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The food contaminant, deoxynivalenol, modulates the Thelper/Treg balance and increases inflammatory bowel diseases.

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ABSTRACT

The incidence of inflammatory bowel diseases (IBD) is increasing in both Western and developing countries. IBD are multifactorial disorders involving complex interactions between genetic, immune, and environmental factors such as exposure to food contaminants. Deoxynivalenol (DON) is the most prevalent mycotoxin that contaminates staple food and induces intestinal breakdown and inflammatory response. To delineate the role of DON oral exposure in IBD, we used a Dextran sulfate sodium (DSS) colitis model in rats fed with a DON-contaminated diet or a control diet for four weeks. Colitis was induced in the third week by increasing concentrations of DSS in the drinking water (0, 2, 3 or 5%). DON exacerbated body weight loss and accelerated the appearance of symptoms in animals treated with DSS. DON increased morphological damage, pro-inflammatory markers (myeloperoxidase, CXCL1 and IL1 β) and immune cell responses. In *lamina propria* of rat with colitis, DON increased adaptive and innate immune responses after anti-CD3/28 or LPS stimulation, respectively. In spleen, DON increased IFN γ secretion and reduced Treg populations. Interestingly, De-epoxy-DON (DOM-1) a detoxified form of DON did not have any consequences on colitis. These results suggest that DON is a risk factor in the onset of IBD.

INTRODUCTION

The incidence of inflammatory bowel diseases (IBD, Crohn's disease; CD and ulcerative colitis, UC) has risen in recent decades in both Western and developing countries (Weimers and Munkholm, 2018). IBDs are characterized by chronic intestinal and systemic inflammation associated with the disruption of the intestinal barrier and microbiota dysbiosis in favor of enterobacteria (Chassaing and Darfeuille-Michaud, 2011; Zhang and Li 2014). Genetic and environmental factors play a major role in the development of these multifactorial diseases; among environmental factors, changes in diet, antibiotic use, microbial exposure or exposure to food contaminants have been identified as risk factors for the development of IBD (Ananthakrishnan et al., 2017; Chassaing et al., 2015).

Trichothecene mycotoxins are fungal secondary metabolites that can contaminate a wide variety of dietary material consumed by humans and animals. Among these mycotoxins, deoxynivalenol (DON), produced by *Fusarium* species is commonly detected in cereals and associated products (flour, pasta or bran). DON is among the most prevalent contaminants in cereal-based food, suggesting regular human exposure (Knutzen et al., 2017; Wang et al., 2014).

Analyses of urine samples indicated that 98% of adults in the United Kingdom had been exposed to DON, while 80% of children in the Netherlands exceeded the tolerable daily intake of this mycotoxin (Pieters et al., 2002; Turner et al., 2008, Vin et al. 2020).

Previous studies by our laboratories and others demonstrated that DON disrupts the intestinal barrier functions (Akbari et al., 2014; Pinton et al 2012; Pinton and Oswald, 2014), induces intestinal inflammatory responses (Pestka, 2010; Cano et al., 2013; Alassane-Kpembé et al., 2017) and exacerbates the genotoxic effect of intestinal microbiota on epithelial cells (Payros et al., 2017). Recently, Vignal et al. observed that chronic ingestion of DON impairs intestinal homeostasis by altering immune response in mesenteric lymph nodes and gut microbiota composition (Vignal et al., 2018). All these data suggest that DON is a risk factor for the development and/or the exacerbation of IBD (Maresca and Fantini, 2010), but this has never been demonstrated. At the molecular level, DON interacts with the peptidyl transferase region of the 60S ribosomal subunit, inducing “ribotoxic stress” that results in the activation of mitogen-activated protein kinases (MAPKs) and their downstream pathways (Lucioli et al., 2013; Pierron et al., 2016).

Using a rat model of colitis induced by increasing doses of Dextran sulfate sodium (DSS), we investigated the role of exposure to DON in the increased incidence of IBD.

MATERIAL AND METHODS

Chemicals

Purified DON was purchased from Sigma (St Quentin Fallavier, France). De-epoxy-DON (DOM-1) a detoxified form, was obtained by transforming crystalline DON (Romer Labs, Tulln, Austria) as previously described (Pierron et al., 2016). DON and DOM-1 were included in the animals' diet premix (Payros et al., 2017). Control and mycotoxin-contaminated diets were manufactured at the INRA UPAE (Jouy en Josas, France) and formulated according to the requirements established for rats (**Supplementary Material Table S1**). Labocea (Plouflagran France) checked DON (8.18 mg/kg) and DOM-1 (8.01 mg/kg) concentrations in the final diet by HPLC-MS/MS.

Animal model of DSS-induced colitis

Male Wistar rats (140-170g body weight, 4 weeks old, Janvier, France) were maintained in the Toxalim animal facility (INRAE, UMR 1331, Toulouse), with free access to food and water throughout the study. The experiments were carried out in accordance with the European Guidelines for the care and use of animals for Scientific Purposes and validated by Toxcomethique Ethics Committee and the French Ministry of National Education Higher Education and Research (APAFIS#4832-2016040716495113). After five days of acclimatization, the animals received the control, DON or DOM-1 contaminated diet (8 mg.kg⁻¹) for four weeks. In the last week, colitis was induced in some animals by adding DSS (0, 2, 3 or 5%) to the drinking water (**Supplementary Material Figure 1**) for seven days and the rats were then euthanized by cervical dislocation and exsanguination. A persistently hunched posture and labored respiration, a weight loss of more than 20% were considered as end-points at which the animals were euthanized.

Clinical scoring of DSS-induced colitis

Body weight, stool consistency and bleeding were monitored daily. Clinical scoring (0-4) was based on the evaluation of the last two parameters (Schultz et al., 2004) (**Supplementary Material Table S2**).

Microscopic scoring analysis

The colon was fixed in 10% buffered formalin for 24h, dehydrated and embedded in paraffin using standard histological procedures. Sections (5µm) were stained with H&E (Hemalun and Eosin). For the analysis of microscopic scoring after DSS-induced colitis, colonic inflammation and damage were graded semi-quantitatively blindly by one or two of the authors according to the method of Tamaki et al. (Tamaki et al., 2006). Each of three histological features (severity of inflammation, extent of inflammation and damage to the epithelium) was scored from 0 to 4. Tissue extent was also taken into account (0 to >75%). The histological score was the sum of these parameters (**Supplementary Material Table S3**).

Myeloperoxidase (MPO) activity

Myeloperoxidase (MPO) is a granule-associated enzyme present in neutrophils and other cells of myeloid origin and is widely used as a marker of intestinal inflammation. Distal segments of colon (1 cm) were sectioned transversely and stored at -80°C until assayed for measurement of MPO activity. MPO is expressed in units (U) per gram of colonic protein, where 1 U corresponds to the activity required to degrade 1 µmol of hydrogen peroxide in 1 min at room temperature. Protein concentrations were determined using the BCA Assay Uptima kit (Interchim, Montluçon, France).

Cytokine measurements

For tissue samples, distal segments of colon (0.5 cm pieces) were stored at -80°C. Tissue proteins were extracted with RIPA buffer (1% Igepal, 0.5% deoxycholic acid and 0.1% sodium dodecyl sulfate in Tris-buffered saline 1X; pH = 7.4) with protease inhibitor cocktail (Roche diagnostic, France). Protein concentrations were determined as above. Clear lysates of colon or cell culture supernatant (see below) were processed for ELISA using commercial ELISA kits (Duoset R&D Systems, Lille, France) for Interleukin-1β (IL-1β), IFNγ, IL-17, IL-10, TNFα and the chemokine CXCL1 (CINC-1, rat Interleukin-8) according to the manufacturer's instructions. Data are expressed as picograms of cytokine per milliliter of cell culture medium or per milligram of tissue protein.

Cell isolation and flow cytometry analysis

Ileal small intestines were washed with cold PBS, cut into 0.5cm pieces, incubated in 3 mM EDTA (Sigma) and digested in Dulbecco Modified Eagle Medium (DMEM) supplemented with 20% fetal calf serum and 100U/mL of collagenase (Sigma) for 40 min at 37°C before filtration and purification by centrifugation using a gradient of 40-80% Percoll. Spleen cells were collected in a 70 µm strainer, washed and separated from red blood cells with ammonium–chloride–potassium (ACK) lysis buffer. Isolated cells from ileal *lamina propria* (iLP) and spleen were seeded on 24-well plates (1.10⁶ cells per well) previously coated or not with 5µg/ml hamster anti-rat CD3 and hamster anti-rat CD28 (G4.18 and JJ319, ebioscience) in CerroTini medium (DMEM supplemented with 20% SVF, 36 mg/l asparagine, 116 mg/l arginine, 10 mg/l folic acid, 1 g/l 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid, 0.05 mmol/l β-mercaptoethanol, 100 U/ml penicillin, 100 Kg/ml streptomycin and 1 µg/ml fungizone). After 72 h of stimulation, culture supernatants were collected and stored at -80°C until cytokine assays were performed. In parallel, lipopolysaccharide (LPS) stimulation (100ng/mL) was performed for 16 h before the supernatants were collected to assess innate immune response. To assess dendritic cell (DC) maturation, cells were stimulated with 40 µg/mL Polyinosinic–polycytidylic acid sodium salt (poly I:C, (Sigma) and 20 µg/mL LPS (Sigma) overnight before cell phenotype analysis by flow cytometry. Antibodies were incubated at 4°C for 30 min. Antibodies directed against CD103 (OX-62, Biolegend, San Diego, USA) and CD11b/c (OX-42, BD Pharmingen, Grenoble, France) were used to determine DC phenotype. CD4 (W3/25, Biolegend) and CD25 (OX-39, Biolegend) were used to identify helper T cells. Intracellular

staining of FoxP3 (JFK-16S, eBioscience) in CD4⁺ T lymphocytes was performed using a Cytofix/Cytoperm Kit Plus (BD Bioscience, CA, USA) to detect regulatory T cells. To quantify the Th17 lymphocytes, cells were stained with anti-TCR $\alpha\beta$ antibody (clone R73, BD Pharmingen) and intracellular cytokine staining was performed with anti-IL-17F (8F5.1A9, Biolegend) antibodies. Cells were stimulated with a cocktail of phorbol 12-myristate 13-acetate (PMA, 15nM, Sigma) and ionomycin (1 μ g/mL Sigma) for 5 h at 37°C. Brefeldin A (10 μ g/mL, Sigma) was added after the first hour of incubation. Cells were harvested, washed and stained with anti-IL-17F antibodies for 30 min at 4°C. Flow cytometry data were collected on MACSQuant Analyzer (Miltenyi Biotec, Paris, France) and analyzed using VenturiOne® (Applied Cytometry, Dinnington, UK) software.

Statistical analysis

Statistical differences between the experimental groups were evaluated by two-way analysis of variance, followed by a Bonferroni post-test (which allows comparison of all pairs of groups). All tests were performed with GraphPad Prism Software (Inc, San Diego, CA). All data are presented as means \pm the standard errors of the mean (SEM). A *p* value of < 0.05 was considered significant.

To evaluate the differences for clinical score and body weight gain, a linear mixed model for repeated measures was fitted. Diet, Time and interaction Time x Effect were set to fixed effects. The time was considered as a repeated measure within the rat, and the rat was assumed as a random effect. The Shapiro-Wilk test was used to assess normality of model's residuals. Differences of Least Squares Means were applied to test pairwise comparisons. Packages lme4 and lmerTest of the R project (<https://www.r-project.org/>) were used to perform these analysis.

RESULTS

DON exposure accelerates the onset and exacerbates DSS-induced colitis in adult rats

To assess the deleterious effect of DON on colitis, a DSS-induced colitis model was used with rats exposed or not to a DON contaminated diet (8 mg.kg⁻¹). As already described (Egger et al., 2000) rodents exposed to DSS in drinking water (2, 3 or 5% DSS) exhibit clinical features of acute colitis including diarrhea and rectal bleeding after seven days in contrast to DSS untreated rats fed a control or DON-contaminated diet (**Figure 1A**). Combined exposure to DON and DSS at all doses tested, significantly accelerated the onset of symptoms (occurrence of diarrhea and blood in feces) and aggravated clinical scores (**Figure 1A, Supplementary Table S4**). The exacerbation of the clinical symptoms in presence of DON was associated with a significant decrease in body weight gain in animals that received 5% DSS (**Figure 1B**), as well as a significant decrease in colon length (**Supplementary Figure S2**). DSS-induced colitis also led to fecal microbiota dysbiosis in favor of *Enterobacteriaceae* (**Supplementary Figure S3**). Taken together, these results indicate that exposure to DON accelerates the appearance of clinical symptoms in animals treated with DSS and aggravates DSS-induced colitis at all doses of DSS tested but with a stronger effect at 5% DSS.

Exposure to DON-contaminated diet exacerbates morphological damage and inflammatory response in the colon of rats with DSS-induced colitis

Morphological damage in the colon were evaluated at the end of the experiment (4 weeks of exposure to DON plus exposure to DSS in the last week of DON exposure). Treatment with 2%, 3% or 5%DSS led to the progressive appearance of multifocal inflammatory cell infiltration, edema, epithelial cell and crypt loss and colonic inflammation (**Figure 2A and 2B**). In animals treated with 3% or 5% DSS, exposure to the DON-contaminated diet further increased the histological scores (**Figure 2A and 2B**). In animals treated with the highest dose

of DSS (5%), the colon displayed loss of crypts and loss of epithelium cell surface. These features, especially loss of crypts, necrotic and apoptotic debris and loss of epithelium cell surface were also observed in animals fed DON-contaminated diet and exposed to lower doses of DSS (2% and 3%) (**Figure 2B**).

The inflammatory status of the colon was then assessed. In rats treated with 5% DSS, exposure to the DON-contaminated diet induced a stronger inflammatory response as evidenced by the increased levels of myeloperoxidase (MPO) (**Figure 2C**), chemokine ligand 1 (CXCL1, a homolog of Interleukin-8) (**Figure 2D**) and IL-1 β (**Figure 2E**). Increased levels of CXCL1 and IL-1 β were also observed in animals treated with 3% DSS due to the exacerbating effect of DON (**Figure 2D and 2E**).

These results demonstrate that DON worsens colitis induced by DSS through the exacerbation of morphological damage and increased production of pro-inflammatory markers.

DON but not DOM-1, a detoxified form of DON, exacerbates colitis induced with 5% DSS

To investigate if the exacerbation of DSS induced colitis was specific to DON, the DOM-1, a detoxified form of DON (Pierron et al., 2018; Bracarense et al., 2020), was used. Exposure to DOM-1 did not increase the severity of the symptoms (**Figure 3A and B**). In 5%DSS treated rats, exposure to DON but not to DOM-1 reduced colon length (**Supplementary Figure 4A**) and increased populations of *Enterobacteriaceae* in feces (**Supplementary Figure 4B**). Similarly, in animals exposed to DSS, the exacerbation of intestinal damages (**Figure 3C-D**) and the increase in MPO in colonic tissue (**Figure 3E**) were observed in animals fed DON contaminated diets but not in animals fed DOM-1 contaminated diets.

DON but not DOM-1 exposure induced an intestinal pro-inflammatory response in 5% DSS-induced colitis in rats

To further understand the mechanism by which exposure to DON exacerbates 5% DSS-induced colitis, local and systemic immune responses were investigated. Immune cells population and functionality were studied in the ileal *lamina propria* of animals exposed or not to the highest dose of DSS (5%) and fed control, or DON- or DOM-1-contaminated diets.

As already reported (Leppkes et al., 2009) colitis increased the percentage of Th17 cells (**Figure 4A and 4B** TCR $\alpha\beta$ ⁺IL-17⁺), and the percentage of helper T cells (Th) (**Figure 4C**, CD4⁺CD25⁺ cells) without influencing the percentage of regulatory T cells (Treg) (CD4⁺CD25⁺ FoxP3⁺ cells) (**Figure 4D**). DSS treatment increased the percentage of colitogenic dendritic cells (CD103⁻ CD11b/c⁺) but had no effect on the percentage of tolerogenic dendritic cells (CD103⁺ CD11b/c⁻) (**Figure 4F and 4G**). When assessing the functionality of the cells, anti-CD3/28 stimulation induced IL17, IFN γ and IL10 secretion. IL17 (**Figure 4H**) and IL10 (**Figure 4J**) secretions were significantly higher in cells isolated from DSS-treated rats and stimulated with anti-CD3/28 whereas IFN γ (**Figure 4I**) secretion was not affected. By contrast, in non-stimulated cells, the secretion of pro- and anti-inflammatory cytokines was not affected by exposure to either DSS or DON (**Figure 4H to 4J**).

When DSS-treated rats were fed the DON-contaminated diet, higher IL17 secretion in response to anti-CD3/28 stimulation was observed with no change in Th17 populations (**Figure 4A, 4B and 4H**). Combined exposure to DON and DSS also reduced the Treg/Th ratio (**Figure 4A and 4E**) associated with a drastic increase in the percentage of helper T cells (**Figure 4C**) and a decrease in IL10 secretion in response to anti-CD3/28 (**Figure 4J**). The combined exposure significantly increased the frequency of pro-inflammatory dendritic cells (CD103⁻CD11b/c⁺) but had no effect on the percentage of tolerogenic dendritic cells (CD103⁺ CD11b/c⁻) (**Figure 4F, 4G**). Upon LPS stimulation, the secretion of IL1 β (**Figure 4L**) increased significantly in rats with colitis and fed the DON-contaminated diet. By contrast, the frequency of activated T cells (CD4⁺ CD44^{high} CD62L^{low} positive cells) was not affected (**Supplementary Figure S5A**).

These cellular inflammatory effects were not observed in DSS-treated rats fed the DOM-contaminated diet. However, in DSS-treated rats, exposure to DOM-1-contaminated feed significantly increased the percentage of tolerogenic DC (CD103⁺ CD11b/c⁻) (**Figure 4G**) and reduced the secretion of TNF α after LPS stimulation (**Figure 4K**).

Taken together, these results indicate that in the *lamina propria* of rats treated with 5%DSS, additional exposure to DON- but not DOM-1-contaminated feed -exacerbates the pro-inflammatory T cell and dendritic cells immune responses and reduces the anti-inflammatory response.

Exposure to DON but not to DOM-1 induced systemic pro-inflammatory response in 5% DSS-induced colitis in rats

To analyze if DON exposure had any consequences for the systemic immune response, the response of spleen cells was investigated. DSS treatment significantly increased the frequency of Th17 cells without modifying the frequency of Treg and T helper or affecting the Treg/Thelper ratio (**Figure 5A to 5D**). No effect was observed on the frequency of colitogenic DC whereas an increase in tolerogenic DC was observed in animals suffering from colitis (**Supplementary Figure 6A and 6B**). In response to anti-CD3/28, the secretion of IL17 (**Figure 5E**) was significantly higher in the DSS-treated group compared to the control group, whereas no effect was observed on the secretion of IFN γ and IL10 (**Figure 5F and 5G**). Upon LPS stimulation, the secretion of IL1 β increased in rats suffering from colitis compared to control animals, whereas no effect was observed on TNF α secretion (**Supplementary Figure 6C and 6D**).

As observed in the intestine, when DSS-treated rats were fed with the DON-contaminated diet, a tendency to an increased number of Th17 (**Figure 5A**) cells as well as an exacerbation of IL17 (**Figure 5E**) and IFN γ (**Figure 5F**) secretion in response to anti-CD3/28 were observed. DON reduced the Treg/Thelper ratio (**Figure 5D**) in DSS treated rats, increased the number of Thelper cells (**Figure 5C**) and reduced the percentage of regulatory T cells (**Figure 5B**) without modifying the secretion of IL10 (**Figure 5G**). In the spleen of DSS treated rats, DC populations were not affected (**Supplementary Figure 6A and 6B**) and DON exposure did not modify the secretion of IL1 β but increased the secretion of TNF α in response to LPS stimulation (**Supplementary Figure 6C and 6D**). No modification of activated T cells was observed (**Supplementary Figure S5B**).

These systemic inflammatory effects were not observed in DSS-treated rats fed the DOM-contaminated diet. However in DSS-treated rats, in response to anti-CD3/28, exposure to the DOM-contaminated feed significantly increased the secretion of IFN γ (**Figure 5F**) and IL10 (**Figure 5G**) in splenocytes whereas the frequency of Th17 cells in the Treg/Th ratio remained unchanged (**Figure 5A and 5D**).

In summary, these data demonstrate that in rat with colitis, exposure to DON contaminated feed modulates the systemic immune response with an increase in IL17 and IFN γ immune response in the spleen.

DISCUSSION

IBD are multifactorial diseases associated with impaired intestinal barrier functions and dysbiosis of intestinal microbiota (Zhang and Li, 2014). The prevalence of IBD has rapidly increased in recent decades especially in Northern Europe and Northern America, and currently affects five million patients worldwide (Weimers and Munkholm, 2018). Besides genetic predisposition, a role for environmental factors has been suggested (Kaplan and Ng, 2017). In this study, the effect of a mycotoxin, DON, was investigated to determine whether it is a risk factor for IBD.

DON was incorporated in the diet to mimic consumer exposure, which occurs mainly through food intake. Animals were exposed to a very low concentration of the food contaminant DON in their diet. Indeed, the concentration of 8.2 mg DON/kg feed is within the no observed adverse effect levels (NOAELs) determined in different studies in rats. Arnold et al. (1986) determined a NOAEL of 5 mg /kg feed in rats exposed to DON for nine weeks (Arnold et al., 1986) whereas Sprando et al. (2005) and Morrissey et al. (1985) determined a NOAEL of 20mg /kg feed in rat exposed to this toxin for four and 12 weeks, respectively (Morrissey et al., 1985; Sprando et al., 2005).

In this study, a rat model of DSS-induced colitis was used to evaluate the potential impact of exposure to low doses of DON on the onset and the exacerbation of IBD. DSS was added to drinking water for a short period and induced reproducible colitis (Ni et al., 1996; Egger et al., 2000). Recent studies investigated the deleterious effect of food additives and food contaminants on the development of IBD. Chassaing et al. showed that food additives such as the synthetic emulsifiers carboxymethyl cellulose and polysorbate 80 are involved in the development of IBD through microbiota dysbiosis and intestinal inflammation (Chassaing et al., 2017). Similarly, Breton et al. demonstrated that short-term exposure to cadmium (Cd, 5 $\mu\text{g}\cdot\text{kg}^{-1}$) accentuated the symptoms of colitis in a DSS mice model (Breton et al., 2016). In the present study, we demonstrated that ingestion of DON-contaminated diet exacerbated the onset and the symptoms of colitis in rats treated with DSS. This effect was specific to DON, as a detoxified form of DON, DOM-1, had no effect.

Among food contaminants, the mycotoxin DON is present in large amounts in cereals especially in wheat and maize (Knutsen et al., 2017). The worldwide incidence of DON contamination in food is high because of increased use of no till farming, non-optimal crop rotation and inadequate fungicide treatments (Larsen et al., 2004; Knutsen et al 2017). Interestingly, this important exposure to DON coincides with higher incidence of IBD, we thus hypothesize that DON plays a deleterious role in IBD onset and/or exacerbation.

There is overwhelming evidence that when present at moderate to high doses, DON, induces a systemic and intestinal inflammatory response in healthy animals (Pestka, 2010; Payros et al., 2016; Robert et al., 2017). In the present study, we observed that a very low dose of DON, below the one inducing the secretion of mediators such as MPO, CXCL1 and IL-1 β , exacerbated intestinal inflammation in colitis induced with 3% and 5% DSS in rats. In clinical studies, IL1 β levels have been reported to correlate positively with IBD severity (Reinecker et al., 1993; Opipari and Franchi, 2015) and to act in concert with other pro-inflammatory mediators to induce Th17 and Th1 immune responses, key mediators of colitis (Ludwiczek et al., 2004; Raza et al., 2012). In animals fed a DON-contaminated diet with DSS-induced colitis, the elevated IL1 β secretion was associated with an increase in the population of Th17 and an increase in IL17 secretion. Patients with ulcerative colitis present a defect in the CD103⁺ DC function with impaired ability to generate Treg and exhibit a colitogenic function inducing Th1/Th17 responses (Matsuno et al., 2017). We observed that DON increased pro-inflammatory DC CD103⁻ CD11b/c⁺ in lamina propria of rats where colitis was induced with 5% DSS. Taken together, our results strengthen the hypothesis of a role for IL1 β and IL17 in DON colitis. Few studies have investigated the effects of DSS induced colitis on systemic immune responses (Abron et al., 2018). The present experiment demonstrated high IL17 secretion as well as a decrease in the Treg/Helper ratio in rats treated with DSS and exposed to DON. Moreover, DOM-1 has no effect on systemic immune population cells but increased the secretion of IFN γ and IL10 confirming that DOM-1 has some immune-modulatory properties in spleen as evidence by increased the number of proliferating cells in this organ and specific antibody response (Pierron et al., 2018).

As mentioned above, IBD are multifactorial diseases. Among the different factors involved, intestinal hyper-permeability also plays a role in the development of colitis (Wyatt et

al., 1993). The disruption of the barrier functions observed in DON treated cells, explants and animals (Lucioli et al., 2013; Garcia et al 2018, Akbari et al., 2014) could be involved in the development of IBD. Similarly, *Enterobacteriaceae* have been identified as possible markers of microbiota instability predisposing to the onset of IBD (Chassaing and Darfeuille-Michaud, 2011; Rizzatti et al., 2017). In the present study, the exacerbation of the colitis in rats fed with a DON-contaminated diet was also associated with an increase in the number of *Enterobacteriaceae* in the feces.

The last half-century has witnessed increased incidence of IBD in Western and developing countries. DON is the most prevalent fungal toxin present in the food chain in Europe and North America (Knutsen et al., 2017). Our results demonstrated that very low doses of DON exacerbated the onset and the symptoms of colitis. These effects were associated with local colonic inflammation, an increase in *Enterobacteriaceae* and a modulation of both mucosal and systemic immune response. As IBD are multifactorial disorders, these findings show that food contaminants including mycotoxins, can exacerbate these disorders. This observation is even more worrying as the effect is observed at very low doses that do not modify intestinal barrier functions. Epidemiological studies are needed to confirm the role of the environmental exposome and more specifically of mycotoxins as risk factors in IBD or other immune-related or intestinal disorders.

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Competing Financial Interests Declaration

The authors declare that they have no conflict of interest.

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Figure 1.

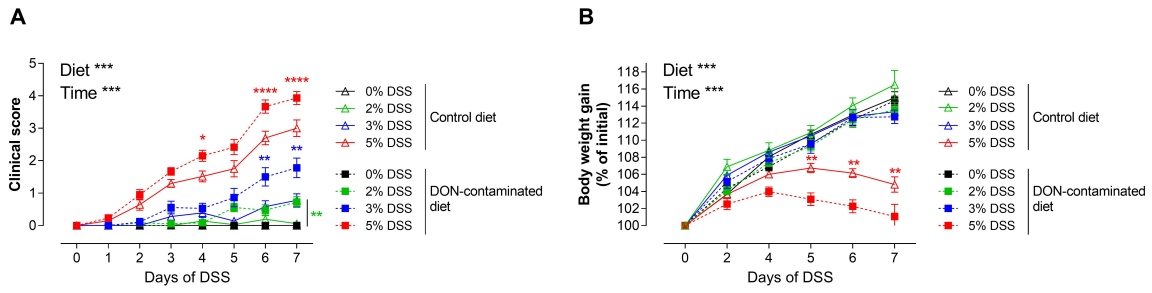


Figure 1: Effect of exposure to DON on the development of DSS-induced colitis in rats with increased doses of DSS. **(A)** Colitis severity in response to 0%, 2%, 3% or 5% DSS. **(B)** Body weight gain (% of initial body weight before DSS treatment). Results are expressed as mean \pm SEM (n = 9-15). One-way ANOVA with Bonferroni's multiple comparison test was used to compare the effect of DON exposure within a group treated with DSS (0, 2, 3 or 5%). Linear mixed model for repeated measures was performed to analyze the effect of the diet (D), the time (T) and the interaction (DxT); * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 2

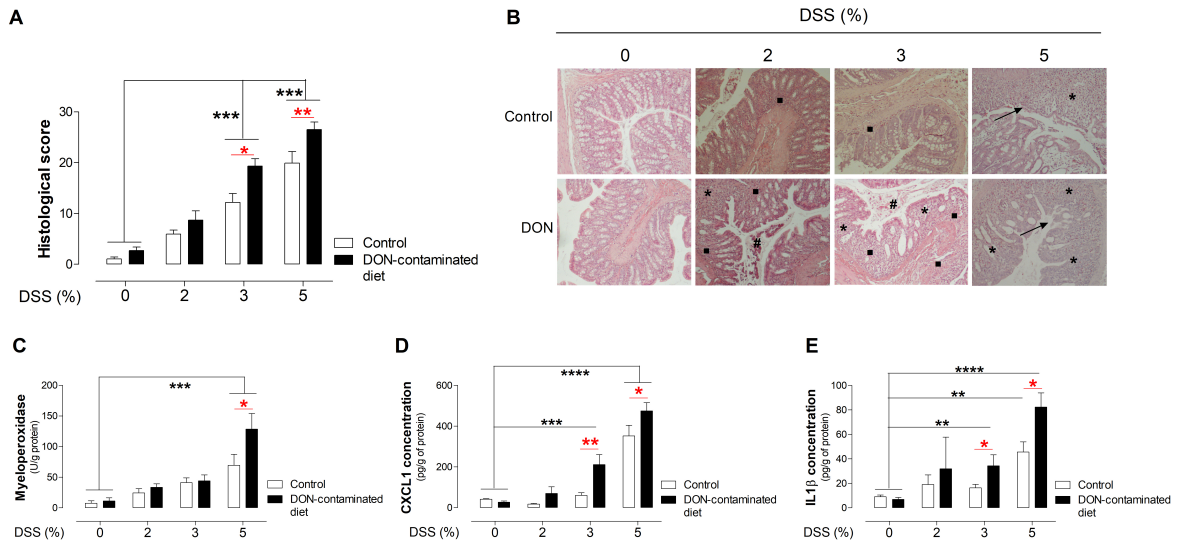


Figure 2. Effect of exposure to DON on morphological damage in the colon of animals treated with increased doses of DSS. **(A)** Histological scores. **(B)** Representative distal colon sections stained with H&E. * loss of crypt, # necrotic and apoptotic debris, ■ immune cell infiltration, (arrow) loss of epithelial cell surface. Images in each row are at the same magnification (x10). Bars = 100 μ m. **(C)** MPO activity. **(D)** Chemokine production CXCL1 (IL-8) and cytokine production IL1 β **(E)**. Results are expressed as mean \pm SEM (n = 9-15). One-way ANOVA with Kruskal-Wallis test, Dunn's multiple comparison test were used to compare the effect of DSS (black stars) or within a group treated with DSS (0, 2, 3 or 5%) the effect of DON exposure (red stars); * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 3.

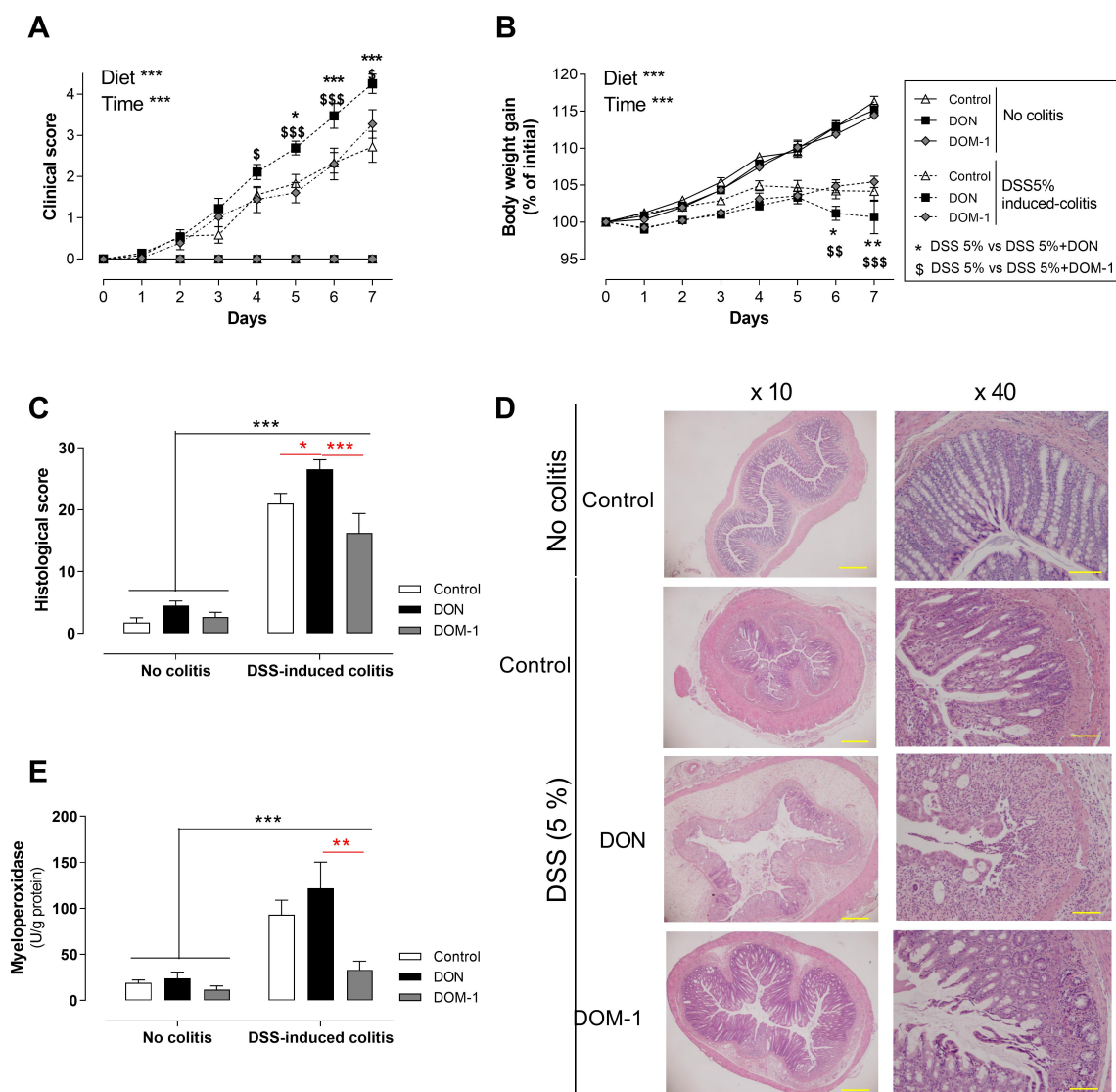
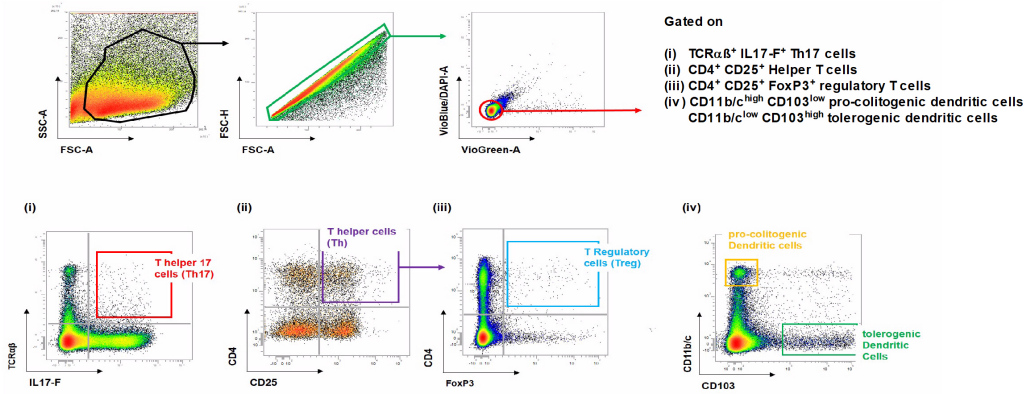


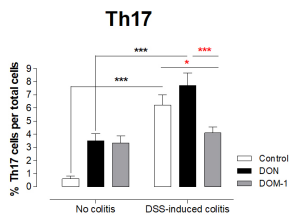
Figure 3. Effect of DON and DOM-1 exposure on the development of colitis and morphological attempts in animals treated with 5% of DSS. **(A)** Disease severity. **(B)** Body weight gain (% of initial body weight before DSS treatment). **(C)** Histological score. **(D)** Representative distal colon sections stained with H&E. Images in each row are at the same magnification: x2 (above) and x10 (below). Bars = 500 μ m (above) or 100 μ m (below). **(E)** MPO activity. Results are expressed as mean \pm SEM ($n = 9-15$). One-way ANOVA with Kruskal-Wallis test, Dunn's multiple comparison test was used to compare two groups. In panels A and B, linear mixed model for repeated measures was performed to analyze the effect of the diet (D), the time (T) and the interaction (D \times T); $^{*+} p \leq 0.05$, $^{**,+} p \leq 0.01$, $^{***,+} p \leq 0.001$.

Figure 4.

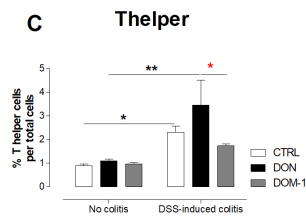
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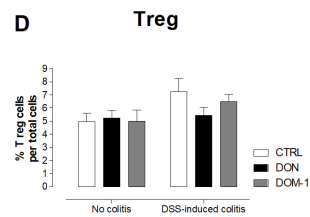
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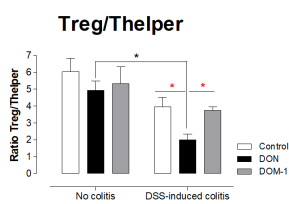
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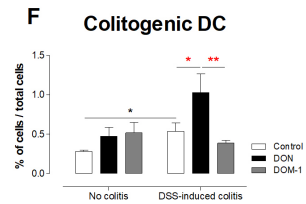
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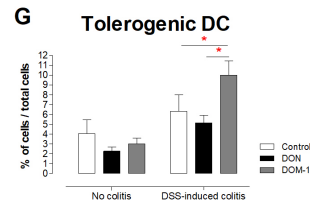
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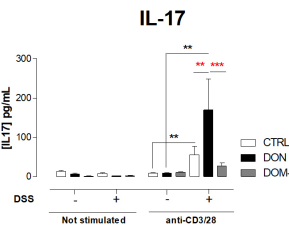
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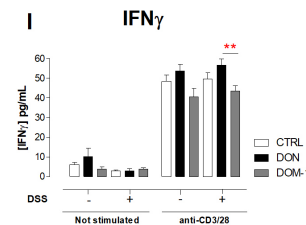
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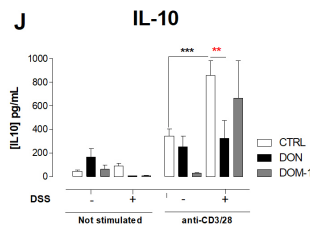
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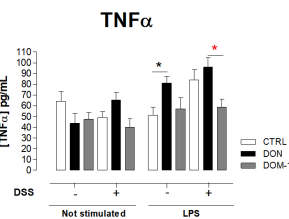
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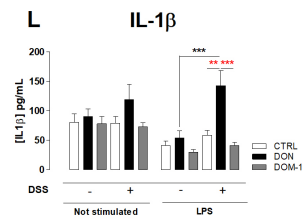


Figure 4. Effect of exposure to DON and DOM-1 on the inflammatory immune response in the ileal lamina propria (iLP) of animals treated with 5% DSS induced colitis. **(A)** Gating strategies for iLP immune cells Th17 cells, regulatory T cells (CD4⁺ CD25⁺ FoxP3⁺ positive cells), helper T cells (CD4⁺ CD25⁺ T cells) pro-colitogenic and tolerogenic dendritic cells. Percentage of Th17 **(B)**, T helper **(C)**, Tregs **(D)**, colitogenic DC **(F)** and tolerogenic DC **(G)**. Ratio of Treg/Thelper cells **(E)**. IL-17 **(H)**, IFN γ **(I)** and IL-10 **(J)** cytokine production after 72 hours of CD3/CD28 T cell stimulation or not. TNF α **(K)** and IL-1 β **(L)** cytokine production after 18 hours of LPS stimulation or not. Data are representative of two batches of experiments. N = 9 animals per group. Mean values \pm SEM are represented. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ between groups of animals fed with control or DON or DOM-1 contaminated diet and treated or not with 5% of DSS.

Figure 5.

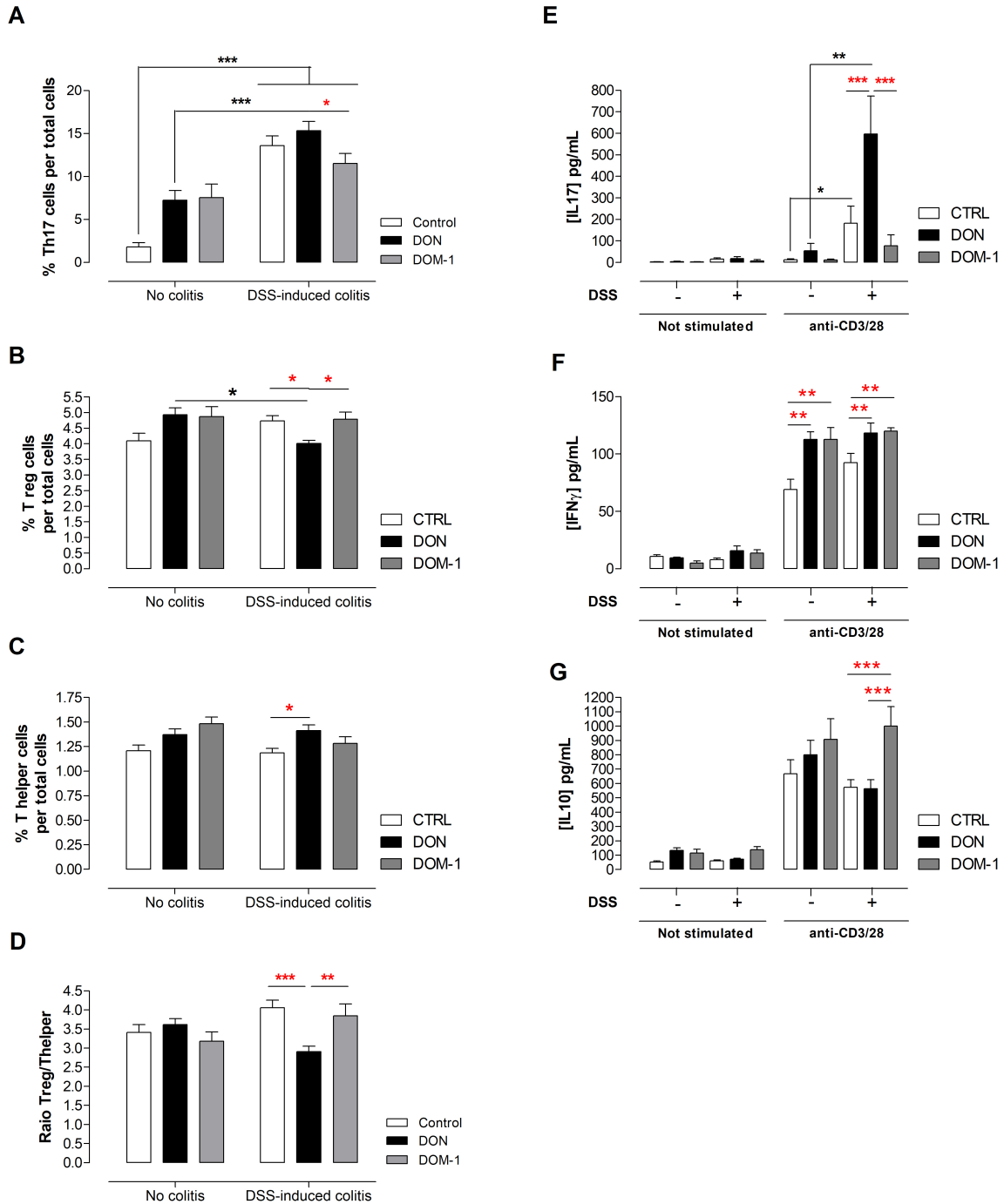
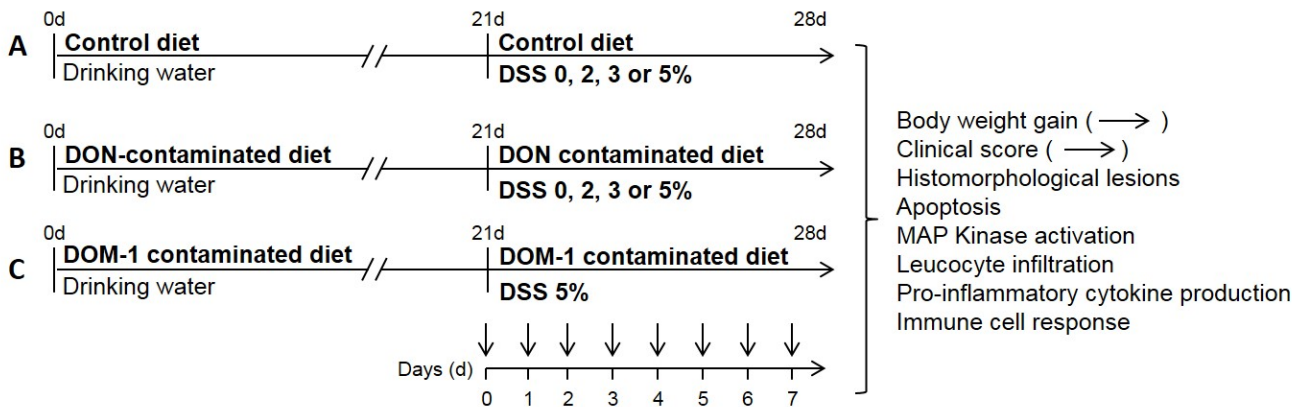


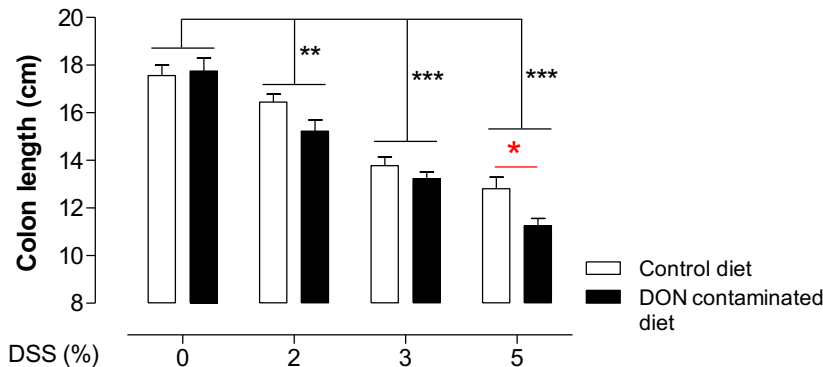
Figure 5. Effect of exposure to DON and DOM-1 on the inflammatory immune response in the spleen of animals treated with 5% DSS induced colitis. Percentage of Th17 (A), Treg(B) and Thelper(C). Ratio of Treg/Thelper cells (D). IL-17 (E), IFN γ (F) and IL-10 (G) cytokine production after 72 hours of CD3/CD28 T cell stimulation or not. Data are representative of two batches of experiments. N = 9 animals per group. Mean values \pm SEM are represented. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ between groups of animals fed with control or DON or DOM-1 contaminated diet and treated or not with 5% of DSS.

Supplementary Figure 1



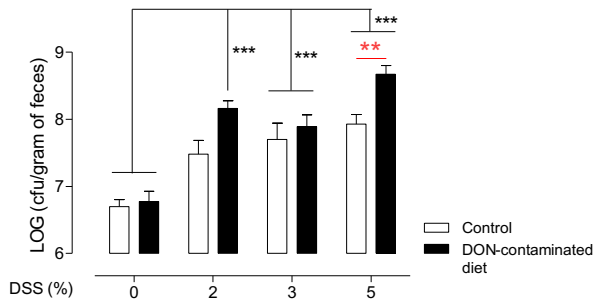
Supplementary Figure 1. Design of rat experimental model for DSS-induced colitis. 4-weeks old male Wistar rats were fed a control (A), DON- (B) or DOM-1 (C) contaminated diet for 4 weeks. During the last week, the animals received different doses of DSS (0, 2, 3 or 5%) in their drinking water to induce acute colitis. Body weight gain and clinical scores were assessed daily during the DSS-treatment. At the end of the experiment, histomorphological lesions, apoptosis, MAP kinase activation, leucocyte infiltration, pro- and anti-inflammatory cytokine production and immune cell response were estimated in the colon, ileal *lamina propria* and the spleen.

Supplementary Figure S2



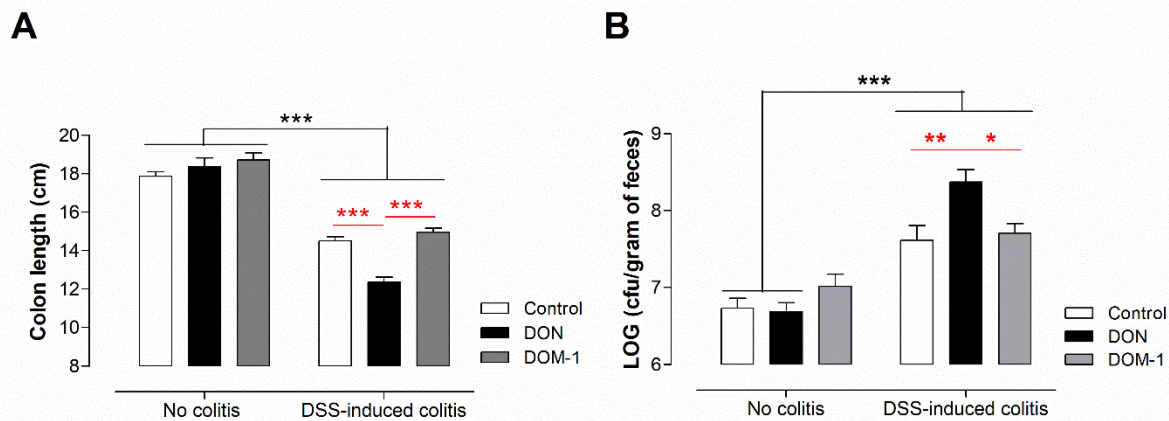
Supplementary Figure 2. DON exposure exacerbated the decrease in colon length in a DSS-induced colitis model. After DSS-induced colitis (day 7), the animals were sacrificed and the colon length was assessed (cm). Colon length depends on the severity of colitis and can be modulated both by the dose of DSS used to induce colitis and by the treatment (control- or DON-contaminated diet). N = 9 to 14 animals per group. Mean values \pm SEM are represented. One-way ANOVA with Bonferroni's multiple comparison test assessed the differences between all the experimental groups, ** $P \leq 0.01$, *** $P \leq 0.001$. A Student's t-test evaluated the differences between animals without colitis and animals treated with 2 %, 3 % or 5 % DSS. * $P \leq 0.05$, between control animals treated with 5% DSS and animals fed with the DON-contaminated diet and treated with 5% DSS.

Supplementary Figure S3



Supplementary Figure 3. Members of the family Enterobacteriaceae were quantified in fecal homogenates at day 7 after treatment, or not, with DSS in animals fed with the control or DON-contaminated diet. N = 9 to 18 animals per group. Mean values \pm SEM are represented. One-way ANOVA with Bonferroni's multiple comparison test assessed the differences between all the experimental groups, *** $P \leq 0.001$ between the group with no colitis and the group with colitis treated with 5 % DSS. ** $P \leq 0.01$ between animals treated with 5 % DSS and fed the DON-contaminated diet and animals treated with 5 % DSS and fed with the control diet.

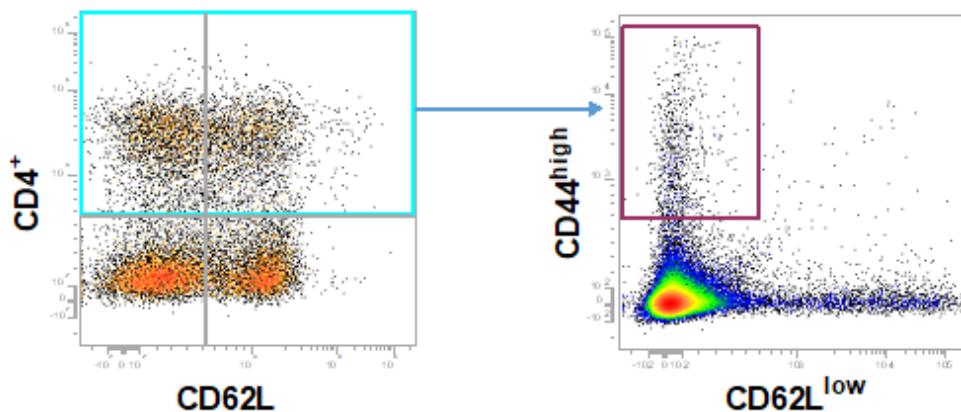
Supplementary Figure 4.



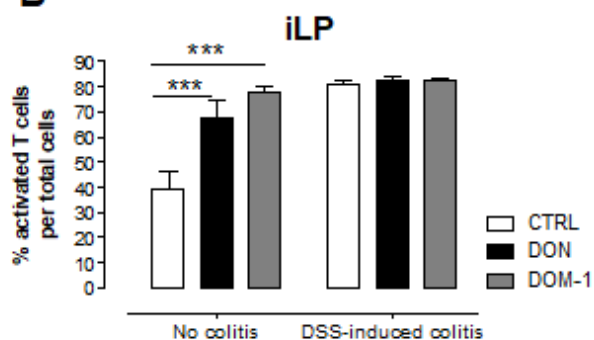
Supplementary Figure S4. Effect of DON or DOM-1 exposure on (A) colon length and (B) members of the family Enterobacteriaceae in fecal homogenates. N = 9 animals per group. Mean values \pm SEM are represented. One-way ANOVA with Bonferroni's multiple comparison test assessed the differences between all the experimental groups, *** $P \leq 0.001$ between groups with no colitis and groups with colitis fed 5 % DSS. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ between all the groups of animals treated with 5 % DSS and fed with control, DON or DOM-1-contaminated diet.

Supplementary Figure S5.

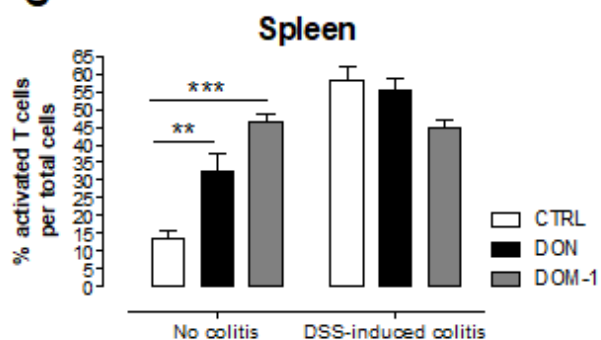
A



B

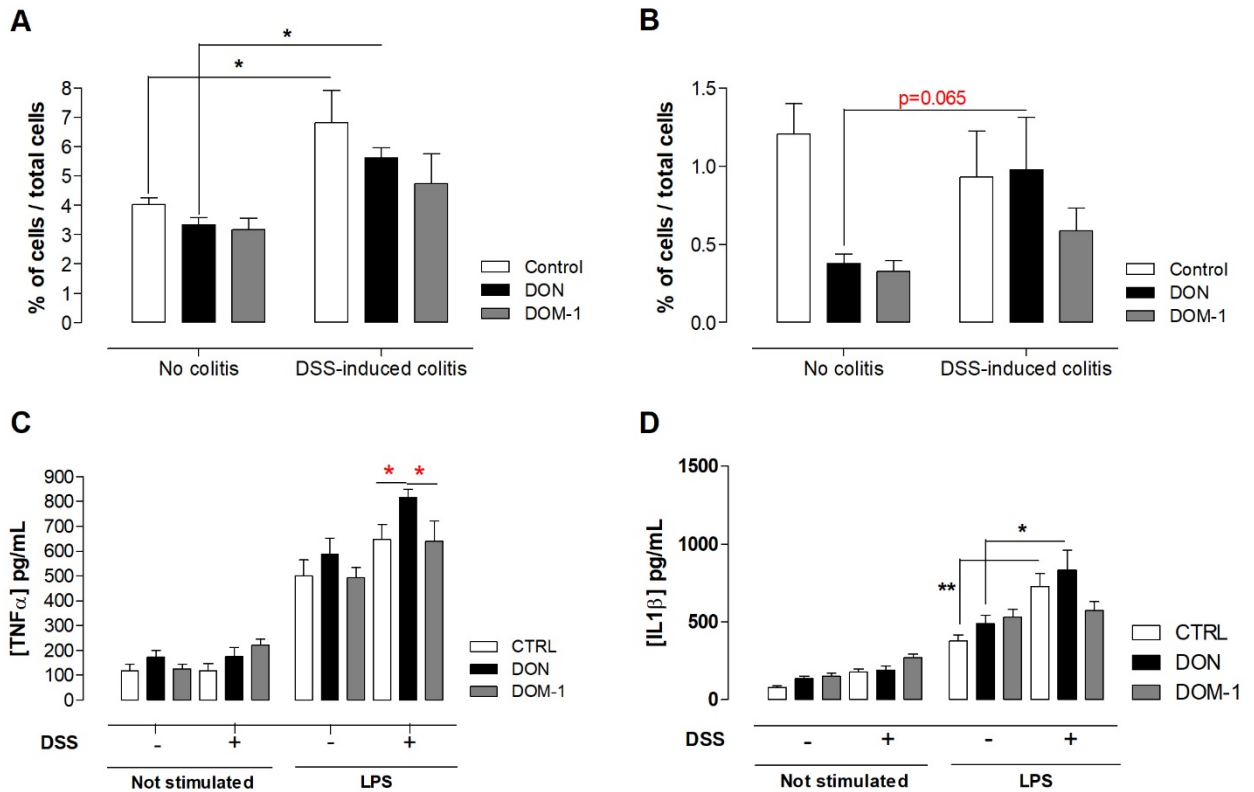


C



Supplementary Figure S5. Effect of DON- or DOM-1-contaminated diet on the activated T cells in the ileal *lamina propria* (iLP) and spleen. **(A)** Gating strategy for activated T cells in iLP. Flow cytometry analysis of percent of CD4⁺ CD44^{high} CD62L^{low} in iLP **(B)** and spleen **(C)** of rats fed with the control, DON or DOM-1 contaminated diet and treated with 5 % DSS.

Supplementary Figure S6.



Supplementary Figure S6. Effect of exposure to DON or DOM-1 on the dendritic cell (DC) populations in the spleen of animals suffering from colitis induced with 5 % DSS. **(A)** colitogenic DC and **(B)** tolerogenic DC. $\text{TNF}\alpha$ **(C)** and $\text{IL-1}\beta$ **(D)** cytokine production after 18 hours of LPS stimulation. Data are representative of two batches of experiments. $N = 9$ animals per group. Mean values \pm SEM are represented. * $P \leq 0.05$, ** $P \leq 0.001$, between groups of animals fed with control or DON or DOM-1 contaminated diet and treated or not with 5 % DSS. * $P \leq 0.05$, between groups of animals treated with 5 % DSS and fed with control or DON or DOM-1 contaminated diet.

Supplementary Table S1.

Composition and mycotoxin contamination of experimental diets

	Diet		
	Control	DON 10mg.kg ⁻¹	DOM-1 10mg.kg ⁻¹
Composition premix			
Cellulose	3.6	3.6	3.6
Caséine chlorhydrique	14.4	14.4	14.4
L Cystine	0.22	0.22	0.22
Mineral compound AIN93G	2.52	2.52	2.52
Vitamin compound AIN93Vx	0.72	0.72	0.72
Bitartrate de choline	0.18	0.18	0.18
Maltodextrine	9.5	9.5	9.5
Maize starch	28.62	28.62	28.62
Sugar	7.2	7.2	7.2
Soya oil	5.04	5.04	5.04
Mycotoxin contamination (mg.kg ⁻¹)			
Deoxynivalenol (DON)	< 0.010	8.18	0.050
De-epoxy-deoxynivalenol (metabolite DOM-1)	< 0.010	< 0.010	8.01
Deoxynivalenol -3-β-D-glucopyranoside (metabolite D3G)	< 0.010	< 0.010	< 0.010
3-acetyl DON	< 0.010	< 0.010	< 0.010
15-acetyl DON	< 0.010	< 0.010	< 0.010
Nivalenol	< 0.010	0.035	< 0.010
Zearalenone	< 0.010	< 0.010	< 0.010

Supplementary Table S1. Composition and mycotoxin contamination of experimental diets. The table shows the composition of the diet and analysis of mycotoxins found in the diet. No mycotoxins were found in the control diet. Only DON was found in the DON-contaminated diet and DOM-1 was found in DOM-1 contaminated diet.

Supplementary Table S2.

Clinical score

Score	Stool consistency	Bleeding
0	Formed stool - normal	No trace of blood
2	Soft stool	Trace of blood in the stool
4	Diarrhea	Rectal bleeding

Supplementary Table S2. Clinical score was based on the evaluation (0 – 4) of the last two parameters: stool consistency and bleeding; 0, formed stool and no trace of blood; 1, diarrhea; 2, diarrhea and trace of blood in the stool; 3, diarrhea and rectal bleeding; 4, diarrhea and rectal bleeding with rectal prolapse.

Supplementary Table S3.

Histological colitis scoring method

Feature scored	Description	Score
<i>Inflammation severity</i>	None	0
	Mild	1
	Moderate	2
	Severe	3
<i>Inflammation extent</i>	None	0
	Mucosa	1
	Mucosa and submucosa	2
	Tansmural	3
<i>Crypt damage</i>	None	0
	Based 1/3 damaged	1
	Based 2/3 damaged	2
	Crypts lost: surface epithelium prese	3
	Crypts and surface epithelium loss	4
<i>Percent involvement</i>	0%	0
	1% - 25 %	1
	25% - 50%	2
	50% - 75%	3
	> 75%	4

Supplementary Table S3. Colonic damage was graded semi-quantitatively & blindly by two different persons (D.P.; M.G.). Each of 3 histological parameters, based on the severity of inflammation, the extent of inflammation and the epithelium damage was scored as absent (0), mild (1% - 25%, 1), moderate (25% - 50%, 2), extended (51% - 75%, 3) or severe (> 75%). The histological score is the sum of these parameters.

Supplementary Table 4.

Colitis score evaluation

Compararison of control animals versus all the groups treated with DSS exposed or not to DON

Diet	DSS treatment (%)	Number of animals	Days of treatment						
			D1	D2	D3	D4	D5	D6	D7
Control diet	0	15	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	2	9	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	3	15	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
	5	15	n.s.	n.s.	***	****	****	****	****
DON-contaminated diet	0	15	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	2	9	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	3	15	n.s.	n.s.	n.s.	n.s.	*	****	****
	5	15	n.s.	***	****	****	****	****	****

Supplementary Table S4. Colitis score evaluation. Significant comparison of control animals versus all the groups treated with DSS exposed or not to DON mycotoxin. N = 9 to 15 animals per group. One-way ANOVA with Bonferroni's multiple comparison test assessed the differences between all the experimental groups, * $P \leq 0.05$, *** $P \leq 0.001$, **** $P \leq 0.001$ between control animals fed with control diet and all the other groups.