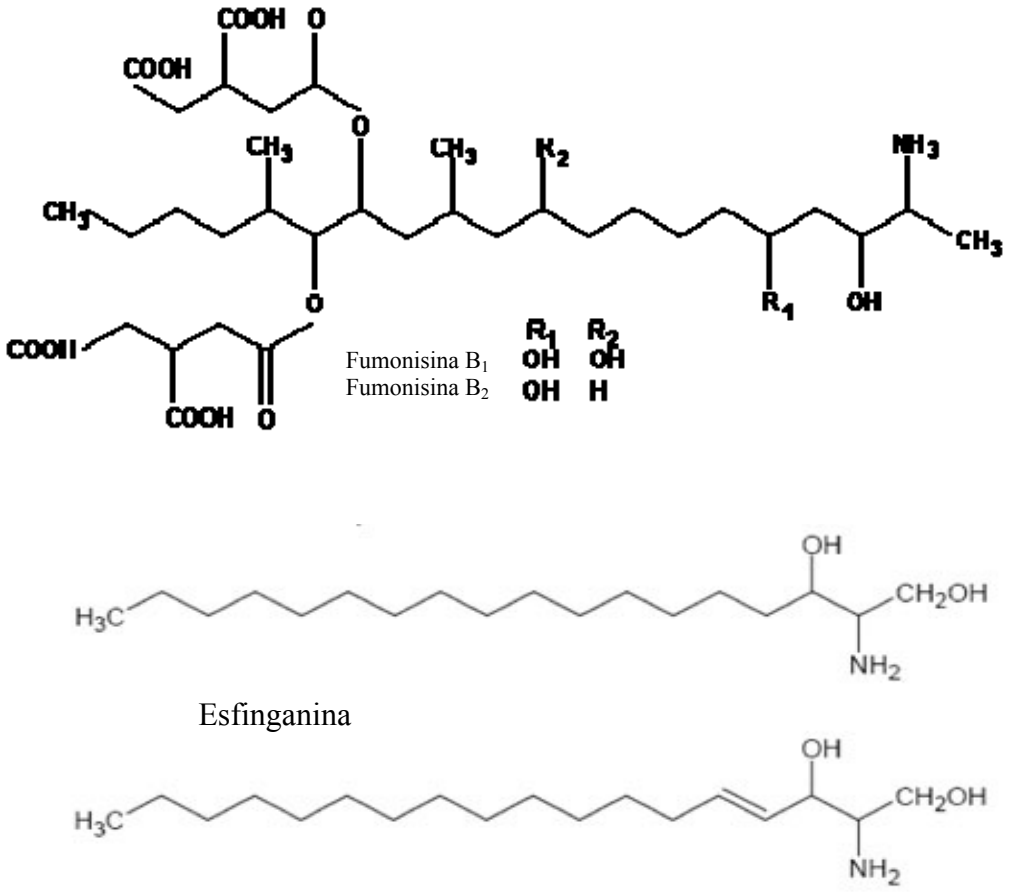
Estado do Paraná, observaram que 97,4% das amostras foram positivas para FB_1 e 4,8% para FB_2. Amostras de milho provenientes dos Estados do Paraná, Mato Grosso do Sul e Goiás, colhidas entre os anos de 1991 e 1992 também foram analisadas para presença de micotoxinas. Com exceção de uma amostra proveniente do Estado de Goiás, as demais estavam contaminadas com FB_1 e FB_2, com níveis que variavam entre 3,25 a 5,45 m/Kg para FB_1 e 2,34 a 5,00 mg/Kg para FB_2 (HIROOKA et al., 1996).

No Rio Grande do Sul, 47,1% das amostras de milho provenientes de colheitas entre 1996 e 1997, estavam contaminadas com média de 8,4 mg/Kg de FB_1 (MALLMANN et al., 1997). No Estado de São Paulo, a ocorrência natural de fumonisinas em amostras de milho híbrido foi de 90,2% para FB_1 e 97,4% para FB_2 (ORSI et al., 2000). Diferenças regionais na concentração de fumonisina foram encontradas analisando-se o mesmo milho híbrido no Estado do Paraná, indicando interferências climáticas na predominância de linhagem toxigênicas (ONO et al., 2001).

2.1.1.1 Estrutura físico-química das fumonisinas

A Fumonisina B_1, o análogo mais abundantemente encontrado (GALVANO et al., 2002), tem a fórmula empírica $C_{34}H_{59}NO_{15}$ e consiste de diéster de propano-1,2,3-ácido tricarbalílico e 2-amino-12,16-dimetil-3,5,10,14,15-pentahidroxieicosano, sendo que os grupos hidroxila dos carbonos 14 e 15 encontram-se esterificados com o grupo carboxila terminal do ácido tricarbalílico (BEZUIDENHOUT et al., 1998). Fumonisinas são moléculas fortemente polares, solúveis em água e em acetonitrila-água e insolúveis em solventes orgânicos (SCOTT, 1993). As formas FB_1 e FB_2 (Figura 1) são os análogos de principal ocorrência natural, sendo a forma FB_1 a mais prevalente na dieta humana, classificada como possível carcinógeno pertencente ao grupo 2B pela Agência Internacional de pesquisa em câncer (IARC, 1993). Estudos realizados com hepatócitos de ratos demonstraram que Fumonisina B_2 é tão eficaz quanto a Fumonisina B_1 na inibição de biossíntese de esfingolipídios (NORRED et al., 1992; WANG et al., 1991).

Figura 1 – Estrutura química da Fumonisina, dos seus análogos B_1 e B_2 e de suas bases esfingóides esfinganina e esfingosina. Fonte: University of Leeds (modificado),



2.1.1.2 Toxicologia das fumonisinas

O estudo sobre a toxicologia das fumonisinas está dirigido ao principal análogo produzido pelo *Fusarium verticillioides*, a Fumonisina B_1.

Esfingolipídios

Os esfingolipídios constituem uma classe de lipídeos que exercem um importante papel na regulação central, sendo encontrados em todas as células eucarióticas (MERRIL et al., 1997, FERRANTE et al., 2002). Em células de mamíferos, a ceramida, a esfingosina, a esfingosina-1-fosfato e a glicosilceramida são os mais importantes esfingolipídios por atuarem na regulação de processos fisiológicos importantes, como a

resposta ao estresse, apoptose, proliferação celular, angiogênese e resistência a quimioterápicos (OBEID et al., 1993; LUBERTO; HANNUN, 1999).

São substâncias com estrutura diversa formada por uma base esfingóide de cadeia longa e um grupo amino, o qual pode ser substituído por um ácido graxo de cadeia longa (ABNET et al., 2001). Os ácidos graxos variam em comprimento, grau de insaturação e presença ou não de grupo hidroxila ligada ao átomo α ou ω (MERRIL et al., 2001). O esqueleto estrutural dos esfingolipídeos é a esfingosina, e as moléculas mais simples dos esfingolipídeos são as ceramidas, que consistem de um ácido graxo ligado ao grupamento amino da esfingosina por uma ligação amida.

Na ceramida, os ácidos graxos variam entre 2 e 28 átomos de carbono na cadeia acila e saturação. A ceramida fornece a base tanto para a síntese como para o catabolismo dos esfingolipídeos complexos e, portanto, também é referido como o centro do metabolismo esfingolipídico (HANNUN; OBEID, 2002).

A esfingosina (So) é a base esfingóide prevalente dos esfingolipídios de mamíferos, sendo a mais freqüente a D-eritro-1,3-diidroxi-2-amino-octadec-4-eno ou 4-trans-esfinganina. As bases esfingóides variam no comprimento da cadeia alquila, posição e número da dupla ligação e presença de outros grupos funcionais, como a hidroxila (MINAMI et al. 2004).

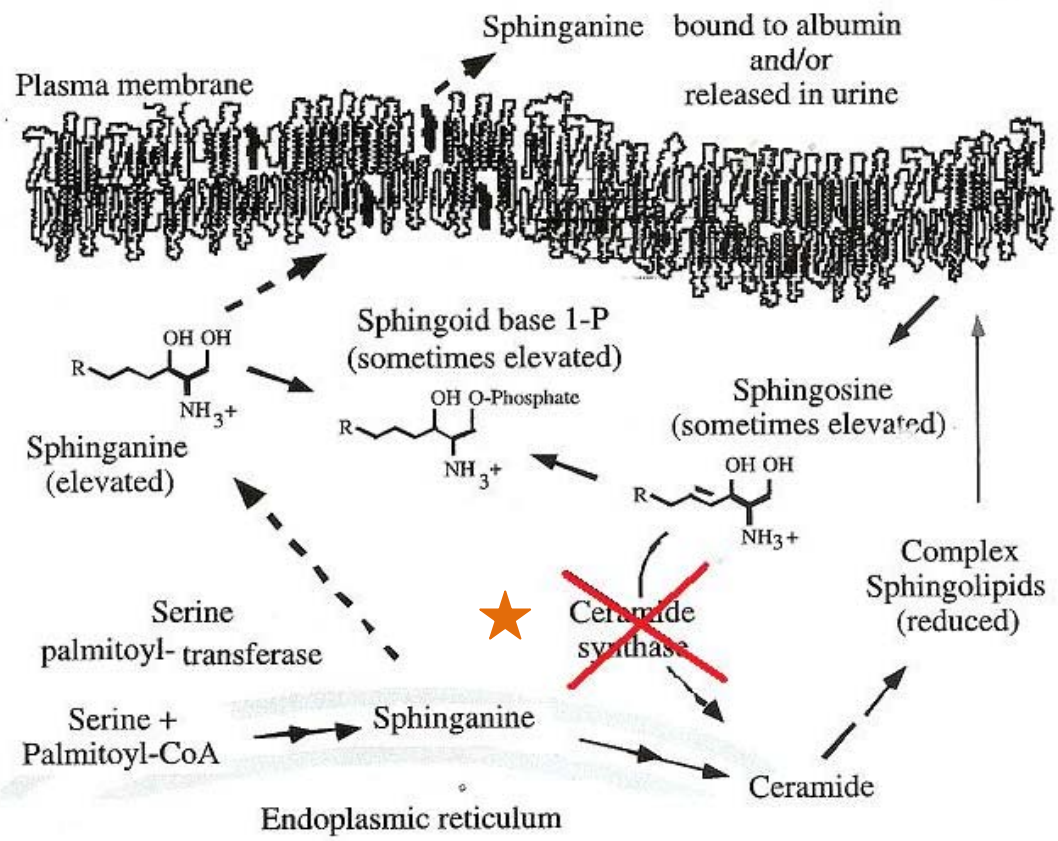
Mecanismo de ação: o mecanismo de ação das fumonisinas ainda não é totalmente compreendido, mas estudos realizados por Soriano et al (2005) propõem que a FB_1 possa intervir na via de biossíntese de esfingolipídios através da inibição da enzima ceramida sintase. Isso se deve ao fato da similaridade da molécula de FB_1 com o complexo aminoálcool esfingosina, que é um dos trinta ou mais aminoálcoois da cadeia longa encontrados nos esfingolipídios de várias espécies. As fumonisinas alteram a biossíntese dos esfingolipídeos, com as maiores alterações nas concentrações das bases esfingóides no rim, fígado, pulmão e coração. O sistema imune específico não é afetado, entretanto FB_1 inibiu a fagocitose e a biossíntese de esfingolipídeos nos macrófagos pulmonares, induzindo um acúmulo de material membranoso nas células endoteliais dos capilares pulmonares. Essa alteração parece ser específica a esse tipo de célula e à espécie suína (DILKIN, 2002).

O metabolismo ocorre em dois estágios: no primeiro, a monoaminoxidase remove o grupo amina e no segundo, as cadeias de ácido tricarboxílico propano são removidas por esterases. Sob ação das esterases ocorre hidrolização da FB_1 que é transformada em um análogo da ceramida, dez vezes mais tóxico que a FB_1 intacta (DESAI et al., 2002). A ligação desse análogo ao sítio catalítico da ceramida sintase constitui o primeiro

evento no processo de bloqueio do metabolismo lipídico (RILEY et al., 2001). A completa inibição da ceramida sintase causa uma elevação rápida da concentração intracelular de esfinganina (Sa) (VAN DER WESTHUIZEN et al., 1999) e depleção dos esfingolipídios complexos nas células (RILEY et al., 1993). Essa inibição faz com que intermediários dos esfingolipídios, a esfinganina (Sa) e a esfingosina (So), se acumulem na circulação sanguínea (Figura 2) (WANG et al., 1992) e na urina (RILEY et al., 1994, WANG et al., 1999) Assim, a elevação das bases esfingóides em urina, soro e tecidos pode ser usada como biomarcador na exposição às fumonisinas (RILEY et al., 1994). Esfingosina (So) e esfinganina (Sa) estão normalmente presentes em concentrações mínimas nos tecidos, porem o nível de Sa livre é sempre menor que o de So livre (RILEY et al., 1993). O acúmulo de Sa e So no sangue pode ser utilizado como indicador de intoxicação por FB_1 (DESAI et al., 2002; SORIANO et al., 2005; MALLMANN; DILKIN, 2007).

A inibição da biossíntese dos esfingolipídios complexos interrompe inúmeras funções celulares, pois estes componentes têm papel fundamental na estrutura da membrana, comunicação celular, interação intracelular/matriz celular e regulação de fatores de crescimento. Atuam também como mensageiros de vários fatores como fator de necrose tumoral, interleucina 1, fator de crescimento de nervos e em vias de sinalização de apoptose e mitose (MERRIL et al. 1993). Em resumo, os esfingolipídios estão diretamente ligados à regulação do crescimento celular, diferenciação e transformação neoplásica.

Figura 2 – Mecanismo de ação das fumonisinas. Pela inibição da acilação da esfinganina e esfingosina pela FB, ocorre a elevação das bases esfingóides e redução dos esfingolipídios complexos. Fonte: Desai et al., 2002 (modificado).



Toxicidade subaguda/aguda: os sinais clínicos de intoxicação aguda por FB_1 em suínos surgem geralmente de três a cinco dias após início de ingestão da ração contaminada. O quadro clínico caracteriza-se por edema pulmonar, hepatotoxicidade, cardiotoxicidade e lesões pancreáticas (HASCHECK et al., 2001; MALLMANN; DILKIN, 2007). Os suínos apresentam dispnéia, fraqueza, anorexia, letargia, vômito, diarréia, icterícia e cianose mais evidentes nas orelhas, focinho, esclera e membranas mucosas. Nas fêmeas gestantes pode ocorrer aborto um a quatro dias após o início dos sinais ou os leitões apresentarem, após o nascimento, sinais de edema pulmonar. Dependendo da gravidade da intoxicação, os animais podem morrer em poucas horas ou em até dez dias após a ingestão da micotoxina (OSWEILER et al.,1992; POZZI et al., 2002; MALLMANN; DILKIN, 2007).

Toxicidade subcrônica/crônica: a intoxicação crônica de suínos por FB_1 ocorre pelo consumo de ração com baixas concentrações da toxina e por um tempo prolongado. Os principais sinais clínicos são inespecíficos e podem ser confundidos com desnutrição, deficiência genética, manejo inadequado e/ou outras afecções que induzem à

clínica do animal (MALLMANN; DILKIN, 2007). Os animais apresentam letargia, perda de apetite, aumento das freqüências cardíacas e respiratórias, cianose na esclera e membranas e pelos eriçados (DILKIN et al., 2004). As lesões e os sinais clínicos dependem da concentração da micotoxina ingerida, idade, sexo e tempo de consumo (POZZI et al., 2002; THEUMER et al., 2002). Não estão bem estabelecidos níveis máximos toleráveis de FB_1 para os animais, mas o Comitê de Micotoxinas da Associação Americana de Diagnóstico Laboratorial Veterinário (CMAADLV) tem recomendado níveis máximos de 5, 10, 50 e 50 μg/g de ração para eqüinos, suínos, bovinos e aves, respectivamente (MUNKVOLD; DESJARDINS et al., 1997).

A intoxicação por FB_1 nos animais causa leucoencefalomalácia em eqüinos, nefrotoxicidade em ovinos, deficiência imunológica e lesões no fígado e nos rins em galinhas, hepatoxicidade e nefrotoxicidade em bovinos e câncer hepático e renal em roedores (SCOTT, 1993; GELDERBLOM et al., 2001; MATHUR et al., 2001; SEGVIC; PEPELJNJAK, 2001; VOSS et al., 2001; DEL BIANCHI et al., 2005). Guzman et al. (1997) constataram que suínos alimentados numa concentração de 140 mg/FB_1/Kg/ração durante nove meses apresentaram diminuição intermitente da ingestão de ração, sem alterações bioquímicas hepáticas e após a necropsia observou-se grave fibrose perilobular hepática.

Em outro estudo, constatou-se que suínos desmamados alimentados com ração contaminada com 100 mg/FB_1/Kg durante dez dias e posteriormente com 190 mg/FB_1/Kg durante 83 dias apresentaram alterações bioquímicas hepáticas. Após a necropsia constatou-se a presença de hiperqueratose, paraqueratose e hiperplasia no esôfago, ulceração na *pars esophagea* do estômago, e no fígado observou-se necrose centrolobular, vacuolização citoplasmática e presença de nódulos hiperplásicos sugestivos de lesões preneoplásicas (CASTEEL et al., 1993). Suínos alimentados com ração contaminada na concentração de 30 mg de fumonisina/Kg durante 28 dias apresentaram edema pulmonar e flacidez dos ventrículos cardíacos, porém não apresentaram lesões significativas no fígado (DILKIN et al., 2004). A FB_1 em intoxicações crônicas altera a resposta humoral e diminui a resposta de anticorpos vacinais em suínos, inibe a fagocitose macrofágica pulmonar, levando ao acúmulo de material membranoso nas células endoteliais dos capilares pulmonares, e induz a apoptose e necrose nos tecidos linfóides (POZZI et al., 2002; PIVA et al., 2005; TARANU et al., 2005).

2.1.1.3 Carcinogenicidade e teratogenicidade

A FB_1 está classificada como possível carcinógeno pertencente ao grupo 2B pela Agência Internacional de pesquisa em câncer (IARC, 1993). Em humanos, o nível de ingestão tolerável de FB_1 é de 2µg/Kg de peso/dia, limite sugerido em 2001 pela *Food and Agricultural Organization* (STOCKMANN-JUVALA; SAVOLAINEN, 2008). A ingestão de alimentos contaminados com FB_1 por mulheres grávidas desencadeia defeitos no fechamento do tubo neural dos fetos, e há evidências de que FB_1 está relacionada ao desenvolvimento de câncer no esôfago (YOSHIZAWA et al., 1994; STEVENS; TANG, 1997; MÜSSNER et al., 2006; STOCKMANN-JUVALA; SAVOLAINEN, 2008).

A Fumonisina B_1 tem demonstrado ser carcinogênica em ratos, exibindo efeitos tanto de promoção como de iniciação. A partir de estudos realizados em ratos que receberam doses de 50 mg/Kg de FB_1, observou-se o desenvolvimento de carcinoma hepatocelular, sendo que em doses acima de 50 mg/Kg ocorreu também a indução de carcinomas de túbulos renais (HOWARD et al., 2001). Em ratos, a teratogenicidade da FB_1 é expressa como uma supressão do crescimento e desenvolvimento ósseo do feto (LEBEPE-MAZUR et al., 1995).

2.1.2 Desoxinivalenol

O Desoxinivalenol (DON) é uma micotoxina pertencente ao grupo B dos tricotecenos, sendo produzida pelo *Fusarium graminearum* (KUSHIRO, 2008). No grupo dos tricotecenos encontramos mais de 180 micotoxinas estruturalmente relacionadas, produzidas por fungos do gênero *Fusarium* spp., que crescem em alimentos, no meio ambiente e em cereais.

A contaminação de produtos agrícolas como trigo, cevada e milho por tricotecenos, em especial pelo DON (BINDER et al., 2007), é um problema cada vez mais comum, possivelmente devido à expansão do uso de plantio direto e mudança nos padrões climáticos (McMULLEN et al., 1997). Sua ocorrência em alimentos para consumo humano e animal representa mais de 90% do número total de amostras (SOBROVA et al., 2010).

Na década de 90, 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 apresentaram 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 750 mg/Kg (SCOOP, 2003).

No sul do Brasil, 24,91% das 297 amostras de trigo utilizadas na alimentação humana apresentaram contaminação por DON, com níveis variando entre 0,6 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, Paraná e Rio Grande do Sul 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 332 µg/Kg (nacional) e 90 µg/Kg (importado) (CALORI-DOMINGUES et al., 2007).

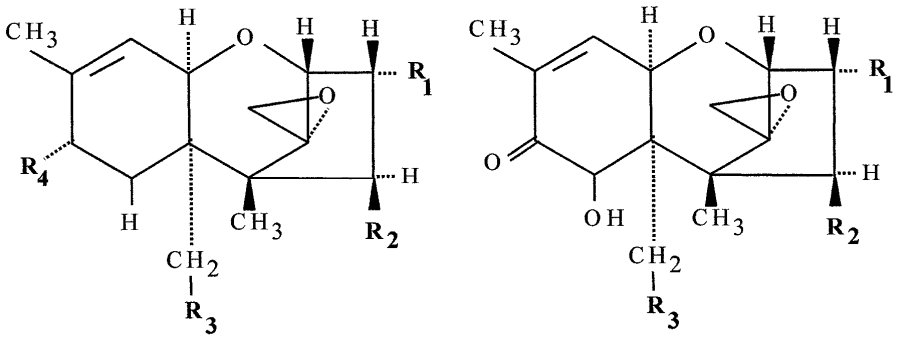
Através da análise de 38 amostras de trigo provenientes de diferentes cultivares e localidades do PR e RS, Santos et al (2011) detectaram a presença de DON em 29 amostras (76.3%) através do teste ic-ELISA (281,6-12291,4 $\mu g.Kg^{-1}$) e em 22 amostras (57,9%) através da técnica LC-MS (155,3-9906,9 $\mu g.Kg^{-1}$). Segundo a ANVISA (2011), a partir de janeiro de 2014 os limites de DON no trigo antes de sua transformação serão de 3000 $\mu g.Kg^{-1}$

2.1.2.1 Estrutura físico-química dos tricotecenos e do desoxinivalenol

Quimicamente, os tricotecenos são sesquiterpenóides de baixa massa molecular, que usualmente contém um anel epóxi em C-12 e C-13 e uma dupla ligação na posição C-9 e C-18, ambas importantes para sua toxicidade (DESJARDINS et al., 1993). Os tricotecenos são divididos em quatro grupos, denominados de A a D de acordo com suas propriedades químicas e fungos envolvidos (UENO, 1977).

Os fungos do gênero *Fusarium* ssp. produzem tricotecenos dos grupos A e B (Figura 3), que são distinguidos pela ausência ou presença do grupo carbonil na posição C-8, respectivamente. Fazem parte do grupo A, tricotecenos como a toxina T-2 e diacetoxiscirpenol (DAS), e do grupo B o desoxinivalenol (DON), 3-acetil-desoxinivalenol (3aDON), 15-acetil-desoxinivalenol (15aDON), fusarenona X (FX) e o nivalenol (NIV) (Quadro 1). As outras duas categorias, C e D, são produzidas por fungos do gênero *Myrothecium* spp., e são caracterizados por um segundo grupo epóxi em C-7 ou C-9,10 (grupo C) ou um anel macrocíclico entre C-4 e C-15 com dois ésteres (grupo D) (UENO, 1985).

Figura 3 – Estrutura química dos tricotecenos dos tipos A e B. Os substituintes R1-R4 estão listados no quadro 1. Fonte: Goyarts, 2006.



Tipo A Tipo B

Os tricotecenos não são degradados durante o processo de industrialização dos alimentos e não são hidrolizados pelo processo de digestão que ocorre no estômago (LAUREN; SMITH, 2001). Entretanto, muitos tricotecenos são solúveis em acetona, clorofórmio e etilacetato. Tricotecenos altamente hidrolisados como DON e NIV são solúveis em solventes mais polares como acetonitrilo, metanol, etanol e água (UENO, 1987).

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).

2.1.2.2 Toxicologia do desoxinivalenol

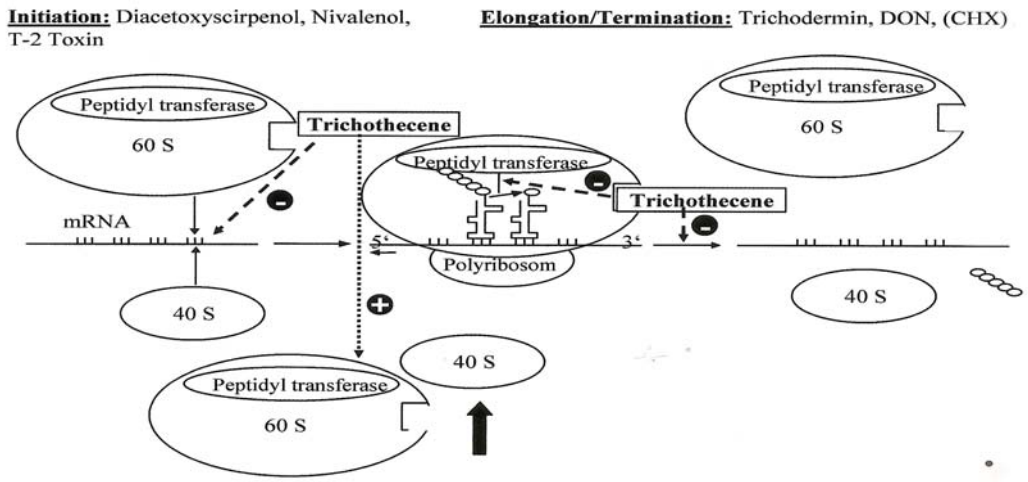
De acordo com Perkowski et al. (1990), a ingestão de DON não representa uma ameaça significativa à saúde pública, apesar de em alguns casos, terem sido registrados náuseas, vômitos, diarréia, dor abdominal, cefaléia, tontura e febre.

A espécie mais sensível a DON e modelo amplamente utilizado para a função do intestino humano é o suíno (NEJDFORS et al., 2000). Neste, o desoxinivalenol é rápida e eficientemente absorvido, provavelmente a partir de partes superiores do intestino delgado, e é excretado principalmente na urina, sem acumular em tecidos (PRELUSKY et al., 1988; ERIKSEN et al., 2003).

O DON é menos tóxico que outros tricotecenos, como a toxina T-2. Contudo, doses extremamente altas podem causar morte por choque. A DL50 para camundongos varia de 49 a 70 mg/Kg (intraperitoneal) (FORSELL et al., 1987) e 46 a 78 mg/Kg (administração via oral) (YOSHIZAWA et al., 1983). Segundo Yoshizawa e Morooka (1973) a DL50 para patos de 10 dias é de 27 mg/Kg, quando a toxina é administrada por via subcutânea.

Mecanismo de ação: Inibição da síntese proteica – os tricotecenos são inibidores bem conhecidos da proteinasintetase. Eles se ligam à subunidade 60S dos ribossomos de células eucarióticas e inibem a ação da peptidil transferase. (FEINBERG; MCLAUGHLIN, 1989). Dependendo dos substituintes, os tricotecenos atuam inibindo tanto a iniciação quanto o alongamento/ terminação da síntese proteica (EHRLICH; DAIGLE, 1987). DON atua inibindo a fase de alongamento/terminação da síntese protéica. Tricotecenos que inibem a iniciação da cadeia peptídica são inúmeras vezes mais potentes do que aqueles que afetam o alongamento/terminação das cadeias peptídicas (EHRLICH; DAIGLE, 1985).

Figura 4 – 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.



Além da inibiçao da síntese proteica, os tricotecenos são 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; UENO, 1985). No entanto, esses efeitos parecem ser secundários a inibição da síntese protéica (THOMPSON; WANNEMACHER, 1990). Como descrito anteriormente, os tricotecenos inibem a síntese protéica através da ligação a peptidil transferase. Inibidores da peptidil transferase podem desencadear uma reação denominada resposta ao estresse ribotóxico que ativam proteínas quinases ativadas por mitogenos (MAPK), as quais são componentes da cascata de sinalização que regula a sobrevivência das células ao estresse (IORDANOV et al., 1997).

As MAPK's constituem uma família de proteínas de transdução de sinal que converte sinais extracelulares como, por exemplo, estresse e fatores de crescimento, para a ativação de uma seqüência de reações intracelulares (HUANG et al., 2010). A especificidade da ativação e função das MAPK's é determinada, em parte, por proteínas que criam complexos multi-enzimáticos, os quais aumentam, diminuem ou redirecionam o fluxo do sinal em resposta a estímulos fisiológicos específicos (AOUADI et al., 2006). A cascata de sinalização das MAPK's é ativada em geral, por diversos estímulos que regulam a produção de citocinas e fatores de crescimento (ROUX; BLEINS, 2004), e respeita uma sequência de fosforilação e desfosforilação, os quais inicialmente ativam uma MAP quinase quinase (MAPKK- Raf), que irão ativar uma MAPK quinase (MAPKK-MKK) e consequentemente ativam MAPK's específicas como ERK, JNK e p38 que são responsáveis pela expressão gênica, levando a uma resposta fisiológica apropriada (MUTALIK; VENKATESH, 2006).

Os membros da família MAPK são classificados dentro de três subfamílias: *extracellular signal-regulated kinase* p44/42 (ERK), p38 e JNK (COBB, 1999). A MAPK p44/42 ERK é de grande interesse, por estar envolvida na modulação da morfologia das células epiteliais e estruturas das junções celulares que regulam a função de barreira celular do trato intestinal. A interação da ERK p44/42 pode mediar a fosforilação de certas proteínas de junção oclusivas ou de moléculas de sinalização associadas a essas e regular a integridade da junção celular e conseqüentemente a função de barreira do epitélio (BASUROY et al., 2006). A sinalização de ERK predomina na resposta proliferativa celular frente a fatores de crescimento e citocinas (MALEMUD, 2007), e sua ativação pode ser observada em processos de proliferação, morte celular e remodelação de citoesqueleto (CHAMBARD et al., 2007, DUMESIC et al., 2009).

A família JNK compreende um subgrupo das MAPK's (JNK1, JNK2 e JNK3) (WAGNER; NEBREDA, 2009), originalmente isolada de pulmões de ratos e ativada em resposta a citocinas, irradiação UV e agentes que danificam o DNA. Estão envolvidas na proliferação celular e apoptose através da ativação de vários alvos (HUANG et al., 2010). Ainda são responsáveis pela regulação da expressão e ativação de mediadores inflamatórios, incluindo TNFα, IL-2, selectina E e metaloproteinases (MANNING; DAVIS, 2003; RINCON; DAVIS, 2009).

A família p38 está relacionada à resposta inflamatória, apoptose e ciclo celular. Quando ativada, a p38 fosforila inúmeros substratos em todos os compartimentos celulares (AOUADI et al., 2006). Demonstrou-se ainda estar envolvida no controle da produção de citocinas, como TNF-α, IL-1β, IL-6 e na patogênese de doenças inflamatórias (WAGNER; NEBREDA, 2009).

O estresse ribotóxico induzido pelo DON ativa membros da família das Src tirosina quinase, que são reguladores "*upstream*" de um grande número de vias de sinalização intracelular (LOWELL, 2004). Eles representam sinais críticos que precedem à ativação das MAPK's e consequentemente a indução de resposta "*downstream*". A localização de c-Src quinase nas junções oclusivas do tecido epitelial sugere que a atividade da Src quinase desenvolve um importante papel na regulação da estrutura e função deste tipo de junção. Em células Caco-2 a c-Src quinase está envolvida na desestabilização das junções oclusivas mediante a exposição a um estresse oxidativo (BASUROY et al., 2006). Esses elementos convergentes sugerem que a via de transdução de sinal, via Src e MAPK, aumentam a permeabilidade das células após a exposição ao desoxinivalenol.

Toxicidade subaguda/aguda: os sinais clínicos da exposição aguda ao desoxinivalenol em espécies sensíveis incluem desconforto abdominal, aumento da salivação, mal-estar, diarréia, emese e anorexia (FORSYTH et al., 1977; YOUNG et al.; 1983, PESTKA et al., 1987; PRELUSKY; TRENHOLM, 1993). Segundo estudos realizados por Pestka e Smolinski (2005), 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. Forsell et al. (1987) demonstraram que a aplicação intraperitoneal de 10-1000 mg/Kg de peso vivo (PV) de DON pura em camundongos resultou em necrose do trato gastrointestinal, medula óssea, baço e timo. Em outro estudo em camundongos com 7,5 mg DON/Kg de peso vivo observou-se atrofia de timo, baço e placas de Peyer (ARNOLD et al., 1986). Ambos os estudos indicam que os tecidos linfóides são particularmente sensíveis ao desoxinivalenol. A ingestão de ração contaminada com doses relativamente baixas de DON (0,05 mg/Kg PV)

pode induzir emese em suínos (FORSYTH et al. 1977; PRELUSKY; TRENHOLM, 1993; PESTKA et al., 1987)

Toxicidade subcrônica/crônica: a exposição prolongada a dietas contaminadas com DON resulta, principalmente, na diminuição do desempenho zootécnico dos animais gerando uma grande perda econômica, especialmente na produção suína (GOYARTS, 2006). Estudos relatam que DON na dose 1 a 2 mg/Kg de ração induz à recusa alimentar parcial em suínos que ingerem alimentos naturalmente contaminados (ROTTER et al., 1994), enquanto que na dose de 12 mg/Kg ocorre a recusa por completo (YOUNG et al., 1983). Além dos efeitos negativos no consumo e ganho de peso, já descrito por outros autores, BergsjØ et al. (1993) descreveram a diminuição na eficiência alimentar em suínos que receberam ração naturalmente contaminada com DON nas doses de 2-4 mg/Kg. Os efeitos anoréxicos e eméticos provocados por desoxinivalenol são, supostamente, mediados pelo sistema serotonérgico, conforme já descrito por Rotter et al (1996), onde DON diminui as concentrações de serotonina e seus metabólitos no fluido cerebroespinhal de ratos e suínos (PRELUSKY et al., 1993).

Em relação aos efeitos sobre os parâmetros sanguíneos, Rotter et al (1994) relataram um aumento da relação albumina/globulina, em suínos que ingeriram concentrações de 0.75-3 mg/Kg de DON, indicando que esta toxina pode alterar o perfil dessas proteínas sanguíneas. Uma diminuição da concentração de proteína e albumina também foi relatada em suínos expostos a dietas contaminadas com 3,5 mg/Kg de DON (BERGSJØ et al., 1992). Döll et al (2003) observaram uma diminuição das proteínas plasmáticas e glutamato desidrogenase (GLDH) em leitões, após a ingestão de milho contaminado com até 3,9 mg/Kg de DON.

Estudos *in vivo* e *in vitro* demonstram que o sistema imune inato é o principal alvo do desoxinivalenol, 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 (PESTK; 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 susceptibilidade a doenças infecciosas (BONDY; PESTKA, 2000; PESTKA; SMOLINSKI, 2005).

Estudos *ex vivo*, utilizando explantes de intestino de suínos demonstraram que após 4 horas de cultivo com 10 mmol/L de DON, importantes alterações morfológicas,

como a coalescência das vilosidades, lise dos enterócitos, edema intersticial e restos celulares, são visualizadas no epitélio intestinal (KOLF-CLAUW et al., 2009).

2.1.2.3 Carcinogenicidade e teratogenicidade

A Agência Internacional de Pesquisa do Câncer (IARC) concluiu em 1993 que « não há evidências em animais experimentais para a carcinogenicidade do desoxinivalenol ». Entretanto, na interpretação de resultados obtidos em literatura, a IARC (1993) concluiu que o 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 ».

Estudos realizados em ratos, camundongos e coelhos não demonstraram efeitos teratogênicos. Contudo, efeitos embriotóxicos foram observados em camundongos e coelhos quando administradas doses ≥ 1 mg/Kg/PV (KHERA et al., 1984). Segundo dados publicados pelo *Scientific Committee on Food* (1999), estudos demonstram que ocorre uma ligeira diminuição na fecundidade em ratos após a administração de uma única dose de 2 mg/Kg, enquanto que outro estudo demonstrou que doses de até 1 mg/Kg não surtem qualquer efeito.

Estudos com leitoas alimentadas com 0,1 a 4,8 mg/Kg DON/ração não demonstraram toxicidade materna ostensiva ou redução do consumo de ração, mas doses de 1 a 2 mg/Kg causam redução no ganho de peso, não apresentando efeitos sobre o tamanho da leitegada, sobrevivência ou deformidades (BERGSJØ et al., 1992).

2.1.3 Epitélio Intestinal e o Sistema Imune

As micotoxinas podem causar imunossupressão e aumentar a susceptibilidade às doenças (BEREK et al., 2001), afetando tanto a imunidade inata como a adquirida. O epitélio intestinal é considerado como barreira física contra patógenos e possui tanto componentes da imunidade inata quanto da adquirida (linfócitos e IgA), pode ser um excelente local para se evidenciar esse efeito.

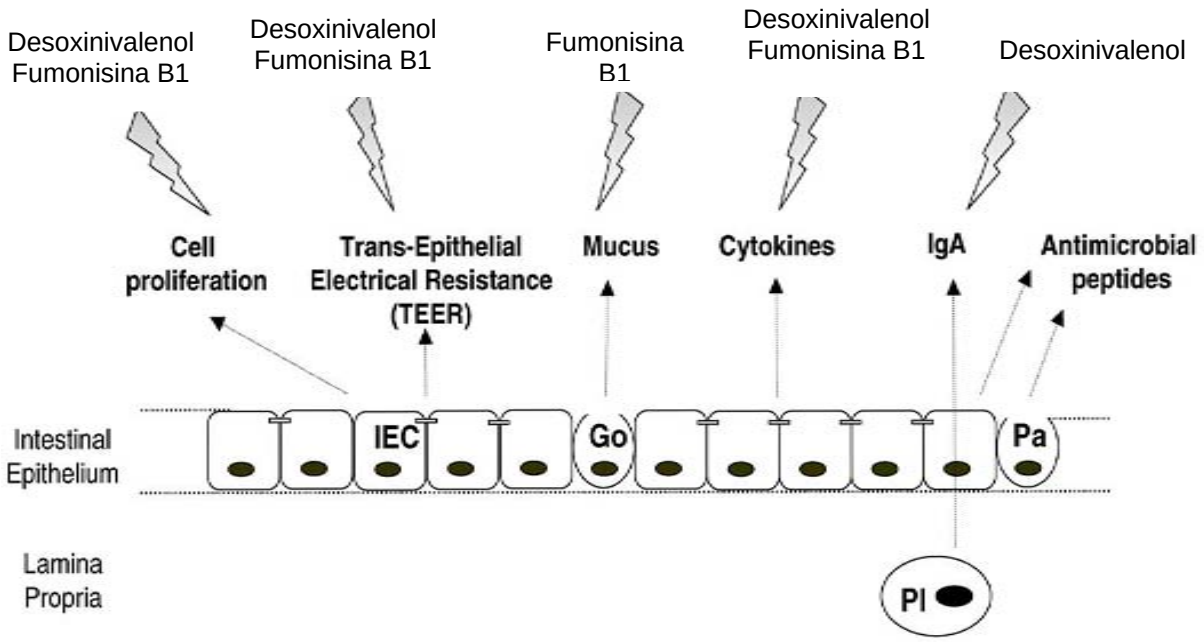
A camada epitelial do intestino é a primeira barreira que previne a entrada de patógenos e a sua integridade é mantida por estruturas intercelulares organizadas (BOUHET; OSWALD, 2005). Ela é formada por uma camada simples de células que atua

como um filtro, permitindo a translocação de nutrientes essenciais da dieta, eletrólitos e água do lúmen intestinal para a circulação. Constitui também a maior e mais importante barreira que impede à passagem de substâncias nocivas, incluindo antígenos estranhos, microrganismos e toxinas do ambiente externo. Alguns fatores químicos como hormônios, neurotransmissores, proteases e micotoxinas podem modificar essa estrutura, e alterar a diferenciação das células do epitélio intestinal (BOUHET; OSWALD, 2005).

Para manter sua efetiva função de barreira celular, o epitélio intestinal precisa estar constantemente se regenerando e mantendo, desta forma, a sua integridade. Células maduras derivadas de células-tronco migram ao longo do eixo criptas-vilosidades em direção ao ápice do vilo, diferenciando-se gradualmente conforme atingem o ápice (BOOTH; POTTEN, 2000). As micotoxinas, assim como as fumonisinas, são descritas como bloqueadoras das fases G0/G1 do ciclo das células epiteliais, diminuindo dessa forma a proliferação celular (BOUHET; OSWALD, 2005), enquanto que DON, em doses baixas, interfere na diferenciação dos enterócitos (KASUGA et al., 1998).

Com a ingestão de alimentos, as células da mucosa intestinal podem ficar expostas a quantidades variáveis de toxinas (PRELUSKY et al., 1996). Estudos *in vitro* e *in vivo* demonstraram que a permeabilidade intestinal é regulada por diversos fatores, entre eles os xenobióticos e as citocinas (GROSCHWITZ; HOGAN, 2009) (Figura 5). Segundo Bouhet e Oswald (2005), a integridade da barreira física do epitélio intestinal pode ser mensurada por meio da resistência elétrica trans-epitelial (TEER) dos enterócitos. Algumas micotoxinas como a fumonisina e o desoxinivalenol são capazes de alterar a resistência trans-epitelial nas células intestinais (BOUHET et al., 2004). O mecanismo de ação não está completamente elucidado, mas segundo McLaughlin et al. (2004) isso ocorre devido à diminuição na quantidade de proteínas nas junções celulares e, de acordo com Leung et al. (2003), a redução na biossíntese de esfingolipídios, que é inibida por ação das micotoxinas, pode alterar a regulação elétrica das células epiteliais.

Figura 5 – Esquema demonstrando os principais efeitos das micotoxinas desoxinivalenol e fumonisina sobre o mecanismo de defesa local desenvolvido pelas células do epitélio intestinal (IEC). Go (Células Goblets), Pa (Células de Paneth) e PI (Plasmócitos secretando imunoglobulinas). Fonte: Bouhet e Oswald, 2005 (modificado).



Foi demonstrado, através de estudo *in vitro*, que em linhagens celulares de suínos (IPEC-1) e de humanos (Caco-2), DON diminui a resistência elétrica trans-epitelial (TEER), aumentando a permeabilidade paracelular (PINTON et al., 2009). Incubando células IPEC-1 com 30 µmol/L DON, ocorre a ativação da via de sinalização das MAPK p44/42 ERK, inibindo-se a expressão de proteína claudina 4 e alterando-se a função de barreira exercida pelo epitélio intestinal (PINTON et al., 2010). No modelo *ex vivo*, utilizando explantes de intestinos de suínos tratados com DON, também observou-se um aumento da permeabilidade do tecido (PINTON et al., 2009).

No que se refere à imunidade inata da mucosa intestinal, sabe-se que a produção de muco a partir das células caliciformes tem importante função na lubrificação e barreira protetora deste epitélio. Sabe-se que quando a mucosa intestinal é "desafiada" ocorre um incremento no número destas células no intestino com o intuito de aumentar a produção de muco. Entretanto, somente um estudo na literatura demonstra que fumonisina induz à hiperplasia de células epiteliais da mucosa intestinal de frangos de corte (BROWN et al., 1992). Portanto, mais estudos neste aspecto são necessários para verificar a influência desta e outras micotoxinas sobre a proliferação de células caliciformes e a produção de muco.

Outro fator importante é a produção de citocinas pelas células epiteliais que desempenham papel fundamental no recrutamento de células inflamatórias para a defesa desta mucosa. De acordo com estudos realizados por Oswald et al. (2003), leitões alimentados com baixos níveis de fumonisina apresentaram menor expressão de IL-8 no íleo, sugerindo que este fato pode ter grande influência na maior susceptibilidade à *Escherichia coli* observada nestes animais quando comparados ao grupo controle.

Esse menor recrutamento de células inflamatórias, ocasionado pela diminuição na expressão de IL-8, segundo os mesmos autores, pode estar associado à ação desta toxina na redução de proliferação celular e integridade da mucosa do intestino, aumentado a susceptibilidade dos animais à colonização bacteriana. Vários estudos investigam os efeitos das diversas micotoxinas sobre a proliferação de células epiteliais intestinais e morfologia intestinal, o que alteraria sua capacidade de proteção do trato intestinal.

2.1.4 Referências

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3 OBJETIVOS

3.1 OBJETIVO GERAL

i. Avaliar os efeitos sistêmicos da contaminação por Desoxinivalenol (DON), Fumonisina (FB) e sua associação em suínos utilizando modelos *in vivo* e *ex vivo.*

3.2 OBJETIVOS ESPECÍFICOS

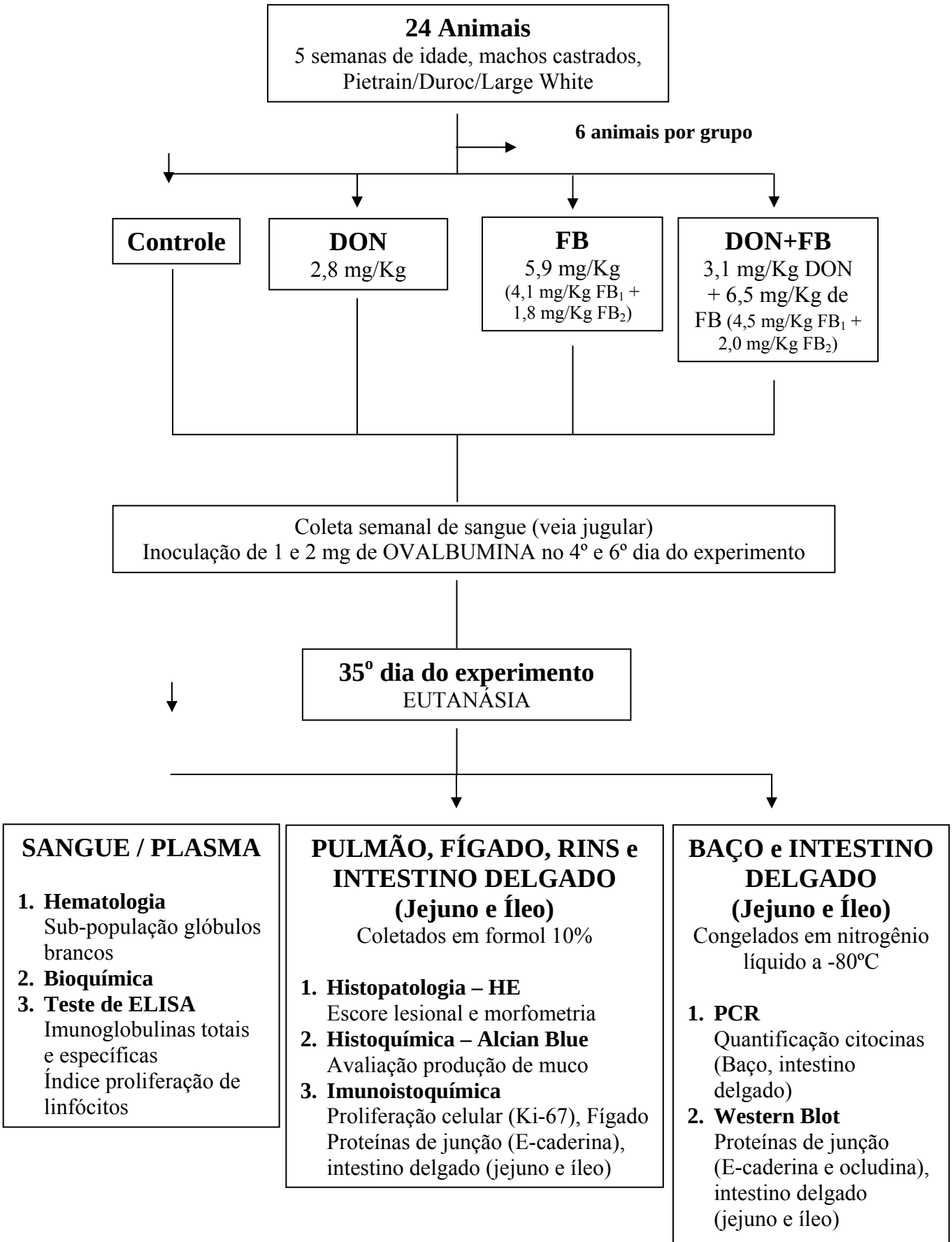
i. Avaliar os efeitos das micotoxinas Desoxinivalenol, Fumonisina B e sua associação sobre:

   - ✓ Desempenho zootécnico.
   - ✓ Parâmetros hematológicos e bioquímicos.
   - ✓ Resposta imune.
   - ✓ Morfologia do fígado, pulmão, rim e intestino.
   - ✓ Proliferação celular e expressão de proteínas de junção.

ii. Avaliar a habilidade do Desoxinivalenol em ativar as MAPK's, utilizando os modelos *ex vivo* (explantes de jejuno expostos ao DON) e *in vivo* (amostras de jejuno coletadas de animais expostos a raçao contaminada por DON).

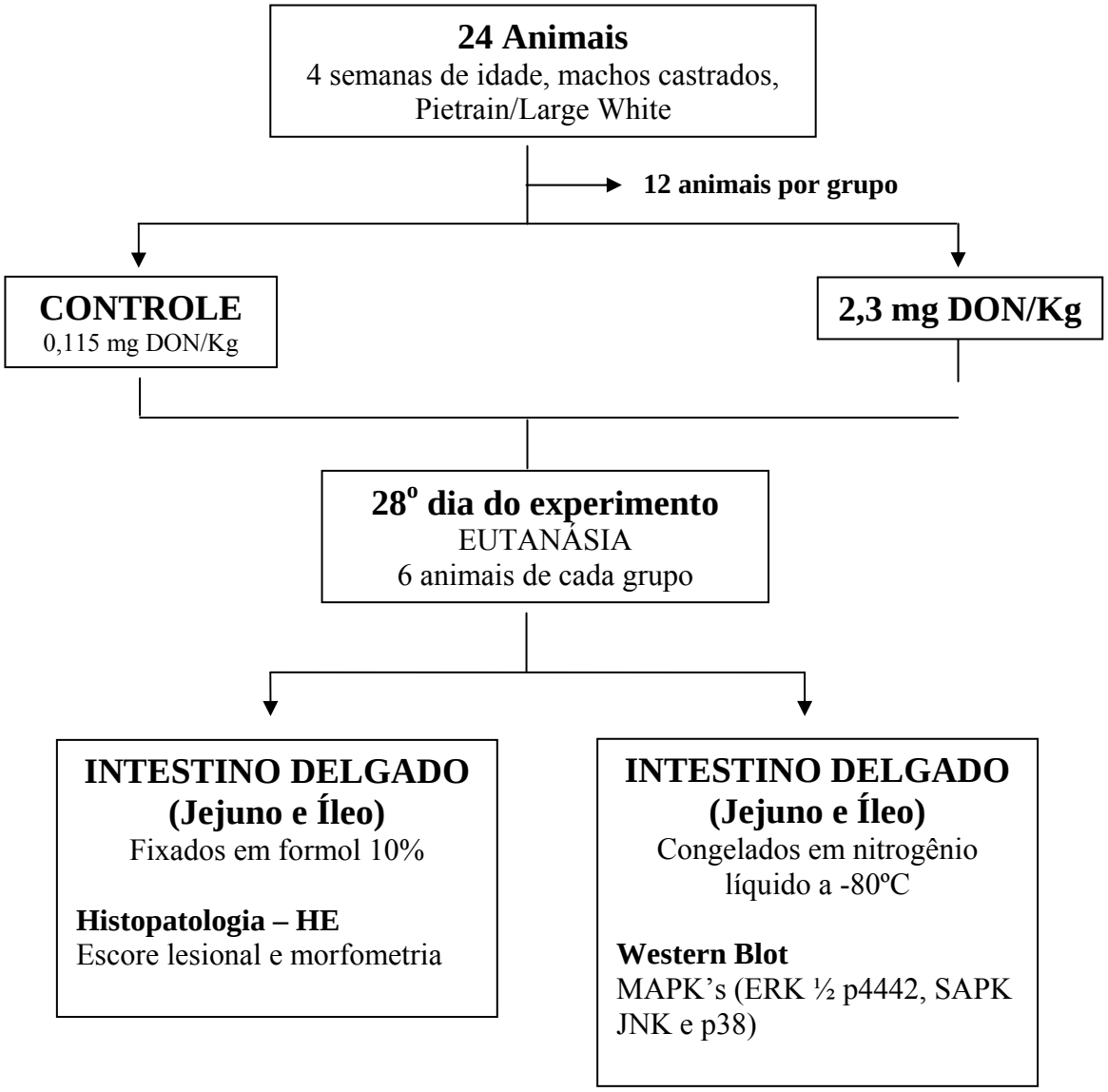
4 DELINEAMENTO EXPERIMENTAL

4.1 EXPERIMENTO *IN VIVO* (ARTIGOS 1 E 2)

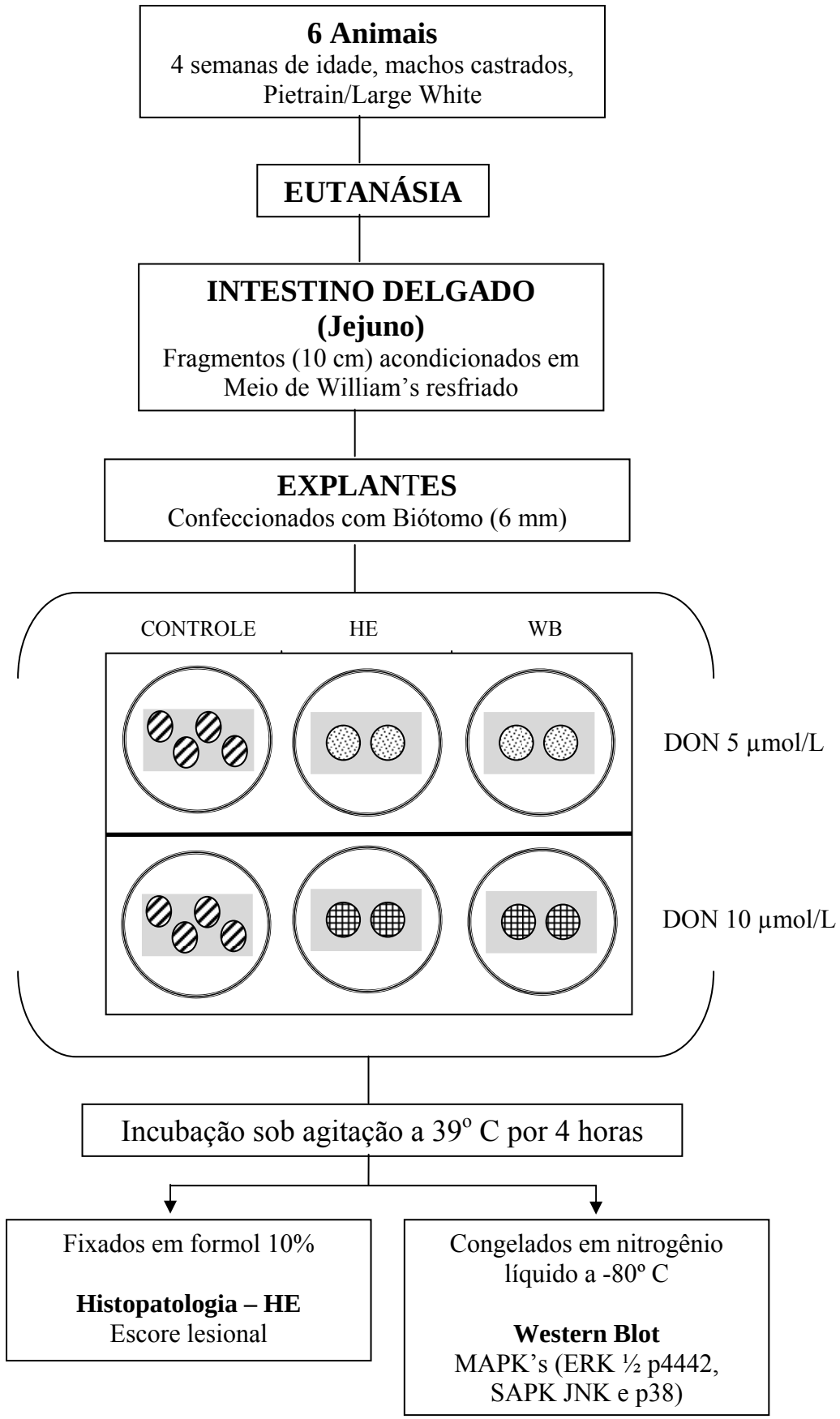


4.2 EXPERIMENTO IN VIVO E EX VIVO (ARTIGO 3)

Modelo In vivo



Modelo Ex vivo



5 ARTIGOS PARA PUBLICAÇÃO

Artigo 1. Individual and combined effects of subclinical doses of deoxynivalenol and fumonisins in piglets.

Artigo 2. Chronic ingestion of deoxynivalenol and fumonisin, alone or in interaction, induces morphological and immunological changes in the intestine of piglets.

Artigo 3. The food contaminant deoxynivalenol activates the mitogen activated protein kinases in the intestine: comparison of *in vivo* and *ex vivo* models

ARTIGO 1

Individual and combined effects of subclinical doses of deoxynivalenol and fumonisins in piglets

Artigo editado de acordo com as normas de publicação da *Molecular Nutrition & Food Research*

Individual and combined effects of subclinical doses of deoxynivalenol and fumonisins in piglets

Bertrand GRENIER[1], Ana-Paula LOUREIRO-BRACARENSE[2], Joelma LUCIOLI[2] GrazielaDROCIUNAS PACHECO[1,2], Anne-Marie COSSALTER[1], Gerd SCHATZMAYR[3], Wulf-Dieter MOLL[3], Isabelle P. OSWALD[4]

ABSTRACT: Deoxynivalenol (DON) and Fumonisin B (FB) are the most frequently toxins from *Fusarium* species and most commonly co-occur in animal diet. These mycotoxins were studied for their toxicity in piglets on several parameters including alteration in plasma biochemistry, organs histopathology and immune response. Twenty four, 5-week-old animals were randomly assigned to four different groups, receiving separate diets for five weeks; a control diet, a diet contaminated with either DON (2.8 mg/Kg) or FB (5.9 mg/Kg), or both toxins. At days 4 and 16 of the trial, the animals were subcutaneously immunized with ovalbumin to assess their specific immune response. The different diets did not affect the animal performance and had minimal effect on hematological and biochemical blood parameters. By contrast, DON and FB induced histopathological lesions in the liver, the lung and the kidney of exposed animals. The liver was significantly more affected when the two mycotoxins were present simultaneously. The contaminated diets also altered the specific immune response upon vaccination as measured by reduced anti-ovalbumnin IgG level in the serum and reduced lymphocyte proliferation upon antigenic stimulation. Because cytokines play a key role in immunity, the expression levels of IL-8, IL-1β, IL-6 and MIP-1β were measured, by RT-PCR at the end of the experiment. The expression of these four cytokines was significantly decreased in the spleen of piglets exposed to multi-contaminated diet. Taken together, our data indicate that ingestion of multicontaminated diets induces greater histopathological lesions and higher immune suppression than ingestion of mono-contaminated diets.

Keywords: *Fusarium* mycotoxins. Combination (co-contamination). Immune system (specific immunity). DON, FB. Swine. Subclinical doses

[1] INRA, Unité de Pharmacologie-Toxicologie, Toulouse, France.
[2] Universidade Estadual de Londrina, Lab. Patologia Animal, Londrina, Brazil
[3] BIOMIN Research Center, Technopark 1, Tulln, Austria
[4] Address correspondence: INRA-Laboratoire de Pharmacologie-Toxicologie; 180 chemin de Tournefeuille BP 93173; 31027 Toulouse Cedex 3;Phone +33561285480; E-Mail : isabelle.oswald@toulouse.inra.fr

1 INTRODUCTION

Mycotoxins are secondary metabolites of fungi that may contaminate animal and human feeds. They are frequently detected in grains, but also in fruits, vegetable, nuts, and silages. The Food and Agricultural Organization estimated that as much as 25% of the world's agricultural commodities are contaminated with mycotoxins and the economic losses due to mycotoxins contamination are estimated in billions dollars annually worldwide [1]. Clinical signs caused by mycotoxins range from acute mortality to slow growth and reduced reproductive efficiency. Consumption of mycotoxins may also result in impaired immunity and decreased resistance to infectious diseases [2].

Worldwide surveys on the occurrence and contamination levels of mycotoxins in raw materials indicate that toxins produced by *Fusarium* species are of concern [3-5]. Among the fusariotoxins, deoxynivalenol (DON) and fumonisin B (FB) are frequently detected with concentrations up to 927 mg DON/Kg and 300 mg FB/Kg. Among cereal samples collected from European countries, 54% were co-contaminated with DON and FB [6]. Similarly, in France, 65% of the maize kernels harvested during 2004-06 were co-contaminated with DON and FB (Arvalis-Institut du vegetal, unpublished data). These two mycotoxins are of major concern not only in terms of their ubiquitous distribution but also because of their effects on human and animal health.

At high concentrations, FB causes equine leukoencephalomalacia and porcine pulmonary edema, and it is nephro and hepatotoxic and carcinogenic in rats and mice. FB1 has been classified as a potential human carcinogen (class 2B) by the International Agency for Research on Cancer. In humans, consumption of FB-contaminated food has been linked with human oesophageal cancer and neural tube defects [7]. Disruption of sphingolipid biosynthesis appears to be one mechanism involved in FB toxicity, with inhibition of ceramide synthase [7] leading to accumulation of sphingoid bases (sphinganine and sphingosine). The effects of ingestion of low doses of FB1 are less documented, but revealed pathological alterations of the lungs and an increase of intestinal colonization by opportunistic pathogenic bacteria in piglets [8-10].

Acute exposure to high doses of DON induces diarrhea, vomiting, leukocytosis, and gastrointestinal hemorrhage. Anorexia, growth retardation and immunotoxicity occur in rodents and pigs following chronic DON ingestion [11]. At the cellular level, DON interferes with the active site of peptidyl transferase on ribosomes, and inhibits protein synthesis [11]. Further, binding of DON to the ribosome in eukaryotic cells

triggers a "*ribotoxic stress response*", which involves phosphorylation of the mitogen-activated protein kinases (MAPK) [12]. MAPK activation modulates expression of genes associated with immune response, chemotaxis, inflammation, and apoptosis. The cellular and molecular mechanisms of the immunomodulating action of DON were described in numerous studies in mice and murine cell lines [13]. Depending on dose and frequency of exposure, DON can be either immunosuppressive or immunostimulatory [11, 14]. Prolonged ingestion of DON produces elevation of serum immunoglobulin A in plasma [13-15] while increasing the susceptibility to infectious diseases [11].

The toxicity of combinations of mycotoxins cannot always be predicted based upon their individual toxicities [1]. Interactions between concomitantly occurring mycotoxins can be antagonistic, additive, or synergistic. The data on combined toxic effects of mycotoxins are limited and therefore, the actual combined health risk from exposure to mycotoxins is unknown. Assessment of the interaction of *Fusarium* mycotoxins has been investigated *in vitro* on immune cells and intestinal epithelial cells [16. 17]. *In vivo* experiments have also been done on mice, pig and poultry using high doses of toxin and looking for animal performance. Among them, few studies were concern with the interaction between DON and FB [18, 19].

The purpose of this study was to compare the effects of low doses of DON and FB1 in pigs when fed individually and in combination with particular emphasis on their effects on the immune response. The experimental design was a factorial assay including control feed and feed contaminated with 3 and 6 mg/Kg DON and FB individually and in combination, respectively. These contaminations levels correspond to levels that occur naturally in cereals [1]. Results have been reported in terms of both general toxicological parameters including weight gain, hematology and plasma biochemistry and organ histology, as well as specific parameters describing immune system responses (total and specific antibody, lymphocyte proliferation, cytokine expression).

2 MATERIALS AND METHODS

2.1 ANIMALS

All animal experimentation procedures were carried out in accordance with the European Guidelines for the Care and Use of Animals for Research Purposes (Directive 86/609/EEC). Twenty-four, 5-week-old weaned castrated male pigs (Pietrain/Duroc/Large-white) were obtained locally. Male pigs were used in this protocol as it was previously demonstrated that a greater effect of DON and FB in male when compared to female pigs [20]. Animals were acclimatized for 1 week in the animal facility of the INRA Laboratory of Pharmacology and Toxicology (Toulouse, France), prior to being used in experimental protocols. Six pigs were allocated to each treatment on the basis of body weight. During the 35-day experimental period, animals were given free access to water and the assigned diet. They were observed daily and weighed weekly.

2.2 EXPERIMENTAL DIETS

Diets were manufactured at INRA facilities in Rennes (France), and formulated according to the energy and amino acid requirements for piglets. Feed composition is detailed in Table 1. Four different batches were prepared, one control batch and three batches artificially contaminated with the mycotoxins. Two strains of *Fusarium*, *F. graminearum* DSM-4528 and *F. verticillioides* M-3125 were used to produce the deoxynivalenol and fumonisins, respectively. These strains were grown separately on rice. Fumonisins were produced as previously described [21]. Deoxynivalenol, was extracted with ethyl acetate, and the extract dried on silica gel 60 (Merck, Darmstadt, Germany). The homogenized extracts contained 24 and 21 g/Kg DON and FB, respectively. The extracts containing the mycotoxins were mixed into the vitamins and minerals supplements and then incorporated into the cereal mixture before granulation.

The feed was analysed for mycotoxin content by Quantas Analytik GmbH (Tulln, Austria) and by using a multi-mycotoxin method [22]. Deoxynivalenol, zearalenone and enniatin were found to be naturally present in the cereals used, resulting in concentrations of 500, 50 and 100 µg/Kg of feed, respectively. All other mycotoxins, including aflatoxins, T-2 toxin, HT-2 toxin and ochratoxin A were below the limit of detection. The mono-contaminated diets contained 2.8 mg de DON/Kg of feed and 5.9 mg of FB/Kg of feed (4.1

mg FB_1/Kg + 1.8 mg FB_2/Kg of feed) while the contaminated diet contained 3.1 mg of DON and 6.5 mg of FB/Kg of feed (4.5 mg FB_1/Kg + 2.0 mg FB_2/Kg of feed).

Table 1 – Composition of the experimental diet

Ingredient (%)	
Wheat	47.50
Soybean meal	24.30
Barley	22.90
Calcium phosphate	1.12
Calcium carbonate	1.00
Vitamin and mineral premix[1]	0.50
Vegetable oil	1.40
Sodium chloride	0.40
Phytase	0.01
Lysine	0.465
Methionine	0.165
Threonine	0.195
Tryptophan	0.045
Composition[2]	
Starch (g)	476.8
Crude protein (g)	218.3
Crude fiber (g)	37.5
Ca (g)	10.5
P (g)	6.5
K (g)	8.7
Net energy (MJ)	15.6

2.3 Experimental Design and Sample Collection

On the 4th and 16th day of the experiment, all piglets were immunized by subcutaneous inoculation with 1 and 2 mg of ovalbumin (OVA) respectively (Sigma, St-Quentin Fallavier, France), dissolved in sterile phosphate buffered saline (PBS) and mixed with incomplete Freund's adjuvant (Sigma). At weekly time intervals, blood samples were aseptically collected from the left jugular vein. Blood was collected in tubes containing sodium heparin or EDTA (Vacutainer®, Becton-Dickinson, USA) for blood culture or blood formula, respectively. Plasma samples were obtained after centrifugation of heparinized blood and stored at -20°C for later analysis. After 35 days of dietary exposure to mycotoxins, immediately after electrical stunning, pigs were killed by exsanguination. Samples of lung,

[1] Vitamin A, 2,000,000 IU/Kg; vitamin D3, 400,000 IU/Kg; vitamin E, 4000 mg/Kg; vitamin C, 8000 mg/Kg; vitamin B1, 400 mg/Kg; vitamin K3, 400 mg/Kg; iron, 20,000 mg/Kg; copper, 4000 mg/Kg; zinc, 20,000 mg/Kg; manganese, 8000 mg/Kg.

[2] Corresponding to 1000 g dry matter/Kg

liver and kidney were collected from all groups and fixed in 10% buffered formalin for histopathological analysis. In addition, a portion of the spleen was collected from euthanatized animals, flash-frozen in liquid nitrogen and stored at -80°C until processed for measurements of cytokine mRNA.

2.4 Hematology and Biochemistry

Hematological analysis was carried out using the impedance coulter LH500 (Beckman Coulter, Villepinte, France). Sub-populations of white blood cells (lymphocytes, monocytes, neutrophils, eosinophils and basophils) were also studied and made manually on 100 leukocytes on May-Grünwald Giemsa stained smears. Plasma concentrations of total proteins, albumin, urea, creatinin, cholesterol, triglycerides and activity of gamma-glutamyl transferase were determined by a Vitros 250 analyzer (Ortho Clinical Diagnostics, Issy les Moulineaux, France) at the Veterinary School of Toulouse (France).

2.5 Histology

The tissue pieces were dehydrated through graded alcohols and embedded in paraffin wax. Sections of 3µm 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 to 2500 μm^2 per slide was observed. As displayed in the Table 2, microscopic observations led to the identification of some different lesions according to the interest organ, and allowed to establish a lesional score per animal. Based on a recent method published [23], we calculated the lesion according to intensity or observed frequency, scored from 0 to 3. For each lesion, the score of the extent was multiplied by the severity factor. For each tissue, the minimal scores were 0 and the maximal scores were 21, 33 and 15 for liver, lung and kidney, respectively (Table 2).

Table 2 –Establishment of a lesional score - endpoints used to assess histological lesions

Tissue	Type of lesions	Severity factor	Total score
LIVER	Disorganization of hepatic cords	1	21
	Hepatic cell vacuolation	1	
	Apoptosis	2	
	Megalocytosis	2	
	Nuclear vacuolation	1	
LUNG	Alveolar edema	2	33
	Interstitial pneumonia	2	
	BALT depletion	2	
	Hypertrophy muscle cell	2	
	Hemorrhage	2	
	Vascular congestion	1	
KIDNEY	Nuclear change	1	15
	Mitosis	1	
	Cytoplasmic vacuolation	1	
	Tubular casts	1	
	Congestion	1	

2.6 Measurement of Hepatocyte Proliferation

The cellular proliferation activity was assessed by counting Ki-67 positive nuclei on formalin-fixed embedded liver section as already described [24]. Briefly, the sections were incubated with the primary antibody (Zymed Ki-67 Clone 7B11 – diluted 1:50) at 4° C overnight in a humidity chamber, then the secondary antibody (Kit Super Picutre™ Zymed, South San Francisco, CA, USA) was applied and followed by the addition of a chromogen (3,3'-diaminobenzidine). Finally, the tissue sections were counterstained with hematoxylin and mounted under coverslips using a permanent mounting medium. The number of Ki-67 positive nuclei among the total of 100 nuclei was counted on the sections under light microscopy at 40x magnification. The proliferative index was calculated by Ki-67 positive cells/total cells x100.

2.7 Measurement of Total and Specific Immunoglobulin Subsets

The total concentration of the immunoglobulin subsets was measured by ELISA as already described [25]. Briefly, the different isotypes were detected with the appropriate peroxidade anti-pig IgA or IgG (Bethyl, Interchim, Montluçon, France) and were quantified by reference to standard curves constructed with known amounts of pig immunoglobulin classes. Titers of specific antibody anti-ovalbumin were also measured by ELISA [14]. Briefly, the anti-ovalbumin antibodies were detected with peroxidase-labeled anti-pig IgG or IgA (Bethyl). Absorbance was read at 450 nm using an ELISA plate reader (Spectra thermo, TECAN, NC, USA) and the Biolise 2.0 data management software.

2.8 Determination of Lymphocyte Proliferative Index

Lymphocyte proliferation was measured on blood samples collected at different times of the experimental period. The quantification was performed in 96 well plates as already described [15, 26]. The results were expressed as stimulating index of lymphocyte proliferation calculated as counts per minute in stimulated culture/cpm in control non-stimulated culture.

2.9 Determination of the Expression of mRNA Encoding for Cytokines by Real-Time PCR

Tissue RNA was processed in lysing matrix D tubes (MP Biomedicals, Illkirch, France) containing guanidine-thiocyanate acid phenol (Extract-All®, Eurobio, les Ulis, France) for use with the FastPrep-24 (MP Biomedicals, Illkirch, France). Concentrations, integrity and quality of RNA were determined spectrophotometrically ($O.D._{260}$) using Nanodrop ND1000 (Labtech International, Paris, France). Besides this inspection, 200 ng of RNA was analyzed by electrophoresis. The reverse transcription and real-time PCR steps were performed as already described [26]. RNA non-reverse transcripted was used as the non-template control for verification of a no genomic DNA amplification signal. Specificity of PCR products was checked out at the end of the reaction by analyzing the curve of dissociation. In addition, the size of amplicons was verified by electrophoresis. The sequences of the primers used are detailed in Table 3. Primers for MIP-1beta, IL-8 and IL-6 detection were designed using Primer Express® software (Applied Biosystems, Courtaboeuf, France). Primers were purchased from Invitrogen (Cergy Pontoise, France). Amplification efficiency and initial fluorescence were determined by DART-PCR method, then values obtained were normalized by both house-keeping genes beta2-µglobulin and ribosomal protein L32 (RPL32) and finally, genes expression was expressed relative to the control group as already described [27].

Table 3 –Nucleotide sequences of primers for real-time PCR

Gene	Primer sequence	Genbank no.	References
RPL32	Forward (300 nM) 5'-TGCTCTCAGACCCCTTGTGAAG-3' Reverse (300 nM) 5'-TTTCCGCCAGTTCCGCTTA-3'	NM_001001636	Flori et al. (2008)
β2-μglobulin	Forward (900 nM) 5'-TTCTACCTTCTGGTCCACACTGA-3' Reverse (300 nM) 5'-TCATCCAACCCAGATGCA-3'	NM_213978	Hyland et al. (2006)
IL-12p40	Forward (300 nM) 5'-GGTTTCAGACCCGACGAACTCT-3' Reverse (900 nM) 5'-CATATGGCCACAATGGGAGATG-3'	NM_214013	Devriendt et al. (2009)
IL-8	Forward (300 nM) 5'-GCTCTCTGTGAGGCTGCAGTTC-3' Reverse (900 nM) 5'-AAGGTGTGGAATGCGTATTTATGC-3'	NM_213867	Present study
IL-1β	Forward (300 nM) 5'-GAGCTGAAGGCTCTCCACCTC-3' Reverse (300 nM) 5'-ATCGCTGTCATCTCCTTGCAC-3'	NM_001005149	Devriendt et al. (2009)
MIP-1β	Forward (300 nM) 5'-AGCGCTCTCAGCACCAATG-3' Reverse (300 nM) 5'-AGCTTCCGCACGGTGTATG-3'	AJ311717	Present study
IL-6	Forward (300 nM) 5'-GGCAAAAGGGAAAGAATCCAG-3' Reverse (300 nM) 5'-CGTTCTGTGACTGCAGCTTATCC-3'	NM_214399	Present study

Notes : RPL32. ribosomal protein L32. IL. interleukin. MIP-1β. macrophage inflammatory protein-1 beta

2.10 STATISTICS

Following the Fisher test on equality of variances, one way ANOVA was used to analyses the different between the different groups of animals at each time point. P values of 0.05 were considered significant.

3 RESULTS

3.1 Individual or Combined Effect of DON and FB on Weight Gain, Hematological and Biochemical Parameters

During the experiment, piglets were weighed weekly and as reported in Table 4, ingestion of individual or combined DON-and-FB-contaminated diets did not significantly impair animal growth.

Table 4 –Individual or combined effect of DON and FB on weight gain

Body weight gain/day (Kg)	*Animal Diets*			
	Control	DON	FB	DON + FB
Days 1 to 14	0.36 ± 0.05^{a}	0.35 ± 0.03^{a}	0.43 ± 0.05^{a}	0.32 ± 0.07^{a}
Days 14 to 35	0.76 ± 0.05^{a}	0.65 ± 0.03^{a}	0.74 ± 0.06^{a}	0.68 ± 0.03^{a}

Notes: results are expressed as mean ± SEM for 5 animals. Means without a common letter differ P<0.05

At the end of the experiment, blood samples were taken from all piglets to investigate the effects of mycotoxins on hematological and biochemical variables (Table 5 and Table 6). Piglets fed either FB or FB+DON-contaminated diets displayed a significant decrease in neutrophils number (Table 5). An increase in creatinin concentration (P= 0.047) and a decrease in albumin concentration (P= 0.015) were also observed in the animal groups fed with FB and DON- contaminated diets, respectively. These alterations were not observed in animals fed with the diet contaminated with both toxins (Table 6).

Table 5 – Individual or combined effect of DON and FB on hematological parameters

Hematological parameters	***Animal diets (Week 6)***			
	Control	**DON**	**FB**	**DON + FB**
White blood cells (thousands/µL)	21.2 ± 1.9^{a}	19.6 ± 2.3^{a}	20.3 ± 2.8^{a}	18.2 ± 1.6^{a}
Lymphocytes (thousands/µL)	12.4 ± 1.9^{a}	11.4 ± 1.4^{a}	14.7 ± 2.1^{a}	12.6 ± 1.0^{a}
Neutrophils (thousands/µL)	7.3 ± 1.1^{a}	$7.0 \pm 1.1^{a,b}$	4.5 ± 0.9^{b}	4.6 ± 0.6^{b}
Red blood cells (thousands/µL)	6.2 ± 0.3^{a}	5.7 ± 0.2^{a}	6.1 ± 0.4^{a}	5.9 ± 0.4^{a}
Mean corpuscular volume (fL)	47.6 ± 0.8^{a}	47.1 ± 0.7^{a}	46.2 ± 0.5^{a}	50.4 ± 1.9^{a}
Hematocrit (%)	29.8 ± 1.6^{a}	27.0 ± 0.5^{a}	28.0 ± 1.9^{a}	29.5 ± 1.6^{a}
Hemoglobin (g/dL)	9.6 ± 0.5^{a}	9.0 ± 0.2^{a}	9.4 ± 0.5^{a}	9.7 ± 0.5^{a}
Mean corpuscular hemoglobin (pg)	15.4 ± 0.2^{a}	15.6 ± 0.2^{a}	15.6 ± 0.2^{a}	16.5 ± 0.8^{a}
Mean corpuscular hemoglobin concentration (%)	32.4 ± 0.4^{a}	33.2 ± 0.3^{a}	33.8 ± 0.6^{a}	32.8 ± 0.4^{a}

Notes: results are expressed as mean ± SEM for 6 animals. Means without a common letter differ P<0.05

Table 6 –Individual or combined effect of DON and FB on biochemical parameters

Biochemical parameters	*Animal diets (Week 6)*			
	Control	**DON**	**FB**	**DON + FB**
Urea (mmol/L)	3.8 ± 0.4^{a}	3.3 ± 0.4^{a}	4.2 ± 0.3^{a}	4.0 ± 0.4^{a}
Creatinin (µmol/L)	102.5 ± 5.3^{a}	98.0 ± 4.1^{a}	120.5 ± 5.6^{b}	101.6 ± 5.5^{a}
Cholesterol (mmol/L)	2.6 ± 0.2^{a}	2.4 ± 0.2^{a}	2.3 ± 0.1^{a}	2.3 ± 0.1^{a}
Triglycerides (mmol/L)	0.51 ± 0.07^{a}	0.34 ± 0.04^{a}	0.39 ± 0.06^{a}	0.41 ± 0.06^{a}
Total proteins (g/L)	59.8 ± 1.0^{a}	57.1 ± 2.1^{a}	59.9 ± 2.5^{a}	57.6 ± 2.5^{a}
Albumin (g/L)	34.3 ± 0.7^{a}	29.2 ± 1.5^{b}	35.1 ± 2.1^{a}	$32.8 \pm 2.1^{a,b}$
GGT (IU/L)	65.4 ± 8.6^{a}	88.6 ± 14.4^{a}	79.4 ± 15.0^{a}	77.0 ± 11.5^{a}

Notes: GGT, Gamma-Glutamyl Transferase. Results are expressed as mean ± SEM for 5 animals. Means without a common letter differ $P<0.05$

3.2 Individual or Combined Effect of DON and FB on Organs Histopathology

Liver, lung and kidney were collected at the end of the trial for histopathological analysis. The lesions observed in these three organs were mild to moderate for animal fed any of the three contaminated diets (DON, FB, DON+FB) (Figure 1).

The main histological lesions observed in the livers, were disorganization of hepatic cords, cytoplasmatic and nuclear vacuolization of hepatocytes, and megalocytosis (Figure 1A and 1B). Piglets fed either DON- or FB contaminated diets displayed significant liver lesions when compared to animal fed control-diet. The lesion score was further increased for animals fed diet contaminated with both toxins. The proliferation of hepatocytes was also assessed by counting Ki-67 positive cells in liver sections. The mean proliferation index were 16.4 ± 1.5 in the control group, 18.8 ± 3.3 in the DON treated group, 22.8 ± 1.7 in the FB treated group and 39.4 ± 12.8 in the DON + FB treated group ($P<0.001$, $P<0.01$ and $P<0.05$, for comparison between DON+FB and control, DON or FB groups, respectively).

In the lung, depletion of bronchiole associated lymphoid tissue and vascular disorders (peribronchiolar, alveolar hemorrhage and congestion) were the most frequent observed lesions (Figure 1C). Of note, that BALT structures were checked and were present in all individual pigs, evaluated in a comparable size and area between experimental groups. Alveolar edema showed a focal presentation (Figure 1D). As demonstrated by the lesional scores, lung lesions were only observed in animals receiving FB or FB + DON contaminated diets. In this later group, a medial hypertrophy of pulmonary arterioles was observed in half of the animals.

Lesions in the kidneys were mild as indicated by low lesion scores. The main observed lesions were degenerative changes in tubular epithelial cells (vacuolation of the cytoplasm and nucleus, Figure 1E and 1F) and interstitial infiltrate of lymphocytes with a

focal or multifocal pattern. These lesions were observed in animals receiving diets contaminated with DON, FB and both toxins.

3.3 Individual or Combined Effect of DON and FB on the Acquired Immune response

The main objective of this study was to assess the individual and combined effect of DON and FB on the immune response in piglets. Ingestion of diets contaminated with individual or combined mycotoxins neither altered the total plasmatic concentration of IgG and IgA nor modulated the lymphocyte proliferation upon concanavalin A stimulation (data not show).

The immunization protocol with ovalbumin (OVA) allowed us to investigate the effects of mycotoxins on antigen specific immunity [14, 26]. The ingestion of diet contaminated with DON or FB individually or in combination significantly altered the production of immunoglobulin after OVA vaccination (Figure 2). Animals fed mycotoxin-contaminated diet displayed a reduced anti-OVA IgG concentration in their plasma. However, because of high variability, the decrease was only significant for animals receiving FB-contaminated feed. This decrease was also observed for animals fed with both toxins. Concerning the effect of mycotoxins on the specific IgA concentration, we only observed a significant increase of this immunoglobulin isotype in piglets fed with DON-contaminated diet. However, when DON was fed in combination with FB, the increase of plasmatic specific IgA concentration was not observed (Figure 2).

Fig 1 – Individual and combined effects of DON and FB on liver, lungs and kidneys. Pigs received a control diet (□), or a DON-contaminated diet (▨), or a FB-contaminated diet (▨), or a diet contaminated with both toxins (■). (A) Hepatocyte cytoplasmatic vacuolization and (B) Hepatocyte megalocytosis (arrow). HE. 40x. (C) BALT depletion and peribronchiolar hemorrhage. HE. 10x and (D) Alveolar edema. HE. 40x. (E) Cytoplasmatic vacuolization of tubular cells and mitosis (arrow) and (F)Nuclear change (arrow) in tubular cells. HE. 40x. Lesion scores were established after histological examination according to the severity and the extent of the lesions. Values are mean ± SEM for 5 animals. Means without a common letter differ $P<0.05$

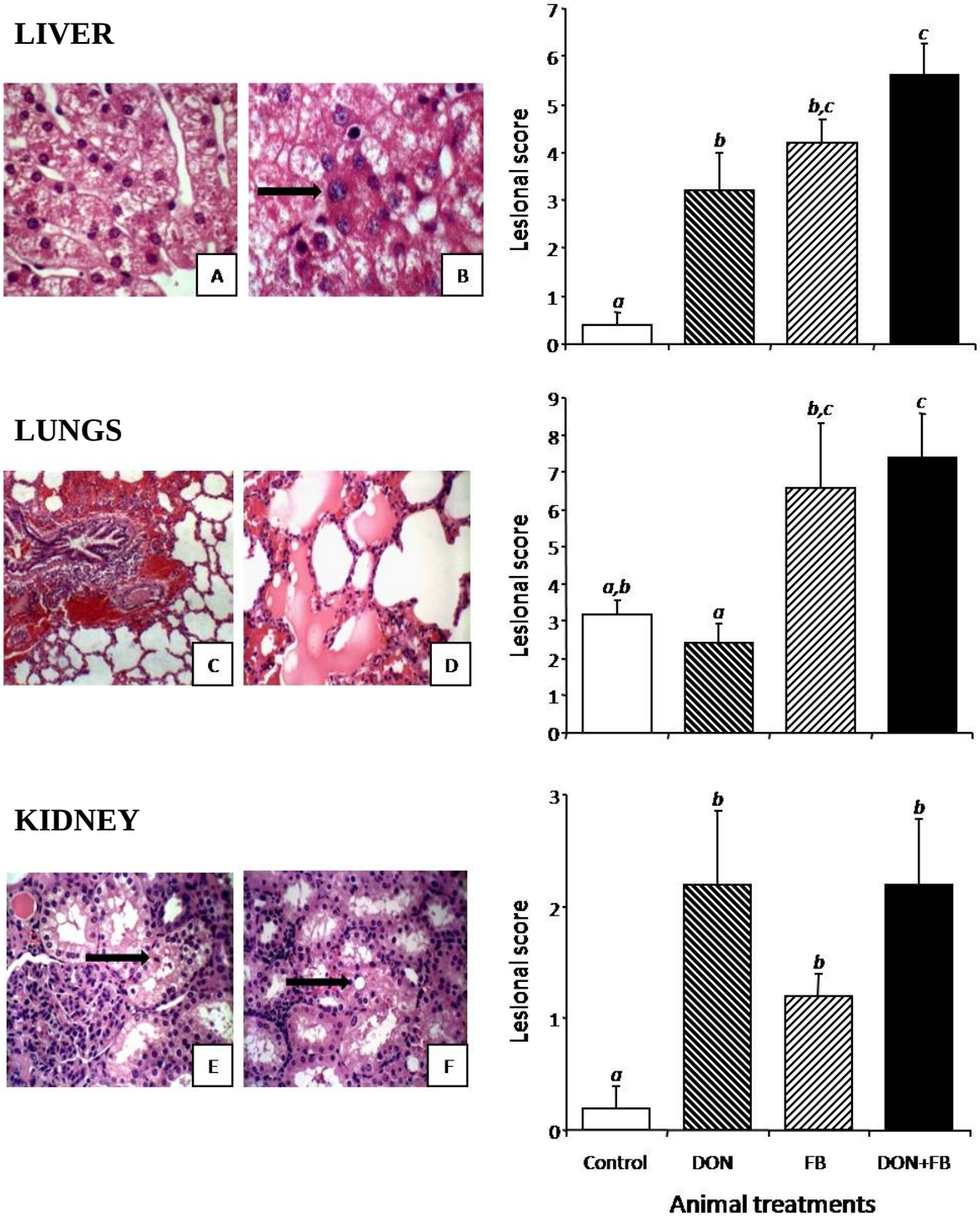
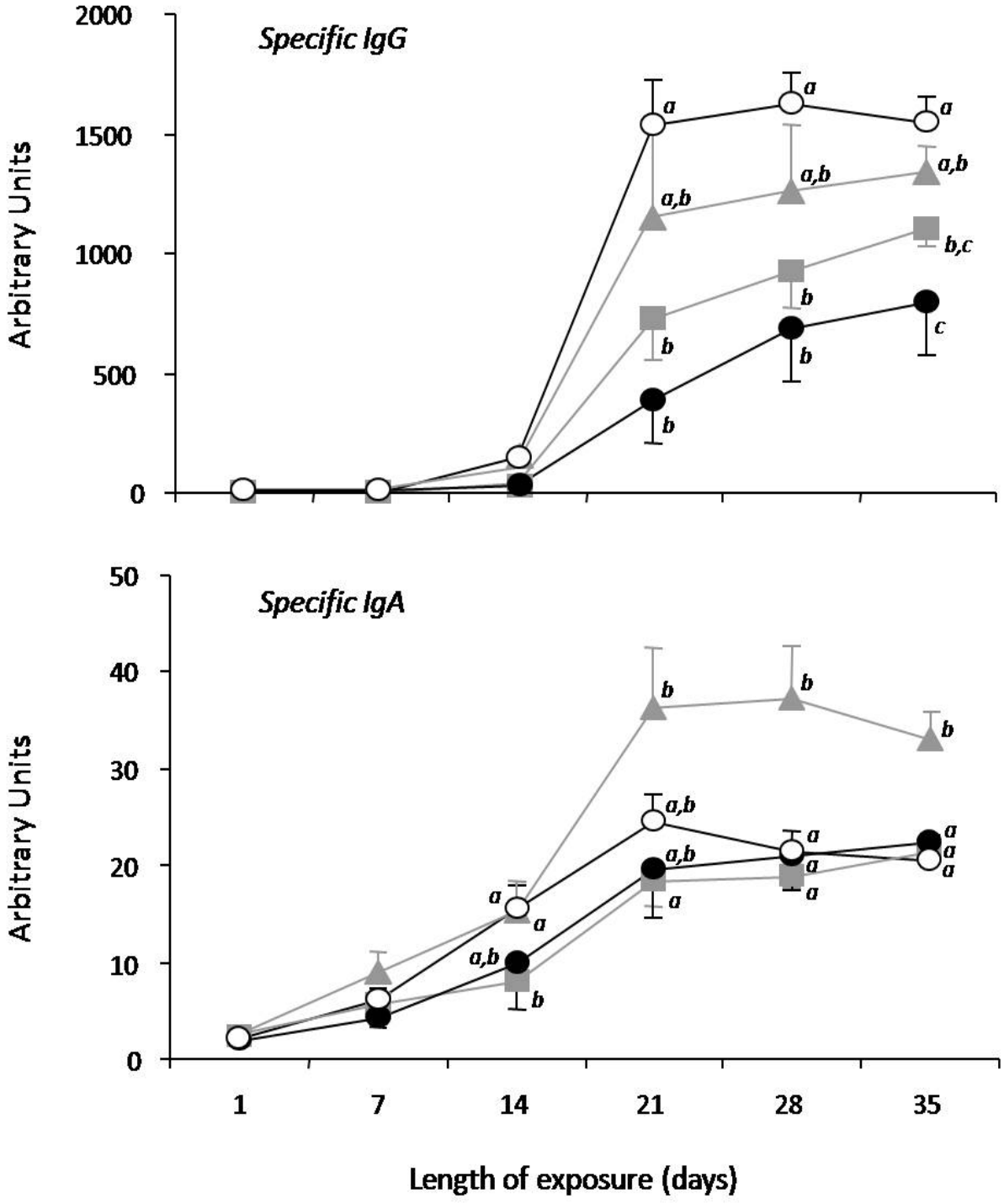
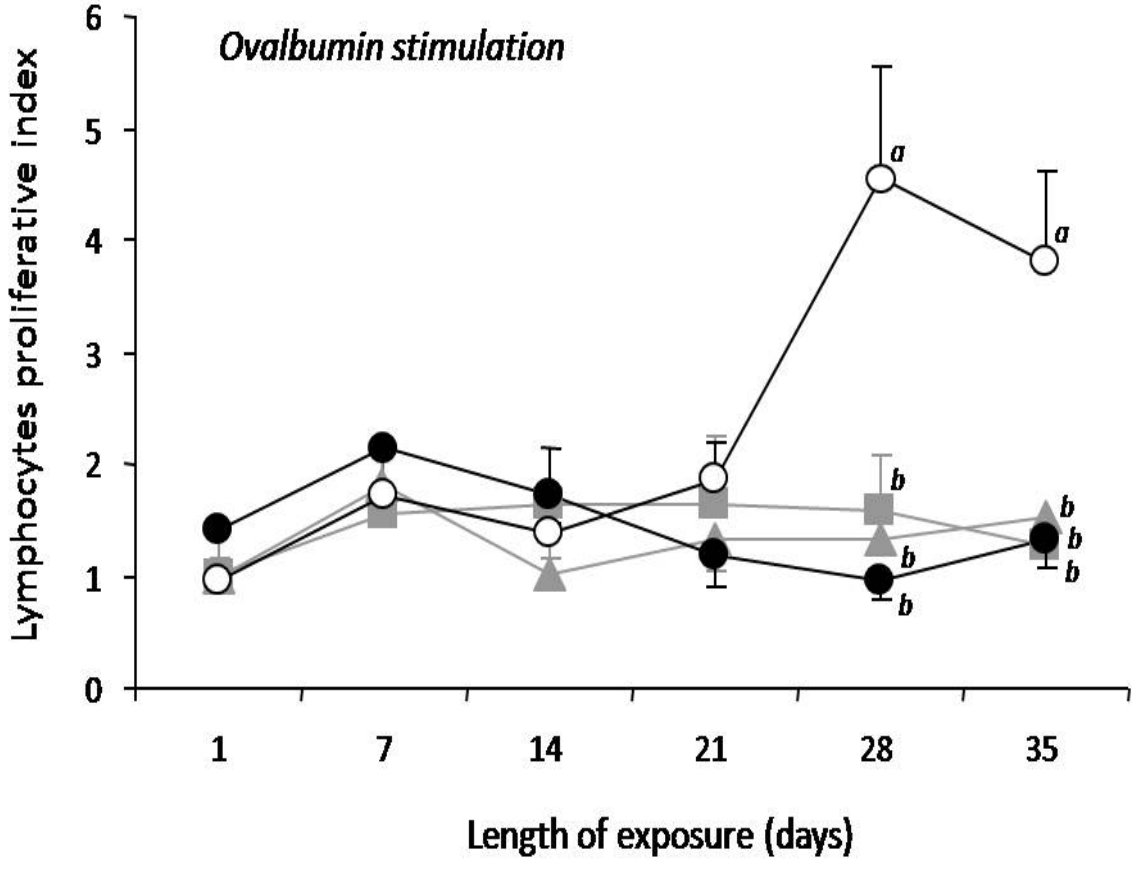


Fig 2 – Individual and combined effects of DON and FB on plasma level of specific immunoglobulins (IgA and IgG) anti-ovalbumin. Pigs received a control diet (○), or a DON contaminated diet (▲), or a FB-contaminated diet (■), or a contaminated diet with both toxins (●). At days 4 and 16 of the trial, animals receiving either control or contaminated feeds were subcutaneously immunized with ovalbumin. Plasma samples were collected weekly and the level of IgA and IgG specific for ovalbumin were determined by ELISA and normalized against a standardized reference plasma. Values are mean ± SEM for 5 animals. Statistics are mentioned when significant changes were observed. Means without a common letter differ $P<0.05$



As already observed [14, 26], the piglets receiving the control diet displayed a significant increase in the lymphocyte proliferation upon OVA stimulation was observed after the second immunization (1.4 fold increase, P=0.191; 3.3 fold increase, P=0,012 and 2.8 fold increase, P=0.020 at days 21, 28 and 35 of the experiment respectively). By contrast, the lymphocyte proliferation upon OVA stimulation in the animals receiving any of the three contaminated diets (DON, FB and DON+FB) remained as low as in control unstimulated lymphocytes (Figure 3).

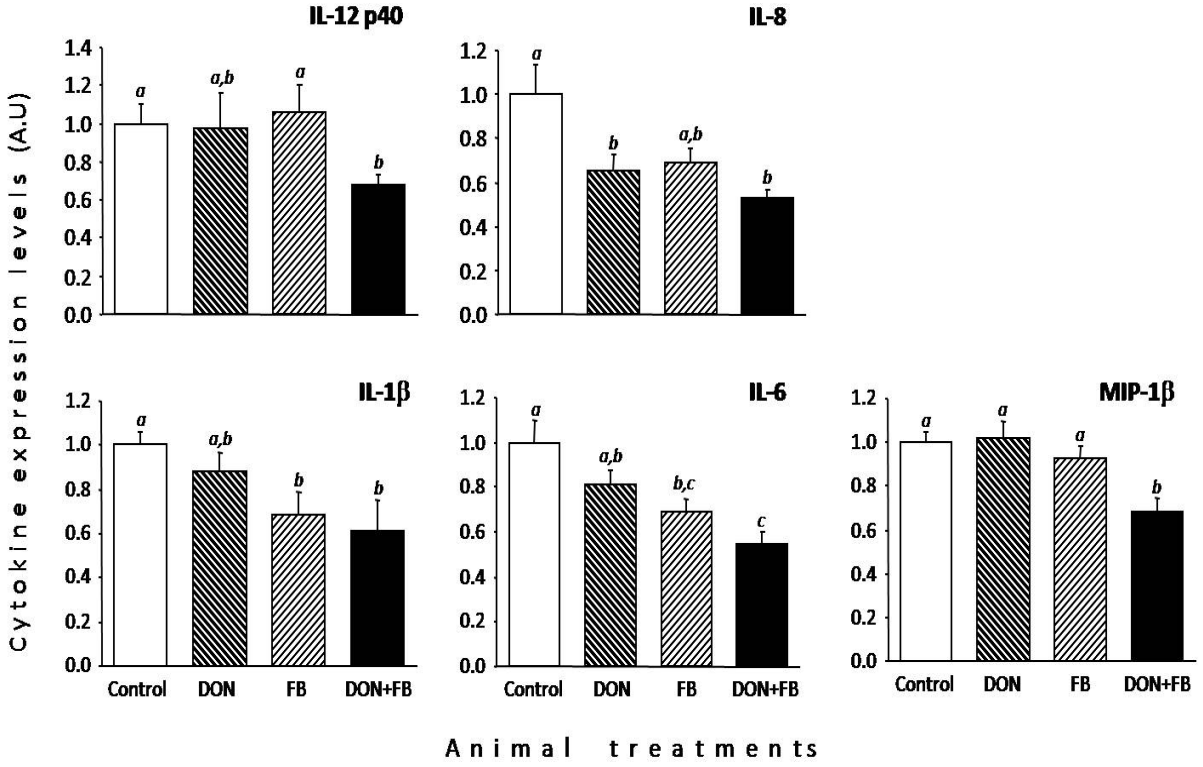
Fig 3 – Individual and combined effects of DON and FB on lymphocyte specific (ovalbumin) proliferation. Pigs received a control diet (○), or a DON-contaminated diet (▲), or a FB-contaminated diet (■), or a contaminated diet with both toxins (●). At days 4 and 16 of the trial, animals were subcutaneously immunized with ovalbumin. Blood samples were taken weekly to measure the lymphocyte proliferation. Results are expressed as stimulating index of lymphocyte proliferation calculated as counts per minute in stimulated culture/cpm in control non-stimulated 432 culture. Values are mean ± SEM for 5 animals. Statistics are mentioned when significant changes were observed. Means without a common letter differ P<0.05



3.4 Individual or Combined Effect of DON and FB on the Expression of Cytokines

Cytokines play a key role in regulating both humoral and cell mediated immunity. The mRNA expression of five cytokines (IL-12p40, IL-8, IL-1β, IL-6 and MIP-1β) was measured by real-time RT-PCR in spleen samples collected at the end of the experiment (Figure 4). Animals fed the diet containing both DON and FB demonstrated a significant decrease in mRNA for all tested cytokines when compared to control pigs (P=0.009 for IL-8; P=0.035 for IL-1β; P=0.004 for IL-6; P=0.031 for IL-12p40; P=0.006 for MIP-1β). Animals fed the diet contaminated with DON only demonstrated a significant decrease in mRNA encoding for IL-8, whereas animals fed the diet contaminated with FB only demonstrated a significant decrease in mRNA encoding for IL-1β and IL-6.

Fig 4 – Individual and combined effects of DON and FB on splenic mRNA expression of cytokines. Pigs received a control diet (□), or a DON-contaminated diet (▨), or a FB-contaminated diet (▨), or a contaminated diet with both toxins (■). Quantification of the relative cytokine mRNA level for each sample is expressed in arbitrary units (A.U). Values are mean ± SEM for 5 animals. Means without a common letter differ P<0.05



4 DISCUSSION

In the present 5 weeks study, piglets were exposed to low doses of two major *Fusarium* mycotoxins, DON and FB, at levels commonly found in crops. Most of the current data concerning the effect of DON and FB on animals, including rodents, have been obtained using highly contaminated feed [9, 12, 13, 28]. It was thus of interest to determine the effect of ingestion of feed contaminated with low level of toxins on zootechnical, hematological, biochemical and immune parameters of piglets.

We did not observe any effect of mycotoxin-contaminated diets (DON, FB, DON+FB) on the body weight gain of the animals. Considering the low contamination levels we are using, these results are not surprising. Indeed, no effect on body weight gain has been reported in pigs and in poultry fed with up to 70 mg FB/Kg feed [18, 29]. The effects of DON on body weight gain are more controversial, especially in pigs. Some studies indicates that dietary concentrations of DON above 1-2 mg/Kg have an effect on weight gain, whereas in other studies no effect is observed for up to 4.5 mg DON/Kg feed [30]. A weight gain reduction has also been described when DON and FB were given together to growing barrows [18]. However, in this study, the dose of FB was 10 fold higher than the one used in the present experiment.

Exposure of piglets to low doses of either DON or FB did not have a major impact on the hematological and biochemical parameters investigated. For blood hematology, only a reduction on neutrophil number was noticed in FB-exposed piglets. This observation is in relation with the reduced viability measured in human neutrophils exposed *in vitro* to FB [31]. For blood biochemistry, a decrease in albumin concentration in DON-exposed animals, and an increased creatinin concentration in FB-exposed piglets were noticed in accordance with previously published studies [18, 20, 32, 33]. Ingestion of diets co-contaminated with DON and FB had less effect on hematology and biochemistry parameters than did mono-contaminated diets. Some studes have already reported a weaker effect on plasma biochemical parameters for piglets fed multi-contaminated diets than for piglets receiving mono-contaminated feeds [18, 19], which suggests an opposite effect of the two mycotoxins.

Despite the absence of effect on zootechnical, hematological and biochemical parameters, ingestion of feed contaminated with low concentrations of DON and FB, induced histopathological lesions in liver, lung and kidney. Toxic effect of FB1 on liver has been reported in several papers using highly contaminated materials [9, 28]. The effects include a disorganization of hepatic cords, hepatocellular vacuolation, megalocytosis,

apoptosis, necrosis and cell proliferation. In the present study, we observed that even when present at 4.1-4.5 mg/Kg in the feed, FB induces similar liver histopathological lesions. Liver lesions, such as hepatic cell vacuolation, were also observed in piglets fed DON-contaminated diet [34]. These lesions were not associated with major biochemical alterations. The biological meaning of the hepatic lesion remains to be determined. Histopathological analysis of the lung confirmed this is a target organ for FB. At high doses (≥ 92 mg/Kg of feed for 4-7 days), FB induces lethal pulmonary edema in swine [9]. In the present study, the low dose of FB also induced pulmonary damages, mainly bronchiole-associated lymphoid tissue depletion and vascular disorders. By contrast, when present at low dose in the diet, DON did not induce any lesion in the lung.

For the three organs investigated, the damages elicited from the ingestion of the diet co-contaminated with DON and FB was equal to or higher than the ones elicited by the ingestion of a single mycotoxin. Very few publications analyze the effects of mixed mycotoxins on histopathological parameters, especially at low doses [35, 36]. The histopathological lesions observed in the lungs of co-exposed piglets were slightly more pronounced that the ones observed in the lungs of FB-exposed animals. In the liver, ingestion of the co-contaminated diet induced significantly higher lesions than ingestion of either of the mono-contaminated feeds as demonstrated by the lesion score and the hepatocyte proliferation. One explanation for the high liver toxicity of DON and FB when present simultaneously could be the higher absorption of FB in the presence of DON. Indeed, DON has recently been shown to decrease the barrier function of the intestine [37]. Thus, ingestion of DON may increase the absorption of FB, mycotoxins already known to be poorly absorbed [7,9].

The main objective of this study was to investigate the effect of low doses of DON and FB ingested separately or in combination on the immune response of piglets. As in previous experiments, it was observed that at low doses, mycotoxins have little or no effect on the total non specific immune responses as measured by lymphocyte proliferation upon mitogenic stimulation and the plasmatic concentrations of immunoglobulin classes. Immunization protocols, as already described, were needed to observe an effect of low doses of mycotoxins, fed either alone or in combination on the immune responses [14, 26, 38].

A very low proliferation index, close to the one observed in unstimulated cells, was obtained in cells isolated from animals fed either DON, FB or DON+FB contaminated diets. This alteration of lymphocyte proliferation might be due to an effect of these toxins on antigen-presentig cells (APC) as suggested by recent *in vitro* studies on

monocytes-derived APC treated with DON [39,40] or *in vivo* studies with piglets acutely exposed to FB [27].

Interestingly, the diet co-contaminated with DON and FB appeared to be able to counteract the increased level of specific IgA observed in animal receiving only the DON-contaminated diet. Indeed, consumption of the DON-contaminated diet increased the level of specific IgA in the plasma [11, 14] whereas ingestion of diet contaminated with both DON and FB did not alter the plasma level of this immunoglobulin isotype. We can hypothesize that FB interfere with the DON-induced IgA elevation at the intestinal level through its action on sphingolipids. Indeed, FB is known to disrupt the sphingolipid metabolism leading to depletion of ceramide and all ceramide-derived complex sphingolipids, such as sphingomyelin [41, 42]. This latter compound has been recently reported to control the amount of IgA in the large intestine [43].

Depending on the mycotoxins, DON or FB significantly impaired the specific IgG concentration and the level of cytokines expression. Nonetheless, the diet co-contaminated with DON and FB led to a strong decrease of specific IgG concentration, greater than the one observed in animals receiving only one toxin. Similar effects were observed for the 5 cytokines investigated, where the impact of the co-contaminated diet was higher than either of the mono-contaminated diets. Several studies investigated cytokines expression during chronic exposure to mycotoxins [14, 15, 25, 27], but none of them concern the co-contamination. Cytokines are important mediators in the immune response. Expressions of Il-8 and MIP-1β, which are involved in cells chemotaxis, were significantly inhibited in animals fed the co-contaminated diet, and it can be anticipated that in these animals, recruitment and migration of antigen-presenting cells to peripheral lymphoid tissue was reduced. Similarly, the decreased mRNA levels of IL-1β and IL-6 mRNA in piglets receiving the co-contaminated diet may lead to a defective antigen presentation and an impaired activation of lymphocytes and may explain the decreased IgG response observed in this study.

Find a mechanism that explains the observed effects after the combination of both toxins is not easy, but at the cellular level, it might be hypothesizes that MAPK's activation could be involved. Indeed both DON and FB have been shown to activate MAPK's [12, 44], and these kinases are well known to modulate numerous physiological processes, such as cell growth, apoptosis or immune response [45].

In conclusion, chronic exposure of low doses of DON or FB, either alone or in combination did not elicit important clinical signs (body weight gain, hematology,

biochemistry), but induced microscopic lesions and altered the immune response, especially when the mycotoxins were fed in combination. The modulation of the immune response was only observed when the immune system was activated. Considering that (i) vaccination or infection by pathogens is a common situation encountered in animal husbandry, and (ii) the natural occurrence of these mycotoxins in feedstuffs, the present experiment suggests a significant disruption in the establishment of an appropriate specific response in animals receiving mycotoxins-contaminated diets. This study also highlights the complexity of mycotoxin interactions; some effects are not enhanced by the combination of toxins (biochemistry, lung and kidney lesions, specific IgA content), while others are (specific IgG content, cytokines expression, liver lesions). These results may have some impact on the current regulation/recommendation that only take into account individual mycotoxins and not multi-mycotoxin contamination.

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ARTIGO 2

Chronic ingestion of deoxynivalenol and fumonisin, alone or in interaction, induces morphological and immunological changes in the intestine of piglets

Chronic ingestion of deoxynivalenol and fumonisin induces, alone or in interaction, morphological and immunological changes in the intestine of piglets.

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Ana-Paula F.L. BRACARENSE[1], Joelma LUCIOLI[1, 4], Bertrand GRENIER[2, 3, 4], Graziela DROCIUNAS PACHECO[1,2] , Wulf-Dieter MOLL[3], Gerd SCHATZMAYR[3] & Isabelle P. OSWALD[2,5]

Running title: DON & FB induce intestinal changes in piglets

ABSTRACT: Deoxynivalenol (DON) and Fumonisins (FB) are mycotoxins produced by *Fusarium* species which naturally co-occur in animal diets. The gastro-intestinal tract represents the first barrier met by exogenous food/feed compounds, the purpose of this study was to investigate the effects of DON and FB, alone and in combination on some intestinal parameters, including morphology, histology, expression of cytokines and junction proteins. Twenty-four 5-wk-old piglets were randomly assigned to four different groups, receiving separate diets for 5 weeks: a control diet, a diet contaminated with either DON (3mg/Kg) or FB (6mg/Kg) or both toxins. Chronic ingestion of these contaminated diets induced morphological and histological changes, as shown by the atrophy and fusion of villi, the decreased villi height and cell proliferation in jejunum, and by the reduced number of goblet cells and lymphocytes. At the end of the experiment, the expression levels of several cytokines was measured by RT-PCR and some of them (TNF-α, IL-1β, IFN-γ, IL-6, Il-10) were significantly up regulated in the ileum or the jejunum. In addition the ingestion of contaminated diets reduced the expression of the adherent junction protein E-cadherin and the tight junction protein occludin in the intestine. When animal were feed with co-contaminated diet (DON+FB), several types of interactions were observed depending on the parameters and segments assessed-synergistic (immune cells), additivie (cytokines and junction proteins expression), less than additive (histological lesions and cytokines expression), and antagonistic (immune cells and cytokines expression). Taken together, the present data

[1] Universidade Estadual de Londrina, Lab. Patologia Animal, Londrina, Brazil
[2] INRA, UMR 1331 ToxAlim, Toulouse, France
[3] BIOMIN Research Center, Technopark 1, Tulln, Austria
[4] These authors equally contribued
[5] Corresponding author. INRA-UMR 1331 ToxAlim; Research Center in Food Toxicology; 180 chemin de Tournefeuille BP 93173; 31027 Toulouse Cedex 3; Phone : +33561285480; E-Mail : isabelle.oswald@toulouse.inra.fr

provide strong evidence that chronic ingestion of low doses of mycotoxins alters the intestine and thus may predispose animals to infections by enteric pathogens.

Key words: Multi-contamination. Mycotoxin. Deoxynivalenol. Fumonisins. Intestine. Swine. Histology. Cellular junctions. Immunity.

1 INTRODUCTION

Mycotoxins are secondary metabolites of various fungi commonly found in feed and foodstuffs. Based on their known and suspected effects on human and animal health, aflatoxin, fumonisin, deoxynivalenol, ochratoxin A and zearalenone are recognized as the five most important agricultural mycotoxins [(1)]. The toxic effects of *Fusarium* mycotoxins in animals include reduced growth, feed refusal, immunosupression, gastrointestinal lesions, and neurological and reproductive disorders [(2)].

Recent surveys demonstrated regular occurrence of low levels of multiple mycotoxins in cereals [(3, 4)]. The toxicity of combinations of mycotoxins cannot always be predicted based upon their individual toxicities [(5, 6)]. Interactions between concomitantly occurring mycotoxins can be antagonistic, additive, or synergistic. The data on combined toxic effects of mycotoxins are limited and therefore, the actual combined health risk from exposure to mycotoxins is unknown [(6)].

The intestinal tract is the first barrier against ingested antigens, including mycotoxins and pathogenic bacteria. Following ingestion of mycotoxin-contaminated food, enterocytes may be exposed to high concentrations of toxins [(7)]. A role of food-associated mycotoxins in the induction or persistence of human chronic intestinal inflammatory diseases has also been suspected [(8)]. Studies focusing on the influence of food-derived antigens on intestinal morphology as an indicator of animal health are common; meanwhile, there are few publications on the effects of chronic exposure to a mycotoxin co-contaminated diet.

Fumonisins (FB) are toxic and carcinogenic mycotoxins produced by *Fusarium verticillioides* and *F. proliferatum,* common pathogens of maize. FB causes porcine pulmonary edema and equine leukoencephalomalacia [(9, 10)]. An association between human esophageal cancer and FB exposure in developing countries has been reported [(11)]. By contrast, the effect of chronic exposition to FB on the intestine has been poorly investigated. *In vitro*, the toxin induces apoptosis, inhibition of proliferation and affects the ability to produce cytokines in cell lines [(12-14)]. *In vivo*, ingestion of FB induces villous fusion and

atrophy, affecting intestinal absorption of nutrients. Also, it has been shown that fumonisin alters the cytokine profile and decreases the specific antibody response [15, 16].

Deoxynivalenol (DON) causes toxic and immunotoxic effects in a variety of cell systems and animal species [17]. DON is produced by *F. graminearum* and *F. culmorum* mainly in wheat, barley and maize. Swine are more sensitive to DON than other species, in part because of differences in the metabolism of DON. Chronic low dietary concentrations induce anorexia, decreased weight gain, and immune alterations, while acute higher doses induce vomiting, hemorrhagic diarrhea and circulatory shock [17-19]. At the cellular level, the main effect is inhibition of protein synthesis via binding to the ribosomes. Low exposure to DON was shown to upregulate expression of cytokines and inflammatory genes with concurrent immune stimulation, whereas high exposure promoted leukocyte apoptosis associated with immune suppression [17, 20]. At the intestinal level DON was shown to increase the permeability of enterocytes and to induce changes in the expression of claudins, a major component of the tight junctions in *in vitro* and *in vivo* models [21-23].

The purpose of this study was to compare the effects of low doses of DON and FB in pigs when fed to pigs individually and in combination with particular emphasis on their effects on the intestine. The experimental design was a factorial assay including control feed and feed contaminated with 3 and 6 mg/Kg DON and FB individually and in combination, respectively. These contamination levels correspond to levels (i) that frequently occur naturally in cereal and (ii) that only induce minimal alteration of zootechnical parameters. We investigated the effect of DON and FB on intestine morphology, on the expression of tight junctions proteins as well as on the intestinal expression of cytokines.

2 Material and methods

2.1 Animals and Diets

A total of 24 crossbred castrated male piglets (10.2 ± 1.89 Kg BW) were used in this study. Pigs were acclimatized for 1 week in the animal facility of the INRA ToxAlim Laboratory (Toulouse, France) prior to being used in experimental protocols. Animals were kept in batch pens for 35 days. Feed and water were provided *ad libitum* throughout the experimental period. The animals were submitted to one of four dietary treatments for 35 days: control diet (0.5 mg DON/Kg of feed, FB below limit of detection), diet containing 2.8 mg DON/Kg of feed, diet containing 5.9 mg FB/Kg of feed (4.1 mg FB_1 +

1.8 mg FB_2) and diet containing 3.1 mg DON plus 6.5 mg FB/Kg of feed (4.5 mg FB_1 + 2.0 mg FB_2). The diets were artificially contaminated with the fungal culture material containing DON and FB as already described [24]. The diet formulations and nutrient contents are described in Table 1. Even if the feed intake of the animals was not measured in the experiment, we can estimate that piglets were exposed to 130 and 260 μg/Kg BW/d of DON and FB, respectively.

Table 1 –Composition of the experimental diet

Ingredient (%)	
Wheat	47.50
Soybean meal	24.30
Barley	22.90
Calcium phosphate	1.12
Calcium carbonate	1.00
Vitamin and mineral premix[1]	0.50
Vegetable oil	1.40
Sodium chloride	0.40
Phytase	0.01
Lysine	0.465
Methionine	0.165
Threonine	0.195
Tryptophan	0.045
Composition[2]	
Starch (g)	476.8
Crude protein (g)	218.3
Crude fiber (g)	37.5
Ca (g)	10.5
P (g)	6.5
K (g)	8.7
Net energy (MJ)	15.6

Deoxynivalenol, zearalenone and enniatin were found to be naturally present in the cereals used, resulting in concentrations of 500, 50 and 100 μg/Kg of feed, respectively. All other mycotoxins, including fumonisin, aflatoxins, T-2 toxin, HT-2 toxin and ochratoxin A were below the limit of deletction.

The experimental design used in this study was entirely randomized with six repetitions (each animal represented one repetition). At the end of the experiment, pigs were

[1] Vitamin A, 2,000,000 IU/Kg; vitamin D3, 400,000 IU/Kg; vitamin E, 4000 mg/Kg; vitamin C, 8000 mg/Kg; vitamin B1, 400 mg/Kg; vitamin K3, 400 mg/Kg; iron, 20,000 mg/Kg; copper, 4000 mg/Kg; zinc, 20,000 mg/Kg; manganese, 8000 mg/Kg

[2] corresponding to 1000 g dry matter/Kg

fasted overnight before being submitted to electrical stunning and euthanized by exsanguination. Samples from mid-jejunum and proximal ileum were collected from each animal from all groups and fixed in 10% buffered formalin solution for histological analysis. In addition, samples from the same regions of the intestine were collected, flash-frozen in liquid nitrogen and stored at -80°C until processed for measurements of junction proteins and cytokine mRNA. The institutional Ethics Committee for Animal Experimentation approved the study.

2.2 Histological Assessment of the Intestine

The tissue pieces fixed in 10% buffered formalin were dehydrated through graded alcohols and embedded in paraffin wax. Sections of 3 µm were stained with hematoxylin-eosin (HE) for histopathological evaluation. A lesional score was designed to compare histological changes. The frequency and severity of each lesion were considered in the score as already described [25]. The following criteria were included in the score: morphology of villi, morphology of enterocytes, interstitial edema and lymph vessels dilation (Table 2). The lesion score was calculated by taking into account the degree of severity (severity factor) and the extent of each lesion (according to intensity or observed frequency, scored from 0 to 3). For each lesion, the score of the extent was multiplied by severity factor.

To evaluate goblet cell density, sections of intestine were stained with alcian blue. Positively stained goblet cells were counted randomly in five fields per sample at 40x magnification, and the means were submitted to statistical analysis.

Villi height and crypt depth were measured randomly on thirty villi using a MOTIC Image Plus 2.0 ML® image analysis system. The numbers of lymphocytes, plasma cells and eosinophils were counted randomly based on morphology on three fields per sample at 40x magnification. The number of mitotic figures was counted in 20 fields per slide using 40x magnification. Each field corresponds to a surface area of 1.5 mm^2. The means of lesional score, intestinal morphometry, number of goblet cells, inflammatory infiltrate and mitosis were utilized for statistical analysis.

Table 2 – Histological criteria used to establish the intestinal lesional score.

Type of lesion	Severity factor	Maximal score
Lymphatic vesses dilation	1	
Cell vacuolation	1	
Cubic enterocytes	2	
Villi flattening	2	39
Villi fusion	2	
Interstitial edema	2	
Villi apical necrosis	3	

Notes: The score for each lesion was obtained by multiplying the severity factor (or degree for severity) 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. 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.

2.3 Immunohistochemical Assessment of the Expression of Junction Molecules

E-cadherin expression was analyzed on formalin-fixed embedded intestinal sections to evaluate intestinal cell adherens junctions. Tissue sections were deparaffinized with xylene and dehydrated through a graded ethanol series. Heat-mediated antigen retrieval was done by heating the sections (immersed in EDTA buffer, ph 9.0) in a microwave oven (750W) for 15 minutes. Endogenous peroxidase activity was blocked by incubation in methanol/H_2O_2 solution. The sections were incubated overnight at 4ºC with the primary antibody (anti-E-cadherin Clone 4A2C7, Zymed, San Francisco, CA, diluted 1:50). The secondary antibody (Kit Super Picture^{TM} Zymed, San Francisco, CA) was applied followed by the addition of a chromogen (3, 3'-diaminobenzidine). Finally, the tissue sections were counterstained with hematoxylin and mounted on coverslips using a permanent mounting medium. Tissue sections were examined, and the proportion of the intestinal section expressing E-cadherin was estimated. Each sample was assessed as showing either normal or reduced staining. Normal staining was considered when a homogeneous and strong basolateral membrane staining of enterocytes was detected. Heterogeneous and weak staining was considered to indicate reduced expression. The results are reported as the percentage of animals showing strong/homogenous immunoreactivity to E-Cadherin.

2.4 Western Blot Analysis of Junction Proteins

Proteins were extracted from ileum and assayed as described previously [(26)]. Briefly, the extraction was carried out on ice in extraction buffer. The protease inhibitors cocktail (antipaïne, pepstatine, benzamidine, aminoethyl benzenesulfonyl fluoride hydrochloride, aprotinin and leupeptin) was added to the extraction buffer just before use.

Extracts of tissue proteins were then separated by SDS-PAGE electrophoresis. Equal amounts of proteins were loaded on a 12.5% acrylamide gel. Migration was conducted in 250 mM Tris buffer (pH 7.6) containing 1% SDS and 1.92 M Glycine. After separation, proteins were transferred onto Optitran BA-S 83 membrane (Whatman®, Germany). In previous studies [22,26] we observed that DON decrease the expression of claudins. In the present study, we extended our knowledge concerning the effect of mycotoxins on junction proteins and evaluated the effect of DON and FB on another tight junction protein (occluding) and on an adherens junction protein (E-cadherin). The antibodies used in this study were E-Cadherin (24E10) Rabbit mAb from Cell Signaling (Cell Signaling Technology, Danvers, MA) diluted 1:500; Rabbit anti-Occludin (672381A) from Invitrogen (Cergy Pontoise, France) diluted 1:500 and β-actin mAB MOUSE (8H10D10) from Cell Signaling. These antibodies are sutiable for the detection of proteins by western-blot. Expression of β-actin was used for checking the equal protein load across gel tracks. Band densities were obtained by scanning the membranes using Odyssey® Infrared Imaging System (LI-COR; ScienceTec, Les Ulis, France). Density data were standardized within membranes by expressing the density of each band of interest relative to that of β-actin in the same lane.

2.5 Determination of the Expression of mRNA Encoding for Cytokines by Real-Time PCR

Tissue RNA was processed in lysing matrix D tubes (MP Biomedicals, Illkirch, France) containing guanidine-thiocyanate acid phenol (Extract-All®, Eurobio, les Ulis, France) for use with the FastPrep-24 (MP Biomedicals, Illkirch, France). Concentration, integrity and quality of RNA were determined spectrophotometrically ($O.D._{260}$) using Nanodrop ND1000 (Labtech International, Paris, France). In addition to this inspection, 200 ng of RNA was analyzed by electrophoresis. The reverse transcription of 2 µg of total RNA was performed using M-MLV reverse-transcriptase, Rnasin® plus (Promega, Charbonnière, France) and random primers (Invitrogen, Cergy Pontoise, France) (5 min at 37°C, 1 hour at 42°C, 15 min at 70°C) as already described [27]. Real-time PCR assays were performed on 8 ng of cDNA (RNA equivalent) in a 25-µl volume reaction per well using Power SYBR® Green PCR Master Mix as the reporter dye and the automated photometric detector ABI Prism 7000 Sequence Detection System for data acquisition (Applied Biosystems, Courtaboeuf, France). The amplification conditions were as follows: 95°C for 10 min

followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. RNA non-reverse transcript was used as a non-template control (NTC) for verification that no genomic DNA amplification signal existed. Specificity of PCR products was checked at the end of the reaction by analyzing the curve of dissociation. In addition, the size of amplicons was verified by electrophoresis. The sequences and concentration of the primers used are detailed in Table 3. Primers for MIP-1β, IL-8 and IL-6 detection were designed using Primer Express® software (Applied Biosystems). Primers were purchased from Invitrogen (Cergy Pontoise, France). Amplification efficiency and initial fluorescence were determined by the DART-PCR method, and the values obtained were then normalized by two housekeeping genes, β2-microglobulin and ribosomal protein L32 (RPL32); and finally, gene expression was calculated relative to the control group as already described [28].

Table 3 – Nucleotide sequences of primers for real-time PCR

Gene	Primer sequence	Genbank no.	References
RPL32	F (300 nM) 5'-TGCTCTCAGACCCCTTGTGAAG-3' R (300 nM) 5'-TTTCCGCCAGTTCCGCTTA-3'	NM_001001636	26
β2-µglobulin	F (900 nM) 5'-TTCTACCTTCTGGTCCACACTGA-3' R (300 nM) 5'-TCATCCAACCCAGATGCA-3'	NM_213978	28
IL-12p40	F (300 nM) 5'-GGTTTCAGACCCGACGAACTCT-3' R (900 nM) 5'-CATATGGCCACAATGGGAGATG-3'	NM_214013	28
IL-8	F (300 nM) 5'-GCTCTCTGTGAGGCTGCAGTTC-3' R (900 nM) 5'-AAGGTGTGGAATGCGTATTTATGC-3'	NM_213867	24
IL-1β	F (300 nM) 5'-GAGCTGAAGGCTCTCCACCTC-3' R (300 nM) 5'-ATCGCTGTCATCTCCTTGCAC-3'	NM_001005149	28
MIP-1β	F (300 nM) 5'-AGCGCTCTCAGCACCAATG-3' R (300 nM) 5'-AGCTTCCGCACGGTGTATG-3'	AJ311717	24
IL-6	F (300 nM) 5'-GGCAAAAGGGAAAGAATCCAG-3' R (300 nM) 5'-CGTTCTGTGACTGCAGCTTATCC-3'	NM_214399	24
IFN-γ	F (300 nM) TGGTAGCTCTGGGAAACTGAATG R (300 nM) GGCTTTGCGCTGGATCTG	NM_213948	51
TNF-α	F (300 nM) ACTGCACTTCGAGGTTATCGG R (300 nM) GGCGACGGGCTTATCTGA	NM_214022	52
IL-2	F (300 nM) GCCATTGCTGCTGGATTTAC R (300 nM) CCCTCCAGAGCTTTGAGTTC	AY294018	53
IL-10	F (300 nM) GGCCCAGTGAAGAGTTTCTTTC R (300 nM) CAACAAGTCGCCCATCTGGT	NM_214041	Present study

Notes : RPL32. ribosomal protein L32. IL. interleukin. MIP-1α. macrophage inflammatory protein-1 alpha

2.6 Statistical Analysis

Data are presented as mean ± SEM. They were analyzed with Statview software, version 5.0 (SAS Institute Inc, Cary, NC), using ANOVA, Tukey and PLSD Fisher test. Data from immunohistochemical analysis were evaluated using Fisher test. P values < 0.05 were considered significant.

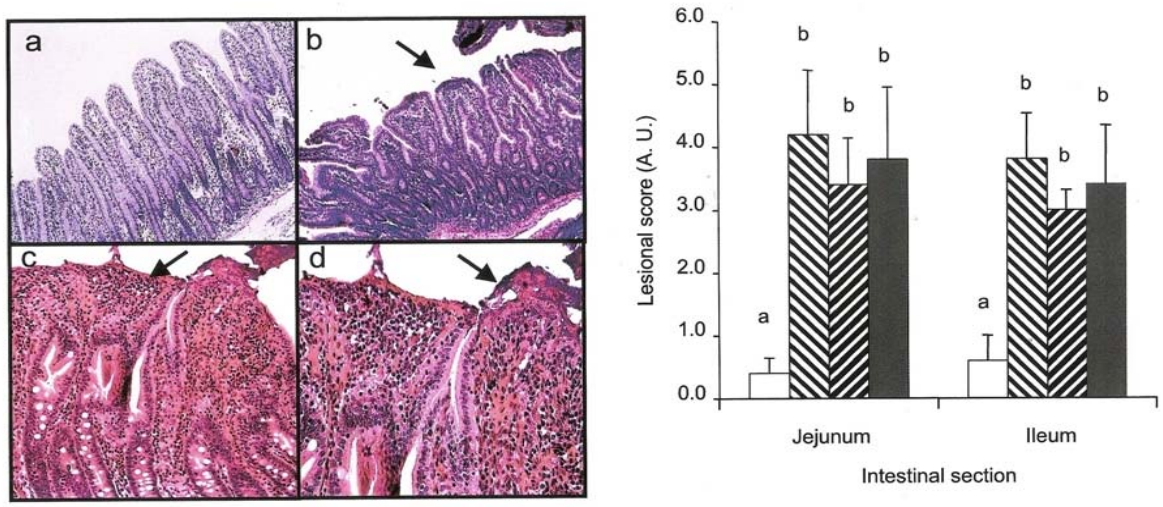
3 RESULTS

3.1 Individual or Combined Effects of DON and FB on the Histology and Morphometry of the Intestine

Ingestion of diet contaminated with DON and FB, alone or in interaction, did not significantly modulate animal weight. The initial and final body weights of animal in the different groups were 9.54 ± 0.99 Kg and 30.50 ± 1.34Kg for the control group, 10.46±1.24 Kg and 28.98±1.75 Kg for the DON treated group, 9.52±0.37 Kg and 31.12±1.63 Kg for the FB treated group and 10.16±0.42 and 28.92±1.91 for the DON+FB treated group (no significant difference). In addition no overall sign of toxicity were observed in animals from the different groups.

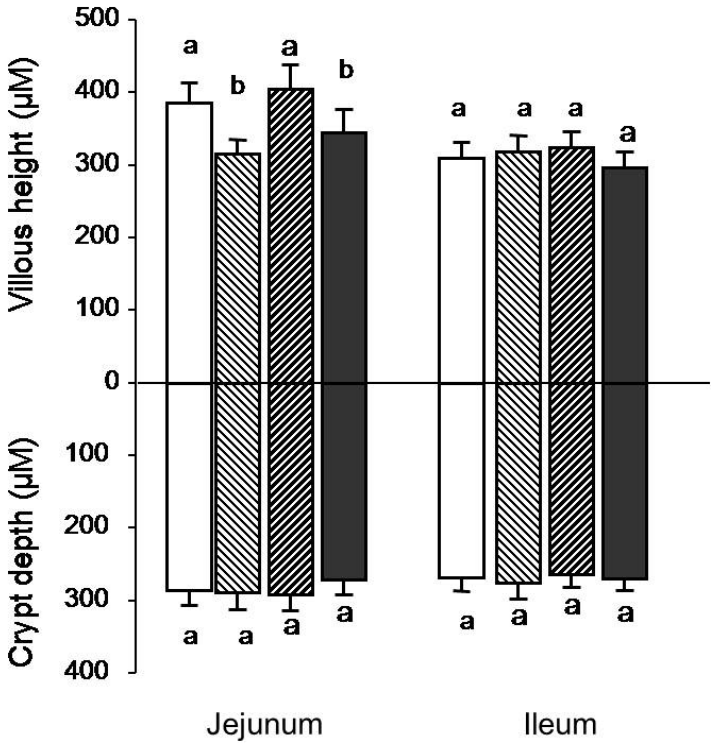
Samples of jejunum and ileum were collected for histomorphometrical analysis. Piglets fed diets contaminated with mycotoxins showed mild to moderate intestinal lesions. The main histological changes observed were multifocal atrophy and villi fusion, apical necrosis of villi, cytoplasmatic vacuolation of enterocytes, and edema of lamina propria. Lymphatic vessel dilation and prominent lymphoid follicles were also observed. As indicated by the lesional scores, piglets fed mycotoxin contaminated diets (DON, FB or DON+FB) displayed significant jejunal and ileal lesions when compared to animals fed the control-diet (Figure 1).

Fig 1 – Effect of individual and combined DON and FB exposure on jejunum and ileum histology. Pigs received a control diet (☐), or a DON-contaminated diet (▨), or a FB-contaminated diet (▨), or a contaminated diet with both toxins (■). (A) Jejunum of a control piglet and (B) DON treated piglet. Villi flattening (arrow). HE. 10x (C) Villi apical necrosis (arrow). HE. 10x and (D) Bacterial adhesion in the area with necrosis (arrow). HE. 40x. Lesional score after histological examination according to the occurrence and the severity of lesions. Values are mean scores ± SEM for 6 pigs. Means without a common letter differ, $P<0.05$



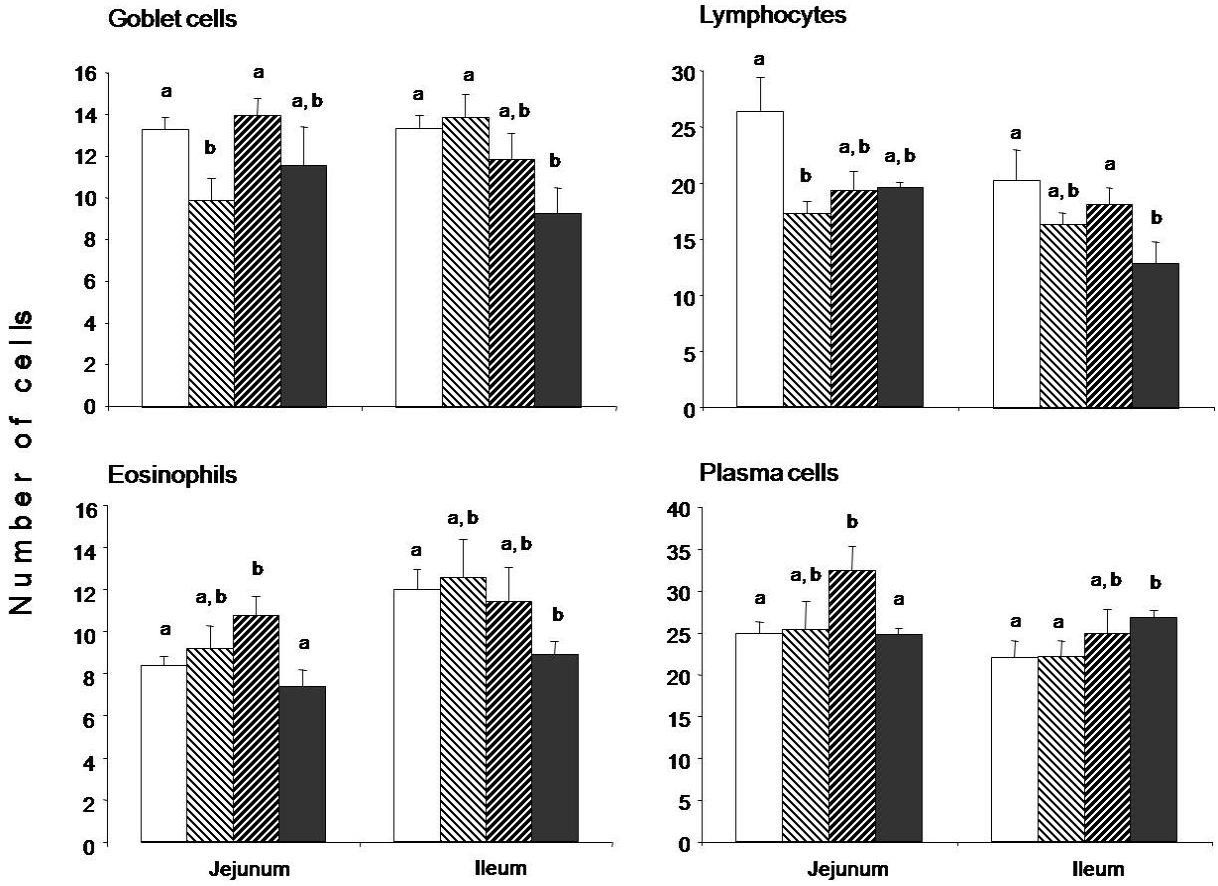
Changes in villous height reflect changes in the balance between epithelial cell proliferation and apoptosis. As shown in Figure 2, villi height decreased significantly in the jejunum of the animals that received DON or DON+FB contaminated diet when compared with control piglets. No change in crypt depth was observed in any intestinal region. 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 DON- and FB+DON-contaminated diet animals respectively (Figure 3).

Fig 2 – Effect of individual and combined DON and FB exposure on jejunum and ileum villi height and crypt depth. Pigs received a control diet (□), or a diet contaminated with DON (▨), FB (▨), or both DON and FB (■). Data are mean height and depth (μm) ± SEM for 6 pigs. Means without a common letter differ, $P < 0.05$.



Increased numbers of lymphocytes, plasma cells and eosinophils was observed in all regions of the intestine. 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 jejunum of DON-treated animals and in the ileum of DON+FB-treated piglets (Figure 3). By contrast, the number of plasma cells and eosinophils in the lamina propria increased significantly in the jejunum of animals fed the FB-contaminated diet.

Fig 3 – Effect of individual and combined DON and FB exposure on the number of inflammatory cells and goblet cells in jejunum and ileum. Pigs received a control diet (□), or a diet contaminated with DON (▧), FB (▨), or both DON and FB (■). Values are mean number of inflammatory and goblet cells per field (1.5 mm^2) ± SE for 6 pigs. Means without a common letter differ, P< 0.05.



3.2 INDIVIDUAL OR COMBINED EFFECTS OF DON AND FB ON INTESTINAL CELL PROLIFERATION

Epithelial cell proliferation was estimated by counting the number of mitosis figures in enterocytes on hematoxylin-eosin stained slides. The mean number of mitosis in the jejunum were 2.36 ± 1.64 in the control group, 1.73 ± 1.35 in the DON treated group, 1.66 ± 1.11 in the FB treated group and 1.91 ± 1.19 in the DON+FB treated group. In the ileum the mean number were 1.75 ± 1.26, 1.78 ± 1.46, 1.62 ± 1.17 and 1.89 ± 1.11 for the control group, DON treated group, FB treated group and DON+FB treated group, respectively. A significant decrease ($P < 0.05$) was observed in the jejunum of the groups fed mono-contaminated diets compared to the control group.

3.3 Individual or Combined Effects of DON and FB on Intestinal Immune Response

To evaluate the mechanisms of porcine intestinal defense against mycotoxin exposure, we quantified the expression of genes coding for pro-inflammatory cytokines. Table 4 describe the expression of 9 cytokines (IFN-β, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p40, MIP-1β, and TNF-α) in the jejunum and the ileum of piglets exposed to DON and FB alone or in combination.

Despite an important variability, all the cytokines assessed showed a tendency and/or significant increase of their expression in intestinal samples from piglets receiving mycotoxin-contaminated diets. However, expression of cytokines revealed different profiles according to treatments and intestinal region (Figure 4). DON induced a significant induction of the expression of IL-1β, IL-2, IL-6, IL-12p40 and MIP-1β in the jejunum, and a significant induction of the expression of TNF-α and IL-1β in the ileum. By contrast, ingestion of FB contaminated feed had only a moderate effect on the expression of cytokines. It induced a significant expression of IL-10 and IFN-γ in the jejunum and the expression of TNF-α and IL-1β in the ileum. When animals were given the DON+FB co-contaminated diet, the expression of TNF-α and IL-1β in their ileum and the expression of IL-10, IFN-γ, IL-1β, MIP-1β, IL-2 and IL-12p40 in their jejunum was not different from the one observed in the intestine of animal fed the mono-contaminated diet. Of note, the expression of IL-6 was only up-regulated after ingestion of DON contaminated diet (+117% in jejunum and +113% in ileum when compared with animal receiving the control feed).

Table 4 –Effect of individual and combined DON and FB exposure on jejunum and ileum mRNA expression of cytokines

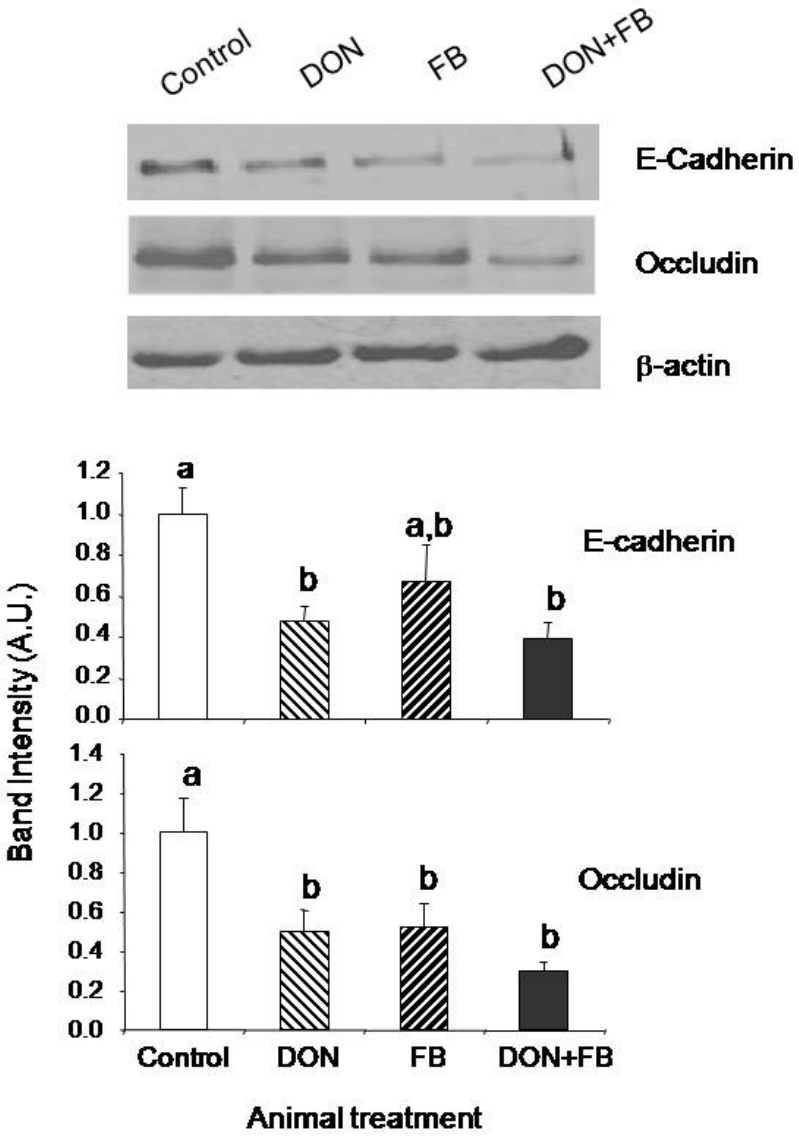
INTESTINAL PORTION	Diet treatment			
Cytokine	**Control**	**DON**	**FB**	**DON+FB**
JEJUNUM				
IL-1β	1.00 ± 0.17^{a}	1.78 ± 0.12^{b}	1.34 ± 0.11^{a}	$1.46 \pm 0.14^{a,b}$
TNF-α	1.00 ± 0.08^{a}	1.29 ± 0.12^{a}	0.88 ± 0.14^{a}	1.56 ± 0.33^{a}
IL-6	1.00 ± 0.12^{a}	2.17 ± 0.30^{b}	1.14 ± 0.11^{a}	1.35 ± 0.14^{a}
IL-8	1.00 ± 0.09^{a}	1.78 ± 0.52^{a}	1.38 ± 0.40^{a}	1.02 ± 0.12^{a}
MIP-1β	1.00 ± 0.08^{a}	1.42 ± 0.14^{b}	$1.36 \pm 0.18^{a,b}$	$1.27 \pm 0.16^{a,b}$
IL-2	1.00 ± 0.16^{a}	1.80 ± 0.25^{b}	$1.74 \pm 0.31^{a,b}$	$1.56 \pm 0.26^{a,b}$
IL-12p40	1.00 ± 0.09^{a}	1.71 ± 0.26^{b}	$1.36 \pm 0.13^{a,b}$	2.01 ± 0.49^{b}
IFN-γ	1.00 ± 0.16^{a}	$1.29 \pm 0.08^{a,b}$	1.43 ± 0.10^{b}	$1.35 \pm 0.17^{a,b}$
IL-10	1.00 ± 0.11^{a}	$1.34 \pm 0.24^{a,b}$	1.51 ± 0.19^{b}	1.63 ± 0.17^{b}
ILEUM				
IL-1β	1.00 ± 0.19^{a}	2.00 ± 0.19^{b}	1.73 ± 0.24^{b}	1.63 ± 0.13^{b}
TNF-α	1.00 ± 0.08^{a}	1.49 ± 0.10^{b}	1.42 ± 0.11^{b}	1.71 ± 0.23^{b}
IL-6	1.00 ± 0.20^{a}	2.13 ± 0.21^{b}	1.02 ± 0.13^{a}	0.96 ± 0.24^{a}
IL-8	1.00 ± 0.18^{a}	1.18 ± 0.08^{a}	1.48 ± 0.38^{a}	1.53 ± 0.30^{a}
MIP-1β	1.00 ± 0.06^{a}	1.50 ± 0.30^{a}	1.19 ± 0.07^{a}	1.42 ± 0.22^{a}
IL-2	1.00 ± 0.12^{a}	1.00 ± 0.22^{a}	1.25 ± 0.16^{a}	1.04 ± 0.11^{a}
IL-12p40	1.00 ± 0.07^{a}	1.04 ± 0.11^{a}	1.09 ± 0.07^{a}	1.34 ± 0.14^{a}
IFN-γ	1.00 ± 0.12^{a}	1.43 ± 0.21^{a}	1.26 ± 0.09^{a}	1.90 ± 0.48^{a}
IL-10	1.00 ± 0.13^{a}	1.14 ± 0.12^{a}	1.23 ± 0.14^{a}	1.57 ± 0.38^{a}

Note: Results are expressed as mean ± SEM for five animals. For each cytokine, means without a common letter differ, $P<0.05$

3.4 Individual or Combined Effects of DON and FB on Intestinal Expression of Junction Proteins

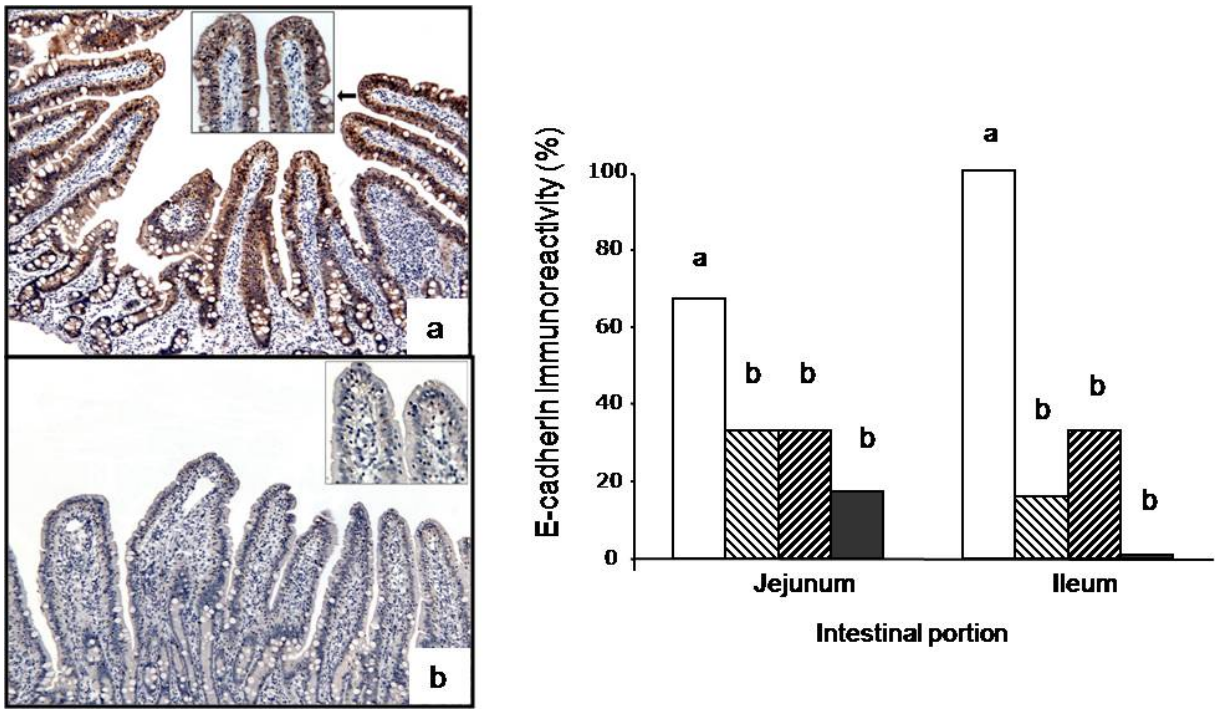
The adherence of enterocytes and permeability of intestinal epithelium is formed to a large extent by multiprotein junction complexes. The expression of two junction proteins, E-cadherin and occludin, was analyzed in the ileum of animals by western blotting. After normalization by the housekeeping gene β-actin, the data revealed a significant decrease of expression of both proteins in animal receiving mycotoxin contaminated diet compared to the animals receiving a control diet (Figure 4).

Fig. 4 – Effect of individual and combined DON and FB exposure on intestinal expression of E-cadherin and occludin. Pigs received a control diet (□), or a diet contaminated with DON (▨), FB (▧), or both DON and FB (■). The upper figure represents the immunoblot. The lower figure represents the expression of the protein estimated by densitometric analyses after normalization with β-actin signal. Values are mean ± SE for 6 pigs. Means without a common letter differ, $P < 0.05$.



As western-blot indicated a significant decrease in the total amount of E-cadherin expression in the ileum, we decided to evaluate the expression of this protein in enterocytes, using an immunohistochemical assay. The expression of E-cadherin in the jejunum and ileum was significantly reduced in the groups that received monocontaminated or co-contaminated diets (Figure 5).

Fig. 5 – Effect of individual and combined DON and FB exposure on intestinal expression of E-cadherin. Pigs received a control diet (□), or a diet contaminated with DON (▨), FB (▨), or both DON and FB (■). (A) Jejunum of a control piglet showing a strong and homogeneous immunoreactivity to E-cadherin. Immunoperoxidase, 20x. (B) Percentage of animal showing a strong immunoreactivity to E-cadherin. Value without a common letter differ, $P<0.05$.



4 DISCUSSION

Co-contamination of grains and feed is frequently reported all around the world and the occurrence of single-mycotoxin contamination seems to be rare [3, 29]. However, most studies to investigate the toxicological effect of mycotoxins have been done with feed spiked with a single mycotoxin at high dose. It was thus of interest to determine the effect of ingestion of feed contaminated with more than one mycotoxin on the intestine. The intestine is the first barrier against mycotoxins and could be exposed to high doses of these toxins [7]. In the present study we have investigated the effect of FB and DON on the intestine of piglets in order to determine if they have additive, synergistic or antagonistic effects. FB and DON act through different mechanisms on the intestinal tract. FB blocks sphingolipid synthesis, which is essential for the formation of cell membranes, while DON inhibits protein synthesis via ribosome binding [17, 30, 31].

We did not observe any effect of mycotoxin-contaminated diets (DON, FB, DON+FB) on the body weight gain of the animals. Considering the low contamination levels we used, these results are not surprising [15,18,24]. The main histological findings observed were villi flattening, apical necrosis and a reduction in the number of goblet cells. Decreased villi height was only significant in the jejunum of piglets fed with the diet mono-contaminated with DON and the diet co-contaminated with DON+ FB. Similar changes were observed during *in vivo* and *ex vivo* exposure of the intestine to DON [25, 33]. The mode of toxic action of DON is inhibition of protein synthesis, thus primarily affecting rapidly dividing cells such epithelial and immune cells [17, 20]. The villi flattening in the jejunum is likely due to impairment of cell proliferation, as could be observed by the decrease in the number of mitotic figures in the same region.

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 endoplasmic reticulum stress [34]. Considering that the decrease in goblet cells density was not related to the villi flattening (data not show), we can hypothesize that DON in mucus-producing cell lines induces a endoplasmic reticulum stress, leading to changes in intestinal cell density. Hyperplasia of intestinal globet cells has been observed in piglets and broiler chicks receiving feed contaminated with 30 and 300 mg FB/Kg feed respectively [35,36] . In the present study, a decrease in the number of goblets cells was observed in piglets fed the DON contaminated and the DON+FB contaminated diets, while in animal receiving the FB-contaminated diet, no effect was observed. The difference in the

effect of FB on goblet cell could be due to the low dose of FB used in the present experiment. Intestinal mucus protects the epithelium against adhesion and invasion by pathogens [30], therefore a reduction in the number of goblets cells can affect the intestinal barrier function. The mechanisms involved in the alterations on the production and composition of the intestinal mucus layer by mycotoxins are still unknown and further studies are required.

Controversial results have been reported with respect to intestinal proliferation in *in vivo* and *in vitro* studies during mycotoxicosis. In this study, we evaluated cell proliferation by counting mitotic figures in intestinal crypts, and we observed a significant decrease of proliferating cells, in the jejunum of the FB and DON-treated groups. Other studies [34, 37] have demonstrated that subchronic exposure to FB or DON and other mycotoxins increases the number of mitotix figures. This difference could be due to the animal model used as well as the dose and duration of the experiment. Despite very different mode of action, both DON and FB have been found to decrease proliferation of intestinal epithelial cells. Indeed, Bouhet et al. (2004) [32] observed an *in vitro* decrease in the proliferation of porcine intestinal epithelial cells treated with FB_1 due to a blockage in the G0/G1 cell cycle phase. Similarly, Van de Walle et al. (2010) [23] detected an *in vitro* decrease in the proliferation, associated with an inhibition of protein synthesis, of human intestinal epithelial cells treated with DON.

Mononuclear and eosinophil inflammatory infiltrate has been reported during FB mycotoxicosis in several species. In addition, proliferation of lymphoid nodules in the ileum and cecum was also observed [35, 37]. We have shown that DON and DON + FB induced a significant decrease in the number of lymphocytes in jejunum and ileum, whereas FB induced a significant increase in eosinophils and plasma cells in the jejunum. Lymphocyte depletion of lymph nodes and spleen was reported in young pigs fed a diet contaminated with DON and zearalenone [38]. Studies of macrophages and lymphocytes have shown that the trichothecene-mediated immunosuppressive effect was associated with induction of apoptosis by activation of c-jun terminal kinase, p38 mitogen-activated protein kinase, and caspases [20, 39]. Because lymphoid cells are constantly renewing, lymphocytes could be particularly sensitive to DON. On the other hand, DON stimulates the production of mucosal antibodies by plasma cells through upregulation of proinflammatory cytokines [17, 18].

In the present study, exposure of piglets to chronic doses of FB, DON or both in the feed induced activation of the proinflammatory cytokine network in the intestine. Increases in the mRNA levels of the nine cytokines evaluated were observed in the jejunum and ileum, however due to high variability the increase was only significant for some of them.

As already described in rodent [17, 20], we observed that DON specifically induced the expression of IL-6. This can be related to the specific effect of DON on the IgA synthesis that was observed on DON-treated animals [18, 24]. A proinflammatory effect was also observed in human enterocytes exposed to DON, as demonstrated by an increased expression of IL-8 [21, 23]. It has been established that the intestine has its own immune network, which can cause localized induction of various cytokines and chemokines [40]. Increases in intestinal cytokine mRNA profile indicative of macrophage and TH1 activation have been reported after DON and FB exposition [41-43]. TNF-α and IL-1β are known to induce apoptosis via the receptor-ligand-mediated mechanism [41, 44]. We hypothesize that, besides the known apoptotic mechanisms [13,20], FB and DON could induce TNF-mediated lymphocyte and epithelial apoptosis in the intestine, which could explain the decrease in the number of these cells observed in exposed pigs. A relationship between clinically relevant concentrations of TNF-α and IL-1β and an increase in intestinal tight junction permeability has been demonstrated in Caco-2 cells [45, 46]. With regard to this association, we can consider that the increased levels of TNF-α and IL-1β observed after the ingestion of DON and FB could also contribute to tight-junction intestinal barrier defects.

In previous studies, we observed that DON decreases the expression of claudins [22, 26]. In the present study, we observed that other junction proteins, such as occludin and E-cadherin were also affected by an exposure to mycotoxins. To the best of our knowledge, this is the first study reporting a reduced expression of E-cadherin in the intestinal tract after ingestion of a mycotoxin-contaminated diet. The reduction of E-cadherin and occludin, suggests a loss of enterocytes' adhesive properties that would correlate with an increased intestinal translocation of toxic luminal antigens, promoting intestinal inflammation [8], with an abnormal delivery of antigens via a paracellular pathway [47, 48], and with an increased susceptibility to enteric infections [21, 30].

One of the aims of this study was to assess the combined effect of DON and FB. The interaction between the toxins can be classified in four different categories: synergistic, additive, less than additive or antagonistic effects [6]. In the present study, we observed synerfistic interactions in the number of goblet cells and eosinophils in ileum, additive interactions in the expression of IL-10, TNF-α and adherent proteins, less than additive interactions in the expression of IFN-γ and in lesion scores, and antagonistic interactions for some cell populations (goblet cells, plasma cells, eosinophils, lymphocytes in jejunum) and some cytokines expression (IL-1β, IL-6). It commonly assumed that mycotoxins with the same mode of action and/or with the same cellular target would have when present

together a synergistic or additive effect [50]. DON and FB quickly result in the activation of MAPK's [20,26,51] that are know to modulate numerous physiological processes, such as cell growth, apoptosis or immune response [52] . This might explain the synergistic and additive interaction we observed at the intestinal level. The effect in the MAPK's network cannot explain the other interactions we observed and we don't have simple hypothesis to propose. Indeed, many different factors may influence the outcome of an interaction, such as the endpoint assessed, the doses and the species used, or the time and route of exposure.

When the same animals, than the ones used in this study, were analyzed for their blood neutrophil counts, their lymphocytes proliferation or their kidney lesion, a less than additive interaction was also observed [24]. An additive effect of the toxins was determined for the liver and the lung lesions, the synthesis of specific antibody and the expression of cytokine in the spleen [24]. In the present study, we also that FB was able to prevent the DON-induced intestinal expression of IL-6. The same antagonistic interaction between these mycotoxins was already detected for the serum level of IgA [24]. Considering that IL-6 is driving the synthesis of IgA [53], it is more than likely that the ability of FB to prevent DON-induced expression of IgA is due to its effect on IL-6 synthesis.

Multi-contamination with low doses of mycotoxins is more likely to occur in natural contamination cereals, but only few studies investigated the effects of co-contaminated mycotoxin diets in pigs [6]. Taken together, the present data provide strong evidences that chronic ingestion of low doses of mycotoxins induces tissue lesions, modulated the immune cells count as well as the cytokine synthesis, and decrease the expression of proteins involved in cell adhesion. This suggests that ingestion of feed contaminated with these toxins may predispose animals to infections by enteric pathogens through an alteration of intestinal barrier function.

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Author's contribution

Conceived and designed the experiments: IPO, APFLB, BG, WDM and GS. Performed the experiments: APFLB, JL, BG and GDP. Analysed the data: APFLB, BG and IPO. Wrote the paper: IPO and APFLB.

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ARTIGO 3

The food contaminant deoxynivalenol activates the mitogen activated protein kinases in the intestine: comparison of *in vivo* and *ex vivo* models

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Joelma Lucioli[1, 2+], Philippe Pinton[1+], Patrick Callu[3], Joëlle Laffitte[1], Francois Grosjean[3], Martine Kolf-Clauw[44], Ana Paula Frederico Rodrigues Loureiro Bracarense[2] and Isabelle P. Oswald[1,5]

ABSTRACT: Mycotoxins are secondary metabolites considered as one of the most hazardous contaminants of concern in food and feed. The purpose of this study was to investigate the ability of DON activate the MAPK following low doses exposure, using *ex vivo* and *in vivo* approaches. The 24 pigs used in the *in vivo* experiment were randomly assigned to two groups and received separate diets during 4 weeks: a control diet and a DON-contaminated diet (2.3mg DON/Kg feed). Six weaning piglets were used for the jejunal explants experiments (*ex vivo* model). Explants were exposed during 4 hours to control, 5 µmol/L or 10 µmol/L of DON. Consumption of DON-contaminated-feed resulted in a decrease in feed intake and body weight gain. No significant changes were observed in the morphology of intestine between the different groups. On the other hand, the jejunum explants incubated with 10 µmol/L of DON showed a significant decrease on the total score morphological compared to the control group and 5 µmol/L-treated explants. We have then demonstrated that the exposure of intestinal tissue to DON *in vivo* or *ex vivo* leads to activation of the Mitogen Activated Protein Kinases. The consequences could impair the homeostasis of intestinal tissue in the aspect of barrier function or immune protection. These data provide also strong evidence that the relationship observed between the two models present the *ex vivo* model of jejunal explants as a good alternative for the study of intestinal tissue.

Key Words*:* Deoxynivalenol. Mycotoxin. Piglet. Swine. Mitogen activated protein kinases

[1] Toxalim UMR 1331-INRA-INP-UPS 180 chemin de Tournefeuille 31027 Toulouse cedex 3, France
[2] Universidade Estadual de Londrina, Departamento de Medicina Preventiva, Laboratório de Patologia Animal, Londrina (Paraná), Brazil
[3] Arvalis Institut du Végétal, Pouline, 41100 Villerable, France
[4] Université de Toulouse, Ecole Nationale Vétérinaire (ENVT), 23 chemin des Capelles, BP 87614, 31300 Toulouse Cedex 3, France
[5] These authors equally contributed to this work. Corresponding author: ioswald@toulouse.inra.fr

1 INTRODUCTION

Mycotoxins are secondary metabolites produced by fungi and detected in various food commodities from many parts of the world. They are presently considered as one of the most hazardous contaminants of concern in food and feed [3]. No region of the world escapes the problem of mycotoxins and according to Lawlor and Lynch [9], until 25% of the world's crops each year are contaminated. The trichothecene deoxynivalenol (DON) contaminates cereals worldwide after grain infestation by *Fusarium* species fungi mainly in field before harvest [13].

DON is resistant to standard processes such as milling and baking and can be found in finished food or feed [18]. DON exhibits toxic effects in humans and all animal species investigated to date [7,18]. Because of the high percentage of wheat in the diets, swine could be at a greater risk of exposure to this toxin. Pigs are particularly susceptible to DON, as they show overt signs of toxicity such as feed refusal, increased salivation and vomiting following ingestion of high doses of DON [5, 26]. More commonly, chronic exposure to lower amounts of DON is of major interest in DON-caused economical losses in animal production due to reduced feed intake and live weight gain, resulting in an increased incidence of infectious diseases and digestive disorders [7, 19, 26].

Trichothecenes inhibit protein synthesis by binding to the ribosomal peptidyltransferase. Inhibitors of the peptidyltransferase reaction can trigger a ribotoxic stress response that activates mitogen-activated protein kinase (MAPK), components of the signaling cascade that regulates cell survival in response to stress [28]. These kinases modulate numerous physiological processes including cell growth, differentiation and apoptosis [4, 14] and are crucial for signal transduction in the immune response [6]. DON activates MAPK's *in vitro* [11, 16, 22, 25, 29] and *in vivo* [29] impairing intestinal nutrient absorption [2, 24] and cell functions affecting the barrier function of the intestine [16, 17]. Intestinal explants represent a relevant and sensitive model to investigate the effects of food contaminants such as DON [8]. However, there is no published data comparing the effects of *ex vivo* and *in vivo* models.

The objective of this study was to investigate the ability of DON to activate the MAPK's after low doses exposure, using the *ex vivo* (jejunal explants exposed to the toxin) and *in vivo* (samples of jejunum from animal exposed to DON contaminated feed) models.

2 MATERIAL AND METHODS

Animals were cared for in accordance with Guidelines National Institute of Health Guide and the French Ministry of Agriculture for the care and use of laboratory. Animals were bred in facilities approved by the French Veterinary Services for animal health and protection (agreement 31-2009-37 obtained April, 02, 2009).

2.1 In vivo Exposure of Pig Intestine to Deoxynivalenol

2.1.1 Animals, Performances and Sample Collection

Twenty four castrated male crossbred pigs (Large White X Pietrain) were used for the experiments. They were acquired locally at 4 week of age, just after weaning, and acclimatized for 20 d in the pig-rearing house of the experimental station of Arvalis Institut du Végétal (Pouline, Villerable, France). Piglets were then distributed within 2 experimental groups according to body weight (9.3 ± 1.4 Kg). Pigs were housed individually with free access to feed and water. They were weighted at day 0, 14 and 28. Feed consumption was also measured. At the end of the experiment, 6 randomly selected animals per experimental groups were submitted to electrical stunning, and euthanized by exsanguination. Samples of jejunum and ileum were collected and fixed in 10% buffered formalin for 24 h for histological analysis. Jejunal samples were collected, snap-frozen in liquid nitrogen and stored at – 80 °C for Western Blot analysis.

2.1.2 Experimental Diet

The experimental diets were prepared locally and formulated according to energy and amino acid requirements for piglets as already described [1]. Two different batches of wheat were used in the diets: one control batch free from mycotoxin contamination and one batch naturally contaminated with DON. Two diets were prepared from the above mentioned wheat batches, soybean meal, amino acids, and a vitamin and mineral premix (Table 1). Dietary contents of DON, 3-acetyl deoxynivalenol, 15-acetyl deoxynivalenol, nivalenol, diacetoxyscirpenol, T-2 toxin, HT-2 toxin were analyzed in the raw materials and in the final diets using GC-MS technique; zearalenone and fumonisin B_1 and B_2 with HPLC

techniques (Qualtech, Vandoeuvre les Nancy, France). Deoxynivalenol content in control and contaminated diet was 0.115 and 2.3 mg DON/Kg respectively.

TABLE 1 – Composition and mycotoxin contamination of experimental diets.

	Diet	
	Control	DON
Composition (%)		
Control wheat	72.44	31.65
Contaminated wheat	0.00	40.00
Soybean meal	20.49	21.30
Soy bean oil	2.00	2.00
Lysine HCl	0.6	0.6
L-Threonine	0.25	0.25
DL-Methionine	0.18	0.18
L-Tryptophane	0.042	0.042
Vitamin and mineral premix*	4.00	4.00
Mycotoxin contamination (mg/Kg)		
Deoxynivalenol	0.115	2.3
3-acetyl DON	n.d.	0.015
15-acetyl DON	n.d.	0.050
Nivalenol	0.075	0.115
DiAcetoxyScripenol	n.d.	n.d.
T2 or HT2 Toxin	n.d.	n.d.
Zearalenone	0.065	0.065.
Fumonisin B1 or B2	0.010	0.010.

* Provided per kilogram of diet: vitamin A, 200 mg; vitamin D_3, 60 mg; vitamin E, 28.8 MIU; vitamin C, 184 mg; vitamin K_3, 2.1 mg; thiamin, 2.1 MIU; riboflavin, 3.2 mg; pantothenic acid, 20.0 mg; niacin, 24 mg; pyridoxine, 5.2 mg; choline, 1.150 mg; folic acid, 2.0 mg; biotin, 0.20 mg; vitamin B_{12}, 0.03 mg; manganese, 61.2 mg; zinc, 179 mg; iron, 101 mg; copper, 17 mg; iodine, 1.24 mg; selenium, 0.20 mg; calcium, 11.340 mg and phosphorus, 3.630 mg. n.d. : not detectable.
Detection limits: 10 µg/Kg for the diacetoxyscirpenol, T-2 toxin, HT-2 toxin, DON, 3-acetyl deoxynivalenol, 15-acetyl deoxynivalenol and nivalenol, 10 µg/Kg for fumonisin B_1 and fumonisin B_2, 15 µg/Kg for zearalenone.

2.2 Ex Vivo Exposure of Pig Intestine to Deoxynivalenol

2.2.1 Animals

Six crossbreed weaning piglets of 4 week-old were used for jejunal explants. Piglets were acclimatized for 1 week in the animal facility of the INRA Toxalim

(Toulouse, France), prior to being used in experimental protocols. Feed and water were provided *ad libitum* through the experimental period. At the end of experiment, piglets were submitted to electrical stunning, and euthanized by exsanguination.

2.2.2 Jejunum Explants Preparation

The explants were obtained as already described [8] with minor modification. Briefly, a 5 cm middle jejunum segments were collected in complete William's Medium E (i.e William's Medium E supplemented with 25 mmol/L glucose, 200 U/mL penicillin, 200 µg/ mL streptomycin and 50 µg/mL gentamicin. These reagents were from Sigma (St. Quentin Fallavier, France). Four to six washes were performed with William's Medium E. Each jejunum segment was then opened longitudinally and pieces of 6 mm diameter were obtained from intestinal tissue with biopsy punches (Kruuse, Centravet, Dinan; France). Two explants/well were deposited villi upward on biopsy sponges in 6 well plates containing control or DON-contaminated medium (Cellstar, Greiner Bio-One). All these operations were achieved in less than 1 h after the piglets were euthanized.

2.2.3 Jejunum Explants Treatment

Explants were exposed to DMSO diluted 1/500 (control) or to 5 or 10 µmol/L DON diluted in complete William's Medium E culture medium at 37 °C under CO_2 controlled atmosphere with orbital shaking for 4 hours. DMSO was used in control explants, as DON stock solution (5 mM) was prepared in DMSO. At the end of the experiment, jejunal explants were collected and either fixed in 10% buffered formalin for 24 h for histological analysis or snap-frozen in liquid nitrogen and stored at – 80 °C for Western Blot analysis.

2.3 Histopathological and Morphometrical Assessment

The fixed tissue pieces were dehydrated through graded alcohols and embedded in paraffin wax. Sections of 3 µm were stained with hematoxylin-eosin (HE) for histopathological evaluation. The resulting slides were viewed independently by two observers at a magnification of x 100. Histological lesions were recorded and a tissue score was established based on the occurrence and severity of lesions as already described [8], with minor modification. The score system, representing a maximum of 12 points, includes both

morphological and lesional information (Table 2). Villi height was measured randomly on thirty villi by MOTIC Image Plus 2.0 ML® image analysis system.

TABLE 2 – Endpoints used to assess histologically the explants in a morphological score (maximal score of 12 points)

Description		End-points	Score
Lesional	Villous fusion	Absent	1
		Present	0
	Zones of lysis	Absent	2
		Localized	1
		Multifocal	0
	Necrosis	Absent	2
		Only at the top of the vili	1
		At the top and at the bottom of the vili	0
	Cellular debris	Absent	1
		Present	0
Morphological	Number of villis	> 10	3
		(5-10)	2
		< 5	1
		0	0
	Epithelial cells	Cylindrical	3
		Cubic	2
		Flat	1
		Absent	0

2.4 TISSUE AND CELLS PROTEIN EXTRACTION, SDS-PAGE, AND IMMUNOBLOTTING

Frozen jejunal samples were washed on ice with PBS-EDTA (0.25 mol/L) with protease inhibitor cocktail (Roche Diagnostics, Meylan, France), lysed on ice in a Potter tissue grinder with lysis buffer (20 mmol/L Tris–HCL pH 8, 5 mmol/L EDTA, 0.02% NaN_3, 1%Triton X100) supplemented with protease inhibitor cocktail. Lysates were homogenized through a 26G needle and sonicated for 30 s. Homogenates were diluted 1/2 with lysis buffer and heated at 100 °C for 10 min before protein quantification. Equal amounts of proteins were loaded a 12.5% acrylamide gel. Migration was conducted in a 250 mmol/L Tris buffer (pH7.6) containing 1% SDS and 1.92 mol/L Glycine. After separation, proteins were transferred onto Optitran BA-S 83 membrane (Whatman®, Germany). The primary antibodies used were Phospho p44/42 ERK MAPK, Phospho SAPK/JNK, Phospho p38 MAPK (diluted 1:500) and □-actin, used as control (diluted 1/1000) (Cell Signaling Technology, Danvers, MA). Membranes were then washed and incubated with secondary antibodies CF™770 goat anti rabbit IgG or CF™770 goat anti mouse IgG (diluted 1:10 000)

obtained from Biotium (Hayward, CA, USA). Band densities were obtained by scanning the membranes using Odyssey® Infrared Imaging System (LI-COR ScienceTec, Les Ulis, France). Fluorescent intensities were determined using LI-COR imaging software after correction for bacKground. The expression of the protein was estimated after normalization calculated by the ratio of the intensity of the band of interest and of the □-actin band.

2.5 Statistical Analysis

The results are presented as means ± SD of independent experiments with different animals. *In vivo* data were analyzed by using Student's test. *Ex vivo* data were compared by one way ANOVA analysis to test the effect of the two concentrations of DON (SYSTAT version 10.0). If significantly different (P values ≤ 0.05), the means were compared by Dunnett's test to control values or by Tukey test for multiple comparisons.

3 RESULTS

3.1 DON Decreases the Zootechnical Performances of Exposed Animals

DON exposure is known to induce in animals, reduction of feed intake, particularly in pigs. A four-week consumption of a contaminated diet (2.3 mg DON / Kg feed) resulted in a decrease of approximately 10% in food intake and body weight gain (Table 3), compared with the control group (P< 0.001).

TABLE 3 – Effect of DON ingestion on animal performances

	Diets	
	Control	**DON 2.3 mg/Kg**
Feed intake (g/d)		
d 1 to 14	750 ± 94	640 ± 65***
d 15 to 28	1211 ± 109	1120 ± 139***
Total period (d 1 to 28)	980 ± 91	880 ± 87***
Weight gain (g/d)		
d 1 to 14	484 ± 67	424 ± 55***
d 15 to 28	751 ± 75	704 ± 92*
Total period (d 1 to 28)	617 ± 59	564 ± 55***

Data are means of 12 piglets (± SD). Comparison between control and DON treated animals P<0.05*; P<0.001***

3.2 DON INDUCES HISTOLOGICAL LESIONS ON THE INTESTINE

The intestinal tissue is a target for DON when contaminated feed is ingested. Our aim was to evaluate the impact of DON on the structure of intestinal epithelium of DON exposed animals as well as *ex vivo*-treated explants. Morphology and lesions were compared between treatments in *in vivo* trial. The scores observed for the morphology of the jejunum were 5.7 and 5.0 for control and DON-contaminated feed group, respectively. The scores evaluating the lesions were of 3.8 and 2.5 for control and DON-contaminated feed group, respectively. In the ileum, a decrease of the morphological (5.8 and 5.2) and lesional (4.7 and 3.3) scores were observed between control group and DON-contaminated feed group respectively. This decrease was not significantly different. On another hand, changes in villous height are indicative of enterocytes loss and impaired absorption of nutrients. The mean villi heights (µm) of the jejunum were 373.72 ± 46.64 and 336.02 ± 29.78 for control and DON-contaminated feed group, respectively. The mean villi heights (µm) of the ileum were 327.25 ± 27.11 and 291.83 ± 39.19 for control and DON-contaminated feed group, respectively (Table 4).

TABLE 4 – Effect of exposure on jejunum and ileum villi height

	Diets	
	Control	**DON 2.3 mg/Kg**
Jejujum	373.73 ± 46.64^{a}	336.02 ± 29.78^{a}
Ileum	327.25 ± 27.11^{a}	291.83 ± 39.19^{a}

Notes: results are expressed as mean ± SE for 12 animals. Means without a common letter differ, $P < 0.05$.

As shown in **Figure 1**, the jejunum explants incubated with 10 µmol/L of DON showed a significant decrease on the total score compared to the control group ($P<0.001$) and 5 µmol/L treated explants ($P<0.05$). The score observed for lesional and morphological changes were 3.3, 1.8 and 1.7, and 4.6, 3.9 and 2.3 for 0, 5 and 10 µmol/L of DON, respectively. After 4 hours of culture with DON flattening of the villi, weak coalescence, necrosis and cellular debris were observed in the slices (Figure 2).

Figure 1 – Effect of 5 and 10 µmol/L DON on the scores of explants after 4 hour of culture. (U.A.: arbitrary unit). Jejunal explants obtained from 4 weeks old pigs were cultured *in vitro* for 4 h with 0, 5 and 10 µmol/L DON before histological examination (lesional and morphological score assessment). For each mycotoxin 2 to 4 explants from the same animal were scored. Data are mean scores ± SD from 6 animals/group. ANOVA analysis was followed by DUNETT (*: P<0.05 / **: P<0.001).

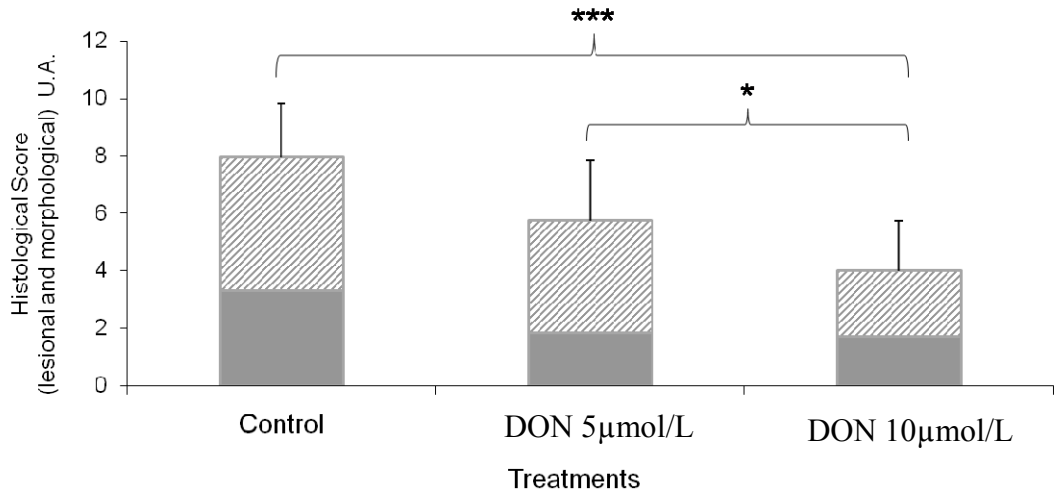
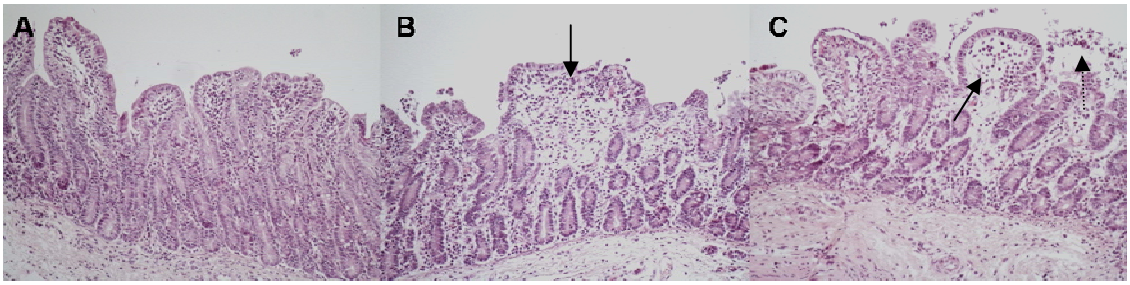


Figure 2 – Effect of 10 µmol/L DON on the morphology of jejunal explants obtained from 4 weeks old pigs, compared to a control explants after 4 hours of incubation. Control explants (A) and 10 µmol/L (B) necrosis and coalescence villi (arrow) and (C) edema (arrow) and debris cellular (dotted arrow). HE, obj. 10x.



3.3 DON Activates the Mitogen-Activated Protein kinase in Vivo and Ex Vivo

The capacity of the ribotoxic stressor DON to induce MAPK phosphorylation was verified using Western Blot analysis. For the jejunal explants it was observed that at concentration 5 and 10 µmol/L, DON induced the phosphorylation of p44/42 ERK ½ and p38 in a dose-dependent manner after 4 hours of incubation compared to the control group. Similar behavior was verified in the jejunal samples of the *in vivo* model at concentration 2.3 mg of DON/Kg of feed. However, in both experimental models the

expression of phospho SAPK/JNK was not altered by the exposition of DON (**Figures 3, 4 and 5**).

Figure 3 – Activation of phospho ERK 1/2 , phospho p38 and phospho SAPK/JNK induced by 5 and 10 µmol/L of deoxynivalenol in jejunal explants after 4 hours of culture. Data are mean scores ± SD from 5-6 different animals/group. ANOVA analysis was followed by DUNETT, asterisk indicates significant difference compared to control explants (*: P<0.05; ***: P<0.001)

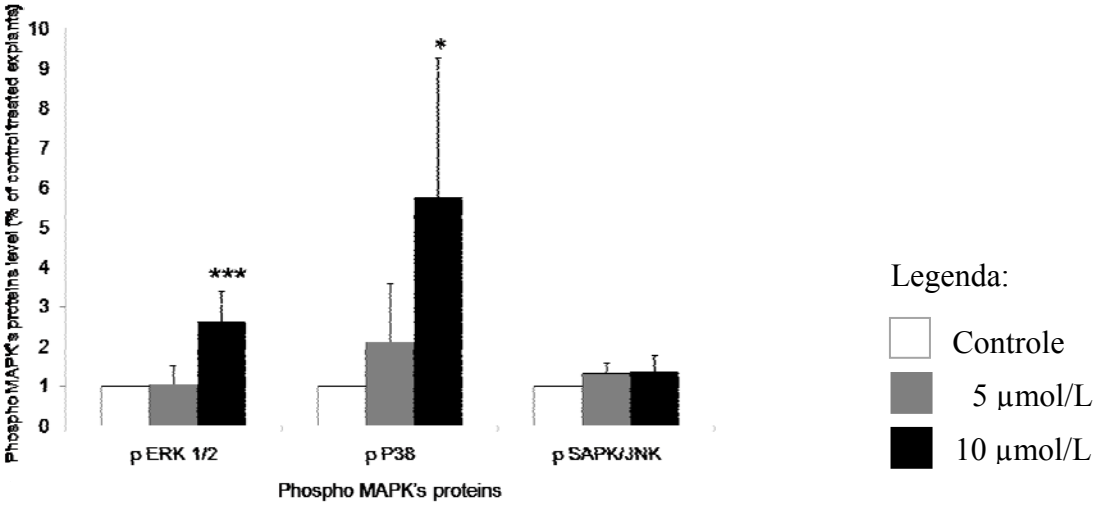


Figure 4 – Expression of MAPK's jejunal explants induced by deoxynivalenol. Jejunal explants obtained from 4-5 weeks old pigs incubated with control and 10 µmol/L of deoxynivalenol for 4 h were subjected to Western blot analysis using phospho ERK1/2, phospho p38 MAPK and phospho SAPK/JNK.

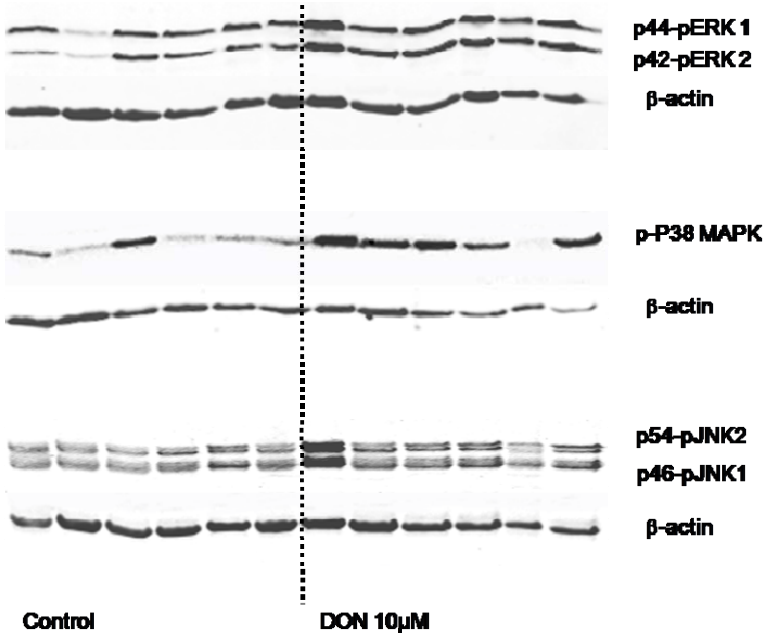
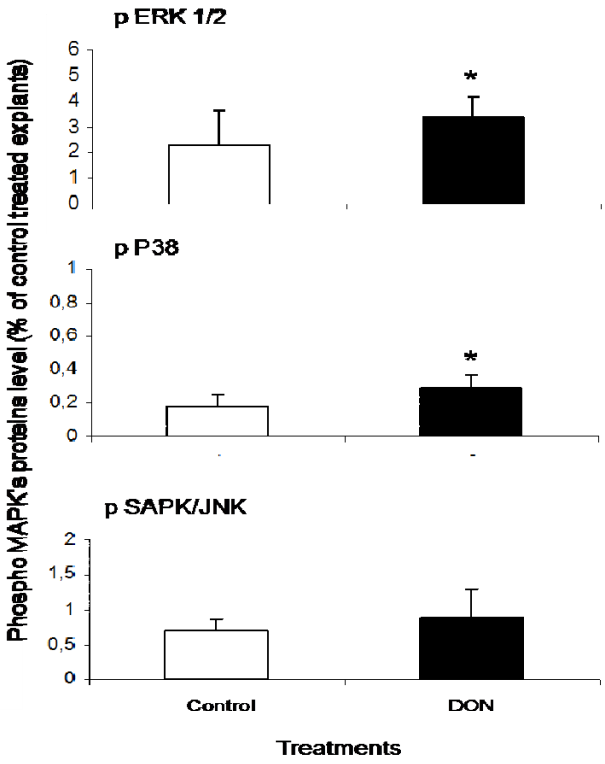


Figure 5 – Activation of phospho ERK ½, phospho p38 and phospho SAPK/JNK induced by 2.3 mg DON Kg feed for a period for 28 days. Data are mean scores ± SD from 6 animals. ANOVA analysis was followed by DUNETT, asterisk indicates significant difference from control values (*: P<0,05; ***: P<0,001)



4 DISCUSSION

The intestinal tract represents the first barrier against ingested chemicals and food contaminants, as mycotoxins, and is also the first line of defense against intestinal infection. Because of their location, intestinal epithelial cells could be exposed to high doses of food contaminants. Deoxynivalenol, one of the most important trichothecenes, is a known inducer of the MAPK pathway via a mechanism called "*Ribotoxic Stress Response*" [15]. At the intestinal tissue, DON alters the intestinal structure [8], affects the nutrient absorption [10] and decreases the expression of the tight junction claudin proteins [17].

To investigate the ability of DON to activate the MAPK's, when administered at low doses, we used two different experimental approaches: the *in vivo* exposure of pigs to DON contaminated feed and the *ex vivo* treatment of jejunal explants. In the *in vivo* study, we demonstrated that MAPK activation occurs in the intestinal epithelium of piglets fed diets contaminated with 2.3 mg DON/Kg feed during of 28 days. The MAPK activation was also observed in jejunal explants exposed to control, 5 and 10 µmol/L of DON during 4 hours. As previously discussed, it is difficult to correlate *in vitro* toxin

concentration with *in vivo* exposure. However, the concentration of toxin in feed and the concentration in culture medium in our experiments are in accordance as 2.3 mg DON/Kg feed corresponds to 7.7 µmol/L [16, 17]. It is particularly interesting to observe that in both *in vivo* and *in vitro* models, there is a good correlation in the increase of MAPK expression. This increase was mainly observed for phospho p44/42 ERK and phospho p38. MAPK p44/42 ERK is of particularly importance because it can be involved in intestinal epithelial cell morphology and in the structure of tight junctions that regulate the barrier function of the intestinal tract [12].

Our findings are similar to those described by Pinton et al. (2010) [16], who investigated the ability of DON to alter the intestinal barrier function through and the interaction with the MAPK signaling pathway in a highly sensitive porcine intestinal epithelial cell line (IPEC-1). They showed that the activation of p44/42 ERK, as a consequence of 30 µmol/L DON exposure, decreases the expression of the tight junction protein claudin-4, which in turn reduces the barrier function in the intestine. In the present study, after 4 hours of culture, the jejunum explants incubated with 10 µmol/L DON showed significant decrease in their total score in comparison to the control group ($P<0.001$) and 5 µmol/L of DON ($P<0.05$).

Alterations of the intestinal epithelium as coalescence of villi, lysis of enterocytes, intersticial edema and cellular debris were associated with exposure to DON at doses of 10 µmol/L. These findings are consistent to those found by Kolf-Clauw et al. (2009) [8], confirming that acute exposure to low doses of DON cause injury to the gut. In our study, piglets fed with 2.3 mg DON/Kg feed contaminated diet *ad libitum* the feed intake and body weight decreased of approximately 10% compared to the control group. This finding is in agreement with Swamy et al. (2002) [23], who also observed a significant decrease of growth performance and feed intake of 34.6 and 32.6 %, respectively, but no significant effect on gain to feed ratio when feeding starter pigs with DON-naturally contaminated feed (5.6 mg DON/Kg feed) over 21 d.

In conclusion, we didn't observed significant reduction in the total score of histopathological and morphometric analysis of villi in the jejunum and ileum of animals fed feed contaminated with DON. The difference in results between the models *in vivo* and *ex vivo* can be explained by the fact that the amount of mycotoxins in food absorbed *in vivo* does not necessarily correspond to the amount absorbed in the *ex vivo* model. Usually in experiments with cultured cells or tissues purified mycotoxins are used, whereas *in vivo* assays are used naturally contaminated foods. However, the response observed for the

expression of MAPK's in both models was very similar, which leads to confirmation of the toxic potential of DON at 10 μmol/L. Also the explant model is a good alternative for this type of study, where the goal is to verify toxigenicity using low doses of substances.

Acknowledgements

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6 CONCLUSÃO

i. A ingestão crônica de dieta contaminada com DON (2,3 mg/Kg) reduziu em 10% o consumo alimentar e ganho de peso.

ii. A alimentação com dietas contaminadas com FB induziu ao aumento da concentração sérica de creatinina e a redução do número de neutrófilos circulantes, enq uanto que a ingestão de DON induziu a redução da concentração sérica de albumina.

iii. A ingestão crônica de dietas contaminadas com doses baixas de DON, FB ou ambas as micotoxinas induziu a redução da resposta imune sistêmica e aumentou a expressão de citocinas no intestino.

iv. A ingestão crônica de dietas contaminadas com doses baixas de DON, FB ou ambas as micotoxinas induziu alterações histológicas no fígado, pulmões, rins e intestinos de leitões.

v. A ingestão crônica de dietas contaminadas com DON, FB ou ambas as micotoxinas diminuiu a expressão de proteínas de junção celular no intestino de suínos.

vi. A exposição de explantes intestinais a 5 e 10 µmol/L DON induziu a alterações histológicas e aumento da expressão de MAPKinases.

CONSIDERAÇÕES FINAIS

Cada vez mais se tem buscado a melhora na qualidade sanitária das dietas dos animais, com o intuito de aumentar os índices de produtividade e também a qualidade do produto final. A contaminação de alimentos e rações por micotoxinas representa um sério problema de saúde para humanos e animais, além de ser um obstáculo à economia de países, como o Brasil, nos quais a balança comercial se baseia nas exportações de *commodities*.

Muito embora nossa legislação estipule limites máximos para diferentes toxinas em alimentos para o consumo humano e animal, faltam ainda estudos mais aprofundados que contemplem a multicontaminação, bem como, a utilização de dosagens mais baixas, próximas as que normalmente são detectadas em nossas culturas. A partir desse estudo, comprovamos os efeitos deletérios ocasionados pela exposição crônica a baixas doses de micotoxinas e a importância desses sobre o *status* sanitário animal.

O reconhecimento dos problemas causados pela ingestão de micotoxinas, seja de forma isolada ou em associação, é sem dúvida o primeiro passo para a implementação de programas que permitam a adoção de medidas apropriadas para a prevenção e redução do

problema. Para tanto se faz necessário a implantação de rotina de inspeção e um maior rigor no cumprimento da legislação. No Brasil, embora sabidamente as micotoxinas sejam responsáveis por expressivos prejuízos na produção de grãos, praticamente não existem estimativas das perdas econômicas associadas a elas.

APÊNDICES

APÊNDICE A

Padronização técnica Imunoistoquímica (IHQ)

Técnica IHQ

Para a padronização do teste de IHQ foram utilizadas amostras de fígado para detecção de Ki-67 e intestinos (jejuno e íleo) para detecção de E-caderina. Como controle positivo para o Ki-67 utilizou-se amostra de tecido mamário canino, positivo para carcinoma, provenientes do Hospital Veterinário/UEL e para a E-caderina amostras de pele humana saudável, provenientes do Laboratório de Patologia do Hospital Universitário/UEL.

1. Lâminas revestidas com **HistoGrip**™ contendo os cortes histológicos seccionados em 3 μm, devem ser colocadas em estufa a 60°C por 3 horas e em seguida desparafinadas em xilol por 20 minutos. Em seguida, os cortes são reidratados em soluções de álcool etílico com concentração decrescente (100%, 95% e 70%), por 10 minutos em cada concentração. Essa etapa é finalizada por uma lavagem em água corrente por 10 minutos, seguida de dois enxágües com água destilada.

2. Para **RECUPERAÇÃO ANTIGÊNICA** as lâminas são colocadas em recipiente plástico com tampa (próprio para microondas) com solução tampão **TRIS EDTA Tween 20 (pH 9,0)**, em quantidade suficiente para cobrir os tecidos. Deixar o recipiente com a tampa entreaberta e realizar 3 ciclos (para amostras Ki-67) e 5 ciclos (amostras E-caderina) de 3 minutos, em potência de 750W em microondas. Em seguida, as lâminas são deixadas com o recipiente entreaberto, esfriando em temperatura ambiente. Após resfriamento, lavar em água corrente por 10 minutos, seguido de dois enxágües com água destilada.

3. Para o **BLOQUEIO DA PEROXIDASE ENDÓGENA**, colocar as lâminas em solução de 140 ml de **METANOL** + 10 ml de **ÁGUA OXIGENADA** 20 volumes (solução deve ser preparada na hora do uso). Deixar em câmara escura por 20 minutos. Em seguida lavar em água corrente por 10 minutos e enxaguar duas vezes com água destilada.

4. Como **ANTICORPOS PRIMÁRIOS** utilizou-se anticorpos monoclonais **Zymed Ki-67 (Clone 7B11)** e Zymed **anti-E-cadherin (Clone 4A2C7)**, ambos na diluição de 1:50. Após secagem individual das lâminas com o auxílio de papel absorvente e colocação de uma gota do anticorpo sobre os cortes, as mesmas são incubadas em câmara úmida a 4°C *overnight.*

IMPORTANTE: Procedimento a ser utilizado quando o anticorpo primário NÃO for diluído com Solução AZIDA - após realizar o bloqueio da peroxidase endógena, secar as lâminas individualmente e colocar uma gota de BSA 5%, cobrindo todo o corte, deixando incubar por 1 hora. Lavar as lâminas com Solução PBS pH 7,2 por 5 minutos, repetir duas vezes. E só então colocar o ANTICORPO PRIMÁRIO.

5. Após incubação, lavar as lâminas com **Solução PBS (pH 7,2)** por 5 minutos por duas vezes.Secar as lâminas individualmente com papel absorvente e colocar uma gota do **ANTICORPO SECUNDÁRIO (**Kit Super Picture^{TM} Zymed, South San Francisco, CA USA), cobrindo todo o corte. Cobrir as lâminas protegendo-as da luz e deixar incubando por 20 minutos.

6. **A partir daqui recomenda-se utilizar LUVAS**. Após incubação com o **ANTICORPO SECUNDÁRIO**, lavar as lâminas com **Solução PBS (pH 7,2)** por 5 minutos por duas vezes. A solução de tetra-hidrocloreto de 3,5-diamino-benzidina (DAB) (Peroxidase Substrat Kit DAB) deve ser misturada imediatamente antes do uso de acordo com a recomendação do fabricante e quantidade suficiente da solução deve ser colocada sobre os cortes (1 gota é suficiente). Deixar reagir por 3 minutos em câmara escura. Em seguida lavar as lâminas em água corrente por 10 minutos, enxaguar em água destilada e, em seguida, contracorar com Hematoxilina de Harris por 1 minuto e novamente lavar em água destilada durante 10 minutos. Após, as lâminas devem ser desidratadas rapidamente em soluções com concentração crescente de álcool etílico (70%, 95% e 100%) e montadas com Entellan.

SOLUÇÕES utilizadas:

Solução HistoGrip^{TM}

3 ml de HistoGrip^{TM} + 150 ml de acetona

Solução PBS pH 7,2

1,98 g de $Na_2HPO_4.7H_2O$ + 0,36 g de $NaH_2PO_4.H_2O$ + 8,17 g de NaCl + 1000 ml de água destilada

Solução TRIS EDTA Tween 20 pH 9,0

0,372 g de EDTA + 1,211 g de TRIS + 200 µl de Tween 20 + 1000 ml de água destilada

APÊNDICE B

Padronização técnica Western Blot (WB)

Etapa 1 – Extração

Em tubos Eppendorfer® de 2 ml, previamente identificados, colocar um fragmento da amostra (± 0,5 cm) e acrescentar 1 ml de Solução de Lise (**Solução 1**). Manter os Eppendorfer's® no gelo durante todo o procedimento. Deixar descansando por 10 minutos e em seguida triturar os fragmentos, em seguida passar pelo sonicador de ponteira por 30 segundos.

Etapa 1 - Preparação das amostras:

Em tubos Eppendorfer® de 1,5 ml, previamente identificados, diluir 50 µl de amostra em 50 µl de solução tampão SBX3 **(Solução 2).** Fechar os tubos e perfurar as tampas com uma agulha e então colocar em banho quente por 10 minutos a 100º C. Em tubos de ensaio, previamente identificados com o número de cada amostra, colocar 260 µl de água Milli-Q® + 30 µl de Tris SDS (**Solução 3**) + 10 µl da amostra + 100 µl de TCA 60% (**Solução 4**). Agitar vigorosamente com VORTEX® para completa homogeneização.

Etapa 2 – Deposição das amostras em membrana de nitrocelulose, leitura e cálculo concentração de proteínas:

Preparação do equipamento: protocolo desenvolvido para um sistema de sucção a vácuo utilizando aparelho Slot Blot® (96 poços). Estabelecer para cada amostra, dois poços destinados ao deposito da amostra. Os depósitos são feitos sobre uma membrana de nitrocelulose OPTITRAN BA-S 83® de 0.45 µ de 11 x 8 cm de superfície. O que permite a deposição de 25 amostras. Não se esquecer de manipular a membrana utilizando uma pinça. O aparelho Slot Blot consiste em uma placa de 96 poços, aonde a parte superior é composta por quatro parafusos e molas que permite uma vedação eficaz.

1. Colocar a membrana de nitrocelulose por 1 a 2 minutos em uma placa de Petri com um pouco de água Milli-Q® (quantidade suficiente para cobrir a membrana)
2. Em seguida, transferir a membrana para outra Placa de Petri com um pouco de TCA 6% (**Solução 5**), em quantidade suficiente para cobrir a membrana.
3. Montar o aparelho, acoplá-lo ao vácuo e colocar uma folha de papel absorvente e sobre esta a membrana de nitrocelulose. Verificar a eficiente do sistema a vácuo distribuindo sobre a membrana 200 µmL de água Milli-Q®. Colocar a tampão com os 96

poços sobre a membrana de nitrocelulose e fechar bem. Utilizando uma pipeta multicanal, distribuir 200 µl de TCA 6% nos poços.

4. Iniciar a deposição das amostras utilizando uma pipeta de 200 µl. Distribuir 190 µl da amostra em cada um dos dois poços, um abaixo do outro. Rinçar o tubo de ensaio com 200 µl de TCA 6% e distribuí-los nos dois poços. Proceder da mesma forma para as demais amostras. Lembrar sempre de agitar bem as amostras antes de distribuí-las e sempre trocar a ponteira da pipeta.

5. Após a distribuição de todas as amostras, rinçar a membrana sob aspiração constante com TCA 6%, com auxílio da pipeta multicanal. Deixar aspirar bem o conteúdo dos pocinhos para então desacoplar o sistema de vácuo, abrir o aparelho e recuperar a membrana de nitrocelulose.

6. Colocar a membrana de nitrocelulose em Placa de Petri com Solução Amido Black (**Solução 6**) por 3 minutos, sob agitação constante. Tomar o cuidado para não inverter a posição da membrana e conseqüentemente a ordem das amostras. A região na membrana onde a amostra está depositada aparece como pequenos círculos de coloração azul intensa.

7. Em seguida lavar a membrana por 30 segundos com água Milli-Q®, em seguida deixar a membrana por 1 minuto em solução de descoloração (**Solução 7**) e novamente lavar a membrana por 30 segundos com água Milli-Q®.

8. Colocar a membrana em uma placa de Petri, sem nenhuma solução, e fazer uma fotocópia da membrana.

9. Colocar em tubos de ensaio, previamente identificados com o número das amostras, 1 ml de Tampão de extração (**Solução 8**).

10. Utilizando luvas, pinça e tesoura, cortar os dois "spots" correspondentes a cada amostra na membrana, dobrar ao meio e colocar no tubo de ensaio com o tampão de extração.

11. Agitar vigorosamente utilizando VORTEX® até que a membrana fique sem nenhum traço da amostra (coloração azul).

12. Utilizando placa de 96 poços, distribuir o conteúdo de cada tubo de ensaio em três poços por amostra.

13. Fazer a leitura espectrofotométrica (absorbância a 630 nm), estabelecer uma média e calcular a concentração em proteínas (µg/ml).

Cálculos:

Concentração da amostra correspondente a µg/ml =

(0.0014 + média espectrofotométrica obtida da amostra) / 0.0233

Quantidade obtida após filtração = [] da amostra correspondente a µg/ml x 1

Concentração de amostra em SBX3 (µg/ml) = quantidade obtida após filtração / 0.01

Volume de proteína desnaturada em SBX3 para 15 µg/ml = 1000 x 15 / Concentração de amostra em SBX3 (µg/ml) → **ESSE É O VOLUME QUE DEVE SER COLOCADO EM CADA UM DOS POÇOS DO GEL DE POLIACRILAMIDA**.

Etapa 3 – Eletroforese em gel de poliacrilamida e transferência sobre membrana de nitrocelulose.

1. Montar o suporte com as placas de vidro aonde será colocado o gel e feito a deposição das amostras. Para verificar se as placas estão bem encaixadas e não existe nenhum tipo de vazamento, preencher o espaço entre as placas de vidro com álcool. Antes de colocar as soluções de géis, escorrer completamente o álcool entre as placas.

2. **Gel de separação 12,5%:** em um Becker misturar 6.22 ml de solução Acrilamida 40% + 5.78 ml de água Milli-Q® + 5 ml de Tris/HCl 1.5 M (pH 8.9) (**Solução 9**) + 2.5 ml Glicerol 80% (**Solução 10**) + 10 µl de EDTA 0.5 M + 200 µl de SDS 20% (**Solução 11**) + 13.3 µl de TEMED + 200 µl de Persulfato de Amônio (**Solução 12**). Acrescentar o Persulfato de Amônio por último, pois quando em contato com o TEMED® inicia-se o processo de polimerização. Misturar bem e com auxílio de uma pipeta P5000 depositar entre as placas de vidro 3.4 ml da solução. Com auxílio de uma piseta acrescentar etanol, até as bordas das placas, para fixação do gel. Deixar polimerizar por 20 minutos.

3. **Gel de concentração**: em um Becker misturar 0.99 ml de solução de Acrilamida 40% + 3.17 ml de água Milli-Q® + 0.63 ml de Tris/HCl 1 M (pH 6.8) (**Solução 13**) + 5 µl de EDTA 0.5 M + 50 µl de SDS 20% + 6 µl de TEMED + 6 µl de azul de bromofenol 1% (**Solução 14**) + 40 µl de Persulfato de Amônio 10%. Misturar bem. Escorrer o álcool restante sobre o gel de separação já depositado entre as placas e com auxilio de uma pipeta P1000 depositar a solução sobre o gel de separação. Delicadamente (para evitar a formação de bolhas), encaixar o pente com as marcações para o depósito das amostras entre as placas de vidro. Limpar com papel absorvente o excesso de gel de concentração. Deixar polimerizar por 15 minutos.

4. Decorridos os 15 minutos, retirar o pente das placas de vidro. Notar a formação de espaços regulares no gel de concentração. Retirar as placas de vidro do suporte e

encaixá-las no aparelho Mini Trans-Blot cell (Bio Rad 170-3930). Colocar o conjunto dentro da cuba de plástico e completar com solução tampão de eletroforese 1X (**Solução 15**).

5. Descongelar as amostras em SBX3. Em capela efetuar a deposição das amostras nos poços formados no gel, conforme quantidades previamente determinadas através da quantificação de proteínas (TABELA). Não se esquecer de agitar bem a amostra antes de fazer o depósito em gel. Identificar bem a seqüência do depósito das amostras.

6. Transferência do gel sobre a membrana de nitrocelulose: a membrana de nitrocelulose deve ser manipulada somente com pinça e jamais entrar em contato com a mãos do manipulador. Cortar uma superfície de 9 x 6 cm e identificar a membrana com auxílio de uma caneta BIC. Deixar a membrana em Placa de Petri com água Miliq (o suficiente para cobri-la) enquanto prepara-se a cuba para transferência. Sobre a parte preta do suporte de transferência será feito o sanduíche de transferência. Aonde iremos colocar uma esponja, papel absorvente WATTMAN®, membrana de nitrocelulose, o gel recuperado da cuba de migração, papel absorvente WATTMAN® e outra esponja (com exceção do gel recuperado, todos previamente molhados em solução tampão de transferência 1X). Para evitar a formação de bolhas durante o processo de transferência, passar uma pequena régua fazendo leve pressão sobre o sanduíche formado. Fechar a placa de transferência, colocar na cuba e completar com a solução tampão de transferência 1X (**Solução 16**).

7. Posicionar a placa de transferência no suporte com os eletrodos e verificar o sentido da migração gel/membrana. Colocar uma pequena barra magnética para auxiliar na agitação. Colocar a cuba de transferência sobre um agitador e dar início a transferência que pode ser feita de duas maneiras: durante 2 horas a 250 mA e 270V ou durante a noite a 50 mA e 200V.

8. Após a transferência recuperar a(s) membrana(s) de nitrocelulose e verificar a presença de proteínas colocando a membrana em solução de Vermelho Ponceau (**Solução 17**) por 10 minutos sob agitação. A marcação permite visualizar a marcação das proteínas sobre a membrana e efetuar a localização das amostras e marcação de pesos moleculares. Pode-se colocar a membrana em isofilme (para proteger a fotocopiadora da umidade) e fazer uma fotocópia.

Etapa 4 – Western Blot

1. Incubar a membrana de nitrocelulose em solução de bloqueio TBST 5% leite (**solução 18**) por 3 horas sob agitação constante, em seguida lavar por cinco minutos com TBST (**Solução 19**) para eliminar todo e qualquer traço de solução de bloqueio.

2. Descongelar a solução de anticorpo primário a ser utilizada.

3. Incubar a membrana com a solução de anticorpo primária, sob agitação constante, por uma hora.

4. Ao fim de uma hora, recuperar a solução de anticorpo primário e recongelar a -20ºC. (Refazer a solução de anticorpo primária a cada cinco utilizações). Lavar a membrana com solução TBST por cinco minutos, sob agitação, por cinco vezes. Efetuar a troca de solução a cada lavagem.

5. Incubar a membrana em cuba protegida da luz com a solução de anticorpo secundário, sob agitação constante, por trinta minutos (o anticorpo secundário deve ser preparado momentos antes de ser utilizado). Passados trinta minutos, desprezar a solução de anticorpo secundário e lavar a membrana com solução TBST, sob agitação, por cinco vezes.

6. Manter a membrana em solução de TBST e efetuar a leitura em aparelho Odyssey® Infrared Imaging System (LICOR ScienceTec)

Soluções Utilizadas:

Solução 1 – Solução de Lise

Coquetel de inibidores de protease

Solução I: dissolver 3 mg de AEBSF, 1 mg de Aprotinina e 1 mg de Leupeptina em 1 ml de água Milli-Q®. Homogeneizar e fazer alíquotas de 20 µl que devem ser congeladas a -20ºC.

Solução II: dissolver 1 mg de Antipaina, 1 mg de Pepstatina A e 15 mg de Benzamidina em 1 ml de DMSO. Homogeneizar bem e fazer alíquotas de 50µl que devem ser congeladas a -20ºC.

Para cada 10 ml de solução de 20 mmol/L Tris HCl pH 8 + 5 mmol/L EDTA + 0,02% NaN3 + 1% Triton X100, colocar uma alíquota de cada solução.

Solução 2 – SBX3

Para 10 ml de solução SBX3: 1.9 ml Tris/HCl 1M (pH 6.8) + 3 ml de glicerol + 1.5 ml de β-mercaptoetanol + 3 ml de SDS + 0.3 ml de Azul Bromofenol + 0.3 ml de Água Milli-Q®.

Solução 3 – Tris SDS

Pesar 12.15 g de TRIS e 1 g de SDS, misturar e dissolver em 70 ml de água Miliq. Ajustar pH 7.4 e completar com água Milli-Q® até 100 ml.

Solução 4 – TCA 60%

Para 100 ml de solução: dissolver 60 g de TCA em 100 ml de água Milli-Q®, misturar bem até completa dissolução.

Solução 5 – TCA 6%

Para 500 ml de solução: dissolver 30 g de TCA em 500 ml de água Milli-Q®, misturar bem até completa dissolução.

Solução 6 – Amido Black

Para 200 ml de solução: pesar 0,2 g de NBB (Naphtol Blue Black) e em Becker de 250 ml acrescentar 90 ml de álcool metílico + 20 ml de etanol absoluto + 90 ml de água Milli-Q®.

Solução 7 – Solução de descoloração

Para 1000 ml de solução: 450 ml de álcool metílico + 100 ml de etanol absoluto + 450 ml de água Milli-Q®

Solução 8 - Solução de Extração de Coloração

Para 100 ml de solução: 2.5 ml de NaOH 1N (1g/100 ml) + 125 µl de EDTA 40mM (14.3mg/ml) + 50 ml de etanol 95% + 47.5 ml de água Milli-Q®.

Solução 9 – Tris/HCl 1.5 M (pH 8.9)

Para 500 ml de solução: dissolver 90.85 g de Tris em 300 ml de água Milli-Q®, ajustar a pH 8.9 e completar até 500 ml com água Milli-Q®.

Solução 10 – Glicerol 80%

Para 100 ml de solução: em proveta calibrada colocar 80 ml de glicerol 100% e completar com água Milli-Q® até 100 ml. Homogeneizar bem a solução. Essa solução deve ser conservada em temperatura ambiente.

Solução 11 – SDS 20%

Para 100 ml de solução: pesar 20 g de SDS e dissolver em 100 ml de água Milli-Q®. Conservar a solução em temperatura ambiente.

Solução 12 – Persulfato de Amônio 10%

Para 100 ml de solução: pesar 10 g de Persulfato de Amonio e dissolver em 100 ml de água Milli-Q®, misturar bem. Fazer alíquotas de 200 µl e congelar a -20º C.

Solução 13 – Tris/HCl 1 M (pH 6.8)

Para 500 ml de solução: dissolver 60.75 g de Tris em 300 ml de água Milli-Q®, homogeneizar bem e ajustar pH a 6.8. Completar até 500 ml com água Milli-Q®.

Solução 14 – Azul de Bromofenol 1%

Para 100 ml de solução: dissolver 1 g de Azul de Bromofenol em 100 ml de água Milli-Q. Conservar em temperatura ambiente.

Solução 15 – Tampão de Eletroforese 1X

Para 1000 ml de solução padrão 10X: em Becker de 1000 ml colocar 30 g de Tris + 144 g de Glicina + 10 g de SDS e acrescentar 700 ml de água Milli-Q® e misturar bem. Recomenda-se colocar o Becker sobre uma placa de agitação e com ajuda de uma barra magnética efetuar a agitação constante até completa dissolução dos reagentes. Após acrescentar os 300 ml de água Milli-Q® restante. Essa solução deve ser conservada em geladeira.

Para preparar 1000 ml de solução Tampão de Eletroforese 1X: diluir 100 ml da solução padrão 10X em 900 ml de água Milli-Q®. Essa solução pode ser conservada em temperatura ambiente.

Solução 16 – Tampão de Transferência 1X

Para 1000 ml de solução padrão 10X: em Becker de 1000 ml colocar 30 g de Tris + 144 g de Glicina e 1 g de SDS e acrescentar 700 ml de água Milli-Q® misturar bem. Recomenda-se colocar o Becker sobre uma placa de agitação e com ajuda de uma barra magnética efetuar a agitação constante até completa dissolução dos reagentes. Após acrescentar os 300 ml de água Milli-Q® restante. Essa solução deve ser conservada em geladeira.

Para preparar 1000 ml de solução Tampão de Transferência 1X: diluir 100 ml da solução padrão 10X em 100 ml de álcool etílico + 800 ml de água Milli-Q®. Essa solução pode ser conservada em temperatura ambiente.

Solução 17 – Solução Vermelho Ponceau

Solução de 0,5% de vermelho Ponceau + 3% de ácido acético

Solução 18 – Solução de Bloqueio TBST 5% leite

Para 100 ml de solução: dissolver 5 g de leite em pó desnatado em 100 ml de solução TBST, agitar bem até completa dissolução.

Solução 19 – Solução TBST

Para preparar 1000 ml de solução TBS 10X: dissolver 24.2 g de Tris + 87.66 g de NaCl em 1000 ml de água Milli-Q®, ajustar o pH a 7.5 com HCL 10N e autoclavar a solução a 120ºC por 20 minutos. Após esfriar conservar em geladeira a -20ºC.

Para preparar 1000 ml de solução TBS 1X: diluir 100 ml de solução TBS 10X em 900 ml de água Milli-Q®. Conservar em temperatura ambiente.

Para preparar 1000 ml de solução TBST: acrescentar uma 1 ml de Tween 20 a 1000 ml de solução TBS 1X. O Tween 20 é bastante viscoso, fazer a aspiração bem lentamente assegurando-se que foi completamente expulso da ponteira da pipeta.

Reagentes utilizados:

Tris (ICN REF.: 819623 CAS: 77-86-1)

HCl (Fisher REF: 1789 CAS: 7547-01-0)

SDS (Sigma REF: L4509 CAS: 151-21-3)

Glicerol (Prolabo 2438829)

B-mercaptoetanol (Pierce REF: 3562 CAS: 60-24-2)

Azul de bromofenol (Sigma REF: B0126 CAS: 115-39-9)

NBB (Sigma N3393)

NaCl (S9625 CAS 7647-14-5)

AEBSF (Sigma A8456)

Aprotinina (Sigma A4529)

Leupeptina (Sigma L2023)

Antipaina (Sigma A6191)

Pepstatina A (Sigma P4265)

Benzamidina (Sigma B6506)

Membrana de nitrocelulose OPTITRAN BA-S 83® (Schleicher & Schuell REF: 10439394)

ANEXOS

ANEXO A

RESOLUÇÃO - RDC N° 7, DE 18 DE FEVEREIRO DE 2011 – ANVISA

Limites máximos tolerados (LMT) em alimentos prontos e em matérias-primas estabelecidos pela ANVISA, através de resolução RDC nº 7, de 18 de fevereiro de 2011, com aplicação imediata.

Micotoxinas	Alimento	MT (µg/Kg)
Aflatoxina M1	Leite fluido	0.5
	Leite em pó	5
	Queijos	2.5
Aflatoxinas B1, B2, G1 e G2	Cereais e produtos de cereais, exceto milho e derivados, incluindo cevada malteada	5
	Feijao	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
	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

Limites máximos tolerados (LMT) em alimentos prontos e em matérias-primas estabelecidos pela ANVISA, através de resolução RDC nº 7, de 18 de fevereiro de 2011, com aplicação a partir de 01/01/2012.

Micotoxinas	Alimento	MT (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

Limites máximos tolerados (LMT) em alimentos prontos e em matérias-primas estabelecidos pela ANVISA, através de resolução RDC nº 7, de 18 de fevereiro de 2011, com aplicação a partir de 01/01/2014

Micotoxinas	Alimento	MT (µg/Kg)
Ocratoxina A	Cereais para posterior processamento, incluindo grao de cevada	20
Desoxinivalenol (DON)	Trigo e milho em graos para posterior processamento	3000
	Trigo integra, trigo para quibe, farinha de trigo integral, farelo de trigo, farelo de arroz, grao de cevada	1500
	Farinha de trigo, massas, crackers, biscoitos de agua e sal e produtos de panificação, cereais e produtos de cereais, exceto trigo e incluindo cevada malteada	1250
Fumonisinas (B1 + B2)	Milho em grao para posterior processamento	5000
Zearalenona	Milho em grao e trigo para posterior processamento	400

Limites máximos tolerados (LMT) em alimentos prontos e em matérias-primas estabelecidos pela ANVISA, através de resolução RDC nº 7, de 18 de fevereiro de 2011, com aplicação a partir de 01/01/2016

Micotoxinas	Alimento	MT

		(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 à 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 subprodutos à base de milho	150
	Trigo integral, farinha de trigo integral, farelo de trigo	200

ANEXO B

Normas para publicação *Molecular Nutrition & Food Research*

Instructions to authors

Authors are requested to follow these instructions carefully. Manuscripts not prepared accordingly will not be accepted.

1 Aims and scope

Molecular Nutrition & Food Research (MNF) is a primary research journal devoted to health, safety and all aspects of molecular nutrition such as nutritional biochemistry, nutrigenomics and metabolomics aiming to link the information arising from the related disciplines Bioactivity & Safety, Immunology, Microbiology and Chemistry.

MNF is published in 12 issues *per* year, including regular issues as well as topical issues. Four categories of scientific contributions are accepted for publication:

(i) research articles,
(ii) reviews,
(iii) educational papers, and
(iv) food & function articles.

⇒ Introducing 'Food & Function' - a new section in MNF:

Manuscripts in which the individual components responsible for any biological activity have not been chemically characterized (e.g. animal studies with an uncharacterized extract of fruits) will not be accepted as full research articles. However, in these cases, authors may submit their manuscript in a shortened form for the new section "Food & Function". In this section, concise contributions describing the functional effects of food without a detailed characterization of the bioactive components will be considered for publication (for further details see Section 4 -Types of contributions).

Our Early View online publication is updated weekly and enables papers to be available online and citable before going into print.

2 General terms of publication

The author vouches that the work has not been published elsewhere, either completely, in part, or in any other form and that the manuscript has not been submitted to

another journal. The submitting author (listed under "Correspondence") accepts the responsibility of having included as coauthors all appropriate persons. The submitting author certifies that all coauthors have seen a draft copy of the manuscript and agree with its publication.

Scientific contributions will be peer-reviewed on the criteria of originality and quality. Following an initial assessment by the Editors, those papers with a high priority rating are sent for external review to experts in the field. To aid in the peer review, we invite authors to suggest potential reviewers for their paper during the online submission procedure. Authors also have the option of naming non-preferred reviewers. Those manuscripts failing to reach the required priority rating or not fitting within the scope of the Journal are not considered further and are returned to authors without detailed comments. On acceptance, papers may be subjected to editorial changes. Responsibility for the factual accuracy of a paper rests entirely with the author.

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3 Online submission of manuscripts

MNF offers a web-based manuscript submission and peer review system. This service guarantees fast and safe submission of manuscripts and rapid assessment. Using this system is obligatory, conventional submission of manuscripts is not accepted.

3.1 General remarks

To submit your manuscript online, please proceed along the following steps:

- Prepare your manuscript and illustrations in the appropriate format, according to the instructions given below (see Sections 4 to 9). Please also make sure that your paper conforms with the scientific and style instructions of **MNF** as given herein. Links for English language assistance also provided here.
- If you have not already done so, create an account for yourself in the system at the submission site, http://mc.manuscriptcentral.com/mnf/ by clicking on the "Create Account" button.
- Let the system guide you through the submission process. Online help is available to you at all times during the process. You are also able to exit/re-enter at any

stage before finally "submitting" your work. All submissions are kept strictly confidential. To monitor the progress of your manuscript throughout the review process, just login periodically and check your Author Center.

If you have any questions concerning the online submission program, do not hesitate to contact Editorial Support at mnf@wiley.com.

3.2 Electronic manuscripts

Please follow the instructions in Section 5 "Organization of manuscripts" when preparing the electronic version of the manuscript and ensure that data are given in the order and the correct style for the journal.

- Main text (incl. front material) as well as figure legends and tables (in this order) should be given in one file, preferably saved in .doc or .rtf format (Word 2003 or older, not .docx).
- Data should be typed unjustified, without hyphenation except for compound words. Use carriage returns only to end headings and paragraphs; spacing will be introduced by the typesetter.
- Do not use the space bar to make indents; where these are required (e. g. tables) use the TAB key.
- If working in Word for Windows, please create special characters using **Insert/Symbol**.
- Figures should preferably be in TIFF, EPS, PPT or the original format. See section 5.9 for details.

All submissions will be converted to PDF format during the upload process. The system automatically generates one PDF file which contains all parts of the manuscript apart from supporting information.

3.3 Revised manuscripts

In revised manuscripts the areas containing the major required changes should be marked and the script color changed. The file(s) with the changes visible on screen should be submitted to the online procedure.

Upon acceptance of the manuscript the final uploaded version will be taken as the basis for copy editing and the subsequent production process.

4 Types of contributions

Three types of scientific contributions are considered for publication:

(i) **Research articles** describing complete investigations. Unsolicited research articles should not exceed 6500 words in total; this includes references, figure legends and tables. Papers of up to 7 printed pages will be published free of charge; for papers exceeding that length a **page charge** (see the journals For Authors page) will be levied. Please note that the length of an article depends greatly on the type of figures and tables provided. Manuscipts must not have been published previously, except in the form of a preliminary communication.

(ii) **Reviews** providing an overview on the current research in a specific field. Review articles should not exceed 8500 words in total including references, figure legends and tables. Review articles of up to 15 printed pages will be published free of charge; for papers exceeding that length a **page charge** (see the journals For Authors page) will be levied.

(iii) **Educational papers** describing and/or explaining a method or technique used in food and nutrition research. They should be written in continuous style with headings (not numbered). An educational paper may be supplemented by multimedia material (e. g. animations or video sequences) which will be only available online.

(iv) **Food & Function articles** describing studies of well-documented functional bioextracts/mixtures exhibiting pharmacological, medical and/or physiological effects, where the bioactive component has not been chemically characterized. However, the work reported has be supported by animal and/or human studies. Research based solely on cell culture will not be considered.

They should be written in a concise and continuous style without subheadings with a maximum of 2500 words (including references as well as figure and table legends) and three display elements (figures and tables). For an example of this type of article format click here. Longer articles will not be accepted for this category. Any additional material pertinent to the study should be provided as Supporting Information online only. This includes e.g any detailed Materials & Methods description. Authors submitting in this category should please make sure that they select 'Food & Function' as article type during submission.

Reviews and educational papers will normally be invited by the Editors. Authors wishing to submit a review or an educational paper should send a brief outline of its

contents to the Editor-in-Chief (schreier@pzlc.uni-wuerzburg.de) before the manuscript is drafted.

5 Organization of manuscripts

Manuscripts must be typewritten with double spacing (including references, tables, legends, *etc*.).

5.1 Contents of first page of manuscript (all types of contributions)

The first page of the manuscript should contain only the following:

1) Title of the paper containing only the keywords pertaining to the subject matter. Standard abbreviations may be used in the title.

2) Full names (including first name) of the authors and the name of the institute. If the publication originates from several institutes the affiliations of all authors should be clearly stated by using superscript numbers after the name and before the institute.

3) Name (and title) and full postal address of the author to whom all correspondence (including galley proofs) is to be sent. E-mail and fax number must be included to speed up communication.

4) A list of abbreviations used in the paper excluding standard abbreviations (see list of "Standard Abbreviations", Section 9).

5) Keywords (max. 5, in alphabetical order).

5.2 Abstract (all types of contributions)

The second page of the manuscript should contain the abstract only. For research articles it should be structured as follows:

Scope

Methods and results

Conclusion (focus on nutritional relevance)

The abstract must be self-explanatory and intelligible without reference to the text. It should not exceed 200 words. Abbreviations, but not standard abbreviations, must be written in full when first used.

5.3 Division into sections (research articles only)

Manuscripts should be divided into the following sections:

"1 Introduction": containing a description of the problem under investigation and a brief survey of the existing literature on the subject.

"2 Materials and methods": for special materials and equipment, the manufacturer's name and location should be provided.

"3 Results"

"4 Discussion"

"5 References"

Sections 3 and 4 may be combined and should then be followed by a short section entitled "Concluding remarks". Subdivisions of sections should be indicated by subheadings.

5.4 References (all types of contributions)

References should be numbered sequentially in the order in which they are cited in the text. The numbers should be set in brackets, thus [2, 18]. References are to be collected in numerical order at the end of the manuscript under the heading "References"; they should also be typed with double spacing throughout. Papers with multiple authors should be limited to listing five authors. Where there are more than five authors, the first four should be listed, followed by *et al*. Please include the title of the manuscript in full, followed by a full stop. Journal names should be abbreviated according to the practice of PubMed. The abbreviated journal name and the volume number should be in italics. Please note the following examples.

Journals:

[1] Keppler, K., Hein, E.-M., Humpf, H.-U., Metabolism of quercetin and rutin by the pig caecal microflora prepared by freeze-preservation. *Mol. Nutr. Food Res.* 2006, *50*, 686-695.

Other serial publications such as "*Advances in Food and Nutrition Research*" should be cited in the same manner as journals.

Books:

[2] Eisenbrand, G., Dayan, A. D., Elias, P. S., Grunow,W., Schlatter, J. (Eds.), *Carcinogenic and Anticarcinogenic Factors in Food*, Wiley-VCH Verlag,Weinheim 2003.

Chapter in a book:

[3] Geis, A., in: Heller, K. J. (Ed.), *Genetically Engineered Food - Methods and Detection*, Wiley-VCH Verlag, Weinheim 2003, pp. 100-118.

Allusions to "unpublished observations", papers "to be published" or "submitted for publication" and the like should be a part of the text, in parentheses. Material "in press" should be entered under references along with the DOI (Digital Object Identifier), if available. Posters and abstracts in meetings books must not be cited unless they are generally accessible. Responsibility for the accuracy of bibliographic references rests entirely with the author. Please note that website addresses must not be included as a reference, but should be inserted in parentheses in the text directly after the data to which they refer.

A link to the latest EndNote style sheet can be found on the journals For Authors page.

5.5 Acknowledgements

Acknowledgements as well as information regarding funding sources should be provided on a separate page and will appear at the end of the text (before the "References").

5.6 Conflict of interest statement

Authors are responsible for disclosing all and any financial and personal relationships between themselves and others that might bias their work. To prevent ambiguity, authors must state explicitly whether potential conflicts do or do not exist. Should such a conflict of interest exist, a statement to that effect must be included in a separate paragraph following on from the acknowledgements section detailing - for each author - the nature of the conflict. Even if there is none, this should also be stated. This is a mandatory requirement for all articles.

5.7 Tables

Tables with suitable captions at the top and numbered with Arabic numerals should be collected at the end of the text on separate sheets (one page *per* table). Column headings should be kept as brief as possible and indicate units (in parentheses). Footnotes to tables should be indicated with a), b), c) *etc*. and typed on the same page as the table.

5.8 Supporting information

Extensive tables should be published online as supporting information. This material will not be typeset so authors should prepare it in the final form (preferably in PDF file format). Also for this reason there will be no galley proofs of this material. Supporting information will be made freely available on the web (similar to the table of contents and the article abstracts). Authors are permitted to place this material on their homepages when they are setting up a link to the full-text version of the article in Wiley Onine LIbrary.

Further, other files may be submitted as supporting information (*e.g.*, animations, video sequences). All supporting information will also undergo the peer-review process. Thus, this material has to be submitted electronically along with the main body of the article. It is in the hands of the Editor-in-Chief to decide which part of the manuscript will be published as supporting information.

5.9 Figures and legends

Please prepare your figures according to the following guidelines:

- Each figure should be given in a separate file and should have the following resolution at their final published size:

Type	Resolution
Graphs	800 -1200 DPI
Photos	400 - 800 DPI
Color (only CMYK)	300 - 400 DPI

- Use the zoom function to check the resolution of the figures: if an image viewed at 400 percent on screen is blurry (pixellated) then the image will not reproduce well in print. An image viewed at 100 percent on screen may look fine but will not necessarily reproduce well as the screen resolution is much lower (72-96 dpi) than that of a printing press.
- Crop, or scale, figures to the size intended for publication; no enlargement or reduction should be necessary. Otherwise figures should be submitted in a format which can be reduced to a width of 50-80 mm or 120-170 mm, with symbols and labels to a height of 2.0 mm (after reduction) and a minimum line weight of 0.3 pt for black lines.

- Photographic images often produce large files. Most software has an option to use LZW compression and this will produce smaller files, especially when the image contains large areas of single color or repeating textures and patterns.
- In electropherograms presented horizontally, the anode should be on the left while in vertical presentations the anode should be at the bottom. Two-dimensional presentations, *e.g.* with isoelectric focusing and sodium dodecyl sulfate-electrophoresis in the two dimensions, are thus presented consistently with the standard coordinate system.
- Figures should be numbered consecutively with Arabic numerals in the order of their appearance.
- Each figure is to be accompanied by a legend which should be self-explanatory. The legends should not appear under the figures but be included after the references.

By supplying high-quality electronic artwork, delays in production can be reduced as follow-up requests for improvement are no longer necessary. Color figures can be reproduced, however, authors will be charged for additional costs incurred for the reproduction of color (see Section 2).

5.10Image manipulation

Manipulation of images is strongly discouraged and all figures must accurately reflect the original data. Information should not be enhanced, eliminated, added, obscured or moved. In cases where manipulation is unavoidable, this should be clearly detailed in the Figure legend. All instruments, software and processes used to obtain the images must be fully detailed in the manuscript either in the Figure legends or the Materials and Methods. Acceptable image manipulation includes uniformly adjusting the contrast of an entire image, and any control images, ensuring that all original data, including the bacKground, remains visible and that no new features are introduced. Cropping of gels, or re-positioning of lanes/fields, is permitted providing that all alterations are clearly indicated by the use of dividing lines in the image itself, vital data are not removed and an explanation of the alterations is included in the Figure legend. Unacceptable manipulation includes, but is not limited to, the enhancement of one feature/band over others, removal of bacKground noise/bands and so on. Authors must be able to produce all data in their raw format upon editorial request.

5.11Biographic material

Corresponding authors of review articles are invited to submit a portrait photograph of themselves and a short biographical text (no more than 80 words) which will appear at the very end of the article.

5.12Structural formulae

Structural formulae should be drawn in the manuscript in the position where they belong. They must be numbered in consecutive order with the other figures.

5.13Equations

Mathematical and chemical equations are to be written in the manuscript at the place in which they belong and should be marked by Arabic numerals in parentheses in the right margin in the order of their appearance.

5.14Abbreviations

Abbreviations are hindrances to a reader working in a field other than that of the author, and to abstractors. Therefore, their use should be restricted to a minimum. Abbreviations should be introduced only when repeatedly used. Abbreviations used only in a table or a figure may be defined in the legend. Standard abbreviations may be used in the title and keywords. If nonstandard abbreviations are used in the Abstract they should be defined in the Abstract, in the list of abbreviations of the manuscript, as well as when first used in the body of the paper.

Section 9 at the end of these instructions contains the list of standard abbreviations which may be used without definition in the articles published in **MNF**.

5.15Ethics

If the manuscript describes experiments using animals, the permission of the national or local authorities (giving the permission or the accreditation number of the laboratory and of the investigator) should be stated. If no such rules or permission are

stipulated in the particular country, this must also be mentioned in the paper. In the case of human studies, it should be stated that local ethical committee approval has been received and that the informed consent of all participating subjects was obtained.

1. Proofs and reprints

Before publication authors will receive page proofs *via* Email in PDF low resolution file format, together with a sheet including instructions and a reprint order form, also as PDF files. The page proofs and the reprint order form should be printed out. The proofs should be carefully corrected following the instructions. In particular, authors should answer any editing queries. The reprint order form should be filled out (even if reprints are not required), and both should be returned as stated in the proof email. Authors will be charged for extensive alterations of their article. Reprints can be ordered at prices shown on the reprint order form. Upon publication (in print) the submitting author (listed under "Correspondence") will receive a complimentary low-resolution pdf of his/her article.

2. Online Open

MNF offers an OnlineOpen service for all authors. Authors have the option of paying a fee to ensure that their articles are available to non-subscribers. For more information go to http://olabout.wiley.com/WileyCDA/Section/id-406241.html .

8. Reporting specific data

8.1 Chemical structures

Structures should be produced with the use of a drawing program such as ChemDraw.

Structure drawing preferences are as follows:

As drawing settings select:

chain angle 120°

bond spacing 18% of width fixed length 14.4 points (0.508 cm, 0.2 in.)

bold width 2.0 points (0.071 cm, 0.0278 in.)

line width 0.6 point (0.021 cm, 0.0084 in.)

margin width 1.6 points (0.056 cm, 0.0222 in.)

hash spacing 2.5 points (0.088 cm, 0.0347 in.)

As text setting select: font, Arial or Helvetica; size, 10 pt.

Under the preferences choose: units, points; tolerances, 3 pixels.

Under page setup choose: paper, US Letter; scale, 100%.

Using the ChemDraw ruler or appropriate margin settings, create structure blocks, schemes, and equations having maximum widths of 11.3 cm (one-column format) or 23.6 cm (two-column format). Note: if the foregoing preferences are selected as cm values, the ChemDraw ruler is calibrated in cm. Also note that a standard sheet of paper is only 21.6 cm wide, so all graphics submitted in two-column format must be prepared and printed in landscape mode.

Use boldface type for compound numbers but not for atom labels or captions. Authors using other drawing packages should, as far as possible, modify their program's parameters to reflect the above guidelines.

8.2 Physical and other data

It is important that novel compounds, either synthetic or isolated/produced from natural sources, be characterized completely and unambiguously. Supporting data normally include physical form, melting point (if solid), UV/IR spectra, if appropriate, ^{1}H and ^{13}C NMR, mass spectral data, and optical rotations or CD information (when compounds have chiral centers).

Reports on flavor constituents should conform to the recommendations made by the International Organization 5 of the Flavor Industry (IOFI). Thus, substances must be identified using the latest analytical techniques. In general, any particular substance must have its identity confirmed by at least two methods; that means, in practice, comparison of chromatographic and spectroscopic data (which may include GC, MS, IR, and NMR) with those of an authentic sample. If only one method has been applied, the identification has to be labeled as "tentative": This is also valid in case of identification performed only by comparison of literature data.

Equations should be numbered consecutively and referred to the text; *e.g.* defined as in Eq. (1).

Physical data should be quoted with decimal points (e. g. 25.8 Jk^{-1} mol^{-1}), and arranged as follows where possible - but in any event in the same order within the manuscript (when measurement conditions remain unchanged they need only be mentioned once, for instance in the column headings): m.p./b.p. 20°C; $[\alpha]_D^{20}$ = -13.5 (*c* = 0.2 in acetone) ^{1}H NMR (200 MHz, $[D_8]$THF, 25°C, TMS): δ = 1.3 (q, ^{3}J (H,H) = 8 Hz, 2 H; CH_2), 0.9 ppm (t, ^{3}J (H,H) = 8 Hz, 3 H; CH_3); IR(Nujol): $\tilde{\nu}$ = 1790 cm^{-1} (C=O); UV/Vis (*n*-hexane):

λ_{max} (ε) = 320 (5000), 270 nm (12000); MS (70 eV): *m/z* (%): 108 (20) [M^+], 107 (60) [M^+ - H], 91 (100) [$C_7H_7^+$]. Plane angles in products of units can have either ° or deg as the unit.

Nomenclature, symbols, and units: The rules and recommendations of the International Union of Pure and Applied Chemistry (IUPAC), the International Union of Biochemistry (IUB), and the International Union of Pure and Applied Physics (IUPAP) should be adhered to.

8.3 Nucleotide and protein sequences

New nucleotide data must be submitted and deposited in the DDBJ/EMBL/GenBank databases and an accession number obtained before the paper can be accepted for publication. Submission to any one of the three collaborating databanks is sufficient to ensure data entry in all. The accession number should be included in the manuscript, e. g. as a footnote on the title page: ‚Note: Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number(s) -'. If requested the database will withhold release of data until publication. The most convenient method for submitting sequence data is by the World Wide Web:

EMBL *via* Webin:

http://www.ebi.ac.uk/embl/Submission/webin.html

GenBank *via* Bankit:

http://www.ncbi.nlm.nih.gov/BankIt/

DDBJ *via* Sakura:

http://sakura.ddbj.nig.ac.jp/

Alternatively, the stand-alone submission tool ‚Sequin' is available from the EBI athttp://www.ebi.ac.uk/Sequin and from NCBI at http://www.ncbi.nlm.nih.gov/Sequin/.

For special types of submissions (e. g. genomes, bulk submissions *etc*.) additional submission systems are available from the above sites.

Database contact information:

EMBL: EMBL Nucleotide Sequence Submissions
European Bioinformatics Institute
Wellcome Trust Genome Campus, Hinxton,
Cambridge CB10 1SD, UK
Tel.: +44 1223 494400; fax: +44 1223 494472
E-mail: datasubs@ebi.ac.uk
http://www.ebi.ac.uk

GenBank: National Center for Biotechnology
Information
National Library of Medicine,
Bldg. 38A, Rm 8 N-803
Bethesda, MD 20894, USA
Tel.: +1 301 496 2475; fax: +1 301 480 9241
E-mail: info@ncbi.nlm.nih.gov
http://www.ncbi.nlm.nih.gov

DDBJ: Center for Information Biology and
DNA Data Bank of Japan
National Institute of Genetics, 111 Yata,
Mishima, Shizuoka 411-8540, Japan
Tel.: +81 559 81 6853; fax: +81 559 81 6849
E-mail: ddbj@ddbj.nig.ac.jp
http://www.ddbj.nig.ac.jp

Protein sequences which have been determined by direct sequencing must be submitted to Swiss-Prot at the EMBL Outstation - The European Bioinformatics Institute. Please note that we do not provide accession numbers, **in advance**, for protein sequences that are the result of translation of nucleic acid sequences. These translations will automatically be forwarded to us from the EMBL nucleotide database and are assigned Swiss-Prot accession numbers on incorporation into TrEMBL.

Results from characterization experiments should also be submitted to Swiss-Prot at the EBI. This can include such information as function, subcellular location, subunit *etc*.

Contact information:

Swiss-Prot: Swiss-Prot submissions,
European Bioinformatics Institute
Wellcome Trust Genome Campus, Hinxton
Cambridge, CB10 1SD, UK
Tel.: +44 1223 494400; fax: +44 1223 494472
E-mail: datasubs@ebi.ac.uk (for sequence submissions); update@ebi.ac.uk (for characterization information)
http://www.ebi.ac.uk

9. Standard abbreviations

The abbreviations as listed below may be used without definition in the articles published in **MNF**. Please refer to Section 5.14 for the correct usage of abbreviations in **MNF**.

A	absorbance
ACN	acetonitrile
A/D	analog to digital converter
amu	atomic mass unit
API	atmospheric pressure ionization
BMI	body mass index
bp	base pairs
BSA	bovine serum albumin
CBB	Coomassie Brilliant Blue
CE	capillary electrophoresis
CEC	capillary electrochromatography
CFE	continuous flowelectrophoresis
CID	collision-induced dissociation
cpm	counts *per* minute
CV	coefficient of variation
CZE	capillary zone electrophoresis
1-D	one-dimensional
2-D	two-dimensional
Da	dalton (molecular mass)
DAD	diode-array detection (or diodearray detector)
2-DE	two-dimensional gel electrophoresis
DMEM	Dulbecco's modified Eagle medium
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
dsDNA	double-stranded DNA
DTT	dithiothreitol

EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis (β-aminoethylether)-*N*,*N*,*N'*,*N'*-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EOF	electroosmotic flow
ER	endoplasmic reticulum
ESI	electrospray ionization
FAB	fast atomic bombardment
FAME	fatty acid methyl esters
FITC	fluorescein isothiocyanate
GC	gas chromatography
GMO	genetically modified organism
HDL	high density lipoprotein
HEPES	*N*-(2-hydroxyethyl)piperazine-2'-(2-ethane-sulfonic acid)
HPCE	high-performance capillary electrophoresis
HPLC	high-performance liquid chromatography
HSA	human serum albumin
HTML	hypertext mark-up language
id	inside diameter
IEF	isoelectric focusing
Ig	immunoglobulin
IL	interleukin
INF	interferon
IT	ion trap
kbp	kilobase pairs
kDa	kilodalton (molecular mass)
LC	liquid chromatography

LDL	low denisty lipoprotein
LOD	limit of detection
LOQ	limit of quantitation
LPS	lipopolysaccharide
mAb	monoclonal antibody
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
Mbp	megabase pairs
MHC	major histocompatibility complex
MOPS	3-(*N*-morpholino)propanesulfonic acid
M_r	relative molecular mass (dimensionless)
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MUFA	monounsaturated fatty acid
m/z	mass-to-charge ratio
NMR	nuclear magnetic resonance
od	outside diameter
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
p*I*	isoelectric point
PMSF	phenylmethylsulfonyl fluoride
PMT	photomultiplier tube
ppm	parts *per* million

PTFE	polytetrafluoroethylene
PUFA	polyunsaturated fatty acid
PVP	polyvinylpyrrolidone
RIA	radioimmunoassay
RNA	ribonucleic acid
RP	reversed phase
rpm	rotations *per* minute
RSD	relative standard deviation
RT-PCR	reverse transcriptase-PCR
SCFA	short chain fatty acid
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SIM	selected ion monitoring
S/N	signal-to-noise ratio
SPE	solid-phase extraction
ssDNA	single-stranded DNA
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TIC	total ion current
TLC	thin-layer chromatography
TOF	time of flight
Tris	tris(hydroxymethyl)aminomethane
URL	uniform resource locator
Vh	volt x hours
VLDL	very low density lipoprotein

ANEXO C

Normas para publicação *British Journal of Nutrition*

Guide for Authors

The *British Journal of Nutrition* is an international peer-reviewed journal that publishes original papers, review articles and short communications in all branches of nutritional science. Short communications will be expedited through the review process. The underlying aim of all work should be, as far as possible, to develop nutritional concepts. The *British Journal of Nutrition* encompasses the full spectrum of nutritional science including 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 case studies or papers on food technology, food science or food chemistry.

As a contributor you are asked to follow the guidelines set out below. Prospective authors may also contact the Editorial Office directly on +44 (0)20 7605 6555 (telephone), +44 20 7602 1756 (fax) or <edoffice@nutsoc.org.uk >(email).

Papers submitted for publication should 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. **The *British Journal of Nutrition* now 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 should 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** (**h**ttp://www.nutritionsociety.org); 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.**

The manuscript must include a statement reporting any conflicts of interest, all sources of funding and the contribution of each author to the manuscript. This statement should be placed at the end of the text of the manuscript before the references are listed. If there are no conflicts of interest this must be stated. If the work was funded, please state "This work was supported by (for example) The Medical Research Council [grant number xxx (if applicable)]". If the research was not funded by any specific project grant, state "This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors." This journal adheres to the Committee on Publication Ethics (COPE) guidelines on research and publications ethics <http://www.publicationethics.org.uk/guidelines>

When substantial revisions are required to manuscripts, authors are 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 3 months, it may, on resubmission, be deemed a new paper and the date of receipt altered accordingly.

The *British Journal of Nutrition* publishes the following: Full Papers, Short Communications, Review Articles, Systematic Reviews, Horizons in Nutritional Science, Workshop Reports, Invited Commentaries, Letters to the Editor/Nutrition Discussion Forums, Book Reviews, Obituaries, Notices, Announcements and Editorials. Full Papers, Short Communications, Reviews, Systematic Reviews, Horizons Articles and Workshop Reports should be submitted to: <http://bjn.msubmit.net/>. Please contact the Editorial Office on <edoffice@nutsoc.org.uk> regarding any other types of article.

Short Communications. Papers submitted as Short Communications should consist of an abstract (250 words maximum), and no more than 3000 words of text (including references). Each Short Communication can include up to two tables or one table and one figure, but these will be at the expense of text (one half-page table or figure is equivalent to about 500 words in two columns or 250 words in one column). A short communication should describe a complete study that examines a specific question of scientific interest and that extends nutritional knowledge and understanding. The nature of the study or question being investigated means that the number of experiments or the amount of data presented is less than would be expected for a full publication. However, all aspects of scientific rigour and evaluation will be of the same standard as for a full publication.

Review Articles/Horizons in Nutritional Science. These will be handled by the Reviews Editor. Please contact the Editorial Office with any queries regarding the submission of potential review articles.

Systematic Reviews. These will be handled by the Systematic Reviews Editor. Please contact the Editorial Office with any queries regarding the submission of potential review articles.

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.

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*. Authors are encouraged to consult the latest guidelines produced by the International Committee of Medical Journal Editors (ICMJE), which contains a lot of useful generic information about preparing scientific papers <http://www.icmje.org/>and also the CONSORT guidelines for reporting results of randomised trials <http://www.consort-statement.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. 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.

(e) *Results*: these should be given as concisely as possible, using figures or tables as appropriate.

(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*: these should be given in a single paragraph after the discussion and include the following information: source of funding, declaration regarding any conflicts of interest and a brief statement as to the contribution(s) of each author.

(h) *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=journal&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:

1. Setchell KD, Faughnan MS, Avades T *et al*. (2003) Comparing the pharmacokinetics of daidzein and genistein with the use of 13C-labeled tracers in premenopausal women. *Am J Clin Nutr* **77**, 411–419.

2. Barker DJ, Winter PD, Osmond C *et al*. (1989) Weight in infancy and death from ischaemic heart disease. *Lancet* **ii**, 577–580.

3. Forchielli ML & Walker WA (2005) The role of gut-associated lymphoid tissues and mucosal defence. *Br J Nutr* **93**, Suppl. 1, S41–S48.

4. Bradbury J, Thomason JM, Jepson NJA *et al*. (2003) A nutrition education intervention to increase the fruit and vegetable intake of denture wearers. *Proc Nutr Soc* **62**, 86A.

5. Frühbeck G, Gómez-Ambrosi J, Muruzabal FJ *et al*. (2001) The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. *Am J Physiol Endocrinol Metab* **280**, E827–E847.

6. Han KK, Soares JM Jr, Haidar MA *et al*. (2002) Benefits of soy isoflavone therapeutic regimen on menopausal symptoms. *Obst Gynecol* **99**, 389–394.

7. Uhl M, Kassie F, Rabot S *et al*. (2004) Effect of common Brassica vegetables (Brussels sprouts and red cabbage) on the development of preneoplastic lesions induced by 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) in liver and colon of Fischer 344 rats. *J Chromatogr* **802**B, 225–230.

8. Hall WL, Vafeiadou K, Hallund J *et al*. (2005) Soy isoflavone enriched foods and inflammatory biomarkers of cardiovascular risk in postmenopausal women: interactions with genotype and equol production. *Am J Clin Nutr* (In the Press).

9. Skurk T, Herder C, Kraft I *et al*. (2004) Production and release of macrophage migration inhibitory factor from human adipocytes. *Endocrinology* (Epublication ahead of print version).

10. Skurk T, Herder C, Kraft I *et al*. (2005) Production and release of macrophage migration inhibitory factor from human adipocytes. *Endocrinology* **146**, 1006–1011; Epublication 2 December 2004.

11. Bradbury J (2002) Dietary intervention in edentulous patients. PhD Thesis, University of Newcastle.

12. Ailhaud G & Hauner H (2004) Development of white adipose tissue. In *Handbook of Obesity. Etiology and Pathophysiology*, 2nd ed., pp. 481–514 [GA Bray and C Bouchard, editors]. New York: Marcel Dekker.

13. Bruinsma J (editor) (2003) *World Agriculture towards 2015/2030: An FAO Perspective*. London: Earthscan Publications.

14. Griinari JM & Bauman DE (1999) Biosynthesis of conjugated linoleic acid and its incorporation into meat and milk in ruminants. In *Advances in Conjugated Linoleic Acid Research*, vol. 1, pp. 180–200 [MP Yurawecz, MM Mossoba, JKG Kramer, MW Pariza and GJ Nelson, editors]. Champaign, IL: AOCS Press.

15. Henderson L, Gregory J, Irving K *et al*. (2004) *National Diet and Nutrition Survey: Adults Aged 19 to 64 Years*. vol. 2: *Energy, Protein, Fat and Carbohydrate Intake*. London: The Stationery Office.

16. International Agency for Research on Cancer (2004) *Cruciferous Vegetables, Isothiocyanates and Indoles. IARC Handbooks of Cancer Prevention* no. 9 [H Vainio and F Bianchini, editors]. Lyon, France: IARC Press.

17. Linder MC (1996) Copper. In *Present Knowledge in Nutrition*, 7th ed., pp. 307–319 [EE Zeigler and LJ Filer Jr, editors]. Washington, DC: ILSI Press.

18. World Health Organization (2003) *Diet, Nutrition and the Prevention of Chronic Diseases*. *Joint WHO/FAO Expert Consultation*. *WHO Technical Report Series* no. 916. Geneva: WHO.

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).

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 (1971) London: The Royal Society, 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, thus: Statistical Analysis Systems statistical software package version 6.11 (SAS Institute, Cary, NC, USA). 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. 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 (SE or SEM) when these vary between means. The standard deviation (SD) is more useful only when there is specific interest in the variability of individual values. The degrees of freedom (df) associated with SED, SEM or SD should also be stated. The number of decimal places quoted should be sufficient but not excessive. Note that pH is an exponential number, as are the log(10) values often quoted for microbial numbers. Statistics should be carried out on the scalar rather than the exponential values.

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. 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.

Where 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.

Figures. 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 beyondexperimentalth 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 HINARI will be exempt from these charges. Refer to a recent copy of the journal for examples of figures.

Plates. The *British Journal of Nutrition* will now also consider the inclusion of illustrations and photomicrographs. The size of photomicrographs may have to be altered in printing; in order to avoid mistakes the magnification should be shown by scale on the photograph itself. The scale with the appropriate unit together with any lettering should be drawn by the author, preferably using appropriate software.

Tables. Tables should carry headings describing their content and should be comprehensible without reference to the text. Tables should not be subdivided by ruled lines. The dimensions of the values, e.g. mg/Kg, should be given at the top of each column. Separate columns should be used for measures of variance (SD, SE etc.), the ± sign should not be used. The number of decimal places used should be standardized; for whole numbers 1·0, 2·0 etc. should be used. Shortened forms of the words weight (wt) height (ht) and experiment (Expt) may be used to save space in tables, but only Expt (when referring to a specified experiment, e.g. Expt 1) is acceptable in the heading.

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 ($P<0{\cdot}05$)'. 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: $*P<0{\cdot}05$, $**P<0{\cdot}01$, $***P<0{\cdot}001$'. The symbols used for *P* values in the tables must be consistent.

Tables should be placed at the end of the text. Each table will be positioned near the point in the text at which it is first introduced unless instructed otherwise.

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.

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 IUPAC-IUB Commission on Biochemical Nomenclature (see *Biochemical Journal* (1978) **169**, 11–14). 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
ANOVA	analysis of variance
apo	apolipoprotein
ATP (GTP)	adenosine (guanosine) 5'-triphosphate
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
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)

Ethics of human experimentation. 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/e/policy/b3.htm), 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 should include a statement that ethical approval has been obtained.

Animal experimentation. The Editors will not accept papers reporting work carried out using inhumane procedures. Authors should indicate that their experiments have been approved by the appropriate local or national ethics committee for animal experiments.

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ANEXO D

Normas para publicação *The Journal of Nutritional Biochemistry*

Guide for Authors

The editors of *The Journal of Nutritional Biochemistry (JNB)*[1] welcome the submission of original manuscripts on experimental and clinical nutrition as it interfaces with biochemistry, molecular biology, physiology, pharmacology, and toxicology. The scope of the journal includes the broad area of *in vitro* and *in vivo* studies of mechanistic aspects of nutritional sciences. The criteria for acceptance of papers submitted for publication are originality, quality and clarity of the content. Each manuscript is internally reviewed and prioritized before a full external review takes place. All contributions must be based on original, unpublished research and will be peer reviewed. All authors bear responsibility for ensuring the integrity and quality of their reported research. It is the author's responsibility to secure permission to use figures or tables that have been published elsewhere.

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University of Kentucky
900 Limestone Street
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Revised manuscripts should also be accompanied by a file (separate from the cover letter) with responses to reviewers' comments. All files should be labeled with appropriate and descriptive file names (e.g., SmithText.doc, Fig1.eps, Table3.doc). The text, tables and graphics must be submitted as separate files. Complete instructions for electronic artwork submission are accessible via the JNB home page (http://journals.elsevierhealth.com/periodicals/jnb/). The web site guides authors through the creation and uploading of the various files. The preferred file format is **Microsoft Word.** Please note that PDF files are not allowed for submission. When the submission files are uploaded, the system automatically generates an electronic (PDF) proof which is then used for review.

Cover Letter

Provide a cover letter indicating the name, mailing address, telephone, fax number, and e-mail address of the corresponding author. The cover letter must state that: all authors listed have contributed to the work, all authors have agreed to submit the manuscript to JNB, no part of the work has been published before, except in abstract form, and all human and animal studies have been reviewed by the appropriate ethics committees. All authors listed in a manuscript submitted to JNB must have contributed substantially to the work, participated in the writing of the manuscript, and seen and approved the submitted version. All individuals who have contributed to the writing of the manuscript must be listed as authors. The editor reserves the right to reject manuscripts that do not comply with the above-mentioned requirements.

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Hennig B, Toborek M, Boissonneault GA. Lipids inflammatory cytokines, and endothelial cell injury. In: Gershwin ME, German JB, Keen CL, editors. Nutrition and Immunology: Principles and Practice. New Jersey: Humana Press Inc.; 2000. pp. 203-20.

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