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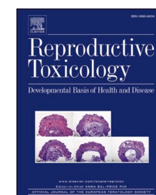

Deoxynivalenol exposure during pregnancy has adverse effects on placental structure and immunity in mice model

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ABSTRACT

Deoxynivalenol (DON), a highly prevalent food contaminant, is known to induce reproductive and immunotoxicity in humans upon exposure. The present study focused on the consequences of exposure to DON during pregnancy for placental barrier and immune function, as well as fetal survival. Female mice received diets contaminated with DON (6.25 and 12.5 mg/kg of diet), starting immediately after mating until the end of the experiment. On day 17 of pregnancy the animals were killed, and maternal and fetal samples were collected for further analysis. Feeding on DON-contaminated diets decreased fetal survival, and DON was detected at significant levels in the fetus. Placentae from DON-exposed mice revealed a reduction in expression of junctional proteins, ZO-1, E-cadherin and claudins, upregulation of AHR mRNA expressions, and increase in IFN-γ, IL-6 and IL-4 production. In conclusion, results of this study demonstrate harmful effects of DON on the course of pregnancy and fetal survival, which might be due to immunological changes in maternal immune organs and placenta. Altogether, these data underline the importance of the quality of maternal diet during pregnancy as they clearly demonstrate the potential harmful effects of a commonly present food-contaminant.

1. Introduction

Pregnancy is a critical period of physiological changes for both the mother and the fetus. Lifestyle, including maternal nutrition, is crucial in long-term health of the infant. Beneficial effects of nutritional supplementation during pregnancy with, for example, prebiotics and polyunsaturated fatty acids on the development of the fetus have been demonstrated previously [1,2]. On the other hand, exposure to various environmental factors and contaminants can interfere with the process of developmental programming, leading to long-term or permanent structural or functional changes in the offspring. Exposure to such stimuli can potentially impose adverse effects on fetal survival and health [3]. The exact mechanisms and causes of many adverse pregnancy outcomes are not fully understood yet, but increasing evidence links pregnancy complications and incidence of some non-communicable chronic diseases in the offspring to the quality of maternal nutrition [4–6]. Moreover, the developing fetus is vulnerable to a wide range of contaminants in maternal food and the environment, which can reach the fetus during pregnancy and lead to adverse developmental outcomes [4,7], including childhood cancers [8], allergic and autoimmune diseases [9,10].

An important group of such nutritional contaminants are mycotoxins, which are secondary metabolites of fungi. Some of the highly prevalent mycotoxins such as aflatoxins, ochratoxins, fumonisins, trichothecenes and zearalenone can induce acute or chronic toxicity upon consumption in humans and animals, even at low concentrations [11]. These mycotoxins are produced by fungal species in the Aspergillus and Fusarium genera, which most frequently contaminate agricultural food products, especially cereal crops [11,12]. As a result of the high prevalence of these fungi in the food chain, mycotoxin exposure is almost ubiquitous and epidemiological studies from different geographical regions indicate that pregnant women and newborns can be easily exposed to mycotoxins [7,13,14]. This potentially harmful exposure during the critical period of pregnancy and early life is associated with various adverse health outcomes, such as skeletal malformation, neural tube defects, growth retardation and neonatal jaundice

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[15,16].

Deoxynivalenol (DON), a trichothecene mycotoxin produced by various different *Fusarium* species, is one of the most common contaminants of cereal crops, especially oats, barley, wheat and maize [17]. Human exposure to DON has been indicated to frequently exceed the stablished safety levels [18–20]. Masked and modified forms of DON, which are not detected by established standard methods, may lead to underreporting of mycotoxin levels in food products [21]. Thus, investigating the toxicity of DON exposure on human health is of utmost importance. The mechanisms by which DON causes toxicity are induction of oxidative stress, DNA fragmentation and inhibition of protein synthesis [22,23]. Exposure to DON elicits pro-inflammatory responses, cytotoxicity and apoptosis in different organs [24–26], which can result in growth impairment and impose adverse effects on gastrointestinal tract, immune regulation and reproduction [27]. In several animal species, DON exposure induced reproductive toxicity and teratogenic effects [28]; the most prominent consequences for the fetus were abnormal skeletal development and lower fetal survival rate [29–31].

Considering that the placenta is the only crossing point between maternal and fetal circulation, fetal exposure to DON can only occur via placental transport. Indeed, DON can be transcellularly transported across BeWo cell monolayers, a trophoblastic cell line used to model the human placental barrier [32]. Moreover, DON transport to the fetus has been measured in different species in vivo [33–35]. Obviously, in these cases the placenta itself is also exposed to DON which can pose additional health risks. As the placenta facilitates the transport of nutrients and elimination of metabolic waste products, defects in its structure and function result in fetal growth restriction and health problems or even fetal death [36,37]. The placenta is also responsible for transmission of maternal immune mediators to the fetus [35]. A healthy development of the fetus depends on the regulation of the immunological milieu within the placental environment. An altered immunological status in the placenta underlies many pregnancy complications such as pre-eclampsia and miscarriage [38]. In a previous study, we demonstrated that DON can disrupt the placental epithelial barrier and induce proinflammatory responses in an in vitro model [39]. Although the evidence for adverse developmental effects of DON exposure is clear, very little is known about the effect of DON on the course of pregnancy and the placenta. Therefore, the present study focused on the consequences of chronic exposure to DON-contaminated diet on pregnancy in mice, with a specific focus on changes in the placental structure, as well as immune modulation in the mother and the fetus.

2. Materials and methods

2.1. Animals

Six-week-old, male and female C3H/HeOuJ mice (50 female and 25 male mice), purchased from Charles River Laboratories (Sulzfeld, Germany) were housed at the animal facility of Utrecht University (Utrecht, The Netherlands) at controlled temperature (21 ± 2 °C) and humidity (50–55%), with a reversed 12:12 h light/dark cycle (lights on from 7.00 pm till 7.00 am) and with ad libitum access to food and tap water. Animals were kept in makrolon cages (22 cm × 16 cm×14 cm, floor area 350 cm2, Tecnilab- BMI, Someren, the Netherlands), in groups of 2 per cage, with wood-chip bedding (Tecnilab- BMI, Someren in the Netherlands), and tissues (VWR, the Netherlands) and cardboard shelters were available. Upon arrival, female mice were randomly allocated to the experimental groups (12 mice per group) and all mice were habituated to laboratory conditions for one week prior to the start of the study. The animals received standard control diet (pelleted food, AIN-93 G, Ssniff Spezialdiäten, Soest, Germany) and routine care for a week upon arrival in the animal facility, before the start of the experiments. This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals of the Utrecht University, and all animal procedures were approved by their Animal Welfare Body under the Ethical license of the national competent authority (Centrale Commissie Dierproeven, CCD).

2.2. Diets

To obtain the experimental diets, semi-purified AIN-93 G soy protein-based diets were composed and mixed with different concentrations of DON (6.25 or 12.5 mg per kg of diet, FERMENTEK Ltd, Jerusalem, Israel) by Ssniff Spezialdiaten GmbH (Soest, Germany). Maximum permitted levels of DON in different cereals and cereal-based products are set by European Commission and United States to be 1–2 mg/kg of food [40]. However, analyzing wheat, corn and other cereal samples from all over the world revealed a considerable percentage of food product exceeding this safety level [41–43], as DON concentration in several commodities were reported to be as high as 20 mg/kg of food [44]. Moreover, consuming high amounts of cereals and cereal-based products, and combined consumption of a wide variety of DON-contaminated food can lead to a significantly higher exposure levels in specific populations [44].

2.3. Experimental design

After 7 days of acclimatization, female mice were fed either AIN-93 G (control) or DON-containing diets (6.25 or 12.5 mg DON/kg of diet) for 2 weeks prior to mating.

In order to avoid DON exposure in the male mice, all animals received control diet during mating. Males and female mice were mated for 4 days by placing one male mouse in the cage with 2 female mice. In order to improve the mating success, part of the bedding from the cage of male mouse was transferred to the cage of female mice. Immediately after separating male mice from the females, the female mice (2 per cage) received control/DON diets until the end of experiment. In order to prevent any additional discomfort and stress during gestation period, the animals were weighed only before mating and at the end of experiment to evaluate the weight gain. The first day after starting the mating was considered day 0 of pregnancy. The female mice were killed by cervical dislocation on day 17 after mating, and samples were collected from mice with live or visible resorbed fetuses (late resorptions) in their uteri. Since the exact day of mating is not monitored in this study, a 3-day variation in gestation day should be taken into account, thus the day of scheduled euthanasia may vary from 14 to 17 days after mating. Breeding success was calculated as ratio of pregnant mice with fetus or visible resorption sites in the uterus on day 17 of pregnancy (late resorptions), to all mated female mice. Isolated placentas were either stored in fixation buffer or snap frozen until further processing. Prior to snap freezing, placentas were cut in half to allow RNA extraction and protein extraction from the same placenta. The male mice were available as surplus for other research after mating.

2.4. FITC-dextran permeability assay

All animals received FITC-dextran (molecular mass 4 kDa; Sigma-Aldrich) by an oral gavage (300 μl of 50 mg/ml FITC-D solution in PBS), 4 h before cervical dislocation. The concentration of FITC-dextran in the serum was determined by measuring fluorescence intensity using a spectrofluorometer (FLUOstar Optima; BMG Labtech) set at excitation and emission wavelengths of 485 and 520 nm, respectively, according to the methods described previously [45].

2.5. RNA Extraction from placenta and fetal intestine samples and Quantitative qPCR

Placental tissues and fetal intestine were weighed and subsequently homogenized with RNA lysis buffer (provided within RNA isolation kit) with 1:1 (w/v) ratio. The clear supernatants of the homogenates were collected for RNA isolation according to the manufacturer's instructions

(SV Total RNA Isolation System, Promega Corporation, Madison, WI, USA). Total RNA content and purity of the samples was measured using the NanoDrop ND-1000 Spectrophotometer (Thermofisher Scientific, Wilmington, DE, USA). cDNA was prepared with use of the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA).

For qPCR, iQSYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and primers for selected genes (Bio-Rad Laboratories, Hercules, CA, USA) were mixed with the samples. Amplifications were performed according to the manufacturer's instructions using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The mRNA quantity was calculated relative to the expression of β-actin reference gene and normalized to the average of control group.

2.6. Western blotting of placenta samples

Half of the placenta collected from each pregnant mouse was weighed and homogenized with RIPA Lysis and Extraction Buffer (Thermo Scientific, Rockford, IL, USA) containing protease inhibitors (Roche Applied Science, Penzberg, Germany) with 1:1 (w/v) ratio. The clear supernatants of the homogenates were used for isolating protein. Total protein concentration was assessed by a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Western blotting was performed according to previously described methods [46]. Primary antibodies for mouse CLDN-3, CLDN-4, ZO-1, OCLD (all from Invitrogen, Carlsbad, CA, USA), and E-cadherin (eBioscience, San Diego, CA, USA), as well as the appropriate secondary antibodies (Dako, Glostrup, Denmark) were used to label the relevant protein bands. Protein bands were visualized by ChemiDoc™ XRS+ System (Bio-Rad Laboratories, Hercules, CA, USA) using ECL reagents mix (Amersham Biosciences, Roosendaal, The Netherlands). The images were analyzed using Image Lab software (version 5.2, 2014, Bio-Rad Laboratories, Hercules, CA, USA). Monoclonal rabbit anti-human β-actin antibody (Cell Signaling, Danvers, MA, USA) was used to evaluate the homogeneity of loading and normalize the optical density of the bands. The protein levels were expressed as the fold change in relation to the average of control group.

2.7. Cytokine measurements of placenta and amniotic fluid

Part of the clear supernatants of the placenta homogenates prepared in RIPA buffer, and amniotic fluid samples collected from amniotic sac of fetuses were used to measure the concentration of cytokines interferon gamma (IFN-γ), interleukin (IL)− 4, IL-6, IL-10, IL-17, IL-1β and tumor necrosis factor alpha (TNF-α) using ProcartaPlex mouse multiplex immunoassays (Thermofisher Scientific, Wilmington, DE, USA) according to the manufacturer's instructions.

2.8. Immunofluorescence staining of placenta

After fixation in 10% formalin, placental samples were embedded in paraffin and 5 µm sections were deparaffinized, and endogenous peroxidase activity was blocked with 0.3% H2O2 (Merck, Darmstadt, Germany) in methanol and rehydrated in a graded ethanol series. Antigen retrieval was performed by incubating the samples in 10 mM citrate buffer (PH 6.0) for 10 min in a microwave. To block non-specific binding sites, 5% goat serum (Dako, Glostrup, Denmark) was used. The sections were incubated with primary antibodies against ZO-1 (1:200, Invitrogen, Carlsbad, CA, USA) or E-cadherin (1:200, eBiosciences, San Diego, CA, USA) followed by Alexa-Fluor conjugated secondary antibodies (1:200, Invitrogen, Carlsbad, CA, USA). Anti-fading mounting medium containing DAPI (ProLong Gold with DAPI, Life Technologies, Thermo Fisher Scientific, Wilmington, DE, USA) was used for nuclear counterstaining and sealing the slides with a coverslip. Localization of the junctional proteins was visualized using the Microscope Leica TCS SP8 X.

2.9. DON measurement in fetus samples

Frozen whole fetus samples were weighed, cut into small pieces and homogenized in PBS at 1:1 v/w ratio. The clear supernatant of the homogenates was used to measure DON concentration by Deoxynivalenol ELISA Assay Kit (Eagle biosciences, Amherst, New Hampshire, USA) according to the manufacturer's instructions.

2.10. Short chain fatty acid concentration in cecum content of pregnant mice

Cecum content of pregnant mice was homogenized in cold PBS at a ratio of 1:10. Clear supernatants of the homogenates were collected and used for measuring the concentration of acetic, propionic, and butyric acids by gas chromatography, as previously described [47], using 2-ethylbutyric acid as internal standard.

2.11. Flowcytometry analysis of spleens and iliac lymph nodes of pregnant mice

Fresh spleen samples collected from pregnant mice were used for isolating splenocytes by methods described previously [48]. Immune cells were labeled using fluorochrome-conjugated monoclonal antibodies against CD4, Tbet (both from BioLegend, San Diego, CA, United States), CD69, CXCR3, CD25, Foxp3, AHR (all from eBiosciences, Thermo Fisher Scientific, San Diego, CA, USA) and T1ST2 (MD Biosciences, St. Paul, MN, USA). A fixable viability dye (eBioscience) was used for assessing cell viability. For detection of intracellular transcription factors, Foxp3 Staining Buffer Set (eBioscience) was used to permeabilize the cells, according to manufacturer's protocol. Results were collected with BD FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed with FlowLogic software (Inivai Technologies, Mentone, VIC, Australia). The gating strategy for selecting specific T cell populations is shown in supplementary figure 1.

2.12. Statistical analysis and sample size calculation

Statistical analyses were performed using GraphPad Prism (version 8, 2018, GraphPad, La Jolla, CA, USA). Results are expressed as the mean ± SEM and differences among groups were statistically determined using one-way analysis of variance (ANOVA) followed by a Bonferroni's multiple comparison post hoc test for selected comparisons. For not normally distributed data Kruskal-Wallis test was performed followed by Dunn's multiple comparisons test. Fisher's exact test was used for comparing differences between proportions. Differences were considered significant at $p < 0.05$.

Sample size was calculated using G*Power v3.1.9, based on a previous in vivo study [31]. The effect size was calculated to be 1.2 based on the expected resorbed implants, and the power was set on 0.9. Correcting alpha based on number of relevant comparisons resulted in the required group size of 6 for each dietary group. Assuming an average breeding success to be 50 (± 5) %, 12–13 female mice were randomly allocated in each dietary group. However, due to the negative effect of DON on breeding success, the number of pregnant mice in groups receiving 12.5 mg DON/kg of diet was lower ($n = 3$), hence the power was recalculated to be 0.8 based on actual number of pregnant mice.

3. Results

3.1. DON exposure during pregnancy significantly affected weight gain and fetal survival

The effects of feeding mice with DON-contaminated diets on pregnancy outcome is summarized in Table 1. A concentration of 12.5 mg DON/ kg diet reduced the breeding success (i.e. the number of pregnant mice) and decreased the fetal survival rate. The lowest concentration of

Table 1
Effect of deoxynivalenol (DON) exposure during pregnancy on breeding success (%) and fetal survival rate (%). ^{ns} p > 0.05, ***p < 0.001 show significant difference compared to control group, using Fisher's exact test.

	Control	DON 6.25 mg/kg	DON 12.5 mg/kg
Number of mated female mice	12	13	13
Number of pregnant mice (% breeding success)	5 (42%)	8 ^{ns} (61%)	3 ^{ns} (23%)
Number of live fetuses per pregnant mouse	6	4.6	2.6
Number of late resorptions per pregnant mouse	1.5	2.8	5.3
Ratio of live/resorbed fetuses	4	1.6^{ns}	0.5***
survival rate (%) = no. of live fetuses / (no. of live + no. of late resorptions) x100	(80%)	(61%)	(33%)

DON induced an apparent increase in breeding success; however, the fetal survival rate was lower compared to control group. Overall, the average number of live fetuses per mouse was decreased as fetal survival was reduced in DON-receiving groups.

Numbers of live and visible resorbed fetuses were counted in the uteri of pregnant animals. The gross macroscopic appearance of non-pregnant and pregnant uteri is depicted in Fig. 1 A, where a clear difference can be observed between the uterus with live fetuses and the uterus with late resorptions. The concentration of DON in fetal homogenates was significantly higher in DON-fed mice, compared to the control group, demonstrating placental transport of DON from maternal to fetal side (Fig. 1B). The tested concentrations of DON in this study did not significantly affect intestinal permeability of pregnant mice, as the concentration of FITC-dextran in the serum was not significantly different between groups (supplementary figure 2).

3.2. DON exposure during pregnancy does not affect SCFA concentrations in the cecum of pregnant mice

Considering the importance of intestinal bacterial colonization in immune system, concentrations of SCFA in the cecum of pregnant mice were measured as a marker for metabolic activity of gut microbiota (Fig. 1C-E). No significant effect on concentrations of different SCFAs was observed between different groups, however, a slight decrease in acetic acid concentration was observed in the cecum of mice fed with DON-contaminated diets (p = 0.06) (Fig. 1C).

3.3. DON exposure during pregnancy alters Th1/Th2 balance in maternal spleen and lymph nodes

Results of flowcytometric analysis showed no significant effect of consuming DON-contaminated diets on Th1, Th2, Th17 and regulatory T cells populations in spleen and ILN isolated from pregnant mice (Fig. 2). Although DON exposure had no significant effect on Th2 in the spleen (Fig. 2 A), a decrease in T1ST2^{+} Th2 cells (p < 0.05) could be demonstrated in the ILNs of animals fed with diet containing 12.5 mg DON/kg (Fig. 2C). Percentages of Th1, Th17 and regulatory T cells in spleen and ILN were not significantly affected by DON exposure (Fig. 2B, D, E-H). However, a significant reduction in AHR $^{+}$CD4^{+} T cells was observed in the spleens of DON-exposed animals, compared to the control group (Fig. 2I).

3.4. DON exposure during pregnancy altered the mRNA and protein expression of junctional proteins in placenta

The mRNA expression levels (Fig. 3A-D) and protein concentrations (Fig. 3E-H) of different junctional proteins were measured in placenta to assess the effect of DON on placental barrier function. Representative protein bands in western blot are shown in supplementary figure 3. Although the mRNA expression level of ZO-1 was not significantly

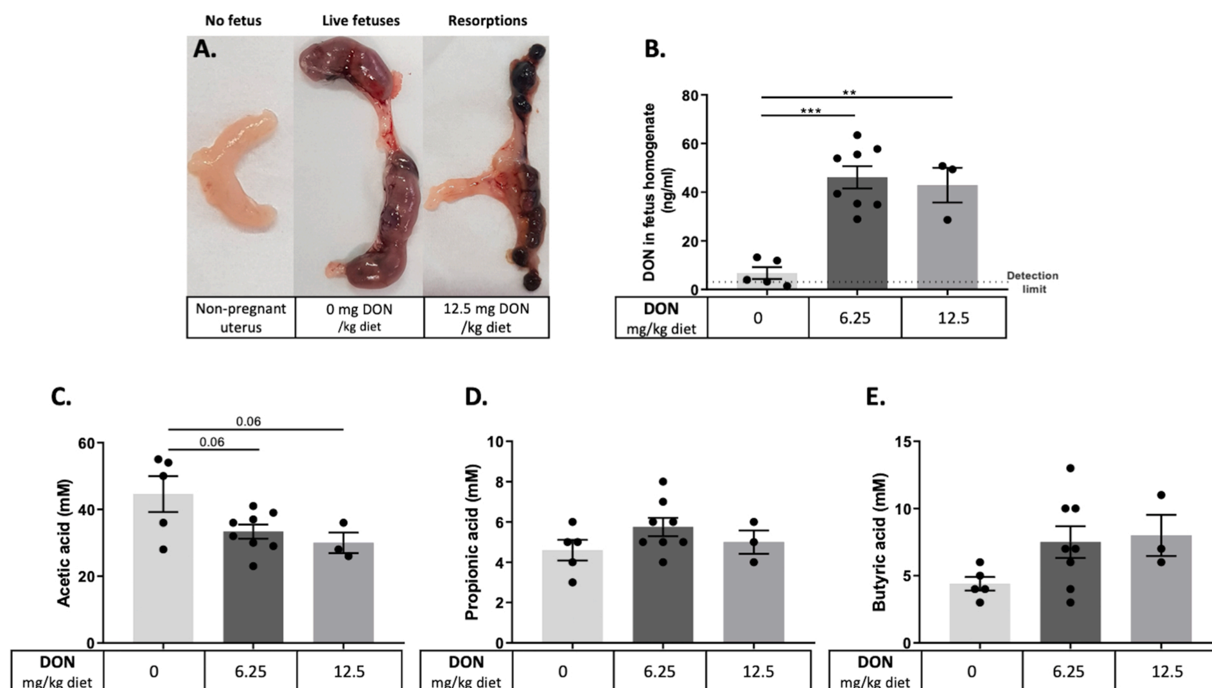

Fig. 1. Deoxynivalenol (DON) exposure negatively affects pregnancy outcome and Short-chain fatty acids (SCFA) concentrations in cecum. Pregnant mice were fed either a control or DON-contaminated diets (6.25 or 12.5 mg/ kg of diet). (A) Representative picture comparing the uterus from a normal non-pregnant mouse, a healthy pregnant mouse on control diet with live fetuses, and a pregnant mouse receiving 12.5 mg DON/kg diet, with resorbed fetuses; (B) the concentration of DON in the whole-fetus homogenates, measured by ELISA. The concentrations of (C) acetic acid, (D) propionic acid, and (E) butyric acid present in the supernatants of homogenates of cecum contents was measured. Data expressed as mean ± SEM, *p < 0.05 and ***p < 0.001 represent the significant difference.

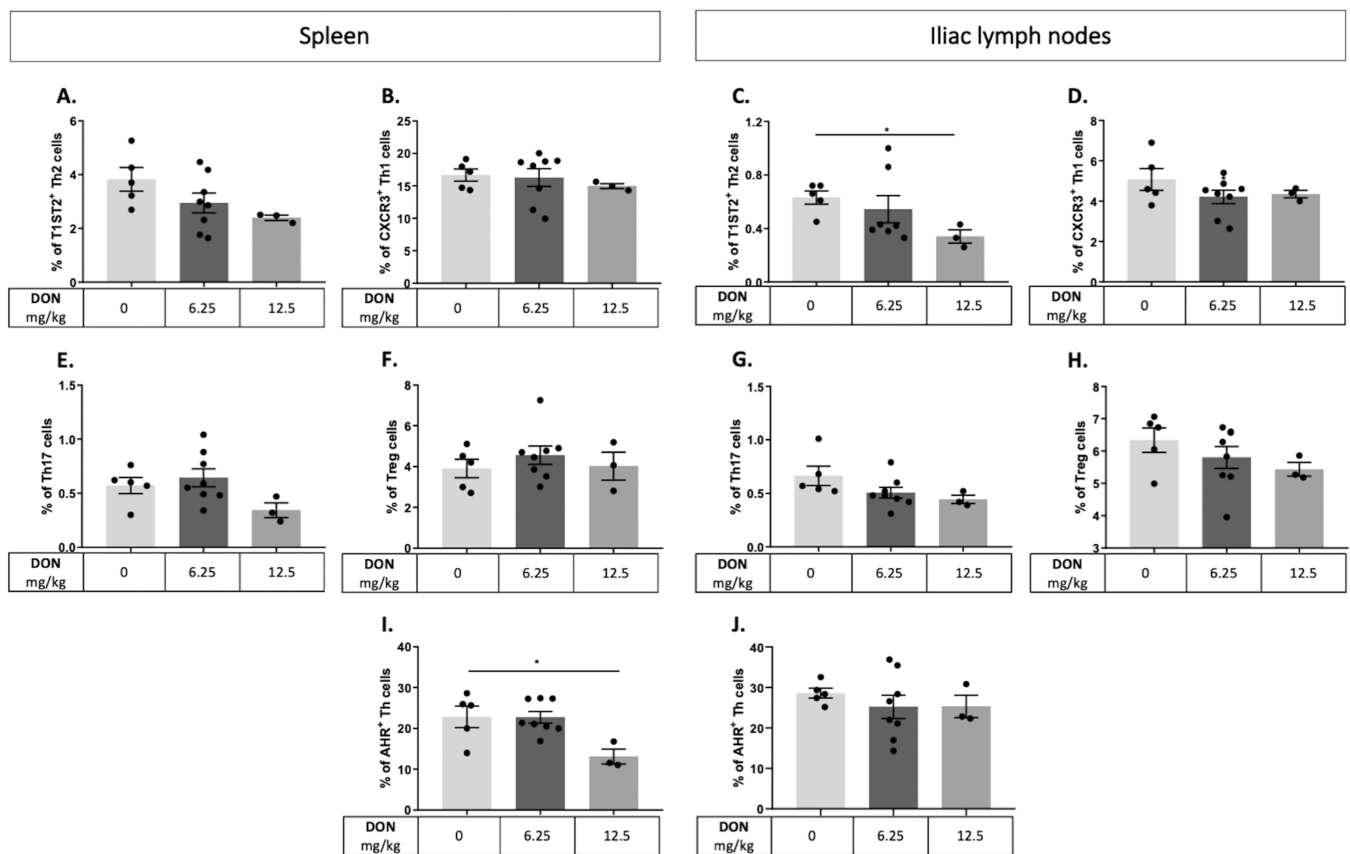

Fig. 2. Deoxynivalenol (DON) exposure negatively affects T cell populations in the spleen and iliac lymph nodes of pregnant mice. Pregnant mice were fed either a control or DON-contaminated diets (6.25 and 12.5 mg/ kg of diet). Percentages of T1ST2^{+} Th2 cells and CXCR3^{+} Th1 cells were calculated out of live CD4^{+} T cells in the spleen (A and B) and iliac lymph nodes (C and D); and percentages of CCR6^{+}RorγT^{+} Th17 cells, CD25^{+}FopxP3^{+} Treg cells, and aryl hydrocarbon receptor (AHR)$^{+}$ T cells were calculated out of live CD4^{+} T cells in the spleen (E, F and I, respectively) and iliac lymph nodes (G, H and J, respectively), using flowcytometry analysis. Data expressed as mean ± SEM, *$p < 0.05$ represent the significant difference.

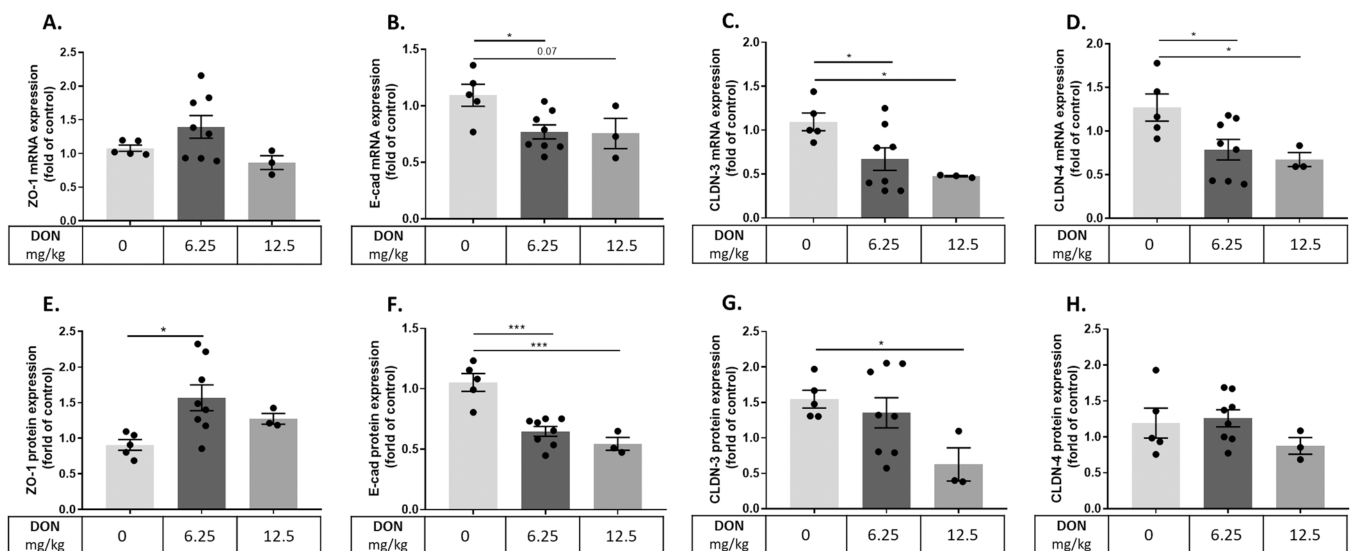

Fig. 3. DON exposure during pregnancy alters the mRNA and protein expressions of junctional proteins in the placenta. Pregnant mice were fed either a control or DON-contaminated diets (6.25 and 12.5 mg/ kg of diet). mRNA and protein expressions of (A, E) zonula occludens protein-1 (ZO-1), (B, F) E-cadherin (E-cad), (C, G) claudin (CLDN)− 3, (D), H CLDN-4 and were measured in placenta homogenates. Data expressed as mean ± SEM of fold of control, normalized to reference gene or protein, *$p < 0.05$ and * **$p < 0.001$ represent the significant difference.

affected (Fig. 3A), its protein expression was increased in group fed 6.25 mg DON/kg in the diet (Fig. 3E). DON-contaminated diets significantly downregulated the mRNA expression of E-cadherin, CLDN-3 and 4 (Fig. 3B, C and D), as well as the protein expression of E-cadherin and CLDN-3 (Fig. 3F and G), compared to the control diet.

To investigate the cellular localization of junctional proteins, immunofluorescence staining was performed on placenta sections for ZO-1 (a tight-junction protein) and E-cadherin (an adherent-junction protein) (Fig. 4). In placental sections from the control group, clear continuous belt-like structures were detectable for both ZO-1 and E-cadherin (Fig. 4A/D, and 4 G/J, respectively). However, in placenta sections of DON-fed mice, especially the higher DON dosage (12.5 mg DON/kg), these belt-like structures for ZO-1 and E-cadherin were not detected (Fig. 4C/F and I/L, respectively).

3.5. *DON exposure during pregnancy alters cytokine levels and aryl hydrocarbon receptor expression in placenta and amniotic fluid*

The concentration of different cytokines in the placenta homogenates and amniotic fluid is depicted in Fig. 5. Presence of DON in the diet, both concentrations of 6.25 mg and 12.5 mg/ kg, significantly increased the concentration of IFN-γ, IL-6 and IL-4 in placental samples

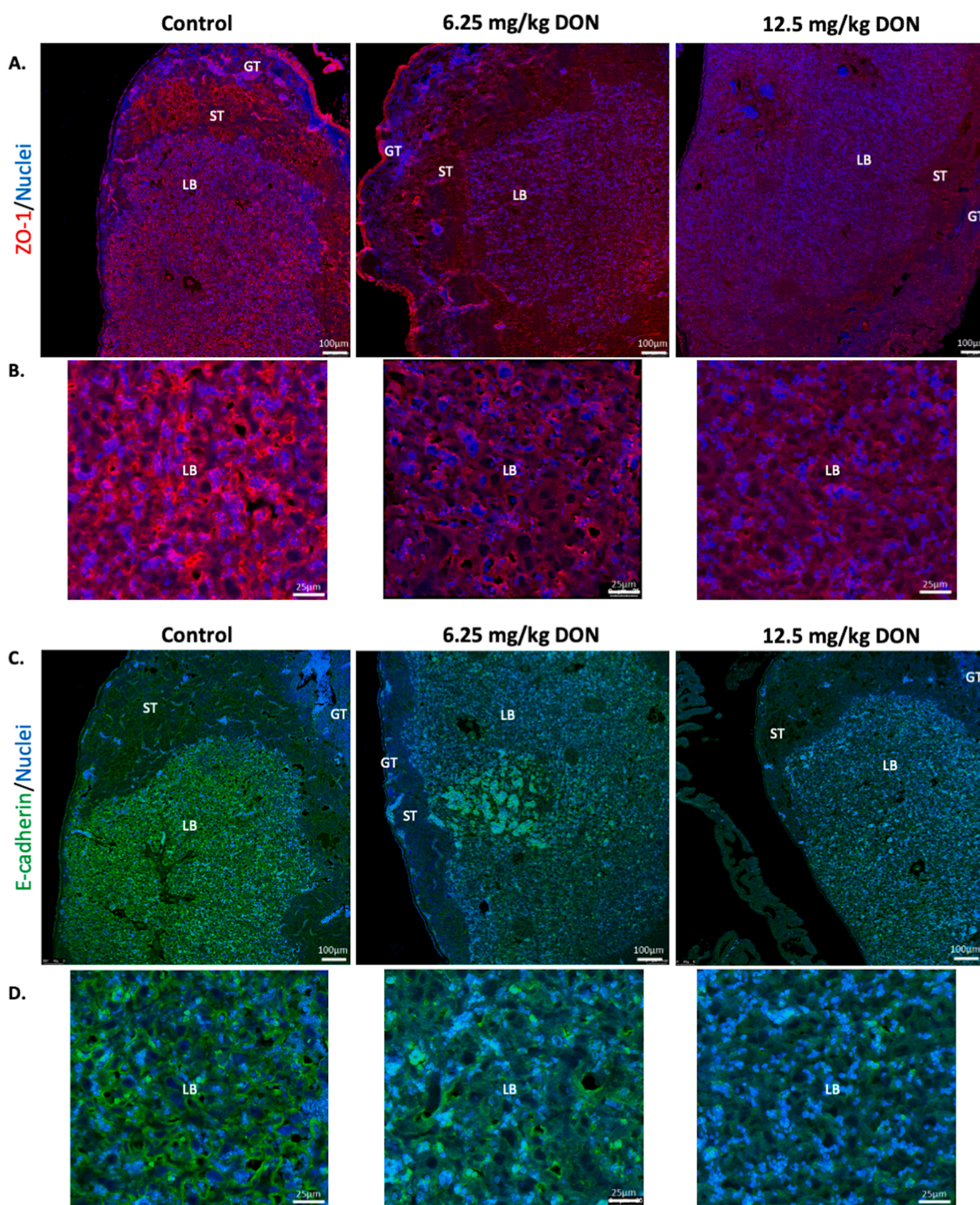

Fig. 4. Deoxynivalenol (DON) exposure during pregnancy alters the cellular localization of zonula occludens protein-1 (ZO-1) protein and E-cadherin in the placenta. Pregnant mice were fed either a control or DON-contaminated diets (6.25 mg/kg and 12,5 mg/kg of diet). The placental sections were visualized by immunofluorescence staining for: A-B) ZO-1 (in red) and nuclei (in blue), and C-D) E-cadherin (in green) and nuclei (in blue). Scale bars represent 100 µm (A, C), and 25 µm (B, D). LB: labyrinth zone, ST: spongiotrophoblasts, TG: Trophoblast giant cells.

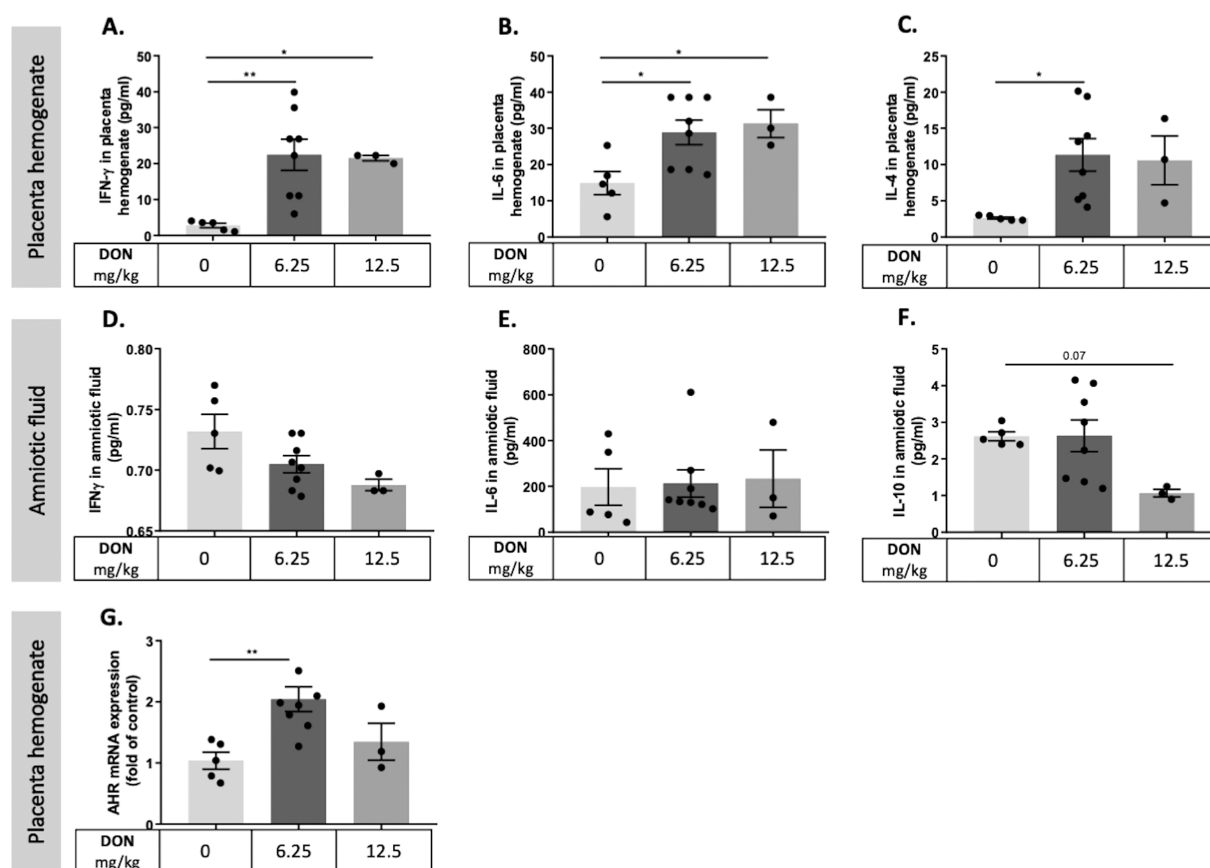

Fig. 5. DON exposure during pregnancy alters cytokine production and AHR expression in placental homogenates and amniotic fluid. Pregnant mice were fed either a control or DON-contaminated diets (6.25 and 12.5 mg/ kg of diet). Concentrations of (A, D) Interferon (IFN)-γ, (B, E) interleukin-6 (IL-6), (C) IL-4, and (F) IL-10 were measured in clear supernatant of placenta homogenates and amniotic fluid, respectively. The mRNA expression (G) aryl hydrocarbon receptor (AHR) was also measured in placenta homogenates. Data expressed as mean ± SEM, $*p < 0.05$ and $***p < 0.001$ represent the significant difference.

(Fig. 5A, B and C), while no effect on IFN-γ and IL-6 concentrations in amniotic fluid were observed (Fig. 5D and E). Although not significantly different ($p = 0.07$), the concentration of IL-10 was decreased in the amniotic fluid of mice fed the 12.5 mg/kg DON diet (Fig. 5F). Concentrations of IL-1β, TNF-α and IL-10 in placenta, and IL-1β, TNF-α and IL-4 in amniotic fluid were below the detection limits of the ELISA kit used in this experiment (data not shown).

Moreover, the mRNA expression levels of AHR, a transcription factor involved in the xenobiotic metabolism, was upregulated the in placental homogenates of DON-fed mice (6.25 mg/kg), compared to the control group (Fig. 5G).

3.6. *DON exposure during pregnancy does not affect junctional protein expression in the fetal intestine*

Considering the adverse effect of direct exposure to DON on intestinal barrier integrity through changing the expression and localization of tight junction proteins [45], gene expression of junctional proteins in intestine samples of the fetuses were measured. However, no significant difference in mRNA expression of ZO-1, OCLD, E-cadherin and CLDN proteins was observed between different groups (supplementary figure 4).

4. Discussion

Pregnant mothers are exposed to a wide variety of environmental toxins and chemicals, which can adversely affect the process of pregnancy and fetal development, either by modulating the maternal parameters such as immune responses, or by passing through the placenta and reaching to the fetus. As the placenta is the most critical organ in maintaining a healthy pregnancy, understanding the effects of xenobiotics on placental structure and function should provide the basis for understanding and predicting aspects of developmental toxicity [49]. DON is a highly prevalent food contaminant known to induce embryotoxicity and reproductive toxicity [50]. In order to gain insight into the possible mechanisms involved in developmental toxicity of DON, the present study investigated the effects of dietary exposure to DON during pregnancy on both maternal and fetal side, as well as the placental structure, focusing on immune and barrier function.

4.1. *DON exposure negatively affects pregnancy and modulates the maternal immune organs and intestinal microbial activity*

Feeding pregnant mice with DON-contaminated diets adversely affected fetal survival, demonstrated by increased fetal mortality and decreases in breeding success. The ratio of live to resorbed fetuses (late resorptions) was considerably decreased with all tested concentration of DON, while the effect of DON on breeding success (i.e. the number of pregnancies) was more prominent with 12.5 mg DON /kg of diet. Similar observations were reported in rats fed with 3–5 mg DON /kg of body weight throughout the gestation period, leading to reduction in weight gain during pregnancy and an increase in number of totally resorbed litters [29,51]. It should be noted that in this study day 0 of pregnancy was calculated from the first day of a 4-day mating period, therefore a 3-day variation in gestational age at which the animals were euthanized could be expected. Moreover, in the current study resorbed implantation in early stages of pregnancy, which did not leave a macroscopic lesion in the uterus on gestation day 17, were not included.

However, considering that the female mice received DON-contaminated diets even before mating and throughout the pregnancy starting immediately after mating, there is a high chance of early resorptions during first days of implantation. Therefore, the ratio used in this study to show the resorption rate is likely to reflect an underestimation of the effect of DON on breeding success. Thus, further experiments are needed to get a more accurate evaluation of the impact of DON on both early and late fetal resorptions. Furthermore, DON is shown to inhibit oocyte maturation, and reduce oocyte quality [52,53]. Considering that in this study female mice received DON-contaminated diets before mating, effects on post-implantation embryos and pre-implantation losses cannot be ruled out, thus the reduction in breeding success and reproductive performance could have resulted from adverse effects of DON on the oocytes and the developing embryos. Further investigations are required to compare the pre- and post-implantation losses and determine the critical window for DON-induced reproductive toxicity.

SCFAs, as products of intestinal bacterial metabolism, are involved in G-protein receptor signaling pathways which are recognized as a molecular link between diet, microflora and immune response [54]. Intestinal SCFAs levels play a critical role during pregnancy, as changes in the intestinal microbial content and metabolism during pregnancy contribute to the occurrence of metabolic changes in the mothers and increase inflammatory markers [55]. In the present study, a tended reduction in concentration of acetic acid in cecum was observed in pregnant mice receiving DON-contaminated diets. Pregnancy increases the concentration of SCFAs in the maternal serum and intestinal lumen [56], and acetic acid is known as the dominant SCFA in both pregnant women and their infants [57]. Gestational serum acetate concentrations, which are most likely mirrored by the acetate production in the intestine, are positively associated with gestational weight gain, maternal adiponectin and leptin levels, as well as neonatal growth [58]. The DON-induced reduction in acetate concentration in cecum may contribute to the adverse health effects in the mother and the neonates.

Changes in the maternal immune system may play a crucial role in reduced fetal survival. In the current study, flowcytometric analysis of maternal spleen and iliac lymph nodes revealed a slight decrease in Th2 cell populations in ILN of mice fed with 12.5 mg/kg of DON, thereby potentially shifting the Th1/Th2 balance towards Th1. During a healthy pregnancy, the maternal immune system shifts towards a more tolerogenic state by downregulating Th1-mediated immune responses and increasing production of regulatory and Th2-mediated cytokines, in order to prevent rejection of the semi-allogeneic fetus [59]. Therefore, an optimal balance of Th1/Th2/Treg immunity, with a slight shift to Th2–type immunity, is required for the maintenance of a successful pregnancy. Although results of the present study showed no significant change in regulatory T cell population in maternal immune organs, a shift in Th1/Th2 balance may have contributed to the increased resorption in DON-exposed mice.

4.2. *DON exposure alters placental structure and immunity*

The placenta, being the only connection between the fetus and the mother, is the most critical organ for maintaining a healthy pregnancy. The main structural components of the placenta are the extensive villous branches forming layers of labyrinth zone, the spongiotrophoblasts zone, and the outer trophoblast giant cells. The maternal blood passes through the spongiotrophoblast via large arterial sinuses, and eventually enters into the labyrinth space where the material exchange between the mother and fetus takes place [60]. In the present study, significant changes in expression and cellular localization of junctional proteins in the placenta of DON-exposed mice were observed, demonstrated by downregulation of protein and mRNA expression levels of E-cadherin and claudin proteins and structural changes in the belt-like intercellular networks of ZO-1 and E-cadherin, which was more obvious in labyrinth zone. The placenta forms a selective barrier at the maternal-fetal interface, mediating transfer of nutrients and metabolic waste products [61], and preventing transmission of pathogens, chemicals and toxins [62]. Therefore, disruption of the integrity of placental barrier can lead to imbalanced maternal-fetal transportation of nutrients and hazardous chemicals. The present study is the first to report effect of DON on placental barrier markers in a murine model. In line with these observations, an earlier in vitro study in our group showed direct exposure to DON induces significant changes in expression level and localization of different junctional proteins in placental epithelial barrier [63].

In addition to the barrier function and critical role in maternal-fetal transportation, the placenta is able to produce and respond to a great diversity of inflammatory stimuli [64], thus a regulated immune response and cytokine production in placenta is required for maintaining a healthy pregnancy. An increase in concentrations of IL-4, IL-6 and IFN-γ was observed in the placenta of DON-exposed mice. Excessive and imbalanced activation of T and NK cells in placenta leads to pro-inflammatory cytokine production, which in turn can cause pregnancy loss through induction of placental infarction and thrombosis [65]. Moreover, we observed a significant reduction in IL-10 concentration in the amniotic fluid of DON exposure animals. Cytokines at the maternal-fetal interface, such as placenta and amniotic fluid, play a crucial role in successful pregnancy. Elevated production of inflammatory cytokines such as IFN-γ and IL-6 can exert detrimental effects in the placenta and induce abortion [66,67], whereas the regulatory cytokine IL-10 is beneficial for maintaining a healthy pregnancy [68] and preventing preterm labor associated with inflammation or infection [69]. In an in vivo study in pregnant mice, beneficial properties of dietary supplementation with proline on enhancing fetal survival and placental development were linked to lower concentration of inflammatory cytokines such as IFN-γ and IL-17, and higher IL-10 production in placenta and amniotic fluid [70]. Furthermore, excessive levels of maternal IL-6 can be transferred to the fetal side [71], which can lead to long-term adverse effects on the offspring, including insulin resistance, elevated stress response as well as hypertension in adulthood [72,73]. Altogether, changes in cytokine production in the placenta of DON-exposed mice reflects an imbalanced immune response in the placenta, which may adversely impact fetal development and pregnancy.

Moreover, the expression of the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor responsible for activating the metabolism of environmental and food-associated xenobiotics [74,75], was measured in the placenta and maternal immune organs. AHR has a crucial role in differentiation and proliferation of regulatory T cells [76], as well as the fetal survival during pregnancy [77,78]. Therefore, a tightly regulated expression of AHR is essential in maintaining a healthy pregnancy and fetal development [79]. To our knowledge, the present study is the first to investigate the effect of DON exposure on the expression of AHR during pregnancy. Interestingly, placental mRNA expression of AHR was increased in mice receiving the diet containing 6.25 mg DON/kg, whereas flowcytometric analysis of maternal immune organs revealed a reduction in AHR^{+} cells in spleens of pregnant mice fed the diet with 12.5 mg DON /kg. It is not yet clear what these outcomes may signify. However, DON-induced alterations in AHR expression in maternal organs and maternal-fetal interface observed in the current study may have contributed to the observed differences in pregnancy outcome, as an increase in AHR expression and activation in the placenta is associated with impaired trophoblast cell proliferation and migration, which can consequently lead to miscarriage and abortion [80,81]. Nevertheless, further experiments with higher sample size are required to fully understand the impact of DON on AHR and its consequences in pregnancy.

4.3. *Direct DON exposure through placenta may contribute to the detrimental effects on the fetus*

Detection of DON in the fetuses of mice fed DON-contaminated diets is consistent with the previous observations, confirming that DON can be transferred across the placenta [82]. Direct exposure of the fetus to DON

may, in part, account for the developmental toxicity of DON during pregnancy, such as induction of fetal skeletal deformities [30,31]. Although DON is known to induce intestinal barrier disruption upon direct exposure, in the present study no significant effect on mRNA expression of fetal intestinal junctional proteins were observed. A possible explanation for this observation could be the low concentration of DON at the fetal side. Similarly, feeding pregnant sows with diets containing 4.42 mg DON/kg diet induced no macroscopic lesions or detectable pathomorphological changes in fetal liver, spleen, lymph nodes, thymus, gut, and bone marrow [83]. However, in another study intrauterine administration of DON in pregnant sows induced a significant impact on T cell populations in piglets after birth, indicated by lower percentages of regulatory and T helper cells in the blood samples and reduced capacity to produce pro-inflammatory cytokines [84]. Considering the well-stablished immunotoxic properties of DON [85], placental transfer of DON can impose adverse immunologic effects on the offspring, as most important immune maturation events occur in early life, interference with the developing immune system can lead to long-term immune dysfunction in adulthood. Indeed, in another recently published paper we were able to demonstrate that the offspring of mice exposed to similar levels of DON during pregnancy and lactation were more susceptible to develop allergic responses in a murine model for ovalbumin-induced food allergy, and vaccination responses to the Influenza vaccine were lower in these mice [86].

4.4. Concluding remarks

Dietary DON exposure during pregnancy has a negative effect on breeding success and fetal survival. Increased early death in DON-exposed mice could be related to the DON-induced imbalanced immune responses in maternal organs, as well as the modifications in the placental barrier and immune function. Moreover, DON reaches the fetus and can induce direct toxicity on the developing fetus. Herein, we are the first to demonstrate changes in placental barrier and immune markers. Overall, results of the present study stress the importance of maternal diet during pregnancy and awareness of the potential harmful effects of commonly present food contaminants on the course of pregnancy and fetal development. Exposure to the toxic compounds such as DON during early stages of development can potentially lead to long-term and persisting adverse health effects in the later stages of life.

Ethical statement

This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals established by the Animal Ethics Committee of the Utrecht University, and all animal procedures related to the purpose of the research were approved under license of the national competent authority (approval number 2016. II.858.014).

CRediT authorship contribution statement

The author's responsibilities were as follow: BL, SB, AH, NST: designed the research; BL, SB, AH, JG, GF: supervised data interpretation; NST, ST: conducted the in vivo experiment; NST: analyzed data and wrote the paper; All authors provided critical intellectual input for data interpretation, read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.reprotox.2022.07.002.

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