ROBERTA ABRAMI MONTEIRO SILVA

AVALIAÇÃO DA SANGUINARINA SOBRE PARÂMETROS INTESTINAIS E SANGUÍNEOS DE SUÍNOS POR MEIO DE DIFERENTES MODELOS EXPERIMENTAIS (*IN VITRO, EX VIVO* E *IN VIVO*)

Londrina
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Orientador: Prof. Dr. Caio Abércio da Silva

Co-Orientadora: Prof. Drª. Ana Paula F. R. L. Bracarense

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Londrina, 18 de Junho de 2014.

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SILVA, Roberta Abrami Monteiro Silva. **Avaliação da sanguinarina sobre parâmetros intestinais e sanguíneos de suínos através de diferentes modelos experimentais (*in vitro, ex vivo* e *in vivo*)**. 2014. 125f. Tese (Doutorado em Ciência Animal) – Universidade Estadual de Londrina, Londrina, 2014.

RESUMO

A plena atividade funcional do trato digestório garante o êxito de desempenho dos leitões em toda sua vida. O intestino, diante dos desafios constantes a que é submetido nos sistemas comerciais de produção, demanda recursos que preservem sua integridade e aumentem a sua capacidade absortiva, melhorando o aproveitamento do alimento ingerido. Para esse fim, o presente trabalho objetivou avaliar os efeitos do extrato vegetal Sanguinarina sobre a integridade de células epiteliais intestinais e na saúde intestinal de suínos no pós desmame e na fase de crescimento. O trabalho foi realizado em três etapas: testes *in vitro, ex vivo* e *in vivo*. Na primeira etapa, a Sanguinarina foi testada sobre células epiteliais intestinais de suínos (IPEC-1), as quais após o processo de divisão celular foram expostas às doses de 0,1; 0,25; 0,5; 0,75; 1,0 e 5,0 µM de Sanguinarina por 48 horas. O teste de citotoxicidade realizado (MTT), apontou 50% de mortalidade celular (IC50) com a dose de 0,65 µM, equivalente a 25 mg/ L de Sanguinarina. Na segunda etapa, a Sanguinarina foi testada em explantes de jejuno de leitões com 24 dias de vida. Após testes para estabelecer a melhor dose de Sanguinarina, optou-se por trabalhar com a dose de 1,0 mg/L. Posteriormente, os explantes foram incubados na presença de Sanguinarina (1,0 mg/L); DON (10 µM) e Sanguinarina (1,0 mg/L) + DON. Os explantes, quando desafiados com DON apresentaram redução ($p<0,05$) no escore tecidual, altura de vilosidades e profundidade de criptas. A Sanguinarina na presença de DON diminuiu de forma significativa as lesões causadas pela micotoxina no epitélio intestinal. Na terceira e última etapa do trabalho, foram utilizados 24 suínos, com idade média de 80 dias e peso médio inicial de 30,36 ± 2,09 kg. Foi realizada coleta de sangue ao abate para quantificação de imunoglobulinas. Após cinco dias de gavagem, com as soluções Controle (água mineral) e Sanguinarina (1,0mg/ L), os animais foram eutanasiados e fragmentos das porções do intestino delgado foram coletadas para avaliações histológicas e morfométricas. O fornecimento de Sanguinarina aumentou a secreção de IgA sérica. A Sanguinarina não mostrou melhora da arquitetura tecidual quando comparada ao grupo controle. A manutenção das funções absortiva e de barreira física do intestino exposto à Sanguinarina não foi eficaz, necessitando estudos posteriores.

Palavras-chave: Alterações histológicas. Barreira seletiva. Células epiteliais. Fitoterápico. Inflamação intestinal. Viabilidade celular.

SILVA, Roberta Abrami Monteiro Silva. **Evaluation of Sanguinarine on the swine's intestinal and blood parameters through different experimental templates (*in vitro, ex vivo* and *in vivo*).** 2014. 125p. Thesis (Ph.D. in Animal Science) - State University of Londrina, Londrina, 2014.

ABSTRACT

The full functional activity of the digestive tract ensures the successful piglets performance throughout their lives. The intestine, due to the continuous challenges is submitted in the commercial systems of production, requires some resources that can preserve its integrity and increase its absorptive ability, enhancing this way the utilization of the ingested food. For that purpose, the present study aimed to evaluate the effects of the plant extract Sanguinarine on the integrity of the swine's intestinal epithelial cells and on their intestinal health after weaning and during their growing phase. The study was carried out in three stages: *in vitro*, *ex vivo* and *in vivo* tests. In the first stage, the Sanguinarine was tested on the swine's intestinal epithelial cells (IPEC-1), which were exposed to doses of 0.1; 0.25; 0.5; 0.75; 1.0 and 5.0 mM of Sanguinarine for 48 hours, as soon after the cell division process. The cytotoxicity assay performed (MTT) showed 50% of cellular death (IC50) at a dose of 0.65 µM, which is equivalent to 25 mg/L of Sanguinarine. In the second stage, the Sanguinarine was tested in jejunal explants of 24 day old piglets. After some tests to determine the ideal Sanguinarine dose, was chose the dose of 1.0 mg/L. Subsequently, the explants were incubated under the presence of Sanguinarine (1.0 mg/L); DON (10 µM); and Sanguinarine (1.0 mg/L) + DON (10 µM). The explants challenged with DON showed reduction ($p<0.05$) in the tissue score, in the villus height and in the crypt depth. The Sanguinarine in the presence of DON significantly decreased the lesions caused by the mycotoxin in the intestinal epithelium. In the third and final stage of the study, were used 24 swine with an average age of 80 days and initial average weight of 30.36 ± 2.09 kg. After slaughter, blood samples were collected for quantification of immunoglobulins. After five days the gavage procedure, with Control (mineral water) and Sanguinarine (1.0 mg/L) solutions, fragments of the small intestine were collected for histological and morphometrical analysis. The providing of sanguinarine increased IgA secretion in the serum. The Sanguinarine did not show some improvement in the tissue architecture when compared to the control group. The maintenance of both absorptive and physical barrier functions of the intestine exposed to Sanguinarine was not effectively achieved, thus requiring further studies.

Keywords: Histological alterations. Selective barrier. Epithelial cells. Phytotherapic. Intestinal inflammation. Cellular viability.

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1 INTRODUÇÃO

Alterações morfológicas no intestino delgado dos leitões, como atrofia das vilosidades e aumento da profundidade das criptas, podem ser associadas à redução do consumo diário de alimento, especialmente durante a primeira semana pós desmame, devido a fatores estressantes como separação dos leitões da matriz, mudança de ambiente e de dieta (CERA et al., 1988; PIERCE et al, 2005).

Essas alterações na fisiologia intestinal juntamente com a imaturidade do sistema digestório interferem negativamente no desenvolvimento dos leitões (HEDEMANN et al, 2006), prejudicando a digestão e absorção de nutrientes, comprometendo o desempenho e predispondo os leitões a problemas entéricos (PLUSKE; HAMPSON; WILLIAMS, 1997).

Em virtude da capacidade digestiva do leitão desmamado ser limitada, a preservação da saúde intestinal é fundamental para minimizar as perdas de desempenho, as doenças ocasionadas por micro-organismos patogênicos e a mortalidade dos leitões. Assim, os ingredientes da dieta devem ser selecionados para estabelecer o equilíbrio do trato digestório, prevenindo distúrbios na sua estrutura e função (MONTAGNE; PLUSKE; HAMPSON, 2003; BUDIÑO; CASTRO JÚNIOR; OTSUK, 2010).

De acordo com Dunsfor, Knabe e Haensly (1989), a intensidade das alterações morfológicas está mais associada à qualidade dos alimentos empregados na formulação das dietas do que à fase em que o animal se encontra. Por isso, há necessidade de se trabalhar com dietas complexas, com ingredientes nobres e altamente digestíveis na fase que sucede o desmame, que maximizam o desempenho dos leitões, diminuindo os problemas de digestão e a má absorção intestinal (QUADROS et al., 2002).

Também em relação à qualidade dos alimentos, deve-se considerar a possível contaminação das matérias-primas destinadas à produção das rações. Os grãos compreendem grande parte da composição das rações e são frequentemente contaminados por fungos que produzem micotoxinas, as quais podem causar danos severos aos órgãos (alterações anatômicas e funcionais), imunossupressão, atraso no desenvolvimento dos animais e consequentemente, diminuição da produtividade (CAST, 2003).

Devido à sua alimentação à base de grãos, os suínos são muito suscetíveis à intoxicação por micotoxinas, especialmente ao desoxinivalenol (DON), apresentando sintomas acentuados após sua ingestão (GOYARTS et al. 2006).

O DON se destaca entre as principais micotoxinas de ocorrência no Brasil e no mundo. A toxicidade aguda, devido à exposição à doses elevadas desta micotoxina,

caracteriza-se pela diarréia, vômitos, leucocitose, hemorragia, choque e morte. A intoxicação crônica com baixas doses, leva à anorexia, redução no ganho de peso, má-absorção de nutrientes, alterações neuroendócrinas e efeitos imunológicos (PESTKA; SMOLINSKI, 2005).

Esses efeitos relacionados à contaminação por DON, além de causarem danos ao sistema digestório, tem outros efeitos prejudiciais aos leitões, visto que no pós-desmame tanto o sistema digestório quanto o sistema imune ainda se encontram imaturos. Dentre esses destacam-se a inflamação intestinal, a resposta pró-inflamatória sistêmica e o aumento nos níveis sanguíneos de leucócitos.

Diante deste cenário, extratos vegetais como aditivos nas rações, estão sendo utilizados para melhorar o desempenho, manipular as funções intestinais e o habitat microbiano dos animais domésticos (PANDA et al., 2000).

O uso de aditivos com ação anti-inflamatória direta ou indireta nas dietas de suínos na fase de creche se identifica com a proposta de melhorar estes aspectos críticos comuns no pós-desmame. Nesse contexto, a Sanguinarina, um dos princípios ativos do Sangrovit®, um alcaloide de origem vegetal que inibe a multiplicação de bactérias, fungos e vírus que comprometem ou levam a danos no trato gastrointestinal, pode ser uma alternativa natural para este fim (SCHMELLER, 1997; MELLOR, 2001). O Sangrovit® é um fitoterápico que poderia assim substituir o uso de antibióticos na nutrição de suínos, cumprindo uma ação anti-inflamatória no trato gastrointestinal, com efeitos paralelos na melhora do apetite e na ingestão alimentar, promovendo melhora no desempenho dos animais (MELLOR, 2001; YAKHKESHI; RAHIMI; GHARIB NASERI, 2011).

Utilizando metodologias *in vitro* (células epiteliais intestinais), *ex vivo* (explantes intestinais) e *in vivo* (suínos em fase de crescimento), o presente estudo propôs avaliar os efeitos da Sanguinarina na estrutura da mucosa intestinal de suínos.

2 REVISÃO BIBLIOGRÁFICA

2.1 FASE PÓS-DESMAME (CRECHE)

Na suinocultura, a fase de creche constitui uma etapa de grande desafio para os nutricionistas pela participação simultânea, após o desmame, de vários fatores estressantes para o leitão, destacando-se, entre outros, a mudança da dieta líquida (leite materno) para uma dieta sólida (ração) (WEARY; JASPERS; HOTZEL, 2008).

A reduzida capacidade digestiva dos leitões no pós-desmame tem efeito direto sobre o desempenho dos animais (SCANDOLERA et al., 2005), devido às alterações histológicas e bioquímicas observadas no intestino delgado, como atrofia dos vilos e hiperplasia das células das criptas, e, consequentemente, redução na absorção dos nutrientes da dieta (XU et al., 2000).

A má absorção ocorre principalmente quando há danos estruturais às células epiteliais, como nas infecções bacterianas, virais ou por protozoários que ocasionam lise celular, que também pode ser causada pela forma física da ração (muito fina ou muito grossa) ou ingestão e ação de determinados produtos tóxicos (ZLOTOWSKI; DRIEMEIER; BARCELLOS, 2008).

No pós-desmame o sistema digestório dos leitões está apto a digerir principalmente o leite materno devido ao fato de seu quadro enzimático apresentar altas concentrações de lactase e baixas concentrações de enzimas como amilases e proteases voltadas para ingredientes não lácteos (LINDEMANN et al., 1986).

Além da insuficiente secreção de enzimas digestivas, há também uma capacidade física limitada (estômago e intestino delgado), não permitindo dessa forma que a digestão e a absorção de nutrientes ocorram de maneira adequada e eficiente (MOLLY, 2001).

Um dos principais fatores do baixo desempenho dos leitões na creche é o fornecimento de rações simples que não contém todos os ingredientes recomendados para leitões recém desmamados, como substitutos lácteos e matérias-primas de alta digestibilidade e palatabilidade (ODGAARD, 2001). Os ingredientes das rações, assim como sua granulometria, são pontos fundamentais dentro da nutrição, uma vez que podem interferir no aproveitamento dos nutrientes pelos animais.

Dessa forma, os problemas relacionados ao baixo desempenho dos leitões após o desmame muitas vezes decorrem do consumo de ingredientes que não estão em nível quantitativo e qualitativo compatíveis com a produção de enzimas no trato gastrintestinal

(SILVA, 2002).

2.1.1 Alimentação de Leitões

A formulação de dietas adequadamente balanceadas para leitões após o desmame tem se constituído num grande desafio para os nutricionistas (HAUPTLI et al., 2012).

O fornecimento de dietas simples, à base de milho e farelo de soja, no período pós-desmame, predispõe o leitão a problemas fisiológicos digestivos, provocando um atraso no crescimento de 8% a 33% em relação aos leitões alimentados com dietas complexas, influenciando, portanto, negativamente o desempenho do animal (AUMAITRE, 2000).

As dietas complexas com alta porcentagem de produtos lácteos, associados a fontes protéicas de origem animal, apresentam maior digestibilidade e resultam maior ingestão, sem predispor os leitões a problemas digestivos (HAUPTLI et al., 2012).

Por isso, a escolha de ingredientes apropriados é tão importante quanto a definição adequada dos níveis nutricionais das dietas para leitões desmamados, pois quanto maior a digestibilidade das matérias-primas utilizadas, maior será o consumo e o aproveitamento da ração pelo animal. Neste sentido, os produtos lácteos, como o leite em pó e o soro de leite, destacam-se pelas qualidades nutricionais e excelente palatabilidade (KUMMER et al., 2009).

O fornecimento de ingredientes lácteos e outras proteínas de origem animal têm sido amplamente utilizados em dietas de leitões. Por suas características, esses ingredientes reduzem os danos decorrentes do desmame, como a diminuição da capacidade absortiva do intestino decorrente da atrofia das vilosidades, e favorecem o consumo de ração (CHAMONE et. al., 2010).

O desafio nutricional imposto pelo desmame é minimizado com a utilização de sucedâneos lácteos, como o soro de leite e o leite em pó, produtos cuja fração protéica (lactoalbumina) não está relacionada somente à sua elevada digestibilidade e palatabilidade, mas também à alta concentração de imunoglobulinas (KUMMER et al., 2009).

A lactose presente nestes ingredientes também contribui com o processo de digestão das proteínas, devido à sua fermentação e consequente acidificação do trato gastrintestinal, melhorando a ação das enzimas digestivas e promovendo o equilíbrio da microflora intestinal por meio da inibição do crescimento de bactérias patogênicas (POWLES e COLE, 1993).

Bertol, Santos Filho e Ludke (2000), ao compararem quatro níveis de inclusão de lactose (0, 7, 14 e 21%) na dieta pré-inicial de leitões desmamados aos 21 dias de idade, observaram que o aumento dos níveis de lactose na dieta acarretou acréscimo linear no ganho

de peso e no consumo diário de ração e redução linear na conversão alimentar no período de 0 a 14 dias após o desmame. O peso médio aos 14 dias e o peso médio aos 28 dias após o desmame também aumentaram linearmente, em consequência do aumento dos níveis de lactose na dieta (BERTOL; SANTOS FILHO; LUDKE, 2000).

Lima et al. (2012) afirmam que o soro de leite e a lactose constituem-se em alimentos utilizados com bons resultados no desempenho pós-desmame. Estes resultados mostram como as dietas complexas, formuladas com ingredientes de melhor digestibilidade, podem possibilitar um melhor desempenho pós-desmame de leitões quando comparados às dietas simples, à base de milho e farelo de soja.

Outra fonte de proteína animal que tem sido estudada nos últimos anos é o plasma animal desidratado pelo método "spray-drying" (PADSD). O produto é rico em imunoglobulinas, principalmente IgG, que permanecem ativas após o processamento (DONZELE; ABREU; HANNAS, 2002).

Apesar das vantagens de se utilizar fontes de proteína animal nas dietas de leitões, o seu emprego em alguns casos é restrito somente às rações fornecidas por um período de uma a duas semanas após o desmame, em razão de seu alto custo e/ou disponibilidade no mercado (DRITZ; TOKACH; GOODBAND, 1994).

Com isso, a definição de um manejo de alimentação que proporcione bom desempenho, com um custo compatível com a atividade suinícola torna-se imprescindível (QUADROS et al., 2002).

Por outro lado, envolvendo também o aspecto econômico, a inclusão do farelo de soja nas rações pré-iniciais e iniciais de leitões é bastante utilizada, extrapolando muitas vezes as quantidades aceitáveis, bem como a total substituição das fontes de proteína de origem animal. Além do farelo de soja, outros produtos derivados da soja, como soja integral desativada (tostada ou extrusada) ou o concentrado de soja, tem sido utilizados. Segundo Bertol, Moraes e Franke (1997), a soja desativada, seja ela extrusada ou tostada, apresenta menor antigenicidade em comparação ao farelo de soja.

Todavia, a inclusão do farelo de soja em dietas para leitões deveria ser limitada devido à presença de fatores antinutricionais (inibidores de tripsina e quimotripsina, urease e lectinas) que reduzem o aproveitamento dos nutrientes, permitindo que seja transportada maior quantidade de proteínas intactas ao intestino delgado, reduzindo a digestibilidade e favorecendo o desenvolvimento de micro-organismos patogênicos pela presença excessiva de substrato (AUMAITRE, 2000). Além disso, esta pode provocar maiores riscos de reação de

hipersensibilidade, alterando a morfologia intestinal, resultando em perda da capacidade de absorção (EASTER; KIM, 2000).

Paralelo ao uso destes produtos de origem vegetal como fontes de proteína nas rações, os cereais tem grande participação como fonte energética nestas rações de leitões desmamados, como também de suínos em outras fases (BERTO; WECHSLER; NORONHA, 2002).

De acordo com Russel, Gahr (2005), a digestão enzimática dos carboidratos é a principal rota pela qual os suínos produzem a glicose que o organismo necessita. Cerca de 70 a 80% da energia consumida por leitões desmamados provém dos amidos de cereais (LINDEMANN et al., 1986), que são incorporados nas dietas com a função de fornecer energia para os diferentes processos metabólicos (WISEMAN; NICOL; NORTON, 2000).

Contudo, o sistema digestório dos leitões ainda está imaturo para digerir eficientemente o amido (PUPA, 2008) em função da baixa atividade da enzima amilase (LINDEMANN et al., 1986). Para isso, algumas técnicas de processamento que envolvem calor e pressão, como extrusão e peletização, são empregadas para promover sua gelatinização, tornando-o mais digestível para os leitões (MOREIRA et al., 2001; EBERT et al., 2005).

Outro recurso empregado para melhorar a digestibilidade das rações e favorecer o desempenho animal é a moagem fina dos ingredientes. Comumente, na alimentação dos suínos, o uso de dietas com partículas finas favorece a utilização dos nutrientes (ZANOTTO; BELLAVER, 1996). Conforme se diminui a granulometria das rações, melhora-se a conversão alimentar e ocorre um incremento no ganho de peso de leitões na fase de creche (OHH et al., 1983). Os autores, trabalhando com leitões desmamados aos 21 dias, observaram que na fase inicial, entre 0 a 14 dias pós-desmame, o ganho de peso e a conversão alimentar melhoraram de forma linear com a redução da granulometria do milho de 919 para 403 micrômetros.

Do ponto de vista nutricional, pode-se considerar que quanto menor o tamanho das partículas do alimento fornecido aos suínos, maior o contato dessas com os sucos digestivos, favorecendo a digestão e a absorção dos nutrientes (ZANOTTO; GUIDONI; MORES, 1999).

2.2 FISIOLOGIA E MORFOLOGIA DIGESTIVA DO SUÍNO

2.2.1 Leitão

A mudança nutricional abrupta do leite materno para uma alimentação sólida (ração) é um evento crítico, que na maioria das vezes pode provocar transtornos digestivos e consequentemente queda no desempenho de leitões desmamados pela alteração na atividade enzimática digestiva (BACH-KNUDSEN; JORGENSEN; CANIBE, 2000).

O fornecimento de ração pode contribuir com as mudanças morfológicas e fisiológicas que ocorrem no intestino delgado após o desmame, dando condições necessárias para a proliferação de bactérias residentes ou que foram ingeridas via ração (KUMMER et al., 2009).

A parede do intestino delgado é composta de quatro camadas, sendo elas: mucosa, submucosa, muscular e serosa (KIERSZENBAUM, 2004). A mucosa apresenta várias estruturas que aumentam sua superfície, incrementando a área disponível para absorção de nutrientes (JUNQUEIRA; CARNEIRO, 2011). Esta camada é composta de pregas circulares, vilosidades, glândulas e microvilosidades (KIERSZENBAUM, 2004), e entre as vilosidades existem pequenas aberturas de glândulas tubulares simples denominadas Criptas de Lieberkuhn (JUNQUEIRA; CARNEIRO, 2011).

As vilosidades e as criptas são recobertas por uma camada contínua de epitélio celular chamado de enterócitos. Os enterócitos das criptas são altamente mitóticos e se regeneram rapidamente. Quando as células das criptas se multiplicam, elas migram para a base da vilosidade, empurrando outras células da vilosidade à frente delas, de modo que há uma progressão contínua de células que migram para as vilosidades. Conforme migram, as células se tornam maduras e passam de células relativamente indiferenciadas nas criptas para células absortivas altamente especializadas nas vilosidades. O comprimento das vilosidades é determinado pela velocidade na qual as células perdidas no ápice das vilosidades são substituídas pelas células das criptas (HERDT, 2004).

Em suínos com mais de três semanas de idade, os enterócitos sofrem renovações periódicas, ocorrendo migração das criptas (células chamadas “imaturas”) para a extremidade das vilosidades (células chamadas “maduras”) em dois a quatro dias. Entretanto, em animais mais jovens, com idades entre duas a três semanas, esse tempo de renovação (migração) é maior, sendo de sete a dez dias no jejuno e íleo (COOK, 1996).

A relação desejável entre vilosidades e criptas intestinais ocorre quando as vilosidades se apresentam altas e as criptas rasas, pois quanto maior a relação altura de vilosidade:profundidade de cripta, melhor será a absorção de nutrientes e menores serão as perdas energéticas com a renovação celular (HANCOCK et al, 1990; LI ,1991; NABUUS, 1995).

Logo após o desmame ocorrem alterações funcionais e estruturais no intestino delgado que compreendem a diminuição na altura das vilosidades, a redução da atividade específica de enzimas digestivas e a piora da capacidade absortiva dos leitões (DONZELE; ABREU; HANNAS, 2002).

Questões como a ausência de imunoglobulinas e de fatores de crescimento (presentes no leite da porca), a presença de componentes antigênicos na ração e a proliferação de certas bactérias no intestino, influenciam estas mudanças morfológicas, que podem tornar o animal mais vulnerável às infecções por certos patógenos, além de contribuir para um atraso na taxa de crescimento (PICKARD; WISEMAN, 2003).

Há uma correlação positiva entre o consumo adequado de alimento e o aumento da altura das vilosidades ao longo do intestino delgado, sendo que o consumo de ração é responsável por 68% da variação na altura das vilosidades (PLUSKE, 1993). Desta forma, pode haver uma diminuição na altura das vilosidades no pós-desmame associada ao baixo consumo de ração apresentado pelos leitões nesta fase.

A fim de minimizar esse problema, estratégias nutricionais devem ser implementadas para estimular o consumo de ração pelos animais. Um estudo realizado por Tse et al. (2010) mostrou que a inclusão de proteína láctea na dieta de leitões desmamados aos 21 dias de idade influenciou positivamente a altura das vilosidades e a profundidade das criptas, refletindo numa melhor conversão alimentar.

2.3 Epitélio Intestinal e o Sistema Imune

A mucosa intestinal está continuamente exposta a antígenos de origem alimentar, toxinas, vírus, bactérias e parasitas. Os mecanismos de proteção imunológica do trato intestinal agem no reconhecimento de substâncias ofensivas e inofensivas, sendo que para as substâncias inofensivas, não deve haver reação imunológica no animal (MOWAT, 2003).

O processo inflamatório é uma reação do organismo à penetração de um agente infeccioso, um antígeno, ou danos celulares. Inflamação é um processo biológico complexo

envolvendo diversas vias que são frequentemente induzidas por produtos da degradação de bactérias, fungos, vírus ou mesmo pelas próprias células após um dano ou apoptose (KULINSKY, 2007).

O estresse e a exposição a antígenos ativa o sistema imune e estimula a produção de citocinas pró-inflamatórias no cérebro, as quais reduzem a ingestão de alimento (KENT et al., 1996) e interagem com hormônios de crescimento e fatores de crescimento similares à insulina (IGF-1) para suprimir o crescimento celular (KELLEY, 2004).

Em relação ao sistema imune, no desmame há grandes mudanças no grau e diversidade de exposição a antígenos ambientais, oriundos do alimento e de organismos potencialmente patogênicos (LALLES et al., 2007).

O desafio antigênico ao desmame ocorre quando o sistema imune da mucosa está desenvolvido a ponto de ativar as respostas imunes. Somado a isso, os componentes imunorregulatorios e imunoprotetores do leite materno estão minimizados. Anticorpos contra antígenos alimentares já podem ser detectados após o desmame, indicando respostas imunes contra eles. Com o tempo as respostas assumem uma forma de tolerância, mas não ocorrem imediatamente (BAILEY et al., 2004).

Por meio de vários mecanismos de defesa, o intestino é a primeira barreira seletiva que impede a entrada de agentes estranhos para os tecidos subjacentes, incluindo proteínas alimentares, toxinas naturais e micro-organismos patogênicos (MACFARLANE; MACFARLANE; CUMMINGS, 2006). Esta função de barreira baseia-se em componentes da imunidade inata e adaptativa como citocinas e imunoglobulinas (WAN; EL-NEZAMI, 2013).

Logo após a ingestão de um alimento contaminado, as células da mucosa intestinal podem ficar expostas às grandes concentrações de contaminantes, incluindo as micotoxinas (PRELUSKY et al., 1996). Como descrito por Bouhet e Oswald (2005), a função de barreira física realizada pelo epitélio intestinal é conseguida através da resistência elétrica trans-epitelial (TEER), que existe na monocamada celular. Algumas micotoxinas, como ocratoxina, patulina e desoxinivalenol, são capazes de diminuir a TEER em células intestinais de humanos. Bouhet et al. (2004) assinalaram que as fumonisinas também alteram a TEER nas células do intestino de suínos, podendo explicar os processos de injúria, descamação e ulceração observados em animais expostos à ingestão de micotoxinas.

Outro aspecto afetado pelas micotoxinas é a produção de citocinas pelas células intestinais, que desempenham papel fundamental no recrutamento de células inflamatórias para defesa desta mucosa. Oswald et al. (2003) relataram que leitões alimentados com baixos níveis de fumonisina diminuem a expressão da interleucina-8 (IL-8) no íleo, sugerindo que

esse fato pode ter grande influência na maior susceptibilidade à *Escherichia. coli,* observada nos animais desafiados quando comparado ao grupo controle. Esse menor recrutamento de células inflamatórias ocasionado pela diminuição na expressão de IL-8, que se associa a ação dessa toxina na redução de proliferação celular e integridade da mucosa do intestino, resulta num aumento da susceptibilidade dos animais à colonização bacteriana (OSWALD et al., 2003).

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 são descritas como bloqueadoras das fases G0/ G1 do ciclo das células epiteliais, diminuindo dessa forma a proliferação celular (BOUHET; OSWALD, 2005), enquanto que o DON, em doses baixas, interfere na diferenciação dos enterócitos (KASUGA et al., 1998).

Todas essas alterações descritas na literatura comprovam que as micotoxinas alteram a resposta imune dos animais, podendo interferir com a resposta vacinal, deixando os animais suscetíveis às infecções inespecíficas.

2.4 MICOTOXINAS

Micotoxinas são metabólitos secundários considerados contaminantes de alimentos de consumo humano e animal, produzidos por certas cepas de fungos filamentosos (MANNON; JOHNSON, 1985; FINKS-GREMMELS, 1999). Os principais gêneros de fungos toxigênicos de campo são: *Fusarium, Alternaria* e *Cladosporium* e os de armazenamento, *Aspergillus e Penicillium* (DRUMMOND, 2012; RODRÍGUEZ, 2010). Dentre estes, destacam- se os gêneros *Aspergillus, Penicillium* e *Fusarium* como os principais produtores de micotoxinas (PEREIRA et al., 2005; ROSA et al., 2006; SIMAS et al., 2007; SKRBIC et al., 2011), podendo ser observados na Tabela 1.

Tabela 1 - Principais espécies fúngicas toxigênicas e micotoxinas de importância mundial.

Espécie de fungo	Micotoxinas
Aspergillus parasiticus	Aflatoxinas B_1, B_2, G_1 e G_2, Aflavininas, Ácido aspergílico, Ácido Kójico
Aspergillus flavus	Aflatoxinas B_1 e B_2, Ácido ciclopiazônico, Aflavininas, Ácido aspergílico, Ácido Kójico, Paspalininas
Aspergillus ochraceus	Ochratoxina A, Ácido penicílico, Xantomegnina, Viomeleina, Ácido Kójico
Aspergillus candidus	Candidulina, Terfenilina, Xantoacina, Ácido Kójico
Aspergillus terreus	Citreoviridina, Citocalasina, Citrinina, Patulina, Ácido terréico, Citreoviridina
Penicillium verrucosum	Ochratoxina A, Citrinina
Penicillium roqueforti	Patulina, Ácido penicílico, Roquefortina
Penicillium citrinum	Citrinina
Penicillium veridicatum	Xantomegnina, Viomeleim, Vioxantina
Fusarium verticillioides	Fumonisina, Bovericina
Fusarium graminearum	Desoxinivalenol, Zearalenona
Fusarium moniliforme	Fumonisina B_1
Fusarium proliferatum	Fumonisina, Moniliformina, Bovericina, Fusaproliferina

Fonte: Algarra (2010).

A contaminação dos cereais por micotoxinas é frequente, podendo ocorrer ainda no campo, durante o armazenamento ou no processamento dos alimentos. Tratamentos tecnológicos usuais de alimentos como tostagem e extrusão, não são capazes de degradar as micotoxinas presentes nos grãos, mantendo-se assim presentes mesmo após a eliminação dos fungos, representando um sério problema para a saúde humana e animal (GAJECKI et al., 2007).

Os alimentos destinados ao homem e aos animais podem estar contaminados por diferentes tipos de micotoxinas, visto que a maioria dos fungos pode produzir mais de uma micotoxina (Tabela 1) (BAKAN et al., 2002; SCUDAMORE; NAWAZ; HETMANSKI, 1998).

Aproximadamente 90% das intoxicações por micotoxinas são crônicas e não apresentam sinais clínicos específicos, e podem portanto, não ser identificadas, ou serem facilmente confundidas com a desnutrição, deficiências de manejo ou outras doenças crônicas que implicam na diminuição da produtividade dos animais (DILKIN, 2002).

A quantidade ingerida de micotoxinas, juntamente com a duração da exposição e a sensibilidade animal, irá determinar os efeitos biológicos desses contaminantes (AKANDE et al., 2006).

De modo geral, as micotoxinas expressam seus efeitos por meio de mecanismos primários, como a redução no consumo de alimento e alterações morfológicas intestinais, que dificultam a absorção de nutrientes (ANDRETTA et al., 2011; HAUSCHILD et al., 2006).

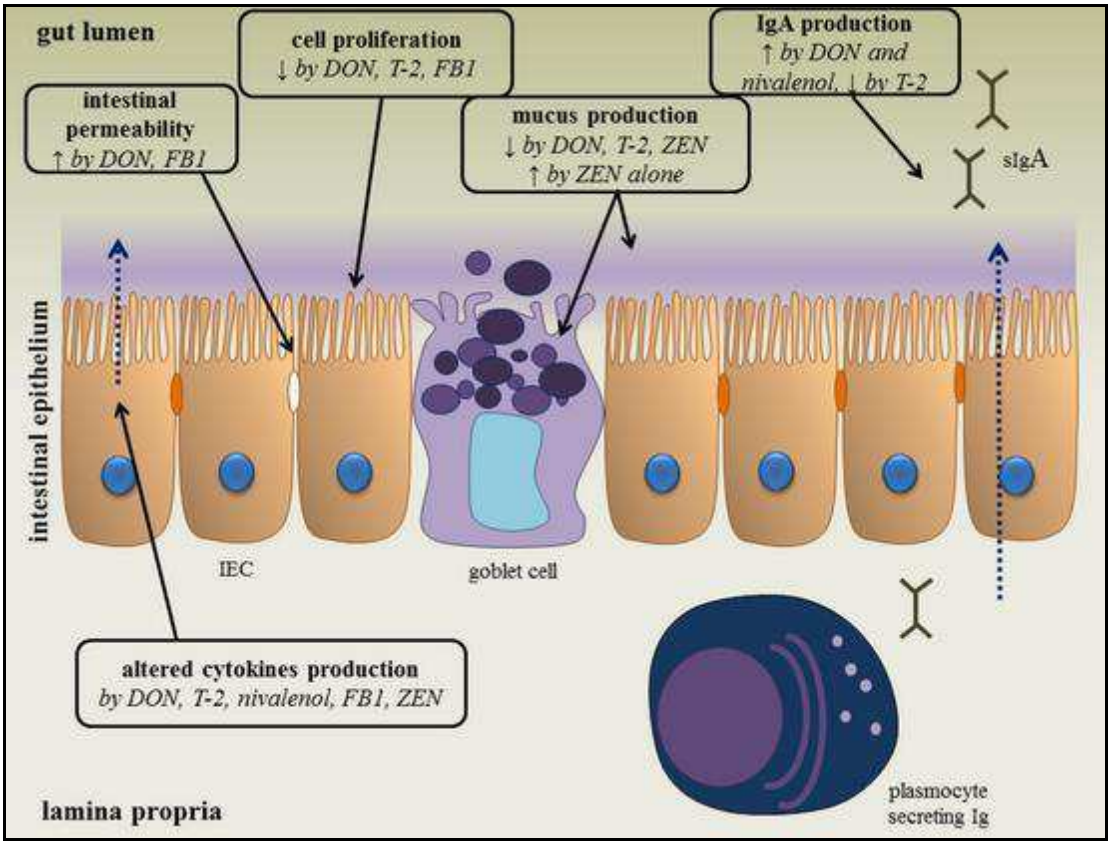
As micotoxinas também podem afetar o sistema imune do animal por meio de uma ação imunossupressora, favorecendo outras infecções por agentes patogênicos, tornando-se difícil o diagnóstico das micotoxicoses (IHESHIULOR et al., 2011).

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 (OSWALD et al., 2005), dentre os quais alguns podem ser tóxicos tanto para o homem quanto para os animais (DRUMMOND, 2012; IAMANAKA; OLIVEIRA; TANIWAKI, 2010).

No animal, a mucosa intestinal atua como a principal barreira protetora, impedindo a entrada de agentes estranhos, incluindo proteínas alimentares, xenobióticos (tais como drogas e toxinas) e micro-organismos patogênicos para os tecidos subjacentes (BOUHET; OSWALD, 2005; OSWALD, 2006). Contudo, a imunidade das mucosas pode ser afetada pelas micotoxinas, comprometendo a defesa do animal (BOUHET; OSWALD, 2005) (Figura 1). Uma variedade de fusariotoxina altera diferentes mecanismos de defesa, incluindo a integridade do epitélio intestinal, a proliferação de células, uma camada de muco, a produção de imunoglobulinas (Ig) e de citocinas (ANTONISSEN et al., 2014).

As fusariotoxinas são particularmente importantes, dentro de todo esse grupo, devido ao seu maior potencial tóxico. Dentre elas destaca-se o desoxinivalenol (DON), também conhecido como vomitoxina, cujos danos envolvem diarreia, redução no crescimento e problemas reprodutivos nos suínos (DILKIN; MALLMANN, 2011).

Figura 1 - Efeito das Fusariotoxinas no Epitélio Intestinal

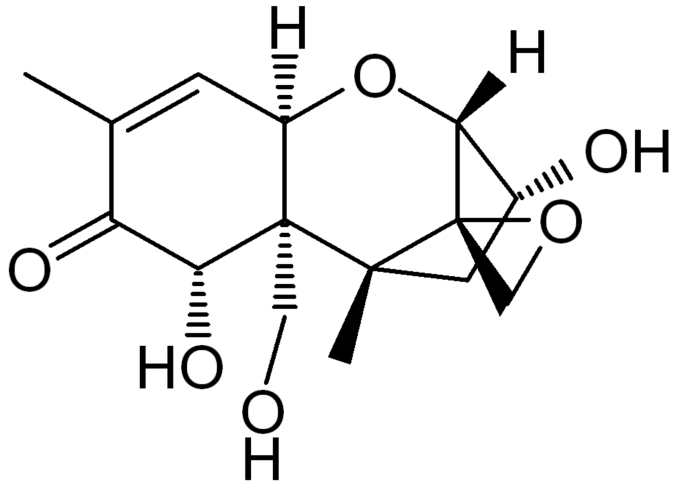


Fonte: Antonissen et al. (2014).

2.4.1 Desoxinivalenol (DON)

A micotoxina desoxinivalenol (DON) ou vomitoxina, metabólito secundário prozuzido pelo gênero *Fusarium,* pertence ao grupo dos tricotecenos não macrocíclico (tipo B). Contém uma dupla ligação entre a posição $C_{9,10}$, um anel epóxido na posição $C_{12,13}$ e um número variável de grupos hidroxila e acetil (Figura 2). São substâncias polares, solúveis em soluções aquosas, metanol e acetonitrila. Apresentam-se quimicamente estáveis, permitindo seu armazenamento em solventes orgânicos sem se decompor (MIDIO; MARTINS, 2000).

Figura 2 - Estrutura molecular do desoxinivalenol (DON)



Fonte: Midio e Martins (2000)

Os tricotecenos crescem e se proliferam em cereais como o milho, cevada, trigo, arroz e aveia, onde encontram um substrato altamente nutritivo para o seu desenvolvimento (PINTO; VAAMONDE, 1996; BÖHM; RAZZAZI-FAZELI, 2005).

O DON surge frequentemente em cereais como o trigo, a cevada, centeio, milho, arroz e sorgo na altura da floração (GAUTAM; DILL- MACKY, 2012).

A contaminação por tricotecenos é o principal problema na região sul do Brasil, em decorrência de suas condições climáticas (chuva abundante, frio, temperaturas entre 6 a 24ºC) (DILKIN; MALLMANN, 2011).

Um estudo realizado com 11.022 amostras de cereais de 12 países europeus mostrou que 57% das amostras foram positivas para DON, e que em somente 6% a concentração de DON foi igual ou superior a 500 µg/kg (GAREIS, 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; NAVAS; SABIN, 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).

Santos et al. (2011), analisando 38 amostras de trigo provenientes de diferentes culturas e localidades do PR e RS, 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 $\mu g.kg^{-1}$).

Em geral, os tricotecenos atuam inibindo a enzima peptil transferase, diminuindo dessa forma a síntese proteica, o que afeta principalmente células em divisão ativa, como as do trato gastrointestinal, pele e células linfóides, eritróides e órgãos vitais. Além disso, essas micotoxinas são imunossupressoras, tóxicas às membranas celulares, induzindo a apoptose e a carcinogênese (DILKIN, 2002; SHIFRIN; ANDERSON, 1999). Particularmente, o DON atua na inibição da fase de alongamento/terminação da síntese proteica, e a via de eliminação mais comum se dá pela urina, podendo ser também eliminado pelas fezes (DILKIN; MALLMANN, 2011).

Existem evidências que sinalizadores celulares são ativados com a presença de 1mg DON/kg de peso vivo, induzindo ativação genética de uma serie de enzimas proteina-quinase, desta forma, levando a célula a apoptose (morte programada da célula) (MURPHY et al., 2006).

Dentre os efeitos tóxicos dos tricotecenos, observam-se injúrias, vômito, diarreia e inflamação intestinal, além de leucopenia, irritação cutânea, refugagem de alimentos, redução no crescimento e falhas reprodutivas (DILKIN; MALLMANN, 2011). Tais efeitos induzidos pelo DON são bem característicos em todas as espécies animais, principalmente nos suínos, que é a espécie mais sucetível à contaminação por essa micotoxina (PESTKA, 2010)

Em estudos de toxicidade aguda e sub-aguda por DON em animais, os efeitos são vômito, anorexia, perda de peso e diarréia. Após esse quadro agudo, foi observada necrose tecidual no trato intestinal, medula óssea e tecidos linfóides (FRANÇA, 2009).

A intoxicação crônica por DON tem sinais clínicos inespecíficos, como diminuição no consumo da ração, depressão e perda de peso. Essas intoxicações induzem alterações imunológicas na produção de imunoglobulinas (DILKIN; MALLMANN, 2011), alterando ainda a resistência elétrica transepitelial na mucosa intestinal, aumentando a permeabilidade intercelular, e consequentemente aumentando os riscos do tecido à infecções (AWAD et al., 2004).

O epitélio intestinal, que constitui a primeira barreira durante a ingestão de alimentos, pode ser exposto às concentrações elevadas de micotoxinas que podem afetar diretamente a capacidade de renovação celular, alterando a capacidade dos enterócitos em assegurar a função de proteção intestinal (BOUHET; OSWALD, 2005).

Estudos utilizando fragmentos de jejuno de suínos, demonstraram que após 4 horas de incubação em meio de cultura com DON a 10 μM, importantes alterações morfológicas foram visualizadas no epitélio intestinal, como a fusão das vilosidades, lise dos enterócitos, edema intersticial e debris celulares (BASSO; GOMES; BRACARENSE, 2013; KOLF-CLAUW et al., 2009; PINTON et al., 2012). Bracarense et al. (2012), em um estudo com leitões aos 35 dias de idade, confirmaram tais efeitos deletérios na morfologia intestinal de leitões que receberam dietas contaminadas com DON. Concomitantemente, diversos estudos *in vitro* também demonstram os impactos do DON em células epiteliais intestinais (MARESCA; FANTINI, 2010; DIESING et al., 2011; PINTON et al., 2012).

O DON também tem atuação nos parâmetros sanguíneos, conforme resultados apontados por Rotter et al. (1994), que relataram um aumento da relação albumina/globulina em suínos que ingeriram concentrações de 0,75 a 3,0 mg de DON/kg, 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 de DON/kg (BERGSJO; MATRE; NAFSTAD, 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 de DON/kg.

Leitões (42 dias de idade) alimentados com dieta contaminada (8,6 mg/kg de DON, 1,2 mg/kg de ZEA) apresentaram uma diminuição significativa no desempenho, aumento do peso do útero e alteração nos parâmetros sorológicos (DOLL et al., 2005). Os autores observaram que as dietas contendo DON superior 300 μg/kg podem reduzir o crescimento e o consumo de alimentos, ao passo que dietas contendo 600 μg/kg de DON podem resultar em alterações imunológica, inflamação sistêmica e danos parciais ao fígado, causando diminuição do crescimento dos suínos (CHAYTOR et al., 2011).

Estudos *in vitro* sugerem que o DON afeta a integridade do epitélio intestinal. Maresca et al. (2002), trabalhando com elevadas concentrações de DON, mostraram alteração na absorção dos nutrientes (as funções de absorção foram afetadas com doses maiores que 10 μM em células intestinais humanas da linha HT-29).

2.4.2 Desoxinivalenol (DON)- Resposta Inflamatória

Como descrito anteriormente, os tricotecenos inibem a síntese protéica por meio da ligação a peptidiltransferase. Esses inibidores podem desencadear uma reação denominada resposta ao estresse ribotóxico, que ativam proteínas quinases ativadas por mitógenos (MAPK), que fazem parte da cascata de sinalização que regula a sobrevivência das células perante situações estressantes (IORDANOV et al., 1997).

As MAPK, compostas por três membros ou subfamílias, ERK (quinases reguladas pelo sinal extracelular), JNK (quinase c-Jun N-terminal) e p38 (JOHNSON; LAPADAT, 2002), constituem uma família de proteínas de transdução de sinal que converte sinais extracelulares, como estresse e fatores de crescimento, para a ativação de uma seqüência de reações intracelulares (HUANG; HAN; HUI, 2010).

A especificidade da ativação e função das MAPK é determinada, em parte, por proteínas que criam complexos multienzimáticos, 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 é ativada por diversos estímulos que regulam a produção de citocinas e fatores de crescimento (ROUX; BLEINS, 2004), respeitando uma sequência de fosforilação e desfosforilação, que culmina na ativação de proteínas como ERK, JNK e p38, responsáveis pela expressão gênica, levando a uma resposta fisiológica apropriada (MUTALIK; VENKATESH, 2006).

A ERK p44/42 está 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 às mesmas, além de regular a integridade da junção celular e, consequentemente, a função de proteção 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), que estão envolvidas na proliferação celular e apoptose através da ativação de vários alvos (HUANG; HAN; HUI, 2010). Diversos estímulos como estresse, citocinas pró-inflamatórias (IL-1β e TNF- α), fatores de crescimento (FGF e TGF) e

ativação de receptores Toll-like (TLR-4 e TLR-9) induzem a ativação das JNK (WESTON; DAVIS, 2007).

Ainda são responsáveis pela regulação da expressão e ativação de mediadores inflamatórios, incluindo TNFα, IL-2, selectina E em etaloproteinases (MANNING; DAVIS, 2003; RINCÓN; 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 à 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.

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 super expressão na produção de citocinas e pela indução da apoptose (ZHOU; YAN; PESTKA, 1998; ZHOU; ISLAM; PESTKA, 2003). Dependendo da dose e frequência de exposição, o DON pode ter ação imunossuspressora ou imunoestimulatória (PESTKA; SMOLINSKI, 2005; PINTON et al., 2008). A exposição crônica a baixas doses de tricotecenos é responsável pela redução na produtividade e proliferação linfocitária, resistência do hospedeiro, função imune humoral e celular e pelo aumento da susceptibilidade a doenças infecciosas (BONDY; PESTKA, 2000; PESTKA; SMOLINSKI, 2005).

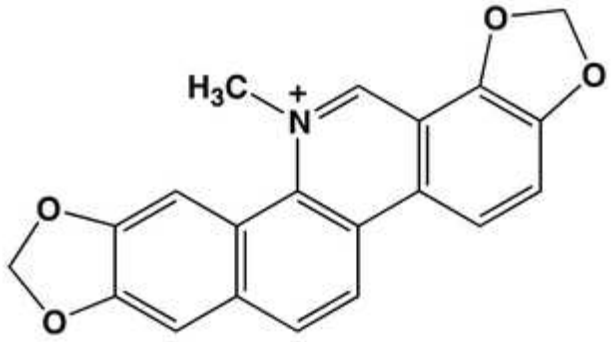
2.5 SANGUINARINA

Os alcaloides representam a maior classe de metabólitos secundários de plantas com uma notável gama de funções farmacológicas, incluindo atividade anti-inflamatórias (HENRIQUES et al, 2004), porém algumas vezes podem ser tóxicos para o homem (TALIB et al, 2010).

A Sanguinarina (SA) é um alcaloide quaternário da benzofenantridina (QBA) (Figura 3), extraído de plantas da família das *Papaveracea* (GRAF et al., 2007), como a *Sanguinaria canadensis*.

Os alcaloides vegetais QBA apresentam propriedades farmacológicas, incluindo efeitos antimicrobianos e anti-inflamatórios (LENFELD et al., 1981; GODOWSKI, 1989; SCHMELLER; LATZ-BRUNING; WINK, 1997; BEURIA; SANTRA; PANDA, 2005;) e devido a essas propriedades, a Sanguinarina vem sendo bastante estudada e utilizada como componente bioativo em cremes dentais (anti-placa) (FRANKOS et al., 1990; WALTEROVA et al., 1995; GIULIANA et al., 1997; HONG; JEONG; SONG, 2005) e como aditivo na alimentação animal (Sangrovit®) (KOSINA et al., 2004; ZDARILOVA et al., 2006), este último com objetivo de aumentar o consumo de ração e melhorar o desempenho de suínos (KOSINA et al., 2004).

Figura 3 - Estrutura molecular da Sanguinarina.



Fonte: Psotová et al. (2006).

Além disso, a Sanguinarina também é conhecida por inibir a proliferação de vários tipos de células tumorais (AHMAD et al. 2000; ADHAMI et al., 2004), exercendo assim, efeitos anti-cancerígenos (LOPUS; PANDA, 2006; CHANG et al., 2007).

Devido à indução de apoptose em células de linfoma primário, a Sanguinarina demonstra atividade anticarcinogênica (CHATURVEDI et al., 1997; HUSSAIN et al, 2007) e de acordo com Eun e Koh (2004) e Basini et al. (2007) ao suprimir o fator de crescimento endotelial vascular, o principal regulador da brotação vasos pré-existentes, a Sanguinarina na dieta leva a um efeito antiangiogênico.

Tem sido demonstrado que a Sanguinarina suprime o crescimento de certas bactérias que causam desconforto gastrointestinal (MAHADRIA et al., 2003), aumenta o apetite e ingestão de alimentos e promove o crescimento do animal (TSCHIRNER; SUSENBETH; WOLFFRAM, 2003).

Além disso, no intestino, ela exerce atividade inibidora da enzima descarboxilase que degrada os aminoácidos aromáticos, suprimindo os efeitos adversos de alguns fungos e bactérias sobre o epitélio intestinal (LENFELD et al., 1981; DRSATA; ULRICHOVA; WALTEROVA, 1996), e devido a esse bloqueio, a alanina, o triptofano e outros aminoácidos são melhores utilizados (DRSATA; ULRICHOVA; WALTEROVA, 1996; TSCHIRNER, 2004).

O bloqueio de parte do mecanismo pelo qual as toxinas são produzidas pode ajudar a prevenir e/ou reduzir a inflamação no intestino. Verificou-se que a Sanguinarina pode melhorar o tempo de recuperação das infecções das paredes intestinais em 60% (por exemplo, C*lostridium* e *E. coli*) poucas horas após o primeiro tratamento. A vantagem para o animal é que a infecção pode ser superada mais rapidamente, e o apetite e a digestão podem ser retomados com o mínimo de interrupções (MELLOR, 2001).

Reiterando estudos que demonstram a ação anti-inflamatória, a Sanguinarina, juntamente com outros alcaloides de origem vegetal, tem sido avaliada como possível substituto ao uso de antibióticos promotores de crescimento (APC) na nutrição animal, visto que numerosos aditivos são utilizados ou propostos como meio para reduzir ou eliminar agentes patogênicos ou melhorar o desempenho dos animais (JOERGER et al., 2002).

A substituição dos APC por substâncias com ação anti-inflamatória se dá pela hipótese de que os antibióticos podem exercer primeiramente uma ação anti-inflamatória, não sendo mais a microbiota intestinal o alvo principal desses compostos. Os APC se acumulam no interior de células inflamatórias fagocíticas, atenuando a resposta inflamatória e diminuindo, consequentemente, a produção de citocinas pró-inflamatórias. Esse processo resulta na diminuição do catabolismo, promovendo indiretamente o crescimento do animal pela preservação de energia e nutrientes para este fim (NIEWOLD, 2007).

Neste contexto, reforça-se a necessidade de alternativas (substâncias) eficazes com propriedades semelhantes para substituírem os APC, já que podem levar à resistência de alguns micro-organismos, sendo banidos em diversos países por esse motivo.

A Sanguinarina, é o principal componente do fitoterápico Sangrovit®, utilizado na alimentação de suínos, visando melhorar o apetite e a ingestão alimentar (MELLOR, 2001), através da preservação da mucosa intestinal desses animais. A Sanguinarina (1% do Sangrovit®), como mencionado anteriormente, é um alcalóide de origem vegetal que inibe a multiplicação de bactérias, fungos e vírus que causam desconforto gastrointestinal (SCHMELLER; LATZ-BRUNING; WINK, 1997; MELLOR, 2001).

Existem informações limitadas sobre a farmacocinética da Sanguinarina, mas devido à grande variedade de efeitos biológicos e o potencial terapêutico deste alcalóide de plantas e ao potencial de utilização em produtos comerciais, seu real modo de ação deve ser melhor investigado (MACKRAJ; GOVENDER; GATHIRAM, 2008).

2.6 MODELO *EX VIVO* NO SISTEMA INTESTINAL

A estrutura intestinal dos suínos, assim como a dos seres humanos, se mostra complexa dificultando a análise morfológica em estudos *in vitro.* Dessa forma, técnicas como o modelo *ex vivo* podem ser alternativas eficazes em pesquisas laboratoriais. Neste modelo se faz possível a simulação de um sistema como o trato intestinal, já que a complexidade das estruturas intestinais quanto aos aspectos morfológicos ou patológicos, faz com que as investigações por meio de modelos de cultura de células *in vitro* sejam dificultadas (RANDALL; TURTON; FOSTER, 2011).

O modelo *ex vivo* consiste no cultivo de explantes e está se mostrando uma técnica eficaz em pesquisas em laboratório. Esse modelo caracteriza-se pelo cultivo de fragmentos de órgãos colhidos com *punch* de biópsia ou bisturi, incubados em placas com meio de cultura em temperatura semelhante à corpórea, simulando as condições do organismo vivo (BANSAL et al., 2009; RANDALL; TURTON; FOSTER, 2011).

Como pontos limitantes, a técnica apresenta um reduzido tempo de incubação compatível com a manutenção da viabilidade celular. Durante a incubação o tecido é submetido à hipóxia, fato que muitas vezes ocasiona autólise do material em estudo. Assim, várias etapas do método têm sido aprimoradas, visando-se diminuir esses efeitos (RANDALL; TURTON; FOSTER, 2011).

Contudo, mesmo com certas restrições, a técnica permite minimizar o uso de animais destinados à pesquisa, uma vez que a partir de um animal são produzidos inúmeros explantes. É possível que fragmentos de órgãos do mesmo doador sejam submetidos a diversos tratamentos, além de se poder controlar as condições ambientais às quais o tecido é submetido (KOLF-CLAUW et al., 2009; RANDALL; TURTON; FOSTER, 2011). Deste modo, a técnica é adequada para estudos toxicológicos, nutricionais e patológicos.

Paralelamente à pesquisa animal, as similaridades anatômicas e fisiológicas entre o homem e o suíno, em particular em relação ao sistema digestório e imunitário, fazem do suíno um modelo excelente de estudo para as pesquisas humanas (ROTHKOTTER; SOWA; PABST, 2002).

Assim sendo, explantes de intestino (KOLF-CLAUW et al., 2009; PINTON et al., 2012; LUCIOLI et al., 2013; BASSO; GOMES; BRACARENSE, 2013; CANO et al., 2013), de pele (DAME et al., 2008), de articulação (OTSUKI et al., 2008), pulmão e brônquios (POUCKE et al., 2010) de suínos têm sido utilizados na experimentação animal com várias finalidades, como a avaliação dos efeitos de substâncias tóxicas, carcinogênicas, terapêuticas, agentes biológicos ou testes de sensibilidade.

Devido às vantagens anteriormente apontadas, Basso, Gomes e Bracarense (2013) confirmam que explantes de jejuno provenientes de suínos de 24 dias incubados por 4 horas demonstraram ser um modelo eficaz na avaliação da toxicidade intestinal.

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3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliação da Sanguinarina sobre a saúde intestinal de suínos através de diferentes modelos experimentais (*in vitro, ex vivo* e *in vivo*)

3.2 OBJETIVOS ESPECÍFICOS

- Avaliar os efeitos da Sanguinarina (Sigma) sobre a viabilidade de células intestinais de suínos utilizando modelo *in vitro.*
- Estudar os efeitos da Sanguinarina (Sangrovit®) sobre a histologia e morfologia de explantes intestinais de leitões expostos ao desoxinivalenol utilizando modelo *ex vivo.*
- Avaliar os efeitos da Sanguinarina (Sangrovit®) sobre a morfologia e morfometria intestinal de suínos aos 80 dias de idade utilizando modelo *in vivo.*
- Analisar os efeitos da Sanguinarina (Sangrovit®) sobre a produção de imunoglobulinas do soro de suínos aos 80 dias de idade utilizando modelo *in vivo.*

4 ARTIGO PARA PUBLICAÇÃO

4.1 AVALIAÇÃO DA SANGUINARINA SOBRE PARÂMETROS INTESTINAIS E SANGUÍNEOS DE SUÍNOS POR MEIO DE DIFERENTES MODELOS EXPERIMENTAIS (*IN VITRO, EX VIVO E IN VIVO*)[1]

[1] Artigo editado de acordo com as normas de publicação da *Revista Brasileira de Zootecnia*

Avaliação da sanguinarina sobre parâmetros intestinais e sanguíneos de suínos por meio de diferentes modelos experimentais (*in vitro, ex vivo e in vivo*).

Resumo

Este estudo foi conduzido com o objetivo de avaliar os efeitos da Sanguinarina sobre parâmetros intestinais e sanguíneos de suínos utilizando-se diferentes modelos experimentais. Inicialmente avaliou-se o efeito de diferentes concentrações de Sanguinarina (0,1; 0,25; 0,5; 0,75; 1,0 e 5,0 μM) sobre a viabilidade de células IPEC-1 de suínos. O teste de citotoxicidade (MTT) revelou 50% de mortalidade celular (IC_{50}) com a dose de 0,65 μM. Na avaliação dos efeitos da Sanguirarina sobre a integridade morfológica intestinal, explantes de jejuno foram expostos por 4 horas aos seguintes tratamentos: meio de cultura (controle), Sanguinarina (1,0 mg/L), desoxinivalenol (DON) (10 μM) e Sanguinarina+DON (1,0 mg/L+10 μM). Os explantes expostos ao DON apresentaram redução significativa no escore tecidual, altura de vilosidades e profundidade de criptas em relação ao grupo controle. Finalmente, realizou-se teste *in vivo*, utilizando-se 24 suínos (30,36 ± 2,09 kg) que foram submetidos, durante cinco dias, aos seguintes tratamentos por meio de gavagem: controle (água mineral) e Sanguinarina (1,0 mg/L, correspondendo a dose de 3 mg/animal/dia). Após esse período, os animais foram abatidos e amostras de sangue foram coletadas para a quantificação de imunoglobulinas, e fragmentos de duodeno, jejuno e íleo foram colhidos para análise histológica e morfométrica. Os explantes tratados com Sanguinarina na presença de DON apresentaram diminuição significativa das lesões causadas pela micotoxina no epitélio intestinal. Nos animais, a Sanguinarina proporcionou melhora do perfil das criptas intestinais e aumento da secreção de IgA sérica. Os efeitos da Sanguinarina nos explantes expostos ao DON e nos animais evidenciaram sua ação na preservação da saúde intestinal.

Palavras-chave: barreira física, células epiteliais, fitoterápico, morfologia intestinal e trato gastrintestinal.

Evaluation of Sanguinarine on the swine's intestinal and blood parameters through different experimental templates (*in vitro, ex vivo and in vivo*).

Abstract

This study was carried out in order to evaluate the effects of the Sanguinarine on the intestinal and blood parameters of pigs using the different experimental templates. At first, was evaluated the effects of different concentrations of Sanguinarine (0.1; 0.25; 0.5; 0.75; 1.0 and 5.0 μM) on the viability of swine's IPEC-1 cells. The cytotoxicity assay (MTT) revealed 50% of cellular death (IC_{50}) at a dose of 0.65 μM. In the evaluation the Sanguinarine effects on the intestinal morphologic integrity, some jejunal explants were exposed for four hours to the following treatments: culture medium (control), Sanguinarine (1.0 mg/L), deoxynivalenol (DON) (10 μM) and Sanguinarine + DON (1.0 mg/L + 10 μM). The explants exposed to DON showed a significant reduction in the tissue score, villus height and crypt depth in relation to the control group. Finally, an *in vivo* test was performed using 24 swines (30.36 ± 2.09 kg) which were submitted for five days to the following treatments by gavage: control (mineral water) and Sanguinarine (1.0 mg/L, corresponding to 3 mg / animal / day). After this period the animals were slaughtered and blood samples were collected for quantification of immunoglobulins, and some fragments of their duodenum, jejunum and ileum were collected for histological and morphometrical analysis. Explants treated with Sanguinarine in the presence of DON showed a significant decrease of lesions caused by the mycotoxins in the intestinal epithelium. In the animals, the Sanguinarine imprived the profile of the intestinal crypts and increased the IgA serum secretion. The effects of Sanguinarine in the explants exposed to DON and in the animals showed its action in preserving intestinal health.

Keywords: physical barrier, epithelial cells, phytotherapic, intestinal morphology, gastrointestinal tract.

Introdução

Nos suínos, os desafios no trato gastrintestinal são contínuos, com a exposição frequente a agentes patogênicos e a substâncias tóxicas que levam à alterações estruturais e funcionais do intestino, comprometendo a digestão e absorção de nutrientes (Silva e Nörnberg, 2003; Gartner e Hiatt, 2007).

Substâncias como as micotoxinas, que acometem a produção dos grãos presentes na composição das rações, são metabólitos que favorecem o desequilíbrio intestinal, com destaque ao desoxinivalenol (DON), uma fusariotoxina que têm grande impacto na saúde animal devido à sua alta toxicidade (Dilkin et al., 2010). O DON, de acordo com a dose ingerida, ocasiona vômito, diarréia, retardo no crescimento e alterações de comportamento, como recusa de alimento em suínos (Minami et al., 2004).

Há uma grande preocupação com a qualidade dos ingredientes empregados nas rações a fim de se preservar a saúde intestinal, sendo comum a utilização de aditivos melhoradores do órgão e, por conseqüência, do desempenho dos animais. Nesse contexto, pela limitação do uso de antibióticos em doses subterapêuticas, os produtos alternativos como os extratos herbais, imunomoduladores e os probióticos vem se destacando (Robbins et al., 2013). Neste rol de aditivos, Windisch et al. (2008) apontam aqueles com ação anti-inflamatória como essenciais para manutenção da saúde e do crescimento animal.

Em relação aos extratos herbais, a Sanguinarina, um alcalóide quaternário benzofenantridino (QBA) presente nas plantas da família *Papaveracea,* apresenta propriedades antimicrobianas, agindo na inibição da multiplicação de bactérias, fungos e vírus que causam danos gastrintestinais (Schmeller et al., 1997; Mellor, 2001; Robbins et al., 2013); ação anti-inflamatória (Vrublova et al., 2010; Khadem et al., 2014) e

também está associada à diminuição da degradação de aminoácidos essenciais (Robbins et al., 2013), efeitos que permitem uma maior disponibilidade de nutrientes, resultando na melhoria da saúde intestinal (Mellor, 2001; Corona et al., 2012).

Os resultados positivos do uso da Sanguinarina para suínos e aves (Corona et al., 2012; Pickler et al., 2013; Khadem et al., 2014) motiva o provimento de mais informações sobre sua ação, sendo objeto deste estudo avaliar, por meio de diferentes modelos experimentais, os efeitos deste princípio nas células e na mucosa intestinal de suínos e suas consequências no perfil imune sérico desta espécie.

Material e Métodos

Foram utilizados três modelos experimentais caracterizados por estudos *in vitro*, *ex vivo* (cultivo de explantes) e *in vivo*.

Modelo *in vitro*

Inicialmente avaliou-se os efeitos de diferentes doses de Sanguinarina sobre a viabilidade de células intestinais de suínos. Para isso, foram utilizadas células da linhagem celular IPEC-1 (Intestinal Porcine Epithelial Cells) obtidas do epitélio intestinal (jejuno e íleo) de leitões recém-nascidos (Bouhet et al., 2004). Seguindo a metodologia descrita por Bouhet et al. (2004), as células foram mantidas e diferenciadas em meio Dulbecco's Modified Eagle's Medium/HAMF12 (DMEM/HAMF F12 Sigma D8062).

Após atingirem a confluência, as células foram tratadas com tripsina e colocadas em placas de cultura celular de 96 poços (Costar, Cambridge, MA, EUA) para a realização do teste de viabilidade celular.

Para definir as doses de Sanguinarina a serem utilizadas, partiu-se da dose de 2,72 µM (Quadro 1), que corresponde à concentração e indicação de Sanguinarina (1%) na apresentação comercial denominada Sangrovit®.

- A Sanguinarina corresponde a 1% do Sangrovit®
- Recomendação Sangrovit® = 100 mg/kg (L)
- Sanguinarina 1 mg/kg (ppm) = 2,72 µM

Massa Molar da Sanguinarina = 367,78 g/mol

$$\frac{1\ \text{mol}}{367{,}78\ \text{g}} \times \frac{1 \times 10^{-3}}{1\text{L}} \longrightarrow 1\ \text{ppm}$$

$$= 2{,}72 \times 10^{-6}\ \text{mol/L}$$

$$= 2{,}72\ \mu\text{M}$$

Quadro 1 – Cálculo da dose referência de Sanguinarina.

As concentrações de Sanguinarina utilizadas foram 0,1; 0,25; 0,5; 0,75; 1,0 e 5,0 µM a partir de uma solução estoque de 5 mM. A Sanguinarina (367,78g/mol) (Sigma) foi dissolvida em água Milli-Q e estocada a -20°C antes da diluição no meio de cultura celular. As amostras controle foram mantidas somente em meio de cultura.

A viabilidade celular foi verificada por meio do método colorimétrico MTT (3-(4,5-dimetiltiazol-2-il) -2,5-brometo de difeniltetrazólio). O método é baseado na conversão do MTT em um sal de formazan, a partir de enzimas mitocondriais presentes somente nas células metabolicamente ativas (Buriol et al., 2009). Desta maneira, a viabilidade celular pode ser determinada pela intensidade da coloração que é proporcional à quantidade de cristais de formazan formados.

As células foram incubadas com as diferentes concentrações de Sanguinarina durante 48 horas, sendo posteriormente incubadas com o MTT durante 24 h a 37 ° C. O teste MTT foi realizado de acordo com Gauthier et al.

(2012), sendo a absorbância determinada em espectrofotômetro Thermo Scan Elisa TECAN em 570 nm. Para cada grupo (diluições) foram realizadas 15 repetições, sendo 3 repetições por teste realizado (5 testes repetidos no tempo).

A citotoxicidade foi determinada pelo cálculo estimado da dose da concentração inibitória de 50% da proliferação celular (IC50) de acordo com a equação estabelecida por Chou (2006): *fa/fu=(D/Dm) m*, em que *D* é a dose de Sanguinarina, *fa* é a fração afetada pelo *D* (por exemplo, percentagem de inibição / 100), e *fu* é a fração não afetada (ou seja *fu* = 1 - *fa*). *Dm* é a dose de efeito médio (por exemplo, IC50), e *m* é o coeficiente da relação dose-resposta.

Modelo *ex vivo*

Foram utilizados nove suínos de 24 dias de idade (6,7 Kg± 1,2) para a colheita de explantes de jejuno. Os explantes de jejuno de quatro suínos foram utilizados para se estabelecer a melhor dose de Sanguinarina quanto à manutenção das estruturas físico-funcionais do intestino, e os explantes dos outros cinco suínos foram utilizados para se testar os efeitos da Sanguinarina frente à contaminação por DON. Os procedimentos experimentais foram realizados em triplicata, sendo que de cada suíno foram coletados 36 explantes, totalizando 324 explantes (nove suínos).

Os animais foram eutanasiados com injeção intravenosa de pentobarbital de sódio (40 mg/ kg de peso vivo). Para a extração dos explantes, fragmentos de 5 cm do jejuno medial foram colhidos imediatamente após a eutanásia dos animais. Os fragmentos foram lavados com solução salina tamponada (PBS) e abertos longitudinalmente. Os explantes foram colhidos com auxílio de *punch* para biópsia (6 mm) e depositados em placas de seis poços (EasyPath), preenchidos com 3 mL de ágar-ágar e contendo meio DMEM acrescido de soro fetal bovino (10%), glutamina (0,02%), gentamicina (0,5%) e

penicilina/estreptomicina (1%). Os explantes foram depositados nas placas com a mucosa voltada para cima (4 explantes/poço), e incubados em estufa a 37^{o}C horas.

No experimento prévio, com explantes de quatro suínos, foram utilizadas as doses de 0,25; 0,50; 1,0; 2,0; 4,0 mg/L de Sanguinarina. As diluições foram obtidas a partir de uma solução estoque de 200 mg/L (10 mg de Sanguinarina diluídos em 50 mL água Milli-Q).

Posteriormente, avaliou-se a eficácia da Sanguinarina na presença ou não do DON sobre os explantes intestinais de cinco suínos. Os explantes foram incubados na presença de Sanguinarina (1mg/L) e DON (10µM) (Sigma-Aldrich), ambos isoladamente e durante quatro horas; e na presença da associação de Sanguinarina e DON. Neste último caso, os explantes permaneceram incubados por vinte minutos em meio de cultura somente com a Sanguinarina (1mg/L), e após esse tempo de incubação foram acrescentados 2µl de DON a 15mM nos poços com 3mL de meio, resultando na concentração de 10µM de DON. Para cada tratamento foram incubados quatro explantes/poço imersos em meio de cultura (grupo controle).

A micotoxina liofilizada DON (Sigma-Aldrich®) foi dissolvida em água Milli-Q e armazenada a -20°C. A partir da solução estoque de 15 mM foi realizada a diluição para uma concentração de10µM. A concentração de 10µM corresponde a 3 mg de DON/kg de alimento, concentração esta que excede o limite máximo tolerado na alimentação animal (0,20 mg/kg de ração). A concentração de 10µM foi determinada pelo conhecido efeito tóxico ao epitélio intestinal (Kolf-Clauw et al., 2009; Bracarense et al., 2012; Pinton et al., 2012; Basso et al., 2013; Lucioli et al., 2013).

Após o período de incubação todos os explantes foram fixados em solução de formalina a 10%, desidratados em soluções crescentes de álcoois e embebidos em parafina. Cortes de 5µm foram corados por hematoxilina e eosina (HE) para análise histológica. As alterações histológicas foram avaliadas utilizando-se escore tecidual, que considera número de vilosidades, morfologia dos enterócitos, edema de lamina própria, desnudamento apical, degeneração celular, presença de microvilosidades e dilatação linfática, considerando a intensidade e severidade das alterações, sendo que o escore máximo (22 pontos) indica a integridade total do intestino (Basso et al., 2013).

Para avaliação morfométrica dos explantes foram mensuradas as alturas de 10 vilosidades e 10 profundidades de criptas com o auxílio do programa Motic image plus 2.0 (Richmond, Canada), utilizando objetiva de 10x.

Os resultados dos testes *ex vivo* foram submetidos à análise de homogeneidade de variância (teste de Levene) e normalidade (Shapiro-Wilk). Os dados que não atenderam a estes parâmetros foram transformados por Log (x). Após constatação da homogeneidade e normalidade, as médias foram submetidas à análise de variância e, constatadas as diferenças, foram comparadas pelo teste de Tukey ($p<0,05$).

Modelo *in vivo*

Nesta etapa foram utilizados suínos em fase de crescimento, a fim de descartar os efeitos negativos do desmame. Foram utilizados 24 suínos machos castrados, com idade média de 80 dias, e peso médio inicial de 30,36 ± 2,09 kg. Os animais foram alojados em duplas, em 12 baias de alvenaria, com piso compacto, equipadas com comedouros metálicos semi-automáticos e bebedouros tipo *nipple*.

O delineamento experimental aplicado foi inteiramente casualizado e cada suíno compreendeu uma unidade experimental. Durante o período de experimento, com

duração de 10 dias, sendo cinco dias de adaptação e cinco dias de tratamento, os animais receberam água e ração à vontade. Foi fornecida uma única ração para os animais, composta basicamente por milho, farelo de soja e núcleo, formulada visando atender as exigências dos suínos na fase de crescimento, atendendo as recomendações mínimas nutricionais estabelecidas pelo NRC (2012).

Os ingredientes, a composição percentual e os valores calculados das dietas experimentais encontram-se na Tabela 1.

Os animais foram submetidos a dois tratamentos experimentais por meio de gavagem: controle (água mineral) e Sanguinarina (1mg/L). A concentração de Sanguinarina foi baseada na recomendação técnica de uso do produto Sangrovit®. Para esta fase foi considerada uma ingestão voluntária de 3 litros de água/animal/dia (NRC, 2012). Dessa forma, a dosagem administrada de Sanguinarina, via gavagem, foi de 3mg/animal/dia.

O procedimento de gavagem foi realizado duas vezes ao dia durante cinco dias, e após esse período os animais foram eutanasiados, utilizando-se insensibilização por meio de corrente elétrica (equipamento da marca Petrovina® IS 2000 com dois eletrodos, utilizando-se 350 volts e 1,3 ampéres) seguida de exsanguinação. No momento do abate foi realizada coleta de sangue para análises imunológicas e na sequência, foram colhidos fragmentos de duodeno, jejuno e íleo para as avaliações histológicas e morfométricas.

Tabela 1 - Composição percentual dos ingredientes e níveis nutricionais calculados da ração experimental

Ingredientes (%)	Ração Crescimento
Milho	66,70
Farelo de Soja	29,70
Óleo de Soja	0,31
Fosfato Bicálcico	1,60
Calcário	0,76
L-Lisina-HCl	0,03
Premix Vit/Min[1]	0,40
Sal Comum	0,50
Total (kg)	100,00
Níveis Nutricionais	
Proteína bruta (%)	19,00
Energia Met. Suínos (Kcal/kg)	3.300
Extrato etéreo (%)	3,20
Fibra bruta (%)	2,90
Cálcio (%)	0,80
Fósforo disponível (%)	0,40
Lisina digestível (%)	0,94

[1]Composição do Premix Vitamínico - mineral: Vitamina A 720.000UI; Vitamina D3 144.000UI; Vitamina E 2.400 UI; Vitamina K3 216 mg; Vitamina B1 96 mg; Vitamina B2456 mg; Vitamina B6 96 mg; Vitamina B12 1.680 mcg; Niacina 2.400 mg; Ácido pantotênico 1.560 mg; Ácido fólico 60 mg; Manganês 5.400 mg; Zinco13,50 g; Ferro 10,50 g; Cobre 2.100 mg; Iodo 150 mg; Selênio 72 mg; Bacitracina de zinco 3.350 mg.
Fonte: Da autora.

As amostras de sangue foram processadas para análises das concentrações de IgG, IgA, IgM do soro, determinadas pelo método ELISA (*Enzyme Linked Immunosorbent Assay*) Quantification Set (Bethyl) seguindo as recomendações do fabricante. Para a determinação das concentrações de IgG, IgA e IgM, as amostras de soro foram diluídas

1:200.000, 1:1000 e 1:10.000, respectivamente. Os valores de absorbância foram obtidos pelo programa Curve Expert 1.3 e transformados para as unidades

As amostras intestinais foram fixadas em solução de formalina tamponada a 10% e posteriormente submetidas ao processamento histológico de rotina e coloração por hematoxilina-eosina (HE). Para a avaliação morfométrica dos fragmentos de intestino foram mensuradas alturas de 30 vilosidades e 30 profundidades de criptas com o auxílio do programa Motic image plus 2.0, utilizando objetiva de 10x.

Os resultados das avaliações histológicas foram submetidos à análise de homogeneidade de variância (teste de Levene) e normalidade (Shapiro-Wilk). Após constatação da homogeneidade e normalidade, as médias foram submetidas à análise de variância e comparadas pelo Teste F ($p<0,05$). Os resultados dos parâmetros sanguíneos foram submetidos ao teste T para a comparação de médias ($p<0,10$).

Resultados e Discussão

Modelo *in vitro*

Por meio do teste de viabilidade celular MTT, baseado na atividade de enzimas mitocondriais, observou-se que as células IPEC expostas a diferentes concentrações de Sanguinarina (Sigma) apresentaram diminuição da viabilidade celular. A análise de regressão não-linear (Sigma Plot) apontou que a Sanguinarina induziu a mortalidade de 50% das células a partir da dose 0,65 μM (equivalente à 0,24 mg de Sanguinarina/kg ração), e que a dose de 2,72 μM (equivalente à 1mg/kg de ração) levou à mortalidade de 80% das células (Figura 1).

Deve-se considerar que a Sanguinarina tem uma ação por contato e transitória, sendo pouco absorvida e majoritariamente excretada pelas fezes (Agarwall et al., 1997; Psotova et al. 2006). Este cenário pode ter contribuído nos resultados observados, que no modelo *in vitro,* levou as células a uma exposição prolongada ao princípio.

As limitadas informações sobre a ação da Sanguinarina em estudos *in vitro*, restringe comparações com outros trabalhos, no entanto, preservadas estas considerações, e compreendendo as diferenças deste cultivo celular, Agarwall et al. (1997), trabalhando *in vitro* com neutrófilos humanos, não verificaram toxicidade da Sanguinarina sob uma concentração 10 vezes maior que a praticada nesta avaliação.

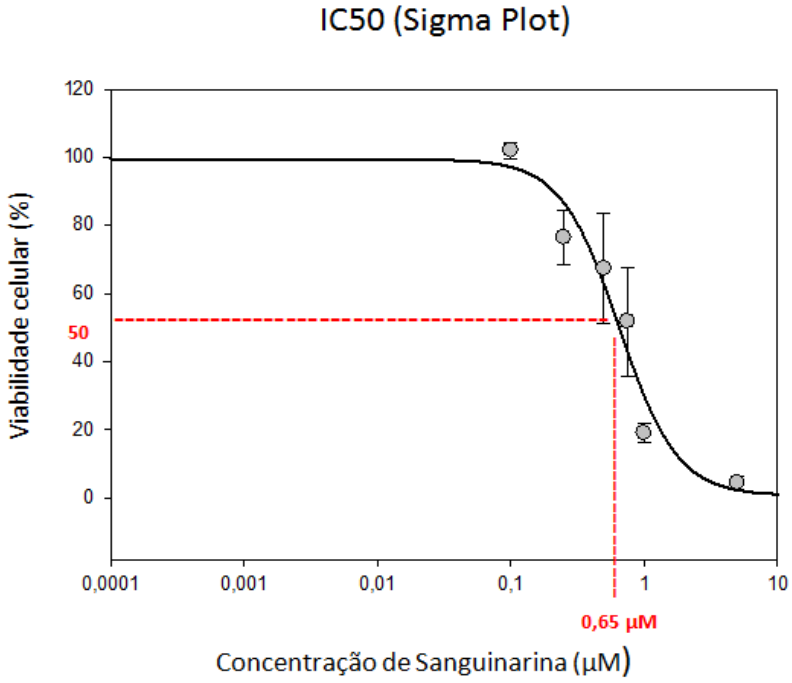

Figura 1 - Viabilidade das células IPEC-1 de suínos incubadas com diferentes concentrações de Sanguinarina (0,1; 0,25; 0,5; 0,75; 1,0 e 5,0 µM). Fonte: Da autora.

A viabilidade celular foi determinada por MTT e expressa como percentagem de células viáveis em relação ao número total de células.

A recomendação do uso da Sanguinarina para suínos é de 1ppm na ração, sendo identificado por Kosina et al. (2004) a segurança deste princípio quando administrado oralmente (1,28ppm) durante 90 dias.

É provável que o efeito observado no teste *in vitro* seja inerente à técnica utilizada, cuja exposição das células epiteteliais à Sanguinarina é direta, o que difere muito do modelo *in vivo*, onde outros componentes do sistema digestório estão envolvidos, reduzindo a concentração de Sanguinarina a que os enterócitos estarão expostos.

Modelo *ex vivo*

Para o estabelecimento da melhor dose de Sanguinarina a ser utilizada no desafio com DON, observou-se que os explantes expostos a 1,0 mg/L de Sanguinarina apresentaram melhor preservação da morfologia intestinal, com redução do edema de submucosa, melhor preservação das microvilosidades e da morfologia dos enterócitos ($p<0,05$), mostrando-se semelhantes ao controle. Essa semelhança do valor do escore tecidual constata a viabilidade tecidual dos explantes expostos a 1,0 mg/L de Sanguinarina (Figura 2). Os grupos submetidos às concentrações de 0,25; 0,50 e 2,0 mg/L de Sanguinarina apresentaram redução significativa (12,8%) do escore tecidual em relação ao grupo controle, isento de Sanguinarina (Figura 2). Os principais achados histológicos foram dilatação de vasos linfáticos, edema de submucosa, atrofia de vilosidades e discreta quantidade de debris celulares (Figura 2). A dose de 2,0mg/L de Sanguinarina resultou na redução significativa da integridade morfológica do intestino de 16,94% em relação ao controle, devido às alterações como severa atrofia de vilosidades, desnudamento apical e diminuição do número de microvilosidades (Figura 2).

A identificação deste efeito negativo da dose de 2,0mg/L de Sanguinarina contrasta com os achados de outros trabalhos que atribuem à Sanguinarina ações antimicrobianas, anti-inflamatórias e imunomoduladoras marcantes (Lenfeld et al., 1981; Agarwall et al., 1997; Chaturvedi et al., 1997; Pickler et al., 2013). Robbins et al. (2013) destacam ainda o efeito da Sanguinarina sobre a integridade das células da barreira intestinal, com melhor taxa de resistência elétrica comparada com a tetraciclina.

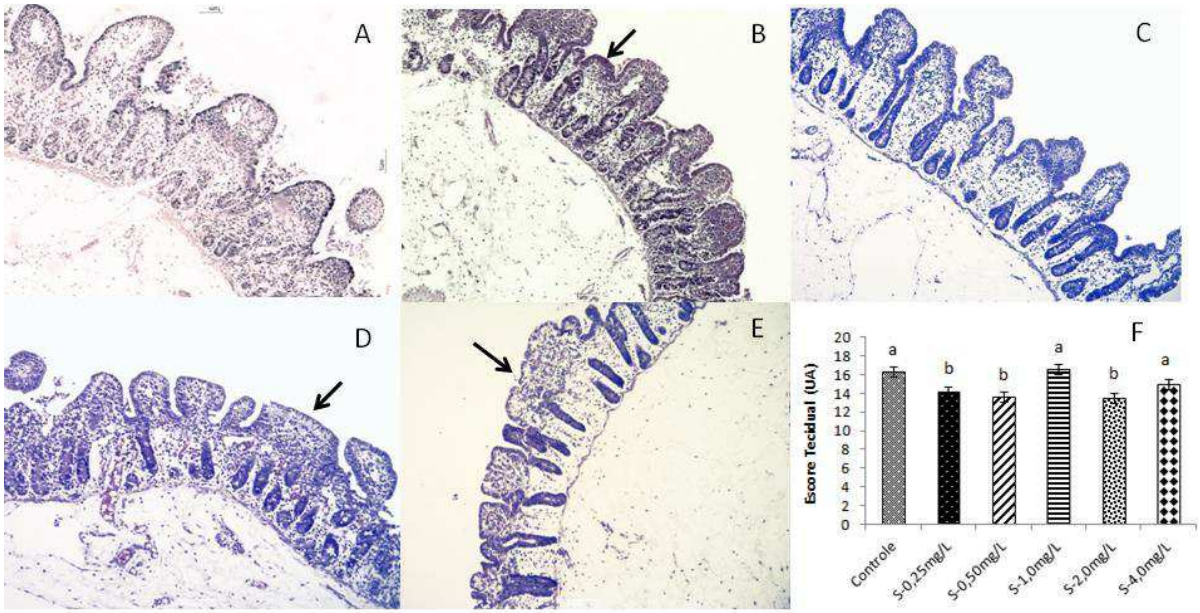

Figura 2 - Achados histológicos e escore tecidual dos explantes intestinais de suínos tratados com diferentes doses de Sanguinarina. (A) Controle. Aspectos histológicos normais; (B) Explantes tratados com 0,50 mg/L de Sanguinarina. Atrofia de vilosidades (seta); (C) Explantes tratados com 1,0 mg/L de Sanguinarina. Vilosidades com aspecto normal; (D) Explantes tratados com 2,0 mg/L de Sanguinarina. Atrofia e fusão de vilosidades (seta); (E) Explantes tratados com 4,0 mg/L de Sanguinarina. Desnudamento focal de ápice de vilosidades (seta). A a E, HE, objetiva 10x; (F) Escore tecidual dos explantes intestinais submetidos a diferentes doses de Sanguinarina (S) (0,25; 0,50; 1,0; 2,0 e 4,0 mg/L. UA=unidades arbitrárias. As médias estão representadas por barras verticais (*n* 4 animais). Os valores representam médias e desvio-padrão. Letras diferentes indicam significância estatística ($p<0,05$). Fonte: Da autora.

A avaliação morfométrica da altura das vilosidades mostrou redução significativa nos explantes tratados com 0,50 e 4,0 mg/L de Sanguinarina em relação aos grupos controle e tratados com 1,0 mg/L de Sanguinarina (Figura 3). Em relação à profundidade de criptas, não se observou diferença entre os tratamentos (Figura 4).

Após as avaliações histológicas para se determinar a dose de Sanguinarina a ser utilizada na sequência do trabalho, optou-se pelo uso da dose de 1,0 mg/L, uma vez seus resultados para o escore tecidual foram semelhantes aos da dose de 4,0 mg/L (Figura 2) e melhores que a dose de 2,0 mg/L para a altura das vilosidades (Figura 3).

Nesta dose pode-se atribuir o benefício da Sanguinarina na preservação das estruturas e funções intestinais, uma característica já demonstrada por Lenfeld et al. (1981) e Agarwall et al. (1991). No intestino do suíno esta ação está provavelmente relacionada ao efeito de contato da droga ao tecido e não à ação pós-absorção, uma vez que este alcalóide é excretado pelas fezes sob valores bastante altos (98%) conforme descreveram Kosina et al. (2004) e Psotova et al. (2006).

A manutenção do equilíbrio homeostático corpóreo está intimamente relacionada à capacidade do intestino em preservar sua função absortiva (Junqueira e Carneiro, 2011). Dessa maneira uma substância que auxilie na manutenção da altura das vilosidades está auxiliando também na homeostase intestinal e corpórea.

A má absorção ocorre principalmente quando há danos estruturais às células epiteliais, como em infecções bacterianas, virais ou por protozoários que ocasionam lise celular, que também pode ser causada pela forma física da ração (muito fina ou muito grossa) ou ingestão e ação de determinados produtos tóxicos (Zlotowski, et al., 2008).

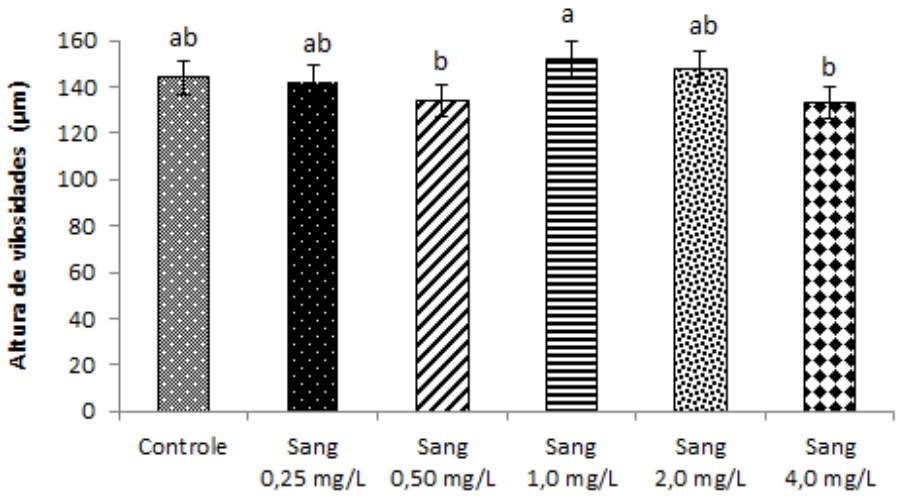

Figura 3 - Altura de vilosidades (µm) dos explantes intestinais de suínos tratados com diferentes doses de Sanguinarina (Sang) (0,25; 0,50; 1,0; 2,0 e 4,0 mg/L). As médias estão representadas por barras verticais (*n* 4 animais). Os valores representam médias e desvio-padrão. Letras diferentes indicam significância estatística (p<0,05). Fonte: Da autora.

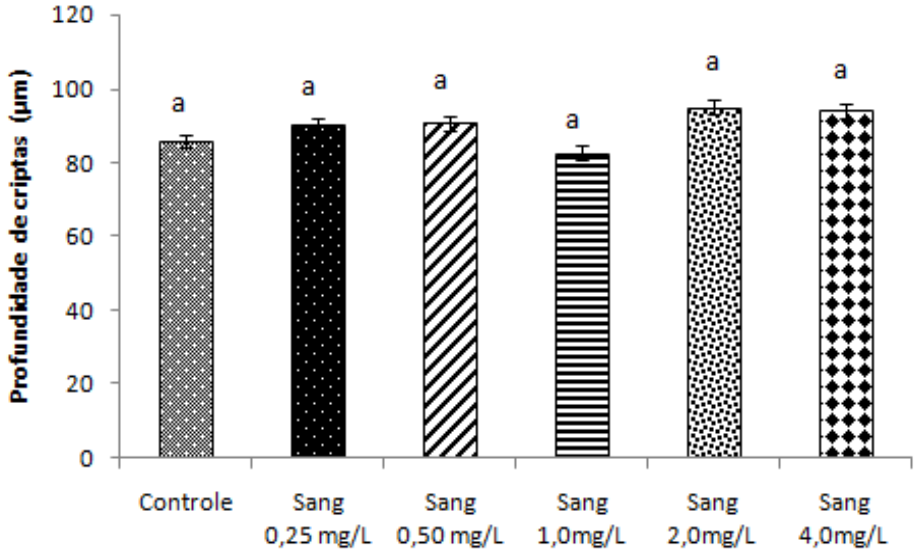

Figura 4 - Profundidade de criptas (µm) dos explantes intestinais de suínos tratados com diferentes doses de Sanguinarina (0,25; 0,50; 1,0; 2,0 e 4,0 mg/L). As médias estão representadas por barras verticais (*n* 4 animais). Os valores representam médias e desvio-padrão. Letras diferentes indicam significância estatística (p<0,05). Fonte: Da autora.

Na avaliação dos efeitos da Sanguinarina na presença ou não do DON, o grupo controle (isento do extrato herbal e da micotoxina) apresentou dilatação de vasos linfáticos, presença de debris celulares e edema de submucosa leves, e atrofia leve à moderada das vilosidades (Figura 5A). Os explantes expostos somente à Sanguinarina apresentaram discreta dilatação de vasos linfáticos e fusão de vilosidades, debris celulares e atrofia das vilosidades de maneira moderada (Figura 5B). A exposição somente à micotoxina desencadeou alterações como desnudamento e atrofia severa das vilosidades com presença acentuada de debris celulares e, consequentemente, a redução no número de vilosidades intestinais (Figura 5C), enquanto que a presença do DON associado à Sanguinarina proporcionou menores danos à integridade da mucosa intestinal, ocasionando alterações morfológicas acentuadas, porém com menor intensidade, como desnudamento apical e dilatação de vasos linfáticos moderada a severa, com degeneração celular, atrofia, fusão de vilosidades com achatamento dos enterócitos e redução no número de vilosidades (Figura 5D).

Esses achados histológicos corresponderam a uma redução de 10,09% nas funções de barreira do intestino no grupo Sanguinarina + DON, quando comparado com o grupo controle ($p<0.05$). Já a piora do escore dos explantes submetidos à incubação na presença de DON foi mais acentuada, ocasionando redução de 34,14% quando comparada ao controle (Figura 5E).

Os resultados evidenciam o papel da Sanguinarina sobre a manutenção e a melhora das estruturas intestinais, confirmando seu potencial de preservação da saúde intestinal (Lenfeld et al., 1981; Agarwal et al., 1997; Chaturvedi et al., 1997; Pickler et al., 2013).

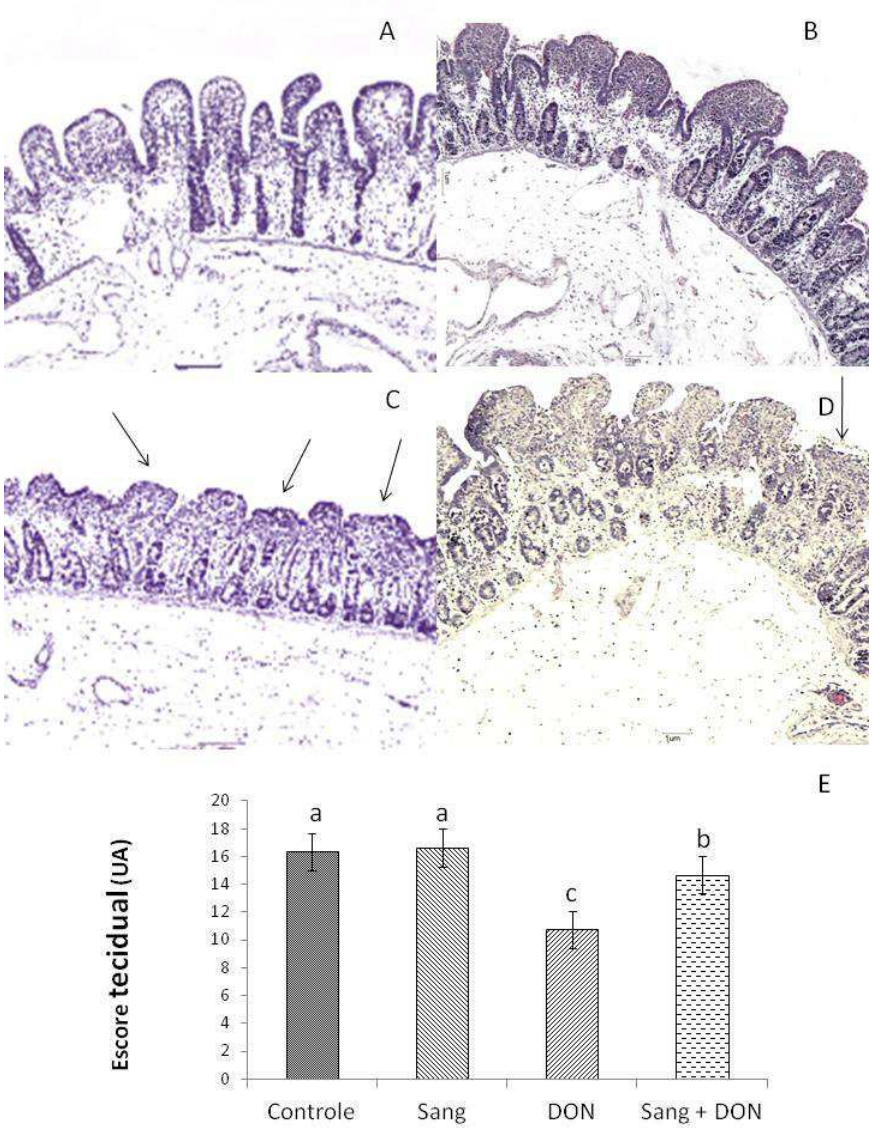

Figura 5 - Achados histológicos e escore tecidual dos explantes intestinais de suínos na presença de Sanguinarina (Sang) (1mg/L), DON (10µM) e Sanguinarina (1mg/L) + DON (10µM). (A) Controle. Aspectos histológicos normais; (B) Explantes tratados com Sanguinarina (Sang) (1mg/L) isoladamente. Aspectos histológicos normais. (C) Explantes expostos ao DON (10µM). Atrofia difusa de vilosidades. (D) Explantes tratados com Sanguinarina (1mg/L) e expostos ao DON (10µM). Desnudamento focal de ápice de vilosidades; (E) Escore tecidual dos explantes intestinais submetidos à Sanguinarina (1mg/L). DON (10µM) e Sanguinarina (1mg/L) + DON (10µM). UA=unidades arbitrárias. As médias estão representadas por barras verticais (*n* 5 animais). Os valores representam médias e desvio-padrão. Letras diferentes indicam significância estatística ($p<0,05$). UA=unidades arbitrárias. Fonte: Da autora.

A análise morfométrica dos explantes (Figura 6) demonstrou que o DON acarretou diminuição ($p<0,05$) da altura das vilosidades quando comparado aos grupos controle e tratados com a Sanguinarina, porém a presença do fitoterápico não controlou completamente os efeitos prejudiciais da micotoxina para este parâmetro. A redução na profundidade de criptas (Figura 7), observada no grupo exposto ao DON, foi de 22,6% quando comparada ao controle, e 23,4 % comparada à Sanguinarina ($p<0,05$). Os resultados do grupo tratado com Sanguinarina + DON não diferiram estatisticamente do grupo controle e do grupo exposto somente à DON, todavia apresentou uma melhora de 16,24% em relação ao último grupo.

Os resultados contradizem os achados de Pickler et al (2013), que verificaram diferença para altura das vilosidades intestinais de frangos submetidos a Sanguinarina via água (1,5 ppm).

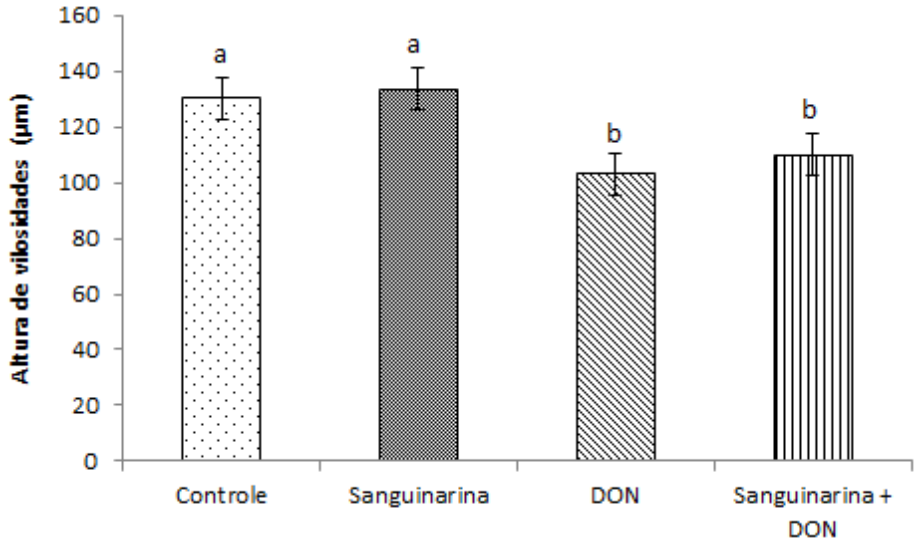

Figura 6 - Altura de vilosidades (µm) dos explantes intestinais de suínos na presença de Sanguinarina (Sang) (1mg/L), DON (10µM) e Sanguinarina (1mg/L) + DON (10µM). As médias estão representadas por barras verticais (*n* 5 animais). Os valores representam médias e desvio-padrão. Letras diferentes indicam significância estatística ($p<0,05$). Fonte: Da autora.

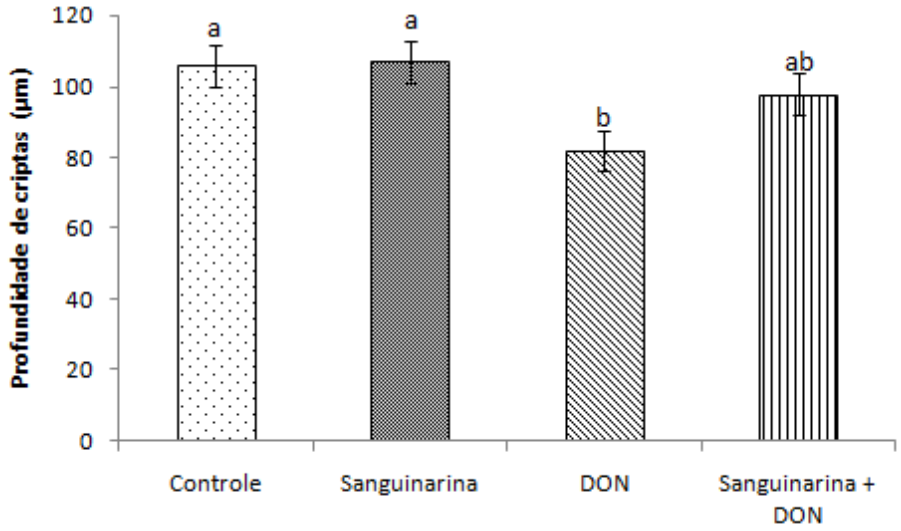

Figura 7 - Profundidade de cripta (µm) dos explantes intestinais de suínos na presença de Sanguinarina (Sang) (1mg/L), DON (10µM) e Sanguinarina (1mg/L) + DON (10µM). As médias estão representadas por barras verticais (*n* 5 animais). Os valores representam médias e desvio-padrão. Letras diferentes indicam significância estatística ($p<0,05$). Fonte: Da autora.

As vilosidades longas estão correlacionadas com a melhora na saúde intestinal, proporcionando melhor uniformidade e integridade da mucosa, além de resultar maior absorção de nutrientes, devido ao aumento da superfície de absorção (Santin et al., 2001; Baurhoo et al., 2007). Porém, a diminuição da altura das vilosidades, em decorrência do aumento na taxa de descamação epitelial, pode ser resultante do incremento da profundidade da cripta para assegurar a adequada taxa de *"turnover"* celular e garantir a reposição das perdas de células da região apical das vilosidades (Araújo et al., 2006; Oetting et al., 2006).

A atrofia dos vilos, normalmente observada no período pós-desmame, pode ser causada tanto pelo aumento na taxa de extrusão como pela diminuição da divisão celular nas criptas. Se o encurtamento do vilo ocorrer via aumento na taxa de extrusão,

então este estará associado a um aumento na proliferação celular da cripta e, geralmente, a um aumento de sua profundidade (Pluske, 2001).

A ocorrência de lesões intestinais em suínos tem sido amplamente relacionada com a exposição às altas concentrações da micotoxina DON nas rações (Bouhet e Oswald, 2005), sendo comuns as alterações na integridade do epitélio, com a penetração de patógenos e o desenvolvimento de processos inflamatórios no órgão (Oswald, 2006). Mesmo sendo consumidas em concentrações moderadas, as micotoxinas causaram impactos no desempenho do animal e levaram a alterações no sistema imunológico, resultando em danos aos órgãos internos (Weaver et al., 2013).

Estudos *in vitro* demonstraram que o DON ocasiona danos celulares e aumento de morte celular do epitélio intestinal associado ao estresse metabólico (Awad et al., 2012). Bracarense et al. (2012) relataram que a ingestão de dieta contaminadas com DON (3 mg /kg) tem efeitos adversos sobre trato intestinal de suínos em crescimento. Os autores observaram um aumento significativo no escore de lesões no jejuno e no íleo e diminuição na altura das vilosidades e no número de células caliciformes e linfócitos. Também verificaram que DON aumentou a expressão de mRNA de diferentes citocinas envolvidas na resposta inflamatória no jejuno e íleo, revelando a presença de inflamação ativa no intestino.

Antonissen et al. (2013), trabalhando com frangos de corte, mostraram efeito negativo do DON na morfologia e integridade do epitélio intestinal, sugerindo uma alteração da função de barreira deste órgão. Diversos estudos apontam tais efeitos deletérios do DON sobre a mucosa intestinal de suínos, especificamente de leitões desmamados (Kolf-Clauw et al., 2009; Grenier et al., 2011; Bracarense et al., 2012; Pinton et al., 2012b; Basso et al., 2013). Esses efeitos implicam em queda no consumo alimentar (Pestka, 2010; Pinton et al., 2010), em baixa absorção de nutrientes (Gartner e

Hiatt, 2007) e aumento da susceptibilidade à doenças ou reações inflamatórias (Maresca e Fantini, 2010; Moon, 2012), levando provavelmente à perdas no desempenho dos animais, como queda no ganho de peso, e consequentemente a prejuízos econômicos, visto que animais com deficiências absortivas devem ingerir maior quantidade de alimento para atender suas exigências.

Modelo *in vivo*

Quanto às análises realizadas nas porções do intestino delgado de suínos em fase de crescimento, os principais achados histológicos observados foram a atrofia de vilosidades, edema de lâmina própria e dilatação de vasos linfáticos. Os resultados dos escores teciduais (Figura 8A) indicam ausência de diferença ($P>0,05$) entre os tratamentos em relação ao escore tecidual e à altura de vilosidades (Figura 8B), identificando-se com os resultados de Vieira et al. (2008), que verificaram não haver influência da Sanguinarina ofertada via ração sobre as vilosidades intestinais de frangos. Os animais tratados com Sanguinarina apresentaram redução significativa ($p<0,05$) na profundidade das criptas em relação ao controle em todas as regiões avaliadas (Figura 8C), contrapondo-se às observações de Vieira et al. (2008). As criptas mais rasas para o grupo tratado com Sanguinarina indicam um intestino eficiente, com menor requerimento de nutrientes para renovação celular. Com diminuição na renovação celular, as células intestinais se tornam mais maduras e consequentemente a produção de enzimas digestivas e absorção de nutrientes se tornam melhores (Ibrahim, 2011). Alguns estudos apontam que o aumento de profundidade das criptas pode indicar alta atividade proliferativa celular, que geralmente ocorre como resposta do epitélio a alguma injúria da mucosa a fim de renovar as células do ápice das vilosidades que estão sendo perdidas (Furlan et al., 2004). Essa renovação, segundo Markovic et al. (2009),

requer energia e proteína, o que pode deprimir o crescimento e desenvolvimento de outros tecidos.

Os resultados sobre a morfometria intestinal, sem vantagens para os grupos experimentais, podem ser decorrentes do curto período de exposição dos animais à Sanguinarina, ou ainda pela forma como esta foi administrada (por gavagem), cuja ação, segundo Kosina et al. (2004), se dá pelo contato com a superfície intestinal, demandando uma contínua presença do produto para a expressão de seus efeitos. Khadem et al. (2014), trabalhando com frangos de corte tratados com Sanguinarina, atribuíram que seus efeitos para a maioria dos parâmetros medidos (morfometria intestinal e de desempenho) foram claramente dose dependentes.

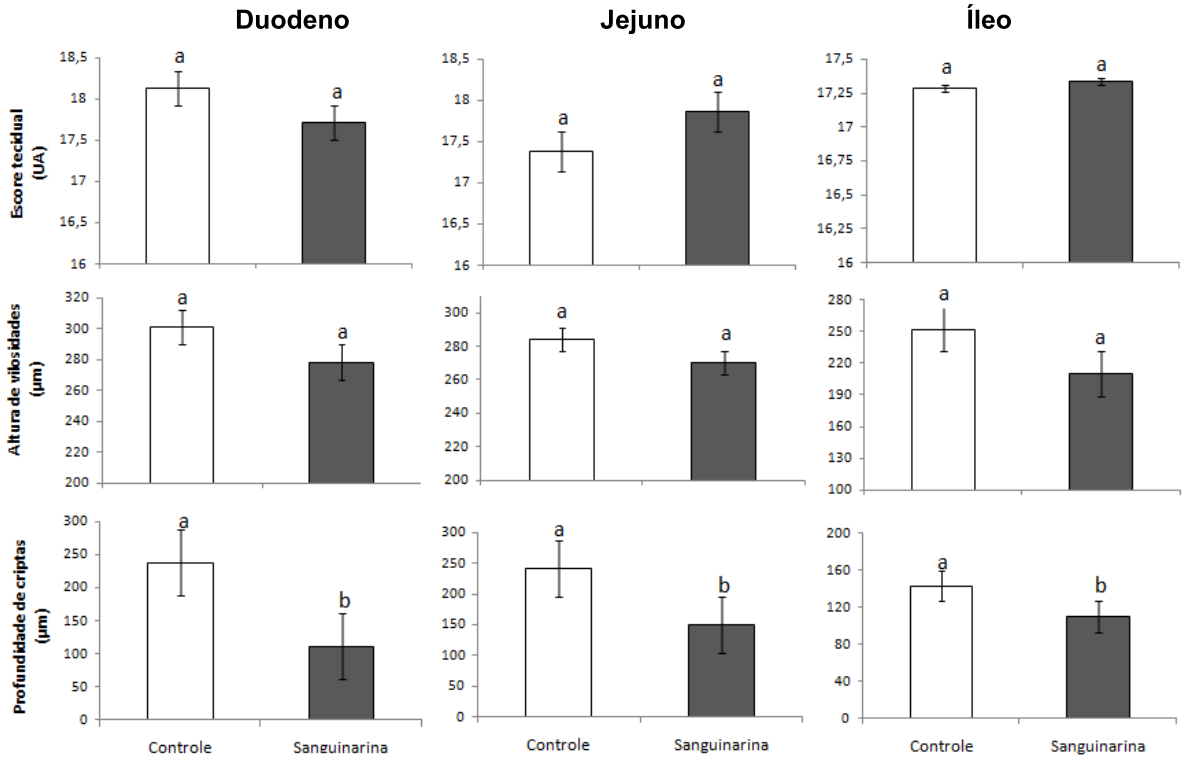

Figura 8 - Escore tecidual (UA), altura de vilosidades (µm) e profundidade de criptas (µm) em fragmentos de duodeno, jejuno e íleo dos suínos submetidos aos tratamentos Controle (□) e Sanguinarina (■). As médias estão representadas por barras verticais (*n* 24 animais). Os valores representam médias e desvio-padrão. Letras diferentes indicam significância estatística ($p<0,05$). UA=unidades arbitrárias. Fonte: Da autora.

Os resultados da quantificação das imunoglobulinas séricas (Figura 9) não indicaram diferença ($p>0,10$) para as concentrações de IgG, e IgM entre os tratamentos. Contudo o nível de IgA foi significativamente maior ($p<0,10$) no soro dos animais tratados com Sanguinarina. A IgA controla o crescimento de bactérias comensais e impede sua adesão às células epiteliais intestinais (IECs) (Mestecky et al., 1999; Fagarasan et al., 2002; Macpherson e Uhr, 2004). Essa ação faz com que a preservação das estruturas do epitélio intestinal seja mantida, podendo diminuir os processos inflamatórios, favorecendo o desempenho do animal. O efeito positivo da Sanguinarina na secreção de IgA pode ser um dos mecanismos que confere à substância propriedades anti-inflamatórias e antimicrobianas (Lenfeld et al., 1981; Agarwall et al., 1991; Pickler et al., 2013).

Neste sentido, Khadem et al. (2014), trabalhando com a cultura de jejuno de frangos de corte submetidos à oxitetraciclina e Sanguinarina, verificaram que ambos os produtos reduziram significativamente a expressão do óxido nítrico sintetase na mucosa do jejuno, indicando sua consistente ação anti-inflamatória e promotora do crescimento.

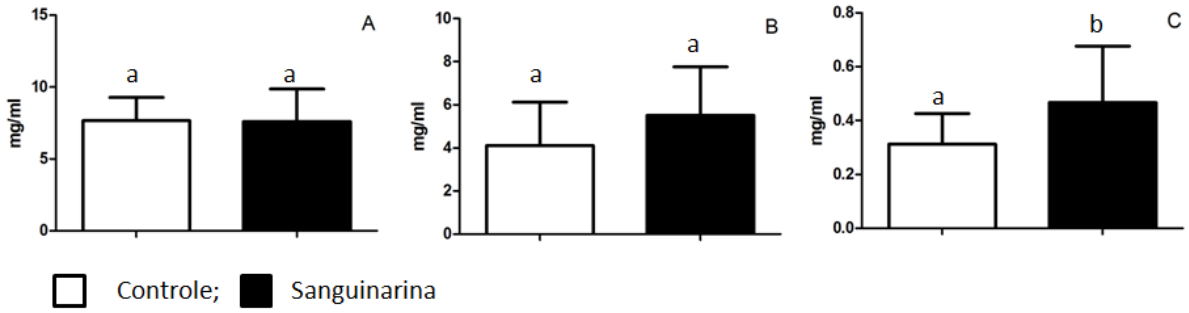

Figura 9 - Quantificação (médias e desvio-padrão) das imunoglobulinas IgG (A), IgM (B) e IgA (C) do soro de suínos submetidos aos tratamentos Controle (□) e Sanguinarina (■). As médias estão representadas por barras verticais (*n* 24 animais). Letras diferentes indicam significância estatística (p<0,10). UA=unidades arbitrárias. Fonte: Da autora.

Conclusões

A Sanguinarina na dose de 0,65 μM resultou em 50% de mortalidade das células IPEC. Explantes expostos a concentrações de 1,0 mg de Sanguinarina/L apresentaram melhor preservação da morfologia intestinal, com efeitos indicativos de prevenção dos danos à integridade da mucosa quando estes foram expostos ao DON. O uso da Sanguinarina *in vivo* resultou num melhor perfil das estruturas das criptas do intestino e aumento do IgA sérico, ratificando sua ação positiva sobre a morfometria intestinal.

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5 CONCLUSÃO GERAL

As respostas à presença da Sanguinarina em explantes de jejuno de leitões foram diferentes das encontradas nas avaliações realizadas nas porções do intestino delgado de suínos em crescimento. Em explantes, a Sanguinarina (Sangrovit®) associada ao DON conseguiu manter as condições de absorção das células epiteliais intestinais próximas ao normal por meio de melhoras no escore lesional, porém com moderada efetividade. A participação da Sanguinarina (Sangrovit®) não exerceu influência sobre a mucosa intestinal de suínos na fase de crescimento, porém aumentou a secreção da imunoglobulina A (IgA).

A manutenção das funções de absorção e de barreira do intestino exposto à Sanguinarina não foi alcançada de maneira eficaz, sendo necessários estudos posteriores para se verificar o mecanismo de ação deste alcaloide.

ANEXOS

ANEXO 1

Artigo publicado em colaboração na revista *PLoSOne*

Deoxynivalenol as a New Factor in the Persistence of Intestinal Inflammatory Diseases: An Emerging Hypothesis through Possible Modulation of Th17-Mediated Response.

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Deoxynivalenol as a New Factor in the Persistence of Intestinal Inflammatory Diseases: An Emerging Hypothesis through Possible Modulation of Th17-Mediated Response

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Abstract

Background/Aims: Deoxynivalenol (DON) is a mycotoxin produced by *Fusarium* species which is commonly found in temperate regions worldwide as a natural contaminant of cereals. It is of great concern not only in terms of economic losses but also in terms of animal and public health. The digestive tract is the first and main target of this food contaminant and it represents a major site of immune tolerance. A finely tuned cross-talk between the innate and the adaptive immune systems ensures the homeostatic equilibrium between the mucosal immune system and commensal microorganisms. The aim of this study was to analyze the impact of DON on the intestinal immune response.

Methodology: Non-transformed intestinal porcine epithelial cells IPEC-1 and porcine jejunal explants were used to investigate the effect of DON on the intestinal immune response and the modulation of naive T cells differentiation. Transcriptomic proteomic and flow cytometry analysis were performed.

Results: DON induced a pro-inflammatory response with a significant increase of expression of mRNA encoding for IL-8, IL-1α and IL-1β, TNF-α in all used models. Additionally, DON significantly induced the expression of genes involved in the differentiation of Th17 cells (STAT3, IL–17A, IL-6, IL-1β) at the expenses of the pathway of regulatory T cells (Treg) (FoxP3, RALDH1). DON also induced genes related to the pathogenic Th17 cells subset such as IL–23A, IL-22 and IL-21 and not genes related to the regulatory Th17 cells (rTh17) such as TGF-β and IL-10.

Conclusion: DON triggered multiple immune modulatory effects which could be associated with an increased susceptibility to intestinal inflammatory diseases.

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Introduction

Mycotoxins are fungal secondary metabolites that commonly contaminate human food and animal feed. Given their global and frequent occurrence, their stability through the food processing chain [1] as well as their known toxic effects, mycotoxins have become a major concern in Europe and North America. Their effects on humans and domestic animals range from decreased resistance to infectious diseases and growth impairment, to cancer induction and death [2,3,4].

Deoxynivalenol (DON) is a mycotoxin of the trichothecenes family mainly produced by *Fusarium graminearum* and *F. culmorum*. Although it is not the most acutely toxic trichothecene, DON is regarded as an important food safety issue since it is the most prevalent trichothecene in wheat, corn, barley and their by-products in Europe and North America [5,6]. Toxicity of DON has been largely demonstrated for humans and all animal species tested [7] but swine, which are readily exposed to this toxin through their cereal-rich diet, are the most sensitive [8]. Therefore they make an excellent research model for animal and even human exposure to DON due to higher similarities concerning the digestive and the immune system than other animal models [9,10]. Acute exposure to high doses of DON results in diarrhea,

vomiting, leukocytosis, gastrointestinal hemorrhage and ultimately death whereas chronical exposure to low doses of this toxin induces anorexia, reduced weight gain and altered nutritional efficiency [7].

This toxin specifically targets dividing cells such as intestinal epithelial cells and immune cells [11]. It can alter the expression of transcription factors by readily binding to the ribosomes and rapidly activating mitogen-activated protein kinases (MAPKs), thus possibly affecting cytokines production and membrane receptors [12].

The intestinal tract is the first physical barrier against food contaminants, chemicals and intestinal pathogens and as such, it plays an important role in the regulation of the immune response to these intrusions. Previous studies have demonstrated that intestinal absorbance of DON takes mainly place in the jejunum both directly from the intestinal lumen to the apical side of the intestinal epithelium and also indirectly through already absorbed toxin reaching the intestinal epithelial cells (IECs) from the blood stream to the basolateral side [13]. Low doses of this toxin can induce morphological and histological impairments of IECs such as atrophy and fusion of the villi [14,15]. It can also decrease the number of goblet cells [14] and alter the intestinal permeability by repressing the expression of tight junction proteins such as claudins 3-4, ZO-1 and occludin [14,16,17,18]. Basolateral exposure to DON recently showed to have a more severe impact on the intestinal barrier integrity and DNA fragmentation than apical exposure [19].

As a defense mechanism, the intestinal epithelial cells are able to produce several pro-inflammatory cytokines such as the tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6 and different chemokines such as IL-8 and the CC-chemokine ligand (CCL)-20 that are crucial for the recruitment and activation of the underlying immune cells of the *lamina propria* [20]. IECs also have a particularly close interaction with dendritic cells (DCs) that play an essential role in the initiation of the adaptive immune response mediated for the most part by $CD4^+$ T helper (Th) cells. Depending on the local intestinal environment, different populations of DCs shape the immune response by activating naive T cells and inducing their differentiation into specific effector T cell populations [21]. Against intracellular infections, Th1 cells are induced to secrete interferon-gamma (IFN-γ) and IL-12 and regulate cellular immunity. DCs are also strongly implicated in the maintenance of tolerance by inducing T regulatory cells (Tregs) that secrete IL-10 and the tumor growth factor beta (TGF-β). Another subset of T helper lymphocytes, the Th17, was recently described as an important mediator of mucosal immunity, defense against extracellular pathogens and autoimmunity [22]. Interestingly, Th17 cells have been related to both pathogenic and regulatory responses, which are generated by two distinct populations of Th17 with a common signature cytokine, IL-17A [23,24]. The dichotomy of Th17 cells results from different stimuli: IL-6, IL-1β and IL-23 promote the expression of pro-inflammatory cytokines by the so-called pathogenic Th17 whereas IL-6 and TGF-β restrain the pathogenic potential of these cells by inducing the production of IL-10 by regulatory Th17 (rTh17) [23,24]. The same plasticity allows induced Tregs to reverse their phenotype and acquire the functions of pathogenic Th17 when cultured with IL-6 [25]. Plasticity of T lymphocytes ensures the adequacy of the immune response to an offensive situation and is critical to maintain the balance between protection and homeostasis. All of this relies on a well-established communication between IECs, DCs and the different T helper cell subsets. The damages caused to the intestinal epithelium by exposure to DON could disrupt these interactions and thus lead to severe disturbances of the intestinal immune system. Taken together, all of this could lead to persistent tissue inflammation and eventually, to chronic intestinal inflammatory diseases such as intestinal bowel disease (IBD) characterized by a dysfunctional intestinal barrier and autoimmune responses. DON could play a role in the induction and/or persistence of such inflammatory diseases [26]. However it is noteworthy that few publications have paid attention to the intestinal effects of DON, and even less to the effect of DON on the above mentioned balance between T cells populations.

The aim of this study was to analyze the influence of a low but relevant concentration of DON on the cytokines levels produced by the different subsets of intestinal T lymphocytes using three methodological approaches: *in vitro*, a non transformed porcine intestinal epithelial cell line (IPEC-1) [16,17] and e*x vivo* porcine intestinal explants [27].

Materials and Methods

Chemicals

Purified DON stock (Sigma Aldrich, Ayshire, UK) was dissolved in dimethylsulfoxide (DMSO) and stored at −20°C before dilution in complete cell culture media. Control samples were treated with equivalent concentrations of DMSO, which were non cytotoxic.

Treatment of the Intestinal Epithelial Cell Line IPEC-1

The Intestinal Porcine Epithelial cell line (IPEC-1) was derived from the small intestine of a newborn unsuckled piglet [28]. IPEC-1 cells are capable of differentiating into mature enterocytes to form a uniform and polarized epithelial layer that suitably mimics the intestinal epithelial barrier and its apico-basolateral discrimination. They have been largely previously used to study bacterial infections, intestinal epithelial integrity, and trans-epithelial transport [18,29,30].

IPEC-1 cells were grown and differentiated as previously described [31]. Briefly, cells were seeded into 4.2 cm^2 polyethylene terephtalate membrane inserts with 0.4 µm pore size (Beckon Dickinson, Pont de Claix, France) at 2 x 10^5 cells per well in 2 ml of growth media. Treatments were applied to the apical compartments of the inserts.

IPEC-1 cells were incubated for 1.5 h, 4 h, 8 h, 12 h or 24 h in the presence of 10 µM of DON or DMSO at 39°C in a humidified atmosphere of 5% CO_2. After treatment, supernatants were collected to evaluate cytokine production and cells were harvested for transcriptional analysis.

Treatment of Explants Cultures

All animal experiments were carried out in accordance with European Guidelines for the Care and Use of Animals for Research Purposes. Jejunal tissue was obtained from six piglets which were 5 week-old, 7–days after weaning. Animals were fed ad libitum prior to the experiment. A 5 cm middle jejunum segment was collected in pre-warmed Williams media (Sigma) supplemented with 200 U/mL penicillin and 200 µg/mL streptomycin (Eurobio, Courtaboeuf, France). It was washed twice and opened longitudinally. Then, the external *tunica muscularis* was removed and explants were made with punch trocards (centravet, Lapalisse, France) and were placed in Williams culture media supplemented with 1% of penicillin/streptomycin, 0.5% of gentamycine (Eurobio), 4.5 g/L of glucose (Sigma), 10% FBS (Eurobio) and 30 mM of amino acid (Ala/Glu) (Eurobio). Pig jejunal explants were incubated with 10 µM of DON or DMSO for 4 h, 8 h or 12 h at 39°C in a humidified atmosphere of 5% CO_2. After treatment, tissues were collected for transcriptional analysis.

IL-8, IL-1 Alpha and IL-17 Cytokine Assays

Concentrations of IL8 and IL-1 alpha were measured by enzyme linked immuno-absorbent assays (ELISA) using specific kits for porcine IL8 and IL-1 alpha (R&D Systems, Minneapolis, MN, USA). Plates were washed with PBS/Tween 20 and then blocked with PBS containing 1% BSA (w/v) for 1 h at room temperature (RT). Supernatant samples were added to the ELISA plate in duplicate and incubated for 2 h at RT. After washing, wells were incubated with biotinylated specific detection antibodies for porcine IL8 and porcine IL1-alpha (R&D Systems) for 2 h at RT. Then, streptavidin-HRP-conjugated antibody (Thermo Fisher Scientific, Courtaboeuf, France) was added for 30 min at 37°C. Positive reactions were revealed by 3,3′,5,5′-tetramethylbenzidine (TMB) (Thermo Fisher Scientific) and reactions were stopped with H_2SO_4 2 N. The optical density (OD) was read at 450 nm.

Cytokine IL-17 was measured using the swine IL-17A VetSet™ ELISA Development kits (Kingfisher biotech, St.Paul, MN, USA).

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

Quantitative real-time PCR (qPCR) was performed to determine the relative mRNA expression levels of chemokines, interleukins, chemokines receptors, enzymes, and several transcription factors involved in the modulation of the intestinal immune response. Total RNA from IPEC-1 cultures and jejunal explants was extracted with Trizol Reagent (Extract all, Eurobio). RNA concentration, integrity and quality were determined spectrophotometrically using Nanodrop ND1000 (Labtech International, Paris, France). RIN of these mRNA fluctuated between 6.80 and 4.70 for 4 hours and 12 hours of culture times, respectively. Then, reverse transcription and real-time qPCR steps were performed as previously described [32]. Non-reverse transcripted RNA was used as non-template control for verification of genomic DNA amplification signal. Specificity of qPCR products was assessed at the end of the reactions by analyzing dissociation curves. Primers were purchased from Invitrogen (Invitrogen, Life Technologies Corporation, Paisley, UK). Specific sequences were specified in tableô 1. Amplification efficiency and initial fluorescence were determined by the ΔΔCt method, then obtained values were normalized using two reference genes, ribosomal protein L32 (RPL32) and cyclophilline A [33]. Stability of these genes was demonstrated previously in intestinal tissues [34]. mRNA expression levels were expressed relative to the mean of the control group at 4 h of exposure to DMSO alone.

Analysis of T Regulatory Cells Phenotype in Lamina Propria Cultured With DON

Leucocytes were isolated from jejunal lamina propria harvested from two pigs by collagenase digestion as described previously [35]. Briefly, pieces of small intestine were incubated for 2 times 45 min in several changes of calcium and magnesium-free Hank's balanced salt solution (HBSS), containing 1 mM of ethylenediamine tetra-acetic acid (EDTA) at 37°C on a shaking platform. This procedure largely removed the epithelial layer. The remaining tissue was then digested in RPMI-1640 containing 0.36 g/ml of collagenase (Collagenase Grade V, Sigma), 10% of FBS, 20 mM of Hepes (Eurobio, Paris, France) and 2000 U/ml of Dnase1 (Roche diagnostics, Mannheim, Germany) at 37°C for 1 h on a shaking platform. Cells were suspended in a 40% Percoll in RPMI solution and underlaid with a 80% Percoll solution (Pharmacia, Uppsala, sweden). Low-density cells were recovered from the 40%/80% gradient interface. Cell suspensions isolated from lamina propria were cultured with different concentration of DON: 0.1 to 10 mM for 48 h at 39°C.

Following 2 days of culture, lymphocytes were evaluated for their expression of CD4, CD25, and FoxP3 using flow cytometry. Briefly, cells were first stained with mouse anti-porcine CD4 (clone MIL17, ACRIS, Herford, Germany) and mouse anti-porcine CD25 (AbD Serotec, Colmar, France). As secondary antibodies isotype-specific fluorochrome- or biotin-labelled goat anti-mouse antibodies (αIgG1-Alexa 488, αIgG2b-biotin (Invitrogen) were used. Biotin-labelled secondary antibodies were further marked with APC-Cy7 streptavidin conjugate (Invitrogen). For intracellular staining, cells were permeabilized with a FoxP3 permeabilization/fixation buffer kit followed by staining with anti-mouse/rat FoxP3-PE that reacts with porcine FoxP3 (eBioscience Inc., San Diego, CA). Flow cytometric analysis was conducted on the lymphocytes using a MACSQuant analyser (Miltenyi biotech, Paris, France) and Venturi solfware. Dendritic cells were excluded based on forward and side scatter.

Statistics

After checking of normal distribution of data, we performed Fisher test and Student's t-test to compare values between multiple groups. The statistical analysis of the data was carried out with Statview software (Statistical Analysis System; SAS for Windows 98; SAS Institute Inc., Cary, NC, USA). Differences were considered to be statistically significant when the p-value was lower than 0.05.

Results

DON potentiated the expression of immune genes and increased protein concentration in differentiated IPEC-1 cells in a time-dependent manner.

We initially aimed to investigate the effects of DON on gene expression and protein production involved in the inflammatory immune response. Characterization of DON immune modulatory effects was first assessed by mRNA expression analysis in intestinal epithelial cells (IPEC-1) (Fig.ô 1). In untreated cells, no statistical difference in mRNA expression was measured between the different times of exposure, reflecting the absence of spontaneous inflammation caused by extended culture time. Exposure to DON significantly increased the expression of pro-inflammatory and regulatory cytokines, chemokines and of the enzyme RALDH (Fig.ô 1A). Early up-regulation in response to DON treatment was observed for IL-8, IL-1α and CCL20. Expression of these genes reached a maximum level after 4 h of DON exposure (100, 14 and 18 fold increase, respectively). mRNA expression levels of these three cytokines decreased in the course of time but remained significantly up-regulated until 24 h of DON exposure. These responses were correlated with IL-8 and IL-1α protein levels which were significantly up-regulated after 8 h of DON exposure (Fig.ô 1B). In addition to these early expressed genes, TGF-β, CX3CL1 and RALDH1 were also significantly increased but only after 8 h of exposure to DON (Fig.ô 1A).

Differentiated IPEC-1 cells were cultured in presence (black squares/bars) or absence (white diamonds/gray bars) of 10 μM of DON for 1.5 h, 4 h, 8 h, 12 h or 24 h. Gene expression was analyzed by RT-qPCR and protein levels by ELISA. Gene expressions were normalized by the mean of two reference genes (Cyclophilin A and RPL32). Data are presented as means +/− SEM of values obtained with three independent experiments. mRNA values are expressed relative to the control group at 4 h. Asterisks denote significant differences between groups: * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Table 1. List of genes, primers sequences (F: Forward; R: Reverse) and accession numbers and references.

Gene Symbol	Gene name	Primer sequence	References
IL1-α	Interleukin 1 - alpha	F: TCAGCCGCCCATCCAA	NC_010445.3
		R: AGCCCCGGTGCCATGT	
IL1-β	Interleukin 1 - beta	F: GAGCTGAAGGCTCTCCACCTC	NM_001005149
		R: ATCGCTGTCATCTCCTTGCAC	
TNF-α	Tumor necrosis factor - alpha	F: ACTGCACTTCGAGGTTATCGG	NM_214022
		R: GGCGACGGGCTTATCTGA	[32]
IL6	Interleukin 6	F: GGCAAAAGGGAAAGAATCCAG	NM_214399
		R: CGTTCTGTGACTGCAGCTTATCC	[68]
IL8	Interleukin 8	F: GCTCTCTGTGAGGCTGCAGTTC	NM_213867
		R: AAGGTGTGGAATGCGTATTTATGC	[14]
IL10	Interleukin 10	F: GGCCCAGTGAAGAGTTTCTTTC	NM_214041
		R: CAACAAGTCGCCCATCTGGT	[14]
IL12-p40	Interleukin 12 p40	F: GGTTTCAGACCCGACGAACTCT	NM_214013
		R: CATATGGCCACAATGGGAGATG	
IL17α	Interleukin 17 - alpha	F: CCAGACGGCCCTCAGATTAC	AB102693
		R: CACTTGGCCTCCCAGATCAC	[69]
IL21	Interleukin 21	F : GGCACAGTGCCCCATAAATC	MN_214415
		R: GCAGCAATTCAGGGTCCAAG	[70]
IL22	Interleukin 22	F: AAGCAGGTCCTGAACTTCAC	AY937228
		R: CACCCTTAATACGGCATTGG	[69]
IL23A	Interleukin 23 - alpha	F: GAGAAGAGGGAGATGATGAGACTACA	[66]
		R: GGTGGATCCTTTGCAAGCA	
TGF-β	Transforming growth factor - beta	F: GAAGCGCATCGAGGCCATTC	X54859
		R: GGCTCCGGTTCGACACTTTC	[69]
CCL20/MIP3 alpha	Chemokine (CCL20)	F: GCTCCTGGCTGCTTTGATGTC	NM_001024589
		R: CATTGGCGAGCTGCTGTGTG	[66]
IFN-γ	Interferon - gamma	F: TGGTAGCTCTGGGAAACTGAATG	NM_213948
		R: GGCTTTGCGCTGGATCTG	[66]
ROR-γ like	Nuclear receptor ROR-gamma-like	F: CCTGGCCCTGGGCATGT	NC_010446.3
		R: TGTTCTAGCAGCGTCCGAAGT	
FoxP3	Forkhead box P3	F: GGTGCAGTCTCTCTGGAACAA	AY669812
		R: GGTGCCAGTGGCTACAATAC	[66]
CX3CL1/Fractalkine	Chemokine (CX3CL1)	F: GCAGCTCCTAGTCCATTAC	EST CK464144
		R: CACCATTCTGACCCAAAG	[66]
XCR1	Chemokine (XCR1)	F: TCTTCTGCAAGCTTCTCAACATC	[66]
		R: GGCTGACCACGGACAGGTA	
CCR6	Chemokine (CCR6)	F: CCTGCACTGCTGCCTCAA	[71]
		R: TTCAGAAAGTAGCTCCGGAA	
STAT3	Signal transducer and activator of transcription 3	F: TGCAGCAGAAAGTGAGCTAC	MN_001044580
		R: CCGGTCTTGATGACTAATGG	[22]
RALDH1	Retinaldehyde deshydrogenase 1	F: TGGAGTGTGTGGCCAGATCA	N–010460–3
		R: GCAGGCCCTATCTTCCAAATG	[72]
T-bet	T-cell beta chain Th17-Th22 like	F: TTTGTGGCCTTTTGCATCCT	Present study
		R: CCTGTGTTTGTGATCTTGTTCCTT	
Cyclo A	Cyclophilin A	F: CCCACCGTCTTCTTCGACAT	MN_214353
		R: TCTGCTGTCTTTGGAACTTTGTCT	[22]
RPL32	Ribosomal Protein L32	F: AGTTCATCCGGCACCAGTCA	MN_001001636
		R: GAACCTTCTCCGCACCCTGT	[16]

doi:10.1371/journal.pone.0053647.t001

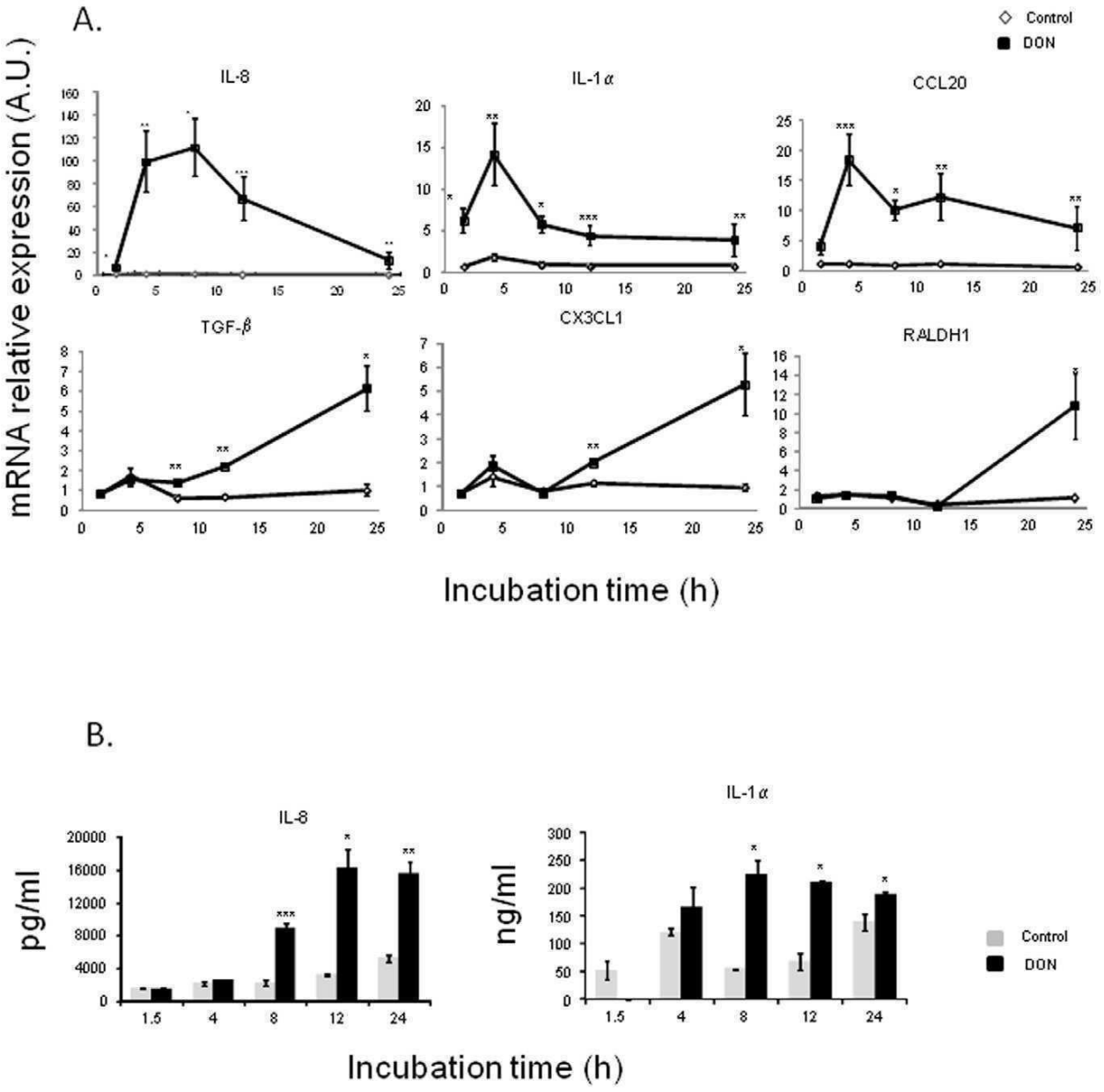

Figure 1. Deoxynivalenol induced cytokines and chemokines mRNA relative expression (A) and protein concentration (B) in IPEC-1 cells.
doi:10.1371/journal.pone.0053647.g001

DON increased relative mRNA Expression Levels of Both pro-Inflammatory Cytokines and DC-Recruiting Chemokines in an *ex vivo* Model of Porcine jejunal Explants

In order to get an insight on the impact of DON on the cross-talk between epithelial cells and immune cells and its impact on the emergence of the mucosal immune response, porcine jejunal explants were exposed to 10 µM of DON. After 4 h, DON exposure induced an up-regulation of mRNA expression of pro-inflammatory cytokines (IL-8, IL-1α and TNF-α) as well as DC-recruiting chemokines (CCL20, CCR6, CX3CL1 and XCR1) (Fig.ô 2). Concerning pro-inflammatory cytokines, two different kinetics appeared again. The increase of both IL-8 and IL-1α was progressive in a time dependent manner and these cytokines reached their highest expression levels after 12 h (12 and 13 fold induction, respectively) (Fig.ô 2A). On the contrary, the significant up-regulation of TNF-α kept more or less steady along the different time points. An additional experiment involving jejunal loops of a 5 week-old pig *in vivo* to 10 µM of DON, suggested a decrease in the expression of these pro-inflammatory cytokines after 24 h of exposure (Fig. S1).

The expression of DC-recruiting chemokines was also measured in our explants model to analyze the effect of DON on CCL20/CCR6-mediated signals which can induce chemotaxis of CCR6-

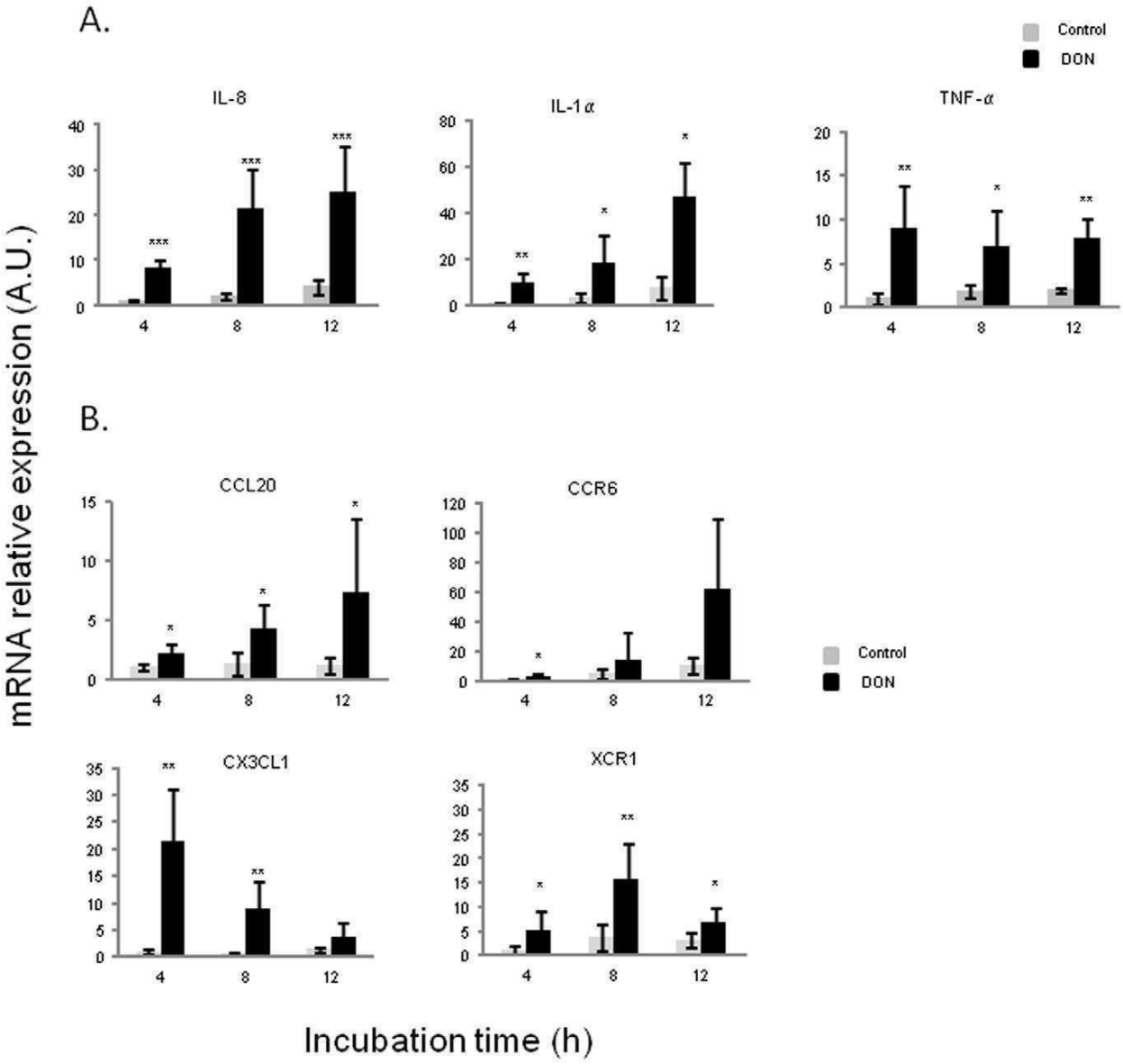

Figure 2. Deoxynivalenol up-regulated pro-inflammatory cytokines (A) and DCs-recruiting chemokines mRNA relative expression (B) in jejunal explants.
doi:10.1371/journal.pone.0053647.g002

expressing DCs and macrophages to sites of tissue damage, driving preferentially the differentiation of naive T-cells towards Th1 or Th17 (Fig.ô 2B). The exposure of jejunal explants to DON significantly increased the expression of CCL20 and CCR6 with a peak after 12 h of exposure (7 and 12 fold induction, respectively). Secondly, we analyzed the expression of CX3CL1 which is known to inhibit Th1 and Th17 differentiation. Opposite to CCL20 and CCR6, CX3CL1 exhibited a maximum peak at 4 h (22 fold induction) and decreased progressively after 8 h and 12 h of DON exposure. XCR1, which is also implicated in DC recruitment, was significantly induced by DON at all exposure times but its maximum level was reached at 8 h of DON exposure (7 fold increase). The expression of both CX3CL1 and XCR1 decreased after their maximal induction peak (after 4 h and 8 h respectively) and this tendency was maintained in the loops experiment after 24 h of exposure (Fig. S1).

Explants were exposed or not with 10 μM of DON for 4 h, 8 h or 12 h. Gene expressions were evaluated by RT-qPCR and normalized with two reference genes (Cyclophilin A and RPL32). Data are presented as means +/− SEM of values obtained with intestinal explants from six different piglets and expressed relative to the control group at 4 h. Asterisks denote significant differences between untreated (gray bars) and DON-treated explants (black bars): * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

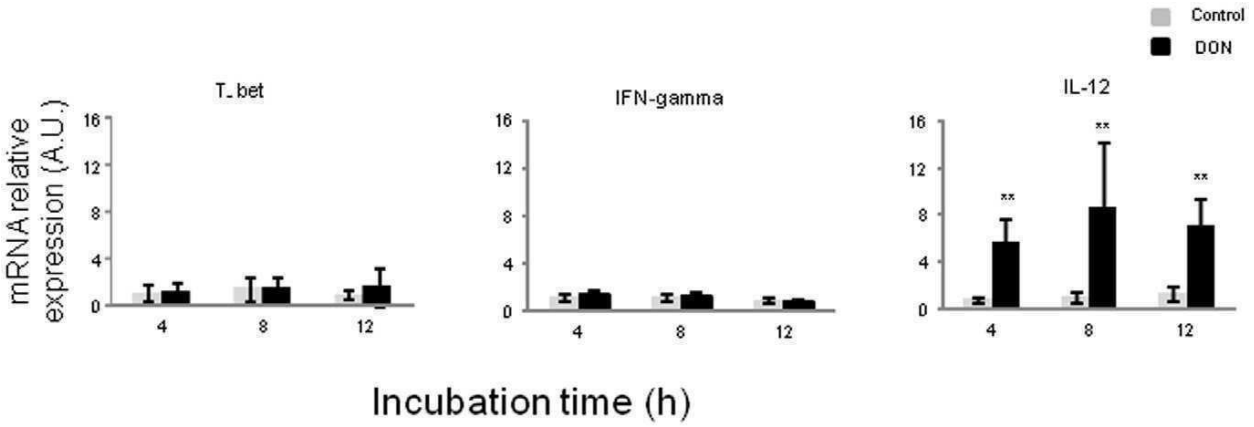

Figure 3. Deoxynivalenol increased IL-12 but not IFN-γ gene expression in jejunal explants.
doi:10.1371/journal.pone.0053647.g003

DON Triggered Th17 Signature genes in Porcine jejunal Explants

In order to examine the effects of DON on naive Th cells differentiation, we analyzed mRNA expression levels of transcription factors and cytokine genes related to Th1 cells (Fig.ô 3), Th17 cells (Fig.ô 4) and Treg cells (Fig.ô 5). DON treatment did not affect expression of T-bet transcription factor nor IFN-γ mRNA levels (Fig.ô 3). Significant increase of IL-12 expression levels was observed at all exposure times (between 6 and 8 fold induction) (Fig.ô 3). DON treatment also induced a time-dependent increase of expression of mRNA encoding for IL-17 A, IL-6 and STAT3 (Fig.ô 4A). Accordingly the protein expression of IL-17A was significantly increased after 12 h of exposure to DON (Fig.ô 4B). IL-1β expression levels were also significantly up-regulated but its levels did not did not vary much in a time dependent manner (16 to 12 fold induction) (Fig.ô 4A). Surprisingly, RORγ-like, an important Th17 transcription factor was negatively regulated by DON treatment (Fig.ô 4A). A different expression profile was observed in genes related to Treg cells. FoxP3 and RALDH1 were not affected by DON treatment after 4 h and 8 h of culture and they were down regulated after 12 h of toxin exposure (Fig.ô 5A). This decrease of Treg cells was also found when analyzing the effect of DON on $CD4^+$ $CD25^+$ $FoxP3^+$ leukocytes extracted from the intestinal lamina propria (Fig.ô 5B). 10 μM of DON significantly reduced the $CD4^+$ $CD25^+$ $FoxP3^+$ cell population of 40%. On the contrary, the preliminary *in vivo* results using the loops experiment suggest an increase of expression of Treg cells related genes (Fig. S1).

Relative mRNA expression of transcription factors and cytokines related to Th1 cells were assessed by RT-qPCR. Explants were exposed or not with 10 μM of DON for 4 h, 8 h or 12 h. Values were normalized with two reference genes (Cyclophilin A and RPL32). Data are presented as means +/− SEM of values obtained with intestinal explants from six different piglets and expressed relative to the control group at 4 h. Asterisks denote significant differences between untreated (gray bars) and DON-treated explants (black bars): * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Relative mRNA expression of transcription factors and cytokines related to Th17 cells (A) and IL-17 A protein concentration (B) were assessed by RT-qPCR and ELISA. Explants were exposed or not with 10 μM of DON for 4 h, 8 h or 12 h. Values were normalized with two reference genes (Cyclophilin A and RPL32). Data are presented as means +/− SEM of values obtained with intestinal explants from six different piglets and expressed relative to the control group at 4 h. Asterisks denote significant differences between untreated (gray bars) and DON-treated explants (black bars): * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

(A) Relative mRNA expression of transcription factors related to T reg cells were assessed by RT-qPCR. Explants were exposed or not with 10 μM of DON for 4 h, 8 h or 12 h. Values were normalized with two reference genes (Cyclophilin A and RPL32). Data are presented as means +/− SEM of values obtained with intestinal explants from six different piglets and expressed relative to the control group at 4 h. (B) The frequency of $CD4^+CD25^+FoxP3^+$ Treg cells were evaluated by flow cytometry after 2 days of co-culture with different concentration of DON (0.1 to 10 μM). Graphs show the mean ± SEM from duplicates from immune cells of jejunal lamina propria isolated 2 different pigs. Data were calculated as relative percentage to control without toxin. Asterisks denote significant differences between untreated (gray bars) and DON-treated explants (black bars): * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

DON Triggered Pathogenic Th17 Cells Subset in Detriment of Regulatory Th17 (rTh17) Cells in Porcine jejunal Explants

To understand which subset of Th17 cells is preferentially induced by DON, pathogenic Th17-related genes (IL-23 A, IL-22 and IL-21) and rTh17-related genes (TGF-β and IL-10) were analyzed (Fig.ô 6). DON significantly increased IL-23A mRNA expression in a time-dependent manner (3, 17 and 31 fold induction at 4, 8 and 12 hours respectively) (Fig.ô 6A). mRNA expression levels of both IL-22 and IL-21 were also significantly induced but to a lesser extent (maximum levels of 10 and 3.5 fold induction respectively) (Fig.ô 6A). Interestingly, upregulation of these genes did not show after 24 h of DON exposure (Fig. S1). On the contrary, TGF-β mRNA expression was not induced by DON treatment (Fig.ô 6B). Interestingly, IL-10 which is produced by both rTh17 and Treg cells showed a significant induction in the presence of DON (6 fold increase) (Fig.ô 6B).

Discussion

Mycotoxins have become an issue of major concern given their global and frequent occurrence as well as their known toxic effects. Among all, DON is of particular interest because it is one of the most prevalent mycotoxins in Europe and North America. The intestinal tract represents the first barrier against DON-contam-

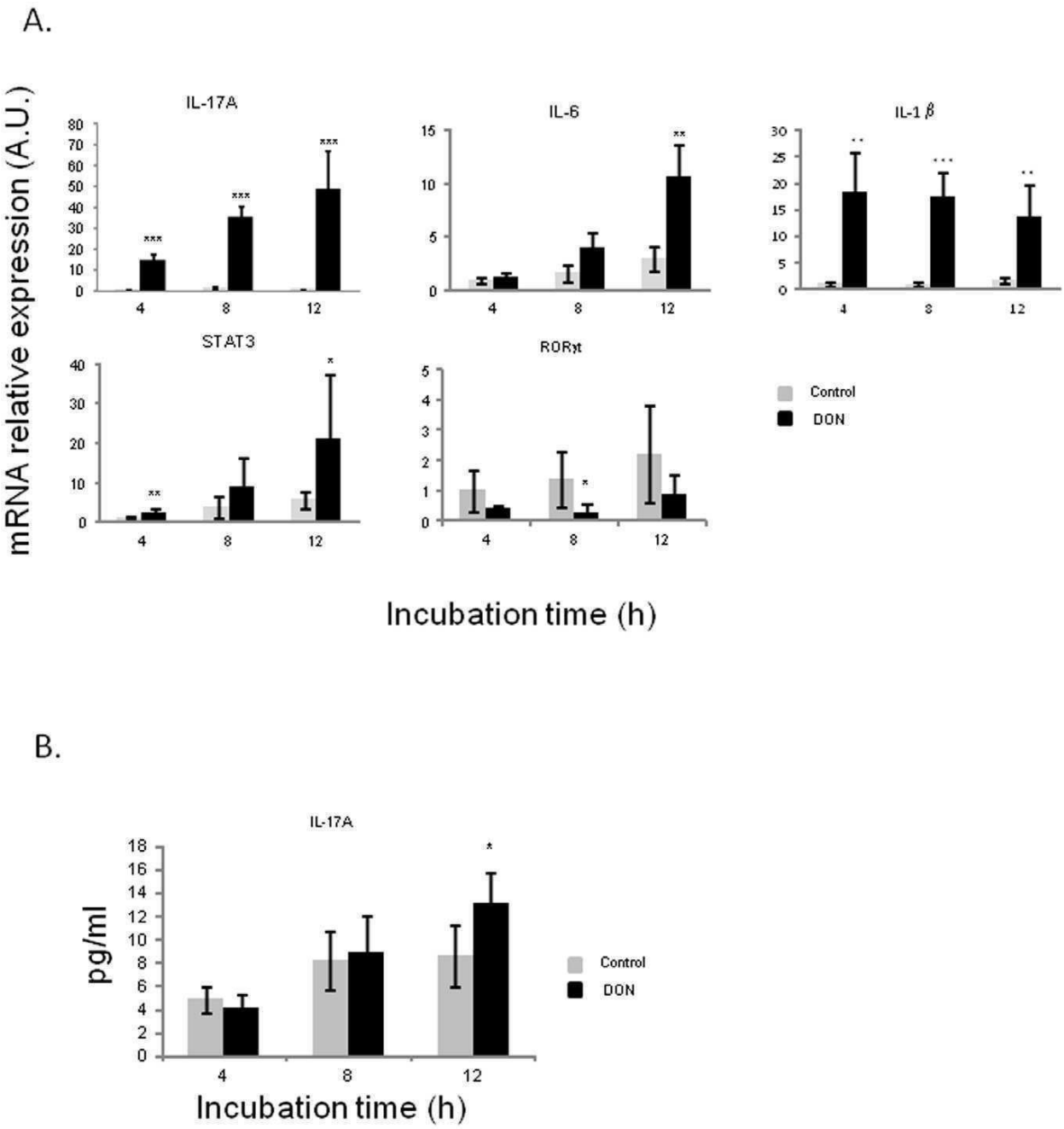

Figure 4. Deoxynivalenol triggered Th17 response in jejunal explants.
doi:10.1371/journal.pone.0053647.g004

inated food and feed. Many authors have already reported Th17 cells to be critical for protection against microbial infections like bacteria, virus and fungi at mucosal surfaces [36,37,38]. The aim of our work was to assess the impact of DON exposure onto the intestinal homeostasis controlled by pro-inflammatory Th17 and the regulatory Treg response. To the best of our knowledge this is the first study showing that DON disrupts the intestinal homeostasis and promotes the Th17 response over the Th1 and T regulatory responses.

Intestinal epithelial cells and jejunal explants were used to investigate the modulation of the intestinal immune response by DON. The choice of using 10 μM of DON was based mainly on the US Federal Grain Inspection Service and the European Union surveys [6,39,40] which evaluated the naturally occurring concentration of this mycotoxin to range between 19 and 50 mg/kg in grain cereals. Assuming that DON would be ingested in one meal, diluted in 1 L of gastrointestinal fluid and would be totally bioaccessible, 10 μM correspond approximately to 3 mg/kg [39,40]. In addition to that, a previous study of our group

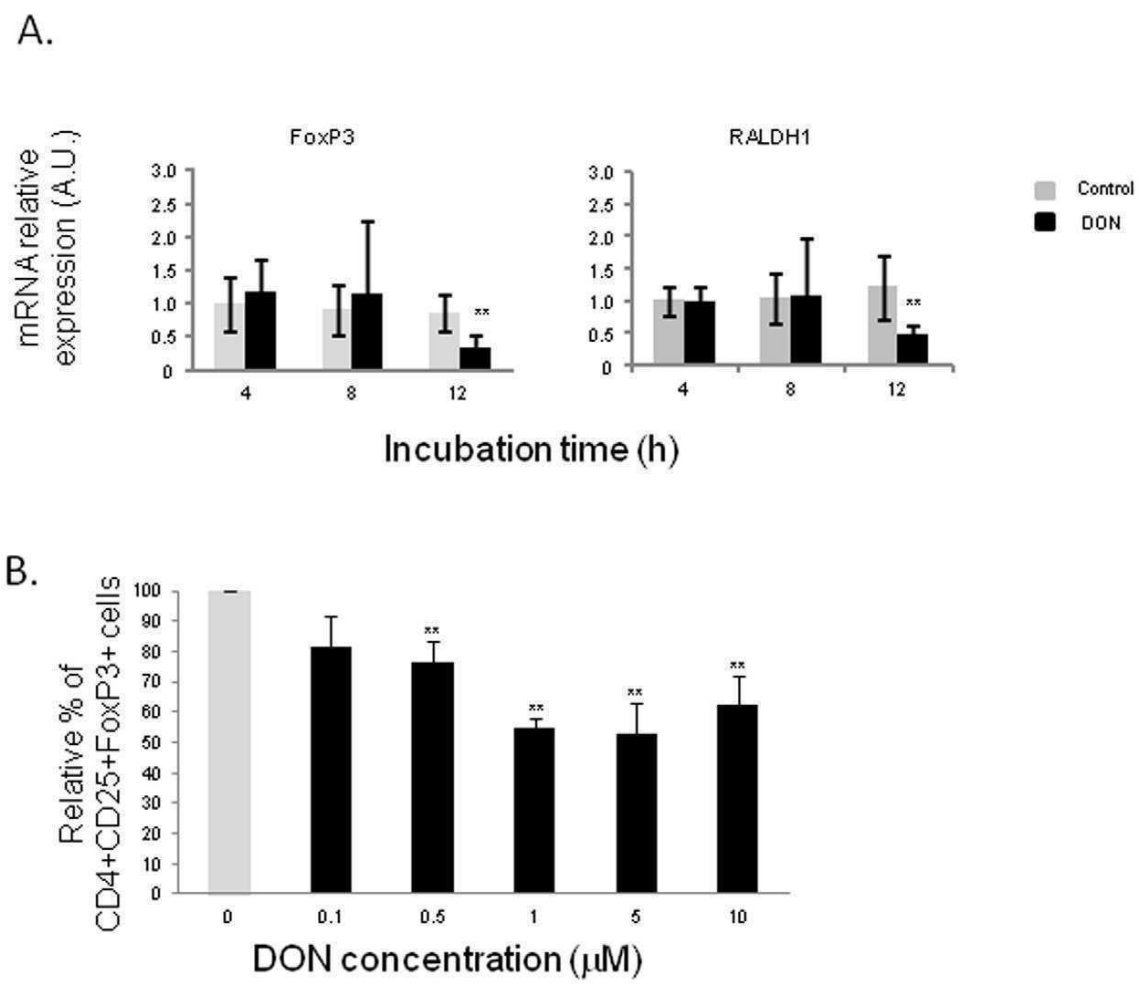

Figure 5. Deoxynivalenol decreased T regulatory response in jejunal explants.
doi:10.1371/journal.pone.0053647.g005

showed that this concentration does not affect cell viability up to 48 h after treatment in the porcine intestinal cell line, IPEC-1 [17] but it does affect the morphology of the epithelial barrier according to another study [27].

In the present experiment, *in vitro* and *ex vivo* exposure to DON led to an early general state of inflammation depicted by mRNA expression increase of IL-8, IL-1α and TNF- α, which is in agreement with previous results of similar studies [41,42]. The inflammation generated at the epithelial level could elicit the activation of the population of Th17 cells by triggering further communication with immune cells of the *lamina propria*. The mode of action by which DON induces such inflammatory response has been described by several authors and it was not the purpose of the present study. Indeed, it has already largely been demonstrated that DON activates ERK1/2 and p38 thus triggering MAPK signaling cascades which upregulate COX-2 and PGE-2, major inductors of the inflammatory response [11]. This molecular mechanism most likely occurs in all immune cellular types, including IECs. Therefore we can also soundly hypothesize that DON-induced MAPK activation in IECs induces chemokine secretion which then stimulates intestinal DCs, thus initiating the classical immune response cascade and lymphocyte activation.

In this study, the exposure to DON induced IL-6, IL-23 and IL-1β expression but did not affect the expression of TGF-β and strongly repressed FoxP3 and RALDH1. These data suggest that in our model DON mainly drives the intestinal immune system towards a Th17 response. In agreement with that, DON strongly induced IL-17 A which is the signature cytokine of these T helper cells, but which can also be produced by other immune cells. However to our knowledge, there is no available antibody for of porcine IL-17 A intracellular labeling which would allow to clarify the source of this cytokine. Despite that, the fact that STAT3, a transcription factor expressed by Th17 cells, was also upregulated in the intestinal explants is a strong indication of the increase of this population in the intestinal tissues. Nevertheless, ROR-γt like, which has been described as the other major transcription factor governing Th17 differentiation in mice [23,43], was not induced but repressed by the presence of DON in explants model. Although this result is surprising, it could be possible that, unlike in the mouse model, in the porcine specie the transcription factor STAT3 plays a more important role than ROR-γt in Th17 differentiation. It would be very interesting to further investigate this point.

The two lineages, Th17 and Tregs share a co-evolutionary origin [44,45] which could explain why they share similarities at

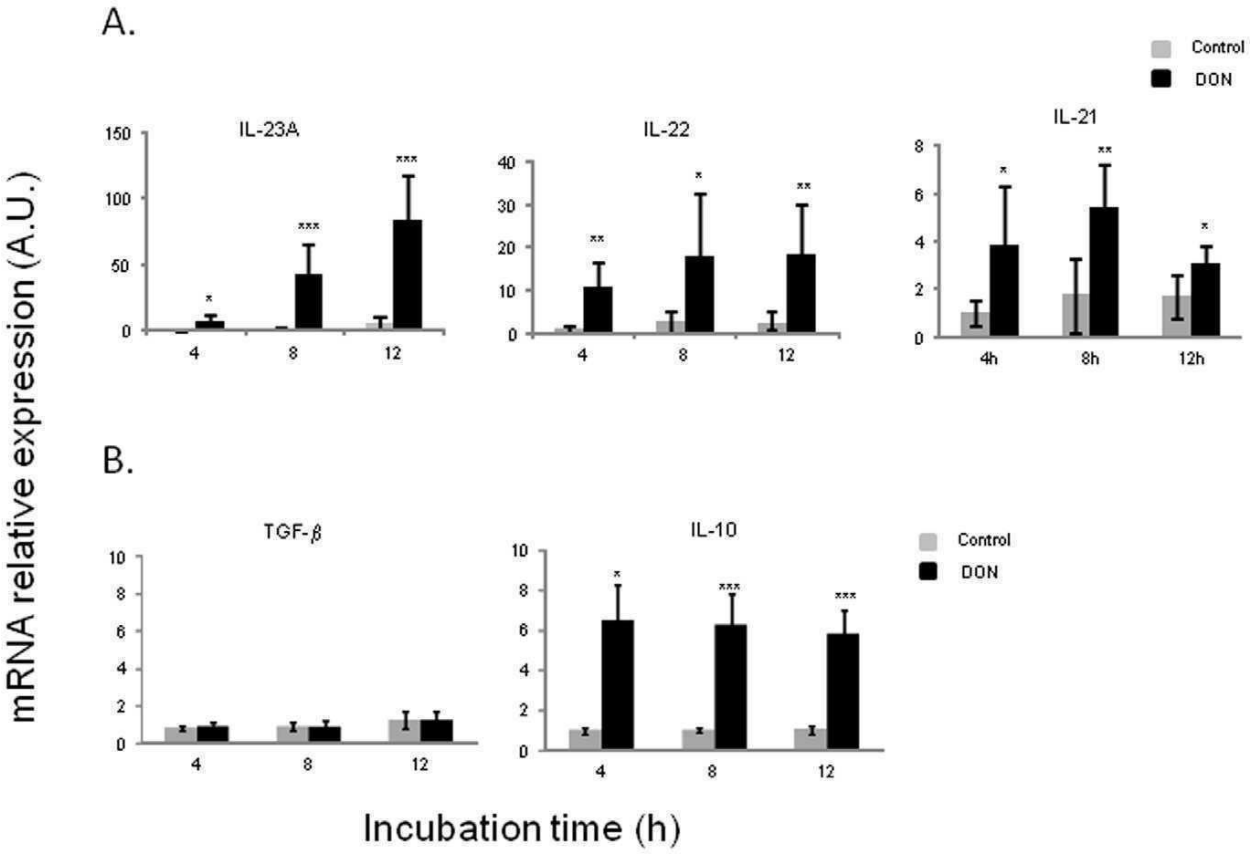

Figure 6. Deoxynivalenol triggered pathogenic Th17 cells subset but not regulatory Th17 (rTh17) cells in porcine jejunal explants.
doi:10.1371/journal.pone.0053647.g006

many levels [46,47,48,49]. In this way, CCR6 appears to be the only chemokine receptor essentially implicated in driving Th17 migration *in vivo* but it is also highly expressed by Tregs [50]. CCL20, the ligand of this receptor drives the conversion of the pathogenic Th17 into the regulatory subsets [23,51]. Our results showed that CCR6 and CCL20 were induced by DON in a time-dependent manner in the intestinal explants. It is therefore tempting to speculate that along the overall pro-inflammatory response both in the intestinal epithelial cells and the intestinal explants, there is a regulatory rTh17 response setting up-rather than the Treg response.

Plasticity of T cells is also reflected in the similarities between rTh17 and Treg cells. Besides their common regulatory action, these two populations share the importance of TGF-β and IL-10 [23,24,51]. TGF-β is involved in the differentiation of these two populations. In this study, there was no significant effect of DON on the expression of this cytokine in the intestinal explants. TGF-β was however secreted by the intestinal epithelial cells after 8, 12 and 24 h of exposure to DON. Further studies will be important to understand the exact role of TGF-β in the differentiation of Tregs in swine but also the impact of DON on the intestinal secretion of this cytokine. However, previous studies have also showed that TGF-β presents minor or absent changes after PMA/Ionomycin or CD3 stimulation [52,53] which might be an indication of the fact that TGF-β might be of less importance in the regulatory response compared to humans and mice. IL-10 is produced by both Tregs and rTh17 to regulate inflammation and has the ability of reversing the pathogenic phenotype of Th17 into rTh17 [24]. In this study, this cytokine was up-regulated in a time independent manner by the presence of DON on explants, which is in contradiction with the absence of increase of FoxP3 and TGF-β. However it is well described that many cells of the innate and adaptive immune system other than Tregs and rTh17 cells express IL-10, such as DCs, macrophages, Th1, Th2 and B cells [54]. Given our results in the intestinal explants on Th1 and Treg, it is most likely for IL-10 to be produced by DCs, macrophages or B cells in that experiment

Taken together, all these results suggest that DON preferentially induces a pro-inflammatory response directed by pathogenic Th17, when CCR6, CCL20 and IL-10 results point to a concomitant protective response most likely lead by rTh17. Supplementary *in vivo* data seems to indicate an increase in the protective response after 24 h of exposure.

Determining whether immune activation or suppression should occur in response to a given pathogen is a critical decision to be taken by the adaptive immune system. Indeed, there is a fine line between inflammation and pathogen elimination and excessive inflammation and tissue damage. Numerous diseases such as rheumatoid arthritis and multiple sclerosis arise from a failure to restore the state of equilibrium [55]. In the intestine, inflammatory bowel diseases (IBD) like ulcerative colitis (UC) and Crohn's disease (CD) are triggered by excessive inflammation of the colon and/or the small bowel leading to recurrent diarrhea and pain [56]. Until recently only excessive Th1 and Th2 responses were accounted for these diseases. Nevertheless, the discovery of the Th17 subset gave a new insight on the causes of these disorders as

IL-17 producing cells were shown to be of major importance in IBD [57]. Besides IL-17, IL-21, IL-22 and IL-23 have also been reported to play an important role in the onset of these diseases [57,58]. Interestingly, all of these cytokines were up-regulated by the presence of DON in our experiments which could imply that chronic exposure to this toxin could be a triggering or enhancing factor of IBD. This hypothesis is supported by a recent study [26] which draws a strong parallel between the alterations caused by exposure to several mycotoxins including DON and the symptoms observed during IBD. In addition to the induction of the above-mentioned cytokines, intestinal permeability is also related to both DON exposure and IBD. This mycotoxin specifically targets claudin expression [16,17] which directly leads to an increase of permeability of the intestinal barrier which could result in increased bacterial translocation, one of the main causes of IBD [56]. The same applies for toll-like receptors (TLRs) impairment, associated with DON exposure [59]. Over activation of dendritic cells (DCs) drawn by abnormal TLR activity could lead to inability to detect bacterial components which is another major source of IBD. Notably, TLR activation disruption has been detected after exposure to T-2 toxin, another member of the trichothecene family [60].

As depicted above, bacterial interactions with the immune cells of the *lamina propria* are a central component of IBD, since inflammation might arise from lack of tolerance to antigens present in commensal bacteria [61]. Besides, loss of the transcription factor T-bet, which regulates TNF-α production, influences bacterial homeostasis by favoring colitogenic microbial populations. The resulting increase of TNF-α production by DCs could trigger breakdown of the intestinal epithelium and facilitate bacterial translocation to the *lamina propria*, increasing risks of IBD [62]. It has already been demonstrated that DON alters the intestinal microbiota of pigs [63]. It thus comes as no surprise that in this study, exposure to DON could possibly lead to induction of colitogenic populations and to strong impairment of the epithelial barrier by the lack of effect on T-bet and significant increase of the expression levels of TNF-α. It is noteworthy that colonization of the small intestine by segmented filamentous bacteria (SFB) of the intestinal microbiota is directly associated with the appearance of Th17 cells and that SFB promote the expression of inflammatory genes linked to IBD [64].

In conclusion, exposure to the mycotoxin DON clearly induced an early intestinal inflammatory response resulting from the interplay of different intestinal cell types and leading to the activation of Th17 cells. These results together with previous observations strengthen the idea that chronical exposure to deoxynivalenol could impair intestinal homeostasis and trigger the appearance of IBD. However, the molecular mechanisms by which this toxin specifically activates Th17 remain unclear. Further research should therefore address the link between MAP kinases activation by DON [11,12] and the herein presented effects on lymphocyte populations. In addition to that, the cellular interplay that takes play among immune cells (IECs – DCs – T cells) upon DON exposure should be better characterized by analyzing for instance cellular migration of DCs, macrophages and T lymphocytes. Finally, more insight should be gained on the role of commensal and pathogenic bacteria on modulating the Th17 response and the impact of DON on such modulation.

Supporting Information

Figure S1 Fold mRNA expression increase of cytokines and transcription factors after DON exposure in an *in vivo* model of jejunal loops. Following the guidelines provided by the French Council for Animal Care (permit number 2011–07–1) and previous published protocols [65,66,67], loops were performed in a 5 week-old pig. Very briefly, 6 consecutive loops (10–0 cm long) were made by surgical ligation in a 2–4 m long segment of the jejunum which was previously thoroughly washed with a solution of metrodinazole, a common antibiotic. PBS was injected in 3 loops as negative control and 10 μM of DON were injected in 3 other loops. The pig was euthanized by barbiturate overdose 24 h post surgery. Jejunal tissues were then collected and snap-frozen in liquid nitrogen before RNA extraction. Relative mRNA expression levels of immune genes related to pro-inflammatory cytokines (A), DC-recrutment chemokines (B), Th1 (C), Th17 (D) and Treg (E) signature and pathogenic/regulatory Th17 cytokines (F) were assessed by RT-qPCR. Gene expressions were normalized by the mean of two reference genes (Cyclophilin A and RPL32). Data are presented as mean +/− SEM of values obtained with three different loops and expressed relative to the control group. Significant differences between untreated loops (gray bars) and treated loops (black bars) with 10 μM of DON are marked with asterisks (* $P<0.05$).
(TIF)

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Author Contributions

Conceived and designed the experiments: LGP IPO. Performed the experiments: PMC JS JC RA. Analyzed the data: PMC JS FM JC RA. Contributed reagents/materials/analysis tools: JC. Wrote the paper: PMC JS.

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ANEXO 2

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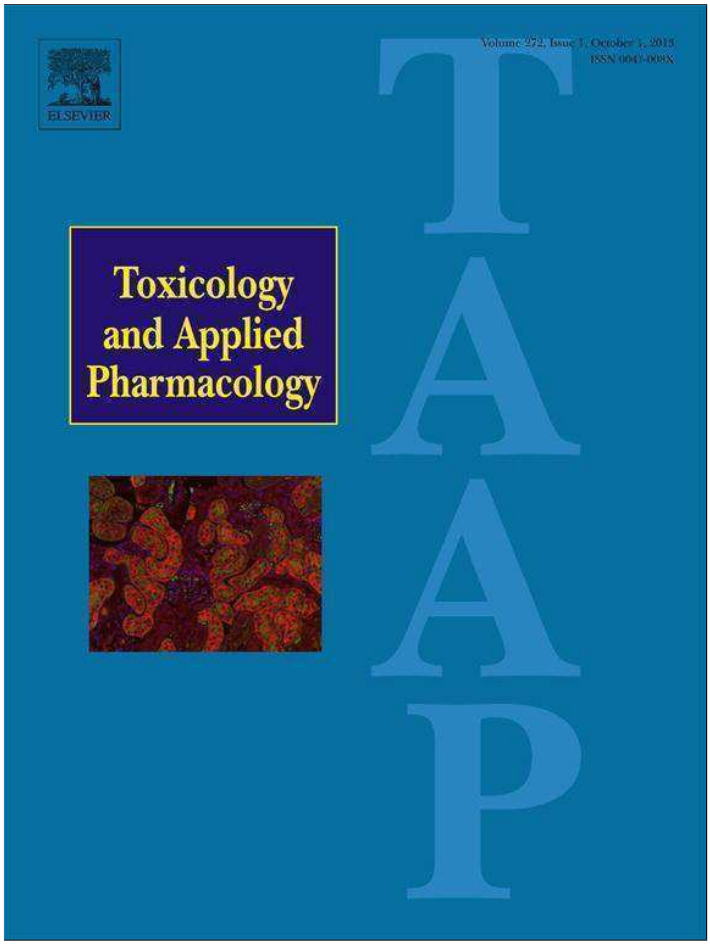
New insights into mycotoxin mixtures: The toxicity of low doses of Type B trichothecenes on intestinal epithelial cells is synergistic.



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New insights into mycotoxin mixtures: The toxicity of low doses of Type B trichothecenes on intestinal epithelial cells is synergistic

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ABSTRACT

Deoxynivalenol (DON) is the most prevalent trichothecene mycotoxin in crops in Europe and North America. DON is often present with other type B trichothecenes such as 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), nivalenol (NIV) and fusarenon-X (FX). Although the cytotoxicity of individual mycotoxins has been widely studied, data on the toxicity of mycotoxin mixtures are limited. The aim of this study was to assess interactions caused by co-exposure to Type B trichothecenes on intestinal epithelial cells. Proliferating Caco-2 cells were exposed to increasing doses of Type B trichothecenes, alone or in binary or ternary mixtures. The MTT test and neutral red uptake, respectively linked to mitochondrial and lysosomal functions, were used to measure intestinal epithelial cytotoxicity. The five tested mycotoxins had a dose-dependent effect on proliferating enterocytes and could be classified in increasing order of toxicity: 3-ADON < 15-ADON ≈ DON < NIV ≪ FX. Binary or ternary mixtures also showed a dose-dependent effect. At low concentrations (cytotoxic effect between 10 and 30–40%), mycotoxin combinations were synergistic; however DON–NIV–FX mixture showed antagonism. At higher concentrations (cytotoxic effect around 50%), the combinations had an additive or nearly additive effect. These results indicate that the simultaneous presence of low doses of mycotoxins in food commodities and diet may be more toxic than predicted from the mycotoxins alone. Considering the frequent co-occurrence of trichothecenes in the diet and the concentrations of toxins to which consumers are exposed, this synergy should be taken into account.

Introduction

Mycotoxins are biologically active secondary fungal metabolites found as contaminants in almost all agricultural commodities worldwide, and they pose a major risk for human and animal health (Wild and Gong, 2010). Among them, the Type B trichothecenes constitute a group of toxins with a keto group at carbon 8 of the parent epoxytrichothecene nucleus. These compounds are produced by *Fusarium graminearum* and *Fusarium culmorum*, the main causal agents of *Fusarium* head blight, an important disease of small grain cereals worldwide.

Considered as phytotoxins, the trichothecenes favor the development of the fungus on the plant, although they are not necessary for the formation of the primary symptoms of the disease (Boenisch and Schafer, 2011). This family of toxins includes, but is not limited to, five closely related congeners: deoxynivalenol (DON), 15-acetyldeoxynivalenol (15-ADON), 3-acetyldeoxynivalenol (3-ADON), fusarenon X (FX; 4-acetylnivalenol) and nivalenol (NIV) (Fig. 1). Due to a high prevalence of *Fusarium* head blight, Type B trichothecenes are the most common contaminants of cereal grains in temperate regions of the word. A large scale data survey indicates that DON, 15-ADON, NIV, FX and 3-ADON are present in 57%, 20%, 16%, 10% and 8%, respectively of food samples collected in the European Union (SCOOP, 2003).

The adverse effects of trichothecenes include emesis, nausea, anorexia, growth retardation, neuroendocrine changes and immunosuppression (Pestka, 2010). In humans, there is a body of evidences suggesting that trichothecenes cause acute illness and are frequently associated with outbreaks of gastroenteritis (Pestka, 2010). At the molecular level, trichothecenes display multiple inhibitory effects on the primary metabolism of eukaryotic cells including the inhibition of proteins, DNA and RNA synthesis (Rocha et al., 2005). This impairment leads to the alteration in cell proliferation in tissue with high rates of cell turnover such as intestinal epithelial cells. Thus intestinal epithelial cells are especially sensitive to trichothecenes and their exposure to these toxins may induce toxicity (De Walle et al., 2010; Pinton et al., 2010). The intestine is also the first barrier to food

Abbreviations: 3-ADON, 3-acetyldeoxynivalenol; 15-ADON, 15-acetyldeoxynivalenol; CI, combination index; DON, deoxynivalenol; DRI, dose reduction index; f_a, fraction affected; FX, fusarenon-X; IC_{50}, inhibitory concentration 50%; MTT, 3,(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide; NIV, nivalenol.

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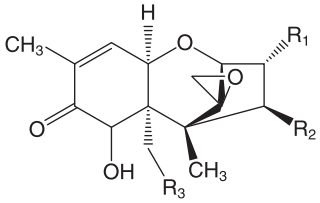

	R_1	R_2	R_3
Deoxynivalenol	OH	H	OH
Nivalenol	OH	OH	OH
Fusarenon X	OH	OAc	OH
15 Acetyl-deoxynivalenol	OH	H	OAc
3 Acetyl-deoxynivalenol	OAc	H	OH

Fig. 1. Chemical structures of DON and NIV and their acetyl derivatives.

contaminants. Following the ingestion of mycotoxin-contaminated food or feed, intestinal epithelial cells can be exposed to high concentrations of toxins (Maresca et al., 2008).

Human and animals are exposed simultaneously to several trichothecenes for at least three different reasons: (i) most *Fusarium* are able to produce a number of mycotoxins simultaneously, (ii) food commodities can be contaminated by several fungi simultaneously or in quick succession and (iii) a complete diet is made up of various different commodities. Humans may also be exposed to multiple trichothecenes via products from animals that have eaten contaminated feed (Streit et al., 2012). Rodrigues and Naehrer (2012) screened 7049 feed and feedstuff samples in a three-year survey on the worldwide occurrence of mycotoxins, and reported 48% to be contaminated by two or more mycotoxins. From a total of 29 wheat samples collected in three EU countries, Monbaliu et al. (2010) reported that 75% of the contaminated samples were positive for more than one type of mycotoxin. In the case of trichothecenes, several studies have shown a co-occurrence of trichothecenes in corn, wheat and barley (Eckard et al., 2011; Hajslova et al., 2007; Kim et al., 1993; Schollenberger et al., 2012).

Because of their natural co-occurrence, there is an increasing concern about the hazard of exposure to mycotoxin mixtures. Unfortunately, the toxicity of mycotoxins when present together, cannot always be predicted based upon their individual toxicities (CAST, 2003; Grenier and Oswald, 2011). Multi-exposure may lead to additive, synergistic or antagonist toxic effects. The data on toxic effects of mycotoxin mixtures are limited and therefore, the actual health risk from exposure to the combination of mycotoxins is unknown. Indeed, there are very few studies addressing the combined effects of mycotoxins and at present the database describing the possible effects of combined exposure of trichothecenes is very sparse and not sufficient to establish either the nature of combined effect or the relative potencies of these toxins.

The aim of the present study was to establish a relative potency scale for five Type B trichothecenes and to assess their combined effects in terms of additive, antagonistic or synergistic toxicity towards human intestinal epithelial cells.

Materials and methods

Toxins

DON, 3-ADON, 15-ADON were purchased from Sigma (St Quentin Fallavier, France); NIV, FX from Waco Pure Chemical Industries LTD (Osaka, Japan). Stock solutions of mycotoxins were dissolved in DMSO to the following concentrations: 15 mM DON and 15-ADON, 20 mM 3-ADON, 10 mM NIV and FX. Stock solutions were stored at −20 °C and working dilutions were prepared in cell culture medium. Final concentration of 0.1% DMSO corresponding to the highest DMSO concentration of working dilutions was tested and results were not significantly different from controls.

Cell culture and reagents

The Caco-2 cell line has been derived from a human colon adenocarcinoma (ATCC HTB-37, Rockville, MD, USA). For these experiments, Caco-2 cells were grown in 75-cm^2 culture flasks in culture medium consisting of Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal calf serum (Perbio Sciences, Bezons, France), 0.1 mM non-essential amino acids, 2 mM L-glutamine, 100 UI/mL penicillin, and 100 μg/mL streptomycin (Eurobio, Courtaboeuf, France) in an atmosphere of 5% CO_2 at 37 °C. Before reaching confluence, the cells were trypsinized and plated in 96-well flat-bottom cell culture plates (Costar, Cambridge, MA, USA) for performing cytotoxicity assay.

Cytotoxicity assays

Two cytotoxicity assays, the MTT test and neutral red uptake, were performed to assess the individual and combined effects of DON, NIV and their acetyl derivatives. Proliferating Caco-2 cells (10 000 cells per well) were seeded in 96-well plates, and incubated for 24 h at 37 °C, before a 48-hour exposure to mycotoxins alone or in mixtures. Negative controls were obtained by the treating cells with the solvent alone (DMSO). The final toxin concentrations tested ranged from 7.5 nM to 6.67 μM. The tested binary combination ratios were: 1/1 for DON/15-ADON, 1/1.67 for DON/3-ADON, 1/0.8 for DON/NIV, 1/0.03 for DON/FX and 1/0.04 for NIV/FX. The tested ternary ratios were: 1/1/1.67 for DON/15-ADON/3-ADON and 1/0.8/0.03 for DON/NIV/FX. These ratios, calculated from preliminary individual cytotoxicity experiments, enabled a similar toxicity to be obtained for each mycotoxin.

The MTT test was performed as described by Gauthier et al. (2012). The neutral red uptake cytotoxicity assay was run according to Repetto et al. (2008).

For both tests, the percentage of viable cells was calculated using the formula:

$$\text{Viability}\,(\%) = 100 \times \frac{\text{Mean OD of mycotoxin(s) treated sample}}{\text{Mean OD of untreated sample}}.$$

Data analysis

Individual mycotoxin cytotoxicity. The dose–response relationships of the individual mycotoxins were biometrically modeled by using the Median-Effect Equation of the Mass Action Law (Chou, 2006): $f_a/f_u = (D\,/\,D_m)^m$ where D is the dose of the toxin, f_a is the fraction affected by D (e.g. percentage of inhibition / 100), and f_u is the fraction unaffected (i.e. $f_u = 1 - f_a$). D_m is the median-effect dose (e.g. IC_{50}), and m is the coefficient signifying the shape of the dose–effect relationship ($m = 1$, $m > 1$, and $m < 1$ indicate hyperbolic, sigmoidal, and flat sigmoidal dose–effect curves, respectively).

We verified that the linear regression correlation coefficients of the median effects plots were greater or equal to 0.95 (Chou, 2011).

Effects of mixtures. Dose–response curves for single mycotoxins, and binary or ternary associations were generated simultaneously. Mycotoxin interactions were analyzed by the isobologram and combination-index methods derived from the median effect principle of Chou and Talalay (1984).

Isobolograms were drawn for a binary combination of mycotoxins at doses inducing 10, 30 and 50% cytotoxicity as previously described elsewhere (Kolf-Clauw et al., in press). The combination-index (CI)

method was also used to analyze the mycotoxin interaction. This index is calculated according to Chou (2011):

$$^{n}(CI)_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j}$$

where $^{n}(CI)_x$ is the combination index for n toxins at x% inhibition, $(D)_j$ is the doses of n toxins that exerts x% inhibition in combination, $(D_x)_j$ is the doses of each of n toxins alone that exerts x% inhibition. CI = 0.9 − 1, CI < 0.9, and CI > 1.1 indicate an additive effect, a synergism, and an antagonism, respectively, regardless of the mechanisms or the units of the drugs.

For all binary and ternary mycotoxin combinations, CI values were generated over a range of fractions of cell viability affected (f_a) from 0.05 to 0.95 (5% to 95% toxicity).

When synergy occurred in the effects of the mixtures, dose reduction indices (DRI) were calculated. The dose reduction index measures how many folds the dose of each mycotoxin in a synergistic combination may be reduced at a given effect level compared with the doses of each mycotoxin alone. The dose reduction index for each drug can easily be obtained by setting the reciprocal of the CI equation (Chou, 2011):

$$^{n}(CI)_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n} \frac{1}{(DRI)_j}$$

and

$$(DRI)_1 = \frac{(D_x)_1}{(D)_1}, \quad (DRI)_2 = \frac{(D_x)_2}{(D)_2} \ldots, \text{etc.}$$

Dose–effect curves analysis. The dose–response relationship analysis for individual mycotoxin cytotoxicity, CI and their 95% confidence interval calculation, the dose reduction index calculation, isobologram plots and f_a–CI plots for combined effects were all performed with Compusyn software version 3.0.1 (ComboSyn Inc., Paramus, NJ, USA).

Statistical analysis. The reported values are the means ± standard deviation (SD) of at least three independent experiments, each with triplicate wells per dose level. Statistical analyses were performed using SigmaPlot version 11.0 (Systat Software Inc., San Jose, CA, USA). Differences between all the mycotoxin treatments were analyzed by the non-parametric Kruskal–Wallis one way analysis of variance on ranks with a critical level of significance set up at $p < 0.001$. Significant differences between groups were analyzed with the Holm-Sidak post hoc multiple comparison procedure. The level of $p < 0.05$ was considered statistically significant.

Results

Comparative cytotoxicity of Type B trichothecenes on Caco-2 cells

Two cytotoxicity assays, based respectively on mitochondrial and lysosomal activities, were used to compare the toxic effect of Type B trichothecenes. Proliferating Caco-2 cells were exposed for 48 h to concentrations of mycotoxins ranging from 7.5 nM to 6.67 μM. The five mycotoxins had a dose-dependent effect on both neutral red uptake and MTT activity (Fig. 2) with mean IC_{50} values ranging from 20 nM to 3 μM (Table 1).

For the overall ranking, mycotoxins were cytotoxic for proliferating enterocytes in the increasing order 3-ADON < 15-ADON ≈ DON < NIV ≪ FX.

Combined cytotoxicity of Type B trichothecenes

The next step was to determine the cytotoxicity of Type B trichothecene mixtures and to characterize the type of interaction between these mycotoxins when they are present together (additivity, synergy or antagonism). The combination ratios, calculated on the basis of the IC_{50} values obtained in individual cytotoxicity experiments, were chosen to obtain an equipotent toxicity for each mycotoxin in a mixture. The cytotoxicity was assessed on proliferating Caco-2 cells using neutral red test, as this assay was more sensitive than the MTT test (Table 1). Both single mycotoxins and binary or ternary mixtures showed a dose-dependent effect (Figs. 3 and 4). Isobolograms were drawn and CI values were calculated for different toxicity levels. When a synergistic effect was observed, the dose reduction indices were calculated to quantify the synergy.

Combined toxicity of DON, 15-ADON and 3-ADON. DON, 15-ADON and 3-ADON, and their binary and ternary combinations, caused a dose-dependent toxicity in Caco-2 cells (Fig. 3). When DON was associated with its acetylated derivatives, an additive effect was observed at the 50% growth inhibition level, while a synergistic effect was noted at lower cytotoxic levels (Figs. 5 and 6).

The Fig. 5 presents the isobologram for the combinations of DON and 3-ADON at three different levels of inhibition (10%, 30% and 50%). In this type of graph, the additive effect follows the diagonal line between the effective concentrations of each single toxin. If the measured combined effect of two toxins is above or below the diagonal line, it indicates an antagonist or a synergistic effect of the combination respectively. In this figure, we can observe the additive effect of DON and 3-ADON at the 50% cytotoxicity level and the synergistic effect at lower cytotoxicity levels. The interaction between DON and its acetylated derivates was further analyzed by calculating CI value at various cytotoxicity levels. The CI/f_a curves for binary and ternary

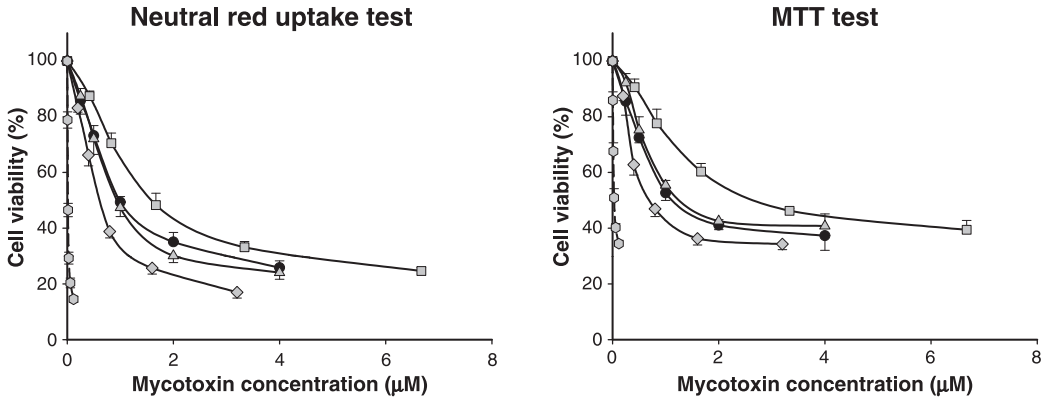

Fig. 2. Comparative cytotoxicity of Type B trichothecenes on proliferating Caco-2 cells. Intestinal epithelial cells were exposed for 48 h to serial dilutions of DON (○), 3-ADON (□), 15-ADON (△), NIV (◇) or FX (⬠). Cytotoxicity was assessed by the MTT and neutral red assays. Data are means ± SD of three to four independent experiments.

Table 1
Comparison of the cytotoxicity of Type B trichothecenes against Caco-2 cells after a 48 h exposure using the MTT and neutral red tests.

Mycotoxin	IC_{50} (μM)	
	MTT	Neutral red
DON	1.39 ± 0.07^{a}	1.19 ± 0.29^{a}
3-ADON	2.94 ± 0.45^{ab}	1.99 ± 0.44^{b}
15-ADON	1.47 ± 0.28^{abc}	1.1 ± 0.38^{ac}
NIV	0.9 ± 0.24^{cd}	0.69 ± 0.16^{cd}
FX	0.04 ± 0.01^{e}	0.02 ± 0.00^{e}

Data are means ± SD of three to four independent experiments.
^{abcde}Means in a column without a common letter differ ($p < 0.05$).

combinations of DON, 3-ADON and 15-ADON are shown in Fig. 6. At low concentrations (f_a between 10% and 30–40%), mycotoxin combinations give a CI < 1 indicating a synergistic toxic effect. At higher concentrations (f_a between 30–40 and 50%), mycotoxin combinations have a CI around 1, indicating an additive effect. Conversely antagonism was observed at 70% cytotoxicity and above (data not shown).

In order to quantify the synergy between DON and its acetyl derivatives, dose reduction indices were calculated for 10% and 30% level of cytotoxicity (Table 2). This latter parameter indicates the ratio between the concentration of tested mycotoxins when used alone or in combination to achieve the same toxicity level. For the ternary mixture DON/15-ADON/3-ADON, the dose reduction indices of the three individual mycotoxins ranged from 6.8 to 10 at 10% toxicity and from 3.4 to 5 at 30% toxicity. For binary mixtures, the dose reduction indices range from 2.8 to 4.3 at 10% toxicity and 2 to 3.2 at 30% toxicity.

Combined toxicity of DON, NIV and FX. The combined toxicity of DON, NIV and FX was also studied on proliferating Caco-2 cells (Figs. 4 and 7). As already observed with DON and acetylated derivatives, when DON was associated with NIV or FX, a synergy was observed at low cytotoxicity levels (Fig. 7). Above 50% toxicity the interaction turned into an additive effect (data not shown). The dose reduction index values were higher at 10% toxicity than at 30% toxicity (Table 2). Compared to single compounds, the toxicity of the DON-FX combination showed a 6-fold concentration reduction for DON and a 3-fold reduction for FX. DON and NIV concentrations showed a 3-fold reduction in a binary mixture. Whatever the toxicity level, the NIV–FX combination gave an additive interaction.

When the three toxins were present together, an antagonism was observed at 10 and 20% of toxicity. The calculated dose reduction index values were 3, 3 and 1.6 respectively for DON, NIV and FX indicating a weaker effect of FX in the mixture compared to that predicted on the basis of additivity.

Discussion

DON is the most prevalent trichothecene mycotoxin present in crops in Europe and North America (CAST, 2003; SCOOP, 2003). Consumers are thus particularly concerned over the exposure to this toxin as indicated by recent survey (Turner et al., 2008). The second French total diet study highlights that exposure to DON significantly exceeds the health-based guidance values (Sirot et al., 2013). DON is often present with other Type B trichothecenes such as 3-ADON, 15-ADON, NIV and FX (Schollenberger et al., 2012). The aim of this

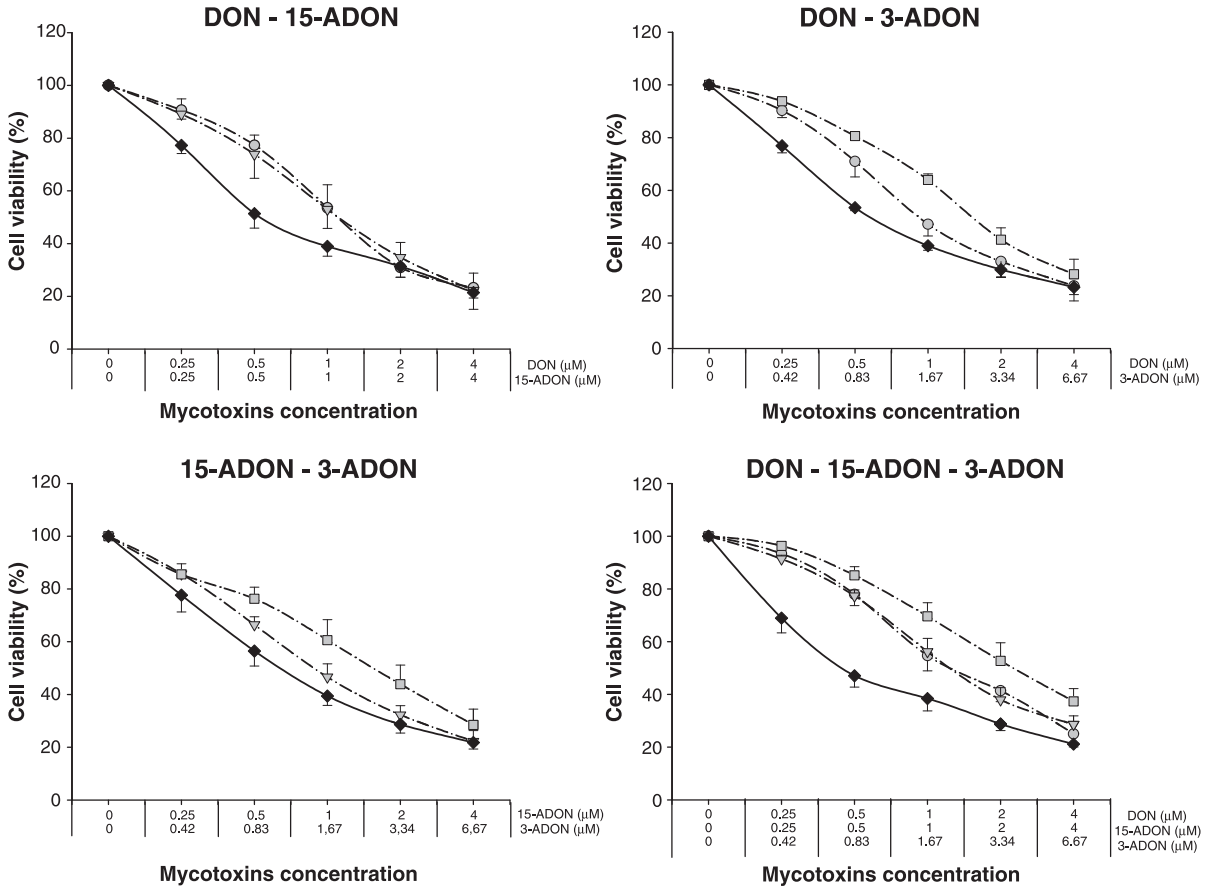

Fig. 3. Comparative toxicity of DON (⌂), 3-ADON (□) and 15-ADON (▽) alone or in binary or ternary mixtures/combinations (◆) on proliferating Caco-2 cells. Intestinal epithelial cells were exposed for 48 h to serial dilutions of toxins alone or in combination and cytotoxicity was assessed by the neutral red assay. Data are means ± SD of three independent experiments.

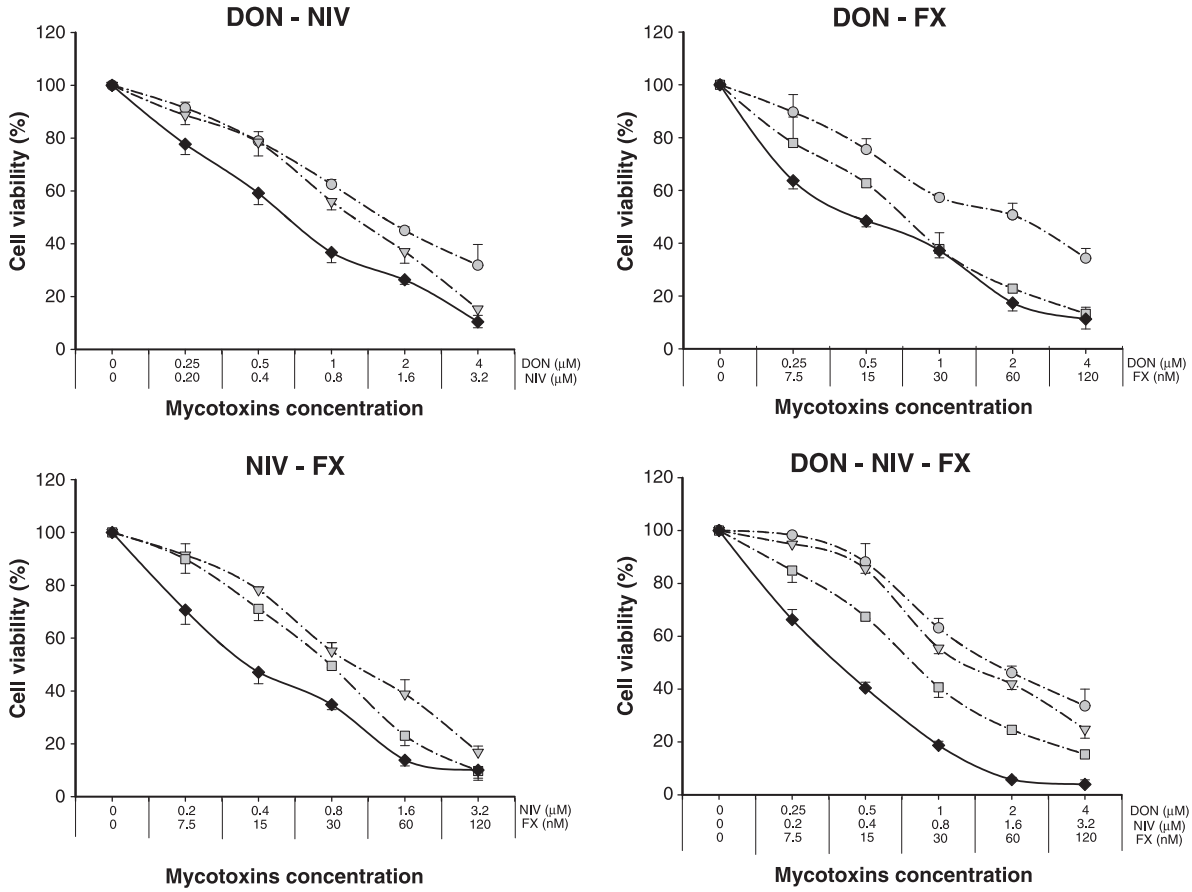

Fig. 4. Comparative toxicity of DON (○), NIV (▽) and FX (□) alone or in binary or ternary mixtures/combinations (♦) on proliferating Caco-2 cells. Intestinal epithelial cells were exposed for 48 h to serial dilutions of toxins alone or in combination and cytotoxicity was assessed by the neutral red assay. Data are means ± SD of three independent experiments.

study was to assess the interactions that occur when there is co-exposure to Type B trichothecenes.

These mycotoxins have been shown to alter several functions of intestinal epithelial cells including cell proliferation, barrier function, nutrient absorption and immune responses (Bianco et al., 2012; Diesing et al., 2012; Maresca et al., 2002). The MTT test and neutral red uptake, respectively linked to mitochondrial and lysosomal functions, were used for the indirect measurement of proliferation and viability of Caco-2 cells. By these two tests, we demonstrated that Type B trichothecenes, alone or in combination, were cytotoxic for intestinal cells in a dose-dependent manner. The IC_{50} and the relative toxicity of the five selected mycotoxins were similar with both cytotoxicity tests.

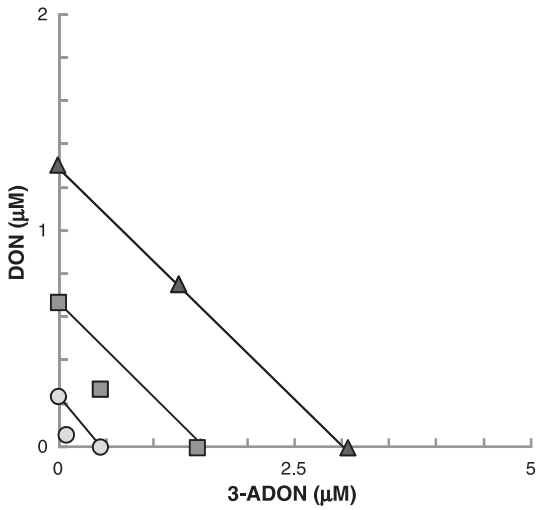

Fig. 5. Isobologram illustrating the combined cytotoxicity of DON and 3-ADON. At concentrations eliciting 10% (○), 30% (■) or 50% (▲) toxicity. The points are mean concentrations of dose–response neutral red cytotoxicity curves for each toxin or toxin combination (Compusyn software analysis).

In this study, we observed that NIV was 20–35 times less toxic to Caco-2 cells than its acetyl derivative FX. A slightly less relative potency (10–20) was reported in the same cell line by Bony et al. (2007). In other cell lines, FX has been found to be more toxic than NIV in unsettled proportions (Forsell and Pestka, 1985; Sundstol Eriksen et al., 2004; Thompson and Wannemacher, 1986). The IC_{50} values calculated in the current study for NIV and DON are in accordance with those obtained by Nielsen et al. (2009) in Caco-2 and other human cell lines, confirming the higher toxicity of NIV compared to DON. By MTT and neutral red uptake assays, 3-ADON was 2-fold less toxic for proliferating Caco-2 cells than DON and 15-ADON. This result confirms the lower toxicity of 3-ADON observed in other previous studies (Daenicke et al., 2011; Pinton et al., 2012; Sundstol Eriksen et al., 2004; Thompson and Wannemacher, 1986; Visconti et al., 1991) even though the difference in toxicity between 3-ADON and the two other trichothecenes varied significantly from one study to another.

Binary and ternary combinations of Type B trichothecenes were tested for interaction using the Chou-Talalay method (Chou, 2006). This method, already used to study other mycotoxin combinations (Jones et al., 1995; Koshinsky and Khachatourians, 1992; Ruiz et al.,

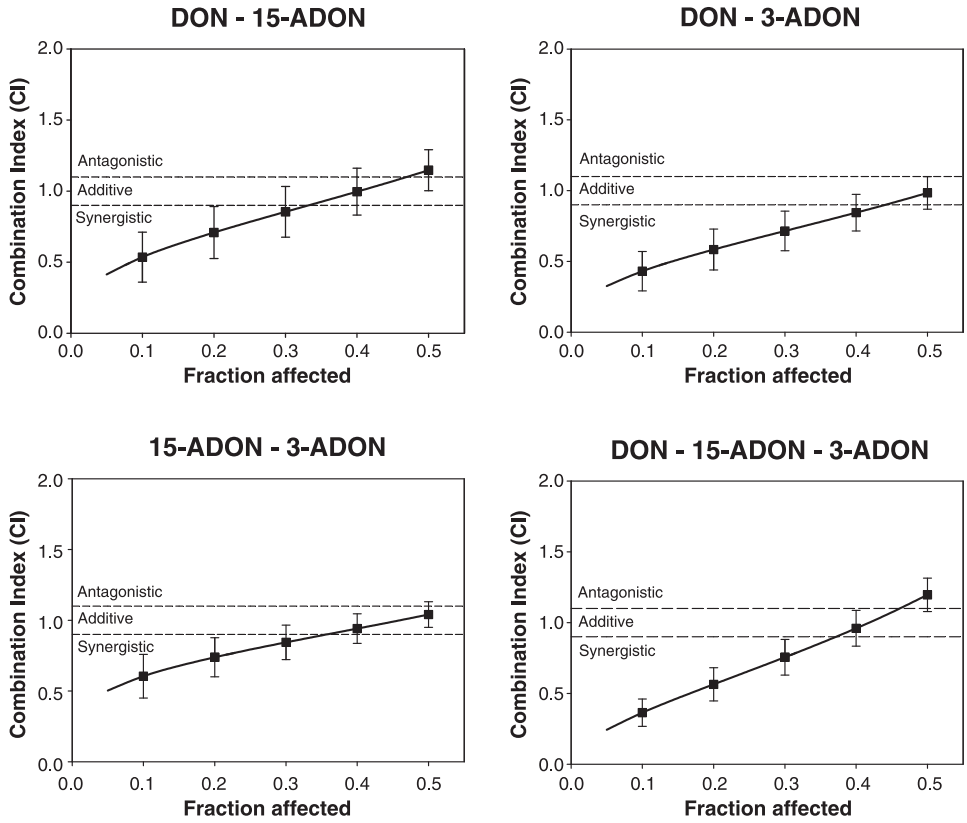

Fig. 6. Combination index — fraction affected curve for binary and ternary combinations of DON, 3-ADON and 15-ADON. CI values were calculated from data obtained from three independent experiments on the basis of equipotent mycotoxin combinations. The vertical bars indicate 95% confidence intervals for CI values based on sequential deletion analysis (Chou, 2006). Horizontal dashed lines correspond to lower and upper limits of the additivity zone.

2011) combines a qualitative assessment of interactions via an isobolographic analysis and a quantification of synergy or antagonism by calculating a combination index and dose reduction index at different effect levels. For the 50% cytotoxicity level, the isobolographic analysis indicates an additive effect of the toxins. This pattern was confirmed by the combination index, calculated for the binary and ternary mixtures. Additive interaction between DON and NIV has already been reported in several cell types such as murine J7741A macrophages (Marzocco et al., 2009), porcine whole blood cells (Luongo et al., 2008), and human lymphocytes (Thuvander et al., 1999). To the best of our knowledge, ours is the first study that assessed globally the combined effect of Type B trichothecenes.

Table 2
Low-dose synergy of Type B trichothecenes for intestinal cytotoxicity.

Mycotoxin	Combination ratio	10% cytotoxicity		30% cytotoxicity	
		CI	DRI	CI	DRI
DON	1:1	0.54	4.2	0.85	2.5
15-ADON			3.4		2.2
DON	1:1.67	0.43	4.3	0.71	2.5
3-ADON			5		3.2
15-ADON	1:1.67	0.61	2.8	0.84	2
3-ADON			4		2.9
DON	1:1.67:1	0.37	8.5	0.76	3.8
3-ADON			10		3.4
15-ADON			6.8		5
DON	1:0.8	0.73	2.8	0.81	2.7
NIV			2.7		2.2
DON	1:0.03	0.48	6	0.66	4.7
FX			3.2		2.3
NIV	1:0.04	0.82	3.5	0.9	3
FX			1.9		1.8
DON	1:0.8:0.03	1.33	–	1.07	–
NIV			–		–
FX			–		–

Dose reduction indices were calculated by comparing the concentration required to reach 10 and 30% cytotoxicity when the mycotoxin was used singly and in combination. DRI > 2 for binary combinations and DRI > 3 for ternary combinations indicate a synergistic effect.

The main results of this paper are the observations that (i) the type of interaction varies with the cytotoxicity level and (ii) below a cytotoxicity level of 50%, the combined effects of binary or ternary mixtures of Type B trichothecenes are synergistic. In the present study a synergistic effect of DON, when combined with other Type B trichothecenes, was observed at IC_{10} and IC_{30}, i.e. at 0.15 to 0.55 μM DON. These findings suggest that the simultaneous presence of low doses of mycotoxins in food commodities and diet may induce greater toxicity than that can be predicted from the mycotoxins alone. This observation is of high biological relevance if we consider the concentrations of mycotoxins to which consumers are exposed. Indeed, DON concentrations of 0.16–2 μg/mL (0.5–7 μM) can be considered as realistic in human gut (Sergent et al., 2006). The lower concentration value corresponds to the mean estimated daily intake of French adult consumers on a chronic basis (Sirot et al., 2013). The higher concentration value simulates level that can be reached after the consumption of heavily contaminated food, as can be occasionally encountered. The dose reduction index, that is a quantitative assessment based on the ratio of observed to predicted doses of trichothecenes in mixtures, permitted the calculation of correction factors that may take the observed low-dose synergies into account. In the present experiments the calculated correction factors ranged from 2 to 10. To the best of our knowledge, this is the first study that mentions the magnitude of mycotoxin synergy. A lack of quantitative estimates of the magnitude of interactions has been pointed as a weakness of chemical combined effect

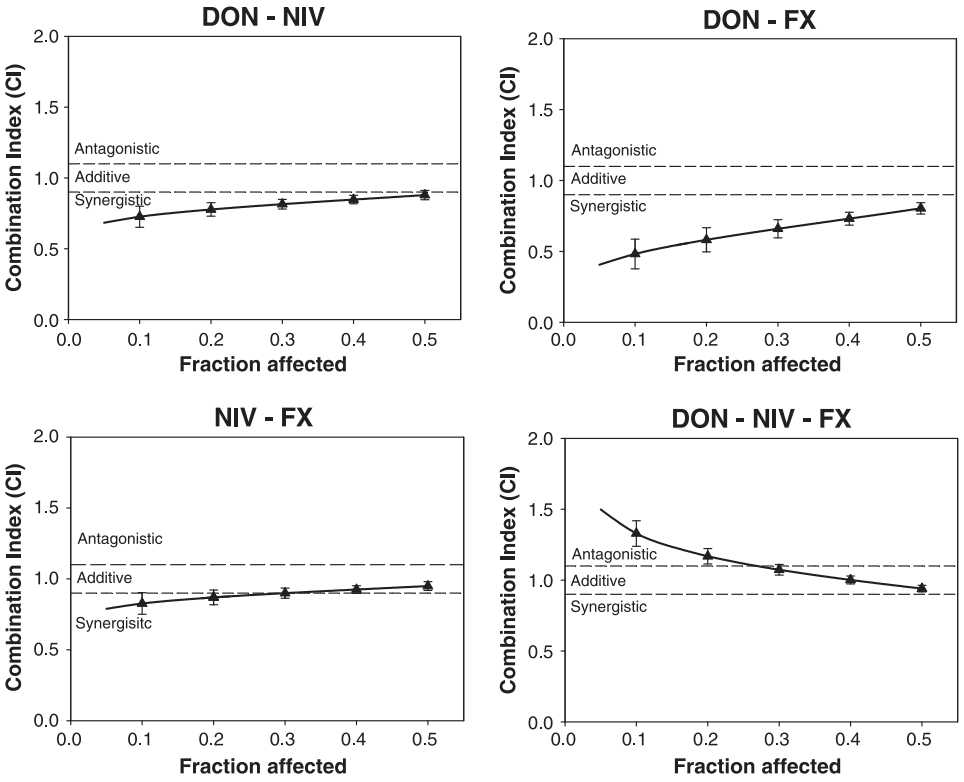

Fig. 7. Combination index — fraction affected curve for binary and ternary combinations of DON, NIV and FX. CI values were calculated from data obtained from three independent experiments on the basis of equipotent mycotoxin combinations. The vertical bars indicate 95% confidence intervals for CI values based on sequential deletion analysis (Chou, 2006). Horizontal dashed lines correspond to lower and upper limits of the additivity zone.

studies. In a critical analysis of 90 papers on mixture toxicity, Boobis et al. (2011) identified only 11 papers that mentioned the interaction magnitude.

The ternary combination of DON–NIV–FX showed antagonism for 10% cytotoxicity. This later antagonistic interaction seems to be linked to a lower toxicity of FX in the mixture as shown by the lower dose reduction index value. The higher toxicity of FX is mainly due to its higher hydrophobicity that facilitates its passage across the apical membrane and a de-acetylation step leading to NIV accumulation in the enterocytes (Ohta et al., 1978; Poapolathep et al., 2003). The reduction of FX toxicity might be due to the competition of DON and NIV at the level of substrate binding sites of the de-acetylase leading to a reduced de-acetylation of FX. This hypothesis deserves to be further investigated.

The Caco-2 cell represents a well-established model for the study of intestinal transport, metabolism and toxicity of nutrients and xenobiotics, and is wildly used in pharmacology and toxicology (Artursson et al., 2001; Boveri et al., 2004). However a number of limitations reported for this model, especially in drug discovery and mechanistic studies, arise critical issues in extrapolating the in vitro results to in vivo situations (Press and Di Grandi, 2008; Sun et al., 2002). In term of cytotoxicity, similar IC_{50} values for DON and NIV were reported for Caco-2 and seven other human permanent cell lines (Nielsen et al., 2009). Nevertheless, the data obtained in this paper, especially the synergy observed at low mycotoxin concentrations should be confirmed using in vivo or ex vivo models (Grenier and Oswald, 2011; Kolf-Clauw et al., 2009).

The present study demonstrates that the effect of a mixture of mycotoxins cannot be predicted solely on the basis of the effect of the individual compounds. Our results clearly indicate that the susceptibility of Caco-2 cells to the mycotoxins differed between the combinations assayed. The mechanism(s) of these interactions deserves further investigations. The synergistic effects observed after cell exposure to a mixture of low concentrations of mycotoxins tested are of practical importance since trichothecenes often occur in combination. The synergistic effect observed could pose a significant threat to public health (Speijers and Speijers, 2004). Considering the co-occurrence of mycotoxins in food, further research into the cytotoxicity of mycotoxin mixtures and their interactions should be addressed. Moreover, given the mycotoxin interactions, government regulatory standards about a great variety of mycotoxins or their mixtures are needed. New risk assessment strategies should take into account the toxicological interactions of mycotoxins in food and feed as already suggested (SCF, 2002).

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Conflict of interest

Authors declare no conflict of interest.

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Thompson, W.L., Wannemacher Jr., R.W., 1986. Structure-function relationships of 12,13-epoxytrichothecene mycotoxins in cell culture: comparison to whole animal lethality. Toxicon 24, 985–994.

Thuvander, A., Wikman, C., Gadhasson, I., 1999. *In vitro* exposure of human lymphocytes to trichothecenes: individual variation in sensitivity and effects of combined exposure on lymphocyte function. Food Chem. Toxicol. 37, 639–648.

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Visconti, A., Minervini, F., Lucivero, G., Gambatesa, V., 1991. Cytotoxic and immunotoxic effects of *Fusarium* mycotoxins using a rapid colorimetric bioassay. Mycopathologia 113, 181–186.

Wild, C.P., Gong, Y.Y., 2010. Mycotoxins and human disease: a largely ignored global health issue. Carcinogenesis 31, 71–82.

ANEXO 3

Normas para publicação da Revista Brasileira de Zootecnia.

Revista Brasileira de Zootecnia

Instructions to Authors – 2014[1]

Topics:

1. Scope

The ***Revista Brasileira de Zootecnia***-Brazilian Journal of Animal Science (RBZ) encompasses all research fields of Animal Science Research. The RBZ publishes original scientific articles in the areas of Aquaculture; Forage; Animal Genetics and Breeding, Animal Reproduction; Ruminant and Non-Ruminant Nutrition; Animal Production Systems and Agribusiness.

2. Editorial policies

2.1. Open access and peer review

The RBZ is sponsored by the Brazilian Society of Animal Science for providing readers or their institutions with free access to peer reviewed articles published online by RBZ. Users have the right to read, download, copy, distribute, print, search, or link to the full texts of articles. The ***Revista Brasileira de Zootecnia*** is included in the Directory of Open Access Journals (DOAJ).

A peer-review system is exerted on manuscripts sent for appreciation to maintain standards of quality, improve performance, and provide credibility. We use the double-blind style of reviewing by concealing the identity of the authors from the reviewers, and vice versa, lest the knowledge of authorship or concern about disapprobation from the author bias the reviewer's judgment. Communication with authors should only be through the Scientific Editor (named as Editor-in-chief). Authors are given the chance to designate names to be considered by the Editor-in-chief as preferred or non-preferred reviewers. Reviewers should notify the editor about conflicts of interest (either positive or negative)

[1] Revised December 2013.

that may compromise their ability to provide a fair and an unbiased review.

2.2. Assurance of contents and assignment of copyright

When submitting a manuscript for review authors should make sure that the results of the work are original, and that the total or partial content of the manuscript, regardless of the language, has not been/ is not being considered for publication in any other scientific journal. Additionally, the authors assure that if they have used the work and/or words of others this has been appropriately cited or quoted warranting absence of plagiarism, which constitutes unethical publishing behavior.

Papers already published or that have been submitted to any other journal will not be accepted. Fractioned or subdivided studies should be submitted together because they will be assigned to the same reviewers.

The content of the articles published by ***Revista Brasileira de Zootecnia*** is of sole responsibility of their authors.

Authors who have a manuscript approved by RBZ are also requested to authorize that the right of total or partial electronic and graphic reproduction (copyright) of the paper be transferred to the Brazilian Society of Animal Science, which ensure us the rights necessary for the proper administration of electronic rights and online dissemination of journal articles.

After completing the submission of the manuscript by using the Manuscript Central™ online system the corresponding author will be asked to upload the file named Assurance of Contents and Copyright and will be responsible for obtaining the signatures of all coauthors. A template with the same name has been already prepared by the Brazilian Society of Animal Science and is available on the journal web site at http://www.rbz.ufv.br/rbz/visao/site/enviarArtigoCPF.php?lingua=2.

The original text of the template must not be altered but only completed with the necessary information. All authors are invited to fill it out properly, sign it, scan and send it by e-mail to RBZ's office at: secretariarbz@sbz.org.br confirming or even disagreeing with their participation in the manuscript.

The manuscript will not be considered for peer reviewing without this form. The deadline will be set allowing a period of 15 days for delivery of forms after which the editorial office act by withdrawing the manuscript.

2.3. Language

Submissions will only be accepted in the English language (either American or British spelling). The editorial board of RBZ reserves the right to demand that authors revise the translation or to cancel the processing of the manuscript if the English version submitted contains errors of spelling, punctuation, grammar, terminology, jargons or semantics that can either compromise good understanding or not follow the Journal's standards. It is strongly recommended that the translation process be performed by native speakers of English.

2.4. Publication costs

The payment of processing fee is a prerequisite for submitting manuscripts to referees. Authors will be charged the amount of R$ 53.00 (Fifty three reais and no cents) per manuscript, which must be done by credit card, accordingly to guidance available on the SBZ website (www.sbz.org.br).

The current charge for publication is different for members and non-members of the BSAS. Considering the full length articles, the fee for members is R$ 160.00 (up to 8 pages in the final format) and R$ 59.00 for each extra page. Once the manuscript is approved, all authors must meet the deadline of current year's membership fee, except for the co-authors who do not work directly in that area, provided they are not the first author and have not published more than one article in the year in question (recurrence). For non-members of BSAS, there is a charge of R$ 128.00 per page (up to 8 pages in the final format) and R$ 251.00 for each page that exceeds it.

2.5. Care and use of animals

The ***Revista Brasileira de Zootecnia*** is committed to the highest ethical standards of animal care and use. Research presented in manuscripts reporting the use of animals must guarantee to have been conducted in accordance with applicable federal, state, and local laws, regulations, and policies governing the care and use of animals. The author should ensure that the manuscript contains a statement that all procedures were performed in compliance with relevant laws and institutional guidelines and, whenever pertinent, that the appropriate institutional committee(s) has approved them before commencement of the study.

2.6. Types of articles

Full-length research article
A full-length research paper provides a complete account of the experimental work. The text should represent the research process and foster its cohesive understanding and a coherent explanation regarding all the experimental procedures and results and must provide the minimal information necessary for an independent reproduction of the research.

Short communication
A succinct account of the final results of an experimental work, which has full justification for publication, although with a volume of information which is not sufficient to be considered a full length research article. The results used as the basis to prepare the short communication cannot be used subsequently, neither partially nor wholly, for the presentation of a full-length article.

Technical note
An evaluation report or proposition of a method, procedure or technique that correlates with the scope of RBZ. Whenever possible, one should show the advantages and disadvantages of the new method, procedure or technique proposed, as well as its comparison with those previously or currently employed, presenting the proper scientific rigor in analysis, comparison, and discussion of results.

Board-invited reviews
Approach that represents state-of-the-art or critical view of issues of interest and relevance to the scientific community. It can only be submitted by invitation of the editorial board of RBZ. The invited reviews will be subjected to the peer review process.

Editorial
Notes to clarify and establish technical guidelines and/or philosophy for designing and making of articles to be submitted and evaluated by RBZ. The editorials will be drafted by or at the invitation of the editorial board of RBZ.

3. Guidelines to prepare the manuscript

3.1. Structure of a full-length research article

Figures, Tables, and Acknowledgments should be sent as separated file and not as part of the body of the manuscript.

The article is divided into sections with centered headings, in bold, in the following order: Abstract, Introduction, Material and Methods, Results, Discussion (or Results and Discussion), Conclusions, Acknowledgments (optional) and References. The heading is not followed by punctuation.

3.1.1. Manuscript format
The text should be typed by using Times New Roman font at 12 points, double-space (except for Abstract and Tables, which should be set in space 1.5), top, inferior, left and right margins of 2.5; 2.5; 3.5, and 2.5 cm, respectively.

The text should contain up to 25 pages, sequentially numbered in arabic numbers at the bottom, leaving the authors to bear the additional costs of publishing extra pages at the time of publication (see publication costs). The file must be edited by using Microsoft Word® software.

3.1.2. Title
The title should be precise and informative, with no more than 20 words. It should be typed in bold and centered as the example: **Nutritional value of sugar cane for ruminants.** Names of sponsor of grants for the research should always be presented in the Acknowledgments section.

3.1.3. Authors
The name and institutions of authors will be requested at the submission process; therefore it should not be presented in the body of the manuscript. Please see the topic Guidelines to submit the manuscript for details.

The listed authors should be no more than eight.

Spurious and “ghost” authorships constitute an unethical behavior. Collaborative inputs, hand labor, and other types of work that do not imply intellectual contribution may be mentioned in the Acknowledgments section.

3.1.4. Abstract
The abstract should contain no more than 1,800 characters including spaces in a single paragraph. The information in the abstract must be precise. Extensive abstracts will be returned to be adequate with the guidelines.

The abstract should summarize the objective, material and methods, results and conclusions. It should not contain any introduction. References are never cited in the abstract.

The text should be justified and typed in space 1.5 and come at the beginning of the manuscript with the word ABSTRACT

capitalized, and initiated at 1.0 cm from the left margin. To avoid redundancy the presentation of significance levels of probability is not necessary in this section.

3.1.5. Key Words

At the end of the abstract list at least three and no more than six key words, set off by commas and presented in alphabetical order. They should be elaborated so that the article is quickly found in bibliographical research. The key words should be justified and typed in lowercase. There must be no period mark after key words.

3.1.6. Introduction

The introduction should not exceed 2,500 characters with spaces, briefly summarizing the context of the subject, the justifications for the research and its objectives; otherwise it will be rerouted for adaptation. Discussion based on references to support a specific concept should be avoided in the introduction.

Inferences on results obtained should be presented in the Discussion section.

3.1.7. Material and Methods

Whenever applicable, describe at the beginning of the section that the work was conducted in accordance with ethical standards and approved by the Ethics and Biosafety Committee of the institution.
A clear description on the specific original reference is required for biological, analytical and statistical procedures. Any modifications in those procedures must be explained in detail.

3.1.8. Results and Discussion

In making this section, the author is granted to either combine the results with discussion or to write two sections by separating results and discussion (which is encouraged). Sufficient data, with means and some measure of uncertainty (standard error, coefficient of variation, confidence intervals, etc.) are mandatory, to provide the reader with the power to interpret the results of the experiment and make his own judgment. The additional guidelines for styles and units of RBZ should be checked for the correct understanding of the exposure of results in tables. The results section cannot contain references.

In the discussion section, the author should discuss the results clearly and concisely and integrate the findings with the literature published to provide the reader with a broad base on which they will accept or reject the authors hypothesis.

Loose paragraphs and references presenting weak relationship with the problem being discussed must be avoided. Neither speculative ideas nor propositions about the hypothesis or hypotheses under study are encouraged.

3.1.9. Conclusions

Be absolutely certain that this section highlights what is new and the strongest and most important inferences that can be drawn from your observations. Include the broader implications of your results. The conclusions are stated by using the present tense.

3.1.10. Acknowledgments

This section is optional. It must come right after the conclusions.

The section acknowledgments must not be included in the body of the manuscript; instead, a file named Acknowledgment should be prepared and then uploaded as an additional document during submission. This procedure helps RBZ to conceal the identity of authors from the reviewers.

3.1.11. Use of abbreviations

Author-derived abbreviations should be defined at first use in the abstract, and again in the body of the manuscript, and in each table and figure in which they are used.
The use of author-defined abbreviations and acronyms should be avoided, as for instance: T3 was higher than T4, which did not differ from T5 and T6. This type of writing is appropriate for the author, but of complex understanding by the readers, and characterizes a verbose and imprecise writing.

3.1.12. Tables and Figures

It is essential that tables be built by option “Insert Table” in distinct cells, on Microsoft Word® menu (No tables with values separated by the ENTER key or pasted as figure will be accepted). Tables and figures prepared by other means will be rerouted to author for adequacy to the journal guidelines.

Tables and figures should be numbered sequentially in Arabic numerals, presented as separate files to be uploaded, and must not appear in the body of the manuscript.

The title of the tables and figures should be short and informative, and the descriptions of the variables in the body of the table should be avoided.

In the graphs, designations of the variables on the X and Y axes should have their initials in capital letters and the units in parentheses.

Non-original figures, i.e., figures published elsewhere are only allowed to be published in RBZ with the express written consent of the publisher or copyright owner. It should contain, after the title, the source from where they were extracted, which must be cited.

The units and font (Times New Roman) in the body of the figures should be standardized.

The curves must be identified in the figure itself. Excessive information that compromises the understanding of the graph should be avoided.

Use contrasting markers such as circles, crosses, squares, triangles or diamonds (full or empty) to represent points of curves in the graph.

Figures should be built by using Microsoft Excel®, or even the software Corel Draw® (CDR extension) to allow corrections during copyediting, and uploaded as separate files, named figures during submission. Use lines with at least 3/4 width. Figures should be used only in monochrome and without any 3-D or shade effects. Do not use bold in the figures.

The decimal numbers presented within the tables and figures must contain a point, not a comma mark.

Mathematical formulas and equations must be inserted in the text as an object and by using Microsoft Equation or a similar tool.

3.1.13. References

Reference and citations should follow the Name and Year System (Author-date)

3.1.14. Citations in the text

The author's citations in the text are in lowercase, followed by year of publication. In the case of two authors, use 'and'; in the case of three or more authors, cite only the surname of the first author, followed by the abbreviation et al.

Examples:
Single author: Silva (2009) or (Silva, 2009)
Two authors: Silva and Queiroz (2002) or (Silva and Queiroz, 2002)
Three or more authors: Lima et al. (2001) or (Lima et al., 2001)

The references should be arranged chronologically and then alphabetically within a year, using a semicolon (;) to separate multiple citations within parentheses, e.g.: (Carvalho, 1985; Britto, 1998; Carvalho et al., 2001).

Two or more publications by the same author or group of authors in the same year shall be differentiated by adding lowercase letters after the date, e.g. (Silva, 2004a,b).

Personal communication can only be used if strictly necessary for the development or understanding of the study. Therefore, it is not part of the reference list, so it is placed only as a footnote. The author's last name and first and middle initials, followed by the phrase "personal communication", the date of notification, name, state and country of the institution to which the author is bound.

3.1.15. Reference section

References should be written on a separate page, and by alphabetical order of surname of author(s), and then chronologically.

Type them single-spaced, justified, and indented to the third letter of the first word from the second line of reference.

All authors' names must appear in the references section.

The author is indicated by their last name followed by initials. Initials should be followed by period (.) and space; and the authors should be separated by semicolons. The word 'and' precedes the citation of the last author.

Surnames with indications of relatedness (Filho, Jr., Neto, Sobrinho, etc.) should be spelled out after the last name (e.g. Silva Sobrinho, J.).

Do not use ampersand (&) in the citations or in the reference list.

As in text citations, multiple citations of same author or group of authors in the same year shall be differentiated by adding lowercase letters after the date.

In the case of homonyms of cities, add the name of the state and country (e.g. Gainesville, FL, EUA; Gainesville, VA, EUA).

Sample references are given below.

Articles

The journal name should be written in full. In order to standardize this type of reference, it is not necessary to quote the website, only volume, page range and year. Do not use a comma (,) to separate journal title from its volume; separate periodical volume from page numbers by a colon (:).

Miotto, F. R. C.; Restle, J.; Neiva, J. N. M.; Castro, K. J.; Sousa, L. F.; Silva, R. O.; Freitas, B. B. and Leão, J. P. 2013. Replacement of corn by babassu mesocarp bran in diets for feedlot young bulls. Revista Brasileira de Zootecnia 42:213-219.

Articles accepted for publication should preferably be cited along with their DOI.

Fukushima, R. S. and Kerley, M. S. 2011. Use of lignin extracted from different plant sources as standards in the spectrophotometric acetyl bromide lignin method. Journal of Agriculture and Food Chemitry, doi: 10.1021/jf104826n (in press).

Books

If the entity is regarded as the author, the abbreviation should be written first accompanied by the corporate body name written in full.

In the text, the author must cite the method utilized, followed by only the abbreviation of the institution and year of publication.

e.g.: "...were used to determine the mineral content of the samples (method number 924.05; AOAC, 1990)".

Newmann, A. L. and Snapp, R. R. 1997. Beef cattle. 7th ed. John Wiley, New York.

AOAC - Association of Official Analytical Chemistry. 1990. Official methods of analysis. 15th ed. AOAC International, Arlington, VA.

Book chapters

The essential elements are: author (s), year, title and subtitle (if any), followed by the expression "In", and the full reference as a whole. Inform the paging after citing the title of the chapter.

Lindhal, I. L. 1974. Nutrición y alimentación de las cabras. p.425-434. In: Fisiologia digestiva y nutrición de los ruminantes. 3rd ed. Church, D. C., ed. Acríbia, Zaragoza.

Theses and dissertations

It is recommended not to mention theses and dissertations as reference but always to look for articles published in peer-reviewed indexed journals. Exceptionally, if necessary to cite thesis and dissertation, please indicate the following elements: author, year, title, grade, university and location.

Castro, F. B. 1989. Avaliação do processo de digestão do bagaço de cana-de-açúcar auto-hidrolisado em bovinos. Dissertação (M.Sc.). Universidade de São Paulo, Piracicaba.

Palhão, M. P. 2010. Induced codominance and double ovulation and new approaches on luteolysis in cattle. Thesis (D.Sc.). Universidade Federal de Viçosa, Viçosa, MG, Brazil.

Bulletins and reports

The essential elements are: Author, year of publication, title, name of bulletin or report followed by the issue number, then the publisher and the city.

Goering, H. K. and Van Soest, P. J. 1970. Forage fiber analysis (apparatus, reagents, procedures, and some applications). Agriculture Handbook No. 379. ARS-USDA, Washington, D.C., USA.

Conferences, meetings, seminars, etc.

Quote a minimal work published as an abstract, always seeking to refer articles published in journals indexed in full.

Casaccia, J. L.; Pires, C. C. and Restle, J. 1993. Confinamento de bovinos inteiros ou castrados de diferentes grupos genéticos. p.468. In: Anais da 30ª Reunião Anual da Sociedade Brasileira de Zootecnia. Sociedade Brasileira de Zootecnia, Rio de Janeiro.

Weiss, W. P. 1999. Energy prediction equations for ruminant feeds. p.176-185. In: Proceedings of the 61th Cornell Nutrition Conference for Feed Manufacturers. Cornell University, Ithaca.

Article and/or materials in electronic media

In the citation of bibliographic material obtained by the Internet, the author should always try to use signed articles, and also it is up to the author to decide which sources actually have credibility and reliability.

In the case of research consulted online, inform the address, which should be presented between the signs < >, preceded by the words "Available at" and the date of access to the document, preceded by the words "Accessed on:".

Rebollar, P. G. and Blas, C. 2002. Digestión de la soja integral en rumiantes. Available at: <http://www.ussoymeal.org/ruminant_s.pdf.> Accessed on: Oct. 28, 2002.

Quotes on statistical software

The RBZ does not recommend bibliographic citation of software applied to statistical analysis. The use of programs must be informed in the text in the proper section, Material and Methods, including the specific procedure, the name of the software, its version and/or release year.

"... statistical procedures were performed using the MIXED procedure of SAS (Statistical Analysis System, version 9.2.)"

3.2. Structure of the article for short communication and technical note

The presentation of the title should be preceded by the indication of the type of manuscript whether it is a short communication or a technical note, which must be centered and bold.

The structures of short communications and technical notes will follow guidelines set up for full-length papers, limited, however, to 14 pages as the maximum tolerated for the manuscript.

Processing and publishing fees applied to communications and technical notes are the same for full-length papers, considering, however, the limit of four pages in its final form. A fee will be charged for publishing additional pages.

3.3. Additional guidelines for style and units – Use of percentage

Because of the intense use of units in the percentage form (%), the Editorial Board of ***Revista Brasileira de Zootecnia*** defines that percentage should be exceptionally and seldom used only for description of relative variations (e.g., variation of a result obtained in a given treatment in relation to other treatment) and not as an absolute unit of measurement.

3.3.1. Chemical or feed composition of diets

Chemical compositions of diets or feedstuffs have to be expressed as mass contents e.g., g kg^{-1} of dry matter or g kg^{-1} as fed.

Examples:

Food composition of the concentrate mixture offered to animals

Item	Incorrect (%)	Correct (g kg^{-1} as fed)
Corn grain	70.0	700
Soybean meal	27.0	270
Urea	1.0	10
Mineral mixture	2.0	20

Chemical composition of corn silage

Item	Incorrect (%)	Correct (g kg^{-1} as fed)
Dry matter[1]	35.23	352.3
Organic matter[2]	95.45	954.5
Crude protein[2]	7.86	78.6
Ether extract[2]	2.35	23.5
Neutral detergent fiber corrected for ash and protein[2]	55.86	558.6
Non-fibrous carbohydrates[2]	29.38	293.8
Non-protein nitrogen[3]	32.45	324.5

[1] Incorrect: percent as fed. Correct: g kg^{-1} as fed.
[2] Incorrect: dry matter percentage. Correct: g kg^{-1} dry matter.
[3] Incorrect: total nitrogen percentage. Correct: g kg^{-1} total nitrogen.

3.3.2. Measures of intake

Measures of intake have to be expressed as mass consumed per mass unit per unit of time.

Example:

Incorrect: "... animals presented average intake of 2.52% of body weight..."

Correct: "... animals presented average intake of 25.2 g kg^{-1} d^{-1} of body weight..."

3.3.3. Units expressed as coefficients

In animal science, it is common to produce variables given by the ratio between two variables. Therefore, because they represent direct measures made at the experimental unit and not relative comparisons among different situations (e.g., among treatments), those variables have to be expressed as mass unit per mass unit.

Most common examples:

Measures of digestibility coefficients:

Incorrect: "... the apparent digestibility coefficient of dry matter was 62.5%..."

Correct: "... the apparent digestibility coefficient of dry matter was 0.625..." (In this example, because it is a fractional measure, it is understood that it is expressed as g g^{-1} or kg kg^{-1}). Another possibility is to express as 625.0 g kg^{-1} of dry matter.

Measures of fractions in degradation assays or body fraction yields or microbial growth

Incorrect: "... estimate of potentially degradable insoluble fraction of protein was 36.2%..."

Correct: "... estimate of potentially degradable insoluble fraction of protein was 36.3 g/100 g..." Another possibility is to express it as 363.0 g kg^{-1} of crude protein.

Incorrect: "...average carcass dressing was 52.1% of body weight..."

Correct: "...average carcass dressing was 52.1 kg/100 kg of body weight..."

Incorrect: "... a microbial yield efficiency of 12.53% in comparison with intake of total digestible nutrients..."
Correct: "... a microbial yield efficiency of 125.3 g of microbial protein per kg of total digestible nutrients..."

Rates or variations over time in enzymatic measures or degradation assays or transit in the gastrointestinal tract
Incorrect: "... passage rate of fibrous material in rumen environment was 3.5%/h..."
Correct: "..... passage rate of fibrous material in rumen environment was 0.035 h^{-1}..." The number of decimal places to be presented should not exceed four; otherwise use scientific notation, i.e. $a \times 10^b$, or change the scale of measurements.

Coefficients of correlation and determination, and descriptive levels of probability
Coefficients of correlation and determination, and levels of probability are fractions and should not be expressed as percentage.

Incorrect: "... the coefficient of determination of the model was 92.53%..."
Correct: "... the coefficient of determination of the model was 0.9253..."

Incorrect: "... variables were strongly correlated (r = -82.39%)..."
Correct: "...variables were strongly correlated (r = -0.8239)..."

Incorrect: "... α = 5%."
Correct: "... α = 0.05."

3.3.4. Correct use of percentages

As previously highlighted, percentage should be used only for description of relative variations. And its use has to be done with parsimony.

Example:

Table 1 - Serum urea nitrogen concentrations (SUN, mg dL^{-1}) ... in grazing cattle

Item	Suplement[1]			CV (%)
	Control	Protein	Starch	
SUN	9.5b	14.3a	9.4b	7.8

[1] Means within rows followed by different letters are different by the Tukey test (P<0.05).

"...protein supplementation increased SUN concentration by 50.5% in relation to the control..."

3.4. Additional guidelines for style and units – Representation of dispersion

The clear, cohesive and correct representation of the results of a research paper is a key component of the characteristics that comprise comprehension, quality and reliability of the scientific publishing process.

However, the direct observation of the manuscripts submitted and the papers published by RBZ enlightens the plurality of the forms of exposure of the indicators of significance and dispersion (measures of uncertainty) of the results presented.

The Editorial Board of RBZ understands that the number of particularities in the form of exposing the results is directly proportional to the number of experimental designs and arrangements, as well as the number of statistical methods utilized.

Nevertheless, standard guidelines should and can be adopted by the authors in order to make the manner of exposure of the results more homogeneous. Thus, the guidelines presented below, which comprise the most common situations, must be followed by the authors for the correct establishment of the publishing style of Revista Brasileira de Zootecnia.

3.4.1. About the representation of the descriptive levels of probability for type I error (P-value)

Following the international trend of results exposure in research papers, the authors are recommended to present P-values from the statistical analyses to the readers, regardless of the critical level of probability adopted in the manuscript (α value). Whatever methods have been applied will not alter the discussion content at all. However, this makes the presentation of results more clear and allows the reader to make "judgments" on the results if they have a different view from that presented by the authors. Reference notes for significance (e.g., use of asterisks) should be avoided.

It is mandatory that the P-value be presented with three decimal places. It must not be displayed with 2 decimal places, for it can generate ambiguity of interpretation (e.g., let us suppose that one assumes α = 0.05. If two variables tested independently present P-values of 0.049 and 0.051, the rounding off for the two decimal places will make a P-value of 0.05 for both; however, one shows significant effect, whereas the other does not.)

3.4.2. About the critical level of probability (the α value) adopted in the manuscript and the significance representation throughout the text

For the right discernment between significance and non-significance in hypothesis testing, according to the Neyman-Pearson school there is the need for establishing a (maximum) critical level of probability acceptable for type I error, from which the differences must be assumed as non-significant, most commonly known as "α value". This must be properly exposed at the end of the description of the statistical procedures, because it is part of the methods set for the research paper.

Example: "...α = 0.05."

The choice of the α value must be done during the experimental planning, considering the factors inherent to the environment and the experimental material and the natural variability of the response variables to be assessed at the assay. Although the α value refers nominally to control of type I error, it must be pointed out that the probability of occurrence of types I and II errors commonly manifest antagonistically. Therefore, more strict α values (e.g., 0.01) represent a great control of type I error, but may reduce the level of control of type II error. This way, it is up to the researcher, after the proper experimental considerations, to define the priorities of control of the statistical errors in their conditions and to adopt the pertinent α level.

If an author chose to make assertions about significance or no significance based on the previous choice of α, the indication of significance must agree with that choice. For instance, let us take a study conducted with α = 0.05. In this study, the analysis of variance showed a P-value of 0.019. When presenting this to the reader in the text, the author must utilize: "...a difference was observed (P<0.05)."

For expressions in the text, use the letter P (capital letter), not in italic and without spaces. Example: "...intake increased (P<0.05), but there was no change in weight gain (P>0.05)." Additionally, for an RBZ's convention, the symbols ≤ or ≥ must not be used. Use only < or >. Do not use the form "P=0.XX".

The basic theory of hypothesis testing shows us the fact that there are two, and only two, distinct regions under a distribution of probability when this is utilized in the test: acceptance region of H0 and rejection region of H0 (or region of no rejection of H0 and region of no acceptance of H0, as some areas would rather use).

This leads us to the warning about two common mistakes involving the interpretation of significance: the use of the term "tendency" or "trend" and the qualification of significance (according to the Neyman-Pearson school).
To illustrate the first mistake, let us suppose that an author is conducting a research project in whose planning α = 0.05. At the analyses, for one of the variables, a P-value of 0.061 was observed. Due to the proximity of this value with the α value, the researcher presents in their text: "...for the X variable there was tendency for difference..."

Considering the summarized idea of tests and hypotheses presented previously, this type of argument is invalid, since there is no region of "tendency for acceptance of H0" or "tendency for rejection of H0". Thus, the value of the statistics calculated can only be included in the regions of "rejection" or "not rejection" of H0. In this sense, the proximity of the value to α does not matter, contrarily to which region the statistics' calculated value suits.

Otherwise, to illustrate the second mistake, let us take a research paper in whose planning α = 0.05. In this case, two variables presented at ANOVA, P-values of 0.035 and 0.002. Some may state that the first result is taken as significant, and the second as "highly" significant, which characterizes qualification. Again, there is the warning: the proximity between the values of P and α does not matter. Hence, there are no "little", "very", "highly" or "poorly" significant results, but only significant or non-significant.

However, there is an increasing tendency among authors worldwide to commingle the Fisher school with the Neyman-Pearson school, i.e., to present significance level and compromise statistical precision with body of evidence in rejecting or not rejecting the null hypothesis. The Fisher school is based on body or strength of evidence, which means that the lower the P-value, the stronger the evidence. By body of evidence we mean that for some reason, such as some experimental conditions that could be controlled but were not, or some variable or variables that are known to interfere on treatment effects but were not dealt with for some particular reason (cost, rain, drought, etc.), a researcher is not forced to conclude in favor of the maintenance of the status quo simply because he (she) found P=0.058. Therefore, we strongly suggest the presentation of the confidence intervals because they combine the magnitude of a treatment effect with the statistical precision and, as such, it circumvents the accept-reject dichotomy of the null hypothesis. Confidence intervals move us away from that dichotomy (Stang et al., 2010)[1].

[1] Stang, A.; Poole, C. and Kuss, O. 2010. The ongoing tyranny of statistical significance testing in biomedical research. European Journal of Epidemiology 25:225-230.

The probability that a continuous random variable equals any one value is ZERO. That's why confidence intervals are built, because instead of making inference about the true value of a parameter, we are now interested in inferring that the true value of the parameter lies within some interval, i.e., the confidence interval. For all practical applications this means that estimates have to be given as the estimate of the mean plus or minus a certain amount (Mood et al., 1974)[2]. Therefore,

$$P\left[\overline{x} - t_{1-\alpha/2}\sqrt{s^2/n} < \mu < \overline{x} + t_{1-\alpha/2}\sqrt{s^2/n}\right] = 0.95$$

means that the probability that the random interval $\left(\overline{x} - t_{1-\alpha/2}\sqrt{s^2/n}, \overline{x} + t_{1-\alpha/2}\sqrt{s^2/n}\right)$ covers the unknown true mean μ equals 0.95. The length of the interval is $2t_{1-\alpha/2}\sqrt{s^2/n}$ and is dependent on sample size (n) and sample variance (s^2). The statistics $t_{1-\alpha/2}$ is some statistics that could be computed from data and on the prior establishment of the significance level(α). Therefore, if authors want to present confidence intervals, they must previously define them. As possible examples we list:

".... the means were presented as $\overline{x}\left(\overline{x} - t_{1-\alpha/2}\sqrt{s^2/n}, \overline{x} + t_{1-\alpha/2}\sqrt{s^2/n}\right)$.";

"... and confidence intervals for the means presented as $\overline{x} \pm t_{1-\alpha/2}\sqrt{s^2/n}$.".

There are statistical softwares that present confidence intervals as outputs, and in such cases, the length of the intervals presented can be calculated as the *upper* minus the *lower* limits of the confidence interval. Therefore, provided that the assumption about the distribution of errors holds true, for a given statistics computed from the data, $t_{1-\alpha/2}\sqrt{s^2/n} = (upper - lower)/2$. For all cases reported above, s^2 = RMS, in which RMS is the residual mean square.

3.4.3. Suggestions of styles for the representation of P-values and dispersion indicators in Tables for the most common experimental designs and arrangements[3]

Balanced experiments with qualitative treatments, conducted without the adoption of experimental arrangements, and considering homogeneous variances among treatments

In these situations, this form of table is recommended:

Table 1 - Voluntary intake of animals fed a diet with different energetic sources

Item	Energetic source[1]			P-value	CV (%)
	Alpha	Beta	Gamma		
		kg d^{-1}			
Dry matter	6.301a	5.302b	5.892ab	0.036	5.3
...					
		g/kg of body weight			
Neutral detergent fiber	12.5a	10.4b	11.2b	0.045	4.8

[1] Means on the same row followed by different letters are different by the Tukey test (P<0.05).

In this example, the coefficient of variation (CV) is calculated as:

$$CV\ (\%) = \frac{\sqrt{RMS}}{\overline{Y}} \times 100$$

in which: RMS = residual mean square; and $\overline{Y}$ = overall mean obtained from all the observations.

Although CV is widely adopted in Brazil, there is a trend for its replacement in the international journals by the standard error of the mean. This also shows as reality for the users of PROC MIXED of SAS, which does not compute CV values for ANOVA. If this is the option for the authors, the tables can be put together as:

Table 2 - Total digestibility coefficients (g g^{-1}) of animals fed diets containing different energetic sources

Item	Energetic source[1]			P-value	SEM
	Alpha	Beta	Gamma		
Dry matter	0.605b	0.612b	0.669a	0.0172	0.035
...					

[1] Means on the same row followed by different letters are different by the Tukey test (P<0.05).

The standard error of the mean must be expressed with the same number of decimal places applied to the means, and can be represented in the table by the acronym "SEM" or by the notation $S_{\overline{x}}$. For the specific case of this example, SEM is calculated as:

$$S_{\overline{x}} = \frac{\sqrt{RMS}}{\sqrt{n}}$$

in which: RMS = residual mean square; and n = number of observations in each treatment.

It is important to emphasize that in case of supposition of homogeneous variances among treatments, only one indicator of variance must be presented; the indication of different standard errors to the different treatments is inconsistent with the presuppositions of the analyses.

[2] Mood, A. M.; Graybill, F. A. and Boes, D. C. 1974. Intoduction to the theory of statistics. McGraw-Hill Kogakusha, LTD., Tokyo.

[3] All the examples herein described are hypothetical. None of them was taken from real experimental situations.

Balanced experiments balanced with qualitative treatments, conducted without the adoption of experimental arrangements and considering heterogeneous variances among treatments

This type of experimental interpretation has become common with the evolution of the statistical software, especially with the utilization of PROC MIXED, from SAS. In this case, as different variances will be assumed among treatments, each treatment must be followed by its respective indicator of dispersion; in this case, the standard error may be used. Another possibility is to present the associated confidence intervals for treatment means.

Table 3 - Characteristics of the metabolism of nitrogen compounds in animals fed different protein sources

Item	Protein source[1]			P-value
	Omega	Pi	Kapa	
Serum urea nitrogen (mg dL^{-1})	12.35±1.36b	17.18±1.75a	18.54±0.98a	0.023
...				

[1] Means on the same row followed by different letters are different by the Tukey-Kramer test (P<0.05).

We stress that the indicator of dispersion presented in Table 1 is inherent to the treatment's mean (thence the association by the symbol ±). In this case, the standard error is mandatory (standard deviation must not be used). The presentation of the confidence intervals may offer a rather comprehensive data description.

Balanced experiments with quantitative treatments, conducted without the adoption of experimental arrangements and considering homogeneous variances among treatments

The differences between quantitative treatments must not be interpreted by means of conventional tests of multiple comparisons (e.g., Tukey, LSD, Duncan, SNK, Dunnett). Utilize appropriate tests of multiple comparisons (e.g., The Williams test) or utilize regression models (linear or nonlinear).

A common and usually efficient form to interpret can be achieved by performing orthogonal decomposition of the sum of squares for treatments in contrasts associated with the different order effects (e.g., linear, quadratic, cubic, etc.). This decomposition can be done through the adjustment of equation of linear regression corresponding to the highest significant order effect[4].

[4] When fitting the linear regression models, use the notation "r^2" (lowercase) for functions with a single independent variable (e.g., simple linear) and "R^2" (capital letter) for the functions with more than one independent variable or for polynomial models (e.g., quadratic).

In the case of orthogonal decomposition, it must be emphasized that experiments carried out with "p" levels (in the case above, four levels of additive in the diet; p = 4) provide evaluation of "p-1" order effects (in the example, p – 1 = 3; linear, quadratic and cubic).

The adoption of the maxim "models of cubic or superior order do not make sense" must be careful, and in some cases, this can distort the presentation and interpretation of results.

Example:

Table 4 - Performance characteristics of animals fed diets containing different levels of additive

Item	Additive (g kg^{-1} of dry matter)				CV (%)	P-value[1]		
	0	3	6	9		L	Q	C
Intake (g)[2]	125	135	147	152	3.8	0.015	0.225	0.567
...								

[1] L, Q and C - linear, quadratic and cubic effects, concerning the inclusion of additive in the diet.

[2] $\hat{Y} = 125.8 + 3.10 \times X$ ($r^2 = 0.976$).

In some cases where high-degree effects are not significant, one can proceed to its grouping in the interpretation of the experiment as "lack of fit", which can reduce the number of columns in the tables.

Example:

Table 5 - Performance characteristics of animals fed diets containing different levels of additive

Item	Additive (g kg^{-1} of dry matter)					CV (%)	P-value[1,2]		
	0	3	6	9	12		L	Q	LF
Intake (g)[3]	125	135	147	152	161	4.1	0.032	0.359	0.603
...									

[1] L and Q - effects of linear and quadratic order concerning the inclusion of additive in the diet.

[2] LF - lack of fit.

[3] $\hat{Y} = 126.2 + 2{,}966 \times X$ ($r^2 = 0.985$).

One example is shown in Figure 1, which simulates the interpretation of the concentration of rumen ammonia nitrogen as function of the time after feeding. Observing the points equivalent to the average concentrations obtained in each period, it can be easily seen that the concentration of ammonia nitrogen rises up to the point of highest concentration more intensely than it declines after this point. So, at the interval evaluated, the elevation and reduction of the concentration of ammoniacal nitrogen are asymmetric in relation to the point of maximum concentration. The interpretation of this by a model of second degree (quadratic) implicitly assumes that elevation and reduction happen with the same intensity, i.e., symmetrically in relation to the point

of maximum concentration (which ends up distorting the location of the maximum point). In this case, as it can be seen in Figure 1, the description is more coherent and logically done by function of the third degree (asymmetric in relation to the maximum point).

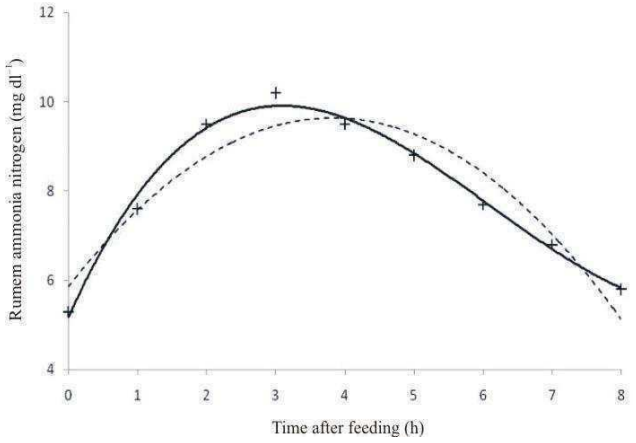

Figure 1 - Concentration of ruminal ammonia nitrogen as a function of the time after feeding (dashed line indicates quadratic function; continuous line indicates cubic function).

Balanced experiments with qualitative treatments, conducted with the adoption of experimental arrangements and considering homogeneous variances among treatments

The adoption of experimental arrangements (e.g., factorial, split plot) is common in experiments in the animal science area, and the information from their application must be adequately exposed to the reader.

As an example, in factorial arrangements the treatments are defined by the combination of the different levels (quantitative or qualitative) of the factors studied. They start to build the aim of studies in terms of their possible interaction or their direct (independent) effects, should they not interact with themselves, on the response variables. Hence, this piece of information (interaction and/or independent effects) must be presented coherently to the reader.

Example:

Table 6 - Voluntary intake in ruminants fed low quality forage and supplemented with nitrogen compounds and/or starch

Item	WN[1]		N[1]		SEM	P-value[2]		
	WS	S	WS	S		N	S	N × S
	g kg^{-1} of body weight							
NDFap[3]	11.2	10.5	12.8	12.0	1.1	0.003	0.046	0.485
...								

[1] WN - without nitrogen compounds; N - with nitrogen compounds; WS -without starch; S - with starch.
[2] N, S and N × S - effects of supplementation with nitrogen compounds, supplementation with starch and their interaction, respectively.
[3] Neutral detergent fiber corrected for ash and protein.

3.5. Additional guidelines for style and units – Abbreviation

The use of defined abbreviations and acronyms by the authors, especially for treatments, should be avoided. When necessary, the abbreviation should be defined the first time it is used in the summary (abstract) and again in the body of the manuscript.

No need to define symbols for chemical elements or simple compounds. Units of weights and measures conform to international standards; therefore it is incorrect to create new abbreviations.

Abbreviations in the titles and tables should be avoided. Long terms or expressions, which aesthetically do not fit as written in tables should be spelled out as footnote of the table or figure.

Example: "Average contents of dry matter (DM), crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (NDF), ether extract (EE), mineral matter (MM), organic matter (OM), total carbohydrates (TC), non-fiber carbohydrates (NFC), and total digestible nutrients (TDN) of the ingredients of the experimental diets."
Suggestion: "Chemical composition of the experimental diets"

Do not start a sentence with an abbreviation, acronym or symbol.
Wrong: "TC is a parameter that influences the final quality of the silage."
Suggestion: Total carbohydrate composition influences the final quality of the silage.

The use of abbreviations and acronyms in the summary should be limited. Too many abbreviations in the text makes it aesthetically cluttered and impairs the comprehension. The description by using abbreviations is appropriate for the author, but difficult to interpret for the reader, who will need to stop reading to verify the descriptions in the text.

Units of measure are not abbreviated when they follow a number in full at the beginning of a sentence.
Wrong: 2 L of water were added to the contents for analysis (...)
Suggestion: Two liters of water were added (...)
All abbreviations are written as singular, although they can be plural in the context (VFA instead of VFAs).
Abbreviations are generally not permitted in either the title or conclusions.

3.5.1. Abbreviations

AA = amino acid
AAI = essential amino acid(s)
ACTH = adrenocorticotropic hormone
ADDM = apparent digestibility of dry matter
ADF = acid detergent fiber
ADFI =average daily feed intake (differs from DMI)
ADG = average daily gain
ADIN = acid detergent insoluble nitrogen
ADL = acid detergent lignin
ADP = adenosine diphosphate
AI = artificial insemination
AIA = acid insoluble ash
AMP = adenosine monophosphate
ANOVA = analysis of variance
ATP = adenosine triphosphate
ATPase = adenosine triphosphatase
avg = average (use only in tables)
BCS = body condition score
BHBA = β-hydroxybutyrate
BLUE =best linear unbiased estimator
BLUP = best linear unbiased predictor
bp = base pair
BSA = bovine serum albumin
bST = bovine somatotropin
BTA = *Bos taurus* autosome
BUN = blood urea nitrogen
BW = body weight
CCW= cold carcass weight
cDNA = complementary deoxyribonucleic acid
CF = crude fiber
CI = confidence interval*
CLA = conjugated linoleic acid
CN = casein
CoA = coenzyme A
Co-EDTA = Cobalt ethylenediaminetetraacetate
CP = crude protein
cRNA = complementary ribonucleic acid
CV = coefficient of variation*
DCAD = dietary cation-anion difference
DE = digestible energy
df = degrees of freedom*
DFD(meat) = dark, firm, and dry
DIM = days in milk
DM = dry matter
DMI = dry matter intake
DNA = deoxyribonucleic acid
DNase = deoxyribonuclease
EBV = estimated breeding value
eCG = equine chorionic gonadotropin
ECM = energy-corrected milk
EDTA = ethylenediaminetetraacetic acid
EE = ether extract
EFA = essential fatty acid
EIA = enzymeimmunoassay
ELISA = enzyme-linked immunosorbent assay
EPD = expected progeny difference
ETA = estimated transmitting ability
FA = fatty acid
FCM = fat-corrected milk
FFA = free fatty acids
FSH = follicle-stimulating hormone
GAPDH = glyceraldehyde 3-phosphate dehydrogenase
GC-MS = gas chromatography-mass spectrometry
GE = gross energy
GH = growth hormone
GHRH = growth hormone-releasing hormone
GLC = gas-liquid chromatography
GLM = general linear model
GnRH = gonadotropin-releasing hormone
h2 = heritability*
hCG = human chorionic gonadotropin
HCW = hot carcass weight
HEPES = N-2-hydroxyethyl piperazine-N'-ethanesulfonic acid
HPLC = high performance (pressure) liquid chromatography
HTST = high temperature, short time
i.d. = inside diameter
i.m. = intramuscular
i.p. = intraperitoneal
i.v. = intravenous
IFN = interferon
Ig = immunoglobulin
IGF = insulin-like growth factor
IGFBP =insulin-like growth factor-binding protein
IL = interleukin
IMI = intramammary infection
IR = infrared reflectance
IVDMD = *in vitro* dry matter disappearance
LA = lactalbumin
LD50 = lethal dose 50%
LG = lactoglobulin
LH = luteinizing hormone
LHRH = luteinizing hormone-releasing hormone
Lig = lignin
LM = *longissimus*(*dorsi*) muscle
LPS = lipopolysaccharide
LSD = least significant difference*
LSM = least squares means*
mAb = monoclonal antibody
ME = metabolizable energy
MEn = metabolizable energy corrected for nitrogen balance
MIC = minimum inhibitory concentration
ML = maximum likelihood
MP = adenosine monophosphate

MP = metabolizable protein
mRNA = messenger ribonucleic acid
MS = mean square*
mtDNA = mitochondrial deoxyribonucleic acid
MUFA = monounsaturated fatty acids
MUN = milk urea nitrogen
n = number of samples*
NAD = nicotinamide adenine dinucleotide
NADH = reduced form of NAD
NADP = nicotinamide adenine dinucleotide phosphate
NADPH2 = reduced form of NADP
NAGase = N-acetyl-ß-D-glucosaminidase
NAN = nonammonia nitrogen
NDF = neutral detergent fiber
NE = net energy
NEFA = nonesterified fatty acids
NEg = net energy for gain
NEl = net energy for lactation
NEm = net energy for maintenance
NEm+p =net energy for maintenance and production
NEp = net energy for production
NFC = nonfiber carbohydrates
NPN = nonprotein nitrogen
NRC = National Research Council
NS = nonsignificant*
NSC = nonstructural carbohydrates
o.d. = outside diameter
OM = organic matter
PAGE = polyacrylamide gel electrophoresis
PBS = phosphate-buffered saline
PCR = polymerase chain reaction
pfu = plaque-forming unity
PG = prostaglandin
PGF2α = prostaglandin F2α
PMNL = polymorphonuclear neutrophilic leukocyte
PMSG = pregnant mare's serum gonadotropin
PSE = pale, soft, and exudative (meat)
PTA = predicted transmitting ability
PUFA = polyunsaturated fatty acids
QTL = quantitative trait loci
r = correlation coefficient*
R^2 = coefficient of determination*
RDP = rumen-degradable protein
REML = restricted maximum likelihood
RFLP = restriction fragment length polymorphism
RIA = radioimmunoassay
RNA = ribonucleic acid
RNase = ribonuclease
rRNA = ribosomal ribonucleic acid
RUP = rumen-undegradable protein
s.c. = subcutaneous
SCC = somatic cell count
SCM = solids-corrected milk
SD = standard deviation*
SDS = sodium dodecyl sulfate
SE = standard error*
SEM = standard error of the mean*
SFA = saturated fatty acids
SNF = solids-not-fat
SNP = single nucleotide polymorphism
sp., spp. = one species, several species
SPC = standard plate count
SS = sums of squares*
SSC = sus scrofa chromosome
SSPE = saline-sodium phosphate-edta buffer
ST = somatotropin
TCA = trichloroacetic acid
TDN = total digestible nutrients
TLC = thin layer chromatography
TMR = total mixed ration
Tris = tris(hydroxymethyl)aminomethane
TSAA = total sulfur amino acids
UF = ultrafiltration, ultrafiltered
UHT = ultra-high temperature
UV = ultraviolet
VFA = volatile fatty acids
wt = weight (use only in tables)

Physical units and other units

× = crossed with, times
°C = celsius (with number)
µ (prefix) = micro
µCi = microcurie
µE = micro-einstein
µF = microfarads
µg = microgram
µg kg^{-1} = parts per billion
µL = microliter
amu = atomic mass unit
atm = atmosphere
bp = base pair
ca. = circa
cal = calorie
cc, cm^3 = cubic centimeter
cfu = colony-forming unit
Ci = curie
cm = centimeter
cM = centimorgan
cm^2 = centimeter, square
cP = centipoise
cpm = counts per minute
cps = counts per second
CPU = central processing unit
cu = cubic

* Use generally restricted to tables and parenthetical expressions.

D = density
d = day(s)
Da = dalton
dL = deciliter
Eq = equivalents
g = gram
g = gravity
h = hour(s)
ha = hectare
Hz = cycles per second (hertz)
IU = international unit
J = joule
K = Kelvin
k (prefix) = kilo
kb = kilobase
Kbp = kilobase pair
KB = kilobyte
kcal = kilocalorie
keV = kiloelectron volts
kg = kilogram
kPa = kilopascal
KU = Klett units
L = liter
ln = logarithm (natural)
log10 = logarithm (base 10)
lx = lux
M (prefix) = mega
m (prefix) = milli
m = meter
M = molar (concentration)
mg kg^{-1} = parts per million
min = minute(s)
mL = milliliter
mM = millimolar (concentration)
mm Hg = millimeters of mercury
mm^3 = cubic millimeter
mmol = millimole (mass)
mo = month(s)
mol = mole (number, mass)
n (prefix)= nano
N = Newton
N = normal (concentration)
ng = nanogram
p (prefix) = pico
P = probability
Pa = Pascal
pfu = plaque-forming unit
pg = picogram
rpm = revolutions per minute
RU = rennet activity unit
s = second(s)
U = unit
use lx = foot-candle
use mmol kg^{-1} = osmolality
V = volt
vol = volume
vol vol^{-1} (use parenthetically) = volume/volume
W = Watt
wk = week(s)
wt vol^{-1} (use parenthetically) = weight/volume
yr = year(s)
Time: The 24h clock should be used, e.g.: 14.00 hours; 14.30 hours

4. Guidelines to submit the manuscript

4.1. The Manuscript Central™ online system

The journal editorial office of ***Revista Brasileira de Zootecnia*** is now using an online system, The Manuscript Central™, to manage the submission and peer review the manuscripts. Manuscript Central™ is a product of the ScholarOne® platform of Thomson Reuters (http://scholarone.com/).

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4.1.1. Authorship

The name and institutions of authors will be asked to be filled in the step 3 of the submission process, named Authors & Institutions; therefore it should not be presented in the body of the manuscript. The corresponding author should provide co-authors' information. Manuscript Central™ will help the corresponding author to check whether an author already exists in the journal's database, just by entering the author's e-mail address and clicking "Find." If the author is found, their information will be automatically filled out.

4.2. The cover letter

It is expected that the corresponding author writes a letter that explains the reasons why the editor would want to publish your manuscript.

See an example of what should go in this letter:

• Inform the title of the manuscript and the last name of the author;

• Primarily it is important to emblazon the relevance of the subject studied in a concise manner.

• If there is any novelty on your work, please report this to the editor. It is also important to stress the originality of the research, if it is the case.

• What is the main finding of the study?

• Additional results but less relevant shall be mentioned then.

• What is the implication of the findings of the study?

• Inform the editor if there is any patent related to your study.

• If any part of this study has already been published, tell the editor that this is the case of preliminary result, or only partial. Also inform the location, the event and the date of such publication. Otherwise, state that this is an original study that has not been published either in part or as a whole.

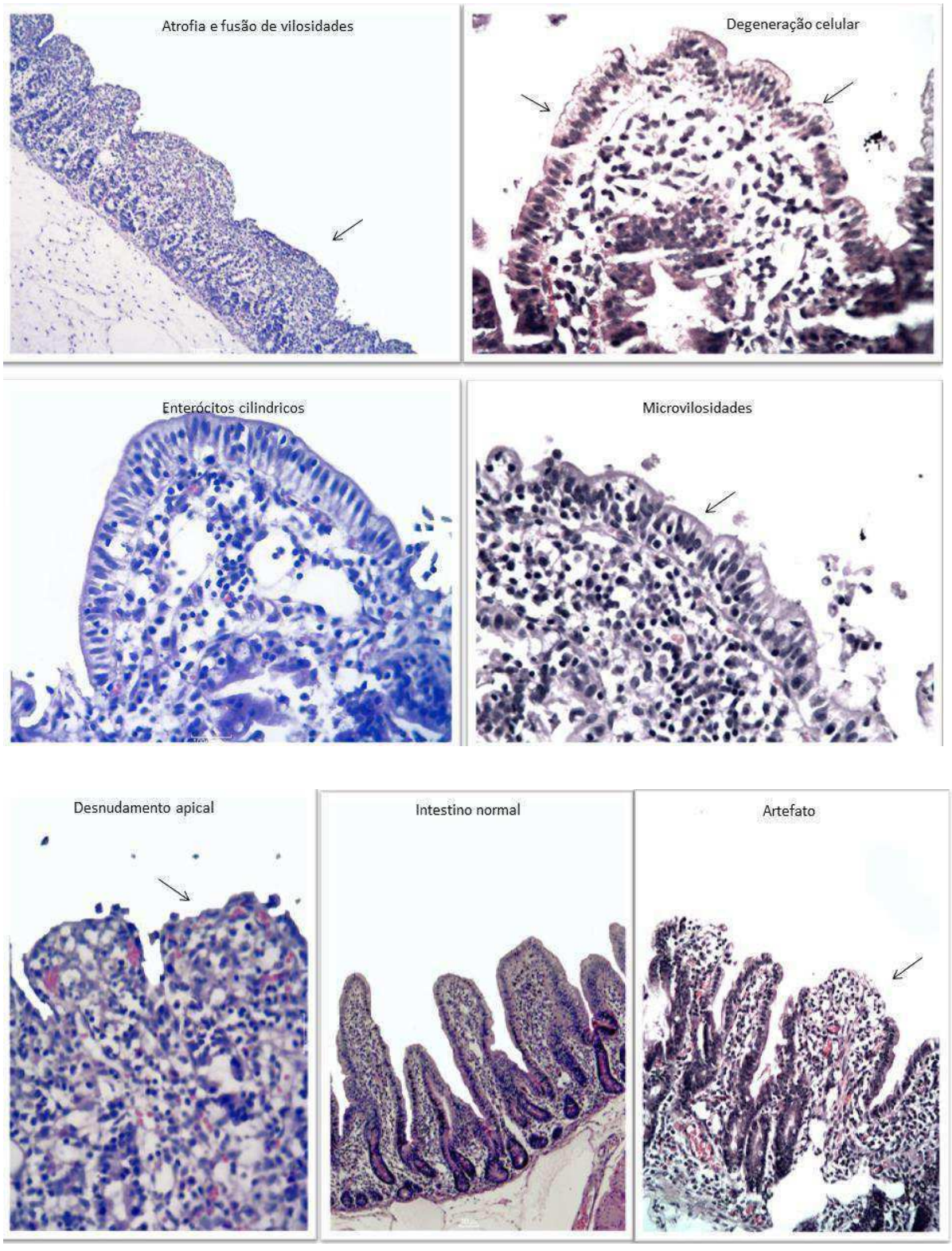
In the step 6 (File Upload) the corresponding author will be asked to upload a file containing the **Cover letter**. In that step of the submission process, please look for File upload, File designation, and then select Supplemental file NOT for review.

Files that ought to be sent besides the Main body: Figures, Tables, and Acknowledgments should be sent as separated file and not as part of the body of the manuscript.

The corresponding author is responsible for obtaining the signatures of all coauthors and send the Assurance of contents and assignment of copyright. Manuscript will not be considered for peer reviewing without this form. The deadline will be set allowing a period of 15 days for delivery of forms after which the editorial office act by withdrawing.

ANEXO 4

Fotos ilustrativas das alterações histológicas observadas para graduação do escore tecidual



Fonte: Basso et al. (2013)