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DÉBORA FURLAN RISSATO

# EFEITOS DA INGESTÃO CRÔNICA DE DIETA CONTAMINADA COM DESOXINIVALENOL SOBRE PARÂMETROS SANGUÍNEOS E JEJUNO DE RATOS WISTAR

Maringá, 2019

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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas (área de concentração Biologia Celular e Molecular) da Universidade Estadual de Maringá para obtenção do grau de Doutor em Ciências Biológicas.

Orientadora: Profª. Dra. Maria Raquel Marçal Natali

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# BIOGRAFIA

**Débora Furlan Rissato** nasceu em Umuarama/Pr em 09/11/1981. Possui graduação em Ciências Biológicas (2004) e mestrado no curso de Pós-Graduação em Ciências Biológicas, área de concentração Biologia Celular e Molecular (2006) pela Universidade Estadual de Maringá. Em 2015 ingressou no curso de Doutorado pelo mesmo programa. É professora assistente no Centro Universitário Ingá desde 2006, onde ministra a disciplina de Biologia Celular e Genética para os cursos de medicina e odontologia. Possui experiência nas áreas de Biologia Celular, Bioquímica, Genética, Histologia e Morfologia com ênfase nos temas: Micotoxinas, Sistema Nervoso Entérico e Morfologia Intestinal.

# DEDICATÓRIA

A todos os pesquisadores que, mesmo em meio a tantas dificuldades, são incansáveis na busca de descobertas que contribuam com o desenvolvimento da sociedade.

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# APRESENTAÇÃO

Esta tese é composta por dois artigos científicos:

O primeiro artigo intitulado por "Chronic ingestion of deoxynivalenol-contaminated diet dose-dependently decreases the area of myenteric neurons and gliocytes of rats", foi submetido à apreciação da revista Toxicology (ISSN: 0300-483X, Fator de impacto: 3,265), conforme carta de submissão em anexo.

O segundo artigo intitulado por: "Efeito pró-inflamatório e atrófico de dieta contaminada com baixas doses de desoxinivalenol sobre o jejuno de ratos" será submetido à apreciação da revista Nutritional Neuroscience (ISSN: 1476-8305, Fator de impacto: 3,313).

Em consonância com as normas do Programa de Pós-graduação em Ciências Biológicas, o primeiro artigo foi redigido de acordo com as normas da revista Toxicology e o segundo de acordo com as normas da revista Nutritional Neuroscience.

## RESUMO GERAL

**INTRODUÇÃO:** O desoxinivalenol (DON), micotoxina pertencente ao grupo B dos tricotecenos, é produzido principalmente por fungos do gênero *Fusarium*. Comumente encontrado em níveis elevados em todos os continentes é a micotoxina mais prevalente em culturas que constituem a base da alimentação humana e que são utilizadas na alimentação de animais. O sistema digestório é o primeiro e o principal alvo do DON, sendo que sua absorção ocorre primariamente no jejuno. Uma vez absorvido, o DON chega rapidamente a outros órgãos, atingindo em ordem decrescente de concentração, fígado, plasma, rim, baço, coração e cérebro. Desta maneira, o DON pode causar efeitos gastrointestinais, hepáticos, hematológicos, renais, esplênicos, cardíacos e neurais. Apesar de o DON afetar primariamente o sistema digestório e possivelmente atingir o sistema nervoso entérico (SNE), não existem estudos dos efeitos do DON sobre este. A ampla distribuição e os elevados níveis de contaminação por esta micotoxina têm levado vários países a regulamentar os níveis máximos de contaminação toleráveis para grãos e seus produtos derivados de consumo humano e animal. Vários trabalhos tem mostrado a ação do DON em doses superiores às regulamentadas como toleráveis para consumo, porém, testes de altas doses, nem sempre podem ser usados para prever os efeitos de baixas doses. **OBJETIVOS:** Os trabalhos que compõem esta tese tiveram por objetivo avaliar os efeitos de uma dieta contaminada com DON em concentrações que se encontram dentro dos valores determinados como limite máximo tolerável (LMT) para consumo. **MATERIAL E MÉTODOS:** No primeiro trabalho avaliou-se a ingestão alimentar, peso corporal, estado oxidativo e análises morfométricas de gliócitos e neurônios de gânglios mientéricos do jejuno de ratos Wistar. Para tanto, 40 ratos machos com 21 dias de idade foram distribuídos em cinco grupos que receberam, durante 42 dias, dieta contaminada com diferentes concentrações de DON (0; 0,2; 0,75; 1,75 e 2 mg/Kg de ração). No segundo trabalho avaliou-se a caracterização morfológica e o estado inflamatório do jejuno, assim como, parâmetros sanguíneos. Para isso, 24 ratos machos Wistar com 21 dias de idade foram distribuídos em três grupos e tratados por 42 dias, sendo, um grupo controle, um grupo que recebeu ração contaminada com DON na menor concentração estabelecida como LMT (0,2 mg/Kg) e um grupo que recebeu dieta contaminada com DON em concentração próxima as máximas estabelecidas como LMT (2 mg/Kg de ração). **RESULTADOS E DISCUSSÃO:** A menor concentração utilizada está próxima dos níveis de contaminação com DON

encontrados em vários países, como na farinha de milho da África (0,294 mg/kg), em 95% das amostras de pães e biscoitos avaliados na China (0,1533 mg/kg) e em 83% das amostras de trigo analisadas na Polônia (0,1402 mg/kg). Todas as concentrações estão dentro dos valores estabelecidos como limites máximos de contaminação permitidos por alguns países. A União Europeia estabelece como LMT de contaminação por DON para trigo duro não processado, 1,75 mg/kg; para cereais não processados, 1,25 mg/kg; para massas e cereais processados destinados ao consumo humano direto, 0,75 mg/kg e para alimentos destinados à crianças, 0,2 mg/kg. No Brasil, atualmente o LMT de DON para trigo não processado é 3 mg/kg, para cereais processados, 1,25 mg/kg, para massas e biscoitos, 1,00 mg/kg, e para alimentos destinados à crianças, 0,2 mg/kg. Durante o período experimental o DON não causou sinais clínicos aparentes de intoxicação como recusa alimentar, redução no ganho de peso e anorexia. Estes achados têm sido relatados na literatura quando são utilizadas concentrações mais altas desta micotoxina. Os animais também não apresentaram sinais clínicos indicadores de alterações gastrointestinais, como diarreia. Entretanto, a ingestão de dieta contaminada com DON, em concentrações consideradas toleráveis, 0,2; 0,75; 1,75 e 2 mg/Kg, promoveu redução na área do corpo celular, dependente da dose, em neurônios HuC/D-IR, nNOS-IR, ChAT-IR, NADH-diaforase positivos e em gliócitos S100-IR do plexo mientérico; alterações morfométricas significativas na parede do jejuno, como redução na altura da parede total, túnica mucosa, altura das vilosidades e profundidade das criptas, redução no número de células caliciformes e aumento da atividade da mieloperoxidase, um indicador de estado inflamatório. Estes dados comprovam que o quadro clínico aparente de intoxicação não revela o real efeito da exposição à micotoxina. A avaliação da densidade de gliócitos mostrou que, a ingestão da dieta contaminada com DON não reduz o número destas células no plexo mientérico de ratos. Esta preservação glial numérica pode ser atribuída à resistência desta população celular e a um mecanismo de defesa exercido pela glia na tentativa de promover a manutenção dos neurônios que permanecem viáveis. Esta hipótese pode ser sustentada, pelo fato de a ingestão da dieta contaminada com DON, nas concentrações utilizadas, não ter promovido redução no número de neurônios entéricos. Em contrapartida, a avaliação do perfil de gliócitos e de neurônios entéricos, mostrou redução na área de tais células de maneira dependente da concentração de DON presente na dieta, o que refletiu em redução na área ganglionar. Isto se deve, provavelmente, ao fato de que esta micotoxina interfere na capacidade de síntese proteica e consequentemente, na atividade metabólica destas células. Nas

concentrações utilizadas no presente trabalho, não houve alteração dos parâmetros oxidativos analisados. Trabalhos demonstrando que as alterações mitocondriais causadas pelo DON, podem alterar o estado oxidativo celular, foram realizados utilizando-se doses superiores às utilizadas em nosso trabalho. O DON geralmente penetra no organismo por via oral e subsequentemente atinge as células epiteliais intestinais, tendo um impacto significativo na integridade da barreira intestinal, o que pôde ser comprovado com a redução da altura da parede total, da túnica mucosa, das vilosidades, da profundidade das criptas e do número de células caliciformes verificadas neste trabalho. Verificou-se, por meio da avaliação da atividade da enzima mieloperoxidase, que a contaminação com 2 mg/Kg de DON também causa efeito inflamatório, pois houve aumento significativo da atividade da MPO e de acordo com a literatura danos causados pelo DON à integridade da parede intestinal podem permitir a passagem de antígenos e bactérias através das células epiteliais, gerando um efeito pró-inflamatório indireto. Entre os indicadores bioquímicos avaliados neste trabalho, somente alanina aminotransferase (ALT) e fosfatase alcalina (ALP) apresentaram alterações, o que está de acordo com a literatura que mostra que doses baixas desta micotoxina não promovem alterações em diversos parâmetros bioquímicos do sangue. No presente trabalho, houve redução nos níveis de ALT e ALP. De acordo com a literatura, estes dados apontam para processos patológicos no fígado no início da exposição à contaminação, com melhora adaptativa ao longo do consumo de dieta contaminada. As concentrações de DON no plasma refletem as concentrações de DON na dieta, porém, no decorrer de nossas análises, não foram observadas alterações quantitativas ou qualitativas nos elementos figurados do sangue. As células sanguíneas circulantes tendem a apresentar baixa sensibilidade à micotoxinas, sendo que, problemas hematológicos observados em casos de intoxicação, são devidos à mielotoxicidade e o DON apresenta baixa mielotoxicidade, o que justifica a sua baixa hematotoxicidade. **CONCLUSÃO:** Estes achados mostram que, mesmo na ausência de sinais clínicos aparentes de intoxicação, a exposição crônica ao DON tem efeitos negativos sobre gliócitos, neurônios e gânglios do plexo mientérico e causa alterações importantes no jejuno, o que poderia comprometer o funcionamento intestinal levando a redução da superfície absortiva e a defesa promovida pelas mucinas.

**Palavras chave:** Micotoxina, Neurônio Entérico, Gliócito Entérico, Estresse Oxidativo, Parede Jejunal, Componentes Sanguíneos, Estado Inflamatório.

## ABSTRACT

**INTRODUCTION:** Deoxynivalenol (DON), a mycotoxin belonging to group B of trichothecenes, is produced mainly by fungi of the genus *Fusarium*. DON is commonly found, at high levels, in all the continents, contaminating cultures that are the basis of human feeding and also used to feed animals. The digestory system is the first and major target of DON, as its absorption takes place primarily in the jejunum. Once absorbed, DON rapidly reaches other organs, at decreasing order of concentration: liver, plasma, kidney, spleen, heart and brain. In this way, DON can cause gastrointestinal, hepatic, hematologic, renal, splenic, cardiac and neural effects. Despite DON affecting primarily the digestion system and possibly reaching the enteric nervous system (ENS), the study of its effects is limited to the Central Nervous System. The wide distribution and the high levels of contamination by DON have prompted many countries to regulate the maximal levels of contamination tolerable for grains (and their derivatives) for human and animal intake. Several studies have been showing the action of DON at doses higher than those considered tolerable for intake; however, the testing of high doses cannot always be used to predict the effects of low doses. **OBJECTIVES:** The investigations of this thesis had the purpose of evaluating the effects of a diet contaminated with DON at concentrations within the maximum tolerable limit (MTL) for intake. **MATERIAL AND METHODS:** In the first work, food ingestion, body weight, oxidative status and morphometric analyses of gliocytes and neurons of jejunal myenteric ganglia were recorded. Male Wistar rats aging 21 days were allotted to five groups that were given, for 42 days, diets contaminated with different concentrations of DON (0, 0.2, 0.75, 1.75 and 2 mg/kg of chow). In the second work, the morphologic characterization and the inflammatory status of the jejunum were assessed, as well as blood parameters. For this purpose, 24 male Wistar rats aging 21 days were distributed in three groups and treated for 42 days: a control group, a group given chow contaminated with DON at the lowest concentration within the MTL (0.2 mg/kg chow) and another group given chow contaminated with DON at a concentration close to the MTL (2 mg/kg chow). **RESULTS AND DISCUSSION:** The lowest concentration is close to the levels of contamination with DON found in the literature for several countries, such as corn flour in Africa (0.294 mg/kg), in 95% of the samples of bread and cookies in China (0.1533 mg/kg) and in 83% of the wheat samples in Poland (0.1402 mg/kg). The concentrations are in accordance with the values established as

maximal limits of contamination by some countries. The European Union established as maximal tolerable limit (MTL) of DON contamination for non-processed wheat 1.75 mg/kg; for non-processed cereals 1.25 mg/kg; for pasta and processed cereals 0.75 mg/kg; for child feeding 0.2 mg/kg. In Brazil, the current MTL of DON for non-processed wheat is 3 mg/kg, for processed cereals 1.25 mg/kg, for pasta and cookies 1 mg/kg and child feeding 0.2 mg/kg. During the experimental period, DON did not cause visible clinical signs of intoxication such as food rejection, decreased body weight gain and anorexia. These findings have been reported in the literature when higher concentrations of the mycotoxin are used. In this work, the animals did not present clinical signs indicative of gastrointestinal alterations, such as diarrhea. However, the ingestion of the DON-contaminated diet, at concentrations regarded as tolerable, 0.2, 0.75, 1.75 e 2 mg/Kg, promoted a dose-dependent decrease in the area of the cell bodies of HuC/D-IR, nNOS-IR, ChAT-IR and NADH-diaphorase positive neurons and in S100-IR gliocytes of the myenteric plexus of this intestinal segment; significant morphometric changes of the jejunal wall were recorded, such as decreased thickness of the wall and of the mucosa, decreased villus height and crypt depth, reduced number of caliciform cells and increased activity of myeloperoxidase (MPO), an indicator of inflammatory status. These data show that the visible clinical picture of intoxication does not reveal the true effect of the exposure to the mycotoxin. In this work, the evaluation of the density of gliocytes showed that the ingestion of the DON-contaminated diet did not decrease the number of these cells in the myenteric plexus of rats. This glial preservation can be attributed to the resistance of the cell population and to a mechanism of defense exerted by the glia in an attempt to promote the maintenance of the still viable neurons. This hypothesis can be reinforced by the fact that the DON-contaminated diet, at the concentrations tested, did not promote a decrease in the number of enteric neurons. On the other hand, the evaluation of the profile of gliocytes and enteric neurons showed a decreased area of these cells that was proportional to the concentration of DON in the chow, with the consequent reduction in ganglion area. Possibly this is due to the fact that this mycotoxin interferes with the capacity of protein synthesis and therefore with the metabolic activity of these cells. At the concentrations used in this work, the oxidative parameters analyzed were not changed. Investigations demonstrating that the mitochondrial changes caused by DON can alter the cell oxidative status were carried out using doses higher than those of this work. DON usually gains access to the organism through oral ingestion and then reaches the

intestinal epithelial cells, where it has a significant impact on the integrity of the intestinal barrier, as indicated by the reduction in the thickness of the total wall and of the mucosa, in villus height, crypt depth and number of caliciform cells, recorded in this study. Through the activity of the enzyme MPO it was verified that the contamination with 2 mg/kg of DON also triggers an inflammatory effect, because there was a significant increase in MPO activity; according to the literature, injuries caused by DON on the intestinal wall may permit the passage of antigens and bacteria through the epithelial cells layer triggering an indirect pro-inflammatory effect. Among the biochemical markers assessed in this work, only alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were decreased, in accordance with literature data showing that low doses of this mycotoxin do not change several blood biochemical parameters. According to other studies, the decreased ALT and ALP point to pathological processes in the liver at the start of the exposure, with an adaptive improvement during the intake of the contaminated diet. The plasma concentration of DON reflects its concentration in the diet; however, during our analyses, quantitative or qualitative changes of blood cells were not observed. Circulating blood cells tend to have low sensitivity to mycotoxins; hematologic alterations observed during instances of intoxication are due to myelotoxicity, which is low for DON, explaining its low hematotoxicity. **CONCLUSIONS:** These findings show that, even in the absence of evident clinical signs of intoxication, the chronic exposure to DON has negative effects on gliocytes, neurons and ganglia of the myenteric plexus and causes important jejunal alterations that may impair intestinal functioning, decreasing the absorptive surface and the defense promoted by mucines.

**Keywords:** Mycotoxin, Enteric Neuron, Enteric Gliocyte, Oxidative Stress, Jejunal Wall, Blood Components, Inflammatory Status.

# CHRONIC INGESTION OF DEOXYNIVALENOL-CONTAMINATED DIET DOSE-DEPENDENTLY DECREASES THE AREA OF MYENTERIC NEURONS AND GLIOCYTES OF RATS

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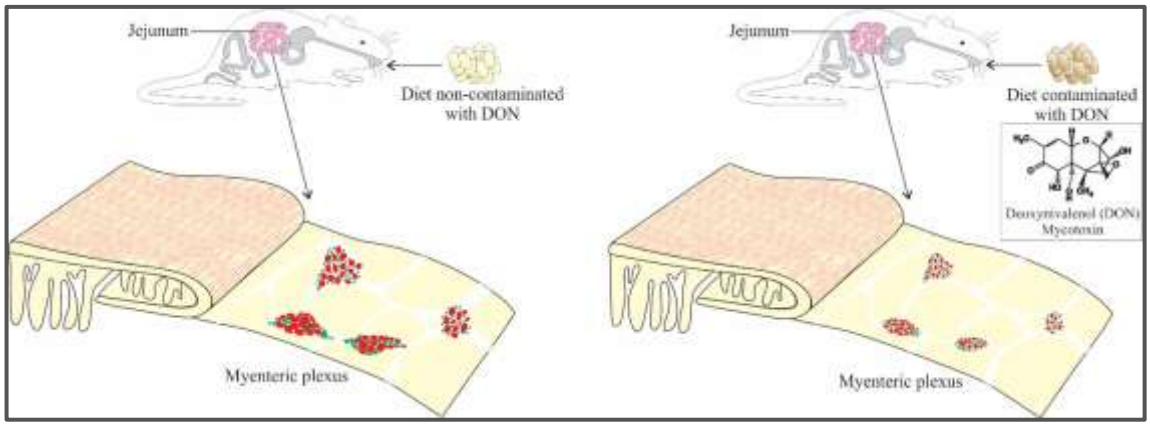
## Graphical Abstract

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**ABSTRACT**

Deoxynivalenol (DON), a mycotoxin produced by *Fusarium spp*, is commonly found in cereals ingested by humans and animals. Its ingestion is correlated with hepatic, hematologic, renal, splenic, cardiac, gastrointestinal and neural damages, according to dose, duration of exposure and species. In this work, the effects of the ingestion of DON-contaminated diet at concentrations considered tolerable for human and animal intake were assessed. Male Wistar rats aging 21 days were allotted to five groups that were given, for 42 days, diets contaminated with different concentrations of DON (0, 0.2, 0.75, 1.75 and 2 mg/kg of chow). Food ingestion, body weight, oxidative status and morphometric analyses of gliocytes and neurons of jejunal myenteric ganglia were recorded. At these concentrations, there was no food rejection, decrease of body weight gain, changes in oxidative status or loss of either neurons or gliocytes. However, even in the absence of visible clinical signs of intoxication, DON decreased gliocyte area, general neuronal population, nitrergic, cholinergic and NADH-diaphorase positive subpopulations and, as a result, ganglion area. It was concluded that, even at a subclinical stage of intoxication, DON had a negative effect on gliocytes and neurons of the myenteric plexus of the rat jejunum.

Keywords: Mycotoxin, Nitrergic Neuron, Cholinergic Neuron, NADH-Diaphorase Positive Neuron, Oxidative Stress.

## 1. Introduction

Deoxynivalenol (DON), a mycotoxin belonging to group B of trichothecenes, is produced mainly by fungi of the genus *Fusarium* (Schaafsma et al., 1998). It is a non-volatile, low-molecular-weight, water-soluble, high-temperature resistant compound that is not degraded by the conventional processes of food industrialization (Yazar and Omurtag, 2008). DON is commonly found, at high levels, in all the continents, contaminating cultures that are the basis of human feeding and also used to feed animals, such as barley, oat, rye, corn, rice and wheat, as well as their derivatives: bread, pasta and cookies (Janaviciene et al., 2018; Jiang et al., 2018; Lauren et al., 1991; Piacentini et al., 2018; Shephard et al., 2010; Sobrova et al., 2010).

The wide distribution and the high levels of contamination by DON have prompted many countries to regulate the maximal levels of contamination tolerable for grains (and their derivatives) for human and animal intake. The European Union established the maximal contaminations levels with DON from 0.2 to 1.75 mg/kg, according to the type of commercialized product (European Union, 2007, 2006). The limits found in these regulations are often extrapolated from experimental data, and the revision of these limits may be necessary as new experimental information is obtained.

The intensity of the effects due to exposure to DON depends on dose, route and duration of exposure and animal species (Bonnet et al., 2012). At extremely high acute doses (unlikely to be found in foods), DON can be lethal (Pestka, 2010). Through intraperitoneal and oral administration, the lethal doses for 50% of the individuals (LD50) are 49 and 78 mg/kg body weight for mice, respectively (Forsell et al., 1987). The chronic exposure to lower doses of this mycotoxin initially leads to decreased food intake, while exposure to higher doses causes food rejection, decreased body weight gain, vomiting and anorexia (Girardet et al., 2011; Reddy et al., 2018). However, these

initial adverse effects vary according to the animal species. Swine are considered the most sensitive to DON exposure, followed in decreasing order of sensitivity by mice, rats, birds and ruminants (Pestka, 2007).

Despite the diversity and complexity of the effects of DON intoxication, the only known binding site for trichothecenes is site A of the peptidyl transferase core of the 60S subunit of the eucariotic ribossome (Garreau de Loubresse et al., 2014). Thus, the primary mechanism of DON toxicity is inhibition of protein synthesis (Ehrlich and Daigle, 1987), triggering several dysfunctions such as impairment of shape and function of organelles, especially mitochondria (Bianco et al., 2012; da Silva et al., 2014; De Chiara et al., 2006), and oxidative stress (Bensassi et al., 2012; Kouadio et al., 2005; Osselaere et al., 2013).

The digestion system is the first and major target of DON, as its absorption takes place primarily in the jejunum (Avantaggiato et al., 2004). Once absorbed, DON rapidly reaches other organs, at decreasing order of concentration: liver, plasma, kidney, spleen, heart and brain (Pestka et al., 2008).

As for the effects of DON on the nervous system, the studies have focused on the search for an explanation for its anorexigenic and emetic effects. It is known that DON had cytotoxic effects on glial cells and can alter the synthesis of neurotransmitters and the activity of Central Nervous System (CNS) neurons (Payros et al., 2016). The enteric nervous system (ENS) is the most complex division of the peripheral nervous system and represents the major neural controlling mechanism of the gastrointestinal functioning (Furness, 2006). Together with the intestinal immune system, the ENS may give the first signs of the existence of hazardous agents in the food (Gonkowski et al., 2015), such as the presence of mycotoxins. In addition, changes in neuronal expression factors can be the first subclinical signs of intoxication by mycotoxins (Gonkowski et

al., 2015; Sousa et al., 2014). Despite DON affecting primarily the digestory system (Avantaggiato et al., 2004) and that its toxic effects described on the CNS possibly affect the ENS, until now no study assessed the effects of this mycotoxin on the ENS.

Taking this into consideration, in this work it was investigated the effects of the ingestion of DON-contaminated diet, at concentrations within the range of maximal tolerated limit (MTL) by the European Union, on body parameters, enteric neurons, enteric gliocytes and oxidative stress of the jejunum of Wistar rats.

## 2. Material and methods

### 2.1 Formulation of the diets

To prepare the basic diet (Table 1), corn, wheat bran and soy bean were ground separately and mechanically mixed to the other ingredients. To prepare the DON-added diets, sterilized culture medium of *F. graminearum* was added to the basic diet. The fungus lineage used was supplied by the Toxicogenic Fungi and Mycotoxins Laboratory of the University of São Paulo and the culture medium was produced at the Microbiology Laboratory of the Federal Technological University of Paraná – campus of Dois Vizinhos (UTFPR-DV). The diets were produced in the Factory of Chow of the UTFPR-DV. They were pelletized and dried in a forced ventilation stove (55 ºC) for 24 hours. The concentration of DON in the culture medium and in the experimental diets was determined by HPLC-MS and was adjusted through dilution and homogenization to produce experimental chows containing the doses of 0.2; 0.75; 1.75 and 2 mg of DON/kg chow.

### 2.2 Animals and experimental design

It was used 40 male Wistar rats (*Rattus novergicus*) aging 21 days and weighing

53,7 ± 4 g, obtained from the Central Animal House of the State University of Maringá. During the experimental period, the animals were kept in polypropylene boxes, with four animals per box, in the Animal House of UTFPR-DV under controlled temperature (23ºC ± 2ºC) and light-dark cycles of 12h-12h. The procedures with the animals followed the guidelines determined by the Brazilian Society of Science in Laboratory Animals (SBCAL/COBEA) and were approved by the Ethics Commission on Animal Experimentation of the UTFPR-DV, protocol 2014-04.

The animals were randomly assigned to five experimental groups, as follows: G0: fed with diet without DON addition (control); G0.2: fed with diet with the addition of 0.2 mg of DON/kg of chow; G0.75: fed with diet added with 0.75 mg of DON/kg of chow; G1.75: fed with diet added with 1.75 mg of DON/kg of chow; and G2: fed with diet added with 2 mg of DON/kg of chow.

Each experimental group had eight animals that were submitted to euthanasia after 42 days being fed as described above. During this period chow and water were freely supplied. Body weight and food intake were recorded weekly. Food intake was calculated by subtracting the amount of chow remaining from the total amount of chow given to the animals. These data were used to calculate the average intake of chow/100 g of body weight. The average intake and the mean values of concentration of DON in the diets were used to estimate the intake of DON/kg of body weight per day.

2.3 Euthanasia and tissue collection

The animals were intraperitoneally injected with Sodium Thiopental (Thionembutal® 120 mg/kg body weight). After laparotomy, the retroperitoneal and mesenteric fats were collected and weighed. The small intestine was removed, and length and width were recorded to calculate intestinal area. Samples of the jejunum of

each animal were collected and destined to immunohistochemical and histochemical analyses of the intrinsic intestinal innervation and evaluation of the oxidative status.

2.4 Analysis of the jejunal intrinsic innervation

2.4.1. Immunohistochemistry

The samples of jejunum were washed in PBS (0.1 M, pH 7.4), filled with paraformaldehyde 4% (pH 7.4), tied at the extremities and immersed on the same fixative for four hours at room temperature. Next, the segments were washed in PBS (0.1 M, pH 7.4) and 0.08% sodium azide (Sigma-Aldrich, St. Louis, USA) was added. In order to obtain whole-mounts, the samples were opened at the mesenteric border and micro-dissected to remove the mucosa and the submucosa. The whole-mount obtained was formed by the external muscular tunica, which harbors the myenteric plexus, and the serosa.

2.4.1.1 Double immunostaining with S100-HuC/D and nNOS-ChAT

The whole-mounts were subjected to double immunostaining with S100-HuC/D to detect HuC/D-immunoreactive (IR) proteins and S100-IR proteins, which stain the general population of immunoreactive neurons (HuC/D-IR) and enteric immuno-reactive gliocytes (S100-IR), respectively.

For analysis of the nitrergic and cholinergic immunoreactive subpopulations (nNOS-IR and ChAT-IR, respectively) the whole-mounts were subjected to double immunostaining (nNOS-ChAT) for the detection of the enzymes nitric oxide synthase (nNOS) and choline acetyl transferase (ChAT) of the myenteric neurons. The whole-mounts were washed twice in PBS (0.01 M, pH 7.4) containing 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, USA) for 10 minutes each wash and immersed for two hours

in a solution containing PBS (0.01 M, pH 7.4), 0.5% Triton X-100, 2% BSA (Sigma-Aldrich, St. Louis, USA) and 20% donkey serum to avoid unspecific links. Then the whole-mounts were incubated at room temperature and constant agitation for 48 hours in a solution containing PBS (0.01 M, pH 7.4), 0.08% sodium azide, 1% BSA, 10% donkey serum and primary antibodies (Table 2). After incubation, the whole-mounts were washed three times in PBS (0.01 M, pH 7.4) containing 0.5% Triton X-100 for five minutes each wash and incubated for two hours at room temperature with the secondary antibodies (Table 2) in a solution of PBS (0.01 M, pH 7.4), 0.08% sodium azide, 1% BSA and 10% donkey serum. The whole-mounts were then washed in PBS (0.01 M, pH 7.4) and placed in slides with coverslips with ProLong® Gold (Life Technologies, Carlsbad, USA) mounting medium for fluorescence immunohistochemistry. For each double staining a negative control was carried out without the primary antibodies.

### 2.4.2 Histochemistry for NADH-diaphorase

For the study of the subpopulation of neurons stained with NADH-diaphorase (NADH-diaphorase positive) it was used the histochemistry for the activity of the mitochondrial enzyme NADH-diaphorase (de Sousa and Neto, 2009; Odorizzi et al., 2010). Samples of jejunum were filled with Krebs solution (pH 7.3) and washed twice in this solution for 10 minutes each wash. Later, the samples were immersed for five minutes in Krebs solution containing 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, USA) and washed twice in Krebs solution for 10 minutes each wash. Next, the samples remained immersed for 45 minutes in an incubation bath containing 25 mL of 0.5% nitro blue tetrazolium (NBT) stock solution (Sigma, Steinheim, Germany); 25 mL phosphate buffer 0.1 M pH 7.3; 50 mL distilled water; and 50 mg of β-NADH (Sigma,

Steinheim, Germany). The reaction was interrupted by fixation of the samples in 10% formalin solution. The samples were kept in this fixative for at least 24 hours before being processed to obtain whole-mounts and mounted as permanent slides.

The samples were sectioned at the mesenteric border and whole-mounts of the external muscular tunica were obtained by micro-dissection of the mucosa and submucosa under stereomicroscope. The whole-mounts were dehydrated in increasing series of ethylic alcohol, cleared in xylene and mounted in slide with coverslip.

### 2.4.3 Morphoquantitative analysis of gliocytes and myenteric neurons

For the morphoquantitative analyses of myenteric gliocytes (S100-IR), general neuronal population (HuC/D-IR), nitrergic (nNOS-IR), cholinergic (ChAT-IR) and NADH-diaphorase positive neuronal subpopulations of the jejunum, the fluorescence microscope Olympus FSX-100 was used. The images were captured under 20x objective, transmitted to a microcomputer and analyzed by an image analysis system (Image Pro Plus 4.5 - Media Cybernetics, Inc., Silver Spring, MD, USA).

The counts of gliocytes and neurons were carried out through sampling in 50 microscopic images (7.22 $mm^2$) per animal which were randomly captured from the intermediate and antimesenteric regions (25 images of each region) of the intestinal circumference. The results were expressed as neurons/$cm^2$ and gliocytes/$cm^2$.

For the morphometric analysis the areas of 100 gliocytes and neuronal cell bodies per animal were measured at random from the intermediate and antimesenteric regions (50 of each cell type in each region). For the morphometric analysis of the ganglia, 50 ganglia (25 from the intermediate and 25 from the antimesenteric region) of the S100-HuC/D immunostained whole-mounts were measured. The results were expressed in $\mu m^2$.

2.5 Determinant parameters of the oxidative status

Samples of jejunum were washed in PBS (0.1 M, pH 7.4), frozen in liquid nitrogen and kept at -80ºC. Later, the samples were weighed and homogenized in 0.6 mL potassium phosphate buffer (200 mM, pH 6.5). Fifty µL of the homogenate were transferred to polypropylene tubes to determine the levels of non-protein sulfhydryl groups (GSH) and the remaining homogenate was centrifuged for 20 minutes at 9,000 x *g*. The supernatant was used to assess the activity of the enzymes superoxide dismutase (SOD) and glutathione s-transferase (GST) and the levels of lipidic hydroperoxides (LOOH).

2.5.1 Enzymatic activity of superoxide dismutase

The activity of SOD was determined according to the method of Marklund and Marklund (1974), based on the capacity of SOD of inhibiting the auto-oxidation of pyrogarol. The reactions were carried out in Tris HCl buffer (200 mM) containing EDTA (2 mM, pH 8.) at room temperature. In a polypropylene tube, 442.5 µL of Tris-HCl-EDTA buffer were added to 20 µL of sample. After vortex agitation, 25 µL of pyrogarol (1 mM) were added and the solution was incubated for 20 minutes. The reaction was interrupted with 12.5 µL of hydrochloric acid (HCl 1 N). The tubes were centrifuged for four minutes at 14,000 g and 300 µL of the supernatant were pipetted in microplaque for spectrophotometric reading (405 nm). The results were compared with the control (Tris-HCl-EDTA buffer with pyrogarol without incubation + medium without sample or incubation), being this value equal to 100%. The amount of protein that inhibited the reaction by 50% (IC50) equals one unit (1 U) of SOD). The results were expressed as U of SOD/mg protein.

2.5.2 Enzymatic activity of glutathione s-transferase

The activity of GST was determined according to the method of Habig et al. (1974). A volume of 225 µL of potassium phosphate buffer (0.1 M, pH 6.5) was added to 25 µL of the supernatant. One-hundred µL of the diluted sample were pipetted and added to 100 µL of the reaction solution in 96-wells plaque. For the preparation of the reaction solution, it was used 4950 µL of potassium phosphate buffer, 150 µL of 1-chloride-2,4-dinitrobenzene (CDNB) and 900 µL of GSH. The reading was carried out in spectrophotometer (340 nm) using 9.6 mM/cm as extinction coefficient. The results were expressed as µmol/min per mg protein.

2.5.3 Levels of non-protein sulfhydryl groups

The levels of GSH were determined by the method of Sedlak and Lindsay (1968). To denaturate and precipitate the proteins of the homogenate, 40 µL of 12% trichloroacetic acid (ATC) were added, agitated in vortex and centrifuged for 15 minutes at 9,700 g. Aliquots of 20 µL of the supernatant or of distilled water (as blank) with 290 µL of Tris 0.4 M buffer (pH 8.9) were placed in 96-wells plaques. The reaction was initiated with the addition of 5 µL of 5,5'-dithiobils-2-nitrobenzoic acid (DTNB) 1 mM five minutes before the reading in spectrophotometer (412 nm). The procedures were carried out at 4ºC and the individual values were interpolated into a standard GSH curve and expressed in µg GSH/g tissue.

2.5.4 Levels of lipidic hydroperoxides

The quantification of LOOH was carried out through the essay of iron II oxidation in the presence of orange xylenol (Jiang et al., 1991). Samples of the supernatant were diluted in methanol PA (1:4) and centrifuged for 30 minutes at 10,000

g (4 ºC). In a 96-wells plaque it was added 60 µL of the supernatant and 240 µL of the reaction medium incubated for 30 minutes in the dark at room temperature. The reading was carried out in spectrophotometer (560 nm) using 4.3 mm/cm as extinction coefficient. The results were expressed as mmol/mg tissue.

2.6 Statistical analyses

The numerical data were evaluated for normality through Kolgomorov-Smirnof or Shapiro-Wilk. Non-parametric data were analyzed through Kruskal-Wallis followed by Dunns *post-hoc* test. Parametric data were subjected to ANOVA followed by Tukey *post-hoc* test. These were carried out with the software Graph Pad Prism® 6.0 (GraphPad Software, Inc., San Diego, California, USA). The significance level adopted was 5% and the results were expressed as mean ± standard error.

## 3. Results

3.1 Food ingestion, body weight and small intestine area

During the experimental period the average daily intake of DON was estimated as 0.0145, 0.0584, 0.141 and 0.157 mg/kg body weight for groups G0.2, G0.75, G1.75 and G2, respectively. During this period none of the animals died or showed apparent clinical signs of intoxication, such as diarrhea, anorexia, food rejection or decreased body weight gain. The contamination with DON had no significant effect on chow intake, body weight, retroperitoneal and mesenteric adipose tissue weight and mean area of the small intestine of the animals of the different groups (Table 3).

3.2 Morphoquantitative analysis of the myenteric plexus

The ingestion of the DON-contaminated diet did not change the ganglionar organization. The S100-HuC/D double immunostaining showed gliocytes located in the interneuronal gaps inside the ganglia (Fig. 1B,C). The nNOS/ChAT double immunostaining evidenced the nitrergic subpopulation predominantly on the peripheral region and the cholinergic subpopulation predominantly on the central region of the same ganglion (Fig. 2G,J).

As for the relative density of the gliocytes (S100-IR), significant differences were not observed across the groups (Fig. 3A). Neither there was alteration of the neuronal density in the general population of neurons (HuC/D-IR) (Fig. 3B), nitrergic (nNOS-IR) (Fig. 2A), cholinergic (ChAT-IR) (Fig. 2B) and NADH-diaphorase positive (Fig. 4A) subpopulations.

However, the ingestion of the DON-contaminated diet, at all the concentrations evaluated, had a negative effect by decreasing the gliocyte area (Fig. 3C) and the cell body area of the general population of neurons (fig. 3D), nitrergic (Fig. 2C), cholinergic (Fig. 2D) and NADH-diaphorase positive neurons (Fig. 4B). This decrease in gliocyte area and cell body area was matched by a significant decrease in ganglion area when the general population of neurons was considered (Fig. 1A).

### 3.3 Tissue oxidative status

The enzymatic activity of SOD and GST, the level of non-protein sulfhydryl groups (GSH) and lipidic hydroperoxides (LOOH) indicate that the ingestion of the diet contaminated with increasing concentrations of DON did not cause changes in the oxidative status of the animals (Fig. 5A,B,C and D).

## 4. Discussion

In this investigation it was used diets contaminated with DON at concentrations of 0.2, 0.75, 1.75 and 2 mg/kg. The lowest concentration is close to the levels of contamination with DON found in the literature for several countries, such as corn flour in Africa (0.294 mg/kg) (Shephard et al., 2010), in 95% of the samples of bread and cookies in China (0.1533 mg/kg) (Jiang et al., 2018) and in 83% of the wheat samples in Poland (0.1402 mg/kg) (Bryła et al., 2018). The concentrations are in accordance with the values established as maximal limits of contamination by some countries. The European Union established as maximal tolerable limit (MTL) of DON contamination for non-processed wheat 1.75 mg/kg; for non-processed cereals 1.25 mg/kg; for pasta and processed cereals 0.75 mg/kg; for child feeding 0.2 mg/kg (European Union, 2007, 2006). In Brazil, the current MTL of DON for non-processed wheat is 3 mg/kg, for processed cereals 1.25 mg/kg, for pasta and cookies 1 mg/kg and child feeding 0.2 mg/kg; in 2019, a new regulation shall be instituted with the aim of decreasing these values to 1 mg/kg for processed cereals and 0.75 mg/kg for pasta, cookies and bakery products (Brazil, 2017).

The animals were exposed for 42 days with DON-contaminated diet, characterizing a chronic exposure to this mycotoxin, once acute exposure is infrequent (Pestka, 2010) and difficult to characterize due to the large amount of food that the animal should ingest to simulate such exposure (Sundheim et al., 2017).

During the experimental period, DON did not cause visible clinical signs of intoxication such as food rejection, decreased body weight gain and anorexia. These findings have been reported in the literature when higher concentrations of the mycotoxin are used, as demonstrated by Payros et al. (2017) using female rats treated for 1-4 weeks with diet contaminated with 10 mg/kg of DON. In that instance, the

animals showed a significant decrease of body weight gain (Payros et al., 2017). The level of contamination with DON without observable adverse effects (NOAEL) for rodents was estimated as 0.1 to 0.15 mg/kg body weight per day (Pestka, 2007), a range that encompasses the highest daily doses (0.141 and 0.157 mg/kg body weight per day) actually ingested by the animals of the present study. Similar results were found in a study with rats exposed to 0.03 mg/kg body weight per day of DON for 14 days, in which food ingestion and body weight gain did not change (Szabó et al., 2017). Swine are considered the most sensitive animals to DON exposure; however, when they chronically ingest a diet contaminated with DON, at concentrations close to those used in this work, they do not exhibit changes in body weight gain (Accensi et al., 2006; Bracarense et al., 2012). This reinforces the notion that the ingestion of low doses of DON has no effect on food ingestion and body weight gain.

The digestory system is the first and major target of DON, especially the jejunum, which is responsible for 44% of the absorption of this mycotoxin (Avantaggiato et al., 2004). In this work, the animals did not present clinical signs indicative of gastrointestinal alterations, such as diarrhea, and the measures of the organ were preserved. However, the ingestion of the DON-contaminated diet, at concentrations regarded as tolerable, promoted a dose-dependent decrease in the area of the cell bodies of HuC/D-IR, nNOS-IR, ChAT-IR and NADH-diaphorase positive neurons and in S100-IR gliocytes of the myenteric plexus of this intestinal segment. Due to the anorexigenic effects under certain circumstances of intoxication by DON, several authors have been studying the effects of the exposure to this mycotoxin on the Central Nervous System (Girardet et al., 2011; Maresca, 2013; Razafimanjato et al., 2011), and little is known about its action on the peripheral nervous system. Although some evidence on the effects of DON on cerebral structures and brain neurochemistry

have been described, the routes signaling the presence of this mycotoxin from the periphery (gastrointestinal tract and blood) to the brain (Bonnet et al., 2012) are poorly understood.

In this work, the evaluation of the density of gliocytes showed that the ingestion of the DON-contaminated diet did not decrease the number of these cells in the myenteric plexus of rats. This glial preservation can be attributed to the resistance of the cell population and to a mechanism of defense exerted by the glia in an attempt to promote the maintenance of still viable neurons. Experimental evidence suggests that the enteric glial cells are essential to keep the homeostasis of the enteric neurons (Aubé et al., 2006; Pereira et al., 2011).

This hypothesis can be reinforced by the fact that the DON-contaminated diet, at the concentrations tested, did not promote a decrease in the number of enteric neurons. This is in accordance with the results of Sousa et al. (2014), that evaluated the effects of Fumonisin, a mycotoxin also produced by fungi from the genus *Fusarium*, on the general population and nitrergic subpopulation of neurons in rats. As myenteric neurons are non-renewable cells (de Souza et al., 1993), it can be stated that feeding with DON-contaminated diet, at the concentrations tested, does not cause neuronal death in the myenteric plexus of rats.

On the other hand, the evaluation of the profile of gliocytes and enteric neurons showed a decreased area of these cells that was proportional to the concentration of DON in the chow, with the consequent reduction in ganglion area. Possibly this is due to the fact that this mycotoxin interferes with the capacity of protein synthesis (Ehrlich and Daigle, 1987; Ueno, 1986) and therefore with the metabolic activity of these cells (Bin-Umer et al., 2011; Kouadio et al., 2005). These data show that, in the myenteric plexus, the gliocytes, the general population of neurons and the cholinergic, nitrergic

and NADH-diaphorase positive subpopulations are affected by the chronic exposure to DON, even at concentrations that do not cause visible clinical signs of intoxication.

Pestka et al. (2008) demonstrated that the concentration of DON found in the brain of mice after exposure to this mycotoxin represents 10% the concentration found in other tissues, and Razafimanjato et al. (2011) found that, even at low doses, DON decreases the viability of cultured glial cells from the Central Nervous System, thus compromising the brain homeostasis in rats. As the enteric glia shares many structural and functional similarities with the astrocytes of the Central Nervous System (Yu and Li, 2014), these investigations give support to our findings of reduced profile of the gliocytes because the jejunum was evaluated, which is the primary site of action of DON.

Studies in animal models demonstrated that the alterations of the glial cells promote changes in the neurochemical coding of the jejunal enteric neurons (Aubé et al., 2006), but the literature lacks data on the direct action of DON in ENS neurons, making it difficult the comparison with the data obtained in this work. Sousa et al. (2014) investigated the action of fumonisin on the ENS, and similarly to our research group working with DON, they found a reduction of the profile of the enteric neurons exposed to the mycotoxin, which was attributed to a negative effect of Fumonisin on neuroplasticity.

According to Scafuri et al. (2017), DON interferes with synaptic plasticity by modulating the activity of acetylcholinesterase. The reduction of the cell body profile of the subpopulation of cholinergic neurons observed in this work is one more contribution to the evidence reported in the literature (Egbunike and Ikegwuonu, 1984) that exposure to mycotoxins interferes with cholinergic transmission.

The subpopulation of nitrergic neurons encompasses inhibitory motor neurons. Therefore, changes in these neurons can be related to changes in intestinal motility (Kunze and Furness, 1999) and to a reduced rate of gastric emptying (Aubé et al., 2006). Fioramonti et al. (1993) verified that DON decreases the rate of gastric emptying in a dose-dependent fashion, and that higher doses of the toxin decrease small intestinal peristalsis through a direct action on the gastrointestinal tract that is independent of the Central Nervous System. However, those authors did not assess the effects of this mycotoxin on the number and profile of the ENS neurons. Therefore, the reduced profile of the nitrergic enteric neurons observed in this work could explain the action of DON of decreasing the rate of gastric emptying.

The work of Gajęcka et al. (2013) sustains the hypothesis above, because these authors verified that the exposure to DON-contaminated diet during 42 days, at levels below the NOAEL, inhibits the gene expression of nitric oxide synthase in the jejunum. They suggest that the exposure to low levels of DON contamination for a prolonged period can modify the gastrointestinal functions because of the diminished concentrations of nitrergic neurotransmitters and that the reduced levels of nitric oxide delay gastric emptying.

The subpopulation of neurons stained with NADH-diaphorase also displayed reduced cell body area. This can indicate a reduced activity and/or expression of the enzyme, NADH diaphorase, as Bin-Umer et al. (2011) and Kouadio et al. (2005) observed that exposure to DON induced, in a time- and dose-dependent manner, a decrease in mitochondrial protein synthesis. Therefore, it is possible to suggest that DON affects mitochondrial metabolism, leading to a decreased metabolic activity of the myenteric neurons and impairing neuronal growth.

In addition, Bin-Umer et al. (2011) showed that the trichothecenes alter the mitochondrial membrane potential and lead to the formation of reactive oxygen species (ROS), but only at doses higher than those affecting mitochondrial translation. At the concentrations used in this work, the oxidative parameters analyzed were not changed. Springler et al. (2017) measured the oxidative status in intestinal cell cultures exposed to DON e did not find increased ROS, although they did observe altered mitochondrial morphology. Investigations demonstrating that the mitochondrial changes caused by DON can alter the cell oxidative status were carried out using doses higher than those of this work. Osselaere et al. (2013) verified that this mycotoxin causes oxidative stress in the small intestine of chicken after exposure to DON at 7.54 mg/kg. Wu et al. (2014) demonstrated, in swine, that the ingestion of chow contaminated with 4 mg/kg of DON causes evident oxidative stress. In this way, the effects of DON on the formation of free radicals and on anti-oxidant defenses depend on dose and length of exposure.

## Conclusion

The ingestion of DON-contaminated diet, at the concentrations regarded as tolerable for human and animal intake, decreases the cell area of gliocytes, of the general population of neurons, of nitrergic, cholinergic and NADH-diaphorase positive subpopulations and, therefore, leads to a decreased ganglion area. These findings show that, even in the absence of evident clinical signs of intoxication, the chronic exposure to DON has negative effects on gliocytes, neurons and ganglia of the myenteric plexus.

Ethic conduct of research

All the procedures with the animals were carried out only after approval by the Ethics Commission on Animal Experimentation of the Institution.

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**Table 1.** Chemical and percentual composition of the experimental chow.

| Component | (%) |
|---|---|
| Corn | 33.63 |
| Soybean meal | 33.10 |
| Wheat bran | 27.20 |
| Soybean oil | 2.30 |
| Calcitic limestone | 2.07 |
| Bicalcic phosphate | 0.73 |
| Salt (iodized) | 0.50 |
| Vitamin premix[1] | 0.10 |
| Mineral premix[2] | 0.35 |
| BHT[3] | 0.02 |

[1]Vitamin supplement, composition/kg: Folic Acid=200mg; Nicotinic Acid=3000 mg; Biotin=20mg; Calcium Pantothenate=1600mg; Chloride Pyridoxin=700mg; Riboflavin=600mg; Chloride Thiamine=600mg; Vitamin A=4000.000UI; Vitamin B12=2500mg; Vitamin D3=100.000UI; Vitamin E=100.000UI; Vitamin K1=75mg.
[2]Mineral supplement, composition/kg: Boron=14.26mg; Calcium=142.94g; Chloride=44.9g; Copper=72.41mg; Chromium=28.65mg; Sulfur=8.6g; Iron=1000mg; Fluorine=28.72mg; Phosphorus=56.9g; Iodine=5.95mg; Lithium=2.85mg; Magnesium=14.48g; Manganese=300mg; Molybdenum=4.32mg; Nickel=14.31mg; Potassium=102.86g; Selenium=4.28mg; Silicon=143.26mg; Sodium=29.38mg; Vanadium=2.87mg; Zinc=860mg.
[3]Botuil hydroxy toluene.

**Table 2.** Characteristics of the primary and secondary antibodies used for immunohistochemical analysis.

| Primary Antibody | Supplier/ Reference | Dilution | Secondary Antibody* | Reference |
|---|---|---|---|---|
| Anti-HuC/D (mouse) | Invitrogen, USA (Cat# A21271, AB_10562207 | 1:400 | Alexa Fluor® 546 (anti-mouse) | Cat#A10036 AB_2534012 |
| Anti-nNOS (rabbit) | Santa Cruz Biotec. (Cat# BIN460169, AB_10789440 | 1:500 | AlexaFluor® 488 (anti-rabbit) | Cat#A21206, AB_10049650 |
| Anti-ChAT (goat) | Millipore (Cat# AB144P, AB_11214092) | 1:200 | Alexa Fluor® 546 (anti-goat) | Cat#A-11056 AB_2534103 |
| Anti-S100 | Sigma, USA (Cat# ABIN337109, AB_10825839) | 1:200 | Alexa Fluor® 488 (anti-rabbit) | Cat#A21206, AB_10049650 |

*The secondary antibodies were used at a dilution of 1:500 and supplied by Invitrogen, USA.

**Table 3.** Food ingestion, body weight and weight of the retroperitoneal and mesenteric fat pads of Wistar rats fed with chow without addition of DON (G0) or with addition of DON at 0.2 mg/kg (G0.2), 0.75 mg/kg (G0.75), 1.75 mg/kg (G1.75) or 2 mg/kg (G2).

| GROUPS | G0 | G0.2 | G0.75 | G1.75 | G2 |
|---|---|---|---|---|---|
| Food ingestion (g/100g body weight per week)[1] | 54.3±2.25 | 50.6±2.69 | 54.5±3.47 | 56.4±3.53 | 55.1±3.23 |
| Initial body weight (g)[2] | 54.2±1,25 | 53.7±0,70 | 53.0±0,75 | 52.8±1,00 | 53.5±0,94 |
| Final body weight (g)[1] | 277.5±4.53 | 266.3±3.73 | 260.5±2.96 | 273.2±3.64 | 267.0±3.77 |
| Body weight gain (%)[1] | 509.5±13.4 | 496.3±1.98 | 497.8±5.72 | 515.8±7.49 | 498.8±5.10 |
| Retroperitoneal fat weight (g)[1] | 1.25±0.11 | 1.22±0.09 | 1.12±0.13 | 1.36±0.08 | 0.96±0.04 |
| Mesenteric fat weight (g)[2] | 1.1±0.05 | 1.12±0.02 | 1.13±0.07 | 1.22±0.07 | 0.96±0.07 |
| Small intestine area ($cm^2$)[2] | 142.7±5.94 | 158.7±20.58 | 147.3±5.29 | 147.0±6.32 | 140.4±5.18 |

[1]One-way ANOVA followed by Tukey *post-hoc* test ($p<0.05$). [2]Kruskal-Wallis followed by Dunns *post-hoc* test ($p<0.05$). Data shown as mean ± standard error (n= 8).

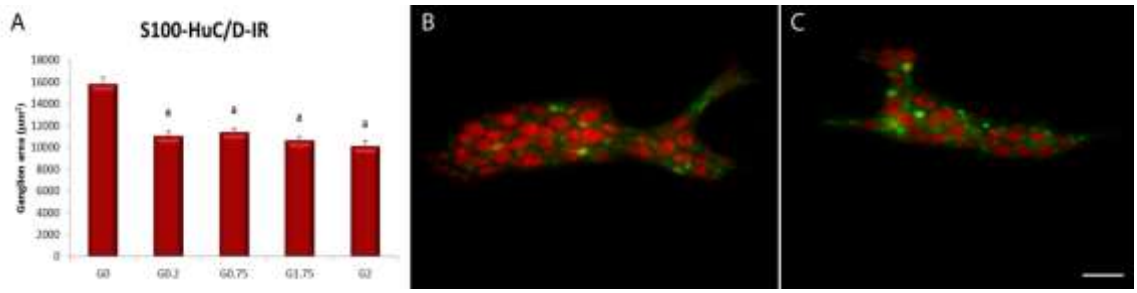


**Fig. 1** S100-HuC/D immunoreactive ganglia of the myenteric plexus of the jejunum of Wistar rats. (A) Mean area of the ganglia. (B) Ganglion from group G0. (C) Ganglion from group G2. Calibration bar = 50 μm. [a]Significant difference with group G0. Kruskal-Wallis followed by Dunns *post-hoc* test ($p<0.05$). Data expressed as mean ± standard error (n=7-8).

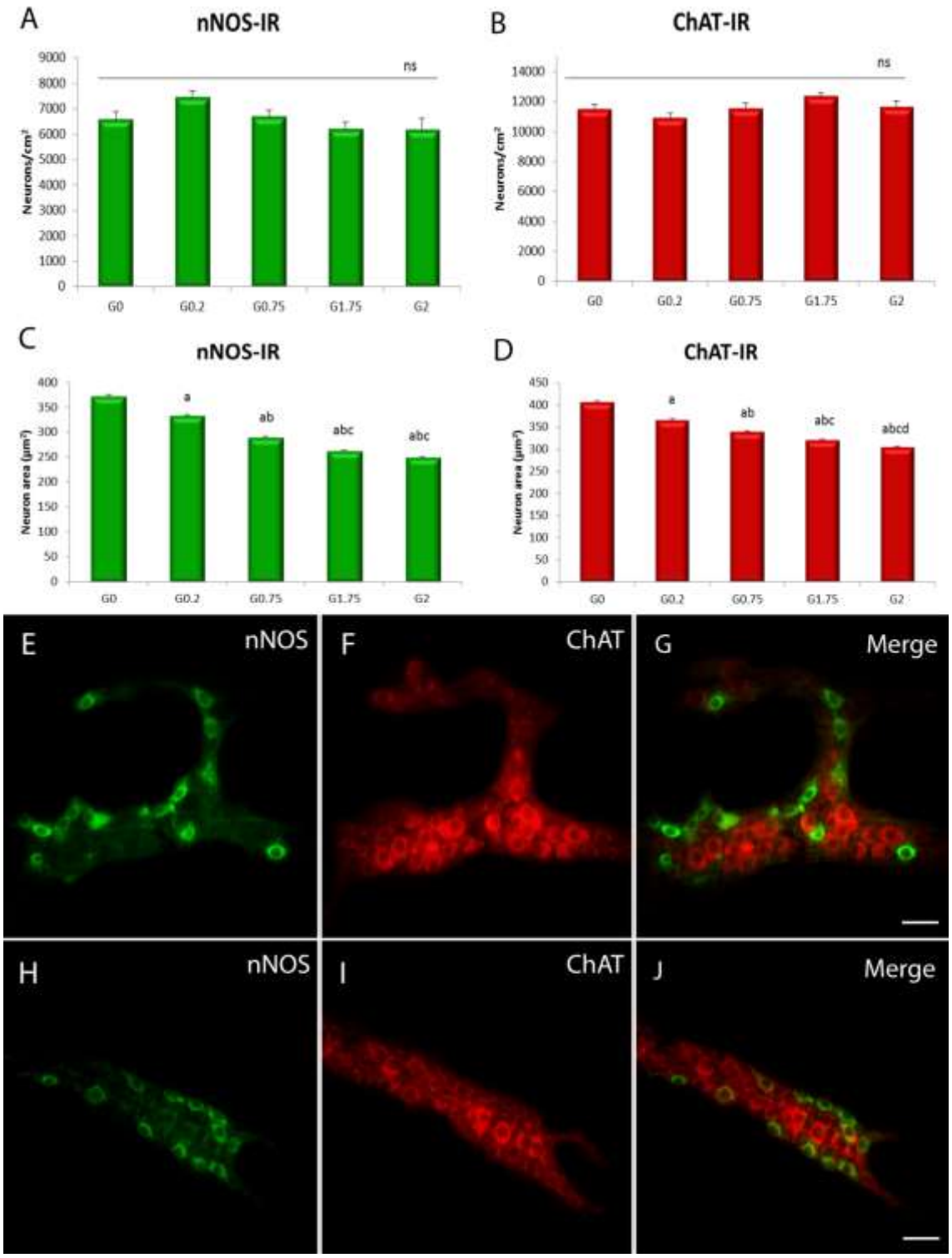


**Fig. 2.** Neurons of the myenteric plexus of the jejunum of Wistar rats. (A,B) Density of the nitrergic[2] (A) and cholinergic[1] (B) subpopulations. (C,D) Mean area of the cell body of neurons of the nitrergic[2] (C) and cholinergic[2] (D) subpopulations. (E,F,G) Nitrergic immunoreactive (E) and cholinergic immunoreactive (F) neurons, and double immunostaining nNOS-ChAT in group G0. (H,I,J) Nitrergic immunoreactive (H) and cholinergic immunoreactive (I) neurons, and double immunostaining nNOS-ChAT in group G2. Calibration bar = 50 µm. [a]Significant difference with group G0. [b]Significant difference with group G0.2. [c]Significant difference with group G0.75. [d]Significant difference with group G1.75. [ns]Non significant. [1]One-way ANOVA followed by Tukey *post-hoc* test. [2]Kruskal-Wallis followed by Dunns *post-hoc* test ($p<0.05$). Data expressed as mean ± standard error (n=7-8).

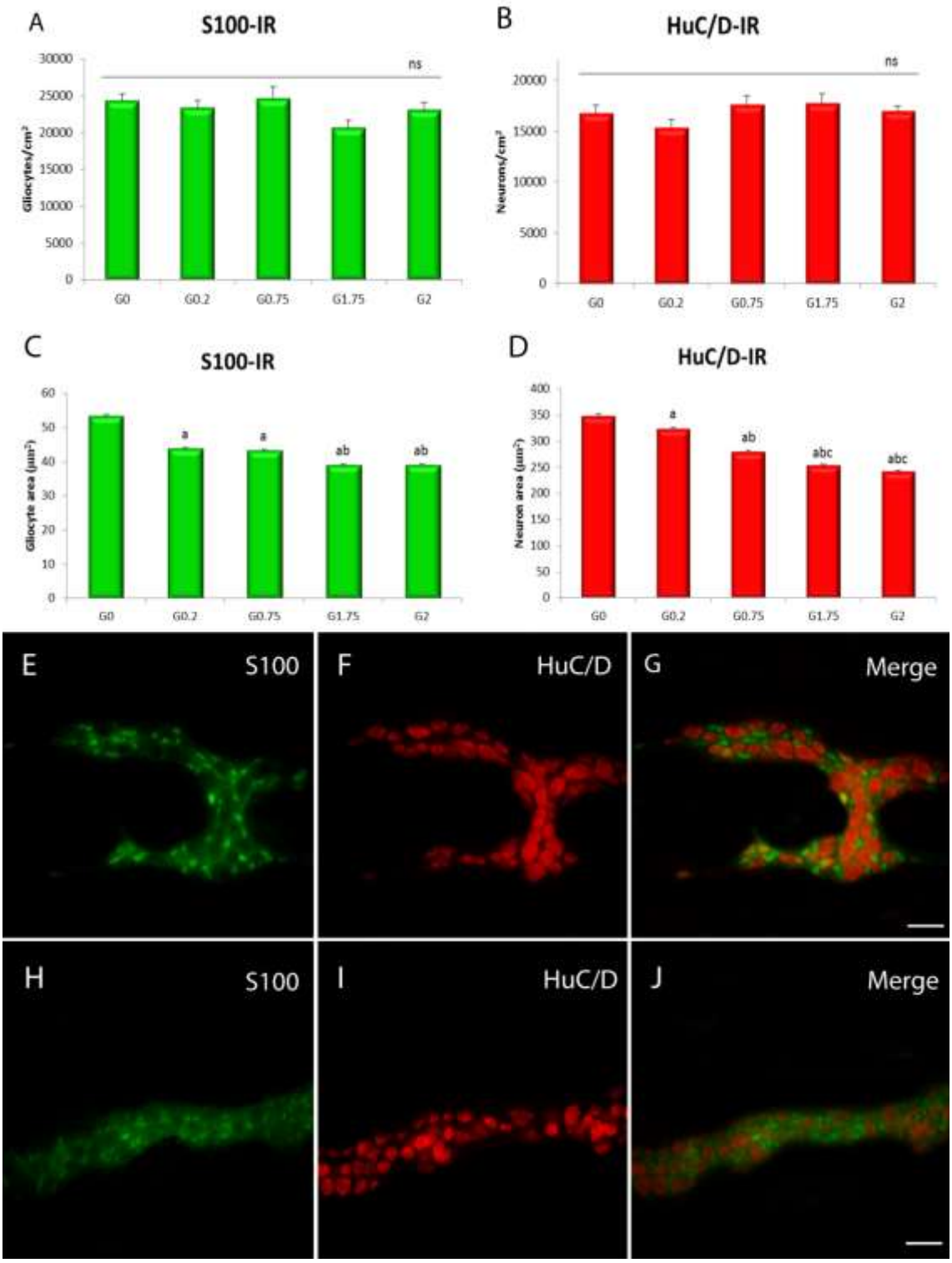


**Fig. 3.** Gliocytes and neurons of the myenteric plexus of the jejunum of Wistar rats. (A,B) Density of glicotyes[1] (A) and of general neuronal population[1] (B). (C,D) Mean area of gliocytes[2] (C) and of the cell body of the general population of neurons[2] (D). (E,F,G) Gliocytes immunoreactive for S100 (E), general population of neurons immunoreactive for HuC/D (F) and double immunostaining S100-HuC/D (G) of group G0. (H,I,J) Gliocytes immunoreactive for S100 (H), general population of neurons immunoreactive for HuC/D (I) and double immunostaining S100-HuC/D (J) of group G2. Calibration bar = 50 µm. [a]Significant difference with group G0. [b]Significant difference with group G0.2. [c]Significant difference with group G0.75. [ns]Non significant. [1]One-way ANOVA followed by Tukey *post-hoc* test. [2]Kruskal-Wallis followed by Dunns *post-hoc* test ($p<0.05$). Data expressed as mean ± standard error (n=7-8).

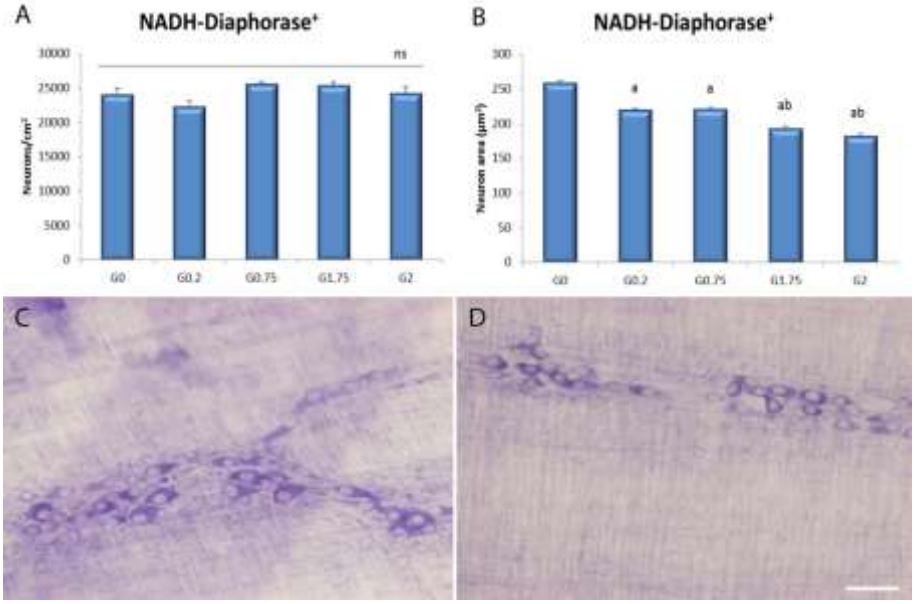


**Fig. 4.** NADH-diaphorase positive neurons of the myenteric plexus of the jejunum of Wistar rats. (A,B) Density[1] (A) and mean area[2] (b) of NADH-diaphorase positive neurons. (C,D) NADH-diaphorase positive neurons of rats of group G0 (C) and group G2 (D). Calibration bar = 50 µm. [a]Significant difference with group G0. [b]Significant difference with group G0.2. [ns]Non significant. [1]One-way ANOVA followed by Tukey *post-hoc* test. [2]Kruskal-Wallis followed by Dunns *post-hoc* test ($p<0.05$). Data expressed as mean ± standard error (n=6).

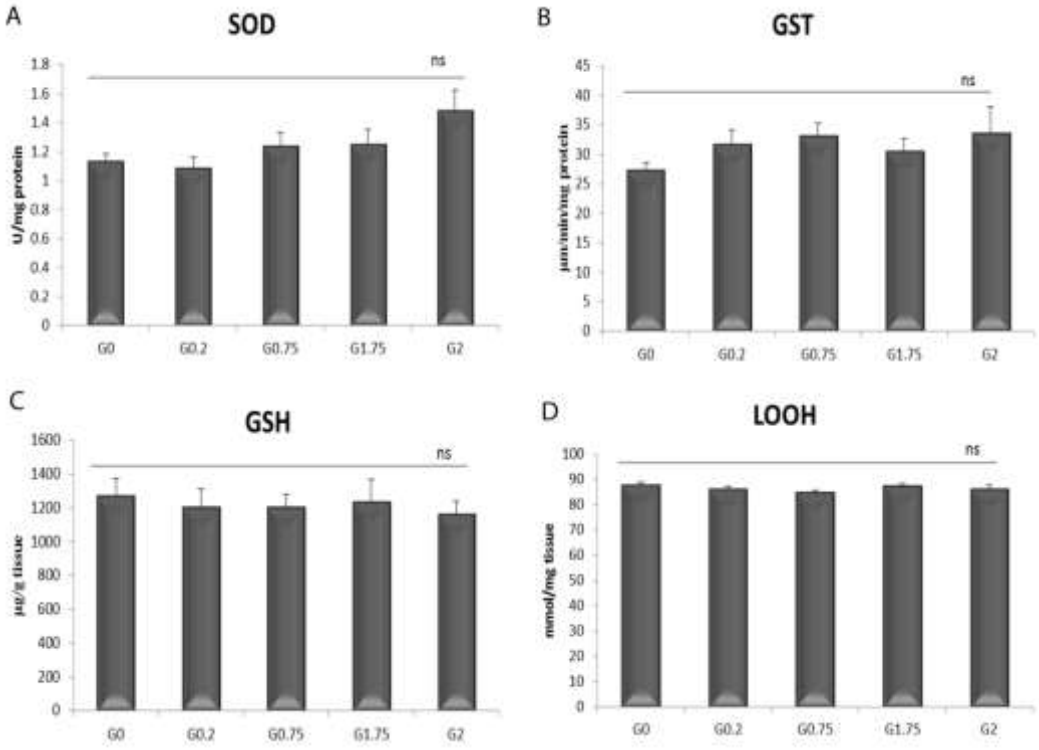


**Fig. 5.** Oxidative status of the jejunum of Wistar rats. (A) Enzymatic activity of superoxide dismutase (SOD)[1]. (B) Enzymatic activity of glutathione s-transferase (GST)[1]. (C) Levels of non-protein sulfhydryl groups (GSH)[1]. (D) Levels of lipidic hydroperoxides (LOOH)[2]. [ns]Non significant. [1]One-way ANOVA followed by Tukey *post-hoc* test. [2]Kruskal-Wallis followed by Dunns *post-hoc* test ($p<0.05$). Data expressed as mean ± standard error (n=6-8).

# EFEITO PRÓ-INFLAMATÓRIO E ATRÓFICO DE DIETA CONTAMINADA COM BAIXAS DOSES DE DESOXINIVALENOL SOBRE O JEJUNO DE RATOS.

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**Resumo**

O desoxinivalenol (DON), micotoxina produzida por *Fusarium spp*, é um contaminante de cereais e alimentos processados em âmbito mundial. Neste trabalho, foram avaliados os efeitos da ingestão de dieta contaminada com DON na menor concentração estabelecida como limite máximo tolerado (LMT) para consumo humano e animal e em concentração próxima as máximas estabelecidas como LMT. Ratos machos Wistar com 21 dias de idade foram distribuídos em três grupos que receberam, durante 42 dias, dieta contaminada com diferentes concentrações de DON (0; 0,2 e 2 mg/Kg de ração). Avaliou-se a ingestão alimentar, o peso corporal, a morfometria e estado inflamatório do jejuno, assim como, parâmetros sanguíneos. Nas concentrações utilizadas, não houve recusa alimentar, redução no ganho de peso, alteração na proliferação celular do jejuno ou alterações no hemograma, leucograma e plaquetograma. Entretanto, ainda que, na ausência de sinais clínicos aparentes de intoxicação, o DON induziu, nas duas concentrações utilizadas, variações morfométricas significativas na mucosa jejunal, tais como, redução na altura desta túnica, das vilosidades, da profundidade das criptas e do número de células caliciformes, e consequente redução da espessura total da parede jejunal. Houve aumento da atividade da mieloperoxidase, um indicador de estado inflamatório. Dentre os parâmetros bioquímicos avaliados no sangue, o DON reduziu os níveis de alanina aminotransferase e fosfatase alcalina. Concluiu-se que, mesmo na ausência de sinais clínicos de intoxicação, o DON gera inflamação e atrofia da mucosa e parede total do jejuno de ratos de forma dose dependente.

**Palavras chave:** Micotoxina, Parede jejunal, Componentes Sanguíneos, Mieloperoxidase.

## 1. Introdução

As micotoxinas são metabólitos secundários [1] produzidas por diversas espécies de fungos filamentosos [2], que podem ser encontradas naturalmente contaminando diversos produtos agrícolas no mundo todo [3-5].

O desoxinivalenol (DON), produzido principalmente por fungos do gênero *Fusarium spp* [6] é a micotoxina mais prevalente em culturas que constituem a base da alimentação humana e de animais [7,8]. É um composto não volátil, resistente a altas temperaturas e não é degradado pelos processos convencionais de industrialização de alimentos [9]. Por isso, é o tricoteceno naturalmente mais abundante e o que é encontrado em maior frequência em grãos e seus produtos derivados [10].

O DON acessa o organismo geralmente por via oral [11] e é absorvido primariamente no jejuno, tendo assim, como alvo principal o sistema digestório [12]. Esta micotoxina pode causar lise dos enterócitos e redução do número de células caliciformes, com prejuízo para a função de barreira do epitélio intestinal, além de promover fusão e redução na altura das vilosidades e redução na profundidade das criptas com comprometimento do processo de absorção de nutrientes [13,14]. Após absorção, o DON chega rapidamente a outros órgãos, atingindo em ordem decrescente de concentração, fígado, plasma, rim, baço, coração e cérebro [15].

Os efeitos e a intensidade da exposição ao DON dependem da dose, da duração do consumo e da espécie [16]. A exposição crônica a baixas doses, leva à redução no consumo de alimento, enquanto a exposição a doses maiores causa recusa alimentar, redução no ganho de peso, vômito e anorexia [17].

Apesar da diversidade e complexidade dos efeitos da intoxicação pelo DON, o único sítio de ligação conhecido para todos os tricotecenos está no sítio A do centro peptidil transferase da subunidade 60S do ribossomo eucariótico [18]. Por isso, o

mecanismo primário de toxicidade do DON é a inibição da síntese de proteínas [19]. Entretanto, a inibição dos ribossomos causada pelos tricotecenos desencadeia uma reação conhecida com “resposta do estresse ribotóxico” [20]. Essa resposta leva a ativação de proteínas quinases ativadas por mitógenos (MAPKs) o que interfere em vias que controlam a sinalização celular, proliferação celular e apoptose [21].

Os elevados níveis de contaminação por DON levou vários países a regulamentar e fiscalizar os níveis máximos de contaminação permitidos para grãos e produtos derivados de consumo humano e animal [22-24].

O presente trabalho teve por objetivo avaliar os efeitos de concentrações próximas ao menor e maior nível de contaminação estabelecido como tolerável por alguns países, sobre o consumo alimentar e ganho de peso, estado inflamatório e comportamento morfométrico do jejuno, além do perfil bioquímico e celular do sangue de ratos Wistar.

## 2. Material e métodos

### 2.1 Formulação das rações

Para o preparo da dieta base (Tabela 1) o milho, farelo de trigo e o farelo de soja foram moídos separadamente e misturados mecanicamente aos demais ingredientes. Para a produção das dietas com adição de DON adicionou-se à dieta base meio de cultivo esterilizado do fungo *F. graminearum.* A linhagem de fungo utilizada foi cedida pelo Laboratório de Fungos Toxicogênicos e Micotoxinas da Universidade de São Paulo e o meio de cultivo foi produzido no Laboratório de Microbiologia da Universidade Tecnológica Federal do Paraná – Campus Dois Vizinhos (UTFPR-DV). As dietas foram produzidas na Fábrica de Rações da Universidade Tecnológica Federal do Paraná – Campus Dois Vizinhos (UTFPR-DV). As dietas foram peletizadas e secas em estufa de

ventilação forçada (55 ºC) por 24 horas. A concentração de DON no meio de cultivo e nas dietas experimentais foi determinada por HPLC-MS e foi ajustada por diluição e homogeneização a quantidade de meio de cultivo adicionado na dieta base de forma a produzir rações experimentais contendo as doses de 0,2 e 2 mg de DON/kg de ração.

2.2 Animais e delineamento experimental

Foram utilizados 24 ratos Wistar (*Rattus novergicus*) machos, com 21 dias de idade e aproximadamente 53,7 ± 4 g de peso corporal, provenientes do Biotério Central da Universidade Estadual de Maringá. Ao longo do período experimental, os animais foram mantidos em caixas de polipropileno, no Biotério Setorial da UTFPR-DV com temperatura controlada (23ºC ± 2ºC) e ciclos de iluminação de claro e escuro de 12 h. Os procedimentos com os animais obedeceram aos princípios determinados pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foram aprovados pelo Comitê de Ética em Experimentação Animal da UTFPR-DV (parecer 2014-04).

Os animais foram distribuídos aleatoriamente em três grupos experimentais: G0- alimentados com ração sem adição de DON (controle), G0.2- alimentados com ração contaminada com 0,2 mg de DON/kg de ração e G2- alimentados com ração contaminada com 2 mg de DON/kg de ração.

Cada grupo foi formado por oito animais submetidos a eutanásia após 42 dias de período experimental. Neste período os animais receberam ração e água *ad libidum.* O peso corporal dos animais e o consumo alimentar foram avaliados semanalmente. Calculou-se o consumo médio de dieta por 100 gramas de peso corporal e o consumo de DON por quilo de peso corporal por dia.

2.3 Eutanásia e coleta dos tecidos

Os animais foram anestesiados com injeção endovenosa de Tiopental Sódico-Thionembutal® (45 mg/kg de peso corporal) e realizada coleta de 5 ml de sangue por punção cardíaca para análise dos componentes sanguíneos. Após, foi injetada uma dose letal do anestésico via intraperitoneal (80 mg/kg de peso corporal) e realizada a laparotomia. O intestino delgado foi removido e mensurado o seu comprimento total. Coletou-se o jejuno, mensurou-se a largura e amostras deste segmento foram destinadas ao processamento histológico.

## 2.4 Avaliação morfométrica do jejuno

### 2.4.1 Fixação, desidratação e inclusão em parafina

Amostras do jejuno foram abertas na borda mesentérica, fixadas em paraformaldeído 4% por 6 horas e armazenadas em álcool 70%. Em seguida, foram desidratadas em série de concentrações crescentes de etanol (80%, 90%, 100%), diafanizadas em xilol e incluídas em parafina para obtenção de cortes histológicos semi-seriados de 6μm de espessura, em micrótomo Leica RM 2145. Os cortes foram colocados sobre lâminas de vidro, desparafinizados em xilol, hidratados em série decrescente de etanol (100%, 90%, 80%, 70%) e submetidos a metodologia descrita nos itens 2.4.2, 2.4.3 e 2.4.4.

### 2.4.2 Coloração com Hematoxilina-Eosina

Os cortes desparafinizados e hidratados foram corados pelo método de Hematoxilina-Eosina (HE), diafanizados e montados entre lâmina e lamínula com resina sintética Permount®. Para avaliar a morfometria da parede total, túnica mucosa, altura de vilos e profundidade das criptas foram capturadas imagens em objetiva de 10X. As imagens foram obtidas em microscópio óptico Olympus Bx41 acoplado a câmera Q

Color 3 Olympus American Inc. Para cada parâmetro foram realizadas 100 mensurações/animal (10 pontos aleatórios/corte) utilizando-se o software de análise de imagem Image Pró Plus® 4.5- Media Cybernetics, Inc. Os resultados foram expressos em μm.

2.4.3 Histoquímica com Ácido Periódico de Schiff (P.A.S).

Cortes destinados a técnica histoquímica com Ácido Periódico de Schiff (P.A.S), foram oxidados com Ácido Periódico 5%, tratados pelo reativo de Schiff, corados com hematoxilina, desidratados, diafanizados e montados entre lâmina e lamínula com resina sintética Permount®. Para quantificar a população de células caliciformes nos vilos, foi realizada contagem (com auxílio de microscópio de luz) de 2500 células do epitélio intestinal por animal em vilos bem preservados, determinando-se o percentual (%) de células caliciformes de acordo com a equação a seguir:

$$\%\ células\ caliciformes = \left(\frac{número\ de\ células\ caliciformes}{número\ total\ de\ células\ nos\ vilos}\right) x100$$

2.4.4 Imunohistoquímica para detecção do Antígeno Nuclear de Proliferação Celular (PCNA).

A detecção das células do epitélio jejunal em processo de proliferação celular foi realizada utilizando-se o kit comercial Histostain® Plus Kits (Invitrogen®), seguindo as instruções do fabricante. Após desparafinização e hidratação, os cortes foram tratados com solução de peroxidase (3% de $H_2O_2$ em metanol por 15 minutos) para bloquear a ação das peroxidases endógenas. Foram feitas 2 lavagens com água destilada por 5 minutos e adicionada solução contendo soro de burro a 10% (10 minutos) para bloqueio de ligações específicas. Posteriormente, os tecidos foram incubados com solução contendo anticorpo primário anti-PCNA na diluição 1:100 por 3 horas. Após duas

lavagens com tampão fosfato salina (PBS) 0,1M (pH 7,4) por 5 minutos, os cortes foram incubados com anticorpo secundário biotinilado por 10 minutos, lavados novamente e, em seguida, tratados com o conjugado estreptavidina-peroxidase (10 minutos).

Após novas lavagens com PBS para remoção do excesso do conjugado enzimático, a reação imunohistoquímica foi revelada por diaminobenzidina (DAB) em PBS e $H_2O_2$ por 15 minutos. Após lavagem em água destilada, os cortes foram contracorados com hematoxilina de Meyer, desidratados em etanol, diafanizados com xilol e montados sob lamínula com resina sintética Permount®. Todos os procedimentos foram conduzidos à temperatura ambiente.

Foram quantificadas 2500 células/animal em criptas bem orientadas, identificando-se aquelas que estavam em proliferação pela coloração marrom. Para obtenção do percentual de células marcadas (em marrom) e não marcadas utilizou-se a equação a seguir:

$$Índice\ proliferativo = \left(\frac{número\ de\ células\ marcadas}{número\ total\ de\ células\ quantificadas}\right) x100$$

2.5 Estado inflamatório do jejuno

Para avaliação da atividade da enzima mieloperoxidase (MPO) amostras do jejuno foram lavadas em PBS (0,1 M, pH 7,4) e congeladas em nitrogênio líquido. Posteriormente, foram homogeneizadas em 0.6 ml de tampão fosfato de potássio (200 mM, pH 6,5) e centrifugado por 20 minutos a 9000 x $g$. O precipitado obtido foi ressuspendido em tampão de fosfato de potássio (80 mM) com 0,5% de brometo de hexadeciltrimetilamio (HTAB pH 5,4). As amostras foram homogeneizadas e centrifugadas por 20 min a 11.000 g a 4 ° C. A reação foi realizada em placa de 96 poços usando tetrametilbenzidina (TMB 18,4 mM). A atividade enzimática da MPO foi

determinada em espectrofotômetro (620 nm). Os resultados foram expressos como unidade de densidade óptica (DO)/min/mg de proteína.

2.6 Parâmetros celulares e bioquímicos sanguíneos

Parte do sangue coletado de cada animal foi colocado em tubo com ácido etilenodiaminotetracético (EDTA) para realização do hemograma. Parte foi adicionada em tubo contendo EDTA fluoretado para a dosagem de creatinina e ureia (plasma) e o restante foi mantido em tubo sem anticoagulante para obtenção do soro que foi utilizado para dosagem de Aspartato Aminotransferase (AST), Alanina Aminotransferase (ALT), fosfatase alcalina (ALP) e albumina. As amostras foram centrifugadas a 3000 rpm por 15 minutos. As análises foram realizadas por meio de kits comerciais (Gold Analisa Diagnostica Ltda, Minas Gerais, Brasil) de acordo com as especificações do fabricante em espectrofotômetro Bioplus 2000. Os dados de albumina, ureia e creatinina foram expressos como grama por decilitro (g/dL), os dados de AST, ALT e ALP foram expressos como unidade de massa atômica por litro (U/L).

2.7 Análise estatística

Os dados foram avaliados quanto à normalidade pelos testes Kolmogorov-Sminov ou Shapiro-Wilk. Dados não paramétricos foram analisados pelo teste de Kruskal-Wallis seguido do pós-teste de Dunn´s. Dados paramétricos foram submetidos à Análise de Variância (ANOVA) seguido do pós-teste de Tukey. Para estas análises foi utilizado o software *Graph Pad Prism*® 6.0 (GraphPad Software, Inc.). O nível de significância adotado foi de 5% e os resultados expressos como média ± erro padrão.

## 3. Resultados

3.1 Parâmetros corporais e ingestão alimentar

Durante o período experimental o consumo médio diário de DON foi estimado em 0,0145 e 0,157 mg/kg de peso corporal, para os grupos G0.2 e G2, respectivamente. Neste período não foram observados sinais clínicos aparentes de intoxicação, como diarreia, recusa alimentar, redução no ganho de peso e anorexia. A contaminação com o DON não alterou significativamente o consumo de ração, o peso corporal, o comprimento do intestino delgado ou a largura do jejuno (Tabela 2).

3.2 Avaliação histológica e morfoquantitativa do jejuno

A análise morfométrica demonstrou que a ingestão de dieta contaminada com DON reduziu a altura da parede total (Figura 1a), da túnica mucosa (Figura 1b) e dos vilos (Figura 1c), de maneira dose dependente, além de reduzir a profundidade das criptas nas duas concentrações utilizadas (Figura 1d). A organização morfológica da parede do jejuno foi preservada entre os diferentes grupos (Figura 1e, f).

A exposição ao DON também reduziu o número de células caliciformes em todos os animais tratados em relação ao grupo controle (Figura 2a, b). Não houve alteração no índice de proliferação celular do epitélio jejunal entre os grupos (Figura 2c, d).

3.3 Estado inflamatório do jejuno

A ingestão de dieta contaminada com 2 mg de DON por Kg de ração promoveu aumento na atividade da mieloperoxidase no jejuno dos ratos (Figura 3).

3.4 Parâmetros celulares e bioquímicos sanguíneos

Análises sanguíneas mostraram que a dieta contaminada com DON nas concentrações de 0,2 mg/kg e 2 mg/kg não alterou quantitativamente ou qualitativamente os elementos figurados do sangue (Tabela 3). Análises bioquímicas indicaram que a exposição ao DON também não alterou os níveis de albumina (Figura 4a), ureia (Figura 4b), creatinina (Figura 4c) e AST (Figura 4d), porém reduziu os níveis de ALT nas duas concentrações utilizadas neste trabalho (Figura 4e) e reduziu de maneira significativa os níveis da fosfatase alcalina na maior concentração utilizada (Figura 4f).

## 4. Discussão

A presença de micotoxinas no sistema digestório pode causar diferentes tipos de respostas, tais como, alterações no contato célula-célula, na produção de muco pelas células caliciformes e inflamação tecidual [25-27]. Há uma tendência em se esperar que todas as doses de um determinado composto tóxico, mesmo estando dentro dos limites toleráveis, como os usados nesse trabalho, produzam efeitos similares e que apenas a magnitude da resposta varie com a dose. Porém, efeitos heterogêneos ou mesmo opostos podem ser observados em doses diferentes, de forma que testes de altas doses não podem ser usados para prever os efeitos de baixas doses [28]. Diversos trabalhos mostram a ação do DON em doses superiores às regulamentadas como toleráveis para consumo humano e animal [21,29,30].

Neste trabalho, foram utilizados níveis de contaminação próximos ao menor e ao maior valor considerados toleráveis para contaminação com DON. A União Europeia estabelece níveis máximos de contaminação com DON de 0,2 a 1,75 mg/kg [22,23] e a Anvisa, no Brasil, estabelece níveis máximos de contaminação entre 0,2 e 3 mg/kg [24] de acordo com o tipo de produto comercializado.

A ingestão diária de 0,0145 e 0,157 mg de DON por kg de peso corporal pelos animais dos grupos G0.2 e G2, respectivamente, não causou sinais aparentes de intoxicação, como, diarreia, recusa alimentar, redução no ganho de peso e anorexia. Resultados semelhantes foram obtidos em experimento com ratos expostos a 0,03 mg de DON por kg de peso corporal por dia, dose equivalente ao valor médio da exposição crônica de animais a esta micotoxina, em países europeus, estimado pela EFSA (Autoridade Europeia para a Segurança de Alimentos) [31]. Os suínos são considerados os animais mais sensíveis à exposição ao DON que os ratos [32], porém, quando estes ingerem cronicamente dieta contaminada com DON, em concentrações próximas às utilizadas no presente trabalho, também não apresentam redução na ingestão alimentar e alterações no ganho de peso [33,34]. Entretanto, nossos resultados revelam alterações morfométricas significativas na parede do jejuno dos animais expostos ao DON, o que mostra que o quadro clínico aparente de intoxicação não revela o real efeito da exposição à micotoxina.

Uma das funções mais importantes do epitélio intestinal é formar uma barreira eficaz contra absorção de patógenos e toxinas [35], e para isso, precisa se regenerar continuamente [29]. O DON geralmente acessa o organismo por via oral e subsequentemente atinge as células epiteliais intestinais, tendo um impacto significativo na integridade da barreira intestinal, bem como na viabilidade celular [11]. A redução da altura da parede total, da túnica mucosa, das vilosidades e da profundidade das criptas verificada em nosso trabalho, reflete alterações no equilíbrio entre proliferação celular e apoptose. Não obtivemos alterações no índice de proliferação celular, o que está de acordo com os resultados de outros trabalhos [14,36]. Estes autores verificaram que a exposição ao DON resulta em um aumento na expressão de caspase-3 o que

indica que as reduções celulares observadas na mucosa intestinal refletem a intensa atividade apoptótica desencadeada por esta micotoxina.

Associado aos enterócitos, o muco produzido pelas células caliciformes constitui a primeira linha de defesa do trato gastrointestinal [37]. Nossas análises comprovam que a ingestão de dieta contaminada com DON reduz significativamente o número de células caliciformes. Toxinas são capazes de se difundir através do muco, bloquear o crescimento de células epiteliais e interromper a produção de muco [38]. Porém, a camada de muco não é uma barreira estática e por meio do retículo endoplasmático, inicialmente, as células caliciformes procuram modular a secreção em resposta a estímulos externos [39,40], liberando rapidamente os grânulos de mucina [13]. Na presença contínua de micotoxinas, esta resposta adaptativa, gera uma grande carga de síntese proteica para estas células secretoras tornando-as suscetíveis ao estresse do retículo endoplasmático, que desencadeia o processo apoptótico [39,40].

As células epiteliais do intestino também contribuem para a regulação de condições inflamatórias [29]. Os danos causados pelo DON ao epitélio intestinal podem permitir a passagem transepitelial de antígenos e bactérias, gerando um efeito pró-inflamatório indireto [41]. Maresca e colaboradores [42] demonstraram que, em cultura de células intestinais, elevadas doses de DON podem alterar a barreira epitelial causando inflamação. No presente trabalho, demonstramos, por meio da avaliação da atividade da enzima mieloperoxidase, que a contaminação com baixa dose de DON (2 mg/Kg) também causa efeito inflamatório, pois houve aumento significativo da atividade da MPO neste grupo e esta enzima, encontrada nos grânulos azurófilos dos neutrófilos, é liberada no espaço extracelular em quadros de inflamação [43]. Resultados semelhantes aos nossos, foram obtidos por Misha e colaboradores [44] que,

avaliaram camundongos submetidos à aplicação tópica de DON e, verificaram aumento nos níveis de MPO, tanto na pele quanto no intestino dos animais.

Após absorção primária no jejuno [12], o DON chega rapidamente ao fígado, plasma, rim, baço, coração e cérebro, atingindo em maior concentração o fígado, seguido pelo plasma [15]. Entre os indicadores bioquímicos avaliados neste trabalho, ALT e ALP apresentaram alterações significativas. Os níveis séricos de ALT e ALP são os biomarcadores utilizados com maior frequência para indicar lesão hepática (45), porém, os dados encontrados na literatura em relação à resposta destas enzimas, frente à exposição ao DON, são controversos. Alguns trabalhos mostram que o DON induz a um aumento de ALT e ALP [45-47], enquanto outros mostram que o DON reduz ALT e ALP [48-50]. No presente trabalho, houve redução nos níveis de ALT para os animais que receberam dieta contaminada com DON, nas duas concentrações utilizadas e redução nos níveis de ALP para animais que receberam dieta contaminada com DON na concentração de 2 mg/kg. Tais variações de valores de ALT e ALP observados quando se compara diferentes trabalhos, provavelmente apontam para processos patológicos no fígado no início da exposição à contaminação, com melhora adaptativa ao longo do consumo de dieta contaminada, como demonstrado no trabalho de Zielonka e colaboradores [51] que encontraram, ao longo de seis semanas de experimento, uma tendência ao decréscimo nos níveis de ALT em suínos contaminados com DON.

As concentrações de DON no plasma refletem as concentrações de DON na dieta [52], porém, em nossas análises, não foram observadas alterações quantitativas ou qualitativas nos elementos figurados do sangue, corroborando com dados da literatura [33,53]. As células sanguíneas circulantes tendem a apresentar baixa sensibilidade à micotoxinas, sendo que, problemas hematológicos observados em casos de intoxicação, são devidos à mielotoxicidade, e o DON apresenta baixa mielotoxicidade [54], o que

justifica a sua baixa hematotoxicidade. Além disso, Chattopadhyay e colaboradores [55] avaliaram cinco tricotecenos, e verificaram que, em comparação aos demais, o DON apresenta o menor potencial hematotóxico.

## Conclusão

A exposição crônica ao DON, em concentrações regulamentadas como toleráveis para consumo humano e animal, não causa sinais clínicos aparentes de intoxicação, porém causa alterações importantes no jejuno, o que poderia comprometer o funcionamento intestinal levando a redução da superfície absortiva e a defesa promovida pelas mucinas.

### Conduta ética de pesquisa

Todos os procedimentos com os animais foram realizados somente após aprovação pelo Comitê de Ética em Experimentação Animal Institucional.

### Apoio financeiro e conflito de interesses

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**Tabela 1.** Composição da dieta base fornecida aos ratos durante o período experimental.

| Componente | (%) |
|---|---|
| Milho | 33,63 |
| Farelo de soja | 33,10 |
| Farelo de trigo | 27,20 |
| Óleo de soja | 2,30 |
| Calcário Calcítico | 2,07 |
| Fosfato bicálcio | 0,73 |
| Sal comum (iodado) | 0,50 |
| Premix vitamínico[1] | 0,10 |
| Premix mineral[2] | 0,35 |
| BHT[3] | 0,02 |

[1]Mistura vitamínica, composição por kg: Ácido Fólico=200mg; Ácido Nicotínico=3000mg; Biotina=20mg; Pantotenato de Cálcio=1600mg; Piridoxina HCl=700mg; Riboflavina=600mg; Tiamina HCl=600mg; Vitamina A=4000.000UI; Vitamina B12=2500mcg; Vitamina D3=100.000UI; Vitamina E=100.000UI; Vitamina K1=75mg.
[2]Mistura Mineral, composição por kg: : Boro=14,26mg; Cálcio=142,94g; Cloro= 44,9g; Cobre=72,41mg; Cromo=28,65mg; Enxofre=8,6g; Ferro=1000mg; Flúor=28,72mg; Fósforo=56,9g; Iodo=5,95mg; Lítio=2,85mg; Magnésio=14,48g; Manganês=300mg; Molibdênio=4,32mg; Níquel=14,31mg; Potássio=102,86g; Selênio=4,28mg; Silício=143,26mg; Sódio=29,38mg; Vanádio=2,87mg; Zinco=860mg.
[3] Botuil Hidroxi Tolueno.

**Tabela 2.** Ingestão alimentar, peso corporal, comprimento do intestino delgado e largura do jejuno de ratos Wistar alimentados com dieta sem adição de DON (G0) e com adição de 0,2 mg/kg (G0.2) ou 2 mg/kg (G2).

| | G0 | G0.2 | G2 |
|---|---|---|---|
| Ingestão alimentar média (g/100g de peso corporal/ semana) | 54,3±2,25 | 50,6±2,69 | 55,1±3,23 |
| Peso inicial (g) | 54,2±1,25 | 53,7±0,70 | 53,5±0,94 |
| Peso final (g) | 277,5±4,53 | 266,3±3,73 | 267,0±3,77 |
| Ganho de peso (%) | 509,5±13,39 | 496,3±1,98 | 498,8±5,10 |
| Comprimento do intestino delgado (cm) | 113,6±2,27 | 125,3±12,75 | 107,3±1,58 |
| Largura do jejuno (cm) | 1,263±0,05 | 1,250±0,03 | 1,313±0,06 |

Os dados foram expressos como média ± erro padrão. One-way ANOVA, seguido pelo pós-teste Tukey ($p<0,05$). n=8

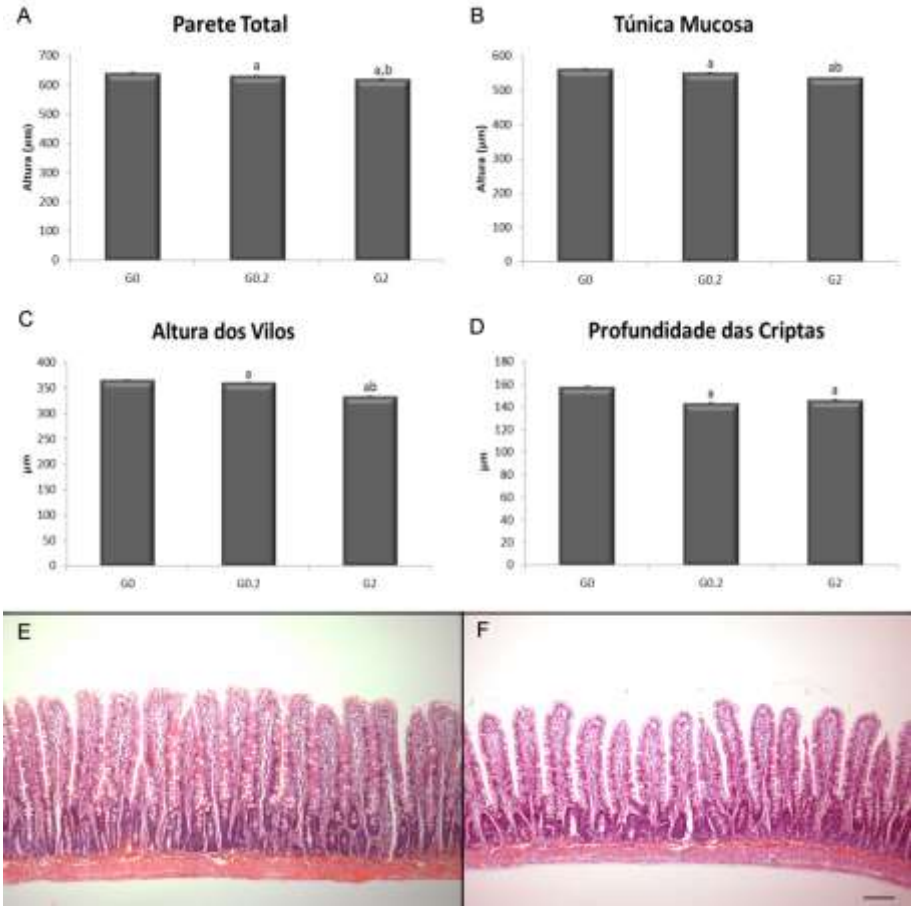


**Figura 1.** Parede do jejuno de ratos Wistar. (A) Altura da parede total. (B) Altura da túnica mucosa. (C) Altura dos vilos. (D) Profundidade das criptas. (E) Jejuno de rato do grupo G0. (F) Jejuno de rato do grupo G2. Barra de calibração = 100 µm. HE. Objetiva 40X. [a]Diferença significativa com o grupo G0. [b]Diferença significativa com o grupo G0.2. Kruskal-Wallis, seguido pelo pós teste Dunn´s ($p<0,05$). Os dados foram expressos como média ± erro padrão (n= 8).

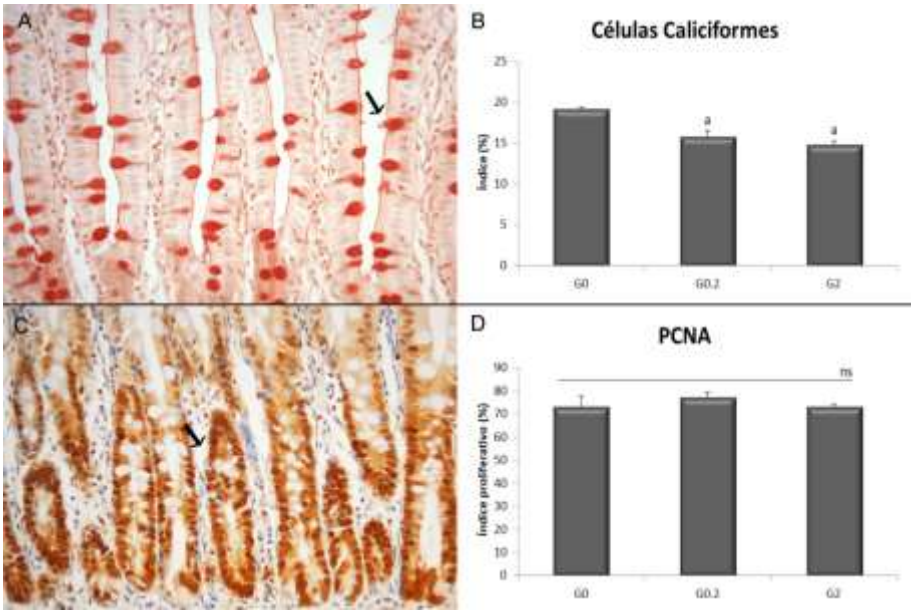


**Figura 2.** Populações celulares do epitélio do jejuno de ratos Wistar alimentados com dieta sem adição de DON (G0) e com adição de 0,2 mg/kg (G0.2) ou 2,0 mg/kg (G2). (A) Células caliciformes P.A.S positivas (seta). (B) Índice de células caliciformes. (C) Células em proliferação PCNA positivas (seta). (D) Índice de células em proliferação. Objetiva 40X. [a]Diferença significativa com o grupo G0. [ns]Não significativo. One-way ANOVA, seguido pelo pós-teste Tukey ($p<0,05$). Os dados foram expressos como média ± erro padrão (n=5).

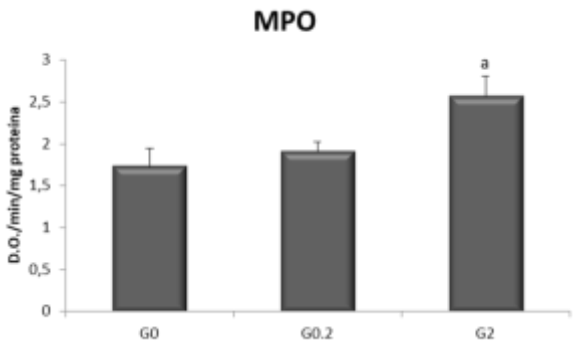


**Figura 3.** Atividade da mieloperoxidase (MPO) no jejuno de ratos Wistar alimentados com dieta sem adição de DON (G0) e com adição de 0,2 mg/kg (G0.2) ou 2,0 mg/kg (G2). One-way ANOVA, seguido pelo pós-teste Tukey ($p<0,05$). Os dados foram expressos como média ± erro padrão (n=5).

**Tabela 3.** Hemograma, Leucograma e Plaquetograma de ratos Wistar alimentados com dieta sem adição de DON (G0) e com adição de 0,2 mg/kg (G0.2) ou 2,0 mg/kg (G2).

| | G0 | G0.2 | G2 |
|---|---|---|---|
| **Hemograma** | | | |
| Eritrócitos(milh/µL)[1] | 6,91±0,2 | 6,82± 0,2 | 7,04±0,19 |
| Hemoglobina(g/dL)[1] | 13,15±0,31 | 12,78± 0,5 | 13,62±0,32 |
| Hematócrito(%)[1] | 37,98±1,14 | 36,92±1,45 | 39,18±1,04 |
| HCM(pg)[1] | 19,05±0,31 | 18,77±0,25 | 19,42±0,11 |
| CHCM(%)[1] | 34,63±0,34 | 34,67±0,33 | 34,86±0,24 |
| PTP(g/dL)[1] | 5,97±0,095 | 5,90±0,14 | 5,78±0,08 |
| **Leucograma** | | | |
| Leucócitos(cels/µL)[2] | 5367±262,9 | 5460±390,6 | 5240±354,6 |
| Neutrófilos segmentados(cels/µL)[1] | 1461±173,3 | 1715±526,3 | 1435±192,2 |
| Eosinófilos(cels/µL)[2] | 18,17±11,55 | 7,167±7,17 | 37,60±12,75 |
| Linfócitos(cels/µL)[1] | 3851±130,3 | 3147±404,6 | 3749±303,1 |
| Monócitos(cels/µL)[1] | 37,17±19,28 | 31,67±19,34 | 90,60±41,98 |
| **Plaquetograma** | | | |
| Plaquetas(cels/µL)[1] | 773000±29705 | 604167±98080 | 549000±98382 |

[1]One-way ANOVA, seguido pelo pós-teste Tukey. [2]Kruskal-Wallis, seguido pelo pós teste Dunn´s ($p<0,05$). Os dados foram expressos como média ± erro padrão (n=6). HCM: hemoglobina corpuscular média. CHCM: concentração da hemoglobina corpuscular média. PTP: concentração de proteína plasmática total.

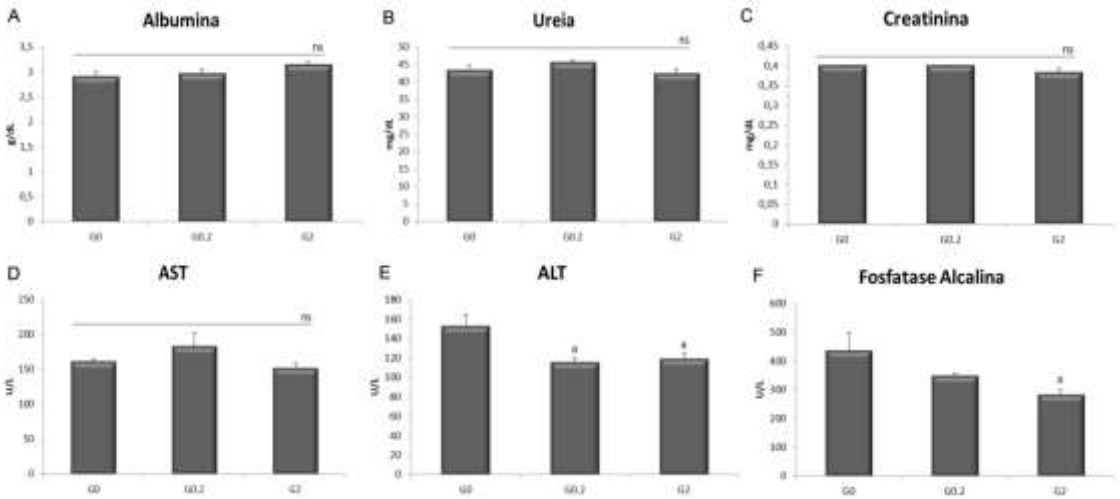


**Figura 4.** Análise sanguínea de proteínas e enzimas de ratos Wistar alimentados com dieta sem adição de DON (G0) e com adição de 0,2 mg/kg (G0.2) ou 2,0 mg/kg (G2). (A) Albumina[1]. (B) Ureia[1]. (C) Creatinina[2]. (D) Aspartato aminotransferase (AST) [1]. (E) Alanina aminotransferase (ALT)[1]. (D) Fosfatase alcalina[1]. [a]Diferença significativa com o grupo G0. [ns]Não significativo. [1]One-way ANOVA, seguido pelo pós-teste Tukey. [2]Kruskal-Wallis, seguido pelo pós teste Dunn´s ($p<0,05$). Os dados foram expressos como média ± erro padrão (n= 6).

# ANEXOS

## Carta de submissão do artigo 1

Ref: TOX_2018_269
Title: CHRONIC INGESTION OF DEOXYNIVALENOL-CONTAMINATED DIET DOSE-DEPENDENTLY DECREASES THE AREA OF MYENTERIC NEURONS AND GLIOCYTES OF RATS
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**Normas da revista Nutritional Neuroscience**

Journal

# Nutritional Neuroscience

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