Prevention of deoxynivalenol toxicity in broiler chickens by means of detoxifying agents

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TESI DOCTORAL

Prevention of deoxynivalenol toxicity in broiler chickens by means of detoxifying agents

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El Dr. ANTONIO JAVIER RAMOS GIRONA, Catedrático de Universidad del Departamento de Tecnología de Alimentos de la Universitat de Lleida, la Dra. ANNA MARIA PÉREZ VENDRELL, investigadora del IRTA y la Dra. VIRGINIE MARQUIS, investigadora de la empresa Phileo Lesaffre.

HACEN CONSTAR

Que, bajo su dirección, la Sra. **Insaf Riahi** ha realizado el trabajo de investigación titulado "Prevention of deoxynivalenol toxicity in broiler chickens by means of detoxifying agents", que presenta para optar al grado de Doctora por la Universitat de Lleida. Considerando que el trabajo realizado constituye tema de Tesis Doctoral, autorizan su exposición y defensa en la Universitat de Lleida.

Y para que así conste, se expide el presente Lleida a veinte de febrero de dos mil veintiuno.

Antonio J. Ramos Girona Anna Mª Pérez Vendrell Virginie Marquis

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Siempre parece imposible hasta que se hace.

Nelson Mandela

RESUM

Les micotoxines es consideren un problema de salut pública molt important a causa dels seus efectes adversos en animals i humans. El deoxinivalenol (DON) és la micotoxina més freqüent en els cereals a tot el món. La contaminació per DON provoca grans pèrdues econòmiques a la indústria avícola degut a les seves dietes a base de cereals. El mètode més utilitzat per a contrarestar l'impacte negatiu de les micotoxines en els animals és l'addició d'agents detoxificants de micotoxines als pinsos. Aquests additius per a pinsos, els anomenats adsorbents o modificadors de micotoxines, adsorbeixen o biotransformen les micotoxines en el tracte gastrointestinal, respectivament. Aquests agents detoxificants han de provar-se en funció de la seva capacitat per unir-se o modificar micotoxines *in vivo*. De moment, no es disposa de models *in vivo* fiables en pollastres per a avaluar l'eficàcia dels detoxificants de micotoxines basats en biomarcadors específics.

L'objectiu d'aquesta tesi va ser, en primer lloc, desenvolupar un model *in vivo,* i després, investigar l'eficàcia dels agents detoxificants, basats en indicadors específics i no específics, en pollastres d'engreix.

La introducció general d'aquesta recerca doctoral comença amb una descripció general de la micotoxina DON. A continuació, s'han reportat biomarcadors específics i inespecífics de toxicitat per DON en pollastres. Finalment, s'ha realitzat una descripció general dels agents adsorbents i biotransformadors davant el DON.

El primer capítol mostra els resultats d'un estudi toxicocinètic de DON en plasma de pollastres d'engreix, realitzat mitjançant bolus oral o injecció intravenosa amb 0.75 o 2.25 mg de DON / kg de pes viu. Aquest estudi toxicocinètic es va realitzar amb l'objectiu d'identificar en quin compartiment biològic era més probable que arribés el DON i especificar en quina forma ho fa (compost inicial o metabòlits). L'anàlisi de plasma per LC-MS / MS va revelar que el DON no es va poder quantificar després de l'aplicació de bolus oral, el que indica la molt baixa biodisponibilitat del DON en pollastres d'engreix. L'avaluació dels paràmetres toxicocinètics després de la injecció intravenosa va revelar la metabolització del DON al DON-3 sulfat, la seva ràpida eliminació i excreció en pollastres d'engreix.

Els capítols 2 i 3 mostren un model *in vivo* per a estudiar l'efecte d'una dieta contaminada amb DON sobre paràmetres específics i no específics en pollastres d'engreix. Es van alimentar quaranta-cinc pollastres d'engreix mascles d'1 dia (Ross 308) durant 42 dies,

distribuidos en 3 grupos experimentales: grupo control (T1), pinso contaminat amb DON (5 mg/kg pinso) (T2) o pinso contaminat amb DON (15 mg/kg pinso) (T3). Les concentracions plasmàtiques, hepàtiques o en excretes de DON i DON-3 sulfat es van utilitzar com indicadors específics **(capítol 3)**. Els paràmetres inespecífics avaluats van ser: paràmetres productius, pes relatiu d'òrgans, morfologia i histologia de l'intestí prim, perfil bioquímic sèric, comportament en front de la por i color de les potes **(capítol 2)**, hematologia sanguínia, resposta a vacunes comuns, IL-8 plasmàtica, expressió gènica relativa d'IL-6, IL-1β, IFN-γ i IL-10 en jejú, índex d'estrès (proporció d'heteròfils a limfòcits) i corticosterona plasmàtica **(capítol 3)**.

El DON només es va quantificar en les excretes, el que suggereix una baixa biodisponibilitat, una acumulació limitada i una ràpida excreció. El DON-3S es va quantificar en totes les matrius biològiques, el que indica que el DON-3S és el metabòlit més adequat d'exposició al DON en pollastres d'engreix. Amb 5 mg de DON/kg pinso, la creatina quinasa va disminuir i la IL-1β, IL-6 i IFN-γ es van regular positivament. Amb 15 mg de DON/kg pinso, l'índex de transformació alimentària es va alterar negativament i el colesterol en sang i els glòbuls vermells van disminuir. A ambdós nivells assajats van augmentar els pesos relatius del pedrer i del timus, la longitud de l'intestí prim i la IL-8 plasmàtica. No obstant això, es va reduir el pes relatiu de l'intestí prim, el còlon i la borsa de Fabrici, la densitat (pes/longitud) de l'intestí prim, l'hemoglobina i la corticosterona plasmàtica. Es pot concloure que els paràmetres específics i no específics afectats pel pinso contaminat podrien ser adequats per avaluar l'eficàcia dels agents detoxificants de micotoxines en pollastres d'engreix.

El capítol 4 mostra el model d'eficàcia *in vivo* utilitzat per provar 3 detoxificants de micotoxines (MFA, IMP i MDE) basats en biomarcadors seleccionats en els **capítols 2 i 3**, així com en altres biomarcadors (DOM-3 sulfat i DOM-1 en excretes, triglicèrids sèrics, expressió relativa d'IL-8 i TNF-α en teixits de jejú i corticosterona en plomes). Es van alimentar 384 pollastres d'engreix mascles (Ross308) d'1 dia d'edat, durant 42 dies, amb dietes formulades com a pinso no contaminat (control), pinso contaminat, control + 0.2% MFA, pinso contaminat + 0.2% MFA, control + 0,0125% IMP, pinso contaminat + 0,0125% IMP, control + 0,15% MDE, o pinso contaminat + 0,15% MDE. El DON va ser la principal micotoxina del pinso contaminat i les concentracions van variar al voltant dels 7 mg/kg pinso. Els biomarcadors estudiats es van avaluar als 10 i 42 dies. Es van detectar DON, DON-3S i DOM-3S a les excretes dels grups amb pinso contaminat.

L'addició de MDE a la dieta contaminada va augmentar l'excreció de DON però va disminuir l'excreció de metabòlit DOM-3S. L'addició de MFA a la dieta contaminada va augmentar l'excreció de DON, la qual cosa suggereix que aquest producte és eficaç per a detoxificar aquesta micotoxina. Al dia 10, el DON va perjudicar l'índex de transformació alimentària, i va augmentar els nivells de colesterol i triglicèrids en sèrum. L'efecte sobre l'índex de transformació alimentària es va evitar mitjançant l'addició d'IMP a la dieta contaminada. L'efecte sobre el nivell de colesterol sèric es va revertir mitjançant la suplementació amb MFA o IMP a la dieta contaminada. A més, al dia 10, el DON va reduir els nivells d'hematòcrit, hemoglobina, glòbuls vermells i monòcits. L'addició d'IMP a la dieta contaminada va contrarestar l'efecte observat sobre els nivells d'hematòcrit i monòcits en sang. Als 42 dies, el DON va millorar l'índex de transformació alimentària, va reduir el pes relatiu del fetge i el nivell de limfòcits en sang. Als 42 dies, a més, el DON va augmentar els recomptes de glòbuls blancs, l'índex d'estrès (proporció d'heteròfils a limfòcits) i corticosterona en plomes. L'efecte sobre l'índex d'estrès va ser contrarestat per l'addició de MFA a la dieta contaminada.

Es pot concloure que els paràmetres específics seleccionats són adequats per a avaluar l'eficàcia dels agents detoxificants de DON en pollastres d'engreix, i que el producte MFA va contrarestar parcialment els efectes negatius de DON.

RESUMEN

Las micotoxinas representan un peligro para la salud animal y humana. El deoxinivalenol (DON) es la micotoxina que más frecuentemente contamina los cereales tanto en países desarrollados como en vías de desarrollo. Debido a sus dietas a base de cereales, la presencia del DON en el alimento de las aves genera serias pérdidas económicas. El método más utilizado para detoxificar las micotoxinas presentes en los piensos es el tratamiento con adsorbentes o la biotransformación de las micotoxinas en el tracto gastrointestinal. Su eficacia real, más allá de los posibles resultados prometedores observados en ensayos *in vitro*, debe ser siempre evaluada en ensayos *in vivo*, poniendo de manifiesto su efecto protector mediante la observación de la evolución de biomarcadores específicos relacionados con la toxicidad de las micotoxinas.

El objetivo de esta tesis fue desarrollar un modelo *in vivo,* y luego evaluar *in vivo* la eficacia de productos detoxificantes de DON, en base a biomarcadores específicos y no específicos, en pollos de engorde.

La introducción general de esta Tesis comienza con una descripción general del DON. A continuación, se han reportado los biomarcadores específicos y no específicos de toxicidad por DON en pollos. Por último, se ha realizado una descripción general de los productos detoxificantes de DON.

El primer capítulo muestra los resultados de la cinética del DON en plasma de los pollos broilers, realizada mediante bolo oral o inyección intravenosa de 0.75 o 2.25 mg de DON/kg de peso vivo. Este estudio de toxicocinética se realizó con el objetivo de identificar a qué compartimento biológico era probable que llegara el DON y especificar en qué forma lo hace (compuesto inicial o metabolitos). El análisis de plasma por LC-MS/MS reveló que el DON no se pudo cuantificar después de la aplicación de bolo oral, indicando la muy baja biodisponibilidad del DON en pollos de engorde. La evaluación de los parámetros toxicocinéticos después de la inyección intravenosa reveló la metabolización del DON en DON-3-sulfato, su rápida eliminación y excreción en pollos de engorde.

Los capítulos 2 y 3 muestran el desarrollo de un modelo *in vivo* en pollos de engorde, que permite evaluar los efectos tóxicos del DON, e identificar los biomarcadores más relevantes (específicos y no específicos). Se realizó un ensayo con 45 pollos broiler machos Ross 308 de un día de vida durante 42 días, distribuidos en 3 grupos

experimentales: grupo control (T1), grupo alimentado con 5 mg/kg de DON (T2) y grupo alimentado con 15 mg mg/kg de DON (T3). El DON y el DON-3 S en plasma, hígado y excretas fueron como biomarcadores específicos **(capítulo 3)**. Los parámetros no específicos evaluados fueron: parámetros productivos, peso relativo de órganos, morfología e histología del intestino delgado, bioquímica de sangre, reacción frente al miedo y el color de las patas **(capítulo 2)**, hematología de sangre, respuesta a las vacunas comunes, IL-8 en plasma, expresión relativa de los genes IL-6, IL-1β, IFN-γ e IL-10 en yeyuno, índice de estrés (proporción de heterófilos a linfocitos) y corticosterona en plasma **(capítulo 3)**.

El DON solo se cuantificó en las excretas, lo que sugiere una baja biodisponibilidad, una acumulación limitada y una rápida excreción. El DON-3S fue cuantificado en todas las matrices biológicas, indicando que el DON-3S es el metabolito más adecuado como biomarcador de exposición al DON en pollos de engorde. Con 5 mg de DON/kg de alimento, la creatina quinasa disminuyó y la IL-1β, IL-6 e IFN-γ aumentaron. Con 15 mg de DON/kg de alimento, el índice de conversión se alteró negativamente, el colesterol en sangre y los glóbulos rojos disminuyeron. A ambos niveles ensayados, los pesos relativos de molleja y timo, la longitud del intestino delgado y la IL-8 plasmática aumentaron. Sin embargo, el peso relativo del intestino delgado, el colon y la bolsa de Fabricius, la densidad del intestino delgado (peso/longitud), la hemoglobina y la corticosterona plasmática disminuyeron. Así, se puede considerar que los parámetros específicos y no específicos afectados significamente por el pienso contaminado podrían ser relevantes para evaluar la eficacia de los agentes detoxificantes de micotoxinas en pollos de engorde.

En el capítulo 4 se evaluó *in vivo* la eficacia de diferentes productos detoxificantes de DON (MFA, IMP y MDE), en base a los biomarcadores previamente seleccionados en los **capítulos 2 y 3**, así como otros biomarcadores añadidos (DOM-3 sulfato, DOM-1 en excretas, triglicéridos en suero, expresión relativa de los genes IL-8 y TNF-α en yeyuno y corticosterona en plumas). Se realizó un ensayo con 384 pollos de engorde machos (Ross 308) de 1 día de edad que recibieron dietas durante 42 días, formuladas como: alimento no contaminado (control), alimento contaminado, control + 0.2% MFA, alimento contaminado + 0.2% MFA, control + 0.0125% IMP, alimento contaminado + 0.0125% IMP, control + 0.15% MDE, o alimento contaminado + 0.15% MDE. El DON fue la principal micotoxina presente en el alimento contaminado y las concentraciones analizadas variaron alrededor de 7 mg/kg de alimento. Los biomarcadores se evaluaron a

los 10 y 42 d. Se detectaron DON, DON-3S y DOM-3S en las excretas de grupos con pienso contaminado. La adición de MDE al pienso contaminado aumentó la excreción de DON, pero disminuyó la excreción del metabolito DOM-3S. La adición de MFA a la dieta contaminada aumentó la excreción de DON, lo que sugiere la eficacia de este producto para desintoxicar esta micotoxina. A los 10 d, el DON perjudicó el índice de conversión, y aumentó los niveles de colesterol y triglicéridos en suero. La adición de IMP a la dieta contaminada contrarrestó el efecto negativo sobre el índice de conversión. El efecto sobre el nivel de colesterol en suero se revirtió mediante la suplementación con MFA o IMP a la dieta contaminada. Además, a los 10 d, el DON redujo los niveles de hematocrito, hemoglobina, glóbulos rojos y monocitos en sangre. La adición de IMP a la dieta contaminada contrarrestó el efecto observado sobre los niveles de hematocrito y monocitos en sangre. A los 42 días, el DON mejoró el índice de conversión, redujo el peso relativo del hígado y el nivel de linfocitos en sangre, mientras que aumentó el recuento de glóbulos blancos, el índice de estrés (relación heterófilos/linfocitos) y el nivel de corticosterona en plumas. La adición de MFA a la dieta contaminada contrarrestó el efecto sobre el índice de estrés.

En conclusión, los parámetros específicos selecionados son adecuados para evaluar la eficacia de agentes desintoxicantes del DON en pollos de engorde, y el producto MFA contrarrestó parcialmente los efectos negativos de DON.

ABSTRACT

Mycotoxins are considered a very important public health issue because of their adverse effects on animals and humans. Deoxynivalenol (DON) is the most frequent mycotoxin in cereals worldwide. DON contamination leads to great economic losses in poultry industry due to their cereal-based diets. The most commonly used method to counteract the negative impact of mycotoxins on animals is the addition of mycotoxin detoxifying agents (mycotoxin detoxifiers) to feed. These feed additives, so-called mycotoxin binders or mycotoxin modifiers, either adsorb or biotransform mycotoxins in the gastrointestinal tract, respectively. These detoxifying agents should be tested not only *in vitro,* but also *in vivo* on their ability to bind or modify mycotoxins. At the time being, no reliable *in vivo* models in chicken are available to evaluate the efficacy of mycotoxin detoxifiers based on specific biomarkers.

The aim of this thesis was first to develop an *in vivo* model then to investigate the efficacy of detoxifying agents, based on specific and nonspecific indicators, in broiler chickens.

The **general introduction** of this doctoral research starts with an overview of DON mycotoxin. Next, specific and nonspecific biomarkers of DON toxicity in chickens have been reported. Finally, an overview of binding and biotransforming agents against DON has been carried out.

The first chapter shows the results of a DON toxicokinetic study in broiler chickens' plasma, performed via oral bolus or intravenous injection of 0.75 or 2.25 mg DON/kg of body weight (BW). This toxicokinetic study was done with the objective to identify which biological compartment the DON was likely to reach and to specify in what form (initial compound or metabolites) it does so. The analysis of plasma by LC-MS/MS revealed that DON could not be quantified after oral bolus application, indicating the very low bioavailability of DON in broiler chickens. The evaluation of toxicokinetics parameters after the intravenous injection revealed the metabolization of DON in DON-3 sulphate, its rapid clearance and excretion in broiler chickens.

Chapter 2 and 3 show an *in vivo* model set-up to study the effect of a DON-contaminated diet on specific and nonspecific relevant biomarkers on broiler chickens. Forty-five 1-day-old male broiler chickens (Ross 308) were fed diets during 42 d, distributed into 3 experimental groups:distribuidos en 3 grupos experimentales: control group (T1), DON-contaminated feed (5 mg/kg feed) (T2), or DON-contaminated feed (15 mg/kg feed) (T3).

Plasma, liver or excreta concentrations of DON and DON-3 sulphate were used as specific indicators (**chapter 3**). The nonspecific parameters evaluated were performance parameters, relative organ weights, morphology and histology of small intestine, serum biochemistry profile, fear behavior and leg color **(chapter 2)**, blood hematology, response to common vaccines, plasma IL-8, relative gene expression of IL-6, IL-1β, IFN-γ and IL-10, stress index (heterophil to lymphocyte ratio), and plasma corticosterone **(chapter 3)**.

DON was only quantified in excreta, suggesting low bioavailability, limited accumulation and rapid excretion of DON. DON-3S was quantified in all biological matrices, indicating that DON-3S is the most suitable metabolite of exposure of DON in broiler chickens. At 5 mg DON/kg feed, creatine kinase decreased and IL-1β, IL-6, and IFN-γ were upregulated. At 15 mg DON/kg feed, feed conversion ratio was impaired and blood cholesterol and red blood cells decreased. At both levels assayed relative weights of gizzard and thymus, the length of small intestine, and plasma IL-8 increased. However, the relative weight of small intestine, colon, and bursa of Fabricius, the density (weight/length) of small intestine, hemoglobin and plasma corticosterone were reduced. It can be concluded that specific and nonspecific parameters affected by the contaminated feed could be suitable to evaluate the efficacy of the mycotoxin detoxifying agents in broiler chickens.

Chapter 4 shows the *in vivo* efficacy model used to test 3 mycotoxin detoxifiers (MFA, IMP and MDE) based on biomarkers selected on **chapters 2 and 3**, as well as other biomarkers (DOM-3 sulphate and DOM-1 in excreta, serum triglycerides, relative expression of IL-8 and TNF-α in jejunum tissues, and corticosterone in feathers). Three hundred eighty-four 1-d-old male broiler chickens (Ross308) were fed for 42 d with diets formulated as non-contaminated feed (control), contaminated feed, control+0.2% MFA, contaminated feed+0.2% MFA, control+0.0125% IMP, contaminated feed+0.0125% IMP, control+0.15% MDE, or contaminated feed+0.15% MDE. DON was the main mycotoxin of the contaminated feed and concentrations varied around 7 mg/kg feed. Studied biomarkers were evaluated at 10 and 42 d. DON, DON-3S, and DOM-3S were detected in excreta from contaminated groups. The addition of MDE to contaminated feed increased the excretion of DON but decreased the excretion of the metabolite (DOM-3S). The addition of MFA to contaminated diet increased the excretion of DON, suggesting that this product is effective to detoxify this mycotoxin. At d 10, DON impaired feed conversion ratio, increased serum cholesterol and triglycerides levels. The effect on feed

conversion ratio was prevented by IMP addition to the contaminated diet. The effect on serum cholesterol level was reversed by MFA or IMP supplementation to the contaminated feed. At d 10, moreover, DON reduced hematocrit, hemoglobin, red blood cells, and monocytes levels. The addition of IMP to the contaminated diet counteracted the effect observed on blood hematocrit and monocytes levels. At 42 d, DON improved the feed conversion ratio, reduced the relative weight of liver, and blood lymphocytes level. At 42 d, furthermore, DON increased white blood cells counts, stress index (heterophils to lymphocytes ratio) and feather corticosterone. The effect on stress index was counteracted by the addition of MFA to the contaminated diet.

It can be concluded that the specific parameters selected are suitable to evaluate the efficacy of DON detoxifying agents in broiler chickens, and the product MFA partially counteracted the negative effects of DON.

RÉSUMÉ

Les mycotoxines peuvent avoir des effets nocifs divers pour la santé et représenter une grave menace pour les êtres humains comme les animaux. Le déoxynivalénol (DON) est la mycotoxine la plus répandue dans les céréales dans le monde. La contamination par le DON entraîne de grandes pertes économiques dans l'industrie de la volaille en raison de leur alimentation basée sur les céréales. La méthode la plus couramment utilisée pour lutter contre l'impact négatif des mycotoxines sur les animaux est l'addition dans les aliments d'agents détoxifiants. Ces additifs alimentaires, appelés capteurs de mycotoxines ou modificateurs de mycotoxines, respectivement adsorbent ou biotransforment les mycotoxines dans le tractus gastro-intestinal, Ces agents détoxifiants doivent être testés sur leur capacité à capter ou à modifier les mycotoxines *in vivo*. Actuellement, aucun modèle *in vivo* fiable chez le poulet n'est disponible pour évaluer l'efficacité des détoxifiants de mycotoxines basés sur des biomarqueurs spécifiques.

L'objectif de cette thèse était d'abord de développer un modèle *in vivo* puis d'étudier l'efficacité d'agents détoxifiants, à partir d'indicateurs spécifiques et non spécifiques, chez les poulets de chair.

L'introduction générale de cette thèse doctorale commence par une description générale de la mycotoxine DON, suivie d'une revue des différents biomarqueurs spécifiques et non spécifiques de la toxicité du DON chez les poulets. L'introduction se termine sur une description générale des capteurs et modificateurs de mycotoxines.

Le premier chapitre présente les résultats d'une étude de toxicocinétique du DON, réalisée par bolus oral ou injection intraveineuse, chez des poulets de chair alimentés avec 0.75 ou 2.25 mg de DON / kg de poids corporel. Cette étude de la toxicocinétique du DON a été réalisée dans le but d'identifier quel compartiment biologique le DON pouvait potentiellement atteindre et de préciser sous quelle forme (composé initial ou métabolites). L'analyse du plasma par LC-MS / MS a révélé que le DON ne pouvait pas être quantifié après administration du DON par bolus oral, ce qui indique la très faible biodisponibilité du DON chez les poulets de chair. L'évaluation des paramètres toxicocinétiques après l'injection intraveineuse a révélé la métabolisation du DON en DON-3 sulfate, ainsi que sa rapide clairance et excrétion chez les poulets de chair.

Les chapitres 2 et 3 montrent un modèle *in vivo* mis en place pour étudier l'effet d'un régime contaminé au DON sur des paramètres spécifiques et non spécifiques sur les

poulets de chair. Quarante-cinq poulets de chair mâles âgés d'un jour (Ross 308) ont été alimentés pendant 42 jours, avec des aliments non-contaminés (témoin), des aliments contaminés par le DON (5 mg /kg d'aliments) ou des aliments contaminés par le DON (15 mg/kg d'aliments). Les concentrations de DON et DON-3S dans le plasma, le foie ou les excreta ont été utilisées comme indicateurs spécifiques (**chapitre 3**). Les paramètres non spécifiques évalués étaient les paramètres de production, le poids relatif des organes, la morphologie et l'histologie de l'intestin grêle, la biochimie du sang, le comportement face au stress (réactions de peur et couleur des jambes) (**chapitre 2**), l'hématologie du sang, la réponse aux vaccins communs, l'IL-8 plasmatique, l'expression relative des gènes de IL-6, IL-1β, IFN-γ et IL-10, l'indice de stress (rapport hétérophile / lymphocyte) et la corticostérone plasmatique (**chapitre 3**).

Le DON n'a été quantifié que dans les excréments, ce qui suggère une faible biodisponibilité, une accumulation limitée et une excrétion rapide. Le DON-3S a été quantifié dans toutes les matrices biologiques, ce qui indique que le DON-3S est le métabolite d'exposition au DON le plus approprié chez les poulets de chair. À 5 mg de DON/kg d'aliment, la teneur en créatine kinase a été diminuée et les niveaux d'IL-1β, 'IL-6 et IFN-γ ont été augmentés. À 15 mg de DON / kg d'aliment, l'indice de conversion a été détorioré et les niveaux de cholestérol sanguin et de globules rouges ont diminué. Aux deux concentrations de DON utilisées, les poids relatifs du gésier et du thymus, la longueur de l'intestin grêle et la teneur en IL-8 plasmatique ont été augmentés. Cependant, le poids relatif de l'intestin grêle, du côlon et de la bourse de Fabricius, la densité de l'intestin grêle, l'hémoglobine et la corticostérone plasmatique ont été réduits. On peut conclure que les paramètres spécifiques et non spécifiques affectés par l'aliment contaminé pourraient être suffisants pour évaluer l'efficacité des agents détoxifiants des mycotoxines chez les poulets de chair.

Le chapitre 4 présente le modèle *in vivo* d'efficacité utilisé pour tester trois détoxifiants de mycotoxines (MFA, IMP et MDE) sur la base des biomarqueurs sélectionnés aux **chapitres 2 et 3**, ainsi que d'autres biomarqueurs (DOM-3 sulphate et DOM-1 dans les excreta, triglycérides sériques, expression relative de l'IL-8 et du TNF-α dans les tissus du jéjunum et corticostérone dans les plumes). Trois cent quatre-vingt-quatre poulets de chair mâles de 1 j ont été alimentés pendant 42 jours avec une alimentation formulée comme suit: aliment non contaminé (témoin), aliment contaminé, témoin + 0.2% MFA, aliment contaminé + 0.2% MFA, témoin + 0.0125% IMP, aliment contaminé + 0.0125%

IMP, témoin + 0.15% MDE, ou aliment contaminé + 0.15% MDE. Le DON était la principale mycotoxine de l'aliment contaminé et les concentrations variaient autour de 7 mg/kg d'aliment. Les biomarqueurs étudiés ont été évalués à 10 et 42 jours. DON, DON-3S et DOM-3S ont été détectés dans les excréments de groupes contaminés. L'addition de MDE au régime contaminé a augmenté l'excrétion de DON, mais a diminué l'excrétion de métabolite DOM-3S. L'ajout de MFA à un régime contaminé a augmenté l'excrétion de DON, ce qui suggère que ce produit est efficace pour détoxifier cette mycotoxine. A j 10, Le DON a altéré l'indice de conversion, a augmenté les taux sériques de cholestérol et de triglycérides. L'effet sur l'indice de conversion a été compensé par l'addition d'IMP au régime alimentaire contaminé. L'effet sur le taux de cholestérol sérique a été inversé par une supplémentation en MFA ou en IMP aux régimes contaminés. A j 10, en outre, le DON a réduit les taux d'hématocrite, d'hémoglobine, de globules rouges, et de monocytes. La supplémentation d'IMP au régime alimentaire contaminé a neutralisé l'effet observé sur l'hématocrite sanguin et les taux de monocytes. À 42 jours, le DON a amélioré l'indice de conversion, a réduit le poids relatif du foie et le taux de lymphocytes sanguins. À 42 jours, en outre, le DON a augmenté les globules blancs, l'indice de stress (rapport hétérophiles/lymphocytes) et la corticostérone des plumes. L'effet sur l'indice de stress a été neutralisé par l'ajout de MFA au régime contaminé.

On peut conclure que les paramètres spécifiques sélectionnés sont appropriés pour évaluer l'efficacité des agents détoxifiants du DON chez les poulets de chair, et que le produit MFA a partillement neutralisé les effets négatifs du DON.

RESEARCH PUBLICATIONS

During the course of this project, a number of research publications have been made which are based on the work presented in this thesis. They are listed here for reference.

- Riahi I, Marquis V, Ramos AJ, Brufau J, Esteve-Garcia E, Pèrez-Vendrell AM. Effects of deoxynivalenol-contaminated diets on productive, morphological, and physiological indicators in broiler chickens. *Animals*. 2020 ; 10 (10) : 1795. https://doi.org/10.3390/ani10101795

- Riahi I, Marquis V, Pèrez-Vendrell AM, Brufau J, Esteve-Garcia E, Ramos AJ. Effects of deoxynivalenol-contaminated diets on metabolic and immunological parameters in broiler chickens. *Animals*. 2021 ; 11 : 147. https://doi.org/10.3390/ani11010147

- Riahi I, Pèrez-Vendrell AM, Ramos AJ, Brufau J, Esteve-Garcia E, Schulthess J, Marquis V. Biomarkers of deoxynivalenol toxicity in chickens with special emphasis on metabolic and welfare parameters. *Toxins*. 2021 ; 13 (3) : 217. https://doi.org/10.3390/toxins13030217

- Riahi I, Pèrez-Vendrell AM, Ramos AJ, Marquis V. A toxicokinetic study reflecting the absorption, distribution, metabolism and excretion of deoxynivalenol in broiler chickens. *Journal of Applied Animal Research*. Submitted.

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15-Ac-DON	15-acetyl- deoxynivalenol
3-Ac-DON	3-acetyl- deoxynivalenol
3-epi-DON	3-epi-deoxynivalenol
3-keto-DON	3-keto-deoxynivalenol
AFB1	Aflatoxin B_1
AFs	Aflatoxins
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate transaminase
$AUC_{0-\infty}$	Area under the concentration-time curve from time zero to infinity
AUC_{0-t}	Area under the concentration-time curve from time zero to the last time point
BW	Body weight
BWG	Body weight gain
CK	Creatine kinase
Cmax	Maximum plasma concentration
DDGS	Dried distillers grains with soluble
DOM-1	Deepoxy-deoxynivalenol
DOM-3S	Deepoxy-deoxynivalenol-3-sulphate
DON	Deoxynivalenol
DON-3G	Deoxynivalenol-3-glucoside
DON-3S	Deoxynivalenol-3-sulphate
EC	European Commission
ECD	Electro capture detection
EFSA	European Food Safety Agency
EGM	Esterified glucomannans
ELISA	Enzyme-linked immunosorbant assay
ERK	Extracellular signal-regulated kinase
FBs	Fumonisins
FCR	Feed conversion ratio
FD	Fluorescence detection
FID	Flame ionization detection
GIT	Gastrointestinal tract
GGT	Gamma-glutamyl transferase
H/L ratio	Heterophil to lymphocyte ratio
HCT	Hematocrit
HGB	Hemoglobin
HPLC	High-performance liquid chromatography
HSCAS	Hydrated sodium calcium aluminosilicate
IAC	Immunoaffinity columns
IBV	Infectious bronchitis virus
IFN-γ	Interferon gamma

IL-1β	Interleukin 1β
IL-2	Interleukin 2
IL-6	Interleukin 6
IL-8	Interleukin 8
IMP	Immunoprotector additive
IS	Internal standard
IUF	Immune ultrafiltration
k_{el}	Elimination rate constant
LAB	Lactic acid bacteria
LC-HRMS	Liquid chromatography high resolution mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LDH	Lactate dehydrogenase
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
MAPKs	Mitogen activated protein kinases
MDE	Mycotoxin degrading enzyme
MFA	Microbial feed additive
MFC	Multifunctional columns
MIPs	Molecularly imprinted polymers
ND	Not detected
NDV	Newcastle disease virus
NF-kβ	Nuclear factor kappa β
NIV	Nivalenol
OTA	Ochratoxin A
PBS	Phosphate buffered saline
RBC	Red blood cell
RW	Relative weight
SPE	Solid-phase extraction
$T_{1/2el}$	Half-life of elimination
TNF-α	Tumor necrosis factor α
UA	Uric acid
UV	Ultraviolet
Vd	Volume of distribution
YCW	Yeast cell walls
ZEN	Zearalenone

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GENERAL INTRODUCTION

1. Mycotoxins: general overview

The name mycotoxin, etymologically, is composed of the Greek word *mykes* (mould or fungus) and the Latin word *toxicum* (poison). Mycotoxins are low-molecular-weight secondary metabolites produced by fungi. Mycotoxigenic fungi could be divided in two classes, field and storage fungi. Field fungi produce mycotoxins on the crop before harvest, such as *Fusarium* species, meanwhile storage fungi produce mycotoxins after crop harvesting, mainly during the storage, such as *Aspergillus* and *Penicillium* species. Temperature and water activity are mainly the most important factors that determine mycotoxin production (Filtenborg et al., 1996). Generally, mycotoxigenic *Fusarium* species are more prevalent in moderate regions, such as Western Europe and North America, and *Aspergillus* and *Penicillium* species are more probably to occur in (sub-) tropical regions.

The main source of exposure to mycotoxins is via ingestion of contaminated food/feed, but also skin contact and inhalation of toxins are considered sources of exposure (Bennett & Klich, 2003). All mycotoxins are of fungal origin, but fungal development does not necessarily mean presence of mycotoxins. Moreover, one fungal specie can produce more than one different mycotoxin and one mycotoxin can be produced by representatives of different fungal genera. Now, there are over 400 chemical compounds classified as mycotoxins. Mycotoxins can cause a variety range of diseases as well as death to both humans and animals. The most important ones associated with human and veterinary diseases are aflatoxins (AFs), citrinin, ergot alkaloids, fumonisins (FBs), ochratoxin A (OTA), patulin, trichothecenes, and zearalenone (ZEN) (Bennett & Klich, 2003). To further complicate the situation, modified and emerging mycotoxins have recently been described (Broekaert et al., 2015) and their importance is increasingly taken into account. The most important groups of mycotoxins produced by *Fusarium* species are: FBs, ZEN, and trichothecenes (mainly, deoxynivalenol {DON}, nivalenol {NIV}, T-2 and HT-2 toxins) (Escrivá et al., 2015). The first cases of mycotoxicosis in animals were described in 1960 in London, England, where approximately 100,000 turkey died due to liver necrosis and biliary hyperplasia (originating a disease that was then called the Turkey 'X' disease) caused by peanut (groundnut) meal contaminated with secondary metabolites from *Aspergillus flavus* (AFs) (Blount, 1961). From that time, investigations have taken place to define different mycotoxins, their toxicology in human

and animals and the possible strategies to counteract their adverse impacts (Haque et al., 2020).

1.1. Deoxynivalenol (DON)

1. 1.1. Origin and structure

DON is a trichothecene mycotoxin produced by *Fusarium* species. Trichothecenes are a group of over 200 toxins, with a molecular weight ranged between 200 and 500 Daltons, mainly produced by species of *Fusarium*, *Myrothecium*, and *Stachybotrys*. All trichothecenes contain a common tetracyclic sesquiterpenoid 12,13-epoxytrichothecene, responsible for their cytotoxicity, and a 9, 10 double bond with various side chain substitutions (Escrivá et al., 2015). Trichothecenes are divided on macrocyclic or non-macrocyclic, based on the presence of a macrocyclic ester or an ester-ether bridge between C-4 and C-15. Trichothecenes are classified into 4 groups (group A, B, C and D) depending on the substitution pattern of the tricyclic 12, 13-epoxytrichothec-9-ene (EPT) core structure. The types A and B are the most prevalent in food and feed, whereas types C and D rarely occurred (Escrivá et al., 2015). The type A group has a hydrogen or ester type side chain at the C-8 position and is composed of T-2 toxin, HT-2 toxin, neosolaniol, and diacetoxyscirpenol, and type B group has a ketone at the C-8 position and is composed of fusarenon-X, NIV, and DON and its derivatives. In summary, DON has a double bond in C9-10, a ketone function at C-8 and a C12-13 epoxy-group (Figure 1).

DON is produced predominantly by *Fusarium graminearum* and *Fusarium culmorum* and, at a lesser extent, by *Fusarium cerealis* and *Fusarium pseudograminearum* under conditions of high moisture and low temperature (Bertero et al., 2018). DON mainly occurs during the field development although could also occur during storage (Ma & Guo, 2008). DON is the most widespread mycotoxin found in grains such as wheat, rye, barley, maize, and oats and their by-products (Creppy, 2002).

DON was described for the first time in 1972 by a Japanese team from *Fusarium*-infected barley (Morooka et al., 1972). The trivial name of DON is vomitoxin, name associated to the vomiting effect observed in pigs fed a DON-contaminated corn based diet (Vesonder et al., 1973). The molecular formula of DON is $C_{15}H_{20}O_6$ and its molecular weight is 296.32 g/mol. Structurally, DON is a polar compound, soluble in water and in some polar solvents (e.g. aqueous methanol, acetonitrile and ethyl acetate) (EFSA, 2004).

DON is composed of 3 free hydroxyl groups (-OH) which also participate on its toxicity (Nagy et al., 2005). DON is a thermostable compound (stable at 120 °C and moderately stable at 180 °C) (EFSA, 2017). The maximum ultraviolet (UV) absorption spectra for DON is 217 nm (Krska et al., 2004). DON has been classified by the International Agency for Research on Cancer (IARC) in group 3 as non-carcinogenic to humans (Ostry et al., 2017).

Modified forms of DON could also be present in foods and feeds, as the acetylated forms 3-acetyl-DON (3-Ac-DON) and 15-acetyl-DON (15-Ac-DON), and the DON-3-glucoside (DON3G), which is defined as the DON modified form mainly present in plants (EFSA, 2017).

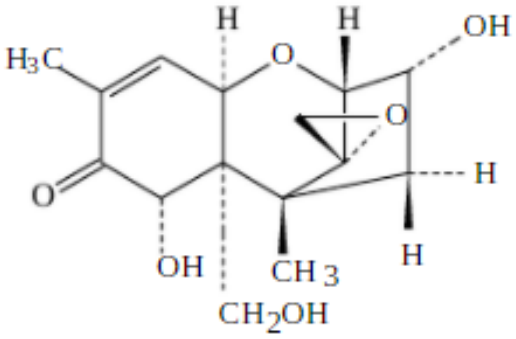

Figure 1. Chemical structure of deoxynivalenol (DON)

1.1.2. Mode of action

The first molecular target of DON and other trichothecenes is the ribosome. DON inhibits protein synthesis via binding to the peptidyl transferase region of the 60S subunit of the ribosome and interfering with the elongation step (Pestka, 2010). Ribosome binding leads first to the activation of ribosomal-associated protein kinase R (PKR) and tyrosine protein kinase (Hck) and, subsequently, the activation of mitogen activated protein kinases (MAPKs) signalling, including p38, Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase 1 and 2 (ERK1/2) (Zhou et al., 2014). The mechanism regulating this activation is named the "ribotoxic stress response" (Pestka, 2010) (Figure 2). The activation of MAPKs by DON *in vivo* and *in vitro* suggests that the ribotoxic stress response mediates DON toxicity (Pestka, 2007). MAP Kinases are involved in cell growth, differentiation and apoptosis (Zhou et al., 2014). Also, it might include leukocytes apoptosis and lymphotoxicity. *In vitro* DON exposure induced

apoptosis in murine T cells, B cells, and IgA^+ cells isolated from spleen, Peyer's patches, and thymus (Pestka et al., 1994). In addition to that, the apoptosis can occur in gastric mucosa, gastric glandular epithelium, and intestinal crypt cell epithelium, and can result in the breakdown of the epithelial barrier and mucus secretion (Bondy & Pestka, 2000). DON is both immunostimulatory and immunosuppressive. At high doses, DON is immunosuppressive and causes leukocytes apoptosis (Pestka, 2008). Exposure to high DON concentrations induces macrophages apoptosis which might innate immune function suppression (Pestka et al., 2004). Furthermore, the acute oral exposure to trichothecene mycotoxins produces adverse effects to actively dividing cells in bone marrow, lymph nodes, spleen, thymus, and intestinal mucosa (Bondy & Pestka, 2000). Additionally, DON induced lymphocyte cytotoxicity (Sharma, 1993). *In vitro* and *ex-vivo* studies demonstrated that trichothecenes have both effect of enhancement or impairment of mitogen-induced lymphocyte proliferation depending of the dose of exposure (Pestka, 2008).

After its activation, p38 and ERK induced gene transcription and enhanced mRNA stability. High expression of the cyclooxygenase-2 (COX-2) gene induced by DON in mouse macrophages (Moon & Pestka, 2002), upregulation of tumor necrosis factor (TNF-α) and interleukin 6 (IL-6) in macrophages, interleukin 8 (IL-8) in monocytes, and interleukin 2 (IL-2) expression in T cells, were reported in a review by Pestka (2008). The induction of mRNA expression genes included also the induction of transcription and post transcription factors such as nuclear factor kappa β (NF-kβ) (Zhou et al., 2014) and leads to promote proinflammatory genes (Pestka, 2010). The induction of proinflammatory gene might contribute to DON induced-anorexia, reduced weight gain, immunostimulation or immunosuppression, and tissue injuries.

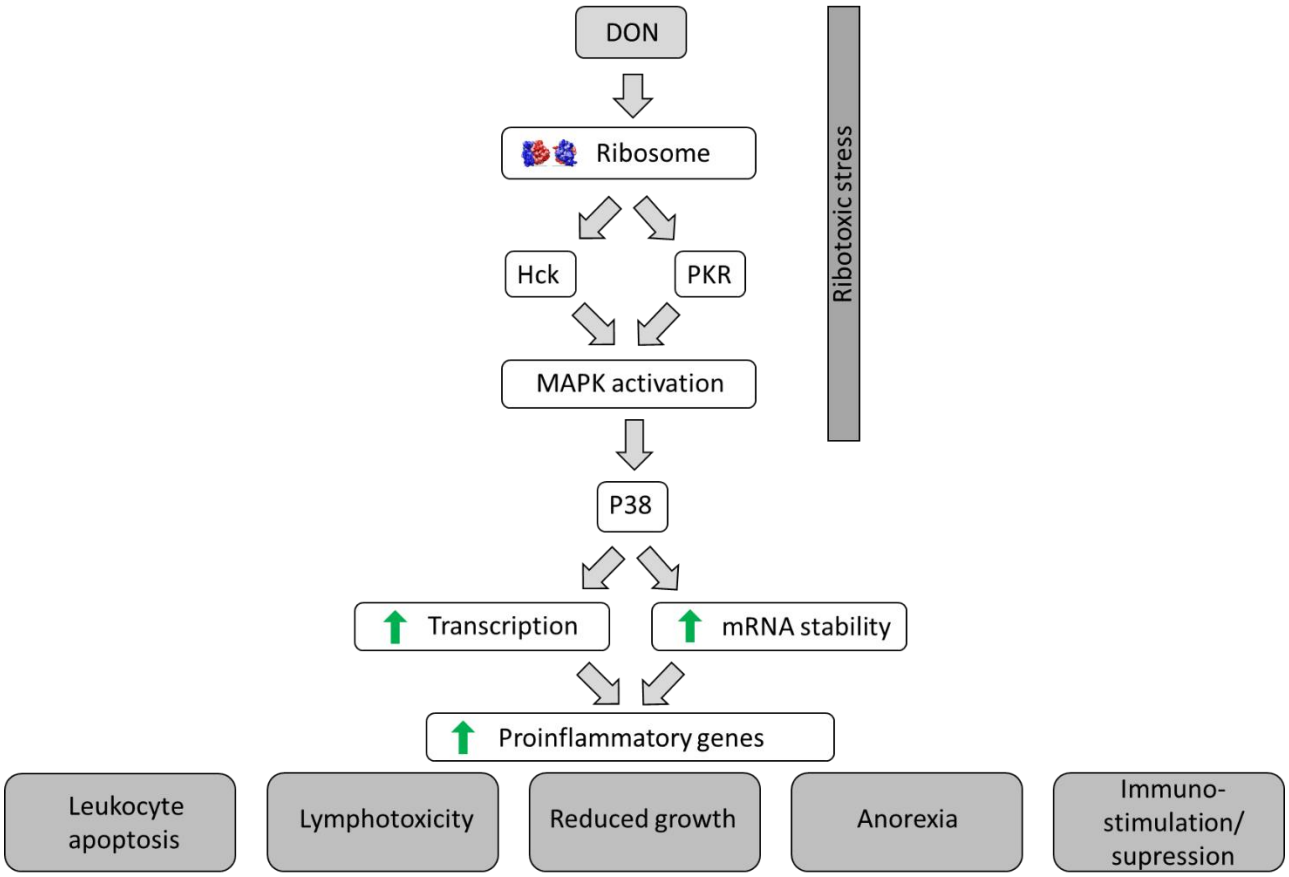

Figure 2. Deoxynivalenol-induced ribotoxic stress response and the effects associated with (Adapted from Pestka, 2008).

1.1.3. Methods of analysis

The methods for the analytical determination of mycotoxins can be classified into chromatographic methods, immunochemical methods and "other" approaches. Liquid chromatography (LC)-tandem mass spectrometry (MS/MS) is the most selective, sensitive, and accurate method which allows the determination of a high number of analytes. Prior to the determination of DON, sampling and sample preparation steps must be followed. For the sampling step, the collection of a representative sample must take into account the heterogeneous distribution of mycotoxins in a batch, mainly in cereals, being the major challenge for this kind of analysis. For this reason, it is highly recommendable to increase the size of samples and subsamples and decrease the particle size in order to minimize the sample variability (Whitaker, 2006). With regard to sample preparation, DON chemically is a polar compound and can be extracted from food or feed with an acidified mixture of water with organic solvents, such as methanol or acetonitrile. Then, centrifugation or filtration step is usually required. The next step is a clean-up of the extract to eliminate interferences from the sample matrix and to improve the

sensitivity and selectivity of the method. This step is required for most of the chromatographic analytical methods. However, for determination of DON, no clean-up is needed after extraction for most of the immunoassay based rapid screening methods, such as enzyme-linked immunosorbent assay (ELISA) and some LC-MS based technologies (Ran et al., 2013). The most employed strategies for clean-up of DON extracts are liquid-liquid extraction (LLE), solid-phase extraction (SPE), immunoaffinity columns (IAC), multifunctional columns (MFC) such as Multisep^{TM} and Mycosep^{TM}, molecularly imprinted polymers (MIPs) and immune-ultrafiltration (IUF) (Ran et al., 2013). Those methods, in spite of their advantages, also have some disadvantages. For example, the SPE is time consuming and not specific, the IAC is expensive and generally detects only one certain mycotoxin, and MFC is vulnerable to the matrix effect (Ran et al., 2013). In addition, the Quick, Easy, Cheap, Effective Rugged and Safe method (QuEChERS-like methods), based on the addition of salt mixtures has been used in DON analysis on phase separation between water or methanol and acetonitrile (Desmarchelier et al., 2010). This method is economic, but it is less sensitive and interferences could be detected in the analysis.

To deal with high number of samples for DON analysis, qualitative methods based on rapid screening methods and quantitative methods based on determination of DON levels have been developed. The screening methods must be rapid, accurate and don't require any clean-up or analyte enrichment steps (Krska et al., 2008), and usually are based on immunochemical techniques such as ELISA. For fast monitoring of the majority of mycotoxins, especially in raw materials, immunochemical methods have become routinely used tools. Membrane-based flow-through immunoassay assays, lateral flow devices and ELISAs are suitable for DON, and are characterized by their excellent repeatability and their good reproducibility.

Although rapid screening methods are widely used due to their easy of operation, low cost and their commercially availability for most of the major mycotoxins, their main disadvantages are their difficulty to quantify the mycotoxin, their lower sensitivity and their cross reactivity, which leads to a risk of false positive or negative results (Ran et al., 2013). On the other hand, the most important and validated analytical methods to determine DON described in literature are chromatography or mass spectrometry-based quantitative methodologies such as high-performance liquid chromatography (HPLC) and LC-MS/MS.

HPLC is considered a suitable technique to detect DON due to the polarity of this toxin and its solubility in organic solvents and water. HPLC coupled with UV detector is the most commonly used method to detect DON (Shephard et al., 2013). However, in general, DON could be combined with fluorescence detection (FD), flame ionization detection (FID), or electro capture detection (ECD). Recently, the trend to determine DON or other mycotoxins simultaneously is LC-MS/MS. This method was widely used to determine DON and/or its metabolite deepoxy-deoxynivalenol (DOM-1) (the nontoxic de-epoxide of DON) in different biological matrices of chickens exposed to DON mycotoxicosis such as plasma, serum, bile, liver, gizzard, cecum, rectum, kidney or excreta (Table 1). In some works, LC-MS/MS required a clean-up step prior the determination of DON or its metabolites (Awad et al., 2011; Lauwers et al., 2019a). However, the most recent studies showed that methods without sample clean-up have been developed (Broekaert et al., 2014; Devreese et al., 2015; Osselaere et al., 2013b).

The disadvantage of chromatographic methods is that results could be affected by matrix effects (Ran et al., 2013). Then, the addition of internal standard substances, such as isotope labelled analytes could minimize the matrix effect. This method is sensitive, for example, DON determination in chicken plasma by LC-MS/MS has limits of quantification (LOQs) for DON ranging from 0.1 to 2.5 ng/mL (Broekaert et al., 2014; Osselaere et al., 2013b). Interestingly, a combination of LC-MS/MS and LC-high resolution mass spectrometry (LC-HRMS) is used to determine not only mycotoxins for which standards are commercially available, but also other phase I and II metabolites for which no commercial standards are available (Lauwers et al., 2019b). This method is useful because phase II metabolites might be the most suitable biomarkers for exposure of some mycotoxins due to the extensive biotransformation by these pathways (Lauwers et al., 2019b). On the other hand, this method was qualitative and untargeted. In conclusion, these chromatography or mass spectrometry-based quantitative methodologies are repeatable, reproducible, rapid, and sensitive. On the other hand, they need clean-up step, are expensive, and can be affected by matrix effects (in the case of LC-MS or LC-MS/MS technique).

Table 1. Methods of analysis of deoxynivalenol (DON) upon its administration to chickens.

Matrix (weight/volume)	Purification method	IS[1] (volume/ concentration)	Reconstitution (solvent/volume)	Injection volume	Analysis method	Method sensibility	Reference
1 mL serum or bile 5 g liver, faeces, gizzard, cecum or rectum	Incubation with β-glucuronidase, extraction with acetonitrile[2] (ACN),Clean-up	0.050 mL U-[$^{13}C_{15}$]-DON[3] (127.0 ng)	495 µL methanol[4] (MeOH)/water (2:8 v/v)	20 µL	LC-MS/MS[5]	LOD[4] = 7 ng/g	(Awad et al., 2011)
2 g liver or kidney Plasma or bile	Extraction with water/ACN mixture, purification	112.5 µL of $^{13}C_{15}$-DON, 1 µg/mL	1 mL water/MeOH (90:10 v/v)	10 µL	LC-MS/MS	LOQ[6] = 1.25 ng/g (liver or kidney) LOQ = 1.25 ng/mL (plasma or bile)	(Osselaere et al., 2012)
Plasma 250 µL	no extraction	12.5 µL of $^{13}C_{15}$-DON, 1 µg/mL	200 µL water-0.1% acid acetic glacial / MeOH (95:5 v/v)	10 µL	LC–MS/MS	LOQ = 1- 2.5 ng/mL	(Osselaere et al., 2013b)
Plasma 250 µL	no extraction	5 µL of $^{13}C_{15}$-DON, 1 µg/mL	200 µL of water	10 µL	LC-MS/MS	LOQ = 0.1-2 ng/mL	(Broekaert et al., 2014)
Plasma 100 µL	no extraction	50 µL of $^{13}C_{15}$-DON, 50 ng/mL	200 µL of water	5 µL	LC–MS/MS	LOQ = 0.1 ng/mL	(Devreese et al., 2015)
Plasma 250 µL	no extraction	25 µL of $^{13}C_{15}$-DON, 0.1 µg/mL	200 µL of water	10 µL	LC–MS/MS and HR-MS[7]	LOQ = 1 ng/mL	(Broekaert et al., 2017)

Table 1. Methods of analysis of deoxynivalenol (DON) upon its administration to chickens.

Matrix (weight/volume)	Purification method	IS[1] (volume/ concentration)	Reconstitution (solvent/volume)	Injection volume	Analysis method	Method sensibility	Reference
Plasma 150 µL	Extraction with ACN and formic acid, clean-up	$^{13}C_{15}$-DON, 1 µg/mL 15 µL of IS (combined working solution (100 ng/mL = 0.1 µg/mL))	150 µL of MeOH/water (85:15 v/v)	5 µL	LC–MS/MS and HR-MS	LOQ = 1 ng/mL	(Lauwers et al., 2019a)
Lyophilized excreta 250 mg		$^{13}C_{15}$-DON, 1µg/mL, (20 µL of IS combined working solution (100 ng/mL = 0.1 µg/mL))	150 µL of MeOH/water (85:15 v/v)				

[1] IS: Internal standard; [2]ACN: acetonitrile; [3]DON: deoxynivalenol; [4]MeOH: methanol; [5]LC-MS/MS: liquid chromatography-tandem mass spectrometry; [6]LOQ: limit of quantification; [7]HRMS: high resolution mass spectrometry.

1.1.4. Legislation

Contamination of food and feed by mycotoxins is a health and economic concern, therefore maximum allowed or recommended levels have been established in several countries. In Europe, these maximum levels have been defined as regulations and recommendations by European Commission (EC). EC Regulation for DON in food have been established by Directive No 1881/2006 on unprocessed cereals and cereal products for human consumption (Table 2). Regarding feed, the regulations have been only established for aflatoxin B1 (AFB1) by Directive 2002/32/EC of the European Council of 7 May 2002 on undesirable substances in animal feed (European Commission, 2002). Meanwhile, the maximum recommended content of DON is described in the Recommendation 2006/576/EC of the Commission of 17 August 2006 (European Commission, 2006a) on certain products intended for animal feed (Table 3).

Table 2. Maximum levels given by the EU for deoxynivalenol (DON) in foodstuffs.

Foodstuffs	**Maximum levels[1] (µg/kg)**
Unprocessed cereals other than durum wheat, oats and maize	1250
Unprocessed durum wheat and oats	1750
Unprocessed maize	1750
Cereals intended for direct human consumption, cereal flour (including maize flour, maize meal and maize grits) bran as end product marketed for direct human consumption and germ, with the exception of processed cereal-based foods and baby foods for infants and young children	750
Pasta (dry)	750
Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals	500
Processed cereal-based foods and baby foods for infants and young children	200
Milling fractions of maize with particle size > 500 µm falling within CN code 1103 13 or 1103 20 40 and other maize milling products with particle size > 500 µm not used for direct human consumption falling within CN code 1904 10 10	750
Milling fractions of maize with particle size ≤ 500 µm falling within CN code 1102 20 and other maize milling products with particle size ≤ 500 µm not used for direct human consumption falling within CN code 1904 10 10	1250

[1]Levels according EU Regulation (EC) No 1881/2006.

Table 3. Guidance levels given by EU for deoxynivalenol (DON) in animal feed (relative to a feedingstuffs with a moisture content of 12%).

Products intended for animal feed	Guidance values[1] (mg/kg)
Cereals and cereal products with the exception of maize by-products	8
Maize by-products	12
Complementary and complete feedingstuffs with the exception of: - Complementary and complete feedingstuffs for pigs Complementary and complete feedingstuffs for calves (< 4 months), lambs and kids	5 0.9 2

[1]Values according EU Recommendation 2006/576/EC.

1.1.5. Occurrence of DON in feed

Both humans and animals are exposed to mycotoxins through contamination of food and feed (Jard et al., 2011). A recent large scale survey monitored the occurrence of mycotoxin from 2008 to 2017 in 74,821 samples of finished feed, maize, maize dried distillers grains with soluble (DDGS), maize silage, soybean grains, soybean meal, wheat, barley, and rice collected from 100 countries for the presence of AFB1, DON, ZEN, FBs, OTA, and T-2 (Gruber-Dorninger et al., 2019). Table 4 and Figure 3 showed the percentage of samples that were positive for all mycotoxins in all samples and for DON in each sample. DON was the most prevalent *Fusarium* mycotoxin followed by FBs and ZEN, mycotoxins were detected in 64%, 60%, and 45% of all samples, respectively. Regarding AFB1, T-2, and OTA, they were detected in 23%, 19%, and 15% of the samples, respectively. The highest median concentrations were found for FBs and DON with 723 and 388 µg/kg, respectively (Table 4). Corn DDGS, finished feed, corn, wheat and barley showed the highest percentage of positive samples for DON, with a percentage of positive samples of 83 %, 70%, 67%, 65% and 61%, respectively (Table 4).

Table 4. The occurrence of deoxynivalenol (DON) in different feedingstuffs.

Mycotoxin[1]/sample	Number of samples	Positive[2] samples					
		Number	Percentage	Median (µg/kg)	1th quartile (µg/kg)	3rd quartile (µg/kg)	Maximum
All samples							
AFB1	51,475	11,941	23	4	2	17	10,918
DON	59,107	37,940	64	388	200	885	84,860

Table 4. The occurrence of deoxynivalenol (DON) in different feedingstuffs.

Mycotoxin[1]/sample	Number of samples	Positive[2] samples					
		Number	Percentage	Median (µg/kg)	1th quartile (µg/kg)	3rd quartile (µg/kg)	Maximum
ZEN	61,413	27,559	45	55	25	147	105,000
FBs[3]	46,477	27,890	60	723	240	1858	290,517
OTA	32,271	4858	15	3	2	7	2000
T-2	27,850	5289	19	22	8	40	3051
DON in Finished feed	18,649	13,004	70	294	134	600	32,893
DON in Corn	12,660	8486	67	520	260	1240	51,374
DON in Corn DDGS	381	316	83	1490	574	2579	84,860
DON in Corn silage	4206	2588	62	474	219	1092	34,861
DON in soybean grains	975	284	29	416	160	640	5500
DON in soybean meal	802	247	31	119	25	424	5600
DON in wheat	5949	3866	65	369	218	865	49,307
DON in barley	4046	2468	61	359	234	750	35,000
DON in rice	226	60	27	266	87	436	3859

[1]AFB1: aflatoxin B_1; DON: deoxynivalenol; ZEN: zearalenone; FBs: fumonisins; OTA: ochratoxin A; T-2: T-2 toxin; [2]Positive samples (> limit of detection= LOD); LOD (AFB1) < 0.5 ng/g; LOD (DON, ZEN, FBs, OTA, T-2 < 1 ng/g); [3]Sum of fumonisins B_1, B_2, and B_3. Adapted from Gruber-Dorninger et al. (2019).

Regional mycotoxin occurrence was monitored in 15 geographic regions (Northern Europe, Central Europe, Southern Europe, Eastern Europe, North America, Central America, South America, Middle East/North Africa, Sub-Saharan Africa, South Africa, Oceania, South Asia, East Asia, Southeast Asia, and Central Asia). DON was the most prevalent mycotoxin in Northern Europe, Central Europe, Eastern Europe, North America, South Africa, Oceania, East Asia, and Central Asia (Figure 3). The levels are considered relatively low by the authors, regarding the EU limits. In the European region, for example, DON with relatively high median level of 504, 428, 324 and 153 µg/kg were found in Northern, Central, Southern, and Eastern Europe, respectively. The lowest EU guidance values for DON were exceeded by 21.5, 20.4, 11.7, and 4.3 % in Northern, Central, Southern, and Eastern Europe, respectively (Gruber-Dorninger et al., 2019). Only 1, 0.9, 0.5, and 0.1 % of the samples did not comply with the highest EU guidance values in the same geographic regions (Gruber-Dorninger et al., 2019). It should be noted that levels lower than the guidance values can already cause adverse effects to the

animals, especially if the contaminated feed is received for longer periods (Kolawole et al., 2020).

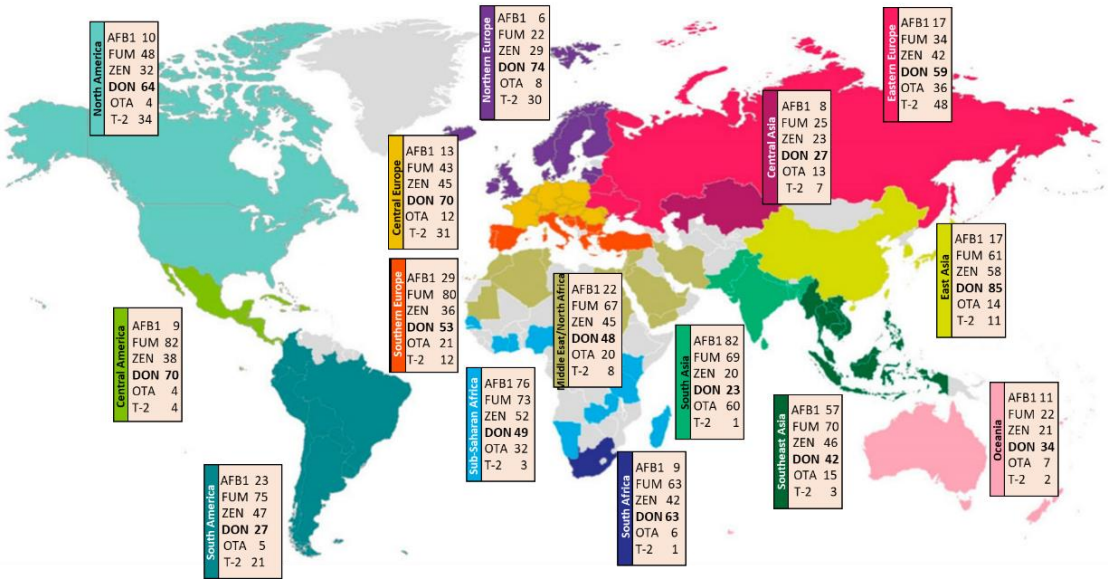

Figure 3. Mycotoxins prevalence in different geographic regions based on percentage of positive samples (> Limits of quantification (LOQs)).

Number of samples analyzed for AFB1, FBs, ZEN, DON, OTA and T-2: Northern Europe: 1,958; Central Europe: 21,036; Southern Europe: 3,527; Eastern Europe: 2,382; North America: 5,471; Central America: 367; South America: 17,332; Middle East/North Africa: 1,075; Sub-Saharan Africa: 208; South Africa: 1,077; Oceania: 1,695; South Asia: 1,136; East Asia: 13,232; Southeast Asia: 4,310; and Central Asia: 15. Adapted from (Gruber-Dorninger et al., 2019).

1.1.6. Co-occurrence of DON in feed

The simultaneous presence of different mycotoxins in the recent survey is the rule rather than the exception (Gruber-Dorninger et al., 2019). Mycotoxins co-occurrence induces additive or synergistic effects which can cause adverse effects even at low mycotoxin concentrations in several animal species (Grenier and Oswald, 2011). The simultaneous presence of DON and ZEN, or DON and FBs were the most observed (48%) in the case of finished feed. It happens in a similar way with corn, with co-occurrences of DON and ZEN in 39%, and DON and FBs in 49% of the samples. The most frequently combination in wheat was DON and ZEN, which was detected in 28% in the samples.

Table 5. Co-occurrence of deoxynivalenol (DON) with other mycotoxins in finished feed, maize, and wheat.

DON[1]Combination	Finished Feed (%)	Maize (%)	Wheat (%)
AFB1[2]	14	15	5
ZEN[3]	48	39	28
FBs[4]	48	49	8
OTA[5]	15	3	6
T-2 toxin	19	10	14

[1]DON: deoxynivalenol; [2]AFB1: aflatoxin B1; [3]ZEN: zearalenone; FBs[4]: fumonisins; OTA[5]: ochratoxin A

2. DON toxicokinetics and biomarkers of exposure

Specific well-characterized biomarkers have shown to predict relevant clinical outcomes across a variety of treatments and populations (Atkinson et al., 2001). Up to date, the use of biomarkers has become commonplace, and biomarker-driven research has been proposed as a successful method (Vidal et al., 2018). The biomarkers of exposure are defined as the metabolites estimated in biological fluids upon the exposure to xenobiotics of individuals (Vidal et al., 2018). The biomarkers of exposure must be specific for each mycotoxin and target species, and the analytical method used for its detection must be validated for each biological matrix considered (EFSA, 2010). As previously mentioned, EFSA has indicated that the relevant biomarkers directly related to exposure of DON are DON and DOM-1, revealing that de-epoxidation is the most important pathway of metabolization of DON (EFSA, 2010). However, research conducted in different species revealed that the metabolization pathway of DON is species-dependent, and de-epoxidation has shown not to be an important pathway of metabolization for poultry species (Devreese et al., 2015).

The metabolization of DON may be changed if birds are exposed to DON in a chronic feeding design or to intravenous injection or oral bolus of the synthetic or labelled DON, also by the biological matrices and the sensitivity of the method of analysis used. The most relevant biomarkers of DON exposure in different biological matrices in chickens are reported in Table 6.

After chronic feeding of DON at 7.54 or 9.5 mg/kg to broiler chickens, DON was quantified as the main metabolite in plasma using LC-MS/MS with LOQ (0.1 to 1.25 ng/mL) (Osselaere et al., 2012; Yunus et al., 2010). However, the concentration of DON in plasma was under the LOD which ranged from 2 to 7 ng/mL when broilers were fed lower or close to the guidance level of DON in poultry feed (Awad et al., 2011; Dänicke et al., 2007; Osselaere et al., 2012).

On the other hand, DON was the only metabolite detected in the plasma when broilers were exposed to the equivalent to guidance level (approximately 0.5 to 0.75 mg/kg BW) intravenously or by oral gavage in a two-way cross-over design, using LC-MS/MS with LOQ (0.1 – 2.5 ng/mL) (Broekaert et al., 2014, 2015; Osselaere et al., 2013b). Furthermore, after 2 h of administration, no DON levels in broilers plasma above LOQ were detected (Osselaere et al., 2013b). The study of toxicokinetic parameters upon quantification of DON in plasma revealed low absolute oral bioavailability (19.3%) or

absorbed fraction (10.6%) due to the poor absorption of DON (Broekaert et al., 2015; Osselaere et al., 2013b) This low absorption may be partially related to the rapid transit time in the gastrointestinal tract (GIT) of birds. It has also been suggested that this poor bioavailability could be associated to the metabolization of DON by the high bacterial content in the GIT of chickens before the main site of absorption (Broekaert et al., 2015). Also, it has been reported that the high clearance with the rapid elimination half-life might be the reasons that poultry are relatively tolerant to DON mycotoxicosis (Osselaere et al., 2013b).

Awad et al. (2011) indicated that after exposure of DON in feed (1 or 5 mg/kg) to broiler chickens for 35 d, DON concentration was recovered around 10-12% in gizzard, 18-22% in cecum and only 6% in excreta of the DON administered. DON was not detected in liver and bile. Moreover, the DON concentration in the content (digesta) of gizzard, cecum, rectum and excreta increased in a dose dependent manner. In contrast, DON could not be quantified in bile, liver, breast meat, and kidney (Dänicke et al., 2007; Osselaere et al., 2012). The analysis of DON and its metabolites using HPLC in broiler chickens receiving an oral administration of tritium-labelled DON at the dose of 2.5 mg/kg BW for 5 days, indicated that DON was transiently distributed and rapidly eliminated in all tissues (Wan et al., 2014). Low DON concentrations were detected in kidney, liver, heart, lung, spleen, and brain and the higher level was detected in small intestine 6 h post-administration.

With regard to DOM-1, several studies analyzed this metabolite in plasma, in different organs, or in excreta after chronic DON feeding, intravenous injection or oral gavage, using LC-MS/MS (Awad et al., 2011; Broekaert et al., 2014,2015,2017; Dänicke et al., 2007; Devreese et al., 2015; Osselaere et al., 2012, 2013b; Schwartz-Zimmermann et al., 2015; Yunus et al., 2010). However, in very few studies could DOM-1be quantified (Osselaere et al., 2012; Yunus et al., 2010).

All studies mentioned followed the guidelines proposed by EFSA and quantified DON and DOM-1 as possible metabolites of exposure of DON in different biological matrices of chickens, but they found inconsistent results. The lack of detection of DOM-1 could be explained by the lack of microbes capable of de-epoxidize DON in poultry species, as hypothesized by Schwartz-Zimmermann et al. (2015).

The use of the analytical method HRMS is a very recent method to identify or detect the untargeted or novel metabolites; biotransformation products and/or modified fungal

metabolites can be identified (EFSA, 2017). Since 2014, and with the improvement of the analytical methods it was reported that the most abundant metabolite in case of broiler chickens after receiving DON mycotoxin was a metabolite phase II named DON-3 sulphate (DON-3S) (Wan et al., 2014). Furthermore, in poultry no DON glucuronidation or sulfonation has been observed (Devreese et al., 2015). Still, the formation of DON-3S was observed also in turkeys, pigeons and laying hens (Antonissen et al., 2016; Devreese et al., 2015; Jurisic et al., 2019; Lauwers et al., 2019a).

DON-3S was identified the first time as a new and major metabolite in poultry in the study of Wan et al. (2014). Among the three new metabolites identified (DON-3S,10-DON-sulfonate, and 10-DOM-1-sulfonate), DON-3S was the major metabolite found in excreta of chickens (88.6%) after the oral administration of tritium-labelled DON at the dose of 2.5 mg/kg BW for 5 days (Wan et al., 2014).

Subsequently, Devreese et al. (2015), using HRMS analysis of plasma of turkeys and broiler chickens, found that DON-3S was the major metabolite after oral gavage as well as after intravenous injection of 0.75 mg DON/kg BW (Devreese et al., 2015). Broiler chickens biotransform or metabolize DON more extensively to DON-3S than turkey poults (Devreese et al., 2015). It has been suggested that sulfation mainly occurred in the enterocytes of the intestinal mucosa (Schwartz-Zimmermann et al., 2015). Similarly, LC-HRMS indicated that DON-3S was mainly present in plasma of broiler chickens dosed at 0.5 mg DON/kg BW after intravenous or oral DON administration, respectively, and DON-3G was not hydrolysed to DON in this species (Broekaert et al., 2017). The authors concluded that chickens are less sensitive to DON toxicity and explained this relative tolerance by the rapid gastrointestinal transit time or by the extensive and rapid sulfation occurred.

Schwartz-Zimmermann et al. (2015) demonstrated that DON-3S was the best biomarker of exposure in all poultry species. In chickens, for example, biological recovery of DON-3S in excreta reached 80% after administration of DON (1.7 mg/kg feed). These authors indicated that DON is rapidly absorbed from the GIT between crop and jejunum. Then, they suggested that after absorption, DON is extensively biotransformed to DON-3S in the intestinal mucosa, liver and/or kidney. The elimination of DON-3S is rapid and complete into the cloaca via urine by the kidney or back into the GIT via bile by the liver (Schwartz-Zimmermann et al., 2015). It has also been demonstrated that the most suitable biomarker for exposure of DON in poultry is DON-

3S detected in different biological matrices (plasma and excreta); after a single intra-crop bolus administration of 0.5 mg DON/kg BW to broiler chickens aged 21 d and weighed 1 kg, DON-3S was detected and no DON was found. The maximum peak area of DON-3S was achieved in plasma after 30 min post administration and after 3-6 h in dried excreta (Lauwers al., 2019a).

In conclusion, DON and DOM-1 are not considered ideal biomarkers in the different biological matrices of chickens. Interestingly, the most abundant metabolite in plasma and excreta of chickens is DON-3S which could be considered the most suitable biomarker of exposure of DON in chickens. This metabolization is a detoxification pathway (Yi et al., 2006). On the other hand, the toxicokinetic behaviour of DON in chickens was characterized by low absorption, rapid gastrointestinal transit time, transient distribution, high clearance and rapid elimination. All these factors may explain the low susceptibility of broilers to DON toxicity.

Table 6. The main metabolites of exposure of deoxynivalenol (DON) in broiler chickens in different biological matrices.

DON[1] dose	Route	Matrix	Metabolites analyzed	Main metabolite	Reference
Toxicokinetics studies					
0.5 mg/kg BW[2]	Intravenous injection or oral gavage	Plasma	DON 3ADON[3] 15ADON[4] DOM-1[5]	DON	(Broekaert et al., 2014, 2015)
0.77 DON-3G[6] mg/kg BW 0.5 DON mg/kg BW	Intravenous injection or oral gavage	Plasma	DON DON-3G DOM-1	DON-3S[7]	(Broekaert et al., 2017)
0.75 mg/kg BW	Intravenous injection or oral gavage	Plasma	DON DOM-1 DON-3S DON-3G 10-DON-sulfonate, DOM- 1 10-DOM-1-sulfonate	DON-3S	(Devreese et al., 2015)
0.75 mg/kg BW	Intravenous injection or oral gavage	Plasma	DON DOM-1	DON	(Osselaere, et al., 2013b)

Table 6. The main metabolites of exposure of deoxynivalenol (DON) in broiler chickens in different biological matrices.

DON[1] dose	Route	Matrix	Metabolites analyzed	Main metabolite	Reference
Acute or chronic administration					
1 or 5 mg/kg	Feed	Serum, bile, liver, digesta of (gizzard, caecum, rectum), and excreta	DON DOM-1	DON	(Awad et al., 2011)
1.5 mg/kg	Feed	Plasma, bile, liver and breast meat	DON DOM-1	-	(Dänicke et al., 2007)
0.5 mg/kg BW	Single intra-crop bolus	Plasma and excreta	DON DON-3S	DON-3S	(Lauwers et al., 2019a)
2.44 or 7.54 mg/kg	Feed	Plasma, liver , kidney, bile	DON DOM-1	DON (plasma and bile) DOM-1 (bile)	(Osselaere et al., 2012)
1.7 mg/kg	Feed	Excreta	DON-3S DOM-3S[8] DON DOM-1 DON sulfonates 1,2,3 DOM sulfonate 2	DON-3S	(Schwartz-Zimmermann et al., 2015)
2.5 mg/kg BW	oral administration	Plasma and organs	DON	DON-3S	(Wan et al., 2014)

Table 6. The main metabolites of exposure of deoxynivalenol (DON) in broiler chickens in different biological matrices.

DON[1] dose	Route	Matrix	Metabolites analyzed	Main metabolite	Reference
			10-DON-sulfonate 10-DOM-sulfonate DON-3S		
9.5 mg/kg	Feed	Plasma	DON DOM-1	DON DOM-1	(Yunus et al., 2010)

[1]DON: deoxynivalenol; [2]BW: body weight; [3]3ADON: 3-acetyl-DON; [4]15ADON: 15 acetyl-DON; [5]DOM-1: deepoxydeoxynivalenol; [6]DON-3G: DON-3-glucoroinde; [7]DON-3S: DON-3-sulphate; [8]DOM-3S: deepoxy-DON sulphate

3. Toxicity of DON and biomarkers of effect

A biomarker of effect is evaluated through a biochemical, physiological, immunological, behavioural, or other organism's alteration (Vidal et al., 2018). Depending upon the magnitude, the biomarker of effect can be recognized as associated with an established or possible health impairment or disease (Vidal et al., 2018). Biomarkers of effect are biomarkers indirectly related to mycotoxin toxicity. The biomarkers of effect of DON toxicity in chickens highlighted in this review are: productive parameters, organ weights, morphology of small intestine, biochemical and haematological parameters, biomarkers related to immune system (common vaccines response as a part of humoral immune response, cellular immune response, and production of proinflammatory cytokines), and biomarkers related to chicken welfare.

3.1. Productive parameters

Studies about the effects of DON on productive parameters in chickens are listed in Table 7. The effect of exposure to DON contaminated diets is highly variable on poultry performance. This effect could depend on the dose of the toxin, the duration of exposure, feed multi-contamination and bird's initial health status. Indeed, the chronic ingestion of DON can cause decrease of feed consumption, body weight gain (BWG) and feed efficiency in chickens (Dänicke et al., 2003). Even at lower concentrations than the recommended guidance value (5 mg DON/kg feed), the presence of DON in chickens feed might have growth inhibitory effects (Chen et al., 2017; Lucke et al., 2017;Yunus et al., 2012a). A decreased BWG of broilers fed 1.68 mg DON/ kg feed at 21 d has been observed (Yunus et al., 2012a). Slow growing chicks fed 2 mg DON/kg feed had lower BWG (Chen et al., 2017). The body weight (BW) and feed intake of broiler chickens have shown to be reduced after feeding 2.5 mg DON/kg feed for 35 d (Lucke et al., 2017). Furthermore, DON exposure from 5 to 14 mg/kg of feed for 35 d had adverse effects on BW, BWG, feed intake and feed conversion ratio (FCR) of chickens (Awad et al., 2019; Dänicke et al., 2003; Ghareeb et al., 2012; Lucke et al., 2017). Broiler chickens exposed to DON from 15 to 18 mg/kg feed for 21 d also showed lower performance compared with controls, according to several studies (Huff et al., 1986; Kuebena et al., 1988,1977; Xu et al., 2011). Recently, Riahi et al. (2020) also reported that the use of DON artificially contaminated feed (15 mg/kg) during 42 d reduced the BWG and impaired the FCR. The poor performance occurred by the presence of DON in feed could be related to the mode of action of this toxin, that is principally the inhibition of protein synthesis at the

elongation or termination steps (Feinberg & McLaughlin, 1989). It has also been shown that DON could have adverse effects on chicken's productive parameters, but this effect appears only during specific parts of their growth cycle (Wang & Hogan, 2019). Awad et al. (2011) noted that the adverse effect of DON was only pronounced at the beginning of the experiment but no effect was observed later on BW, BWG, feed consumption and FCR. In fact, they found that feeding DON at 1 or 5 mg/kg to broiler chickens decreased the feed intake in a quadratic manner during the first week and BW and BWG during the second week (Awad et al., 2011). Moreover, a reduction of feed intake was observed only at the third week of the trial after DON (5 mg/kg) exposure to broilers (Awad et al.,2006b). Adverse DON temporary effect on growth performance was also observed in chickens fed 1.68 or 12.20 mg DON/kg feed during the 21 d of exposure, and this effect disappear later (Yunus et al., 2012a). BWG was only reduced during the second week in the study conducted by Kubena et al. (1989), which used 16 mg DON/kg of feed during 21 d. The meta-analysis conducted by Andretta et al.(2011) reported that the effect of mycotoxins on broiler's growth was greater in young broilers.

It has been hypothesized that the temporally effect produced especially at the early stages of chicken development is related to the capacity of birds to later adapt to DON mycotoxicosis.

In contrast, few studies found that broilers are more sensitive to DON-contaminated diets during later stage of growth. Feeding DON from 5.9 to 9.5 mg/kg feed to broilers reduced BWG and feed intake during the grower period (21 to 42d) (Swamy et al., 2004a). Broiler chickens fed 7.90 mg DON/kg feed from 21 to 34 d had lower BW, BWG, feed intake, and impaired FCR (Wang & Hogan, 2019). The authors concluded from this study that after chronic exposure to DON- contaminated diets, the adverse effects on growth performance are not necessarily cumulative (Wang & Hogan, 2019).

However, other reports indicated that DON at low, moderate, high or extremely high levels had no adverse effects on poultry performance. The exposure to naturally contaminated DON diets, containing 1.5 to 1.87 mg DON/kg to broiler chickens for 21 or 28 days had no effects on BW, BWG, feed consumption, and FCR (Hulan & Proudfoot, 1982; Liu et al., 2020). No adverse effect was indicated on broiler performance feeding the guidance value for 15, 21 , 28 or 42 d, respectively (Antonissen et al., 2017; Liu et al., 2020; Riahi et al., 2020; Yu et al., 2018). BW, BWG, feed intake and FCR were not adversely affected by the inclusion of 10 mg DON/kg in broiler diets for 35 d or 42 d

(Awad et al., 2004; Awad et al., 2006a; Ghareeb et al., 2014, 2016). Productive parameters of birds exposed to 16 mg DON/kg feed for 21 d were not adversely affected (Harvey et al., 1997). Even high level of an artificially DON contaminated diet (116 mg/kg) did not adversely affect BWG and feed consumption of broiler chickens from 6 to 11 days (Moran et al., 1982). Failure to observe significant differences on growth parameters might be suggestive of adaptation of birds to mycotoxins over time, and that poultry are relatively tolerant to DON mycotoxicosis compared with other species, especially pigs, due to the differences in DON absorption, distribution, metabolism and elimination (ADME) (Broekaert et al., 2015).

Furthermore, it has been reported that DON could have growth stimulatory effects. Some studies indicate that DON in poultry feed enhance growth. Feeding 4.6 mg DON/kg feed to Leghorn and broiler chicks for 4 weeks (between 7 and 35 days of age) increased their daily BWG (Hamilton et al., 1985). In the same manner, BW of male Leghorn chicks was increased when fed 9 mg DON/kg feed at 35 d and 18 mg DON/kg feed at 21, 28 and 56 d (Kubena et al., 1985; Kubena & Harvey, 1988).

In addition, the inclusion levels of 4.7 or 8.2 mg DON/kg in broiler chickens feed increased BWG and feed intake in a significant quadratic manner in the finisher period (Swamy et al., 2002). A contaminated diet with DON at 1.5 mg/kg for 35 d enhanced broiler performance by increasing the BWG and reducing the FCR (Dänicke et al., 2007). Taiwan country chickens exposed to 5 mg/kg of DON in feed during 16 wk showed a growth promoting impact in comparison with chickens receiving 2 mg DON/kg (Chen et al., 2017). It has been suggested that this concentration near to or equal to 5 mg/kg could have a stimulator and promoting effect on growth of broilers and it was described as an hormesis phenomenon (Swamy et al., 2002). Hormesis is characterized by low-dose stimulation and high-dose inhibition (Swamy et al., 2002).

In summary, the effect of DON on chicken's performance has shown to be quite variable. This variability among studies depends on the dose and the duration of exposure, the co-occurrence of different mycotoxins with DON in contaminated grains, the source of mycotoxin used (naturally *vs* artificially), the climatic factors, the strain of the birds used, the methods of analysis of mycotoxins and the adaptability and the tolerance of chickens to DON mycotoxicosis (Liu et al., 2020; Murugesan et al., 2015). This tolerance may be due to the biotransformation of DON to DON-3S in chickens, which is much less toxic than DON itself (Wan et al., 2014).

Table 7. Effects of deoxynivalenol (DON) on productive parameters of chickens.

DON[1] (mg/kg) diet	Exposure duration (d)	Reported Effects	Reference
1.5	35	↑ BWG[2] and ↓ FCR[3]	(Dänicke et al., 2007)
up to 1.87	28	none	(Hulan & Proudfoot, 1982)
4.6	15	none	(Antonissen et al., 2017)
4.6	28 (from d 7 to 35)	↑ BWG and feed intake	(Hamilton et al., 1985)
1 or 5	35	↓ feed intake (7 d) and BW [4]and BWG (14 d)	(Awad et al., 2011)
1.5 or 5	21	none	(Liu et al., 2020)
5	21	↓ feed intake (14-21d)	(Awad et al.,2006b)
5	28	none	(Yu et al., 2018)
7.90	34	↓ BW, BWG and feed intake and ↑ FCR (21-34)	(Wang & Hogan, 2019)
4.7or 8.2	56	Feed intake and BWG responded quadratically (42 to 56 d)	(Swamy et al., 2002)
5.9 or 9.5	56	↓ BWG and feed consumption (d 21 to 42)	(Swamy et al., 2004a)
2, 5 or 10	112	BWG at 2 mg/kg lower than BWG at 5 mg/kg	(Chen et al., 2017)
2.5 ,5 or 10	35	Overall the trial: ↓ BW at 2.5 mg/kg and 5, ↓ BWG at 5 mg/kg and ↓ feed intake at all doses. At week 5: ↓ BW at 5 mg/kg and feed intake at 5 and 10 mg/kg	(Lucke et al., 2017)
5 or 10	35	↓ BW, BWG and feed intake and ↑ FCR	(Awad et al., 2019)
10	35	↓ feed intake (d 21 to d 35) and ↓ BW and BWG at d 35 and ↑ FCR at 21, 28 and 35 d	(Ghareeb et al., 2012)
10	35	none	(Ghareeb et al., 2014)
10	35	none	(Ghareeb et al., 2016)
10	42	none	(Awad et al., 2004)
10	42	none	(Awad, et al., 2006a)
1.68 or 12.20	35	↓ feed intake and BWG (21 d)	(Yunus et al., 2012a)

Table 7. Effects of deoxynivalenol (DON) on productive parameters of chickens.

DON[1] (mg/kg) diet	Exposure duration (d)	Reported Effects	Reference
Up to 14	35	↓ feed intake and BWG and ↑ in feed to gain ratio	(Dänicke et al., 2003)
5 or 15	42	↓ BWG and ↑ in FCR at 15 mg/kg	(Riahi et al., 2020)
15	21	↓ BWG	(Kubena et al., 1997)
16	21	↓BWG, ↑FCR	(Huff et al., 1986)
16	21	↓BWG, ↑FCR	(Kubena et al., 1988)
16	21	↓BWG, ↑FCR (d 8 to 14)	(Kubena et al., 1989)
16	21	none	(Harvey et al., 1997)
9 or 18	35	↑ BW (at 18 mg/kg d 2↑ BW (at 9 mg/kg d 35)	(Kubena et al., 1985)
Up to 18	21	↓ feed intake and BWG	(Xu et al., 2011)
18	84	↑ BW at 28 and 56 d	(Kubena & Harvey, 1988)
Up to 216	From 6 to 11	none	(Moran et al., 1982)

[1]DON: deoxynivalenol; [2]BWG: Body weight gain; [3]FCR: feed conversion ratio; [4]BW: Body weight; ↑, increase; ↓, decrease

3.2. Relative weight of organs

The effect of DON on organ weights of chickens is summarized in Table 8. Regarding DON toxicity, this biomarker is highly variable and results are contradictory. The weight of organs expressed as a percentage of the body weight of the bird are named relative weight (RW). Some organs seemed to be the most affected in different studies as, for example, the organs with high turnover of protein such as liver, immune organs and small intestine (Feinberg & McLaughlin, 1989). The RW of liver decreased in birds fed 18 mg DON/kg feed during 5 weeks (Kubena et al., 1985), but this parameter remained unaltered using the same dose of toxin during 12 weeks, and this may be explained by the difference on the duration of exposure (Kubena & Harvey, 1988). Indeed, the time to toxin exposure may be a significant factor as the organ initially swells with toxin exposure followed by shrinkage (Swamy et al., 2004a). Similarly, in broiler chickens exposed to DON at different levels (up to 10 mg/kg), a significant decrease of liver RW is observed at week 3 of the trial (Lucke et al., 2017). However, in another trial, this parameter increased

temporally in the second week of exposure for broilers fed a low DON diet (1.68 mg DON/kg) (Yunus et al., 2012a). The changes observed on the RW of the liver might be associated to lipid metabolism alterations (Kubena et al., 1997).

It has been previously observed that 10 mg of DON/kg diet for 35 d decreased the RW of kidneys of broiler chickens and it has been suggested that DON alter the indicated organ cells (Awad et al., 2014). In contrast, other reports indicated that the RW of kidneys remained unaltered (Dänicke et al., 2003; Kubena et al., 1985,1988; Kubena & Harvey, 1988).

The RW of gizzard increased in several studies after DON exposure of broiler chickens (Awad et al., 2014; Huff et al., 1986; Kubena et al., 1985, 1988; Kubena & Harvey, 1988; Riahi et al., 2020). The increase of RW of gizzard might be directly related to a difference in the density of the diets, or might be a result of the irritation of the upper GIT (Kubena et al., 1985).

The RW of small intestine of broilers fed 5 mg/kg DON for 21 d decreased (Awad et al., 2006b). In the same manner, Yunus et al. (2012b) reported a reduction in the RW of duodenum and jejunum, and they suggested that this decrease could be due to the reduction of villus height which then resulted in decreased digestion of nutrients and energy.

Regarding the immune organs, different results have been reported using the same DON concentration during the same exposure time; the RW of bursa of Fabricius of birds did not change after feeding 16 mg DON/kg of feed for 21 d (Kubena et al., 1988). Using the same trial conditions, an increase of the RW of bursa of Fabricius was found (Kubena et al., 1989). Results of these studies are highly variable, suggesting that organ weights might not be a good indicator of DON toxicity (Awad et al., 2008). An increase of the RW of spleen for broilers fed a low level of DON (1.68 mg/kg) at wk 4 was reported (Yunus et al., 2012b). Similarly, the RW of spleen was higher in the chickens fed 5 mg/kg of DON contaminated diet and this increase was interpreted as a consequence of the irritation of chickens immune system by the toxin, the swelling of this organ and some alterations on a cellular level (Chen et al., 2017). Nevertheless, the indicated immune organ was reduced in Lohman chickens fed up 14 mg DON /kg for 5 weeks (Dänicke et al., 2003). In other studies, the RW of spleen was not affected by DON chicken's diets (Table 8). In our recent study, a significant increase of thymus of birds exposed to DON at 5 or 15 mg/kg for 42 d was reported (Riahi et al., 2020).

Due to the contradiction between results, it can be considered that RW of organs might not be a very precise biomarker of DON toxicity.

Table 8. Effects of deoxynivalenol (DON) on relative organ of weights of chickens.

DON[1](mg/kg) diet	Exposure duration (d)	Reported Effects	Reference
up to 1.87	28	No effect on crop, proventriculus, gizzard, intestines, heart, liver, pancreas, kidneys, testes, adrenals, and thyroids	(Hulan & Proudfoot, 1982)
1 or 5	35	No effect on [2]RW of heart, proventriculus, gizzard, pancreas, liver, small intestine, cecum, colon, thymus ,spleen and bursa of Fabricius	(Awad et al., 2011)
5	21	↓ RW of small intestine, = RW of heart, gizzard, pancreas, caecum, colon and spleen	(Awad et al., 2006b)
5.9 or 9.5	56	No effect on RW of liver, kidney, spleen and bursa of Fabricius	(Swamy et al., 2004a)
2,5 or 10	112	↑ RW of spleen (at 5 mg/kg)	(Chen et al., 2017)
2.5, 5 or 10	35	↓ RW of liver	(Lucke et al., 2017)
10	35	No effect on RW of bursa of Fabricius, spleen and thymus	(Ghareeb et al., 2012)
10	35	↑ RW of gizzard, ↓ RW of kidneys, = RW of brain, heart, pancreas, liver, lung, thymus, spleen and bursa of Fabricius	(Awad et al., 2014)
1.68 or 12.20	35	↑ RW of liver and spleen, = RW of heart and thymus	(Yunus, et al., 2012a)
1.68 or 12.20	35	↓ RW of duodenum and jejunum, = RW of proventriculus and gizzard	(Yunus et al., 2012b)
Up to 14	35	↑ RW of heart, ↓ RW of spleen, = RW of proventriculus, gizzard, liver kidneys , small intestine, and bursa of Fabricius	(Dänicke et al., 2003)
5 or 15	42	↑ RW of gizzard and thymus, ↓ RW of colon and small intestine, = RW of heart, proventriculus, pancreas, liver, kidneys, cecum, spleen and bursa of Fabricius	(Riahi et al., 2020)
15	21	↑ RW of gizzard heart and bursa of Fabricius, = RW of proventriculus, liver and kidney	(Kubena et al., 1997)
16	21	↑ RW of gizzard, = RW of proventriculus, spleen, liver, and kidney	(Huff et al.,1986)

Table 8. Effects of deoxynivalenol (DON) on relative organ of weights of chickens.

DON[1](mg/kg) diet	Exposure duration (d)	Reported Effects	Reference
16	21	↑ RW of gizzard, = RW of proventriculus, liver, kidneys, spleen and bursa of Fabricius	(Kubena et al., 1988)
16	21	↑ RW of gizzard and bursa of Fabricius , = RW of proventriculus, pancreas, liver , kidneys and spleen	(Kubena et al., 1989)
16	21	no effect on RW of liver, left kidney, heart, spleen, pancreas, proventriculus, gizzard, and bursa of Fabricius	(Harvey et al., 1997)
9 or 18	35	↓ RW of liver, = RW kidney, heart, proventriculus, testes, spleen, or bursa. , and ↑ RW of gizzard	(Kubena et al., 1985)
18	84	↑ RW of gizzard, = RW liver, kidney, heart, proventriculus, spleen, bursa of Fabricius, and testes	(Kubena & Harvey, 1988)

[1]DON: deoxynivalenol; [2]RW: Relative weight; ↑: increase; ↓: decrease; =: unaffected

3.3. Intestinal morphology

As all trichothecenes, DON is a small molecule that have the capacity to passively cross through cell membranes and to be easily absorbed by the epithelium cells of the intestine. These epithelium cells can be exposed to high concentrations of DON following ingestion of contaminated diets (Pinton & Oswald, 2014). On the other hand, the intestinal tract is a very important barrier to ingested feed contaminants and is also the first line of defence against various gut antigens (Ghareeb et al., 2015). Moreover, it has been reported that DON alter intestinal morphology (Awad et al., 2011). The studies reporting the impairment of intestinal morphology parameters in chickens are listed in Table 9.

The density of the intestine was identified as the ratio between the absolute weight of small intestine and its length. Birds fed DON-contaminated diets from 1 to 21 d had lower jejunum density (Wang & Hogan, 2019). In broilers exposed to 1.68 or 12.20 mg/kg for 35 d, DON exposure has shown to produce a reduction of the weights of duodenum and jejunum and an increase of length of both segments and, consequently, DON decreased the small intestine density, in a dose- and time-dependent manner (Yunus, et al., 2012b). Also, the length was lower and the density of the small intestine was higher in broilers receiving 5 and 15 mg DON/kg compared with controls (Riahi et al., 2020). Contaminated DON feed at concentration (3 to 4 mg/kg) which is below the European maximum guidance level resulted in a decreased duodenal villus height (Antonissen et

al., 2014). Jejunal and ileum histological analysis revealed that broiler chickens exposed to the DON diets for 34 d had shorter villi and reduced crypt depth than control birds (Wang & Hogan, 2019). Additionally, it has been shown that chickens consuming DON (5 or 10 mg/kg feed) had shorter and thinner villi compared to chickens ingesting control diets (Awad et al., 2006a,b ; Wu et al., 2018; Yang et al., 2017).

It has also been reported a significant reduction of villus height, villus surface area and muscularis thickness in jejunum of broiler chickens upon feeding naturally contaminated diets with DON (1 or 5 mg/kg feed) (Awad et al., 2011). Data reported by Yunus et al. (2012b) indicated that feeding 12.20 mg/kg of DON contaminated diet to broiler chickens resulted in a reduced villus height in the duodenum and jejunum. Similarly, feeding DON contaminated feed (7.54 mg/kg) for 21 d decreased the villus height and crypt depth both in duodenum and jejunum of broiler chickens (Osselaere et al., 2013a). In the same manner, villus height and villus height to crypt depth ratio in the duodenum were decreased in chickens receiving the guidance value for 4 weeks (Yu et al., 2018). Only one study shows opposite results regarding intestine morphology, as they observed that feeding diets at a concentration up to 18 mg of DON/kg of feed linearly increased broiler villi heights (Xu et al., 2011). In recent reports villus height was unaffected by the dietary inclusion of DON (Chen et al., 2017; Riahi et al., 2020).

The decrease of the villus height may be due to the villus contraction and leads to the impairment of nutrient transport and utilization, resulting in the alteration of gain weight of animals fed DON (Alizadeh et al., 2015). Furthermore, Robert et al. (2017) indicated that the shortening of villus height produced by DON may be explained by the implication of this mycotoxin in the balance between epithelial cell proliferation and apoptosis. As such, the adverse effect of DON on the villus height can be linked with an impaired nutrient digestion due to a reduced number of differentiated epithelial cells (Grenier et al., 2013). After a mechanical or toxic damage, more enterocytes need to be generated to migrate progressively along the villus toward the tip, which induced the change in crypt depth (Haschek et al., 2010). In addition, change in crypt depth in birds fed DON could be attributed to the capacity of DON to reduce cell proliferation (Osselaere et al., 2013a). The crypt-depth to villus-height ratio was calculated to assess the intestinal architectural changes after the treatment (Haschek et al., 2010). To conclude, changes observed in morphology and histology of small intestine could be due to the irritant effects of DON on the upper GIT and the inhibition of protein biosynthesis, leading to the alteration of

the absorption and digestion mechanisms which negatively affect bird growth (Ghareeb et al., 2015; Osselaere et al., 2013a).

Table 9. Effects of deoxynivalenol (DON) on morphological parameters of small intestine of chickens.

DON[1](mg/kg) diet	Exposure duration (d)	Reported Effects	Reference
1 or 5	35	↓ villus height of jejunum,↓ villus surface area	(Awad et al., 2011)
2.88 to 4.38	23	↓ villus height of duodenum	(Antonissen et al., 2014)
5	21	↓ height and width of villi in duodenum	(Awad et al.,2006b)
5	28	↓ villus height and villus height to crypt depth ratio in the duodenum	(Yu et al., 2018)
7.54	21	↓ villus height and crypt depth	(Osselaere et al., 2013a)
6.62 to 7.90	21	↓ density of small intestine, ↓ villus height in the jejunum, ↓ villus height and crypt depth in the ileum	(Wang & Hogan, 2019)
2, 5, or 10	112	= villus height of jejunum and ileum	(Chen et al., 2017)
10	21	↓ villus height, ↑ crypt depth, ↓ villus height to crypt depth ratio in the duodenum	(Wu et al., 2018)
10	35	= length and density of different segments of GIT	(Ghareeb et al., 2016)
10	42	↓ villus height of jejunum, , = crypt depth and villus height and crypt depth	(Yang et al., 2017)
10	42	↓ villus height in the duodenum and jejunum	(Awad et al., 2006a)
1.68 or 12.20	35	↓ villus height, ↑ length of duodenum and jejunum	(Yunuset al., 2012b)
5 or 15	42	↑ length and ↓ density of small intestine, = villus height and crypt depth	(Riahi et al., 2020)
Up to 18	21	↑ villus height in the mid-ileum No effects on crypt depth and goblet cells per villi counts	(Xu et al., 2011)

[1]DON: deoxynivalenol; ↑: increase; ↓: decrease; =: unaffected

3.4. Biochemical and hematological parameters

Biochemical and hematological parameters are used to infer the health status of animals. The most significant results of the effect of DON on biochemical blood parameters are indicated in Table 10. Dietary DON may affect the enzymes reflecting

liver and kidney activities such as lactate dehydrogenase (LDH), alanine aminotransferase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), creatine kinase (CK) and creatinine. Broilers exposed to 15 mg DON/kg feed during 21 d had higher AST activity in blood (Kubena et al., 1997). However, the same dietary level in chickens feeding during 42 d did not affect this enzymatic activity (Riahi et al., 2020). ALP and AST activities increased in broilers exposed to 2.95 mg DON/kg feed for the 2 last week of the trial (Klapácová et al., 2011). Faixová et al. (2007, 2006) showed an elevation of serum ALT activity in broilers exposed to 3 mg DON/kg feed for 6 weeks. However, broilers fed DON (10 mg/kg) for 35 d had lower ALT (Ghareeb et al., 2016). Swamy et al. (2002) reported that DON at 4.6 mg/kg increased the levels of GGT activity. These results were in accordance with a previous report of chickens fed 15 mg DON/kg (Kubena et al., 1997). Contaminated diet with DON at 5 mg/kg decreased the serum CK level in broiler chickens aged 42 d (Riahi et al., 2020), reflecting the reduction of this enzyme from the circulation (Husic & Suelter, 1983). Generally, the changes obtained in enzymatic activities might be due to hepatic disorders, chronic liver damage, leakage of the enzymes into the blood, biliary obstruction, or kidney affliction (Ghareeb et al., 2016; Klapácová et al., 2011; Swamy et al., 2002).

For biochemical parameters reflecting lipid metabolism, higher cholesterol level and triglycerides were observed in broilers fed DON at 10 mg/kg for 35 d (Ghareeb et al., 2016). The increased cholesterol level may suggest liver or kidney damage function and a high stress status of the bird (Ghareeb et al., 2016). The increased level of triglycerides might be related to lipid metabolism alteration and or to biliary obstruction (Ghareeb et al., 2016). These results are in contrast with the previous findings which observed decreased cholesterol and triglycerides levels in broilers (Faixová et al., 2006,2007; Ghareeb et al., 2012; Huff et al., 1986; Kubena et al., 1985; Riahi et al., 2020). This is in agreement with the meta-analysis report of Andretta et al.(2012) indicating that broilers fed mycotoxins (T2, FBs, DON, OTA, and ZEN) had lower total cholesterol (-14 %) and lower triglycerides (-39 %) compared to negative control birds. It was suggested that these changes in indicated biochemical parameters could be explained by involvement of liver and a shift of concentrations from the blood to the liver (Kubena et al., 1987).

Regarding biochemical parameters reflecting protein metabolism, it has been described that the total plasma protein and albumin levels of chickens fed a contaminated diet with 2.95 mg DON/kg feed during 2 weeks of exposure were decreased compared to

control (Klapácová et al., 2011). The reduction of total protein level after feeding DON to broilers was observed also in previous reports (Faixová et al., 2007,2006; Ghareeb et al., 2012). This decrease could be the result of protein and DNA synthesis inhibition induced by DON (Feinberg & McLaughlin, 1989). In addition, the decreased plasma protein level may be related to the decrease of feed intake. DON toxicity is also expressed through a change in the profile of uric acid (UA) (Ghareeb et al., 2012a; Swamy et al., 2002). The decreased level of UA may be attributed to the efficiency of amino acid utilization, changes in enzyme systems, altered renal filtration, and reabsorption rates (Kubena et al., 1987).

Blood hematological parameters serve as indicators of the physiological state of birds (Chowdhury et al., 2005). The absence of significant effect of DON exposure on hematology of broiler chickens exposed to different DON concentrations (up to 16 mg/kg feed) at 21 d or 35 d has been shown (Dänicke et al., 2003; Harvey et al., 1997). Even 50 mg/kg of DON did not change these indicators in broiler chickens aged 21 d (Harvey et al., 1991). In other studies, a significant decrease on hemoglobin (HGB) concentration was observed accompanied or not with a decrease on hematocrit (HCT) and a decrease on red blood cells (RBC) count of birds exposed to DON mycotoxin (Harvey et al., 1991; Huff et al., 1986; Kubena et al., 1988; Kubena & Harvey, 1988; Swamy et al., 2002). In their meta-analysis, Andretta et al. (2012) reported that the presence of mycotoxins in broiler diets decreased the HCT and the HGB concentration by 5 % and 15 % respectively. The decreasing of HCT hypothesized that hematopoietic tissue may be affected by DON toxin (Chattopadhyay et al., 2013). However, no hemorrhage or hemolysis was observed in broilers. The decrease of HGB and RBC does not necessarily mean that DON induces anemia in poultry, because values are within the range of reference (Chowdhury et al., 2005). Moreover, the decrease of HGB and RBC is a marker of bone marrow malfunction, and the immune system alteration.

Blood biochemistry and hematology could be a useful indicator to evaluate the DON effect on the physiological and hemostatic state of chickens. For this matter, further studies are needed to clarify this impact in poultry.

Table 10. Effects of deoxynivalenol (DON) on blood biochemistry and hematology of chickens.

DON[1] (mg/kg) diet	Exposure duration (d)	Reported Effects[2]	Reference
2.95	28	↓levels of Tot Prot, Alb ↑ALT, AST and ALP.	(Klapácová et al., 2011)
3	42	↓ levels of Tot Prot, mg, Trig and free glycerol, ↑ALT activity	(Faixová et al., 2006)
3	42	↓ Tot Prot, Trig and free glycerol, ↑ALT activity	(Faixová et al., 2007)
4.7or 8.2	56	Quadratic responses in serum concentrations of Alb and GGT, ↓ lipase activity, ↑UA, hemoglobinemia and erythrocytosis	(Swamy et al., 2002)
10	35	↓of plasma Tot Prot and UA, ↓plasma Trig level (tendency),	(Ghareeb et al., 2012)
10	35	↓ level of ALT,↑ serum Chol and Trig	(Ghareeb et al., 2016)
Up to 14	35	= Glu, Tot Prot, , HCT and HGB	(Dänicke et al., 2003)
5 or 15	42	↓ CK (at 5 mg/kg) and ↓ level of Chol (at 15 mg/kg)	(Riahi et al., 2020)
15	21	↑ activities of AST, LDH and GGT.	(Kubena et al., 1997)
16	21	↓Trig, Alterations in RBC count, HGB and HCT	(Huff et al., 1986)
16	21	↓ Glu level	(Kubena et al., 1988)
16	21	No effect on Glu, ALT,AST, Creat and Hgb	(Harvey et al., 1997)
9 or 18	35	↓ Glu, Chol, Trig and LDH, ↑ Creat, ↓ in HGB and HCT	(Kubena et al., 1985)
18	63	↓ HGB concentration, RBC count and HCT	(Harvey et al., 1991)
18	84	↓ in HGB concentration at 28 d	(Kubena & Harvey, 1988)
50	21	No effect on hematological parameters	(Harvey et al., 1991)

[1]DON: deoxynivalenol; [2]Tot Prot: total protein; Alb: albumin; UA: uric acid; Chol: cholesterol; Trig: triglycerides; ALT: alanine transferase; AST: aspartate transaminase; ALP: alkaline phosphatase; GGT: gamma-glutamyl-transferase; LDH: lactate deshydrogenase; Glu: glucose; Creat: creatinine; CK: creatine kinase; HGB: hemoglobin; ↑: increase; ↓: decrease; =: unaffected.

3.5. Biomarkers related to immune system

Few studies have been conducted regarding DON effects on poultry immune system, despite that the first outcome of DON toxicity is immune system functions and responses.

As the immune system has rapidly proliferating cells and tissues with high rates of protein turnover, it is consequently considered the most susceptible system to DON and trichothecenes mycotoxicosis (Feinberg & McLaughlin, 1989). Responses to common vaccines as Newcastle disease virus (NDV) and infectious bronchitis virus (IBV) are considered as humoral immune response parameters (Dänicke et al., 2003; Ghareeb et al., 2012). Dänicke et al. (2003) suggested that the antibody titers against NDV could be a relevant biomarker for DON effects on protein synthesis inhibition due to the reduction of antibody titers to NDV when broiler chickens fed up 14 mg of DON/kg feed for 5 weeks. In the same manner, broilers exposed to a mixture of *Fusarium* mycotoxins, included DON, presented a decreased NDV titers at 28 and 42 d of age (Li et al., 2012). Also, NDV antibody titers decreased in White Leghorn chicks fed 18 mg of DON/kg of feed at 18 wk (Harvey et al., 1991). However, the exposure to 12.2 mg of DON/kg of feed of broilers for 5 weeks increased titers against NDV at week 2 and week 4, but decreased numerically at week 5 (Yunus et al., 2012a). As trichothecenes, DON is both immunostimulatory and immunosuppressive, depending on the level and the length of exposure (Pestka et al., 2004). The enhancement or the depression of the response to NDV observed may be due to the ability of DON to modulate the immune response during chronic exposure. Concerning the antibody titers against IBV, it was concluded that is also a good indicator of depression of protein synthesis induced by the exposure of DON (Ghareeb et al., 2012). Antibody titers to IBV decreased in broiler chickens receiving DON at 10, 12.2 or 12.6 mg/kg for 5 weeks (Ghareeb et al., 2012; Yunus et al., 2012a). Meanwhile, no effect on the IBV titers was found following exposure of broiler chickens to different concentrations of DON (4.7 or 8.2 mg/kg of feed) for 21 d and 42 d (Swamy et al., 2002). Furthermore, no effects were found of a concentration of 12.2 mg DON/kg on antibody against IBV titers in broiler chickens after 14 and 28 d Yunus et al., 2012a).

The discrepancies in results of these biomarkers of effect might indicate that the humoral immune response of broilers fed DON is variable, and further studies should be undertaken (Yunus et al., 2012a).

Leukocytes apoptosis and lymphocytes proliferation are considered as cellular immune responses to DON detrimental effects (Ghareeb et al., 2012; Pestka et al., 2004). DON has the ability to promote *in vitro* apoptosis in chicken spleen lymphocytes and tissue (Chen et al., 2017; Ren et al., 2015). Ingestion of DON from 5.9 to 9.5 mg/kg feed reduced the B cells and T cells counts in broiler chickens (Swamy et al., 2004a). In the *in*

vivo study conducted by Ghareeb et al. (2012) blood lymphocytes count was decreased in broilers receiving DON (10 mg/kg feed) for 35 d.

Furthermore, the production of proinflammatory cytokines could be used as an indicator to evaluate the effects of DON contaminated diets exposure on immune system competence or modulation (Lucke et al., 2018). After MAPKs activation, p38 and ERK induced gene transcription and enhanced mRNA stability (Pestka, 2010). The induction of mRNA expression genes included also the induction of transcription and post transcriptional factors, such NF-kβ, and leads to promote proinflammatory genes (Pestka, 2010; Zhou et al., 2014). Proinflammatory cytokines such as interleukin 1β (IL-1β), IL-6, IL-8, TNF-α, and interferon gamma (IFN-γ) are released predominantly by cells of the innate immune system or macrophages activated by molecular components of the pathogens. These cytokines are involved in the coordination of a local and systemic inflammation and the regulation of immune responses leading to deep physiological and behavioural changes (Bruno et al., 2015). The gene expression of IFN-γ in the cecal tonsils of chickens fed *Fusarium* mycotoxins challenged with coccidian was upregulated (Girgis et al., 2008). Broiler chickens exposed to a mixture of mycotoxins, in which DON concentration was approximately 2 mg/kg, for 42 d had higher expression of (IL1-β) and IL-6 and lower expression of IFN-γ in spleen tissues (Li et al., 2012). In a recent study conducted by Lucke et al. (2018) , the mRNA gene expression of IL-6 increased in broilers receiving up 5 mg/kg for 35 d.

The upregulation of cytokine gene expression by DON was explained by the ability of this mycotoxin to inhibit synthesis of labile protein repressors of mRNA expression (Girgis et al., 2008; Yan et al., 1997). Zhou et al. (1997) confirmed that the induction of mRNA gene expression of cytokines is a result of DON-induced transient expression of specific mRNAs responsible for impairment of synthesis of high turnover proteins. It has also been suggested that this induction of immune related genes by DON mycotoxin is associated to a higher transcription factor's binding activity in leukocytes at the transcription level and to a higher mRNA stability at the post-transcription level (Girgis et al., 2008; Pestka et al., 2004). In addition to that, the upregulation of cytokines production is linked to the transcription factor NF-κB activation (Zhou et al., 2014).

However, in response to DON (10 mg/kg feed), broiler chickens had lower TNF-α in plasma and lower IL-1β, IFN-γ and transforming growth factor beta receptor I (TGFBR1) in jejunum, and no effect was observed on plasma IL-8 and TNF-α nor on IL-8 and NF-

kB1 in the jejunum (Ghareeb et al., 2013). Grenier et al. (2016) reported a down-regulation of IL-6 in cecal tonsils in broiler fed 1.6 mg DON/kg feed. No effect on TNF-α and IL-1β in spleen and bursa of Fabricius tissues was observed in broilers exposed to 5 mg/kg DON for 28 d (Yu et al., 2018).The differences obtained among studies regarding the DON effect on cytokines production depends on the biological matrix. For example, the determination of cytokines in serum could be a non-suitable indicator to evaluate the cytokine production due to their short-half-life or due to the low sensitive method used (Zhou et al., 1997). Another explanation could be that the modulating effect of DON on the innate immune response (Ghareeb et al., 2013). The ability of DON to upregulate cytokines release is associated to the effect of DON on the innate immune response, leading to the impairment of chicken‘s resistance to infectious disease (Ghareeb et al., 2013). Furthermore, DON-induced proinflammatory cytokines can be useful in the underlying cause of DON-induced feed refusal. Infection induced anorexia could be explained by the upregulation of relative gene expression of IL-1β, IL-6, TNF-α and IL-8 (Tachibana et al., 2017).

The effect of DON on immune system functions and responses through the evaluation of those indicators mentioned above are reviewed in Table 11.

Table 11. Effects of deoxynivalenol (DON) on immune-biomarkers of chickens.

DON[1] (mg/kg) diet	Exposure duration (d)	Reported Effects[2]	Reference
1.6	34	↓ IL-6, = IFN-γ, IL-1β, IL-17, and IL-10 in cecal tonsils	(Grenier et al., 2016)
2	42	↓ NDV titers at 28 d and 42 d,↑mRNA expression of IL-lß and IL-6,↓ mRNA expression of IFN-γ in spleen	(Li et al., 2012)
Up to 3.8	70	↑ IFN- γ gene expression in cecal tonsils	(Girgis et al., 2008)
Up to 5	35	↑mRNA expression of IL-6 in the duodenum, ↓ mRNA expression of IL-8 and IL-10 in the jejunum (quadratic trend)	(Lucke et al., 2018)
5	28	No effect on TNF-α and IL-1β in spleen and bursa of Fabricius tissues	(Yu et al., 2018)
4.7or 8.2	56	= IBV	(Swamy et al., 2002)
5.9 or 9.5	56	↓ B cells and T cells	(Swamy et al., 2004a)
2, 5 or 10	112	Apoptosis in chicken spleen lymphocytes	(Chen et al., 2017)

Table 11. Effects of deoxynivalenol (DON) on immune-biomarkers of chickens.

DON[1] (mg/kg) diet	Exposure duration (d)	Reported Effects[2]	Reference
10	35	↓ lymphocytes and ↓ IBV	(Ghareeb et al., 2012)
10	35	In plasma: ↓TNF-α, = IL-8, in jejunum: ↓ IL-1β, IFN-γ, TGFBR1, = TNF-α. IL-8 and NF-kB1	Ghareeb et al., 2013)
10	35	↓ IBV titers	(Ghareeb et al., 2016)
12.2	35	↑ NDV(14 d / 28d), ↓ IBV(35d)	(Yunus et al., 2012a)
3.5 to 14	35	↓ NDV titers	(Dänicke et al., 2003)
18	63	No effect on NDV	(Harvey et al., 1991)
18	126	↓ NDV	(Harvey et al., 1991)

NDV: Newcastle disease virus; IBV: infectious bronchitis virus; IFN-γ: interferon gamma; IL-1β: interleukin 1β; IL-6: interleukin 6; TNF-α: tumor necrosis alpha; TGFBR1: transforming growth factor beta receptor I; NF-kβ: nuclear factor kappa β; CCK-8: cell counting kit-8; IL-10: interleukin 10: IL-17: interleukin 17; ↑: increase; ↓: decrease; =: unaffected.

3.6. Biomarkers related to welfare parameters

To evaluate welfare related-parameters induced by DON toxicity in chickens, the indicators linked with physiological, hormonal, and behavioural welfare are highlighted in Table 12.

Only a few studies have been carried out on the impact of DON contamination of poultry feed on biomarkers related to physiological, hormonal, and behavioural welfare. Onbaşilar and Aksoy (2005) reported that the impairment of the number of circulating leukocytes, such as heterophils and lymphocytes, is part of the physiological stress. Therefore, the heterophil to lymphocyte ratio (H/L) was used as a good indicator to evaluate stress in poultry (Gross & Siegel, 1983).

The stress index was elevated in broilers fed DON (10 or 18 mg/kg) for 21 or 35 d (Ghareeb et al., 2012, 2014; Xu et al., 2011). However, Dänicke et al. (2003) did not report significant differences on H/L ratio in broilers fed a contaminated diet with a concentration of 14 mg/kg of DON per kg. The elevation of circulating levels of corticosterone is included also within the physiological stress biomarkers (Onbaşilar & Aksoy, 2005). Antonissen et al. (2017) showed that broilers exposed to a DON (4.6 mg/kg) contaminated diet for 15 days had higher mean plasma corticosterone levels than

chickens fed control diet. Similarly, Ghareeb et al. (2014) observed that feeding broilers with 10 mg/kg of DON for 35 d, increased the plasma corticosterone level. The elevation of corticosterone was related with the upregulation of gene expression of IL-1β , IL-6 and TNF-α proinflammatory cytokines (Islam & Pestka, 2003). The increase in corticosterone might be associated with the mycotoxin induced increase in susceptibility to infectious diseases (Antonissen et al., 2014).

The fear response is a welfare-related behaviour evaluated by tonic immobility reaction, and can provide more information on chicken's stress status (Campo et al., 2005; Ghareeb et al., 2014). The duration of tonic immobility was longer in broilers exposed to 10 or 15 mg DON/kg (Ghareeb et al., 2014). This effect could be related to the adverse effect of DON on brain regional neurochemistry. Indeed, the inclusion of contaminated grains with *Fusarium* mycotoxin in the diet of broiler chickens for long-term (1 to 56 d) increased the concentration of 5-hydroxytryptamine (5 HT) in the pons and in the cortex, which is responsible in the regulation of fear (Swamy et al., 2004b). Stress-induced by DON altered brain neurochemistry of broilers by increasing the levels of serotonin (strong satiety neurochemical) which suggests that DON resulted in the partial feed refusal and growth suppression (Swamy et al., 2004b). Consequently, these adverse effects on bird's welfare might be associated with the mycotoxin-induced increase in sensitivity to infectious diseases.

Table 12. Effects of deoxynivalenol (DON) on welfare related parameters in chickens.

DON (mg/kg) diet	Exposure duration (d)	Reported Effects	Reference
4.6	15	↑ Plasma corticosterone	(Antonissen et al., 2017)
10	35	↑ H/L ratio	(Ghareeb et al., 2012)
10	35	↑ Plasma corticosterone, H/L ratio, and duration of tonic immobility reaction.	(Ghareeb et al., 2014)
Up to 14	35	No significant effect on H/L ratio	(Dänicke et al., 2003)
Up to 18	21	↑ H/L ratio	(Xu et al., 2011)

[1] DON: deoxynivalenol; H/L: heterophil to lymphocyte ratio; ↑: increase; ↓: decrease; =: unaffected.

4. Detoxification of DON contaminated feed

4.1. Detoxification strategies

The presence of mycotoxins in food and feed is practically unavoidable due to their ubiquitous presence and stability. Therefore, in order to minimize the risks posed by mycotoxins, pre-and post-harvest control management methods of mycotoxin prevention have been developed (Awad et al., 2010). The most important pre-harvest strategy is the application of Good Agriculture Practices (GAP). These common practices include crop rotation, tillage, use of soil fertilizers, adequate irrigation, selection of high quality seeds, avoiding high plant densities, insect control as well as the use of resistant varieties (Awad et al., 2010; Jouany, 2007). To assess the mycotoxin risk at the field level, prediction models integrating some of these field parameters and weather input have been developed (Jouany, 2007). Furthermore, to reduce fungal infestation, timing of harvest should be selected carefully, and the humidity levels of grains before and during the storage should be carefully controlled (less than 15%), and the temperature during storage should also be controlled via combined cooling and drying operations associated with ventilation systems (Kabak et al., 2006). Even if the best management of these strategies was accomplished, mycotoxin contamination cannot be totally eradicated (Jouany, 2007).

Post-harvest strategies to prevent mycotoxin contamination can include the use of methods of physical treatment of grains, such as sorting, washing, dehulling, thermal treatment, grain milling and irradiation (Jouany, 2007). Regarding chemical treatment of grains, ammoniation process, nixtamalization, combination of heat treatment with $NaHCO_3$ and H_2O_2 alone or with $Ca(OH)_2$, sodium bisulfite solutions and ozone treatments were reported as detoxification process of mycotoxins (Jouany, 2007). Regarding DON, 85% of this toxin was transformed into a non-toxic metabolite in pigs, the DON-sulfonate conjugate, when contaminated maize (4.4 mg DON/kg) was treated with sodium bisulfite solutions at 80 °C for 18 h (Young et al., 1987). However, other chemical treatments with hydrochloric acid, hydrogen peroxide, sodium hypochlorite, ascorbic acid and ammonium carbonate, were not efficient to decontaminate DON (Jouany, 2007). It should be noted that the use of chemical products is not exempt from health risks, since it can produce the formation of derivatives of unknown toxicity. For this reason, the chemical methods of detoxification of mycotoxins are specifically forbidden in the EU (European Commission, 2006b). Thus, taking into account the high thermostability of mycotoxins and the prohibition of using chemical detoxification

methods, few strategies are available to counteract the toxic effect of mycotoxins in feed. One of them is the use of feed additives, known as mycotoxins detoxifiers, the most commonly used strategy for feeds today (Kolosova & Stroka, 2011).

Mycotoxin detoxifiers, also named mycotoxin-detoxifying agents, are defined as compounds added to feed to reduce the effect produced by mycotoxin or to counteract the toxic impact on the animal (Jard et al., 2011). Mycotoxin detoxifiers were established as a new group belonging to the category of technological feed additives and are defined by Regulation 2009/386/EC as: 'substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action' (European Commission, 2009). It should be pointed out that the use of such products does not mean that the animal feed exceeding the established maximum limits may be used. Mycotoxin detoxifiers are divided into two groups: mycotoxin binders and mycotoxin modifiers. Investigations are required to demonstrate the efficacy and safety of these feed additives. The efficacy of these products has been based on nonspecific parameters in several literature reports, such as productive parameters, organ weights, and immunological parameters (Ghareeb et al., 2012). However, EFSA stated that only the specific biomarkers of exposure must be taken into account to prove the efficacy of these feed additives (EFSA, 2017). Such biomarkers, specific to mycotoxin toxicity, are the presence of the parent mycotoxins and their derived metabolites in different biological matrices, determined by instrumental techniques as LC-MS/MS and LC-HRMS. Regarding DON, EFSA proposed DON and its metabolites in serum as the specific biomarkers of DON exposure (Rychen et al., 2018).

4.1.1. Binders

4.1.1.1. Definition and classification

Mycotoxin binders, also named adsorbing agents, binding agents, adsorbents or sequestering agents, are large molecular weight compounds able to bind the mycotoxins, even under existing conditions in the GIT of animals. The toxin-adsorbing agent complex passes through the animal digestive system and is eliminated via the faeces. The binder limits the mycotoxin bioavailability after ingestion and prevents or minimizes their exposure to animals (Devreese et al., 2013b; Vila-Donat et al., 2018). The classes and subclasses of these agents are detailed in Figure 4. The adsorption process is attributed via different types of interactions between mycotoxins (the solute molecule or adsorbate)

and the adsorbing agent (non-dissolved solid or adsorbent). These interactions are hydrophobic bindings (Van der Waals forces), hydrogen bonds, electrostatic attractions or repulsions and coordinated bonds (Di Gregorio et al., 2014). The complex mycotoxin-adsorbing agent must not dissociate and be stable in the GIT. Its stability mainly depends on pH and is related with the physical properties of the adsorbing agent. The physical properties implicated in the process of adsorption are the size and distribution of the pores, the total charge and charge distribution, and the accessible surface area (Di Gregorio et al., 2014). On the other hand, different properties of the adsorbed mycotoxins also play a significant role such as polarity, shape, size, low surface area and solubility, as well as uncoupling and charge distribution (in the case of ionized compounds) (Huwig et al., 2001).

Mycotoxin binders are divided into two groups: inorganic and organic binders. The inorganic binders are mainly aluminosilicates, which are the largest class of mycotoxin adsorbing agents and the most used binders employed to counteract mycotoxicosis. Aluminosilicates are subdivided in two classes: the phyllosilicates, group composed of bentonites, montmorillonites, hydrated sodium calcium aluminosilicates (HSCAS), smectites, kaolinites and illites. The other subclass is the tectosilicate, mainly composed by zeolites. The ability of aluminosilicates to bind mycotoxins has commonly been shown in the case of AFs *in vitro* as well as *in vivo* (Ramos & Hernández, 1996; Vila-donat et al., 2019, 2020). However, it must be highlighted the risk of the use of those adsorbing agents in adsorbing other essential nutrients such as minerals and vitamins, or the risk of contamination of natural clays with dioxins, or the risk of interaction with antibiotic (Devreese et al., 2013a; Huwig et al., 2001; Jouany, 2007; Osselaere et al., 2012). They have also shown to be less efficient in binding *Fusarium* mycotoxins such as FBs, ZEN and trichothecenes, as well as OTA (Avantaggiato et al., 2005; Kabak et al., 2006).

Active carbon or activated charcoal is another group of inorganic mycotoxin binders. Active carbon is a non-soluble powder formed by pyrolysis of several organic compounds. Its manufacture can imply processes of activation, developing in this case a highly porous structure (Galvano et al., 2001). The specific surface area of active carbon varies from 500 to 3500 m^2/g (Ramos et al., 1996b). The ability of activated carbon to efficiently bind DON, ZEN, AFB1, FB1 and OTA has been reported (Avantaggiato et al., 2004; Cavret et al., 2010; Devreese et al., 2012; Huwig et al., 2001). However, the disadvantage of the use of activated carbon is its nonspecific binding ability; showing its

efficacy *in vitro* but the correlation with in *vivo* studies is poor. Often the product is not effective *in vivo* resulting in a reduction of nutrient absorption of minerals and vitamins, as well as the impairment of feed nutritional value (Avantaggiato et al., 2004; Ramos et al., 1996b).

Polymers are another inorganic binders, with examples as the polyvinylpyrrolidone and the cholestyramine. Polyvinylpyrrolidone is a highly polar amphoteric polymer that has been successfully tested on ZEN adsorption (Alegakis et al., 1999; Ramos et al., 1996a). Cholestyramine is an insoluble, quaternary ammonium anion exchange resins which strongly binds anionic compounds (Underhill et al., 1995). It is used as human drug for reducing cholesterol through adsorbing bile acids in the GIT. It has proven to be effective in *in vitro* binding FB1, OTA and ZEN (Avantaggiato et al., 2003, 2005; Döll et al., 2004; Ramos et al., 1996b). The use of polymers in animal feed is limited due to their highly cost (Kolosova & Stroka, 2011).

Regarding the organic binders, the cell wall components derived from *Saccharomyces cerevisiae* yeasts are the most used materials. Yeast cell walls (YCW) are mainly composed of β-glucans and mannan oligosaccharides and showed ability of binding across a wider spectrum of mycotoxins both *in vitro* and *in vivo*. It has been reported that the β-glucan fraction of YCW is directly involved in the binding strength (Yiannikouris et al., 2004). The β-D-glucan fraction of YCW is directly correlated in the binding process with ZEN, through different binding mechanisms (hydrogen and Van der Waals interactions) (Shetty & Jespersen, 2006; Yiannikouris et al., 2004). Likewise, YCW based fractions (β-glucans and mannans) has shown its capacity to *in vitro* bind various mycotoxins such DON, ZEN, AFB1 and OTA, as well as *in vivo* (Cavret et al., 2010; Fruhauf et al., 2012; Pfohl-Leszkowicz et al., 2015). Esterified glucomannans (EGM) have demonstrated to be effective in mitigating the toxic effects of different mycotoxins induced simultaneously (Aravind et al., 2003; Avantaggiato et al., 2005; Li et al., 2012; Mohaghegh et al., 2017). The interaction mechanism between yeasts and mycotoxins has been discussed by Luo et al. (2020) and can be summarized as follows: first, the mycotoxin adsorption by yeasts is determined by the three-dimensional structures and compositions of their cell walls. Moreover, the interaction forces between mycotoxins and yeasts were identified as both hydrophobic and electrostatic interactions with weak hydrogen and van der Waals bonds.

Another group of organic mycotoxin binders is lactic acid bacteria (LAB). As yeasts, LAB occur as part of natural microbial population in spontaneous food fermentation and as starter cultures in the food and beverage industry (Shetty & Jespersen, 2006). LAB are gram-positive, non-sporulating and tolerant to acid bacteria. Lactic acid was produced by these bacteria as the main end-product of carbohydrate fermentation. The main genera of LAB are *Lactococcus*, *Lactobacillus, Leuconostoc* and *Pediococcus*. Some LAB strains such as *Lactobacillus rhamnosus* have shown their ability to *in vitro* detoxify mycotoxins like DON, T-2, ZEN, FB1, AFB1 and OTA (El-Nezami et al., 1998, 2002 a,b ; Niderkorn et al., 2006; Piotrowska & Zakowska, 2005). Moreover, the strength of mycotoxin-LAB interaction could be affected by the amino acid composition of the peptidoglucan structure (Dalié et al., 2010).

Micronized fibers originated from different plant materials (wheat, barley, oat, pea hulls) are also used against mycotoxicosis. They are composed principally of cellulose, hemicellulose and lignin. The micronized fibers have been used as mycotoxin binders due to the favourable gut adsorption as well as enhanced fecal excretion (Aoudia et al., 2009). For example, micronized wheat fibers showed to be an effective binder against OTA (Aoudia et al., 2009). Regarding complex bio-sorbents, dehydrated grape pomace, apple pomace or humic acids have been proven to adsorb different mycotoxins (Avantaggiato et al.,2014; Gutzwiller et al., 2007; Santos et al., 2011).

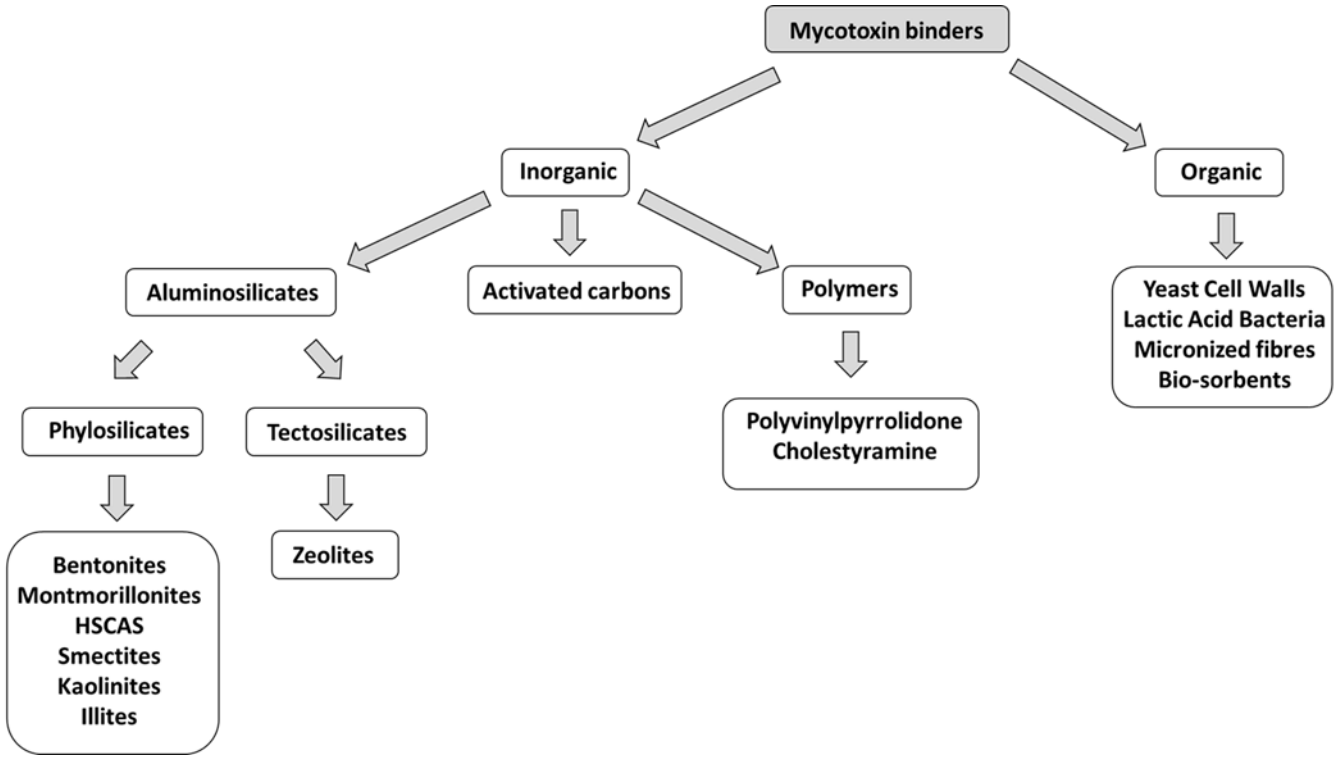

Figure 4. Mycotoxin binder classification (adapted from EFSA, 2009)

4.1.1.2. *In vitro* studies

The *in vitro* process of mycotoxin adsorption is mainly dependent on the physicochemical properties of the binder and the mycotoxin, as well as the pH of the test medium. The adsorbing efficiency of the binder is highly influenced by the kind of matrix where adsorption takes place (including gastric or intestinal juices), the mycotoxin concentration, and the presence of different mycotoxins at the same time, so in some cases efficiency is hard to be predicted. Binding studies could also be affected by feed structure, moisture content and oxygen availability during testing. All these factors have been reported in the review by Wielogorska et al. (2016).

Generally, DON adsorption is low, DON is adsorbed in a very small proportion by the most commonly used adsorbents due to its structure. Indeed, DON is a hydrophilic and non-ionisable molecule with a bulky epoxy-group that does not aid adsorption to plane surfaces (EFSA, 2009).

Galvano et al. (1998) tested 15 experimental activated carbons obtained from olive residues at 2 g/L each for 4 µg DON/mL and they found that the efficacy of binding ranged from 1.83 to 98.9%. Furthermore, using the same concentration of binder and mycotoxin, they found that three commercial activated carbons had the capacity to bind 89% to 98%,

but a fourth one showed a low adsorption capacity and bound only 14% of the toxin. Similarly, HSCAS and sepiolite showed also low DON adsorptions, as they bound only 3.9 and 4.5%, respectively. The *Lactobacillus rhamnosus* strain GG showed a binding capacity of 52% of DON, however the *Lactobacillus rhamnosus* strain LC-705 bind only 1% of DON (El-Nezami et al., 2002b). Moreover, Tomašević-Čanović et al. (2003) indicated that organo-zeolite and zeolite at 10 g/L were not able to bind DON (2 μg/mL). *In vitro* screening test of 14 adsorbents evaluated by Avantaggiato et al. (2004), revealed that active carbon is the unique product that showed efficacy to bind DON (Table 13). Active carbon at 1 g/L bound 84-95% and 52-59% of DON at 2 and 10 μg/mL, respectively. The rest of adsorbing agents showed poor ability to bind DON, usually less than 18%. According to the adsorption isotherms, active carbon showed to be effective to bind DON with a capacity of binding (35.1 μmoL/g). Same authors later *in vitro* tested seven other adsorbents and found that standard Q/FIS adsorb 50-53% of DON at 2 μg DON/mL, but only 13-18% at 10 μg DON/mL (Avantaggiato et al., 2005). However, the other adsorbing agents tested showed poor efficiency to bind DON, generally less than 11% (Avantaggiato et al., 2005). Cavret et al. (2010) identified six products (activated charcoal, cholestyramine, mannans, algal β-glucan, fungal β-glucan, leguminous plant short bladed) with potential capacity to adsorb DON *in vitro* (see Table 13 for adsorption percentage). These studies have been conducted with buffer solution (pH 7 and 3) containing increasing concentrations of DON (1, 2, 6, 8, 10 and 12 μg/mL). The adsorption rate decreased as DON concentration increased. Sabater-Vilar et al. (2007) found that the *in vitro* screening of different mineral clays, humic substances and YCW derived products assessed by HPLC and bioassay at different pH showed poor adsorption of DON and the only product that proved the ability to bind DON was the active carbon. The active carbon was showed to be an effective adsorbing agent of DON *in vitro*. However, its practical application is restricted due its nutritional limitations such as adsorption of minerals, vitamins and other nutrients. Regarding, the poor efficiency of clays against DON, it has been hypothesised that the lower polarity of DON is the reason (Sabater-Vilar et al., 2007). Kong et al. (2014) reported that among the adsorbing agents tested *in vitro*, even the active carbon was not effective to bind DON. *Lactobacillus paracasei* LHZ-1 strain, isolated from yogurt, showed that the cell wall of this LAB can remove only up to 40.7% of 50 μg/mL of DON (Zhai et al., 2019a).

The efficacy of active carbon in binding DON by using a dynamic gastrointestinal model was also tested (Avantaggiato et al., 2004). The *in vitro* intestinal absorption was 51% of the 170 µg DON provided through contaminated spiked wheat and the major absorption occurred in the jejunum among the other compartments of small intestine. An *in vitro* model simulating the condition of the porcine GIT was developed to study the efficacy of 10 commercially available mycotoxin detoxifying agents to detoxify DON (Döll et al., 2004). The results indicated that active carbon was able to bind DON, whilst the other products tested were not effective in detoxifying DON.

Table 13. *In vitro* efficacies of deoxynivalenol (DON) binders.

DON[1] concentration	Binder	Binder concentration	*In vitro* conditions	Efficacy (% binding)	Reference
Single concentration studies					
4 µg/mL	HSCAS[2]	2 g/L		3.9	(Galvano et al., 1998)
	Sepiolite			4.5	
	3 commercial active carbon			89.1-97.8	
	Commercial active carbon			14.0	
	15 experimental active carbon			1.8-99	
20 µg/mL	*Lactobacillus rhamnosus* GG	1×10^{10} cfu/mL	Buffer pH 7.3	52	(El-Nezami et al., 2002b)
	Lactobacillus rhamnosus LC705			1	
2 µg/mL	Organo-zeolites	10 g/L	Buffer pH 3	< 1-9	(Tomašević-Čanović et al., 2003)
	Zeolite			1	
0.5-20 µg/mL	Active carbon	1 g/L	Isotherme Buffer pH 7	The binding capacities 35.1µmoL/g	(Avantaggiato et al., 2004)
2 µg/mL	Active carbon	1 g/L	Buffer pH 3-7-8	84 to 95	(Avantaggiato et al., 2004, 2005)
10 µg/mL				52 to 59	
2-10 µg/mL	Bentonite	1 g/L	Buffer pH 3-8	2-13	
	Celite			1-10	
	Zeolite			2-5	
	Cholestyramine			4-10	
	Glucomannan			1-12	

Table 13. *In vitro* efficacies of deoxynivalenol (DON) binders.

DON[1] concentration	Binder	Binder concentration	*In vitro* conditions	Efficacy (% binding)	Reference
	Mycosorb			3-18	
	Flo Bond			9-12	
	Florisil			5-11	
	Microton			3-11	
	Myco AD A - Z			1-11	
	Ryfix- Toxal			0-16	
	Tixolex			1-16	
	Mycofix Plus			1-13	
2 µg/mL	Standard Q/FIS	1 g/L	Buffer pH 3-7-8	50 to 53	
10 µg/mL	Satandard Q/FIS			13 to 18	
2-10 µg/mL	Amberlite XAD-2	1 g/L	Buffer pH 3-8	2-11	(Avantaggiato et al., 2005)
	Amberlite IRA-900			2-11	
	Amberlite MB-150			2-8	
	Dowex 1 - X8			2-5	
	Dowex Marathon MSA			1-10	
	Dowex MR-3			0-9	
1 mg/mL	3 minerals	5 g/L	Buffer pH 2.5-8	5-21	(Sabater-Vilar et al., 2007)
	6 humic acid			0-21	
	4 yeasts			1-19	
	Active carbon			70-93	
1-12 µg/mL	Active carbon	1 g/L	Buffer pH 7	96-99	(Cavret et al., 2010)
	Cholestyramine			56-69	
	Mannans			61-88	
	Algal β-glucan			67-84	
	Fungal β-glucan			80-92	

Table 13. *In vitro* efficacies of deoxynivalenol (DON) binders.

DON[1] concentration	Binder	Binder concentration	*In vitro* conditions	Efficacy (% binding)	Reference
	Leguminous plant short bladed			47-55	
	Active carbon		Buffer pH 3	97-99	
	Cholestyramine			4-24	
	Mannans			86-93	
	Algal β-glucan			78-90	
	Fungal β-glucan			78-98	
	Leguminous plant short bladed			2-24	
250 ng/mL	Bentonite	5 g/L	Buffer pH 6.8	7	(Kong et al., 2014)
	Cellulose			17	
	YCW[3]			23	
	Active carbon			14	
	Mixture of all above			4	
50 µg/mL	Cell wall of *L. paracasei* LHZ-1	1×10^{10}cfu/mL	Phosphate-buffered saline (PBS; 0.05 M, pH 7.2)	up to 40.7	(Zhai et al., 2019a)
Experimental GIT[4] model studies					
2.8 mg/kg (170 µg DON)	Active carbon	5, 10, 20 g/L	GIT model GIT solutions wheat matrix pH 7 to 2 Tº 37	28-51	(Avantaggiato et al., 2004)

Table 13. *In vitro* efficacies of deoxynivalenol (DON) binders.

DON[1] concentration	**Binder**	**Binder concentration**	***In vitro* conditions**	**Efficacy (% binding)**	**Reference**
3.3 mg/kg	Modified aluminosilicate	0.82 g/L	Buffer pH 3- 5- 6- 7 37 °C	17	(Döll et al., 2004)
	Bentonite			1	
	Active carbon			67	
	Cholestyramine			10	
	Mykosorb			24	
	Toxisorb			1	
	Klinosan			0	
	Mycofix Plus			1	
	Fix A TOX			21	
	Likratox			2	

[1]DON: deoxynivalenol; [2]HSCAS: Hydrated sodium calcium aluminosilicates; [3]YCW: yeast cell wall; [4] GIT: Gastrointestinal tract.

4.1.1.3. *In vivo* studies in chickens:

To proof the efficacy of detoxifying agents, the *in vivo* experiments are indispensable (EFSA, 2009) (Table 14). According to this, Swamy et al. (2002) investigated the toxicity of feeding blends of grains naturally contaminated with *Fusarium* mycotoxins (containing DON at 4.7 to 8.4 mg/kg) on productive and metabolic indicators in broilers and evaluated the efficacy of EGM (0.2%) in counteracting adverse mycotoxin-induced effects. EGM addition did not have any effect on productive parameters but mitigated the DON toxicity on most of the blood parameters, and decreased breast muscle redness.

Later, the same group investigated the impact of diets with blends of grains naturally contaminated with *Fusarium* mycotoxins (containing DON at 5.9 to 9.5 mg/kg) on growth, immunological parameters and, on brain regional neurochemistry of broiler chickens and examined the efficacy of glucomannan polymer (0.2%) in mitigating negative mycotoxin-produced effects. This product only counteracted the effect produced on β-cells count and hypothalamic 5HIAA:5HT ratio, among many immunological and brain regional neurochemistry parameters evaluated (Swamy et al., 2004 a,b).

Faixova et al. (2006, 2007) studied in separate investigations the effects of feeding DON contaminated diets (3 mg DON/kg) on blood biochemistry in broilers and the effect of a glucomannan mycotoxin adsorbent (0.2%) or a selenium-enriched yeast (1.4 mg/kg) in preventing negative effects. Glucomannan or selenium supplementation efficiently reversed the adverse effects produced by DON on most of the plasmatic biochemical parameters. Klapácová et al. (2011) also evaluated the effect of lignin (0.5%) on blood biochemistry in broilers exposed to a DON contaminated diet (2.95 mg DON/kg). Dietary lignin ameliorated the negative effect on total protein, albumin, potassium, and ALP activity but not in magnesium and cholesterol. The authors considered that this feed additive was not effective in detoxifying DON mycotoxin.

Li et al. (2012) examined the efficacy of YCW (0.2%) on physiological, biochemical and immunological parameters of broilers exposed to feed-borne *Fusarium* mycotoxins (including DON at 2 mg/kg feed). This binder considerably improved the RW of some organs and alleviated the mycotoxins induced effects related to immune functions.

A smectite-type clay mineral (illite–ambrosite) was unable to counteract the negative effect produced by DON on zootechnical parameters of broiler chickens, altering the pharmacokinetic parameters (Osselaere et al., 2012). Later, the same authors tested the

same feed additive on the oxidative stress and histological markers in broiler chickens fed 7.54 mg DON/kg feed (Osselaere et al., 2013a). The results revealed that this clay-based mycotoxin-adsorbing agent (illite-ambrosite clay) only counteracted the adverse effect of DON on small intestine histology and altered the parameters related to oxidative stress on liver and small intestine.

Yang et al. (2017) demonstrated the antioxidant effect of *Lactobacillus plantarum* JM113 and its ability on counteracting the stress oxidative induced by DON mycotoxin and protecting the intestinal barrier in Arbor Acres broilers.

Wu et al. (2018) tested the efficacy of the same feed additive (*Lactobacillus plantarum* JM113) on the intestinal toxicity of DON (10 mg/kg) in Arbor Acres broilers. It has been reported that this additive was effective to recover the damages observed in intestinal morphology and intestinal barriers, as well as increased the abundance of beneficial bacterium and regulated the balance of gut microbiota.

The addition of *Lactobacillus* spp. have shown to alleviate significantly the negative effect induced by DON on intestinal and liver lesion score of broilers, on small intestinal histology indicators, oxidative stress response in the intestine and liver, and also on the antioxidant capacity in these tissues (de Souza et al., 2020).

Table 14. *In vivo* efficacies of various deoxynivalenol (DON) binders in chickens.

DON[1] or DON in mixture levels, (mg/kg)	**Binder**	**Binder inclusion (%)**	**Duration (d)**	**Effects reported**	**References**
DON (4.7-9.7)	EGM[2] from *Saccharomyces cerevisiae*	0.2	56	Counteract most of the blood parameter alterations caused by the *Fusarium* mycotoxin-contaminated grains and reduce breast muscle redness.	(Swamy et al., 2002)
DON (5.9-9.5)	Polymeric glucomannan mycotoxin adsorbent (GM polymer)	0.2	56	No effect on zootechnical parameters. Preventing mycotoxin-induced decreases in B-cell counts. Counteract the effect of DON on hypothalamic 5HIAA:5HT ratio.	(Swamy et al., 2004 a,b)
DON (3.0)	Modified glucomannan (Mycosorb®)	0.2	42	Decrease plasma ALT[3] and ALP[4]activities. Counteract plasma levels of magnesium, triglycerides, free glycerol and total protein produced by dietary DON. Did not alleviate adverse effect on calcium metabolism.	(Faixová et al., 2006)
DON (3.0)	Dietary selenium (selenium-enriched yeast) Selplex®	0.00014	42	Mitigate plasma levels of calcium, magnesium and ALT activity in chicks produced by dietary DON. Did not alleviate negative effect on phosphorus, albumin, cholesterol	(Faixová et al., 2007)

Table 14. *In vivo* efficacies of various deoxynivalenol (DON) binders in chickens.

DON[1] or DON in mixture levels, (mg/kg)	**Binder**	**Binder inclusion (%)**	**Duration (d)**	**Effects reported**	**References**
				levels ALP, AST[5], LDH[6] activities, glycerol, and triglycerides.	
DON (2.95) ZEN (1.95)	Lignin	0.5	28 (the last 14 d inclusion)	Counteract the effect on total protein, albumin, potassium, and ALP activity but not in magnesium and cholesterol.	(Klapácová et al., 2011)
AF (0.10) ZEN (0.28) FUM (5.87) DON (2.03)	YCW (Detoxza)	0.2	42	Counteract the effect on the RW of liver and spleen at 21 d and on the RW of liver, bursa of Fabricius and thymus at 42 d. Positive protection effect (alleviate) on anti-ND[7] titers, IL-1β[8], IL-6[9] and IFN-γ[10] but did not reach the levels in the control.	(Li et al., 2012)
DON (2.44 to 7.54)	Smectite-type clay mineral (illite-ambrosite)	0.15	21	No effect on zootechnical parameters and altered pharmacokinetics.	(Osselaere et al., 2012)

Table 14. *In vivo* efficacies of various deoxynivalenol (DON) binders in chickens.

DON[1] or DON in mixture levels, (mg/kg)	Binder	Binder inclusion (%)	Duration (d)	Effects reported	References
DON (7.54)	Clay-based mycotoxin adsorbing agent (illite-ambrosite clay)	0.15	21	Not only DON but also the adsorbing agent, both alter the mRNA expression of oxidative stress markers in liver, jejunum and ileum barrier function and ileum inflammatory reaction. Shifted the adverse effects induced on the gut wall morphology in duodenum and jejunum.	(Osselaere et al., 2013b)
DON (10)	*Lactobacillus plantarum* JM113	1×10^9cfu/kg	42	Alleviate the negative effects of DON on oxidative stress parameters and protect the integrity of the intestinal barrier in broilers challenged with DON.	(Yang et al., 2017)
DON (10)	*Lactobacillus plantarum* JM113	1×10^9cfu/kg	42	Shift the adverse effect on digestion, absorption, and metabolic functions of the gut.	(Wu et al., 2018)
DON (19.3)	*Lactobacillus* spp.		14	Improve the intestinal health (morphology and histology) and reduce the oxidative stress damage on jejunum and liver.	(de Souza et al., 2020)

[1] DON: deoxynivalenol; ZEN: zearalenone; AF: aflatoxin; FUM: fumonisins; [2] EGM: esterified glucomannans [3]ALT: alanine transferase; [4]ALP: alkaline phosphatase; [5]AST: aspartate transaminase; [6]LDH: lactate deshydrogenase; [7]anti-ND titers: anti Newcastle disease titers; [8]IL-1β: interleukin 1β; [9]IL-6: interleukin 6; [10]IFN-γ: interferon-gamma

4.1.2. Biotransforming agents

4.1.2.1. Definition and classification

Mycotoxin-biotransforming agents, also called mycotoxin modifiers, are defined as microorganisms or their enzymes able to degrade or biotransform the mycotoxins into a less or non-toxic compound(s) through routes such as (de) acetylation, oxygenation, ring/side chain cleavage, deepoxidation, isomerization or glycosylation (Wielogorska et al., 2016). They are classified into bacteria, yeasts and fungi, or their enzymes. They could act on the contaminated material or in the intestinal tract of animals prior to the absorption of mycotoxins (Devreese et al., 2013b). In order to be considered a good biotransforming agent, it must be able to get a rapid degradation to less or non-toxic metabolites under different conditions or in complex environments. Furthermore, it must preserve the nutritive and organoleptic properties of the feed, being stable along the intestinal tract at different pH levels, all this while showing safety in its use, and a practical and economic feasibility (Awad et al., 2010; Devreese et al., 2013b).

DON-degrading bacteria have been isolated from rumen and intestinal microbiota, soil and water. The main bacterial degradations of DON are the deepoxidation to DOM-1, generally by rumen or intestinal anaerobic bacteria. Then, the oxidation to 3-keto-deoxyinvalenol (3-keto-DON), along with isomerization to 3-epi-deoxynivalenol (3-epi-DON), by aerobic bacteria. DON-degrading bacteria have demonstrated *in vitro* their abilities to degrade DON mycotoxin. These DON-degrading bacteria are listed in Table 15.

The microorganism strain DSM 11798 belonging to the family of the *Coriobacteriaceae* has shown to be able of degrading DON and trichothecenes mycotoxins, both *in vitro* and *in vivo*, by reducing the 12,13-epoxide group of representative trichothecenes (Fuchs et al., 2002; Schatzmayr et al., 2006). This microorganism is a live, gram-positive, strictly anaerobic bacteria, with a temperature optimum of 37° C and a pH-optimum (6-7.5). The trade name of this microorganism is Biomin® BBSH 797 feed additive is the first and only microorganism authorized in Europe as a substance for reduction of the contamination of feed by DON (technological feed additives) since 2013 (EU, 2013).

Some microorganisms produce enzymes responsible for degradation of mycotoxins. Enzymes are another group of biotransforming agents. The epoxidases (enzymes

produced by the microorganism strain DSM 11798) have shown to have the potential to detoxify trichothecenes mycotoxins. Their mode of action is based on the biotransformation of the epoxy-group into a diene group (Schatzmayr et al., 2006). A lipase isolated from *Aspergillus niger* has shown to have the capability of biotransformation of more than 70% of DON (Yang et al., 2017). Moreover, the use of epimerases to detoxify DON has been reported (Hassan et al., 2017). DON epimerization is a two-step enzymatic DON detoxification; firstly, DON oxidation to 3-keto-DON, followed by selective reduction of 3-keto-DON to 3-epi-DON (Hassan et al., 2017; He et al., 2017).

Another group of biotransforming agents is yeasts. *Trichosporon mycotoxinivorans* is a mycotoxin-degrading yeast that was isolated from the hindgut of the lower termite *Mastotermes darwiniensis*. Mycofix®Plus is a toxin deactivator containing the yeast *Trichosporon mycotoxinivorans* which owns the lactonase activity degrading zearalenone and ochratoxin A and the DSM 11798 strain owning the epoxidase activity degrading DON (Avantaggiato et al., 2005; Dänicke et al., 2003). Under laboratory conditions, certain baker's yeast (*Saccharomyces cerevisiae*) strains were able to inhibit the production of DON and other mycotoxins (ZEN and OTA) (Armando et al., 2013).

Fungi are another group of biotransforming agents and, interestingly, have been used to degrade mycotoxins. *Aspergillus oryzae*, *Rhizopus oryzae* and *Aspergillus tubingensis* have been shown to degrade DON in less toxic compounds (Garda-Buffon et al., 2011; Ito et al., 2013).

4.1.2.2. *In vitro* studies for DON

Previous studies found that DON can be transformed into metabolites less or non-toxic through acetylation, glycosylation, deepoxidation, oxidation or epimerization (Table 15). Generally, these reactions of transformation occurred under relative high temperature, over 30 ºC, and in neutral medium (Guan et al., 2009).

Deepoxidation

As with other trichothecenes, DON toxicity resides in the 12, 13-epoxide ring, and if the cleavage of that ring is achieved, the mycotoxin becomes less toxic. The deepoxidation route has been extensively documented and several authors described this deepoxidation reaction in ruminal, intestinal microbiota or environment (Ahad et al., 2017; Fuchs et al., 2002; He et al., 1992; Kollarczik et al., 1994). Yoshizawa et al. (1983)

were the first to characterize the DOM-1 under anaerobic conditions, in rat urine and feces. Microbial DON deepoxydation under aerobic or anaerobic conditions and at low or high temperatures has been reported in several cases (Table 15).

Microorganisms from ruminant animals transform DON to DOM-1. For example, after *in vitro* DON incubation (up to 10 ppm) with rumen fluid from cow, 84% and 89% of DON was transformed in DOM-1 within 24 h and 48 h, respectively (King et al., 1984). When the initial concentration of the toxin increased to 100 ppm, the transformation rate was only 37% in 24 h (King et al., 1984). The deepoxidation of DON by rumen fluid of steer was also described by Cote et al. (1986). Moreover, microorganisms from fistulated dairy cow rumen fluid were found to have a deepoxidation function (Swanson et al., 1987). The partial microbial transformation of DON to DOM-1 was obtained within 12 h after DON was incubated with these microorganisms, and DOM-1 concentration increased over time (36 h). Nevertheless, the parent DON was the major metabolite present at all sampling times (Swanson et al., 1987).

As previously explained, the microorganism DSM 11798 strain, isolated from bovine rumen fluids, is able to efficiently degrade DON to DOM-1, both *in vitro* and *in vivo* by reduction of the epoxide ring (Fuchs et al., 2002; Schatzmayr et al., 2006). An *in vitro* model with pig intestine was developed to prove the biotransformation of DON by the microorganism DSM 11798 to DOM-1. The rate of transformation was 100 % in the anterior part of the small intestine. The authors concluded that the biotransformation of DON by the microorganism DSM 11798 was favoured by the intestinal environment including the gut microbiota and the physiological conditions at this placement (pH, redox potential, and oxygen content) (Schatzmayr et al., 2006).

Deepoxidation also occurred with microorganisms from non-ruminant animals. DON was transformed to DOM-1 by chickens, and swine microorganisms (He et al., 1992; Kollarczik et al., 1994). The microorganisms in the digesta of the large intestines of chickens were able to completely transform DON to DOM-1 and the activity was conducted in a pH range from 5.72 to 6.91 (He et al., 1992). At pH lower pH than 5.2, the DON transformation activity is completely inhibited (He et al., 1992). Additionally, chicken intestinal bacterial isolates have also shown to be effective in degrading DON into DOM-1 (Young et al., 2007; Yu et al., 2010). The approach with PCR-DGGE guided microbial selection was promising in isolating DON-transforming bacteria from chicken intestines, and the bacterial isolates able to transform DON to DOM-1 isolated belong to

four different bacterial groups, *Clostridiales*, *Anaerofilum*, *Collinsella*, and *Bacillus* (Yu et al., 2010). Furthermore, a bacterial specie *Eggerthella* sp**.** DII-9 isolated from chicken intestines has been reported to have deepoxidation activity under anaerobic conditions with temperatures (37-40ºC) and pH of 6-10 (Gao et al., 2018). More recently, Gao et al. (2020) isolated another strain from chicken intestines with deepoxidation activity the *Slackia sp. D-G6.*Deepoxidation activity was achieved also when DON was mixed with the bacterial gut microbiota of pigs (Kollarczik et al., 1994). To compare the toxicity of DON and its metabolite DOM-1, the authors used a MTT (3-(4,5-dimethylthia-zol-2-yl)-2,5-diphenyltetrazolium bromide) test and demonstrated that the transformation of DON was correlated with a loss of cytotoxicity. This result was confirmed also by Schatzmayr et al. (2006), which reported that DOM-1 is 500 times less toxic than DON.

It was noted that the deepoxidation function of the intestinal microorganisms of chickens or pigs showed a great variability. This variation depends essentially on the samples collected from different animals strains individuals and intestinal regions (He et al., 1992; Kollarczik et al., 1994).

Transformation of DON to DOM-1 by microbes from the intestinal tract of fish has been also reported (Guan et al., 2009). A microbial culture (C133) obtained from one brown bullhead (*Ameiurus nebulosus*), completely transformed *in vitro* DON to DOM-1 at 15 °C after 96 h incubation. This high transformation ability was maintained by the microbial culture C133 over a broad range of temperatures (from 4 to 25 °C) and pH values (from 4.5 to 10.4). Moreover, the human fecal microbiota showed to degrade DON, leading to the appearance of DOM-1 in urine (Gratz et al., 2013).

DON was transformed also to DOM-1 by microorganisms from other environments. A mixed microbial culture from one composite soil sample was capable to biotransform completely DON to DOM-1 after 60 h of incubation under aerobic conditions and moderate temperatures (Islam et al., 2012). A bacterial consortium, PGC-3, isolated from soil, demonstrated a deepoxidation activity under aerobic conditions, temperatures (20–37 ◦C), pH (5–10) (He et al., 2016). A microbial consortium (contains of strains of *Stenotrophomonas* and *Alkaliphilus-Blautia* species), DX100 with deepoxidation activity was isolated from soil. DX100 was able to biotransform DON into DOM-1 under aerobic and anaerobic conditions (Ahad et al., 2017).

The fungi *Aspergillus oryzae* and *Rhizopus oryzae* isolated from rice were able to decrease DON levels as the peroxidase activity increased (Garda-Buffon et al.,

2011).These fungi species degraded DON to DOM-1 by a peroxidase enzyme under aerobic conditions (Garda-Buffon et al., 2011).

Oxidation

The oxidation pathway of transformation of DON has also been described (Table 15). The C-3 hydroxyl in DON was transformed to oxygen forming 3-keto-DON produced by *Agrobacterium-Rhizobium* E 3-39 (Shima et al., 1997). Likewise, only one mixed culture was able to transform DON into 3-keto-DON among 1285 microbial cultures obtained from farmland soils, cereal grains, insects and other sources, but, the microorganisms responsible for the biotransformation have not been identified (Völkl et al., 2004). *Devosia insulae* sp. A16 (from soil) *and* a microbial consortium C20 (from soil, rice, corn, wheat, rice panicles, and fresh corn leaves) have the ability to degrade DON via oxidation pathway to 3-keto-DON under aerobic conditions (Wang et al., 2019; Wang et al., 2020). The optimal temperature and pH for DON degradation were 30-35°C and pH 7-8., respectively (Wang et al., 2019; Wang et al., 2020).

Epimerization

On the other hand, several studies reported that bacteria isolated from soil samples such as *Nocardioides bacterium* WSN05, *Devosia mutans* 17-2-E-8*, Paradevosia shaoguanensis* DDB001*,* and a microbial consortium LZ-N1 have shown to be able to degrade DON in 3-epi-DON under aerobic conditions (Table 15). Furthermore, *Lactobacillus rhamnosus* isolated from human milk degrade DON in 3-epi-DON under anaerobic conditions.

The biotransformation pathway of DON by targeting C3-OH, via oxidation and epimerization, which results in the formation of 3-keto-DONand 3-epi-DON, has been reported (Table 15). Zhou et al. (2008) reported that the *Barpee* bacterium strain isolated from a soil sample transformed DON (87%) mainly to 3-epi-DON and to 3-keto-DON in a lesser extent, under aerobic conditions. A bacterium *Devosia mutans* 17-2-E-8 was capable of completely transforming DON into a major product 3-epi-DON and a minor product 3-keto-DON which were less toxic than DON (He et al., 2015). The bacterial strain, *Sphingomonas* S3-4, isolated from wheat, showed the ability to degrade completely DON into non-toxic compounds 3-keto-DON and 3-epi-DON, via two sequential reactions (He et al., 2017). In addition, the bacterium, *Devosia mutans* 17-2-

E-8, demonstrated its capability to transform DON into its non-toxic stereoisomer 3-epi-DON by the formation of 3-keto-DON intermediate (Hassan et al., 2017).

A strain of the aerobic fungi *Aspergillus tubingensis* (NJA-1) was isolated from soil (He et al., 2008) . An unknown compound in NJA-1 showed the ability to convert DON into another product with a conversion rate of 94.4% (He et al., 2008).

A cytochrome P450 system from *Sphingomonas* sp. strain KSM1 (isolated from lake water) was capable of catabolizing DON into a less toxic product identified as 16-hydroxy-DON (Ito et al., 2013).

Table 15. Microbial transformation of deoxynivalenol (DON) and produced degradation metabolites.

Microorganism	Origin	Transformation reaction/	Transformed product	Condition	Reference
Rumen microorganisms	Cow	Deepoxidation	DOM-1	Anaerobic	(King et al., 1984)
Rumen microorganisms	Steer	Deepoxidation	DOM-1	Anaerobic	(Cote et al., 1986)
Rumen microorganisms	Dairy cow	Deepoxidation	DOM-1	Anaerobic	(Swanson et al., 1987)
The microorganism DSM 11797	Rumen fluid	Deepoxidation	DOM-1	Anaerobic	(Fuchs et al., 2002)
The microorganism DSM 11797	Bovine rumen fluid	Deepoxidation	DOM-1	Anaerobic	(Schatzmayr et al., 2006)
Intestinal microorganisms	rat	Deepoxidation	DOM-1	Anaerobic	(Yoshizawa et al., 1983)
Chicken gut		Deepoxidation	DOM-1	Anaerobic	(He et al., 1992)
Pig gut		Deepoxidation	DOM-1	Anaerobic	(Kollarczik et al., 1994)
Bacterial isolates LS100 and SS3	Chicken digesta	Deepoxidation	DOM-1	Anaerobic	(Young et al., 2007)
Microbial culture C133	Fish digesta	Deepoxidation	DOM-1	Aerobic	(Guan et al., 2009)
Bacillus sp. LS100 + *Clostridiales, Anaerofilum, Collinsella,* and *Bacillus*	Chicken intestine	Deepoxidation	DOM-1	Anaerobic	(Yu et al., 2010)
Human microbiota	Fecal slurry	Deepoxidation	DOM-1	Anaerobic	(Gratz et al., 2013)

Table 15. Microbial transformation of deoxynivalenol (DON) and produced degradation metabolites.

Microorganism	Origin	Transformation reaction/	Transformed product	Condition	Reference
Eggerthella sp. DII-9	Chicken intestines	Deepoxidation	DOM-1	Anaerobic	(Gao et al., 2018)
Slackia sp. D-G6	Chicken intestines	Deepoxidation	DOM-1	Anaerobic	(Gao et al., 2020)
Consortium of Bacteria *(Serratia, Clostridium, Citrobacter, Enterococcus, Stenotrophomonas* and *Streptomyces)*	Agricultural soil isolates	Deepoxidation	DOM-1	Aerobic	(Islam et al., 2012)
PGC-3 consortium comprising 10 bacterial genera including *Desulfitobacterium*	Soil	Deepoxidation	DOM-1	Aerobic	(He et al., 2016)
Microbial consortium DX100 with *Stenotrophomonas* and *Alkaliphilus-Blautia* species complex	Soil	Deepoxidation	DOM-1	aerobic and anaerobic	(Ahad et al., 2017)
Rhizopus oryzae / *Aspergillus oryzae*	Rice	Deepoxidation by a peroxidase	DOM-1	Aerobic	(Garda-Buffon et al., 2011)
Agrobacterium Rhizobium group strain E3-39	Enriched soil	Oxidation	3-keto-DON	Aerobic	(Shima et al., 1997)

Table 15. Microbial transformation of deoxynivalenol (DON) and produced degradation metabolites.

Microorganism	Origin	Transformation reaction/	Transformed product	Condition	Reference
Mixed culture D107		Oxidation	3-keto-DON	Aerobic	(Völkl et al., 2004)
Devosia insulae A16	soil sample	Oxidation	3-keto-DON	Aerobic	(Wang et al., 2019)
Microbial consortium C20	Soil, rice, corn, wheat, rice panicles, fresh corn leaves from China	Oxidation	3-keto-DON	Aerobic	(Wang et al., 2020)
Bacterium strain *Barpee*	Soil isolate	Oxidation	3-keto-DON		(Zhou et al., 2008)
Devosia mutans 17-2-E-8	Agricultural soil isolates (alfalfa soil sample)	Epimerization and oxidation	3-epi-DON (major product) 3-keto-DON (minor product)		(Hassan et al., 2017) (He et al., 2015) (He et al., 2016)
Sphingomonas S3-4	*Fusarium*-infected wheat grains	Oxidation and epimerization	aldo-keto reductase / 3-oxo-DON 3-epi- DON	Aerobic	(He et al., 2017)
Nocardioides bacterium WSN05-2	Soil isolates	Epimerization	3-epi-DON	Aerobic	(Ikunaga et al., 2011)
Nocardioides and *Devosia*	Soil and wheat leaves	Epimerization	3-epi-DON	Aerobic	(Sato et al., 2012)
Paradevosia shaoguanensis DDB001	wheat field soil	Epimerization	3-epi-DON	Aerobic/	(Wang et al., 2017)
Microbial consortium LZ-N1 (*Pseudomonas* sp. Y1 and *Lysobacter* sp. S1)	Soil samples	Epimerization	3-epi-DON	Aerobic	(Zhai et al., 2019b)

Table 15. Microbial transformation of deoxynivalenol (DON) and produced degradation metabolites.

Microorganism	Origin	Transformation reaction/	Transformed product	Condition	Reference
Lactobacillus rhamnosus (7 strains tested, better degradation rate for SHA113)	Human milk	Epimerization	3-epi-DON	Aerobic	(Qu et al., 2019)
Aspergillus tubingensis NJA-1	Soil	-	-	Aerobic	(He et al., 2008)
Cytochrome P450 system from *Sphingomonas* sp. Strain KSM1	lake water	-	16-hydroxy-deoxynivalenol	Aerobic	(Ito et al., 2013)

DON: deoxyinvalenol; ADON: acetyl-deoxynivalenol; DOM-1: de-epoxy-deoxynivalenol; epi-DON: epimerize-deoxynivalenol.

5.1.2.3. *In vivo* studies with DON

A complete information of the safety of biotransforming agents for target species, consumers and environment is decisive. Likewise, studying their stability in the varying conditions of the GIT, their toxicity and their effect on nutritive and organoleptic properties of the feed is determining (Awad et al., 2010; Wielgorska et al., 2016). Similarly, to *in vitro* studies, the *in vivo* studies also suffer from variability even within the same species. These variations included the mycotoxin level, the detoxifier level, the age, nutritional and health status, and sensitivity of animals (Neeff et al., 2013). The *in vivo* efficacy studies of DON biotransforming agents in chickens are listed in Table 16.

Dänicke et al. (2003) tested the efficacy of Mycofix®Plus to counteract the adverse effects of DON. However, they reported a decrease in performance of broilers receiving this product. Later, in a study conducted by Osselaere et al. (2012), the supplementation of the same detoxifying agent did not affect the performance of broilers, but altered the DON pharmacokinetics parameters by increasing the plasma levels of DON in the group of birds receiving the naturally contaminated feed with mycotoxin-detoxifying agents. Similar results were found in the study conducted by Devreese et al. (2012), which found a significantly higher area under the plasma concentration–time curve ($AUC_{0-\infty}$), maximal plasma concentration (Cmax) and relative oral bioavailability in the groups of broilers receiving the detoxifying agent with or without DON, compared with the control group. Plasma concentration of DOM-1 was not detected in these two studies (Devreese et al., 2012; Osselaere et al., 2012).

The microorganism DSM 11798 showed to be effective in preventing DON mycotoxicosis in broilers exposed to 10 mg DON/kg feed. The supplementation of this probiotic counteracted the histological and absorptive alterations caused by DON (Awad et al., 2004, 2006a).

In recent years, the group of Awad and Ghareeb evaluated the efficacy of the microbial feed additive: The microorganism DSM 11798 at 2.5 kg/ton feed in broilers exposed to 10 mg/kg of DON during 35 d on productive, biochemical, immunological, genotoxic, and welfare parameters. The microbial feed additive considerably overcame the adverse effect on performance (Ghareeb et al., 2012), on indicators related to immune functions (Ghareeb et al., 2013), on stress index and biomarkers related to welfare status

of birds (Ghareeb et al., 2014), and on genotoxic effect induced by DON (Awad et al., 2014).

Concluding, at the moment the commercial biological transformation of DON is only based on *micro-organism* DSM 11798. In practice, the use of biotransforming agents as a way of DON detoxification is limited. This limitation is related to the lack of information about mechanisms of transformation and their effects on feeds nutritional values and safety of animals, and on toxicity of transformed products.

Table 16. *In vivo* efficacies of various DON biotransforming agents in chickens.

DON[1] or DON in mixture levels, (mg/kg)	Biotransforming agent	Biotransforming agent inclusion (g/kg)	Duration (d)	Effects reported	References
DON (3.5-14) ZEN[2] (0.06-0.26)	The microorganism DSM 11798 / Yeast	2.5	35	Reduce performance and alter some biochemical serum parameters independently of the dietary mycotoxin concentration.	(Dänicke et al., 2003)
DON (10)	The microorganism DSM 11798	2.5	42	Counteract the toxic effects of DON on intestinal glucose transport.	(Awad et al., 2004)
DON (10)	The microorganism DSM 11798	2.5	42	Alleviate the histological alterations induced by DON	(Awad et al., 2006a)
DON (10)	The microorganism DSM 11798	2.5	35	Counteract the adverse effect on BW[4] and BWG[5] and led to normal physiological, immunological and welfare parameters.	(Ghareeb et al., 2012)
DON (2.44 to 7.54)	Bentonite - montmorillonite upgraded with a yeast	1.5	21	No effect on zootechnical parameters and altered pharmacokinetics	(Osselaere *et al.*, 2012)
DON (10)	The microorganism DSM 11798	2.5	35	Counteract the effect on: TNF-α[6] in plasma of broiler chickens and mRNA of IL-1β[7] and TGFBR 1[8] in jejunum tissue.	(Ghareeb et al., 2013)

Table 16. *In vivo* efficacies of various DON biotransforming agents in chickens.

DON[1] or DON in mixture levels, (mg/kg)	Biotransforming agent	Biotransforming agent inclusion (g/kg)	Duration (d)	Effects reported	References
DON (10)	The microorganism DSM 11798	2.5	35	Prevent the DNA damage of blood lymphocytes. Counteract the effects of DON on RW [9]of gizzard. No counteracting effect was shown on thiobarbituric acid reactive substances (TBARS)[10].	(Awad et al., 2014)
DON (10)	The microorganism DSM 11798	2.5	35	Counteract the effect of DON on welfare indicators (corticosterone plasma level, stress index and fearfulness response) and ameliorate the negative impact on performance of broilers.	(Ghareeb et al., 2014)
DON (10)	The microorganism DSM 11798	2.5	35	Overcome the effect on antibody titers to the IBV however, no mitigating effect on biochemical parameters (cholesterol, triglycerides or ALT[11]) was observed.	(Ghareeb et al., 2016)

[1]DON: deoxynivalenol; [2]ZEN: zearalenone; [3]NSP: Nonstarch polysaccharide; [4]BW: Body weight [5]BWG: Body weight gain; [6]TNF-α: tumor necrosis alpha; [7]IL-1β: Interleukin 1beta: [8]TGFBR 1: Transforming growth factor, beta receptor I; [9]RW: relative weight; [10]TBARS: thiobarbituric acid reactive substances; [11]ALT: alanine transferase.

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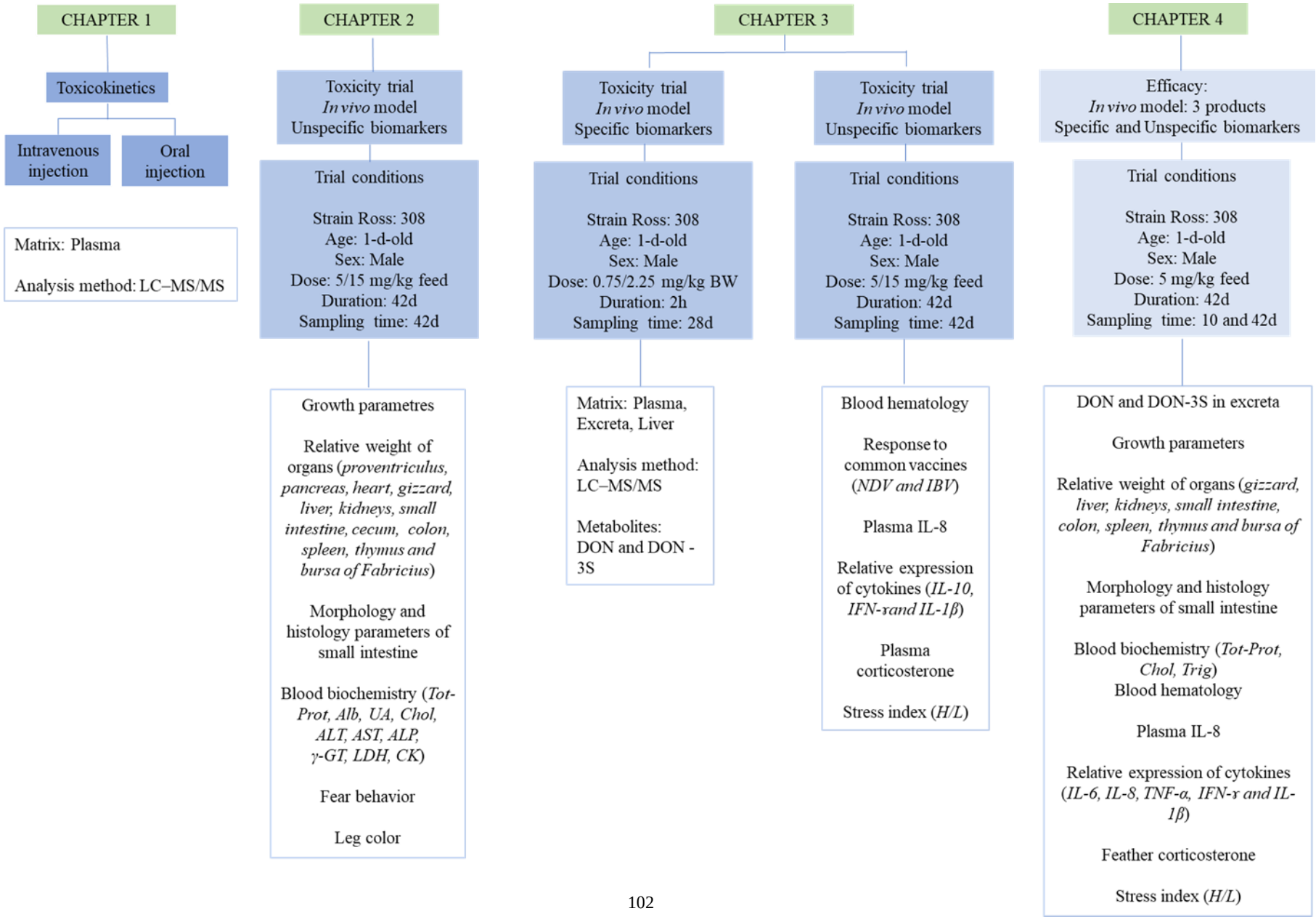
EXPERIMENTAL AIMS

The general aim of this thesis is to set up an *in vivo* model in broiler chicken to study the efficacy of detoxifying agents of DON. For this reason, firstly, an *in vivo* model has to be developed in broiler chicken to follow the toxic effects of DON and to define the relevant biomarkers of effect of toxicity and thereafter, an *in vivo* trial will be conducted in presence of different DON detoxifying agents in order to evaluate their efficacy.

The first chapter focuses on the identification of the specific biomarkers of the *in vivo* model. The objectives being to determine the relevant and suitable biomarkers of exposure of DON in broiler chickens through the determination of DON and it(s) metabolite(s) in plasma by a validated and sensitive method of analysis. For this end, a toxicokinetic study was performed with broiler chickens. DON was administrated by oral and intravenous bolus. The calculated toxicokinetic parameters allowed to understand the mode of action of the mycotoxin and to evaluate chicken health risks. Next, sensitive methods were validated to extract, detect and quantify DON and metabolites in plasma liver and excreta after chronic feeding.

The second and the third chapter of this thesis focus on the research of biomarkers of effects in chickens exposed to DON and on the determination of the most significant biomarkers of toxicity. Based on bibliography, trials' conditions such as age of the chickens, sex, duration of the trial, DON concentration in feed (should be enough to be able to detect mycotoxins in biological fluids but not too much to stay close to the natural conditions), and sampling time have been determined and adapted for our last trial based on what we observed. The indirect biomarkers evaluated in the second chapter were growth parameters, relative weight of organs, morphology and histology parameters of small intestine, blood biochemistry, fear behaviour, and leg color. Regarding the third chapter, the biomarkers of effect examined were blood hematology, response to common vaccines (NDV and IBV), plasma IL-8 concentration and relative expression of cytokines (IL-10, IFN-γ and IL-1β), plasma corticosterone level and stress index.

In the fourth chapter, an *in vivo* efficacy study was conducted through studying the effect of three different feed additives on biomarkers selected as most relevant in the three precedent chapters.


CHAPTER 1
Toxicokinetics
Intravenous injection
Oral injection
Matrix: Plasma
Analysis method: LC–MS/MS
CHAPTER 2
Toxicity trial
In vivo model
Unspecific biomarkers
Trial conditions
Strain Ross: 308
Age: 1-d-old
Sex: Male
Dose: 5/15 mg/kg feed
Duration: 42d
Sampling time: 42d
Growth parametres
Relative weight of organs (proventriculus, pancreas, heart, gizzard, liver, kidneys, small intestine, cecum, colon, spleen, thymus and bursa of Fabricius)
Morphology and histology parameters of small intestine
Blood biochemistry (Tot-Prot, Alb, UA, Chol, ALT, AST, ALP, γ-GT, LDH, CK)
Fear behavior
Leg color
CHAPTER 3
Toxicity trial
In vivo model
Specific biomarkers
Trial conditions
Strain Ross: 308
Age: 1-d-old
Sex: Male
Dose: 0.75/2.25 mg/kg BW
Duration: 2h
Sampling time: 28d
Matrix: Plasma, Excreta, Liver
Analysis method: LC–MS/MS
Metabolites: DON and DON - 3S
Toxicity trial
In vivo model
Unspecific biomarkers
Trial conditions
Strain Ross: 308
Age: 1-d-old
Sex: Male
Dose: 5/15 mg/kg feed
Duration: 42d
Sampling time: 42d
Blood hematology
Response to common vaccines (NDV and IBV)
Plasma IL-8
Relative expression of cytokines (IL-10, IFN-γand IL-1β)
Plasma corticosterone
Stress index (H/L)
CHAPTER 4
Efficacy:
In vivo model: 3 products
Specific and Unspecific biomarkers
Trial conditions
Strain Ross: 308
Age: 1-d-old
Sex: Male
Dose: 5 mg/kg feed
Duration: 42d
Sampling time: 10 and 42d
DON and DON-3S in excreta
Growth parameters
Relative weight of organs (gizzard, liver, kidneys, small intestine, colon, spleen, thymus and bursa of Fabricius)
Morphology and histology parameters of small intestine
Blood biochemistry (Tot-Prot, Chol, Trig)
Blood hematology
Plasma IL-8
Relative expression of cytokines (IL-6, IL-8, TNF-α, IFN-γ and IL-1β)
Feather corticosterone
Stress index (H/L)

METHODOLOGY

1. Animal trials

Table 17 shows the global experimental conditions for all chicken trials conducted during this Thesis. For all experiments, 1-d-old male broiler chickens Ross 308 were obtained from a commercial hatchery (Granja Crusvi, Montblanc) from Catalonia (Spain), and reared in battery cages located at the IRTA facilities at Mas Bové experimental farm (Constantí, Tarragona, Spain). Birds were initially maintained at 34°C, and the temperature was gradually lowered by 3 °C/week to 21 °C by the end of week 6. During the first 2 days, continuous lighting was provided after which the duration of light was gradually decreased to 18 h (7 d) and 14 h afterwards. All the birds in the 3 experiments carried out were fed maize and soybean meal-based diets. Nutrient concentrations in the basal diets (starter or grower) met or exceeded minimum requirements according to the National Research Council (1994). The composition of the control feed diet used in all trials is presented in Table 18. Feed in mash form and water were provided *ad libitum*.

Three experimental designs were planned, depending on whether it was a toxicokinetic, a toxicity or an additive efficacy study.

For the toxicokinetic study, 24 birds were used. Birds were distributed into six cages (4 birds per cage) for 28 d and fed the basal starter diet. A stock solution of 2.5 mg DON/mL was prepared from a DON pure standard (Fermentek, Jerusalem, Israel) in acetonitrile (ACN) (Honeywell, Seelze, Germany). From this stock solution, single doses of 0.75 or 2.25 mg DON/kg of chicken BW were intravenously or orally administrated to the chickens (4 birds for each application and for each dose). The intravenous injection was performed by a 25G needle (0.5 × 16 mm, Terumo Europe, Leuven, Belgium) in the wing vein of the bird.

For the *in vivo* toxicity study, 45 birds were used. Chickens were vaccinated against infectious bronchitis virus (IBV) at the hatchery and against Newcastle disease virus (NDV) at the farm at start of the trial. Birds were divided into fifteen cages (3 birds per cage) for 42 d, and fed diets containing 0, 5 or 15 mg/kg of DON (fifteen birds per treatment). Broiler chickens fed the starter diet from 1 to 21 d and a grower diet from 21 to 42 d.

For the *in vivo* additive efficacy study, a total of 384 birds were used divided into 96 cages (4 birds per cage) for 42 d, and fed one of the following diets: control, DON

(5 mg/kg), control microbial feed additive (MFA), DON+MFA, control immuno-protector additive (IMP), DON+IMP, control mycotoxin degrading enzyme (MDE), DON+MDE. Broiler chickens were fed the starter diet from 1 to 21 d and a grower diet from 21 to 42 d.

The mycotoxin used in this study was produced by inoculating wheat with *Fusarium graminearum* strain I159 (Metayer et al., 2019). After four weeks of growth, the cultured Fusarium species were dried at 90 ◦C for 3 h, ground and sieved to powder. DON contaminated diets for the *in vivo* toxicity or additive efficacy studies were prepared by replacing a determined amount of basal diet with contaminated wheat at different levels. Dry matter, ash, ether extract, crude fiber, gross energy, starch, sodium chloride, and crude protein content in feed were determined by proximate analysis according to the standard procedures of Association of Official Analytical Chemists (2004) (Table 18).

To analyze DON concentrations in the feed, samples were taken at different locations in the batch and subsequently pooled. All diets were analyzed for the content of DON and other mycotoxins with a validated liquid chromatography-tandem mass spectrometry method (LC-MS/MS) (Riahi et al., 2021). DON levels of the experimental feeds used in the *in vivo* toxicity trial and in the *in vivo* additive efficacy trial were shown in Table 19 and Table 20 respectively. The concentrations and limit of detection of other mycotoxins tested in these feeds are also reported in Table 19 and Table 20. DON tested was either absent or present in very low concentration in control feeds of the different trials. Similarly, other mycotoxins were absent or in very low concentrations in all experimental feeds. For the *in vivo* toxicity assay, mycotoxin analysis in feeds were performed by the Applied Mycology Unit of the Food Technology Department of the University of Lleida (Spain). For the *in vivo* additive efficacy assay these analyses were carried out by Phytocontrol laboratory (Nimes, France).

Table 17. Overview of the experimental conditions of the different studies.

	Toxicokinetic study	**Toxicity study**	**Additive efficacy study**
Target specie	Broiler chickens		
Strain	Ross 308		
Age	1-d-old		
Sex	Male		
Housing	Cages		
Feeding type	Acute	Chronic	Chronic
Duration	2 h	42 d	42 d

Table 17. Overview of the experimental conditions of the different studies.

	Toxicokinetic study	**Toxicity study**	**Additive efficacy study**
Sampling time	28 d	42 d	10 and 42 d 21 d (for excreta samples)
Feed based	Maize and soybean		
Feed form	Mash		
Feed phase	Starter	Starter and grower	Starter and grower
Contamination source and application	DON standard (via intravenous or oral bolus injection)	Cultured material (via feeding)	Cultured material (via feeding)
DON[1]concentration	0.75 mg/kg BW[2] 2.25 mg/kg BW	5 mg/kg feed 15 mg/kg feed	5 mg/kg feed
Experimental design[3]	T1: Control	T1: Control	T1: Control
	T2: DON (0.75 mg/kg BW)	T2: DON (5 mg/kg feed)	T2: DON (5 mg/kg feed)
	T3: DON (2.25 mg/kg BW)	T3: DON (15 mg/kg feed)	T3: MFA[4]
			T4: DON+MFA
			T5: IMP[5]
			T6: DON+IMP
			T7: MDE[6]
			T8: DON+MDE

[1]DON, deoxynivalenol; [2]BW: Body weight**;** [3]T: treatment; [4]MFA, microbial feed additive, [5]IMP, immuno-protector additive; [6]MDE, mycotoxin-degrading enzyme

Table 18. Formulation and proximate analysis of control diets.

Ingredients (%)	**Starter: control 1-21 days**	**Grower: control 21-42 days**
Maize	54.00	59.49
Soya-meal 48 %	36.93	31.02
Soyabean oil	4.91	5.73
Monocalcium phosphate	1.42	1.30
Calcium carbonate	1.23	1.13
Sodium chloride	0.19	0.21
Sodium bicarbonate	0.27	0.24
DL-methionine	0.30	0.26
L-Lysine HCl	0.23	0.18
Noxyfeed antioxidant	0.02	0.02
Premix[1]	0.49	0.44
Calculated content (%)		
Metabolizable energy (kcal/kg)	3050	3150
Crude protein	22.0	19.5

Table 18. Formulation and proximate analysis of control diets.

Ingredients (%)	Starter: control 1-21 days	Grower: control 21-42 days
Ether extract	7.01	7.92
Crude fibre	2.36	2.25
Lysine	1.38	1.18
Methionine + cysteine	0.91	0.87
Threonine	0.81	0.70
Tryptophan	0.21	0.18
Calcium	0.90	0.82
Inorganic phosphorus	0.64	0.59
Sodium	0.16	0.16
Analysed contents (%): *in vivo* toxicity trial		
Dry matter	88.9	89.1
Crude protein	21.8	19.6
Gross energy (kcal/kg)	4094	4186
Ether extract	6.75	7.84
Ash	5.56	4.98
Crude fibre	2.40	2.24
Sodium chloride	0.30	0.31
Analysed contents (%): *in vivo* additive efficacy trial		
Dry matter	88.0	88.3
Crude protein	22.0	20.0
Gross energy (kcal/kg)	4093	4168
Ether extract	6.41	7.59
Ash	5.33	4.61
Crude fibre	2.72	2.34
Sodium chloride	0.29	0.34

[1]Vitamin premix provided following nutrients per kg of diet: vitamin A: 13,500 IU; vitamin D3: 4800 IU: vitamin E: 67 IU; vitamin B1: 3 mg; vitamin B2: 9 mg; vitamin B6: 4.5 mg; vitamin B12: 16.5 µg; vitamin K3, 3 mg; calcium pantothenate: 16.5 mg; nicotinic acid, 51 mg; folic acid: 1.8 mg, biotin: 30 µg; Fe: 54 mg; I: 1.2 mg; Co: 0.6 mg; Cu: 12 mg; Mn: 90 mg; Zn: 66 mg; Se: 0.18 mg; Mo: 1.2 mg.

Table 19. Mycotoxin analysis of experimental feeds of the toxicity study.

Mycotoxin[1] (µg/kg)	Control group		DON group (5000 µg/kg)		DON group (15,000 µg/kg)		LOD[2] (µg/kg)
	Starter	Grower	Starter	Grower	Starter	Grower	
DON	65	73	4760	4650	14,390	15,120	50
ZEN	< LOD	< LOD	84.4	85.9	242	259	1.75
FBs	142	225	257	216	216	275	25
OTA	0.94	1.59	0.90	1.11	1.21	1.10	0.5
AFB1	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	0.3

[1] DON: deoxynivalenol; ZEN: zearalenone; FBs: fumonisins; OTA: ochratoxin; AFB1: aflatoxin B_1; [2]LOD: limit of detection.

Table 20. Mycotoxin analysis of experimental feeds of the additive efficacy study.

Treatment / mycotoxins [1](µg/kg)	DON (µg/kg)	FB1[3] (µg/kg)	Other mycotoxins[4]
Starter			
Control	<LOD[2]	33 ± 7	<LOD
DON	7048 ± 846		<LOD
MFA	<LOD		<LOD
MFA + DON	8402 ± 1008		<LOD
IMP	<LOD		<LOD
IMP + DON	7445 ± 893		<LOD
MDE	<LOD		<LOD
MDE + DON	5367 ± 644		<LOD
Grower			
Control	<LOD	81 ± 18	<LOD
DON	8865 ± 1064		<LOD
MFA	<LOD		<LOD
MFA + DON	7067 ± 848		<LOD
IMP	<LOD		<LOD
IMP + DON	6000 ± 720		<LOD
MDE	<LOD		<LOD
MDE + DON	5607 ± 673		<LOD

[1]DON: deoxynivalenol; [2]MFA: microbial feed additive; IMP: immuno-protector additive; MDE: mycotoxin degrading enzyme; [3]<LOD: below limit of detection (DON LOD: 15 µg DON/kg); [4]FB1: fumonisin B_1 (LOD: 7.5 µg/kg); [5]Aflatoxins (B_1, B_2, G_1, G_2) and ochratoxin A (LOD: 0.3 µg/kg); zearalenone (LOD: 3 µg/kg), fumonisin B_2 (LOD: 7.5 µg/kg); HT-2 toxin, T-2 toxin, 15-acetyl-deoxynivalenol, 3-acetyl-deoxynivalenol, fusarenon X and nivalenol (LOD: 15 µg/kg); deoxynivalenol-3-glucoside (LOD: 30 µg/kg).

2. Specific biomarkers: DON and metabolites analysis

2.1. Sample collection

Table 21 shows a summary of the samples collected in the three chicken trials conducted in this Thesis.

Regarding the toxicokinetic study, after intravenous or oral DON administration, blood samples were taken in heparinized tubes at different time points: 0 (pre-administration) and 3, 6, 10, 15, 30, 60, and 120 minutes post-administration. 112 samples were centrifuged at 2851 × *g* for 10 min and plasma was stored at -20 ºC until analysis of DON and DON-3S in plasma.

Regarding the *in vivo* toxicity study, blood was collected at 42 d from twelve birds per treatment into-heparinized tubes to obtain plasma. After birds' euthanasia, liver samples (n =5) were excised, weighed, and stored at -20 ºC until lyophilization. Excreta samples were collected fresh for each cage (a pool of excreta of 3 birds) (n =5) for the

whole trial (from 1 to 42 d) and stored at -20 ºC until lyophilization. Lyophilized liver and excreta samples were stored at darkness at ambient temperature until DON and DON-3 analysis.

For the *in vivo* additive efficacy study, excreta samples (pool of excreta of 4 birds) (n = 6) from 19 to 21 d and from 35 to 42 d were collected. After lyophilization, samples were stored at darkness at ambient temperature until DON, DON-3S, DOM-1, and DOM-3S determination.

Table 21. Sample collection for DON and its metabolites determination.

		Toxicokinetic study		**Toxicity study**		**Additive efficacy study**	
Sample	**Mycotoxin**	**Date**	**n[1]**	**Date**	**n**	**Date**	**n**
Plasma	DON[2]	28	112	42	5		
	DON-3S[3]	28	112	42	5		
Liver	DON			42	5		
	DON-3S			42	5		
Excreta	DON			42	5	21/42	6
	DON-3S			42	5	21/42	6
	DOM-3S[4]					21/42	6
	DOM-1[5]					21/42	6

[1]n: number of samples; [2]DON: deoxynivalenol; DON-3S: deoxynivalenol-3-sulphate; DOM-1: deepoxydeoxynivalenol.

2.2. DON and DON metabolites extraction from samples

2.2.1. Chemicals, products and reagents

The analytical standard of DON was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Internal standard (IS) isotope ($^{13}C_{15}$-DON) was purchased from Biopure (Bioser, Barcelona, Spain). All standards were stored at -15 ºC. Acetic acid (LC-MS gradient grade), sodium sulphate, sodium acetate and hexane were obtained from PanReac Química SLU (Barcelona, Spain). Ammonium formate was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). ACN and Methanol (HPLC gradient grade, MeOH) were purchased from Honeywell (Seelze, Germany). Nylon syringe filters (0.20 µm pore size, 25 mm diameter) were obtained from Agilent (Santa Clara, CA, USA).

2.2.2. Preparation of standard solution

A standard stock solution of DON was prepared in ACN (1 mg/mL), and then diluted with HPLC-grade ACN to obtain an individual working standard solution of

5µg DON/mL. A standard stock solution of $^{13}C_{15}$-DON, as 1.2 mL of a solution of 25 µg/mL in ACN, was purchased. An individual working standard solution of 5 µg/mL was prepared by diluting the above stock solution of $^{13}C_{15}$-DON with HPLC-grade ACN. The stock solutions were stored at -15 °C.

2.2.3. Plasma extract

Plasma pretreatment was performed as described by Broekaert et al. (2014). Briefly, 10 µL of $^{13}C_{15}$-DON IS solution (5 µg/mL) and 750 µL of ACN were added to 250 µL of chicken plasma, followed by vortex mixing (1 min) and a centrifugation (8517 × g, 10 min). Then, the supernatant (1 mL) was transferred to another tube and was evaporated to dryness under nitrogen flow over a heating block. The dry residue was reconstituted in 1 mL of an ammonium formate 5 mM/MeOH (50:50, v/v) solution. A volume of 10 µL was injected for HPLC-MS/MS analysis.

2.2.4. Liver and excreta extracts

10 µL of 5 µg/mL of IS working solution ($^{13}C_{15}$-DON) and 10 mL of ACN:water:acetic acid (79:20:1, v/v/v) were added to 1 g of lyophilized liver or excreta, followed by a vortex mixing for 2 min. Sodium sulfate (4 g) and sodium acetate (1.5 g) were added followed by a vortex mixing (5 min) and then with orbital shaker (IKA™ KS 260, Fisher Scientific, Madrid, Spain) for 20 min. Next, centrifugation step was performed (2716 × g, 10 min). Supernatants (5 mL) were transferred to a new tube, added with hexane (5 mL), and vortex mixing. After separation of two phases, the hexane phase was removed. An aliquot (5 mL) of the extract was evaporated to dryness under nitrogen flow over heating block. The dry residue was reconstituted in 0.5 mL of ammonium formate (5 mM) in water and methanol (50:50, v/v). The sample was passed through Nylon syringe filters (0.20 µm) and injected directly in HPLC-MS/MS.

2.2.5. HPLC-MS/MS analysis

The HPLC-MS/MS analysis was performed with a Transcend 600 LC (Thermo Scientific TranscendTM, Thermo Fisher Scientific, San Jose, CA, USA) coupled to an Orbitrap (ExactiveTM, Thermo Fisher Scientific, Bremen, Germany) with an electrospray ionization (ESI) source (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA). The chromatographic separation was carried out in a Zorbax Plus C18 column (1.8 µm particle size, 2.1 × 100 mm; Agilent, San Jose, CA, USA). Gradient elution was

established with a mobile phase consisting of 5 mM ammonium formate in water (eluent A) and methanol (eluent B) at a flow rate of 0.2 mL/min. The gradient elution started at 95% B at 1 min, and it was decreased to 0% B during 4 min (from 8 to 12 min). The composition of the mobile phase returned to the initial conditions in 0.5 min and was maintained during 1.5 min. The temperature of the column was set at 25 ºC, and the injection volume was 10 µL. Compounds were ionized by electrospray ionization in the positive and negative mode and measured first in full scan and then in targeted MS/MS mode at a collision energy of 30 eV (both in the range from m/z 50-500). MS analyses were carried out using selected reaction monitoring (SRM) mode with positive and negative electrospray ionization (ESI +/-). The spray voltage was set at 4 kV. Sheath and auxiliary gas were N2 (> 95%). Skimmer, capillary and tube lens voltage were set at 18 V, 35 V and 95 V, respectively. Heater and capillary temperature were 305 and 300ºC, respectively. The capillary and nozzle voltage were 4000 V and 95 V (-95 V in ESI-), respectively. All compounds were detected in negative electrospray ionization mode (ESI−). The limit of quantification (LOQ) for DON and DOM-1 was 5 ng/mL and 15 ng/mL, respectively. Since, no standard solutions are commercially available for DON-3S and DOM-3S accurate quantitative analysis cannot be performed.

Finally, the data were processed using XcaliburTM version 3.0 (Quanbrowser and Qualbrowser) and Mass FrontierTM 7.0. The chromatographic analysis was performed in the laboratory of Department of Chemistry and Physics (Research group “Analytical Chemistry of Contaminants”. University of Almeria, Spain).

Table 22. HPLC-MS/MS parameters.

Analyte	Retention time (min)	characteristic ion (m/z)	Fragment ion (m/z)
DON[1]	8.12	341.1242 $[M+CH_3COO]^-$	201.0747
DON-3S[2]	5.80	375.0744 $[M-H]^-$	345.0640
$^{13}C_{15}$-DON	8.12	310.1659 $[M-H]^-$	243.1310
DOM-3-S[3]	5.82	359.0806 [M-H]-	328.9606

[1] DON: deoxynivalenol; [2]DON-3S: deoxynivalenol 3-sulphate; DOM-3-S: deepoxy-deoxynivalenol-3 sulphate.

Summarizing, DON and DON-3S were analyzed in plasma of the toxicokinetics study and the *in vivo* toxicity trial. DON and DON-3S were analyzed in liver and excreta

of the *in vivo* toxicity trial. DON, DON-3S, DOM-1, DOM-3S were determined in excreta of the *in vivo* additive efficacy trial (Table 24).

3. Nonspecific biomarkers

3.1. Sampling and tissue collection

For the *in vivo* toxicity trial, samples were taken at 42 d, however for the *in vivo* efficacy trial, biological samples were taken at 10 and 42 d (Table 23). Blood samples were collected into non-heparinized tubes for hematological and serological parameters. Then, blood serum was separated by centrifugation at 4500 × *g* for 10 min to determine response to common vaccines (NDV and IBV). Blood samples also were collected in heparinized tubes and centrifuged at 1000 × *g* for 15 min for plasma IL-8 and corticosterone determination. The quantity of blood was 4-5 mL/bird. Immediately, after euthanasia by cervical dislocation, distal parts of the jejunum were collected for evaluation of the intestinal histology and determination of relative gene expression of cytokines (interleukin 6 (IL-6), interleukin 10 (IL-10), interleukin 1β (IL-1β), interferon-gamma (IFN-γ), interleukin 8 (IL-8) and tumor necrosis factor-α (TNF-α) by quantitative real-time PCR (qRT- PCR). Regarding histological analysis, samples were fixed in neutral-buffered formalin, and processed afterwards using standard protocols for hematoxylin and eosin staining of paraffin sections. Regarding the relative gene expression of cytokines, samples were taken in RNA later (Sigma-Aldrich, Barcelona, Spain) and stored for 24 h, at ambient temperature. Then, RNA later was removed and samples were stored at -80 ºC until further analysis. For the additive efficacy *in vivo* trial, at 42 d, bird's feathers (control and IMP groups) were taken from wings and stored at -20 ºC until corticosterone analysis.

Table 23. Sample collection and analysis for the toxicity and additive efficacy studies.

		Toxicity study		**Additive efficacy study**	
Sample	**Determination**	**Date**	**n**[1]	**Date**	**n**
Blood	Hematology	42 d	12	10/42 d	12
Serum	Biochemistry	42 d	12	10/42 d	12
	Response to NDV[2] and IBV[3]	42 d	12		
Plasma	IL[4]-8	42 d	12	10 d	6
	Corticosterone	42 d	12		
Jejunum	Histology	42 d	12	42 d	12
	Cytokines:				

Table 23. Sample collection and analysis for the toxicity and additive efficacy studies.

		Toxicity study		Additive efficacy study	
Sample	**Determination**	**Date**	**n[1]**	**Date**	**n**
	IL-8			10/42 d	6
	TNF-α[5]			10/42 d	6
	IL-6	42 d	12	10/42 d	12
	IL-1 β	42 d	12	42 d	12
	IFN-γ[6]	42 d	12	42 d	12
	IL-10	42 d	12		
Feather	Corticosterone			42 d	6

[1] n: number of samples per treatment; [2]NDV: Newcastle disease virus; [3]IBV: infectious bronchitis virus; [4] IL: interleukin; [5]TNF-α, tumor necrosis α; [6]IFN-γ; interferon gamma.

3.2. Biomarkers evaluated (*in vivo* toxicity trial) and biomarkers selected (*in vivo* additive efficacy trial)

For the *in vivo* toxicity trial, biomarkers were evaluated at 42 d. The most relevant biomarkers were selected to evaluate in the *in vivo* additive efficacy trial. Other biomarkers were added also to be evaluated in the *in vivo* additive efficacy trial. All parameters for the *in vivo* additive efficacy trial were evaluated at 10 and 42 d (Table 24).

3.2.1. Productive parameters

Body weight gain (BWG) of all birds and feed intake were evaluated at d 21, 35 and 42 for the *in vivo* toxicity trial and at d 10, 21 and 42 d for the *in vivo* additive efficacy trial. Feed conversion ratio (FCR) was calculated as follows: FCR (period a-b) = [(cumulative feed intake (period a-b)/ BWG (period a-b) per lot/number of birds per lot)].

3.2.2. Relative organ weights

For the *in vivo* toxicity trial, the weights of organs, including the heart, proventriculus, pancreas, cecum, gizzard, liver, kidneys, small intestine, colon, spleen, bursa of Fabricius and thymus, were recorded. The weight of each organ was converted to a value relative to the BW. For the *in vivo* additive efficacy trial, gizzard, liver, kidneys, small intestine, colon, spleen, bursa of Fabricius and thymus were recorded and their relative weights were calculated.

3.2.3. Blood biochemistry

For the *in vivo* toxicity trial, serum concentrations of total protein, albumin, glucose, cholesterol, uric acid and activities of gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST), alanine transferase (ALT), alkaline phosphatase (ALP), creatine

kinase (CK), lactate dehydrogenase (LDH) were evaluated using an automatic biochemical analyzer (Olympus AU5800, Beckman Coulter, Brea, CA, USA). For the *in vivo* additive efficacy trial, serum concentrations of total protein, cholesterol and triglycerides were determined.

3.2.4. Blood hematology

Complete blood cell counts (hematocrit, hemoglobin, red blood cells, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) and differential leukocyte counts (white blood cells, eosinophils, basophils, monocytes, lymphocytes, and heterophils) were performed by hematologic analyzer (CELL-DYN 3700, Abbott, Chicago, USA). Heterophil to lymphocyte ratio was determined and defined as an index of stress. These parameters were determined for both *in vivo* toxicity and additive efficacy trials.

Blood biochemistry and hematology analysis were performed in an external laboratory (Echevarne, Barcelona, Spain).

3.2.5. Response to vaccines

Antibody titers against Newcastle disease virus (NDV) was determined by Haemagglutination inhibition test. Antibody titers against IBV was determined by Haemagglutination inhibition test and by ELISA test kit (Idexx®, Westbrook, Maine, USA) following the instructions of the kit. Response to vaccines was evaluated only for the *in vivo* toxicity trial. This analysis was carried out in an external laboratory (Centre de Sanitat Avicola de Catalunya i Aragó, Reus, Spain).

3.2.6. IL-8 in plasma

IL-8 was determined in plasma using a commercially available enzyme-linked-immunosorbent assay (ELISA) kit (MyBioSource, San Diego, California, USA), according to manufacturer's instructions. Briefly, 96-well plates were pre-coated with anti-IL-8 antibody and the biotin conjugated anti-IL-8 antibody was used as detection antibodies. The standards, test samples and biotin conjugated detection antibody (50 µL each one) were added to the wells subsequently, and washed with wash buffer. Streptavidin-Horseradish peroxidase enzyme (HRP) (50 µL) was added, and unbound conjugates were washed away with wash buffer. TMB (3, 3', 5, 5' tetramethylbenzidine) substrates were used to visualize the HRP enzymatic reaction. TMB was catalyzed by

HRP to produce a blue color product that changed into yellow after adding an acidic stop solution. As the density of yellow was proportional to the IL-8 amount of sample captured in plate, the optical density absorbance at 450 nm in a microplate reader (Anthos, Labtec instruments, Austria) was read, and the concentration of IL-8 was calculated. This parameter was determined for both *in vivo* toxicity and additive efficacy trials.

3.2.7. Small intestine morphology, morphometry and cytokines production

Immediately after euthanasia of birds, small intestine samples were taken. The weight of the segment from the duodenum to the ileo-cecal junction was measured, after removal of the intestinal content by squeezing. Length of this segment was measured, and density was calculated as the ratio of the absolute weight to the length of the small intestine. Morphometry analysis of jejunum sections was performed as reported previously (Nofrarías et al., 2006). Briefly, formalinized jejunal sections were dehydrated and embedded in paraffin. Sections, 5 µm thick, were stained with hematoxylin and eosin. Morphometric measurements were performed in 10 well-oriented villi and crypts selected from each intestinal segment, using a light microscope (BHS, Olympus, Barcelona, Spain). The villus height and crypt depth were evaluated by a linear ocular micrometer (Olympus, Microplanet). Villus:crypt ratio was calculated by dividing villus height by crypt depth. The morphological and morphometric parameters were determined for both *in vivo* toxicity and additive efficacy trials. The cytokines determination in jejunum tissues was performed as previously described (Reid et al., 2016). Briefly, total RNA was isolated from approximately 20 mg of tissue using RNeasy minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Isolated RNA was eluted into 50 µL RNase-free water and stored at -80 °C. The yield of RNA was determined by spectrophotometry (BioPhotometer, Eppendorf, Hamburg, Germany). For the *in vivo* toxicity trial, expression of mRNA for the cytokines IL-6, IL-10, IL-1β, and IFN-γ in jejunum tissues was measured by qRT-PCR (Reid et al., 2016). For the *in vivo* additive efficacy trial, mRNA expression for IL-6, IL-8 and TNF-α was measured at 10 and 42 d, and the mRNA expression for IL-1β and IFN- γ was measured at 42 d of the experiment. The mRNA expression of each gene was normalized to its glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression in the same sample. Threshold cycle (Ct) values were obtained, and relative gene expression was calculated using the formula $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001). Relative expression of IL-6, IL-10, IL-1β, and IFN-γ determination was carried out in the Centre de Recerca en Sanitat Animal, (Barcelona,

Spain), and relative expression of IL-8 and TNF- α were performed in Phileo by Lesaffre (Marcq-en Baroeul, France).

3.2.8. Corticosterone determination, fear level and leg color

For the *in vivo* toxicity trial, corticosterone level in plasma was determined using a commercially available ELISA kit according to manufacturer's instructions (Cusabio, Houston, USA). For the additive efficacy trial, feather corticosterone (FCORT) concentrations were measured by an ELISA kit (Corticosterone ELISA kit; Neogen Corporation, Ayr, UK), as previously described by Carbajal et al. (2014). Only samples from control and IMP groups were determined. Corticosterone in plasma analysis was carried out in Echevarne laboratories (Barchelona Spain), and corticosterone in feather analysis was performed at the Universitat Autonoma de Barcelona (Laboratori d'Anàlisi d'Indicadors Hormonals, d'Estrès, Benestar i Reproducció Animal, Barcelona, Spain).

Fear level and leg color were evaluated only for the *in vivo* toxicity trial. To test bird's fear level, tonic immobility reaction was evaluated as described previously by Ghareeb et al. (2014). In a separate room, bird was placed on its back on a laboratory table and was restrained for 45 s on its sternum by experimenter hand while holding the head and neck with the other hand. Next, the experimenter removed his hand gradually. If the bird remained immobile for 20 s a stopwatch was started to record the time until the bird righted itself. The latency until righting was recorded and is defined as the duration of tonic immobility reaction. If the bird righted itself in less than 20 s, the tonic immobility reaction had not been induced, and another induction was started. In the case that the bird did not right itself over 10 min, a maximum score of 600 s was given for the tonic immobility duration.

Footpad color was determined using a Minolta CR-300 with CIE Lab color system (illuminant D65, 2^{o} standard observer, 8 mm aperture Ø): L* corresponds to lightness, a* to green-red color, and b* to blue-yellow color.

4. Data evaluation

Regarding the toxicokinetic study, toxicokinetic parameters were analyzed using non-compartmental analysis. The area under the concentration-time curve from time zero to the last time point (AUC_{0-t}), the area under the concentration-time curve from time zero to infinity ($AUC_{0-\infty}$), maximum plasma concentration (Cmax), elimination rate constant

(k_{el}), half-life of elimination ($T_{1/2el}$), volume of distribution (Vd) and clearance were determined.

Normality of the data and homogeneity of variance were verified. The in vivo toxicity study or the in vivo additive efficacy study were designed as a completely randomized design. Data were analyzed by ANOVA using the GLM procedure of SAS (SAS 9.4, SAS institute, Cary, NC, USA). Data from the toxicity study were subjected also to orthogonal polynomial contrasts (linear or quadratic) to examine the responses to increasing dietary DON in broiler chickens. Data from the additive efficacy study were subjected to orthogonal contrasts to determine the nature of the response exhibited on evaluated variables to the feeding of DON contaminated feed with or without feed additive. Differences between treatment means were tested using Duncan test. Results in tables are reported as least square means and differences were considered statistically significant at $p \leq 0.05$ and tendency at $p \leq 0.1$.

Table 24. Determination of DON biomarkers of toxicity in broiler chickens.

	***In vivo* toxicity trial**	***In vivo* additive efficacy trial**	
Biomarkers	**Evaluated**	**Selected**	**Added**
Specific biomarkers			
DON[1] and metabolites			
DON in plasma			
DON in liver			
DON in excreta			
DON-3S[2] in plasma			
DON-3S in liver			
DON-3S in excreta			
DOM-3S[3] in excreta			
DOM-1[4] in excreta			
Nonspecific biomarkers			
Productive parameters			
BWG[5]			
Feed intake			
FCR [6]			
Relative weight of organ			
Heart			
Proventriculus			
Pancreas			
Cecum			
Gizzard			
Liver			

Table 24. Determination of DON biomarkers of toxicity in broiler chickens.

	***In vivo* toxicity trial**	***In vivo* additive efficacy trial**	
Biomarkers	**Evaluated**	**Selected**	**Added**
Kidneys			
Small intestine			
Colon			
Spleen			
Bursa of Fabricius			
Thymus			
Morphology of small intestine			
Length			
Density			
Villus height			
Crypt depth			
Villus height to Crypt depth			
Blood biochemistry			
Total protein			
Cholesterol			
Triglycerides			
CK[7]			
Albumin			
AST[8]			
ALT[9]			
ALP[10]			
GGT[11]			
LDH[12]			
Uric acid			
Glucose			
Blood hematology			
Hematocrit			
Hemoglobin			
Red blood cell			
MCV[13]			
MCH[14]			
MCHC[15]			
White blood cell			
Eosinophils			
Basophils			
Monocytes			
Lymphocytes			
Heterophils			
Response to common vaccines			
NDV[16]			
IBV [17]			
Cytokines			

Table 24. Determination of DON biomarkers of toxicity in broiler chickens.

	***In vivo* toxicity trial**	***In vivo* additive efficacy trial**	
Biomarkers	**Evaluated**	**Selected**	**Added**
IL[18]-8 in plasma			
IL-8 in jejunum			
IL-1β			
IFN- γ [19]			
TNF-α[20]			
IL-10			
IL-6			
Welfare parameters			
H/L ratio [21]			
Plasma corticosterone			
FCORT [22]			
Fear behaviour			
Leg color			

[1]DON: deoxynivalenol; [2]DON-3S: DON-3 sulphate; [3]DOM-3S: deepoxyDON-3 sulphate; [4]DOM-1: deepoxyDON; [5]BWG: body weight gain; [6]FCR: feed conversion ratio; [7]CK: creatine kinase; [8]ALT: alanine transferase; [9]AST: aminotransferase; [10]ALP: alkaline phosphatase; [11]GGT: gamma-glutamyltransferase; [12]LDH: lactate dehydrogenase; [13]MCV: mean corpuscular volume; [14]MCH: mean corpuscular hemoglobin; [15]MCHC: mean corpuscular hemoglobin concentration; [16]NDV: Newcastle disease virus; [17]IBV: infectious bronchitis virus; [18]IL: interleukin; [19]IFN-γ, interferon-gamma; [20]TNF-α: tumor necrosis factor; [21]H/L ratio: heterophil to lymphocyte ratio; [22]FCORT: feather corticosterone.

Parameters determined in the *in vivo* toxicity trial

Parameters determined in the *in vivo* additive efficacy trial

Parameters determined in the *in vivo* additive efficacy trial

5. References

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EXPERIMENTAL STUDIES

CHAPTER 1

A toxicokinetic study reflecting the absorption, distribution, metabolism and excretion of deoxynivalenol in broiler chickens

A toxicokinetic study reflecting the absorption, distribution, metabolism and excretion of deoxynivalenol in broiler chickens

Abstract

To identify the specific biomarkers of exposure of DON in chickens, a toxicokinetic study was performed via oral or intravenous application of deoxynivalenol (DON). Doses of 0.75 and 2.25 mg DON/kg of body weight (BW) were administrated intravenously or orally to the chickens. Next, blood samples were collected at several time points and plasma was obtained. Liquid chromatography tandem mass-spectrometry (LC-MS/MS) was used to quantify plasma levels of DON and its metabolite DON-3-sulphate (DON-3S). A non-compartmental analysis was performed to study the main toxicokinetic parameters after intravenous or oral application of the toxin. Regarding oral administration, DON plasma level was below the limit of detection (LOD) of the method (1.5 ng/mL) and DON-3S could not be identified. After intravenous administration of DON at 0.75 and 2.25 mg DON/kg BW, the elimination half-life was 57.1 and 47.7 min, respectively, indicating the rapid elimination of DON. The metabolite DON-3S was found in plasma of broilers exposed to DON intravenously. The absence of DON in chicken plasma after oral bolus application suggests the low absorption of this mycotoxin. The presence of DON-3S in plasma, indicates that this metabolite could be the appropriate biomarker of DON exposure in chickens.

Keywords: deoxynivalenol; broiler chickens; toxicokinetic; deoxynivalenol-3-sulphate; plasma.

1. Introduction

The occurrence of mycotoxins in animal feed presents a threat for animal health and can result in important economic loss. Deoxynivalenol (DON) is a mycotoxin, mainly produced by *Fusarium graminearum* and *Fusarium culmorum* that belongs to the trichothecenes family. DON is the most commonly detected mycotoxin in contaminated food and feedstuffs worldwide (Gruber-Dorninger et al., 2019). Poultry species are highly exposed to this mycotoxin due to cereal based- diets. The maximum recommended level of DON in poultry feed was fixed by the European Union in complete feed at 5 mg/kg whilst in pigs was fixed at 0.9 mg/kg (European Commission, 2006).

At molecular and cellular levels, DON inhibits protein biosynthesis and induces ribotoxic and oxidative stress and apoptosis (Payros et al., 2016). The toxic effects of DON are manifested by feed intake reduction, alteration of intestinal functions and immunotoxicity in poultry species (Aguzey et al., 2019). To better understand the mode of action and to evaluate animal and human health risks, knowledge on the kinetic parameters of toxins is essential. The toxicokinetic studies have to be performed to elucidate the absorption, distribution, metabolism and excretion (ADME) pathways of the target mycotoxin in the target species.

In general, poultry species are considered to be tolerant to DON *Fusarium* mycotoxin, what could be explained by differences in toxicokinetic properties. Indeed, the absorption and distribution of DON by poultry species are limited and its elimination is rapid (Payros et al., 2016). With regard to the metabolism, the analysis of suitable metabolites of exposure in blood plasma using validated analytical methods, upon toxicokinetics studies, revealed the formation of DON-3-sulphate (DON-3S) as a metabolite phase II in poultry species (Devreese et al., 2015).

The aim of this study was to assess the toxicokinetics of DON at two concentrations (0.75 and 2.25 mg DON/kg BW), after intravenous or oral bolus administration, in broilers plasma and to determine the most suitable metabolite (s) of exposure of DON.

2. Materials and Methods

2.1. Animals and experimental design

A total of twenty-four one-day old male Ross 308 broiler chickens were randomly distributed into six cages at four chickens per cage for 28 days. The study was approved by the Ethical Committee for Animal Experimentation of IRTA, and carried out in accordance with current regulations on the use and handling of experimental animals (Decree 214/97, Generalitat de Catalunya, Catalonia, Spain). Birds were housed at temperatures of 34 ºC for the first two days and temperature decreased gradually 3 ºC per week until reaching 22 ºC at 28 d. The applied light program was 24 hours of light per day the first two days, 18 hours of light per day until 7 days, and 14 hours of light per day afterwards. Birds were received water and starter blank feed *ad libitum* during the whole trial. The diet was formulated according the nutrient requirements for Ross 308 strain broilers and based on maize and soybean meal (Table 1). The DON and aflatoxin B1 (AFB1) contents of the feed provided were evaluated by high performance liquid

chromatography (HPLC) method (Table 1). The determination of other mycotoxins such as: zearalenone (ZEN), total fumonisins (FBs) and ochratoxin A (OTA) was performed by the Ridascreen® Zearalenon, Ridascreen® Fumonisin and Ridascreen® Ochratoxin A enzyme-linked-immunosorbent assay (ELISA) kits (R-Biopharm), following the manufacturer's instructions, with detection limits of 1.75, 25, and 2.5 μg/kg for ZEN, FBs and OTA, respectively.

Table 1. The formulation and the proximate analysis of the blank control feed.

Ingredients (%)	**Starter: blank control diet 1–28 days**
Maize	54.00
Soybean meal 48 %	36.93
Soybean oil	4.91
Monocalcium phosphate	1.42
Calcium carbonate	1.23
Sodium chloride	0.19
Sodium bicarbonate	0.27
DL-methionine	0.30
L-lysine HCl	0.23
Noxyfeed	0.02
Premix[1]	0.49
Calculated content (%)	
Metabolizable energy (Kcal/kg)	3050
Crude protein	22.0
Ether extract	7.01
Crude fibre	2.36
Lysine	1.38
Methionine + cysteine	0.91
Threonine	0.81
Tryptophan	0.21
Calcium	0.90
Inorganic phosphorus	0.64
Sodium	0.16
Analysed content (%)	
Dry matter	88.9
Crude protein	21.8
Gross energy (Kcal/kg)	4094
Crude fiber	2.40
Ether extract	6.75
Ash	5.56
Sodium chloride	0.30
Mycotoxins (µg/kg)[2]	
DON	65
ZEN	< 1.75
FBs	142
OTA	0.94
AFB1	< 0.3

[1]Vitamin premix provided following nutrients per kg of diet: vitamin A: 13,500 IU; vitamin D3: 4800 IU: vitamin E: 67 IU; vitamin B1: 3 mg; vitamin B2: 9 mg; vitamin B6: 4.5 mg; vitamin B12: 16.5 µg; vitamin K3, 3 mg; calcium pantothenate: 16.5 mg; nicotinic acid, 51 mg; folic acid : 1.8 mg, biotin: 30 µg; Fe: 54 mg; I: 1.2 mg; Co: 0.6 mg; Cu: 12 mg; Mn: 90 mg; Zn: 66 mg; Se: 0.18 mg; Mo: 1.2 mg. [2] DON: deoxynivalenol; ZEN: zearalenone; FBs: fumonisins; OTA: ochratoxin; AFB1: aflatoxin B1; limit of detection of DON, ZEN, FBs, OTA, and AFB1: 50, 1.75, 25, 0.5, 0.3 µg/kg, respectively.

At d 28, birds were weighed individually. From all animals, sixteen birds were used and divided into 4 groups. DON used in the trial was obtained from Fermentek (Jerusalem, Israel) and was dissolved in acetonitrile (ACN) (Honeywell, Seelze, Germany) to obtain 2.5 mg/mL DON as stock solution. Next, single doses of 0.75 or 2.25 mg DON/kg of BW were intravenously or orally administrated to the chickens (4 birds for each application and for each dose), taking into account that the daily feed intake of broilers was 150 g feed/kg BW. The intravenous injection was applied in the wing vein using a 25G needle (0.5 x 16 mm, Terumo Europe, Leuven, Belgium). After the toxin administration, blood samples were taken in heparinized tubes at different time points at (0) pre-administration and at 3, 6, 10, 15, 30, 60, and 120 minutes post-administration. Samples were centrifuged at 2851 × *g* for 10 min and plasma was stored at -20 ºC until further analysis.

2.2. Compounds, Standards and solutions

DON analytical standard was supplied by Sigma-Aldrich Chemie GmbH (Steinheim, Germany). DON standard was dissolved in ACN as stock solution (1 mg/mL), and then diluted with HPLC-grade ACN to obtain an individual working standard solution of 5 µg/mL. The stable internal standard (IS) isotope ($^{13}C_{15}$-DON) was obtained from Biopure (Bioser, Barcelona, Spain) as 1.2 mL of a solution of 25 µg/mL in ACN. An individual working standard solution of 5 µg/mL was prepared by diluting the above stock solution of $^{13}C_{15}$-DON with HPLC-grade ACN and was stored at -15 ºC.

2.3. Instrumentation, materials and reagents

The separation of the analytes was performed on a Zorbax Plus C18 (1.8 µm × 2.1 × 100 mm) column from Agilent (San Jose, CA, USA). Chicken plasma was analyzed on a Transcend 600 LC (Thermo Scientific TranscendTM, Thermo Fisher Scientific, San Jose, CA, USA) coupled to an Orbitrap (ExactiveTM, Thermo Fisher Scientific, Bremen, Germany) with an electrospray ionization (ESI) source (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA). Ammonium formate was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). ACN and Methanol (MeOH) (HPLC gradient grade) were purchased from Honeywell (Seelze, Germany).

2.4. Sample preparation for DON and DON-3S determination in broilers plasma

Preparation of the plasma extract was carried out according to the method of Broekaert et al. (2014). Briefly, for calibrator samples, to 250 µL of blank plasma, 10 µL

of a 5 µg/mL internal standard working solution ($^{13}C_{15}$-DON), 10 µL of a 5 µg/mL of the standard working solution of DON, and ACN was added up to a volume of 1 mL.

For incurred samples, to 250 µL of chicken plasma, 10 µL of $^{13}C_{15}$-DON IS solution (at 5 µg/mL) and 750 µL of ACN were added. ACN was added to precipitate plasma proteins. The samples were vortexed for 1 minute approximately. Afterwards, the samples were centrifuged at 8517 × g for 10 min. The supernatant (1 mL) was transferred to a new tube and was evaporated to dryness under nitrogen flow over a heating block and reconstituted in 1 mL of ammonium formate 5 mM/MeOH (50:50, v/v) solution, and 10 µL were injected for (HPLC-MS/MS) analysis.

2.5. LC-MS/MS analysis

Gradient elution was established with a mobile phase consisting of 5 mM ammonium formate in water (eluent A) and methanol (eluent B) at a flow rate of 0.2 mL/min. The gradient elution started at 95% B at 1 min and it was decreased to 0% B at 8 to 12 min afterwards it increased to 95% at 12.5 min and maintained up to 14 min. The column temperature was set at 25ºC and the injection volume was 10 µL. MS analyses were performed using selected reaction monitoring (SRM) mode with positive and negative electrospray ionization (ESI±). The settings on the spectrometer were as follows: compounds were ionized by electrospray ionization in the positive and negative mode and measured first in full scan and then in targeted MS/MS mode at a collision energy of 30 eV (both in the range from m/z 50-500). ESI parameters were as follows: spray voltage, 4 kV; sheath gas (N2, > 95%), 35 (adimensional); auxiliary gas (N2, > 95%), 10 (adimensional); skimmer voltage, 18 V; capillary voltage, 35 V; tube lens voltage, 95 V; heater temperature, 305 ºC; capillary temperature, 300 ºC. The capillary and nozzle voltage were 4000 V and 95 V (- 95 V in ESI-), respectively. Finally, the data were processed using XcaliburTM version 3.0 (Quanbrowser and Qualbrowser) and Mass FrontierTM 7.0. The retention time and MS/MS parameters are indicated in (Table 2).

Table 2. HPLC-MS/MS parameters.

Analyte	Retention time (min)	characteristic ion (m/z)	Fragment ion (m/z)
DON[1]	8.12	341.1242 $[M+CH_3COO]^-$	201.0747
DON-3S[2]	5.80	375.0744 $[M - H]^-$	345.0640
$^{13}C_{15}$-DON	8.12	310.1659 $[M-H]^-$	243.1310

[1] DON, deoxynivalenol; [2]DON-3S; deoxynivalenol 3-sulphate.

2.6. Toxicokinetic analysis and data evaluation

The toxicokinetic parameters were analyzed using non-compartmental analysis. The area under the concentration-time curve from time zero to the last time point (AUC_{0-t}), the area under the concentration-time curve from time zero to infinity ($AUC_{0-\infty}$), maximum plasma concentration (Cmax); elimination rate constant (k_{el}), half-life of elimination ($T_{1/2el}$), volume of distribution (Vd) and clearance were determined.

3. Results

After a single oral bolus of DON at 0.75 and 2.25 mg/kg BW, no plasma above the LOQ could be observed for DON and its metabolite DON-3S. Plasmatic DON concentration *vs* time profiles after intravenous injection of DON at both doses (0.75 and 2.25 mg/kg BW) are shown in Figure 1. Moreover, from 2 h p.a. no levels above LOQ could be detected for DON (Figure 1).

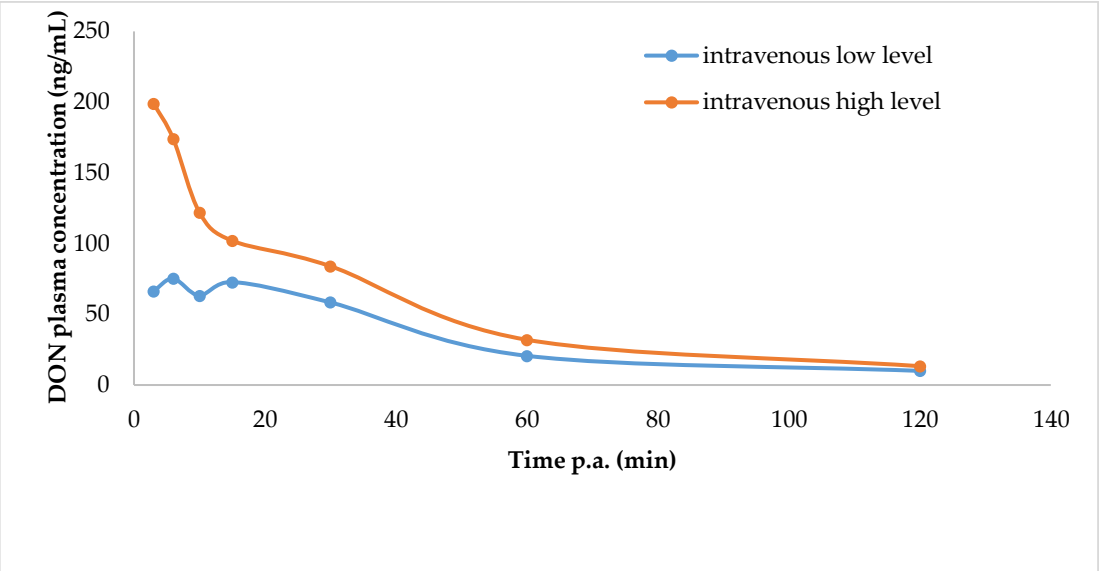

Figure 1. Plasma concentration-time profile of deoxynivalenol (DON) after a single intravenous administration of deoxynivalenol of 0.75 (n=5) and 2.25 mg DON/kg BW (n=4) to broiler chickens.

The HPLC-MS/MS coupled with orbitrap analysis revealed the presence of DON-3S in plasma (Figure 2). Since no commercial standard solution of DON-3S is available, accurate quantitative analysis cannot be performed, and the results were evaluated using the absolute chromatographic peak areas. Average chromatographic peak areas were calculated per treatment and per time (Figure 2).

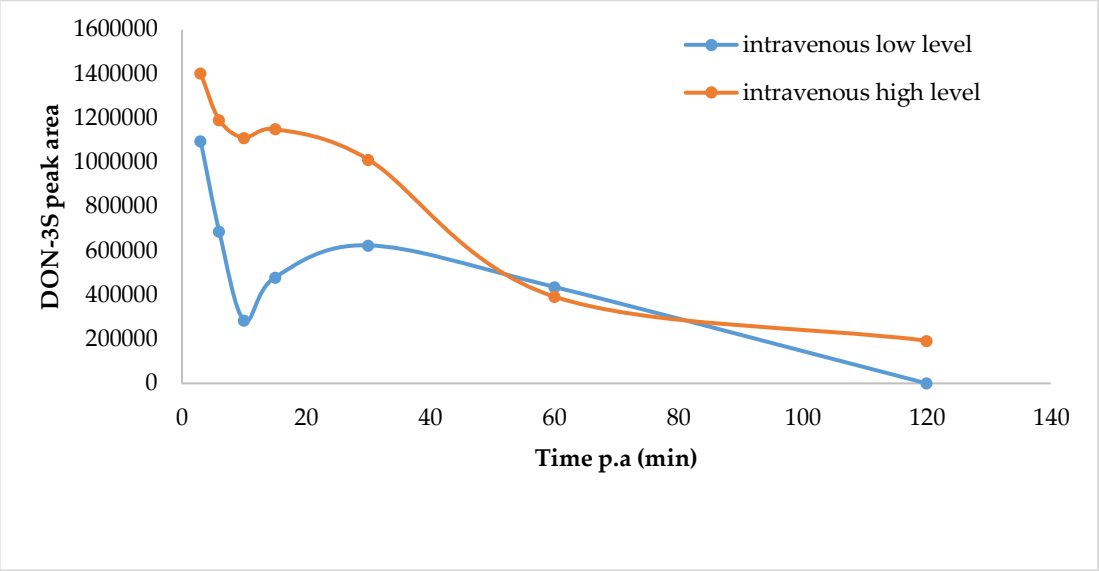

Figure 2. Peak area of deoxynivalenol-3-sulphate (DON-3S) at different times after intravenous (IV) administration of 0.75 (n=5) and 2.25 mg DON/kg (n=4) BW to broiler chickens.

The toxicokinetic analysis of both metabolites was performed based only in data obtained after intravenous administration (Table 3). The study of the main toxicokinetic parameters revealed that the AUC_{0-t} and $AUC_{0-\infty}$ after IV administration at 2.25 mg/kg BW were much higher than after IV administration at 0.75 mg/kg BW, which correlates with the doses administered (Table 3). The toxin clearance from plasma of broilers intravenously exposed to DON (0.75 or 2.25 mg/kg BW) is rapid (0.16 or 0.33 L/min kg). Furthermore, DON showed a remarkably shorter elimination half-life ($T_{½elimination}$ of 57.1 or 47.7 min) after intravenous injection for both doses.

Table 3. Toxicokinetic parameters of deoxynivalenol (DON) and deoxynivalenol-3 sulphate (DON-3S) after intravenous injection of 0.75 (n=5) and 2.25 mg DON/kg BW(n=4) to broiler chickens.

	DON[1] (intravenous low level)	**DON (intravenous high level)**
Toxicokinetic parameters of DON[2]		
(AUC_{0-t}) (ng min/mL)	3726	5838
($AUC_{0-\infty}$) (ng min/mL)	4550	6755
C_0 (ng/mL)	66.4	199
Cmax (ng/mL)	75.2	199
k_{el} (1/min)	0.012	0.014
$T_{1/2\ el}$ (min)	57.1	47.7
V_d (L/kg)	13.1	22.7
Clearance (L/min kg)	0.16	0.33
Toxicokinetic parameters of DON-3S[3]		
(AUC_{0-t}) (peak area * min)	754,444	1,110,110
($AUC_{0-\infty}$) (peak area * min)	754,444	1,111,082
C_0 (ng/mL) (peak area)	1,097,515	1,403,403

[1] DON, deoxynivalenol; [2]AUC0-t, area under the concentration-time curve from time zero to the last time point; AUC0-∞, area under the concentration-time curve from time zero to infinity, Cmax, maximum plasma concentration; k_{el}, elimination rate constant; $T_{1/2el}$, half-life of elimination Vd, volume of distribution; [3]DON-3S, deoxynivalenol 3- sulphate.

4. Discussion

To understand the sensitivity of broiler chickens to the toxic effects of DON, knowledge of the toxicokinetic properties is necessary. Toxicokinetics deals with what the body does with a DON mycotoxin when given a relatively high dose (2.25 mg/kg BW) relative to the guidance dose (0.75 mg/kg BW). The absorption, distribution, metabolism and elimination termed ADME processes are involved in toxicokinetics.

No detection of DON mycotoxin and its metabolite phase II DON-3S in plasma of birds received the toxin orally even at high level (2.25 mg/kg BW) was observerd, presumably due to the low oral bioavailability of this mycotoxin in broilers. (Osselaere et al., 2013). Prelusky et al. (1986) found low levels of radioactivity in plasma (less than 1% of the dose administrated), when, orally administered 2.2 mg/bird/day of radiolabelled DON (14C), to White Leghorn hens by crop intubation. The absolute oral bioavailability of DON mycotoxin was found to be low and accounts to only 19.3% in broiler chickens, 20.9% in Turkeys, and 30.4% in pigeons (Antonissen et al., 2016; Devreese et al., 2015; Osselaere et al., 2013). Furthermore, Broekaert et al. (2015) showed that the absorbed fraction of DON in broiler chickens, after oral DON administration was only 10.6%,

however, in pigs, the absorbed fractions were 100% for DON, which may explain the difference of susceptibility to DON mycotoxicosis between these two species. The concentration of DON in plasma was under LODs for the applied LC–MS/MS method, when broilers fed naturally or artificially contaminated diet up to 5 mg DON/kg diet for 21 or 35 d (Awad et al., 2011; Dänicke et al., 2007; Osselaere et al., 2012).

Lun et al. (1988) reported that DON disappeared from the gastrointestinal tract between the crop and the jejunum, and no DON was detected in the blood from the portal vein or heart of laying domestic chickens after intubation with feed containing contaminated corn.

It has been reported that DON has been widely and transiently distributed into different tissues of broiler chickens (plasma, muscle, abdominal fat, stomach, large and small intestine, liver, kidney, heart, lung, skin, spleen, brain, testes, ovary and adrenals) after using oral administration of radio-labelled DON for 5 days (Wan et al., 2014). In the current study, the low Vd may imply the transient distribution of DON in broiler chickens. The transient distribution may be related to the rapid elimination of DON (Wan et al., 2014).

In regard to the metabolization of DON in broiler chickens, the biotransformation of DON on DON-3 S has been showed in the current study. Devreese et al. (2015) revealed that after using high-resolution mass spectrometry (HR-MS) analysis of plasma of turkeys and broiler chickens, DON-3S was the major metabolite after oral gavage, as well as after intravenous injection of 0.75 mg/kg BW of DON. In the same way, after a semi-quantitative analysis using HR-MS, it has been seen that DON is metabolized to DON-3S in pigeons after intravenous or oral bolus administration of the parent mycotoxin (Antonissen et al., 2016). After a single intra-crop bolus administration of 0.5 mg/kg body weight DON to broiler chickens aged 21 d and weighed 1 kg, DON-3S was detected and no DON was found (Lauwers et al., 2019). . According to the recent study of Jurisic et al. (2019), after feeding a dose of 3.8 and 7.5 mg DON /kg of DON feed to laying hens, the analysis of DON in plasma and eggs by LC–MS/MS revealed that DON-3S is the main metabolite, with an average concentration of 6.8 and 10 ng/mL in plasma. Furthermore, the other metabolites, DOM-1, DON-3α-glucuronide, DON-10-sulfonate, DOM-1-10-sulfonate, were only detected at traces or were not detectable in chickens and Turkey poults (Devreese et al., 2015; Schwartz-Zimmermann et al., 2015).

DON-3S was also detected in excreta after chronic feeding of both levels. Wan et al. (2014) indicated that after the administration of radioactive DON, DON-3S was accounting about 88.6% in excreta of broiler chickens. DON is extensively metabolized to DON-3S in excreta. The conversion of DON to DON-3S could occur in the enterocytes of the intestinal epithelium, in liver and kidney, as hypothesized by Schwartz-Zimmermann et al. (2015). These authors found that DON-3S is the major metabolite in excreta of broiler chickens and its biological recovery accounted 80%. Lauwers et al. (2019) found that the maximum peak of area of DON-3S was achieved in dried excreta after 3-6 h post administration.

DON-3S was reported to be a suitable biomarker of DON exposure in broilers. Sulphation was considered as a detoxification pathway (Yi et al., 2006). Then, this pathway of biotransformation may explain the low susceptibility of broilers to DON toxicity (Wan et al., 2014).

Next to the extensive metabolization on DON-3S, DON is rapidly eliminated in broiler chickens. The results of rapid clearance and short elimination of DON found in the current study were comparable with a previous report in which they found a total body clearance of 0.12 L/min kg and an elimination half-life of 27.9 min after intravenous administration of 0.75 mg/kg BW DON to broiler chickens (Osselaere et al., 2013). Similarly, clearance of DON was 0.11 L/min and $T_{1/2elimination}$ was 18.6 min in broiler chickens exposed to the DON mycotoxin at 0.5 mg/kg BW intravenously (Broekaert et al., 2017). This is again comparable to turkeys with a clearance of 0.13 L/min and is rapidly eliminated ($T_{1/2elimination}$ = 37.2 min) upon intravenous DON injection at 0.75 mg/kg BW (Devreese et al., 2015). Likewise, the toxicokinetic study of DON in pigeon revealed that upon intravenous administration of 0.3 mg/kg BW, the total body clearance was 0.20 L/min kg and the elimination half-life was 20.4 min (Antonissen et al., 2016). The rapid elimination is probably linked with the polarity of DON and the lower volume of distribution. The rapid clearance and elimination might be the reason that poultry are less sensitive to DON compared with other species (Antonissen et al., 2016).

5. Conclusions

In conclusion, from the results of this research, it could be confirmed the low absorption, the limited accumulation and the rapid clearance and excretion of DON in broilers. DON was metabolized in DON-3S and this latter phase II metabolite can be

considered the suitable biomarker of DON exposure in broiler chickens. The characteristics of ADME process of DON in broiler chickens imply the tolerance of this specie to the toxic effects of this mycotoxin.

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CHAPTER 2

Effects of deoxynivalenol-contaminated diets on productive, morphological, and physiological indicators in broiler chickens

Effects of deoxynivalenol-contaminated diets on productive, morphological, and physiological indicators in broiler chickens

Abstract

The present study with 1-day-old male broilers (Ross 308) was conducted to evaluate the effects of deoxynivalenol (DON) at different levels (5 and 15 mg/kg feed) on growth performance, relative weight of organs, morphology of the small intestine, serum biochemistry, and welfare parameters of broiler chickens. Forty-five broiler chicks were randomly divided into three different experimental groups with five replicates each: (1) control group received a non-contaminated diet, (2) contaminated diet with 5 mg DON/kg of feed, and (3) contaminated diet with 15 mg DON/kg of feed for 42 days. Results showed that feed artificially contaminated with DON at guidance level (5 mg/kg diet) did not affect growth performance parameters. However, 15 mg/kg reduced body weight gain and altered feed efficiency. DON at two assayed levels significantly increased the absolute and relative weight of thymus and the relative weight of gizzard and decreased the absolute and the relative weight of the colon. Compared to controls, both doses affected small intestine morphometry parameters. In terms of biochemical indicators, DON at 5 mg/kg reduced the creatine kinase level and at 15 mg/kg DON reduced the cholesterol level. Furthermore, DON at 15 mg/kg induced more fear in broilers compared to broilers fed the guidance level. It was concluded that even the guidance level of DON did not affect the chickens' performance. However, its toxic effect occurred in some organs and biochemical parameters.

Keywords: deoxynivalenol; broilers; guidance level; performance; organ weights; small intestine; blood biochemistry; welfare

1. Introduction

Deoxynivalenol (DON) is a secondary metabolite produced by *Fusarium* fungi. DON is the most widespread mycotoxin found in grains such as wheat, rye, barley, maize, oats, and their byproducts (Creppy, 2002; Escrivá et al., 2015). A recent survey, reported that DON is the most frequent contaminant of feedstuffs in Europe (Biomin, 2018). From 4311 samples evaluated, 63% were positive, with an average positive level of 0.6 mg/kg and a maximum of 40.7 mg/kg (Biomin, 2018). For poultry feeding stuffs, the recommended maximum level for DON is set at 5 mg/kg (European Commission, 2006), although lower doses of DON could cause adverse effects on performance (Chen et al.,

2017; Lucke et al., 2017). On the contrary, other reports indicated that DON at higher than the recommended tolerance value did not cause adverse effect on zootechnical parameters. Body weight gain, feed intake, and feed conversion ratio of broiler chickens were not affected by the inclusion of naturally or artificially contaminated diets with DON at 10 mg/kg DON for 35 and 42 d of age (Awad et al., 2004, 2006a; Ghareeb et al., 2014, 2016). Furthermore, an important list of investigations indicated that this toxic effect could be observed when birds fed concentration of DON greater than 15 mg/kg (Huff et al., 1986; Kubena et al., 1989).This suggests that chickens may be relatively tolerant to this *Fusarium* toxin. However, it has been observed that DON-contaminated feed could affect organ weights, blood biochemical, and immunological parameters (Ghareeb et al., 2013; Kubena et al., 1997). Interestingly, it was reported that broilers exposed to dietary DON had a higher stress index (heterophil to lymphocyte ratio) (Ghareeb et al., 2012; Xu et al., 2011), meanwhile there are few studies regarding the effects of DON on stress of animals. The fear response is a welfare-related behaviour as expressed by tonic immobility reaction and can provide more information on bird's stress status (Campo et al., 2005; Ghareeb et al., 2014). However, lack of information was reported on the effect of DON feeding on this indicator.

The first purpose of this study herein was to test two doses of DON mycotoxin to better understand tolerable DON level in broilers feed, to explore potential damages a lower level might cause, and to see if, in the practice conditions, broilers could tolerate up 15 mg/kg of DON through evaluating some indicators of effect of toxicity such as productive, morphological and physiological parameters. Furthermore, due to the lack of information about DON effect on bird's welfare, we hypothesized that both doses can affect the welfare status in the terms of fear response and footpad color.

2. Materials and Methods

2.1. Ethical approval

All animal care procedures were approved by the Ethical Committee for Animal Experimentation of IRTA, in accordance with current regulations on the use and handling of experimental animals (Decree 214/97, Generalitat de Catalunya, Catalonia, Spain).

2.2. Experimental design, birds, and diets

A total of forty-five 1-d-old male chicks (Ross 308) was randomly distributed in fifteen battery cages from 1 d to 42 d of age. Temperature in the first two days was 34 °C,

then the temperature was gradually decreased 3 °C weekly to 21 °C until the end of the experiment. Twenty-four hours of light was provided during the first two days with a reduction to 18 h until 7 days and 14 h afterward. Chickens were fed a starter diet from 1 to 21 days and a grower diet from 22 to 42 days. Diets were formulated to meet or exceed broilers requirements according to NRC (1994). Chickens of treatment 1 received a starter and grower basal diet composed of maize (54 and 59%), soybean meal (36 and 31%), soy oil (4.9 and 5.7%), monocalcium phosphate (1.42 and 1.30%), minerals, amino acids, and a premix with vitamins and minerals (0.49 and 0.44%). Chickens of treatments 2 and 3 received basal diet contaminated with DON at 5 mg/kg feed or 15 mg/kg feed, respectively. Each treatment had 5 replicated pens with each pen containing 3 birds. Feed in mash form and water were provided for *ad libitum* consumption.

2.3. Analyses

Representative feed samples for each group were analyzed for the content of dry matter, crude protein, gross energy, crude fiber, ether extract, crude ash, and sodium chloride (AOAC, 2004) (Table 1). For the trial, DON was produced by growing *in vitro Fusarium graminearum* strain I159 on wheat, in accordance to the protocol described by (Metayer et al., 2019) (ENVT, Toulouse, France), and was mixed into the experimental feed. The powdered culture material was included to obtain 5 or 15 mg DON/kg feed. DON levels were confirmed by liquid chromatography mass spectrometry. In the basal starter diet, DON concentration was 65 µg/kg feed, while in DON-contaminated starter diets, DON was 4760 and 14,390 µg/kg for treatments 2 and 3, respectively. The level of DON in the grower basal diet was 73 µg/kg feed, and 4650 and 15,120 µg/kg for treatment 2 and 3, respectively. HPLC analysis also confirmed that aflatoxin B1 (AFB1) was below the limit of detection (0.3 µg/kg). Zearalenone (ZEN), fumonisins (FBs) and ochratoxin A (OTA) were determined using Ridascreen® and Elisa kits (R-Biopharm) following the manufacturer's instructions. The limit of detection (1.75, 25, and 2.5 µg/kg for ZEN, FBs, and OTA, respectively) (Table 1).

Table 1. Analyzed composition and mycotoxin contamination of the experimental diet.

Item	Control Group	DON [1] Group (5 mg/kg)	DON Group (15 mg/kg)
Broiler starter			
Dry matter (%)	88.9	88.9	89.0
Crude protein (%)	21.8	21.3	21.5
Gross energy (Kcal/kg)	4094	4147	4170
Crude fiber (%)	2.40	2.20	2.48
Ether extract (%)	6.75	7.13	7.24
Ash (%)	5.56	5.65	5.65
Sodium chloride (%)	0.30	0.32	0.32
Mycotoxins (µg/kg)			
DON	65	4760	14,390
ZEN	< 1.75	84.4	242
FBs	142	257	216
OTA	0.94	0.90	1.21
AFB1	< 0.3	< 0.3	< 0.3
Broiler grower			
Dry matter (%)	89.1	89.1	89.1
Crude protein (%)	19.6	19.8	19.6
Gross energy (Kcal/kg)	4186	4208	4240
Crude fiber (%)	2.24	2.37	2.48
Ether extract (%)	7.84	7.94	8.02
Ash (%)	4.98	4.97	4.96
Sodium chloride (%)	0.31	0.35	0.33
Mycotoxins (µg/kg)			
DON	73	4650	15,120
ZEN	< 1.75	85.9	259
FBs	225	216	275
OTA	1.59	1.11	1.10
AFB1	< 0.3	< 0.3	< 0.3

[1] DON : deoxynivalenol; ZEN :zearalenone; FBs :fumonisins; OTA :ochratoxin; AFB1 : aflatoxin B1; limit of detection of DON, ZEN, FBs, OTA, and AFB1: 50, 1.75, 25, 0.5, 0.3 µg/kg, respectively.

2.4. Productive parameters and organ weights

Chickens were tagged in their wing and weighed individually, and feed consumption for each pen was determined at 21 d, 35 d, and 42 d of age. Body weight gain and feed intake were calculated and feed conversion ratio was calculated as the ratio between feed intake and body weight gain for 21 d, 35 d, and 42 d. The incidence of mortality was recorded daily. On d 42, chickens were weighed individually, and 15 birds in each treatment were humanely euthanized according to IRTA ethics instructions. Proventriculus, pancreas, heart, gizzard, liver, kidneys, small intestine, cecum, colon, spleen, thymus, and bursa of Fabricius were excised and weighed. Organ weights were

presented as direct measured and defined as absolute organ weights. Relative organ weights were expressed as a percentage of body weight.

2.5. Morphological and histological traits of small intestine

The gastrointestinal tract of chickens (12/treatment) euthanized at 42 d (from the duodenum to the ileocecal junction) was weighed after removal of the content by gentle squeezing. Absolute weight and length were determined and relative weight and density were calculated. Density of the intestines was calculated as the ratio between the absolute weight in grams and the length of the intestine in centimeters.

The jejunum was considered the segment between the end of the duodenal loop and Meckel's diverticulum. Intestinal jejunum samples were taken close to the junction of Meckel's diverticulum and then sampled and fixed in 10% neutral-buffered formalin solution for at least 48 h. Each tissue sample was dehydrated in serial alcohol baths, cleared in xylene, and embedded in paraffin wax using an automatic tissue processor system (Leica, TP 1020, Barcelona, Spain). Tissue blocks were mounted, sectioned at 4 μm, and stained with hematoxylin and eosin. Intestinal jejunum samples were observed with a light microscope (BHS, Olympus, Barcelona, Spain). Villus height and crypt depth were measured. Measurements were taken in 10 well-oriented villi and crypts from each intestinal section of each animal (Nofrarías et al., 2006). Villus height and crypt depth were measured using a linear ocular micrometer (Olympus, Microplanet, Barcelona, Spain). Results are expressed in μm. Villus: crypt ratio was calculated dividing villus height by crypt depth.

2.6. Blood biochemistry

At d 42, blood samples (1 mL/bird, 3 birds/pen) were collected by cardiac puncture into non heparinized tubes for serum biochemistry. Serum samples were obtained by centrifugation at 978 × g for 10 min. Total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholesterol, alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), glucose, lactate dehydrogenase (LDH), uric acid, and creatine kinase (CK) were determined using an automatic biochemical analyzer (Olympus AU5800, Beckman Coulter, Brea, CA, USA).

2.7. Fear behaviour and leg color

Fear levels (tonic immobility reaction) were determined according to the method described by (Campo et al., 2005; Ghareeb et al., 2014). Briefly, at d 28 and 35 of the

trial, 15 birds per treatment were treated individually to determine the fear test. In a separate room, bird was placed on its back on a laboratory table and was restrained for 45 s on its sternum by experimenter hand. Then, the experimenter removed his hand gradually. If the bird remained immobile for 20 s. a stopwatch was started to record the time until the bird righted itself. The latency until righting was recorded and is defined as the duration of tonic immobility reaction. If the bird righted itself in less than 20 s, the tonic immobility reaction had not been induced, and another induction was started. In the case that the bird did not right itself over 10 min, a maximum score of 600 s was given for the tonic immobility duration.

At the end of the trial the footpad color of 3 chickens per replicate was determined by means of a Minolta CR-300 with CIE Lab color system: L* corresponds to lightness, a* to green–red chromaticity, and b* to blue–yellow chromaticity.

2.8. Statistical analysis

Data were analyzed as a completely randomized design and were showed as means ± RMSE. Each cage was considered the experimental unit. Data were analyzed by one way analysis of variance (ANOVA) using the General Linear Model Procedure of SAS software (SAS 9.4, SAS Institute, Cary, NC, USA) to examine the effect of different treatments. Orthogonal polynomials were used to determine linear and quadratic dose responses. To test the normal distribution of data, Kol–Mogrov–Smirnov test (named after Andrey Kolmogorov and Nikolai Smirnov) was used. All statements of differences were considered significant at $p \leq 0.05$ and trends were considered at $0.05 < p < 0.10$.

3. Results

3.1. Growth performance and organ weight

The results of productive parameters and mortality are listed in (Table 2). After 42 days of trial, DON presence at 5 mg/kg did not affect these parameters compared with the control group ($p > 0.05$). However, a tendency of decrease in a linear way ($p = 0.08$) was observed in broilers fed 15 mg/kg feed on body weight gain (BWG) which was 6% lower than broilers fed the control diet. In addition, DON at 15 mg/kg altered the feed conversion ratio ($p = 0.03$) in a linear way. No significant effects of dietary treatments were observed on mortality. The results of absolute and relative organ weights are presented in (Table 3). Compared to control group, DON treatments elevated the absolute weight of thymus ($p = 0.0002$) and decreased the absolute weight of colon ($p = 0.01$).

Moreover, an increase in the relative weight of gizzard ($p = 0.006$) and thymus ($p < 0.001$) and a reduction in the relative weight of colon ($p = 0.03$) was observed after feeding different levels of DON (5 and 15 mg/kg) to chickens aged 42 d.

Table 2. Effects of deoxynivalenol (DON)-contaminated feed (5 and 15 mg/kg) on growth performance of broiler chickens.

Dietary Treatment [1]							
Item	**Control**	**DON(5 mg/kg)**	**DON(15 mg/kg)**	**[2]RMSE**	***p*-Value**	**Linear**	**Quadratic**
BWG (g/d/bird)							
d 0 to 21	29.1	30.7	30.4	4.42	0.83	0.70	0.65
d 0 to 35	50.2	50.7	48.5	3.71	0.67	0.46	0.60
d 0 to 42	58.5	57.4	54.7	3.10	0.22	0.08	0.94
Feed intake (g/day/bird)							
d 0 to 21	42.4	43.0	40.4	2.76	0.38	0.24	0.43
d 0 to 35	72.4	72.4	69.7	4.73	0.64	0.38	0.73
d 0 to 42	85.3	86.9	88.0	6.29	0.80	0.53	0.85
Feed conversion ratio (g:g)							
d 0 to 21	1.46	1.41	1.40	0.12	0.73	0.53	0.65
d 0 to 35	1.44	1.42	1.43	0.03	0.94	0.77	0.86
d 0 to 42	1.45	1.51	1.55	0.06	0.09	0.03	0.49
Mortality (%)							
d 0 to 42	13.3	19.9	13.3	18.2	0.80	0.90	0.52

[1] DON: deoxynivalenol; [2]RMSE: root mean square error (n = 5); [a,b]: means values with different superscripts with the same row differ ($p \leq 0.05$).

Table 3. Effects of deoxynivalenol (DON)-contaminated feed (5 and 15 mg/kg) on organ weights of broiler chickens.

Dietary Treatment [1]							
Item	**Control**	**DON (5 mg/kg)**	**DON (15 mg/kg)**	**[2]RMSE**	***p*-Value**	**Linear**	**Quadratic**
Gizzard (g)	39.3	42.6	39.4	4.89	0.19	0.76	0.07
Liver (g)	53.3	56.5	52.6	8.85	0.53	0.70	0.31
Kidneys (g)	13.4	14.0	13.3	2.03	0.71	0.81	0.43
Colon (g)	2.89^{a}	2.04^{b}	2.12^{b}	0.74	0.01	0.03	0.03
Thymus (g)	9.58^{b}	16.5^{a}	14.2^{a}	3.81	0.0002	0.02	0.0003
Bursa of Fabricius (g)	6.44	5.87	5.15	1.86	0.26	0.10	0.83
Gizzard (%)	1.46^{b}	1.65^{a}	1.62^{a}	0.14	0.006	0.04	0.01
Liver (%)	1.99	2.19	2.08	0.27	0.25	0.61	0.11
Kidneys (%)	0.51	0.54	0.53	0.07	0.58	0.60	0.36
Colon (%)	0.11^{a}	0.08^{b}	0.08^{b}	0.02	0.03	0.09	0.04
Thymus (%)	0.33^{b}	0.63^{a}	0.57^{a}	0.13	< 0.0001	0.001	< 0.0001
Bursa of Fabricius (%)	0.24	0.22	0.19	0.07	0.22	0.08	0.95

[1] DON: deoxynivalenol; [2]RMSE: root mean square error (n = 5); [a,b]: means values with different superscripts with the same row differ ($p \leq 0.05$).

3.2. Morphological traits of small intestine

Density of the small intestine was defined as the ratio of the absolute weight of small intestine to the length. A reduction in the absolute weight ($p = 0.05$) and a reduction trend of the relative weight of the small intestine and small intestine (a trend) ($p = 0.09$) was observed after exposure to DON in broilers. Furthermore, the small intestine was longer ($p = 0.01$) for birds fed dietary DON compared to control chickens resulting in a lower density ($p = 0.001$) for birds fed DON (Table 4). No significant effect was observed on villus height, crypt depth, and ratio of villus height to crypt depth (Table 5).

Table 4. Effects of deoxynivalenol (DON)-contaminated feed (5 and 15 mg/kg) on small intestine morphology of broiler chickens.

Dietary Treatment [1]							
Item	**Control**	**DON (5 mg/kg)**	**DON (15 mg/kg)**	**[2]RMSE**	***p*-Value**	**Linear**	**Quadratic**
Small intestine (g)	67.7^{a}	59.9^{b}	59.3^{b}	8.11	0.02	0.02	0.10
Small intestine (%)	2.57^{A}	2.34^{B}	2.34^{B}	0.29	0.09	0.09	0.09
Length (cm)	192^{b}	206^{a}	208^{a}	14.9	0.01	0.01	0.10
Density (g/cm)	0.34^{a}	0.29^{b}	0.28^{b}	0.04	0.001	0.002	0.02

[1] DON: deoxynivalenol; [2]RMSE: root mean square error (n = 5); ^{a,b}: means values with different superscripts with the same row differ ($p \leq 0.05$). ^{A,B}: means values with different superscripts with the same row differ ($p < 0.01$).

Table 5. Effects of deoxynivalenol (DON)-contaminated feed (5 and 15 mg/kg) on small intestine histology of broiler chickens.

Dietary Treatment [1]							
Item	**Control**	**DON (5 mg/kg)**	**DON (15 mg/kg)**	**[2]RMSE**	***p*-Value**	**Linear**	**Quadratic**
Villus height (µm)	956	921	928	169	0.90	0.79	0.76
Crypt depth (µm)	128	102	99	31.6	0.12	0.11	0.31
Villus height-to-crypt depth ratio	7.84	9.46	9.74	2.11	0.14	0.11	0.36

[1] DON:deoxynivalenol; [2]RMSE: root mean square error; (n = 5).

3.3. Blood biochemistry

There was no effect of DON on blood biochemical values ($p > 0.05$), except for a decline in creatine kinase at 5 mg/kg DON in quadratic way ($p = 0.04$) and cholesterol level at 15 mg/kg DON ($p = 0.004$) (Table 6).

Table 6. Effects of deoxynivalenol (DON)-contaminated feed (5 and 15 mg/kg) on blood biochemistry of broiler chickens.

Dietary Treatment [1]							
Item[2]	**Control**	**DON (5 mg/kg)**	**DON (15 mg/kg)**	**[3]RMSE**	***p*-Value**	**Linear**	**Quadratic**
Total protein (g/L)	29.7	29.6	30.0	2.10	0.92	0.74	0.81
Albumin (g/L)	10.1	10.0	9.84	0.76	0.56	0.29	0.90
AST (U/L)	332	285	314	64.3	0.24	0.71	0.10
ALT (U/L)	2.46	2.16	2.23	0.74	0.57	0.52	0.41
Cholesterol (mg/dL)	147 [a]	137 [ab]	127 [b]	14.8	0.004	0.001	0.47
ALP (U/L)	5807	4849	4893	2239	0.48	0.37	0.43
GGT (U/L)	23.7	22.5	20.8	5.22	0.36	0.16	0.90
Glucose (mg/dL)	252	249	252	10.6	0.74	0.72	0.50
LDH (U/L)	3894	3689	3200	1142	0.32	0.13	0.95
Uric acid (mg/dL)	5.63	5.01	4.70	1.36	0.21	0.10	0.52
CK (U/L)	9532	4412	7527	5571	0.10	0.63	0.04

[1] DON: deoxynivalenol; [a,b]: means values with different superscripts with the same row differ ($p \leq 0.05$). [2]AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; GGT: gamma-glutamyl transferase; CK: creatine kinase; [3]RMSE: root mean square error (n = 5).

3.4. Fear behaviour and leg color

The effects of experimental treatments on tonic immobility reaction and footpad color are shown in (Table 7). Birds fed DON at 15 mg/kg showed longer tonic immobility duration than birds fed 5 mg/kg at 35 d ($p = 0.05$). Furthermore, no marked differences among the diets groups were detected for the footpad color.

Table 7. Effects of deoxynivalenol (DON)-contaminated feed (5 and 15 mg/kg) on welfare parameters of broiler chickens.

Dietary Treatment [1]							
Item	**Control**	**DON (5 mg/kg)**	**DON (15 mg/kg)**	**[2]RMSE**	***p*-Value**	**Linear**	**Quadratic**
Fear behaviour							
Tonic immobility duration (s)							
28 d	132	103	118	127	0.85	0.86	0.59
35 d	169 [ab]	85.7 [b]	246 [a]	151	0.05	0.09	0.06
Number of inductions							
28 d	1.61	1.50	1.84	1.09	0.72	0.52	0.62
35 d	1.38	1.25	1.53	0.72	0.61	0.49	0.47
Footpad color[3]							
L	78.0	77.8	77.6	1.78	0.87	0.62	0.88
a	3.24	2.87	3.13	1.18	0.74	0.94	0.45
b	30.6	33.7	31.9	5.36	0.35	0.70	0.16

[1] DON: deoxynivalenol; [2]RMSE: root mean square error (n = 5); [a,b]: means values with different superscripts with the same row differ ($p \leq 0.05$); [3]L: lightness, a: green–red color; b: blue–yellow color.

4. Discussion

DON mycotoxin is frequently detected in poultry feed and chickens are considered relatively tolerant to DON in terms of growth and performance parameters (Ghareeb et al., 2016), although several studies show highly variable effect of DON on these parameters. Growth performance parameters of broiler chickens were not affected by DON guidance value (5 mg/kg) inclusion in experimental groups in present study. Likewise, lack of effect of DON at 5 mg/kg in feed on body weight gain, feed intake and feed conversion ratio was reported by researchers (Awad et al., 2006a; Yu et al., 2018). Even at 10 mg/kg tested in broiler diets, body weight, bodyweight gain, feed intake, and feed conversion ratio were not adversely affected for 35 d (Ghareeb et al., 2014, 2016) and for 42 d (Awad et al., 2004, 2006a). These results observed might be suggestive of adaptation of birds to mycotoxins over time and that poultry are relatively tolerant to DON mycotoxicosis compared with other species especially pigs due to the differences in DON absorption, distribution, metabolism, and elimination (ADME) (Broekaert et al., 2017). Osselaere et al. (2013) found that the absolute oral bioavailability in broilers chickens was accounting for only 19.3%, and therefore contributing to the poor absorption of DON. Furthermore, they reported that DON is characterized by its high clearance and rapid elimination. The tolerance of broilers to DON could also be explained by the extensive metabolization of DON in DON-3 sulphate; this phase II metabolite is

much less toxic than DON itself (Schwartz-Zimmermann et al., 2015). Interestingly, DON at 15 mg/kg tended to decrease the BWG and altered the feed conversion ratio at the final of the experiment. This finding is consistent with the finding of (Kubena et al., 1997). The differences occurred by the presence of 15 mg/kg DON in feed could be justified by the mode of action of this toxin that is principally the inhibition of protein synthesis at the elongation or termination steps (Feinberg & Mclaughlin, 1989).

Previous studies suggested that the organ weights could be influenced by mycotoxins (Awad et al., 2014). The most susceptible tissues to trichothecenes mycotoxicosis, including DON, are those with high protein turnover rates, such as the immune system (bone marrow, lymph nodes, spleen, and thymus), the liver, the intestinal mucosa, and the small intestine (Feinberg & Mclaughlin, 1989). Consequently, it was expected that DON might affect immune organs (such as thymus, spleen, and bursa of Fabricius), liver, and small intestine weights as organ targets. In the current study, DON treatments increased significantly the relative weight of gizzard. Similarly, an increase in the relative weight of this organ after DON exposure (up to 16 mg/kg feed) was observed in other studies with broiler chickens (Awad et al., 2004; Kubena et al., 1989). The increase in relative weight of gizzard may be related to a difference in the density of the diet, or may be a consequence of irritation of the upper gastrointestinal tract (Kubena et al., 1985). Birds exposed to highly DON-contaminated diets (82.8 mg/kg) for 27 d had small erosions in the gizzard mucosa (Lun et al., 1986). The changes observed in weights of thymus and colon were not mentioned in previous reports. However, the relative weight of thymus of broilers exposed to DON (from 1 to 12.20 mg/kg for 5 wk) did not change significantly (Awad et al., 2011; Yunus et al., 2012a). Similarly, the relative weight of colon was unchanged in broilers fed dietary DON at different levels 1, 5, and 10 mg/kg for different experimental periods (3, 5, and 6 wk) (Awad et al., 2006 a,b, 2011). The increase in relative weight of thymus induced by DON treatments showed in this study was expected as thymus is a lymphoid organ and with high protein turnover. From this current study, a reduction in absolute weight and a trend of the reduction in relative weight of small intestine were noted. Relative weight of small intestine of broilers fed 5 mg/kg DON for 21 d decreased (Awad et al., 2006 b). Similarly, Yunus et al. (2012 b) tested two levels of DON in broiler chickens, low level (1.68 mg/kg feed) and high level (12.20 mg/kg feed), and reported a reduction in the relative weight of small intestine segments (duodenum and jejunum). This reduction may be strongly related with other morphological changes

observed when birds were fed DON such as the decrease in villus height (Yunus et al., 2012b). Thus, the higher relative weights of gizzard and thymus and the reduction in the relative weights of colon and small intestine after DON intoxication suggests that DON caused a non-direct effect on chickens. It may include either enlargement or atrophy of the internal organs, probably due to irritation or cell damage, or it is an indicative of the animal's necessary immune responses to dietary contaminations (Awad et al., 2014).

The gastrointestinal tract (GIT) is the first barrier against ingested chemicals, feed contaminants, and natural toxins (Awad et al., 2008). The intestinal epithelium cells can be exposed to high concentrations of DON following ingestion of contaminated diets (Sharma, 1993). The morphology of the intestine could give some information on gastrointestinal development (Xia et al., 2019). Therefore, as intestinal morphology indicators of toxicity of DON, parameters included in the absorption process were evaluated such as the length of small intestine, villus height, and crypt depth. Birds fed DON in the current investigation had longer small intestines and lower density than birds fed the control diet. Consistent with results found by Yunus et al. (2012b), the length of duodenum and jejunum increased, and therefore, the density of small intestine decreased significantly in broilers fed DON low level (1.68 mg/kg feed) and DON high level (12.20 mg/kg feed). We speculate that this may be explained by the decrease in villus height induced by dietary DON (numerical decrease in this study), which could be accompanied by lower electrophysiological properties, which resulted in low absorption of glucose in the small intestine (Awad et al., 2004). Furthermore, Yunus et al. (2012b) indicated that with the increase in length and the density decrease in the small intestine, higher absorption of DON, reduction in the absorption of nutrients, and therefore, a decrease in performance was expected.

It was reported that DON could reduce villus height, which was explained by villus contraction and resulted in nutrient transport and utilization impairment and, therefore, could impair zootechnical parameters (Alizadeh et al., 2015; Yu et al., 2018). However, in the current study, this decrease did not reach statistical significance, probably due to the difference of number of samples between treatments. It has been reported that diets containing DON at different levels (2, 5, and 10 mg/kg feed) for 16 weeks have no significant effects on villus height of jejunum and ileum of Taiwan country chickens (Chen et al., 2017). In addition to that, crypt depth of broiler chickens was not affected by DON treatments (1 and 5 mg/kg) during 35 d (Awad et al., 2011). Similar results

regarding crypt depth were observed when broiler chickens were fed with 18 mg DON/kg feed for 21 d (Xu et al., 2011). However, decreased villus height of small intestine of broilers exposed to DON mycotoxin has been described in other studies (Yu et al., 2018; Yunus et al., 2012b).

From the present study, it is clear that high DON levels decreased serum cholesterol level. This change could be related to possible damage in liver function induced by DON mycotoxin. Similarly, Kubena et al. (1987) observed a reduction in the serum cholesterol level of White Leghorn chickens fed 18 mg/kg of DON-contaminated grains for 28 d. The authors explained this decrease to liver involvement and a shift of concentrations from the blood to the liver. In their meta-analysis, Andretta et al. (2012) reported that broilers fed challenged mycotoxins (T2, FBs, DON, OTA, and ZEN) showed lower total cholesterol compared to negative control birds and that this reduction was about 14%. In contrast, other authors have described increases in cholesterol levels. Thus, higher cholesterol levels in broilers fed DON (10 mg/kg) for 35 d was found by (Ghareeb et al., 2016). The authors suggested also that this increase may be due to liver or kidney function damage or stress. Additionally, DON-contaminated diet at 5 mg/kg decreased the creatine kinase level, and this reduction could reflect the rates of the loss of this enzyme from the circulation (Husic & Suelter, 1983) or cell damage with leakage of the contents into the blood (Andretta et al., 2012). Andretta et al. (2012) indicated that broilers fed challenged mycotoxins (T2, FBs, DON, OTA, and ZEN) had lower CK than the negative control birds (−27%).

Few studies suggested that the stressful effect of *Fusarium* mycotoxins included DON appeared through the alteration of the brain regional neurochemistry. For example, the inclusion of contaminated grains with *Fusarium* mycotoxin in the diet of broiler chickens increased concentrations of 5-hydroxytryptamine (5 HT) in the pons and in the cortex (Swamy et al., 2004). Knowing that the 5-HT system takes place in the regulation of fear, we suggested that DON could affect the fear level in broilers. Therefore, stress-related behaviour in the current study was evaluated through evaluating the duration of tonic immobility at week 4 and 5, which was prolonged in birds fed a high DON level compared with birds fed a low DON level. These results indicated that chickens fed a higher level of DON had a higher level of fear and probably reinforce the poor performance induced at this level.

5. Conclusions

In conclusion, broiler chickens could tolerate dietary contaminated DON in terms of growth at the guidance value but not at 15 mg/kg. However, the effect of DON is apparent from 5 mg/kg to 15 mg/kg on organ weights such as the gizzard, thymus, and colon and on the small intestine morphology. In addition, the effect of both doses was apparent on the physiological and behaviour status of the birds through impacting the blood cholesterol, creatine kinase level, and fear response. The changes obtained on parameters evaluated appear as indirect response to the dietary DON and give a better understanding of the DON-tolerable level in poultry feeding. Further studies should evaluate relevant biomarkers of toxicity of DON related to the health and welfare of chickens.

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CHAPTER 3

Effects of deoxynivalenol-contaminated diets on metabolic and immunological parameters in broiler chickens

Effects of deoxynivalenol-contaminated diets on metabolic and immunological parameters in broiler chickens

Abstract

The current study was conducted to examine the effects of deoxynivalenol (DON) at different levels (5 and 15 mg/kg feed) on the metabolism, immune response and welfare parameters of male broiler chickens (Ross 308) at 42 days old. Forty-five one- day-old broiler chickens were randomly distributed into three different dietary treatments: 1) control, 2) DON-contaminated diet with 5 mg DON/kg of feed (guidance level), and 3) DON-contaminated diet with 15 mg DON/kg of feed. Five replicated cages with three birds each were used for each treatment in a randomized complete block design. The results showed that DON was detected in excreta of birds fed contaminated diets compared with controls. The metabolite DON-3 sulphate (DON-3S) was detected in plasma and excreta in both treated groups, as well as in the liver (but only at 15 mg/kg feed). The increase in the level of DON decreased the hemoglobin concentration ($p < 0.001$), whereas the erythrocyte counts were only decreased at 15 mg DON/kg feed. No effect of DON on the responses to common vaccines was observed. In plasma, interleukin 8 levels in both contaminated groups were significantly higher than in the control group. The expression of interleukin 6, interleukin 1β and interferon-γ increased in jejunum tissues of broilers fed 5 mg/kg of DON compared with controls. The stress index (heterophil to lymphocyte ratio) was not affected by DON-contaminated diets compared with controls. The plasma corticosterone level was significantly lower in both DON groups compared with controls. In conclusion, DON-3S could be used as a specific biomarker of DON in different biological matrices, while the immune response in broiler chickens is stimulated by the presence of DON at the guidance level, but no adverse effect was observed on physiological stress parameters.

Keywords: deoxynivalenol; broiler chickens; deoxynivalenol-3-sulphate; blood hematology; immune response

1. Introduction

Deoxynivalenol (DON) is a secondary toxic metabolite mainly produced by *Fusarium* species that belongs to the trichothecenes family. DON frequently occurs in cereals, including wheat, maize, barley, rye, and oats (Creppy, 2002). A 10-year survey from 2008 to 2017 of the global mycotoxin occurrence in feed revealed that DON was

the most prevalent of mycotoxins and was detected in 64% of 74,821 samples collected from 100 countries (Gruber-Dorninger et al., 2019). Therefore, DON is considered to be the most frequently found mycotoxin in poultry feed, as chicken diets consist of high levels of cereals. The guidance level of DON in poultry feed is 5 mg/kg (European Commission, 2006). In terms of productive parameters, it has been reported in some studies that poultry could tolerate up to 15 mg/kg feed (Harvey et al., 1997; Kubena et al., 1997). This tolerance could be related to the metabolism of DON in this specie (Broekaert et al., 2017). The metabolism of DON or other mycotoxins is defined as the conversion of the native toxin (DON) to various degradation metabolites in the organism and in the digestive tract by microbes (Dänicke & Brezina, 2013). The analysis of the metabolites in the plasma of broiler chickens after a single intravenous injection or oral bolus of the synthetic or labelled DON at the guidance level, revealed that the main metabolite is DON-3-sulphate (DON-3S) of all metabolites (Broekaert et al., 2017; Devreese et al., 2015). However, the determination of DON-3S in plasma, liver and excreta of broiler chickens fed chronic DON at the guidance level has not been evaluated to date. Knowledge on the metabolites of mycotoxins is essential to test the efficacy of detoxifying agents *in vivo* afterwards (EFSA, 2017).

On the other hand, the immune system is a target of DON mycotoxicosis (Bondy & Pestka, 2000). As with other trichothecenes, DON can induce either immunostimulation or immunosuppression, depending on the dose and the duration of exposure (Pestka et al., 2004). A low to moderate concentration induces the upregulation of cytokines, whereas a high concentration induces the apoptosis of immune cells (Pestka, 2010). However, results are not conclusive regarding the effect of DON on parameters related to the poultry immune system. Furthermore, DON-induced physiological stress has been observed in chickens, but few studies have evaluated the related stress indicators (Antonissen et al., 2017; Ghareeb et al., 2012).

The purposes of this study were to investigate the metabolism of DON in broiler chickens after chronic feeding and to better understand its effects on the chicken immune response at both 5 mg/kg and 15 mg/kg levels. We also tested the hypothesis that DON can affect the physiological stress parameters of birds.

2. Materials and Methods

2.1. Ethical approval

All animal care procedures were approved by the Ethical Committee for Animal Experimentation of IRTA, in accordance with current regulations on the use and handling of experimental animals (Decree 214/97, Generalitat de Catalunya, Catalonia, Spain).

2.2. Birds, diets, and experimental design

Birds, management, the production of DON and contaminated diets, and experimental design for this current study have already been detailed in a recently published paper (Riahi et al., 2020). Forty-five 1-day-old male chicks (Ross 308) were randomly allotted to 15 battery cages (0.62 m x 0.62 m x 0.41 m). Birds were vaccinated against infectious bronchitis virus (IBV) at the hatchery and against Newcastle disease virus (NDV) at the farm at the start of the trial. At the farm, the lighting program was 24 hours of light per day for the first two days, 18 hours of light per day until 7 days, and 14 hours of light per day afterwards. The birds were maintained at 34 °C for the first two days and the temperature was gradually decreased by 3 °C per week until reaching 21 °C, and then was maintained. Chickens were fed starter diets from 1 to 21 days and grower diets from 22 to 42 days based on corn, soybean meal, soy oil, and a premix with vitamins, minerals, and amino acids; diets were formulated according the nutrient requirements for Ross 308 strain broilers (Table 1). Feed was provided in mash form ad libitum, in a metal feeder connected to each battery cage. Unlimited access to water was provided from individual nipple drinkers. Three dietary treatments with five cages per treatment and three birds per cage were used. The treatment 1 received a non-contaminated diet, and treatments 2 and 3 received DON contaminated diets at 5 mg/kg feed or 15 mg/kg feed, respectively for 42 d. DON used in this trial was produced by inoculating wheat with *Fusarium graminearum* strain I159 as reported by Metayer et al. (2019) (ENVT, Toulouse, France).

Table 1. Formulation and proximate analysis of control diet.

Ingredients (%)	Starter: control 1–21 days	Grower: control 21–42 days
Maize	54.00	59.49
Soybeanmeal 48 %	36.93	31.02
Soybean oil	4.91	5.73
Monocalcium phosphate	1.42	1.30
Calcium carbonate	1.23	1.13
Sodium chloride	0.19	0.21
Sodium bicarbonate	0.27	0.24
DL-methionine	0.30	0.26
L-lysine HCl	0.23	0.18
Noxyfeed	0.02	0.02
Premix[1]	0.49	0.44
Calculated content (%)		
Metabolizable energy (Kcal/kg)	3050	3150
Crude protein	22.0	19.5
Ether extract	7.01	7.92
Crude fibre	2.36	2.25
Lysine	1.38	1.18
Methionine + cysteine	0.91	0.87
Threonine	0.81	0.70
Tryptophan	0.21	0.18
Calcium	0.90	0.82
Inorganic phosphorus	0.64	0.59
Sodium	0.16	0.16

[1]Vitamin premix provided following nutrients per kg of diet: vitamin A: 13,500 IU; vitamin D3: 4800 IU: vitamin E: 67 IU; vitamin B1: 3 mg; vitamin B2: 9 mg; vitamin B6: 4.5 mg; vitamin B12: 16.5 µg; vitamin K3, 3 mg; calcium pantothenate: 16.5 mg; nicotinic acid, 51 mg; folic acid : 1.8 mg, biotin: 30 µg; Fe: 54 mg; I: 1.2 mg; Co: 0.6 mg; Cu: 12 mg; Mn: 90 mg; Zn: 66 mg; Se: 0.18 mg; Mo: 1.2 mg.

2.3. Analysis of mycotoxins in experimental feeds

The presence of DON and other mycotoxins in the feeds used in the assay was evaluated. Regarding DON analysis, 5 g of ground feed from each diet was mixed with 40 mL of distilled water and stirred for 10 min at 600 rpm. Thereafter, the mixture was filtered through Whatman paper no 4, and 2 mL was passed through a DONPREP® immunoaffinity column (R-Biopharm, Rhone LTD Glasgow, UK) which was cleaned up with 5 mL of MiliQ water. To eluate DON, 3 mL of HPLC-grade methanol was passed through the column and evaporated to dryness under a gentle stream of nitrogen. Dry extract was reconstituted in 1 mL of HPLC-grade mobile phase and 100 µL was analyzed

by HPLC using a Waters (Milford, MA, USA) Module Alliance 2695®, coupled to a UV/Visible dual λ absorbance Detector Waters 2487®. A Waters Spherisorb® 5 µm ODS2, 4.6 x 250 mm column was used. Absorption wavelength was set at 220 nm. The mobile phase was methanol:acetonitrile:water (4:4:92, v/v/v) and was set at a flow rate of 1.2 mL min^{-1}, and the column temperature was set at 40 °C. The limit of detection (50 µg/kg) was considered to be three times the signal of the blank.

With regard to Aflatoxin B_1 (AFB1) analysis, 5 g of ground feed sample from each diet was mixed with 15 mL of 60% methanol and stirred for 10 minutes at 600 rpm. Thereafter, the mixture was filtered through Whatman paper no 4, and 2 mL of filtrate was added to 14 mL of phosphate buffered saline (PBS) and mixed well. The whole solution was passed through an Easy-extract® Aflatoxin immunoaffinity column (R-Biopharm), which was cleaned up with 20 mL of PBS. To eluate AFB1, 1.5 mL HPLC-grade methanol and 1.5 mL MiliQ water were sequentially passed through the column and 100 µL of the joint eluates was analyzed by HPLC coupled with a fluorescence detector (FLD). The chromatographic equipment and column were the same as those used for DON analysis, but coupled to a Multi λ Fluorescence Detector Waters 2475®. The excitation wavelength was set at 365 nm and the emission wavelength was set at 465 nm. The derivatization of AFB1 was obtained using a post-column photochemical derivatization device (UVE^{TM} Derivatizer LC Tech). The mobile phase consisted of a solution of water:methanol:acetonitrile (70:17:17) set at a flow rate of 1.2 mL min^{-1} and the column temperature was 40 °C. The limit of detection (0.3 µg/kg) was considered to be three times the signal of the blank.

The detection of zearalenone (ZEN), total fumonisins (FBs) and ochratoxin A (OTA) in feed samples was carried out using the Ridascreen® Zearalenon, Ridascreen® Fumonisin and Ridascreen® Ochratoxin A enzyme-linked-immunosorbent assay (ELISA) kits (R-Biopharm), following the manufacturer's instructions, with detection limits of 1.75, 25, and 2.5 µg/kg for ZEN, FBs and OTA, respectively. All mycotoxin analyses were carried out by the Applied Mycology Unit of the Food Technology Department of the University of Lleida (Spain).

2.4. Sampling and analysis

At 42 days of age, blood samples (3 mL/bird, 3 birds/pen) were collected by cardiac puncture in non-heparinized tubes for the hematological and serological analysis. Blood samples (3 mL/bird, 3 birds/pen) were collected also into heparinized tubes for

interleukin 8 (IL-8), corticosterone, DON and DON-3S determination (Table 2). Blood serum of each bird was separated by centrifugation at 4500 × g for 10 min. Plasma was separated by centrifugation at 1000 × *g* for 15 min for IL-8 and corticosterone determination and at 2851 × g for 10 min for DON and DON-3S determination. Samples were stored at -20 ° C until further analysis. Mortality rates were 13 %, 20% and 13 % for the control treatment, and treatments 2 and 3, respectively. Twelve birds in each treatment were individually weighed and humanely euthanized according to IRTA ethics instructions at 42 days. Immediately, the entire intestine was carefully removed and the distal part of the jejunum (5 cm taken from Meckel's diverticulum) was collected for each bird, rinsed in PBS, and subsequently stored in RNAlater (Sigma-Aldrich, Barcelona, Spain) for 24 h, at ambient temperature. Then, samples were stored at -80°C without RNAlater until quantitative real-time PCR (qRT- PCR) analysis. Liver samples were excised, weighed, and stored at -20° º C until lyophilization. Fresh excreta samples from each cage were collected daily (day 1 to day 42) and stored at -20° C until lyophilization. One cage sample was a pool of excreta of three birds. Lyophilized liver and excreta samples were stored at darkness at ambient temperature until further analysis.

Table 2. Collection of biological samples.

Sample	**Analysis**[1]
Blood	Hematology
Serum	Response to common vaccines
Plasma	IL-8 Corticosterone DON and DON-3S
Small intestine (Jejunum)	IL-6, IL-1β, IL-10, IFN-γ
Liver	DON and DON-3S
Excreta	DON and DON-3S

[1]IL-8:interleukin 8; DON: deoxynivalenol; DON-3S: deoxynivalenol 3- sulphate; IL-6: interleukin 6 ; IL-1β interleukin- 1β: IL-10: interleukin 10; IFN-γ : interferon gamma.

2.5. DON and DON-3S determination in different biological matrices (plasma, liver, and excreta)

2.5.1. Chemicals, products and reagents

The DON analytical standard was supplied by Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The DON standard was dissolved in acetonitrile (ACN) as stock solution (1 mg/mL), and then diluted with HPLC-grade ACN to obtain an individual working standard solution of 1 µg/mL. The stable internal standard (IS) isotope ($^{13}C_{15}$-

DON) was obtained from Romer labs (Bioser, Barcelona, Spain) as 1.2 mL of a solution of 25 µg/mL in ACN. An individual working standard solution of 5 µg/mL was prepared by diluting the above stock solution with HPLC-grade ACN and was stored at -15 °C. Acetic acid (LC-MS gradient grade) was purchased from Montplet & Esteban SA (Barcelona, Spain). Sodium sulfate, sodium acetate and hexane were from PanReac Química SLU (Barcelona, Spain). Ammonium formate was from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Methanol (HPLC gradient grade, MeOH) was purchased from Honeywell (Seelze, Germany).

2.5.2. In plasma

Plasma extract preparation was carried out according to the method of Broekaert et al. (Broekaert et al., 2014). Briefly, 5 µL of $^{13}C_{15}$-DON IS solution (at 1 µg/mL and 750 µL of ACN were added to 250 µL of chicken plasma. ACN was added to precipitate plasma proteins. The samples were vortexed approximately for 1 min. Afterwards, the samples were centrifuged at 8517 × g for 10 min. The supernatant (1 mL) was transferred to a new tube, evaporated to dryness under nitrogen flow over a heating block and reconstituted in 1 mL of ammonium formate 5 mM/MeOH (50:50, v/v) solution, and 10 µL was injected for HPLC-MS/MS analysis.

2.5.3. In liver and excreta

Samples of lyophilized liver or excreta were weighed (1 g) in 50 mL centrifugation tubes. In total, 5 µL of 1 µg/mL of IS working solution ($^{13}C_{15}$-DON) and 10 mL of ACN: water: acetic acid (79:20:1, v/v/v) were added. Samples were vortex mixed for 2 min. Four grams of sodium sulphate and 1.5 g of sodium acetate were added and then each tube was vortex mixed for 5 min and then with an orbital shaker for 20 min (IKA™ KS 260, Fisher Scientific, Madrid, Spain). After extraction, a centrifugation was made at 2716 × g for 10 min, and supernatants were transferred to another tube and 5 mL of hexane was added and vortexed. After the separation of two phases, the hexane phase was removed. A 5 mL aliquot of the extract was evaporated to dryness under nitrogen flow over a heating block and reconstituted in 0.5 mL of water ammonium formate (5 mM):MeOH (50:50, v/v) and then filtrated through nylon syringe filters (0.20 µm) from Agilent (Santa Clara, CA, USA) and injected directly in HPLC-MS/MS.

2.5.4. LC-MS/MS analysis

The HPLC-MS/MS analysis was carried out with a Transcend 600 LC (Thermo Scientific TranscendTM, Thermo Fisher Scientific, San Jose, CA, USA) coupled to an Orbitrap (ExactiveTM, Thermo Fisher Scientific, Bremen, Germany) with an electrospray ionization (ESI) source (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA). Chromatographic separation was achieved using a Zorbax Plus C18 (1.8 µm × 2.1 × 100 mm) column from Agilent (San Jose, CA, USA). Gradient elution was established with a mobile phase consisting of 5 mM ammonium formate in water (eluent A) and methanol (eluent B) at a flow rate of 0.2 mL/min. The gradient elution started at 95% B at 1 min and was decreased to 0% B at 8 to 12 min afterwards; it increased to 95% at 12.5 min, which was maintained up to 14 min. The column temperature was set at 25 °C and the injection volume was 10 µL. MS analyses were performed using a selected reaction monitoring (SRM) mode with positive and negative electrospray ionization (ESI±). The settings on the spectrometer were as follows: compounds were ionized by electrospray ionization in the positive and negative mode, measured first in full scan, and then in targeted MS/MS mode at a collision energy of 30 eV (both in the range from m/z 50–500). ESI parameters were as follows: spray voltage, 4 kV; sheath gas (N2, > 95%), 35 (adimensional); auxiliary gas (N2, > 95%), 10 (adimensional); skimmer voltage, 18 V; capillary voltage, 35 V; tube lens voltage, 95 V; heater temperature, 305 °C; capillary temperature, 300 °C. The capillary and nozzle voltage were 4000 V and 95 V (-95 V in ESI-), respectively. Finally, the data were processed using XcaliburTM version 3.0 (Quanbrowser and Qualbrowser) and Mass FrontierTM 7.0.

2.6. Blood hematology

Hemoglobin (HGB, g/dL) and erythrocytes (Red Blood Cells, RBC/µL) were measured using a CELL-DYN 3700 hematology analyzer (Abbott, Chicago, IL, USA). A blood sample was collected in microcentrifuge (Haematokrit 200, Helltich Zentrifugen, Tuttlingen, Germany) capillary tubes for hematocrit (HCT, %) determination, which was performed in a Neubauer chamber (Brand, Germany). The mean corpuscular volume (MCV, fL) and mean corpuscular hemoglobin (MCH, pg) were determined by the hematologic analyzer CELL-DYN 3700 (Abbott, Chicago, IL, USA), and mean corpuscular hemoglobin concentrations (MCHC, g/dL) were calculated as MCHC = hemoglobin/hematocrit. Leukocytes per µL and the differential leukocyte count

(heterophils, lymphocytes, monocytes, eosinophils and basophils, %) were also measured using a hematologic analyzer CELL-DYN 3700 (Abbott, Chicago, IL, USA).

2.7. Response to common vaccines (NDV and IBV)

Antibody titers against NDV or IBV were determined by the hemagglutination inhibition (HI) test using standard protocols by the World Organisation for Animal Health (OIE). Serial twofold serum dilutions were made in PBS and 0.025 mL was added to the wells. Four hemagglutination (HA) units of the test antigen were added to each dilution and incubated at room temperature for 30 min. An equal volume of 1% chicken red blood cells (RBCs) in PBS was then added to the wells until agglutination occurred in the negative control sample. All plates included NDV or IBV-negative and NDV or IBV-positive control sera. The highest dilution of serum displaying the inhibition of agglutination was designated as the reciprocal log2 HI titer for that serum sample. Moreover, IBV was determined in serum using an ELISA test kit (Idexx®, Westbrook, Maine, USA), according to the protocols specified by the supplier. Briefly, 96-well plates were coated with viral antigen; after the incubation of the test sample (100 µL) in the coated well, an IBV-specific antibody formed a complex with the coated viral antigens. After washing away unbound material from the wells, a conjugate (100 µL) was added which bound to any attached chicken antibody in the wells. Unbound conjugate was washed away and 100 µL of substrate solution (TMB) was added to the wells and incubated for 15 min at ambient temperature. Subsequent color development was directly related to the amount of IBV antibody present in the test sample. The color development was stopped with a stop solution (100 µL) and the absorbance values were measured and recorded at 650 nm. Then, the amount of antibody to IBV present in the test sample was calculated.

2.8. Plasma IL-8 determination

IL-8 was determined in plasma using a commercially available ELISA kit (chicken IL-8 ELISA kit) according to the manufacturer's instructions (MyBioSource, San Diego, California, USA). Briefly, 96-well plates were pre-coated with anti-IL-8 antibody, and the biotin conjugated anti-IL-8 antibody was used for the detection of antibodies. The standards, test samples, and biotin-conjugated detection antibody (50 µL each) were subsequently added to the wells and washed with wash buffer. Streptavidin–horseradish peroxidase enzyme (HRP) (50 µL) was added and unbound conjugates were washed away with wash buffer. TMB (3, 3', 5, 5' tetramethylbenzidine) substrates were used to

visualize the HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue-color product that changed to yellow after adding an acidic stop solution. As the density of yellow was proportional to the IL-8 amount of the sample captured on the plate, the optical density absorbance at 450 nm in a microplate reader (Anthos, Labtec instruments, Salzburg, Austria) was read, and the concentration of IL-8 was calculated.

2.9. Gene expression by quantitative Real-Time PCR (qRT-PCR)

Total RNA from the tissue samples of the distal jejunum (20 mg) was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was eluted into 50 µL of Rnase free water and stored at -80 °C. The yield of RNA was determined by spectrophotometry (BioPhotometer, Eppendorf, Hamburg, Germany). Cytokine gene expression (IL-6, IL-1β, IFN-γ and IL-10) were evaluated as previously described by Reid et al. (2016). The mRNA quantification of cytokines was determined by qRT-PCR using QuantiTect™ SYBR® Green one-step RT-PCR Kit (QIAGEN, Hilden, Germany). The PCR amplification was performed using 7500-Fast Real-time PCR (Applied Biosystems, Ca, USA) (Reid et al., 2016).The threshold cycle values (Ct) were t normalized to the reference gene (glyceraldehyde-3-phosphate dehydrogenase (GAPDH)). The average ΔCt of the control samples was used to calculate the target gene expression according to the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). This relative quantification related the PCR signal of the target transcript gene in a treatment group to the average signal of untreated control. Duplicate samples were used.

2.10. Physiological stress related-parameters

2.10.1. Stress index (heterophil to lymphocyte ratio)

The heterophil to lymphocyte ratio (H/L), considered as an indicator of stress, was calculated by dividing the number of heterophils by the number of lymphocytes (Ghareeb et al., 2012; Xu et al., 2011).

2.10.2. Plasma corticosterone determination

The plasma level of corticosterone was measured with a commercially available ELISA kit (chicken corticosterone ELISA kit) according to the manufacturer's instructions (Cusabio, Houston, TX, USA). Briefly, the microtiter plate provided in this kit was pre-coated with an antigen. Standards, samples, antibody specific for corticosterone (CORT) (50 µL each), and HRP-conjugate (100 µL) were added to the

appropriate microtiter plate wells. The competitive inhibition reaction was launched between pre-coated CORT and CORT in samples. Then, 100 µL of substrate solution (TMB) was added to the wells, incubated for 15 min at 25 °C, and protected from light. The color development was stopped with an acid solution (50 µL) and the intensity of the developed yellow color was measured. A microplate reader (Skanit, Thermo Fisher Scientific, Madrid, Spain) capable of measuring absorbance at 450 nm was used.

2.11. Statistical analysis

Statistical analysis was performed by SAS software (SAS 9.4, SAS Institute, Cary, NC, USA). After the determination of normality and variance homogeneity, data were evaluated as a completely randomized design by a one-way analysis of variance (ANOVA) using the General Linear Model Procedure to test the effect of different treatments. Response to common vaccine parameters were logarithmically transformed to maintain the homogeneity of variance. Each cage was considered to be an experimental unit. Results were shown as means ± standard error of the means (RMSE). Orthogonal polynomials were used to determine linear and quadratic dose responses. To test the normal distribution of data, a Kol–Mogrov–Smirnov test (named after Andrey Kolmogorov and Nikolai Smirnov) was used. The significance level was set at $p \leq 0.05$. A trend was defined as p-value between 0.05 and 0.10 ($0.05 < p \leq 0.10$).

3. Results

3.1. Dietary mycotoxin concentrations

The DON levels found in control diets were 65 and 73 µg/kg in the starter and grower diets respectively. The concentrations of DON in contaminated feeds were close to 5 and 15 mg/kg, as expected. Diets also included lesser amounts of ZEN, FBs and OTA. AFB1 was not detected in all experimental diets (Table 3).

Table 3. Mycotoxin analysis of experimental feeds.

Mycotoxin[1] (µg/kg)	**Control group**		**DON group (5000 µg/kg)**		**DON group (15000 µg/kg)**	
	Starter	Grower	Starter	Grower	Starter	Grower
DON	65	73	4760	4650	14,390	15,120
ZEN	< LOD	< LOD	84.4	85.9	242	259
FBs	142	225	257	216	216	275
OTA	0.94	1.59	0.90	1.11	1.21	1.10
AFB1	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD

[1] DON: deoxynivalenol; ZEN: zearalenone; FBs: fumonisins; OTA: ochratoxin; AFB1: aflatoxin B1; [2]limit of detection (LOD) of DON, ZEN, FBs, OTA, and AFB1: 50, 1.75, 25, 0.5, 0.3 µg/kg, respectively.

3.2. DON and DON-3S determination in plasma, liver, and excreta

After a chronic DON feeding of broilers at a low (5 mg/kg) or high level (15 mg/kg) for 42 days, DON and DON-3S were analyzed in plasma, liver and excreta. Results showed that, in the non-contaminated control treatment, DON was below the limit of quantification (LOQ) (5 ng/mL) and DON-3S could not be identified. Similarly, DON was below the LOQ (5 ng/mL) in plasma and liver in broilers fed a contaminated diet at both levels but was detected only in excreta. DON-3S was detected in plasma and excreta at both levels and was significantly lower in the DON low dosage group. Interestingly, DON-3S was also detected in liver but only at the highest level assayed (Table 4).

Table 4. Average concentrations of deoxynivalenol (DON) and deoxynivalenol-3 sulphate (DON-3S) (peak area) in plasma, liver and excreta of broilers fed low deoxynivalenol level (5 mg/kg feed) and high deoxynivalenol level (15 mg/kg feed)[1,2,3]

Dietary treatment/ biological matrix	DON [1]	DON-3S[2] (× 10^6)
Plasma (ng/mL)		
Control	ND[3]	ND
DON low level (5 mg/kg)	ND	0.27 ± 0.01^{b}
DON high level (15 mg/kg)	ND	0.62 ± 0.15^{a}
SEM	-	0.11
p-value	-	0.01
Liver (ng/g)		
Control	ND	ND
DON low level (5 mg/kg)	ND	ND
DON high level (15 mg/kg)	ND	0.70 ± 0.37
Excreta (ng/g)		
Control	ND	ND
DON low level (5 mg/kg)	22.0	110^{b}
DON high level (15 mg/kg)	24.1	295^{a}
SEM	11.9	22.2
p-value	0.81	0.0001

[1] DON: deoxynivalenol; [2] DON-3S: deoxynivalenol 3-sulphate; [a,b] within the same column: different superscripts are significantly different (p value < 0.05); [3]ND = not detectable (limit of detection (LOD) = 1.5 ng/mL).

3.3. Hematological indices

The results of blood hematology parameters are reported in (Table 5). DON contaminated feed decreased the HGB level in a dose dependent mannner ($p = 0.0002$). Furthermore, the presence of DON at 5 mg/kg did not affect RBC, MCV, and MCHC ($p > 0.05$). However, 15 mg/kg DON in broiler diets significantly reduced RBC and MCHC and increased MCV blood level ($p < 0.05$). Leukogram data were not affected by the different dietary treatments ($p > 0.05$).

Table 5. Effects of deoxynivalenol (DON)-contaminated feed (5 and 15 mg/kg) on hematological parameters of broiler chickens.

Dietary Treatment [1]							
Item[2]	**Control**	**DON (5 mg/kg)**	**DON (15 mg/kg)**	**[3]RMSE**	***p*-Value**	**Linear**	**Quadratic**
HCT (%)	31.9	30.1	30.2	2.59	0.16	0.15	0.20
HGB (g/dL)	12.1[a]	11.1[b]	10.1[c]	1.01	0.0002	<0.0001	0.54
RBC (× 10^6 µL)	2.3[a]	2.3[a]	1.9[b]	0.13	<0.0001	<0.0001	0.06
MCV (fL)	132[b]	133[b]	151[a]	1.42	0.004	0.001	0.29
MCH (pg)	50.1	49.4	50.5	12.7	0.14	0.22	0.12
MCHC(g/dL)	37.8[a]	37.3[a]	34.1[b]	2.90	0.004	0.001	0.50
Leukocyte (×10^3 µL)	15.8	18.3	19.2	6.40	0.37	0.20	0.55
Eosinophil (%)	6.00	4.66	7.07	3.23	0.33	0.27	0.32
Basophil (%)	7.84	7.08	6.61	4.60	0.51	0.26	0.76
Lymphocyte (%)	41.2	37.7	37.1	9.74	0.52	0.33	0.55
Monocyte (%)	2.15	2.75	0.92	2.43	0.19	0.17	0.21
Total heterophils (%)	44.5	48.7	48.3	11.0	0.64	0.43	0.61

[1] DON: deoxynivalenol; [a,b,c]: means values with different superscripts with the same row differ ($p \leq 0.05$); [2] HCT: hematocrit; RBC: red blood cell; HGB: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; [3]RMSE, root mean square error (n = 5).

3.4. Response to common vaccines (NDV and IBV)

No significant effects of dietary treatments were observed on titers against NDV and IBV in broilers at 42 days ($p > 0.05$) (Table 6).

Table 6. Effects of deoxynivalenol (DON)-contaminated feed (5 and 15 mg/kg) on antibody titers against Newcastle disease virus (NDV) and infectious bronchitis virus (IBV) in broiler chickens.

Dietary Treatment [1]							
Item[2]	**Control**	**DON (5 mg/kg)**	**DON (15 mg/kg)**	**[3]RMSE**	***p*-Value**	**Linear**	**Quadratic**
Titers against NDV (HA)	0.38	0.30	0.25	0.54	0.82	0.56	0.84
Titers against IBV (HA)	2.92	3.20	3.00	1.03	0.81	0.95	0.52
Titers against IBV (ELISA)	874	851	786	889	0.31	0.13	0.76

[1] DON: deoxynivalenol; [2] NDV: Newcastle disease virus; HA: haemagglutination inhibition; IBV: infectious bronchitis virus; [3]RMSE, root mean square error (n = 5).

3.5. Plasma IL-8 production and relative mRNA expression of immune genes

In plasma, IL-8 was significantly upregulated in all broiler chickens receiving DON (5 and 15 mg/kg) compared to the control group ($p = 0.001$) (Figure 1). DON feeding at 5 mg/kg significantly stimulated the mRNA relative expression of IL-6, IFN-γ, and IL-1β in the jejunal tissues of broiler chickens ($p < 0.05$). However, the mRNA expression of these genes was comparable to the control group when the diet was contaminated with 15 mg/kg feed (Figure 2).

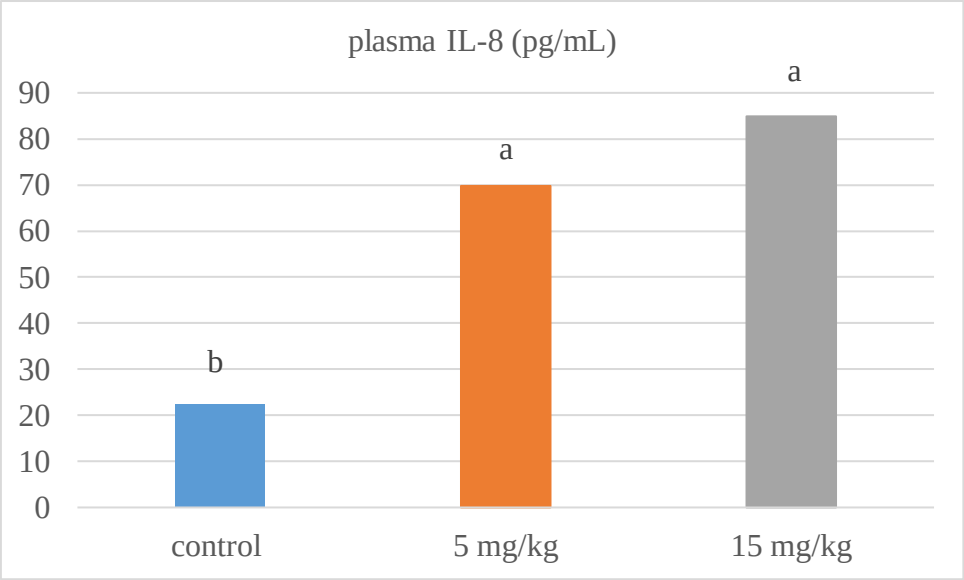

Figure 1. Effects of deoxynivalenol (DON)-contaminated feed (5 and 15 mg/kg) on plasma IL-8 levels in broiler chickens (n = 5); [a,b] values with different superscripts for each cytokine differ ($p \leq 0.05$).

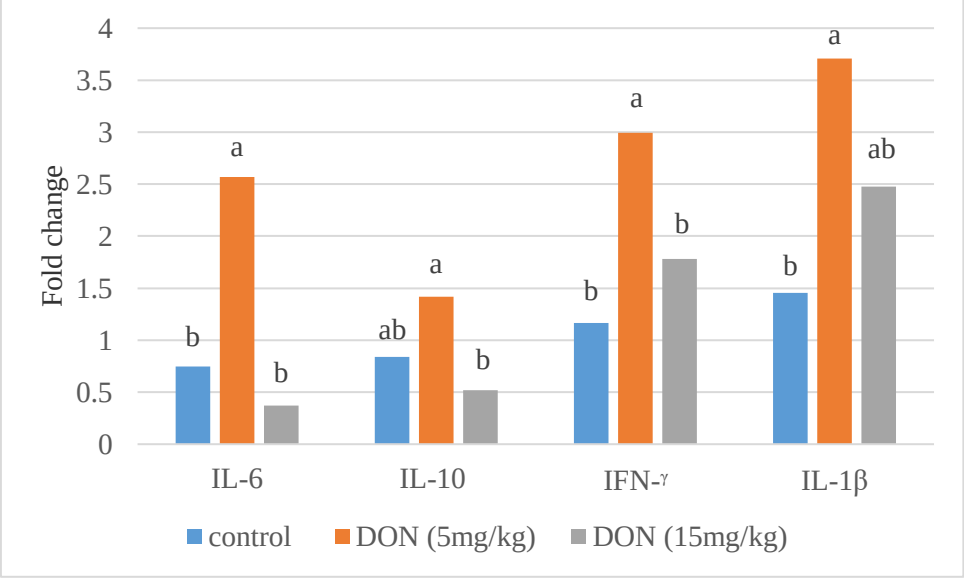

Figure 2. Effects of deoxynivalenol (DON)-contaminated feed (5 and 15 mg/kg) on the relative mRNA expression of immune genes (IL-6, IL-10, IFN-γ, and IL-1β) in the jejunal tissues of broiler chickens determined by qRT-PCR (n = 5); [a,b] values with different superscripts for each cytokine differ ($p \leq 0.05$).

3.6. Physiological stress parameters

The effects of experimental treatments on welfare-related indicators are presented in Table 7. No significant differences among the diet groups were detected for the stress index (H/L ratio). However, birds fed DON at both levels (5 and 15 mg/kg) showed lower plasma corticosterone level than birds fed a control diet ($p = 0.03$).

Table 7. Effects of deoxynivalenol (DON)-contaminated feed (5 and 15 mg/kg) on the heterophils to lymphocytes ratio (H/L) and plasma corticosterone level of broiler chickens.

Dietary Treatment [1]							
Item[2]	**Control**	**DON (5 mg/kg)**	**DON (15 mg/kg)**	**[3]RMSE**	***p*-Value**	**Linear**	**Quadratic**
H/L ratio	1.11	1.33	1.36	0.54	0.47	0.30	0.50
Plasma corticosterone (ng/mL)	2.93[a]	2.34[b]	2.44[b]	0.15	0.03	0.07	0.04

[1] DON: deoxynivalenol; ; [a:b]: values with different superscripts with the same row differ ($p \leq 0.05$); [2] H/L ratio: heterophil to lymphocyte ratio, [3]RMSE, root mean square error (n = 5).

4. Discussion

In practice, it is almost impossible to find feed that does not have a basal DON contamination, given the frequent contamination of cereals with this mycotoxin. Thus, the dietary concentration of DON was 65 and 73 µg/kg in the starter and grower control feed, respectively. This level was too low to cause adverse effects in broiler chickens (European Commission, 2006). Dietary ZEN concentrations ranged between below the LOQ and 259 µg/kg and increased as DON increased in the diet. DON and ZEN can be found alone or together (Cote et al., 1985). According to literature reports, the content of ZEN in the present study was not enough to produce synergism with the amounts of DON that negatively affect the health of broiler chickens (Swamy et al., 2002). Besides, FBs and OTA levels were below the guidance value established for poultry feed (20 and 0.1 mg/kg for FBs and OTA, respectively) (European Commission, 2006). Furthermore, the levels of AFB1 were below the limit of detection in all feeds. Therefore, the effects observed in the evaluated indicators in the current study were not attributable to AFB1, ZEN, FBs, and OTA, and thus only DON was responsible for these effects.

Although other authors have stated that the concentrations of DON chosen for this study should not have major effects on performance (Harvey et al., 1997; Kubena et al.,

1997), our results revealed that 15 mg/kg reduced body weight gain and altered the feed conversion ratio of broiler chickens at 42 days (Riahi et al., 2020).

Regarding the pathway of metabolization, DON was not detected in the plasma of birds even at 15 mg/kg feed. This result could be explained by the low absorption of this mycotoxin into plasma. The absolute oral bioavailability at the guidance value in chickens fed orally is poor, amounting to only 19.3% (Osselaere et al., 2013). Similarly, DON was not detected in the liver. This result could be related to its rapid metabolism and excretion. Other researchers have explained the lack of quantification of DON in plasma and liver by the protective effect of the hepatic/renal first pass effect. In fact, poultry are characterized by the protective hepatic/renal first pass effect. This effect is controlled by gut and liver enzymes, which induce the oxidation, reduction or hydrolysis (phase I reactions), and/or conjugation (phase II reactions) of toxins (Ghareeb et al., 2015). However, our results revealed that DON could be quantified in excreta of birds fed DON-contaminated diets**.** The detection of DON in excreta may be attributed to the rapid clearance of this toxin into excreta. The mentioned findings were previously also described when broilers were fed DON at 5 mg/kg by Awad et al. (2011).

Toxicokinetic studies performed in chickens (after single intravenous or oral bolus injection of labelled or synthetic DON) have demonstrated that DON-3S is the most abundant metabolite in plasma and excreta (Broekaert et al., 2017; Devreese et al., 2015; Lauwers et al., 2019; Wan et al., 2014). In addition, only one chronic study evaluated DON-3S in excreta of broiler chickens after chronic feeding of DON at a lower concentration than the maximum recommended (1.7 mg/kg) (Schwartz-Zimmermann et al., 2015). In the current study, DON-3S was detected in plasma and excreta of broilers exposed to recommended levels and at 15 mg/kg, suggesting the extensive metabolization of DON to DON-3S in broiler chickens. Interestingly, DON-3S was detected in the liver, but only in broilers exposed to the highest concentration tested. This result could be associated with the bird's cholesterol metabolism, as the blood cholesterol level was significantly affected in birds fed 15 mg DON/kg (Riahi et al., 2020). This result could correlate with the adverse effect of 15 mg DON/kg on performance found in a recent research work from our group (Riahi et al., 2020), and may confirm that, if feed contamination is lower (5 mg/kg), birds continue to have sufficient capacity for excretion and a limited deposition into the liver. In conclusion, DON-3S is a suitable biomarker for DON exposure in broilers; this biotransformation could be considered as a detoxification

pathway (Yi et al., 2006) and may explain the low susceptibility of broilers to DON at guidance levels (Wan et al., 2014).

It has been suggested that the impairment of the immune system is the most important outcome of DON toxicity (Bondy & Pestka, 2000). Consequently, it was expected that DON might affect blood hematological parameters, response to common vaccines (NDV and IBV), and cytokine production. In fact, the results of the current study showed that DON at higher levels (15 mg/kg) slightly more prominently affected hematological indices than 5 mg/kg feed. A significant dose-dependent decrease in hemoglobin concentration and a significant effect of a dietary dose of 15 mg DON/kg feed on RBC values, MCV, and MCHC were observed. The loss of HGB concentration and RBC count induced by DON mycotoxicosis could be a marker of bone marrow malfunction (Prelusky et al., 1994). In fact, bone marrow is an immune organ that is susceptible to DON mycotoxin because cells rapidly divide in this organ. On the other hand, the values of hematological parameters observed were still within the range of reference values, and no anemia was induced (Chowdhury et al., 2005). The feeding of chickens with contaminated diets containing 9 or 18 mg of DON/kg of feed significantly decreased the hemoglobin concentration and the RBC count (Harvey et al., 1991; Kubena et al., 1985). Moreover, it has been reported that DON could affect the humoral immune response by reducing the antibody titers against NDV and IBV (Dänicke et al., 2003; Ghareeb et al., 2012). However, in the current research, neither 5 nor 15 mg/kg of DON in broilers feed affected the vaccinal immune response after regular vaccination with NDV and IBV in broilers ($p > 0.05$). Yegani et al. (2006) observed no effect of feeding broiler breeder hens with grains naturally contaminated with *Fusarium* mycotoxins based on DON (12.6 mg/kg) for 12 weeks on antibody titers against NDV. This result was also in agreement with those of Harvey et al. (Harvey et al., 1991), who reported no effect on NDV antibody titers in White Leghorn chicks fed 18 mg of DON/kg of feed for 9 weeks. Regarding the antibody titers against IBV, Swamy et al. (Swamy et al., 2002) found no effect on IBV titers with different concentrations of DON (4.7 and 8.3 mg/kg of feed) in broiler chickens exposed for 21 days and 42 days. Similar findings were reported by Yegani et al. (2006), after feeding broiler breeder hens a concentration of 12.6 mg DON/kg for 28 and 56 days. Furthermore, no effects were found with a concentration of 12.2 mg DON/kg on antibodies against IBV titers in broilers chickens after 14 and 28 days (Yunus et al., 2012). The failure to observe significant results in response to common vaccines and the

variability between literature reports suggests that those parameters could not be a relevant biomarker for DON toxicity in poultry.

IL-8 is a proinflammatory cytokine involved in pathogen defense and immune regulation, and it is considered as an early biomarker of the inflammation process (Sallusto & Baggiolini, 2008). In this study, DON presence in chicken feed at 5 and 15 mg/kg resulted in an increase of IL-8 production in plasma, suggesting that DON could have an effect on the innate immune response and inflammation process. The upregulation of IL-8 has been shown in *in vitro and in vivo* studies in humans, rodents and farm animals (Lessard et al., 2015; Maresca et al., 2008). To our knowledge, the upregulation observed of the plasma IL-8 of broiler chickens after DON exposure is new information. The duration of exposure of DON is an important variable, as with a shorter duration (35 days), no significant differences between control and contaminated feeding groups were observed in a previous feeding trial with broiler chickens (Ghareeb et al., 2013).

In the actual study, the effects of DON on the gene expression of cytokines in jejunum tissues of broilers were evaluated as markers of the intestinal immune system. The addition of 5 mg DON/kg feed significantly upregulated the mRNA expression of the proinflammatory cytokines IL-6, IFN-γ, and IL-1β, suggesting that DON at the guidance level is immunostimulatory in broilers aged 42 days. These results are in agreement with previous results indicating that DON exposure from 2 to 5 mg/kg upregulated the proinflammatory cytokines in broiler chickens, such as IL-6 in jejunum, IL6 and IL-1B in spleen, and IFN-γ in cecal tonsils (Girgis et al., 2008; Grenier et al., 2016; Li et al., 2012). The upregulation of the mRNA gene expression of cytokines is due to the ability of DON, as a protein-synthesis inhibitor, to impair the synthesis of high turnover proteins and, as a result, to induce a transient expression of specific mRNAs (Zhou et al., 1997). In a similar manner, it has been suggested that this induction of immune-related genes by DON mycotoxin is due to the increasing of the binding activity of transcription factors in leukocytes such as the nuclear factor κB (NF-κB) at the transcription level, and to the increasing stability of the mRNA at the post-transcription level (Girgis et al., 2008; Pestka et al., 2004). However, mRNA expression of IL-6, IFN-γ, and IL-1β was comparable to a control group when broilers were fed 15 mg/kg, suggesting that this dose did not induce immune-suppression in broilers aged 42 days. The mRNA level of the anti-inflammatory IL-10 was not statistically affected, suggesting that DON did not affect anti-inflammatory

cytokines, as previously reported in some studies (Girgis et al., 2008; Grenier et al., 2016). IL-10 is involved in the cross-regulation of IFN-γ gene expression (Moore et al., 2001). The upregulation of IFN-γ gene expression, therefore, may not be necessary to correlate the changes in IL-10 gene expression (Girgis et al., 2008).

The physiological stress includes the elevation of the stress index (H/L ratio), defined as a result of the impairment of the number of circulating heterophils and lymphocytes, and an elevation of the circulating levels of corticosterone (Onbaşilar & Aksoy, 2005), although the effect of DON on the H/L ratio and plasma corticosterone level in poultry has not been extensively documented (Antonissen et al., 2017; Ghareeb et al., 2014). As no significant effect was observed on blood heterophils and lymphocytes, therefore, dietary DON did not affect the stress index H/L ratio. Similarly, Dänicke et al. (2003) did not find significant differences in the H/L ratio of broilers fed a contaminated diet with a concentration of 14 mg/kg of DON per kg.

Corticosterone is the primary glucocorticoid secreted by the adrenal glands in birds and is involved in immune reactions and stress responses (El-Lethey et al., 2003). Corticosterone was selected as a physiological stress marker as the effects of feeding a contaminated diet containing DON has previously been shown to increase this glucocorticoid in the plasma of broiler chickens (Antonissen et al., 2017; Ghareeb et al., 2014). The results of this study showed that the plasma levels of corticosterone decreased in broilers fed 5 and 15 mg/kg of DON compared to the controls. The differences between these results may be attributed to the very marked circadian cycle of the plasma corticosterone measurement. In fact, the difference of sampling time between treatments could affect the circadian cycle and thus could mask the real effect on the plasma corticosterone level. Furthermore, blood sampling is stressful to the animal and can mask the effect of the stressor under study.

5. Conclusions

In conclusion, DON and DON-3S in excreta are suitable metabolites of DON exposure in broilers fed the guidance level (5 mg/kg). Moreover, DON at this level induced proinflammatory cytokine production, suggesting that DON is immunostimulatory in broiler chickens at 5 mg/kg for 42 days. The effect of this high dose (15 mg/kg) was observed on the deposition of DON-3S in the liver and on the reduction in hematological parameters, suggesting that DON affects the health status of

birds. Further studies are required to directly elucidate DON and DON-3S in excreta as relevant endpoints for the efficacy testing of detoxifiers, and further investigations will need to pay closer attention to the most important immune system indicators that could be affected by DON mycotoxicosis in poultry.

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CHAPTER 4

The effects of deoxynivalenol (DON) and three detoxifying agents in broiler chickens based on specific and nonspecific parameters

The effects of deoxynivalenol (DON) and three detoxifying agents in broiler chickens based on specific and nonspecific paramters

Abstract

This study was conducted to evaluate the effect of three feed additives in diets contaminated with 7.42 mg/kg of deoxynivalenol (DON), alone or in combination, on broiler metabolism, performance, health and welfare. A total of 384 male broilers aged 1-42 days were used. Birds were randomly distributed into eight treatments with twelve replicates of four broilers each, as follows: 1) control diet; 2) contaminated diet (5mg/kg DON); 3) control diet + 2 kg microbial feed additive (MFA)/ton feed; 4) contaminated diet + 2 kg MFA/ton feed; 5) control diet + 0.125 kg immuno-protector additive (IMP)/ton feed; 6) contaminated diet + 0.125 kg IMP; 7) control diet + 1.5 kg mycotoxin degrading enzyme (MDE)/ton feed; 8) contaminated diet + 1.5 kg MDE/ton feed. Productive parameters, organ weights, morphology and histology of small intestine, blood biochemistry and hematology, cytokines production, and welfare related parameters were evaluated. In addition, DON and metabolites generated after DON exposure were determined in excreta. Results showed that DON had an inhibiting temporary effect on the feed conversion ratio at 10 d, and the addition of IMP counteracted this effect. From 21 d, feed conversion ratio was improved in response to DON. Liver relative weight tended to decrease in the 42-d-old DON-fed broilers compared to that in the control-fed birds. Supplementation with IMP showed to reverse this effect. There were no significant differences in morphological and histological parameters of the small intestine of broilers fed the diet containing DON compared with control group. However, DON contaminated feed slightly affected the lipid metabolism of birds by increasing the serum cholesterol and triglycerides levels at 10 d old. Addition of IMP or MFA to the DON-contaminated feed of broilers effectively alleviated serum cholesterol level Moreover, feeding a DON-contaminated diet reduced the hematocrit, hemoglobin and monocytes level in broilers at 10 d. This effect was prevented by IMP but not by MFA nor MDE. The relative gene expression of pro-inflammatory cytokines was not affected by the different experimental treatments except a down-regulation of tumor necrosis factor-alpha (TNF-α) in DON contaminated group. Moreover, contamination of the broiler diet with DON increased the stress related parameters, as the heterophil to lymphocyte ratio (stress index), and the feather corticosterone, suggesting that DON elevates the physiological stress responses of broilers. However, the dietary MFA supplementation tended to alleviate the

mycotoxin-related effects on the stress index. Addition of MFA to contaminated feed increased the excretion of DON, suggesting that this feed additive is effective in counteracting the adverse effect of DON. DON, DON-3-sulphate (DON-3S) and deepoxy-DON-3-sulphate (DOM-3S) were detected in all excreta of contaminated diets. The results of the present trial indicate that the addition MFA or MDE showed to be effective on the specific parameters by increasing the excretion of DON. MFA supplementation was effective also on DON effect on cholesterol level and stress index. IMP showed to reverse some DON induced nonspecific effects such as productive, biochemical and hematological parameters. However, MDE product was not effective against DON mycotoxin.

1. Introduction

Due to their cereal- based diets, broiler chickens are highly exposed to mycotoxins. Deoxynivalenol (DON) is the most frequently occurring natural feed contaminant (Gruber-Dorninger et al., 2019). A low to moderate long-term exposure to DON in broilers could adversely affect their production, physiology, immune system, and welfare parameters (Ghareeb et al., 2012; Riahi et al., 2020).

Despite several prevention approaches at field and appropriate grain transport and storage, the presence of mycotoxins in food and feed is often unavoidable. Consequently, different post-harvest control strategies have been developed to prevent livestock species, included poultry, against the deleterious effects of mycotoxins.

The use of feed additives as detoxifying agents against mycotoxins is one of these measures. The detoxifying agents are subdivided into two groups: mycotoxins binders and mycotoxins modifiers, which adsorb or biotransform these toxins in the gastro-intestinal tract, respectively (Jard et al., 2011). In the case of DON, adsorption has generally proven not to be a good strategy as binders targeting DON have mostly low efficiency. However, the use of biotransforming-agents seems to be more effective. On the market, few products claim to biodegrade DON, except for one having an authorization dossier. Technical information (mode of action, derived metabolites produced, an efficacy) of other proposed biotransforming agents is very scarce at the moment.

To establish the efficacy of an anti-mycotoxin additive many *in vitro* methods, such as single-concentration studies, isotherm studies (binder concentration fixed, toxin concentration increasing) and experimental gastro-intestinal tract models, have been

developed to test mycotoxin detoxifiers. However, the European Food Safety Authority (EFSA) has stated that *in vitro* studies were not enough to proof the efficacy of mycotoxin-detoxifying agents (EFSA, 2010) and that *in vivo* trials should be performed. Since the effect of the products is not produced in the feed directly, but when it is ingested and acts in the digestive tract of the animal.

Although the efficacy and safety of the detoxifying agents have been demonstrated by using biomarkers of effect such as zootechnical, morphological, and immunological parameters (Ghareeb et al., 2012), EFSA (2017) stated that detoxifying agents have to be evaluated *in vivo* based essentially on specific biomarkers related to mycotoxin exposure on the target specie, such as the contents of native mycotoxin and its metabolites in a biological matrix (blood, urine, milk or organs), determined by accurate methods of analysis (EFSA, 2017). Regarding DON, EFSA proposed DON and deepoxy-DON (DOM-1) in serum as the most specific indicators of DON exposure (EFSA, 2017). However, in poultry species included broiler chickens, DON-3 sulphate (DON-3S) metabolite has shown to be the most suitable biomarker of exposure (Wan et al., 2014).

Therefore, the purpose of the current research was to evaluate the effect of DON assayed at the EU maximum guidance level for poultry in an artificially contaminated feed, together with the presence or the absence of 3 detoxifying agents (microbial feed additive, immune-protector additive, and mycotoxin degrading enzyme), in order to detect differences in the DON-induced specific (DON, DON-3S, DOM-1, and DOM-3S metabolite production) and nonspecific effects (productive, immunological and welfare parameters) originated by the presence of those feed additives. A microbial feed additive (microorganism) or mycotoxin degrading enzyme claiming to biotransform DON on its less toxic metabolites have been used. The use of immune-protector additive claims to protect animals from the immunotoxic effects of DON.

In this study, and with the objective to test the model, three anti-mycotoxin detoxifier with different mode of action were tested:

- The first one, noted MFA for microbial feed additive claimed to be a complete anti-mycotoxin solution comprising a microorganism with DON degradation capability;

- The second one was used for its immune properties and called IMP;

- The last one, is also a complete anti-mycotoxin solution comprising mycotoxins degrading enzymes and called MDE.

2. Materials and Methods

2.1. Birds and housing

Three hundred eighty-four 1-d-old male broiler chickens (Ross 308) were weighed on arrival and randomly distributed in 96 cages (8 treatments with 12 replicates), each cage containing 4 birds for 42 days. The birds were housed under a controlled environment room. In the first two days of age, room temperature was set at 34 ºC with a gradual reduction of 3 ºC weekly to 21 ºC afterwards. For lightening, the first two days chicks were provided with 24 h of light, with a reduction to 18 h until 7 days and 14 h afterwards until the end of the trial.

2.2. Mycotoxin concentration, diets and experimental design

DON was produced at the National Veterinary School of Toulouse (France) by *in vitro* growing of *Fusarium graminearum* strain I159 on wheat, as described by Metayer et al. (2019), and then was powdered and mixed to the experimental feed to obtain a 5 mg DON/kg feed contamination.

Maize and soybean-based feeds were previously analyzed for multi-mycotoxins contents (Phytocontrol, Nimes, France) to know any previous mycotoxin contamination. The ingredients were mixed to obtain a maize-soybean-based diet (Table 1), according to Ross 308 broiler strain recommendations. A microbial feed additive (MFA), or a nutritional additive (IMP), or a mycotoxin degrading enzyme (MDE), and DON contaminated wheat were homogenously added, according to the treatment group.

The birds were fed eight starter diets from 1 to 21 days old and grower diets from 22 to 42 days old (Table 1), being treatments 1, 3, 5 and 7 the uncontaminated diets and treatments 2, 4, 6 and 8 the DON-contaminated diets (5 mg/kg feed). The eight experimental diet treatments were prepared as follows:

Treatment 1: control diet (T1).

Treatment 2: T1 + 5 mg DON/kg feed (T2).

Treatment 3: T1 + 2 kg microbial feed additive (MFA)/ton feed (T3).

Treatment 4: T1 + 5 mg DON/kg feed + 2 kg microbial feed additive (MFA)/ton feed (T4).

Treatment 5: T1 + 0.125 kg immuno-protector additive (IMP)/ton feed (T5).

Treatment 6: T1 + 5 mg DON/kg feed + 0.125 kg immuno-protector additive (IMP)/ton feed (T6).

Treatment 7: T1 + 1.5 kg mycotoxin degrading enzyme (MDE)/ton feed (T7).

Treatment 8: T1 + 5 mg DON/kg feed + 1.5 kg mycotoxin degrading enzyme (MDE) /ton feed (T8).

Diets were prepared in mash form. Diets and water were provided *ad libitum*. Representative feed samples for each phase and treatment were analyzed for the content of nutrients (Table 1) and mycotoxins (Table 3). DON, aflatoxins B_1, B_2, G_1, and G_2, ochratoxin A, zearalenone, fumonisins B_1 and B_2, HT-2 and T-2 toxins concentrations were analyzed in the basal feed (T1). For the other experimental diets (T2-T8), the contents of DON, 15-acetyl DON, 3-acetyl DON, DON-3-glucoside, fusarenon X, and nivalenol were determined. All mycotoxin analysis in experimental diets were carried out by Phytocontrol (Nimes, France). The limits of detection were 0.3 µg/kg for aflatoxin B_1, B_2, G_1, G_2 and ochratoxin A; 3 µg/kg for zearalenone; 7.5 µg/kg for fumonisin B_2; 15 µg/kg for HT-2 toxin, T-2 toxin, 15- acetyl-deoxynivalenol, 3- acetyl-deoxynivalenol, fusarenon X, and nivalenol; 30 µg/kg for deoxynivalenol-3-glucoside.

Table 1. Composition of the basal diet.

Ingredient (%)	Starter	Grower
Maize	54.00	59.49
Soybean meal 48 %	36.93	31.02
Soybean oil	4.91	5.73
Monocalcium phosphate	1.42	1.30
Calcium carbonate	1.23	1.13
Sodium chloride	0.19	0.21
Sodium bicarbonate	0.27	0.24
DL-methionine	0.30	0.26
L-lysine HCl	0.23	0.18
Noxyfeed	0.02	0.02
Vitamin-mineral premix[1]	0.49	0.44
Calculated composition (%)		
Metabolizable energy (Kcal/kg)	3050	3150
Crude protein	22.00	19.50
Ether extract	7.01	7.92
Crude fibre	2.36	2.25
Lysine	1.38	1.18
Methionine + cysteine	0.91	0.87
Threonine	0.81	0.70
Tryptophan	0.21	0.18
Calcium	0.90	0.82
Inorganic phosphorus	0.64	0.59
Sodium	0.16	0.16
Analyzed composition (%)		
Dry matter	87.9	88.0
Crude protein	21.3	19.7
Starch	35.9	39.9
Ether extract	6.14	7.04
Ash	5.01	4.99
Crude fibre	2.72	2.34
Sodium chloride	0.29	0.34

[1]Vitamin premix provided following nutrients per kg of diet: vitamin A: 13,500 IU; vitamin D3: 4800 IU: vitamin E: 67 IU; vitamin B1: 3 mg; vitamin B2: 9 mg; vitamin B6: 4.5 mg; vitamin B12: 16.5 µg; vitamin K3, 3 mg; calcium pantothenate: 16.5 mg; nicotinic acid, 51 mg; folic acid: 1.8 mg, biotin: 30 µg; Fe: 54 mg; I: 1.2 mg; Co: 0.6 mg; Cu: 12 mg; Mn: 90 mg; Zn: 66 mg; Se: 0.18 mg; Mo: 1.2 mg.

2.3. Performance parameters

The weight of chickens and feed consumption per each pen were measured at 10, 21 and 42 d of experiment. Body weight gain (BWG), feed intake and feed conversion ratio (FCR) were determined at those periods. Mortality was recorded on a daily basis.

2.4. Sample collection and analysis

At d 10 and at the end of the trial (42 d), one bird per pen (12 birds/treatment) were weighed and euthanized by cervical dislocation. Blood was sampled prior euthanasia (6 mL/bird). Blood samples were collected in non-heparinized tubes for biochemical and hematological analysis. Serum of each bird was separated by centrifugation at 978 × g for 10 min for blood biochemistry determination. Blood samples collected into heparinized tubes were centrifuged at 1000 × g for 15 min to obtain plasma samples for IL-8 determination. After the removal of small intestine contents, proximal section of jejunum were collected and rinsed with phosphate buffered saline (PBS) for histological and pro-inflammatory cytokines determination. For histological determination, tissues were fixed in 10% neutral-buffered formalin for 48 h, at 4 ºC, then trimmed and processed for hematoxylin and eosin staining (Nofrarías et al., 2006). For proinflammatory cytokines, tissues were immersed in RNA-later (Sigma Aldrich, Barcelona, Spain) for 24 h, at ambient temperature and then samples were frozen at -80 ºC until quantitative real-time PCR (qRT- PCR) analysis. Samples of excreta were collected fresh for each cage at 21 d and for 35 to 42 d and stored at -20 ºC until lyophilization. Samples were lyophilized, homogenized and stored at ambient temperature until DON mycotoxin and its metabolites analysis. At the end of the experiment, feathers from the wings of birds of control and IMP groups were taken and stored at -20 ºC until corticosterone analysis (Table 2).

Table 2. Sample collection and analysis.

Sample	Determination	Sampling time	Number of sample
Excreta	DON and metabolites	21/42	6
Serum	Biochemistry	10/42	12
Blood	Hematology	10/42	12
Plasma	IL-8[1]	10/42	12
Jejunum	Histomorphology	10/42	12
Jejunum	Proinflammatory cytokines	10/42	12
Feathers	Corticosterone (control and IMP[2]groups)	42	6

[1] IL-8: interleukin 8; [2] IMP : immuno-protector additive

2.5. Organ weights and morphometric traits of small intestine

After weighing, organs including gizzard, liver, kidneys, colon, spleen, thymus, and bursa of Fabricius of one bird per pen (12 birds/treatment) were weighed. The weight of

organs was expressed on a relative body weight basis (g/kg). The gastrointestinal tract of chickens euthanized (from the duodenum to the ileocecal junction) was removed of the content by gentle squeezing. Length, absolute and relative weight of this section were measured, and, subsequently, the density was calculated, that is, the ratio of the absolute weight in grams to the length of the small intestine in centimeters, at 10 and 42 d.

2.6. Measurement of villus height and crypt depth

Histomorphology analysis of jejunal sections was performed as described by Nofrarías et al. (2006). Briefly, formalinized jejunal sections were sectioned at 4 µm, stained with hematoxylin and eosin and observed using a light microscope (BHS, Olympus, Barcelona, Spain). The measurements of villus height and crypt depth were done with a linear ocular micrometer (Olympus, Microplanet, Barcelona, Spain) and the ratio villus height to crypt depth was calculated.

2.7. Blood biochemistry and hematology

Total protein, cholesterol and triglycerides levels in serum were determined by an automatic biochemical analyzer (Olympus AU5800, Beckman Coulter, Brea, CA, USA). For hematological measurements, hemoglobin (HGB), red blood cells, (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), leukocytes and differential leukocyte count (heterophils, lymphocytes, monocytes, eosinophils and basophils) were determined by the hematologic analyzer (CELL-DYN 3700, Abbott, Chicago, USA). Blood for hematocrit (HCT) determination was collected into microcentrifuge (Haematokrit 200, Helltich Zentrifugen, Germany) capillary tubes, which was made in a Neubauer chamber (Brand, Germany). Mean corpuscular hemoglobin concentrations (MCHC, g/dL) was calculated by dividing the hemoglobin by the hematocrit values. Heterophil to lymphocyte ratio (H/L) is defined as a stress index (Ghareeb et al., 2012). H/L ratio was determined at 10 and 42 d of the trial.

2.8. Determination of plasma cytokine level and quantification of mRNA of immune genes by quantitative real time PCR (qRT-PCR)

The determination of interleukin 8 (IL-8) in chicken's plasma was performed using a commercially available ELISA kit according to manufacturer's instructions (MyBioSource, San Diego, California, USA).

RNA from samples (20 mg of the distal jejunum) was isolated using RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. Total RNA

was diluted in 50 µL in Rnase free water. Next, total RNA was quantified by spectrophotometry (BioPhotometer, Eppendorf, Hamburg, Germany). For the calculation of the relative mRNA expression, GAPDH was used as house-keeping gene and the expression of genes of interest was normalized using GAPDH. Cytokine gene expression is expressed as fold gene expression (Delta-Delta-Ct) (Livak & Schmittgen, 2001). Cytokines gene expression interleukin 1 beta (IL-1β), interleukin 6 (IL-6), IL-8, interferon gamma (IFN-γ) and tumor necrosis factor (TNF-α) were evaluated (Reid et al., 2016).

Table 3. Cytokines evaluated in plasma and jejunum.

Cytokine	Matrix	Sampling date	Number of samples/ treatment
IL[1]-6	Jejunum	42 d	12
IL-8	Plasma Jejunum	10 and 42 d	6
IFN-γ[2]	Jejunum	42 d	12
IL-1β	Jejunum	42 d	12
TNF-α[3]	Jejunum	10 and 42 d	6

[1]IL: interleukin; [2]IFN-γ: interferon gamma; [3]TNF-α : tumor necrosis factor.

2.9. Corticosterone determination

Feather corticosterone (FCORT) concentrations were determined using an ELISA kit (Corticosterone ELISA kit; Neogen Corporation, Ayr, UK), as previously described by Carbajal et al. (2014). Only samples from T1, T2, T5, and T6 were determined. The results were expressed in pg/mg (Bortolotti et al., 2008).

2.10. Analysis of DON and its metabolites in excreta by LC-MS/MS

1 g of sample was mixed with 10 µL $^{13}C_{15}$-DON (Biopure, Bioser, Barcelona, Spain) (5 µg/mL) and 10 mL of acetonitrile:water:acetic acid (79:20:1, v/v/v). After vortex mixing for 2 min, 4 g of sodium sulphate and 1.5 g of sodium acetate were added. The samples were vortex mixed again for 5 min, followed by mixing for 20 min with an orbital shaker (IKA™ KS 260, Fisher Scientific, Madrid, Spain). Then, a centrifugation step (10 min, 2716 × g) was performed. The supernatant was transferred to a new tube and 5 mL of hexane was added and vortexed. Two phases were separated, the hexane was removed and the extract was evaporated to dryness under nitrogen flow over a heating block. Dry extract was then reconstituted in 0.5 mL of 5 mM ammonium formate in

water:MeOH (50:50, v/v) and then filtrated through PTFE 0,45 µm filter and analyzed by means of liquid chromatography–tandem mass spectrometry (LC–MS/MS).

The LC–MS/MS analysis was performed with a Transcend 600 LC (Thermo Scientific Transcend^{TM}, Thermo Fisher Scientific, San Jose, CA, USA) coupled to an Orbitrap mass analyzer (Exactive^{TM}, Thermo Fisher Scientific, Bremen, Germany) with an electrospray ionization source (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA). Chromatographic separation was carried out on a Zorbax Eclipse Plus C18 Rapid Resolution High-Definition column (2.1 × 100 mm, 1.8 µm particle size, Agilent, San Jose, CA, USA) at a flow rate of 0.2 mL/min, using gradient elution (1 min: 95% B, 8 min: 0% B, 12 min: 0% B, 12.5 min: 95% B, 14 min: 95% B). Mobile phase A was ammonium formate in water (5 mM), and mobile phase B was methanol. The column temperature was set at 25 ºC and the injection volume was 10 µL. Compounds were ionized by electrospray ionization in the positive and negative mode and measured first in full scan and then in targeted MS/MS mode at a collision energy of 30 eV (both in the range from *m/z* 50-500). Electrospray ionization was carried out at a spray voltage, 4 kV; sheath gas (N_2, >95%), 35 (adimensional); auxiliary gas (N_2,> 95%), 10 (adimensional); skimmer voltage, 18 V; capillary voltage, 35 V; tube lens voltage, 95 V; heater temperature, 305 ºC; capillary temperature, 300 ºC. The capillary and nozzle voltage were 4000 V and 95 V (-95 V in ESI-), respectively. Data acquisition was achieved in Xcalibur^{TM} version 3.0 (Quanbrowser and Qualbrowser) and Mass FrontierTM 7.0. The limit of quantification (LOQ) for DON and DOM-1 was 5 ng/mL and 15 ng/ mL, respectively.

2.11. Statistical analysis

To test the normal distribution of data, Kolmogorov-Smirnov test was used. Then, ANOVA using the GLM procedure of SAS software (SAS 9.4, SAS institute, Cary, NC, USA) in a completely randomized design was performed between dietary treatments. Orthogonal contrasts were used to determine the nature of the response exhibited on evaluated variables to the feeding of DON contaminated feed with or without feed additive. DON and DON-3S in excreta data were transformed into logarithm for statistical analyses. The data expressed as percentage were transformed using angular transformation. The probability level of $p \leq 0.05$ was declared significant and a trend was reported if $p \leq 0.10$. In this chapter results were not analyzed following the factorial design because the different detoxifying agents used have different mode of action.

3. Results

3.1. Dietary mycotoxin concentrations

Natural DON contamination in the control feed was under the limit of detection of the method (15 µg/kg). The level of the other mycotoxins was also under the limits of detection However, fumonisin B_1 was detected in control feed and the concentrations were 33 and 81 µg/kg for starter and grower feed, respectively. These concentrations were under the maximum level (20000 µg/kg) recommended by the European Union (European Commission, 2006) in poultry feed. On the other hand, the contaminated diets were formulated to theoretically result in 5000 µg DON/kg. The verified levels of DON in the DON-contaminated feed was between 5367±673 and 8865±1064 µg DON/kg (Table 4).

Table 4. Analysis of mycotoxins in experimental diets.

Treatment	DON[1] (µg/kg)	Feed [2] additive	DON (µg/kg)	FB1[4] (µg/kg)	Other mycotoxins[5]
Starter					
1	0	0	<LOD[3]	33 ± 7	<LOD
2	5000	0	7048 ± 846		
3	0	MFA	<LOD		
4	5000	MFA	8402 ± 1008		
5	0	IMP	<LOD		
6	5000	IMP	7445 ± 893		
7	0	MDE	<LOD		
8	5000	MDE	5367 ± 644		
Grower					
1	0	0	<LOD	81 ± 18	<LOD
2	5000	0	8865 ± 1064		
3	0	MFA	<LOD		
4	5000	MFA	7067 ± 848		
5	0	IMP	<LOD		
6	5000	IMP	6000 ± 720		
7	0	MDE	<LOD		
8	5000	MDE	5607 ± 673		

[1]DON: deoxynivalenol; [2]MFA: microbial feed additive; IMP: immuno-protector additive; MDE : mycotoxin degrading enzyme; [3]<LOD: below limit of detection ; (DON LOD : 15 µg DON/kg); [4]FB1: fumonisin B1 (LOD: 7.5 µg/kg); [5]Aflatoxins (B_1, B_2, G_1, G_2) and ochratoxin A (LOD: 0.3 µg/kg); zearalenone (LOD: 3 µg/kg), fumonisin B_2 (LOD: 7.5 µg/kg); HT-2 toxin, T-2 toxin, 15-acetyl-deoxynivalenol, 3-acetyl-deoxynivalenol, fusarenon X and nivalenol (LOD: 15 µg/kg); deoxynivalenol-3-glucoside (LOD: 30 µg/kg).

3.2. Determination of DON and its metabolites in excreta

As expected, DON was quantified in all excreta of birds fed DON at 21 d as well as 42 d, in diets with or without additive ($p < 0.05$), as can be seen in Table 5. Interestingly, DON concentrations in excreta were higher in birds exposed to DON and MFA than in birds exposed to DON alone at 21 d (70.6 vs 22.5 ng/g, $p < 0.08$) and at 42 d (153 vs 60.7 ng/g, $p < 0.0001$). Similarly, DON concentrations in excreta were higher in birds exposed to DON and MDE compared with birds exposed to feed contaminated by DON alone at 42 d ($p = 0.03$). However, DON concentrations in excreta were slightly lower in birds exposed to DON and IMP compared with birds fed DON contaminated diet at 42 d ($p = 0.06$).

Regarding DON-3S, the peak areas of this metabolite were significantly higher in all birds receiving DON diets (T2, T4, T6, and T8) ($p < 0.05$). Supplementation with a feed additive did not have a significant influence on the mycotoxin concentration in the excreta of any measured analytes ($p > 0.05$) (Table 5). DOM-1 was not detected in any excreta sample. DOM-3S peak areas in excreta were lower in birds exposed to DON added with MDE in comparison with birds exposed to DON contaminated feed at 42 d ($p = 0.05$).

Table 5. Effect of experimental diets on deoxynivalenol (DON) and metabolites in excreta of broiler chickens.

Dietary treatments[1]/Item			**21 d**			**42 d**		
Treatment	**DON (mg/kg)**	**Feed additive**	**DON (ng/g)**	**DON-3S[2] (×10[6]) peak of area**	**DOM-3S[3] (×10[6]) peak of area**	**DON (ng/g)**	**DON-3S (×10[6]) peak of area**	**DOM-3S (×10[6]) peak of area**
1	0	0	<5	3.57[b]	ND	<5	1.20[b]	ND
2	5	0	22.5[b]	170[a]	3.15	60.7[c]	241[a]	3.99
3	0	MFA	<5	2.57[b]	ND	<5	5.86[b]	ND
4	5	MFA	70.6[a]	172[a]	2.73	153[a]	211[a]	3.37
5	0	IMP	ND	3.64[b]	ND	<5	3.41[b]	ND
6	5	IMP	26.4[b]	200[a]	3.11	51.5[d]	234[a]	3.22
7	0	MDE	<5	1.61[b]	ND	<5	5.30[b]	ND
8	5	MDE	24.0[b]	197[a]	3.10	75.1[b]	207[a]	2.39
p- Value			**<0.0001**	**<0.0001**	0.54	**<0.0001**	**<0.0001**	0.25
RMSE[3]			25.9	42.6	0.56	12.0	44.0	0.13
p- Value (Orthogonal contrasts)								
0 *vs* DON (T1 *vs* T2)			**0.01**	**<0.0001**	-	**<0.0001**	**<0.0001**	-
DON *vs* DON + MFA (T2 *vs* T4)			***0.08***	0.93	0.21	**<0.0001**	0.25	0.42
DON *vs* DON + IMP (T2 *vs* T6)			0.62	0.23	0.89	***0.06***	0.79	0.33
DON *vs* DON + MDE (T2 *vs* T8)			0.64	0.27	0.87	**0.03**	0.20	**0.05**

[1]DON: deoxynivalenol; MFA: microbial feed additive; IMP: immuno-protector additive; MDE: mycotoxin degrading enzyme; [2]DON-3S: deoxynivalenol-3 sulphate; [3]DOM-3S: deepoxyDON-3 sulphate.

[4]RMSE: root mean square error; n: 6.

[a,b,c] Means within a column with different superscripts differ significantly. Significant *p* values ($p \leq 0.05$) and significant tendency ($p \leq 0.10$) are italicized in bold.

3.3. Zootechnical parameters

The results of productive parameters are presented in Table 6. The BWG of broiler chickens was not influenced by the dietary treatments at 10 and 42 d ($p > 0.05$). Birds exposed to DON contaminated feed combined with MFA had lower BWG than birds receiving DON contaminated feed alone ($p = 0.06$) at 21 d. Similarly, birds fed DON contaminated diet combined with MDE had lower BWG than birds exposed to DON contaminated feed alone ($p = 0.03$) at 21 d.

The different dietary treatments did not affect ($p > 0.05$) the feed intake at 10 and 42 d. At 21 d, feed intake was reduced (tendency) ($p = 0.07$) in the group DON + MFA compared to the DON group.

The DON-contaminated feed impaired the FCR in broiler chickens at 10 d ($p = 0.03$), while feed supplementation with IMP has a significant tendency to counteract this negative effect ($p = 0.06$). At 21 and 42 d, DON contamination of the control feed significantly improved the FCR ($p < 0.05$). The addition of IMP to the DON diet at 21 d resulted in a tendency of impairment of FCR ($p = 0.08$). In the same way, the combined diet of DON with MDE impaired the FCR compared to the FCR in birds exposed to DON contaminated diet ($p < 0.05$) at 21 and 42 d.

Table 6. Effect of experimental diets on performance parameters.

Dietary treatments[1]/Item			0-10 d			0-21 d			0-42 d		
Treatment	**DON (mg/kg)**	**Feed additive**	**BWG[2] (g/d/bird)**	**Feed intake (g/d/bird)**	**FCR[3] (g:g)**	**BWG (g/d/bird)**	**Feed intake (g/d/bird)**	**FCR (g:g)**	**BWG (g/d/bird)**	**Feed intake (g/d/bird)**	**FCR (g:g)**
1	0	0	16.9	23.0	1.361^{c}	36.5	53.1	1.455^{ab}	48.4	77.8	1.611^{ab}
2	5	0	16.1	22.9	1.418^{ab}	38.7	54.2	1.401^{b}	50.9	80.6	1.583^{c}
3	0	MFA	17.0	23.3	1.373^{bc}	38.8	54.9	1.418^{b}	52.0	81.8	1.572^{c}
4	5	MFA	16.3	22.3	1.375^{bc}	34.6	49.9	1.442^{b}	47.8	75.9	1.587^{bc}
5	0	IMP	16.8	23.4	1.395^{bc}	37.1	53.1	1.431^{b}	51.3	80.8	1.575^{c}
6	5	IMP	16.7	22.9	1.368^{bc}	36.2	52.1	1.449^{ab}	49.7	79.2	1.595^{bc}
7	0	MDE	16.2	23.5	1.464^{a}	37.1	52.5	1.420^{b}	50.7	80.7	1.593^{bc}
8	5	MDE	15.5	22.5	1.454^{a}	34.1	50.4	1.501^{a}	48.0	77.8	1.624^{a}
p- Value			0.39	0.72	**0.0002**	0.28	0.41	**0.015**	0.31	0.49	**0.0011**
RMSE[4]			1.68	1.84	0.06	5.01	5.56	0.06	5.01	7.09	0.03
p- Value (Orthogonal contrasts)											
0 *vs* DON (T1 *vs* T2)			0.31	0.92	**0.03**	0.32	0.65	**0.05**	0.21	0.32	**0.03**
DON *vs* DON + MFA (T2 *vs* T4)			0.84	0.46	0.10	***0.06***	***0.07***	0.14	0.14	0.11	0.78
DON *vs* DON + IMP (T2 *vs* T6)			0.40	0.99	***0.06***	0.25	0.39	***0.08***	0.54	0.61	0.35
DON *vs* DON + MDE (T2 *vs* T8)			0.33	0.56	0.16	**0.03**	0.10	**0.0003**	0.16	0.32	**0.001**

[1] DON = deoxynivalenol; MFA = microbial feed additive; IMP = immuno-protector additive; MDE = mycotoxin degrading enzyme; [2] BWG = body weight gain; [3]FCR = feed conversion ratio.

[4]RMSE = root mean square error; n = 12.

[a,b,c] Means within a column with different superscripts differ significantly. Significant *p* values ($p \leq 0.05$) and significant tendency ($p \leq 0.10$) are italicized in bold.

3.4. Mortality

No significant differences in the mortality were observed among the experimental groups except that there was a tendency to increase in birds fed DON combined with MDE compared with DON contaminated feed ($p < 0.08$) (Table 7).

Table 7. Effect of experimental diets on mortality.

Dietary treatments[1]/Item			**42 d**
Treatment	**DON (mg/kg)**	**Feed additive**	**Mortality (%)**
1	0	0	2.08
2	5	0	2.08
3	0	MFA	0.00
4	5	MFA	0.00
5	0	IMP	4.16
6	5	IMP	8.33
7	0	MDE	2.08
8	5	MDE	8.33
p- Value			0.14
RMSE[2]			9.13
P- Value (Orthogonal contrasts)			
0 *vs* DON (T1 *vs* T2)			1.00
DON *vs* DON + MFA (T2 *vs* T4)			0.56
DON *vs* DON + IMP (T2 *vs* T6)			0.11
DON *vs* DON + MDE (T2 *vs* T8)			***0.08***

[1]DON: deoxynivalenol; MFA: microbial feed additive; IMP: immuno-protector additive; MDE: mycotoxin degrading enzyme.

[2]RMSE: root mean square error; n: 12.

Significant tendency ($p \leq 0.10$) are italicized in bold.

3.5. Relative weight of organs

The results of RW of organs at 10 and 42 d are shown in Table 8 and Table 9, respectively. The RW of organs evaluated were not affected by dietary treatments at 10 d ($p > 0.05$).

At the end of the experiment, the RW of livers of birds receiving DON contaminated feed for 42 d tended to decrease compared with broilers fed control diet ($p = 0.09$). However, the addition of IMP to the DON contaminated feed counteracted this effect and increased the RW of this organ ($p = 0.004$). The broilers exposed to DON and MFA had higher RW of small intestine compared with those fed MFA only ($p = 0.05$). In the same manner, the weight of the small intestine of the broilers exposed to control group was higher than that of broilers fed MDE ($p = 0.05$). Regarding RW of bursa of Fabricius, a tendency of decrease was observed upon feeding broilers IMP plus DON diets in comparison with those receiving control diet added with IMP ($p = 0.09$). Furthermore, the birds fed DON contaminated feed supplemented with MDE had a higher weight of the bursa of Fabricius (a tendency $p = 0.09$) than birds fed basal diet supplemented with MDE.

Table 8. Effect of experimental diets on relative weight (RW) of organs in broiler chickens at d 10[1].

Dietary treatments[2]/Item			**10 d**							
Treatment	**DON (mg/kg)**	**Feed additive**	**Gizzard (%)**	**Liver (%)**	**Kidneys (%)**	**Small intestine (%)**	**Colon (%)**	**Thymus (%)**	**Spleen (%)**	**Bursa of Fabricius (%)**
1	0	0	3.63	3.43	0.94	7.22	0.19	0.58	0.072	0.22
2	5	0	3.42	3.53	0.98	7.28	0.19	0.65	0.067	0.22
3	0	MFA	3.43	3.35	0.92	7.65	0.20	0.63	0.082	0.20
4	5	MFA	3.65	3.42	1.01	7.47	0.21	0.62	0.055	0.23
5	0	IMP	3.75	3.52	1.02	7.49	0.21	0.65	0.075	0.23
6	5	IMP	3.42	3.38	0.97	7.41	0.17	0.61	0.061	0.23
7	0	MDE	3.60	3.40	0.94	7.01	0.19	0.66	0.072	0.25
8	5	MDE	3.65	3.55	0.95	7.40	0.20	0.59	0.082	0.24
p- Value			0.20	0.85	0.57	0.67	0.72	0.83	0.25	0.81
RMSE[3]			0.37	0.37	0.13	0.80	0.05	0.15	0.02	0.04
p- Value (Orthogonal contrasts)										
0 *vs* DON (T1 *vs* T2)			0.17	0.55	0.48	0.86	0.81	0.24	0.66	0.97
DON *vs* DON + MFA (T2 *vs* T4)			0.12	0.48	0.65	0.55	0.38	0.65	0.35	0.75
DON *vs* DON + IMP (T2 *vs* T6)			0.98	0.35	0.76	0.69	0.37	0.48	0.62	0.89
DON *vs* DON + MDE (T2 *vs* T8)			0.12	0.86	0.55	0.71	0.83	0.32	0.21	0.27

[1] RW: relative weight; [2] DON: deoxynivalenol; MFA: microbial feed additive; IMP: immuno-protector additive; MDE: mycotoxin degrading enzyme.

[3]RMSE: root mean square error; n: 12.

Table 9. Effect of experimental diets on relative weight (RW) of organs in broiler chickens at d 42[1].

Dietary treatments[2]/Item			42 d							
Treatment	**DON (mg/kg)**	**Feed additive**	**Gizzard (%)**	**Liver (%)**	**Kidneys (%)**	**Small intestine (%)**	**Colon (%)**	**Thymus (%)**	**Spleen (%)**	**Bursa of Fabricius (%)**
1	0	0	1.69	2.01	0.64	3.16^{ab}	0.102	0.59	0.123	0.089^{AB}
2	5	0	1.65	1.78	0.63	3.15^{ab}	0.125	0.62	0.124	0.088^{AB}
3	0	MFA	1.70	1.99	0.62	2.96^{bc}	0.124	0.57	0.108	0.091^{AB}
4	5	MFA	1.74	1.97	0.61	3.26^{a}	0.106	0.61	0.107	0.085^{AB}
5	0	IMP	1.72	2.15	0.65	3.05^{abc}	0.113	0.53	0.121	0.099^{B}
6	5	IMP	1.61	2.18	0.67	3.10^{ab}	0.100	0.57	0.105	0.077^{B}
7	0	MDE	1.54	2.03	0.59	2.79^{c}	0.094	0.58	0.102	0.077^{B}
8	5	MDE	1.70	2.01	0.64	3.06^{abc}	0.113	0.61	0.129	0.102^{A}
p- Value			0.21	0.13	0.39	**0.05**	0.55	0.68	0.16	***0.09***
RMSE[3]			0.18	0.30	0.07	0.33	0.04	0.11	0.02	0.02
p- Value (Orthogonal contrasts)										
0 *vs* DON (T1 *vs* T2)			0.63	***0.09***	0.74	0.93	0.19	0.60	0.94	0.92
DON *vs* DON + MFA (T2 *vs* T4)			0.26	0.17	0.64	0.42	0.26	0.88	0.16	0.73
DON *vs* DON + IMP (T2 *vs* T6)			0.55	**0.004**	0.22	0.72	0.14	0.27	0.10	0.22
DON *vs* DON + MDE (T2 *vs* T8)			0.56	0.10	0.63	0.40	0.48	0.83	0.70	0.16

[1] RW: relative weight; [2] DON: deoxynivalenol; MFA: microbial feed additive; IMP: immuno-protector additive; MDE: mycotoxin degrading enzyme.

[3]RMSE: root mean square error; n: 12.

[a,b,c] Means within a column with different superscripts differ significantly; Significant *p* values ($p \leq 0.05$).

[A,B] Means within a column with different superscripts tended to be differ significantly different; Significant tendency ($p \leq 0.10$) are italicized in bol

3.6. Intestinal morphological parameters

The results of the morphology and histology of small intestine at 10 and 42 d are presented in Table 10. Small intestine length did not change by dietary treatments. However, the small intestine density at 10 d was higher in birds fed with MFA with DON compared to birds fed DON contaminated feed alone **(***p* = 0.02). Additionally, the evaluation of the small intestine histology at 42 d revealed that the villus height, crypt depth and the ratio villus height to crypt depth were not affected by dietary treatments (*p* > 0.10).

Table 10. Effect of experimental diets on small intestine morphometry.

Dietary treatments[1]/Item			10 d		42 d		42 d		
Treatment	**DON (mg/kg)**	**Feed additive**	**Length (cm)**	**Density (g/cm)**	**Length (cm)**	**Density (g/cm)**	**Villus height (µm)**	**Crypt depth (µm)**	**Villus height to crypt depth ratio**
1	0	0	100	0.15^{B}	173	0.37	998	102	10.2
2	5	0	100	0.15^{B}	182	0.38	921	104	9.82
3	0	MFA	97.5	0.17^{A}	184	0.35	1080	123	9.55
4	5	MFA	97.9	0.17^{A}	183	0.36	1010	96.8	10.9
5	0	IMP	100	0.15^{B}	188	0.34	998	99.0	11.1
6	5	IMP	103	0.16^{AB}	183	0.37	1008	93.5	11.6
7	0	MDE	99.7	0.16^{AB}	180	0.35	1078	112	10.2
8	5	MDE	97.9	0.16^{AB}	175	0.35	957	111	9.56
p- Value			0.72	***0.08***	0.12	0.70	0.62	0.18	0.66
RMSE[2]			7.95	0.01	13.3	0.05	176	21.1	2.42
p- Value (Orthogonal contrasts)									
0 *vs* DON (T1 *vs* T2)			0.82	0.97	0.11	0.87	0.36	0.81	0.70
DON *vs* DON + MFA (T2 *vs* T4)			0.51	**0.02**	0.80	0.34	0.38	0.50	0.39
DON *vs* DON + IMP (T2 *vs* T6)			0.39	0.44	0.78	0.90	0.36	0.31	0.14
DON *vs* DON + MDE (T2 *vs* T8)			0.52	0.58	0.20	0.27	0.69	0.52	0.83

[1]DON: deoxynivalenol; MFA: microbial feed additive; IMP: immuno-protector additive; MDE: mycotoxin degrading enzyme.

[2]RMSE: root mean square error; n: 12.

[A,B] Means within a column with different superscripts tended to be differ significantly different; Significant tendency ($p \leq 0.10$) are italicized in bold.

3.7. Blood biochemical parameters

The results of blood biochemical parameters at 10 and 42 d are shown in Table 11. The contaminated diet with DON increased the serum cholesterol level ($p = 0.04$) and triglycerides ($p = 0.03$) of broiler chickens at 10 d. The addition of MFA (tendency) ($p = 0.09$) or IMP ($p = 0.05$) counteracted the mycotoxin-induced effect on cholesterol but not on triglycerides level. The serum total protein levels were not affected by the experimental treatments ($p > 0.05$) at both time-points.

Table 11. Effect of experimental diets on serum biochemistry.

Dietary treatments/Item[1]			10 d			42 d		
Treatment	**DON (mg/kg)**	**Feed additive**	**Cholesterol (mg/dL)**	**Total protein (g/L)**	**Triglycerides (mg/dL)**	**Cholesterol (mg/dL)**	**Total protein (g/L)**	**Triglycerides (mg/dL)**
1	0	0	154	23.5	140	144	29.5	106
2	5	0	168	23.8	167	145	28.8	100
3	0	MFA	153	22.4	174	137	29.7	96.2
4	5	MFA	156	22.6	158	148	30.2	96.8
5	0	IMP	161	23.8	152	147	30.1	102
6	5	IMP	154	23.0	153	139	29.2	103
7	0	MDE	159	23.5	154	136	28.2	112
8	5	MDE	157	22.7	157	144	30.2	97.5
p- Value			0.44	0.57	0.27	0.39	0.42	0.85
RMSE[2]			17.1	2.08	30.9	14.3	2.25	28.7
p- Value (Orthogonal contrasts)								
0 *vs* DON (T1 *vs* T2)			**0.04**	0.71	**0.03**	0.91	0.53	0.61
DON *vs* DON + MFA (T2 *vs* T4)			***0.09***	0.18	0.48	0.61	0.20	0.73
DON *vs* DON + IMP (T2 *vs* T6)			**0.05**	0.35	0.27	0.32	0.71	0.80
DON *vs* DON + MDE (T2 *vs* T8)			0.11	0.22	0.43	0.82	0.17	0.78

[1]DON: deoxynivalenol; MFA: microbial feed additive; IMP: immuno-protector additive; MDE: mycotoxin degrading enzyme.

[2]RMSE: root mean square error; n: 12.

Significant *p* values ($p \leq 0.05$) and significant tendency ($p \leq 0.10$) are italicized in bold.

3.8. Blood hematological parameters

The results of blood hematological parameters at 10 and 42 d are shown in Table 12 and Table 13.

3.8. 1. Blood count

The presence of DON (T2) in broiler diet at 10 d of age reduced significantly HCT (p = 0.01), HGB (p = 0.008) and tended to decrease RBC level (p = 0.08). At 10 d, the addition of IMP to DON contaminated diet relatively increased the HCT level compared to broilers fed DON contaminated diet alone (p = 0.08). At 42 d, DON contaminated feed (T2) tended to increase blood MCV level (p = 0.09, but MDE supplementation overcome this effect (p = 0.04). MFA or MDE significantly reduced MCH (p = 0.008) regarding control group at 42 d. Lower levels of MCH (p = 0.008) were observed in the blood of broilers at 42 d when fed MFA compared with broilers fed DON and MFA.

3.8. 2. Leukogram

DON contaminated feed (T2) tended to reduce the monocytes presence at 10 d (p = 0.07). The addition of IMP to DON contaminated diet shifted the DON-induced effect on blood monocytes at 10 d (p = 0.009).

At 42 d, DON contaminated feed increased leukocytes contents (p = 0.05) and tended to reduce the lymphocytes level (p = 0.07).

When the DON diet was supplemented with MDE, the lymphocytes presence on broilers 10 d old were reduced (p = 0.05).

Birds fed DON combined with MDE showed a trend in reduction of eosinophils compared to birds exposed to DON contaminated feed at 42 d (p = 0.06). Also, the combination of DON and IMP tended to decrease lymphocytes regarding the IMP group (p = 0.07) and increased the blood monocytes regarding DON contaminated feed at 42 d (p = 0.05. Similarly, the monocytes increased when DON diet was added with MDE compared with DON contaminated feed at 42 d.

Table12. Effect of experimental diets on blood count.

Dietary treatments[1]/Item			10 d						42 d					
Treatment	**DON (mg/kg)**	**Feed additive**	**HCT[2] (%)**	**HGB[2] (g/dL)**	**RBC[2] (×10^6 /µL)**	**MCV[2] (fL)**	**MCH[2] (pg)**	**MCHC[2] (g/dL)**	**HCT (%)**	**HGB (g/dL)**	**RBC (×10^6 /µL)**	**MCV (fL)**	**MCH (pg)**	**MCHC (g/dL)**
1	0	0	30.9	11.8	2.05	148	56.9	38.4	30.1	13.4	2.59	116	52.6^a	44.5
2	5	0	28.5	10.9	1.93	147	56.4	38.3	30.0	12.8	2.49	120	53.3^a	43.6
3	0	MFA	29.9	11.1	1.99	149	56.1	37.5	31.1	12.5	2.66	116	50.3^b	43.5
4	5	MFA	29.2	11.2	1.99	147	56.4	38.5	29.1	12.9	2.45	118	52.7^a	44.6
5	0	IMP	28.5	10.8	1.92	148	56.5	38.1	29.4	12.7	2.46	119	52.5^a	43.9
6	5	IMP	30.3	11.3	1.98	152	57.2	37.6	29.9	13.2	2.56	118	52.7^a	44.0
7	0	MDE	29.7	11.2	2.00	147	56.6	38.1	28.2	12.3	2.43	115	50.4^b	43.5
8	5	MDE	29.4	11.2	2.02	145	55.6	38.2	29.7	13.1	2.52	115	51.8^{ab}	44.5
p- Value			0.21	0.15	0.57	0.78	0.48	0.93	0.49	0.61	0.20	0.39	**0.008**	0.81
RMSE[3]			2.37	0.86	0.15	9.01	1.68	2.17	2.84	1.35	0.20	5.60	2.07	2.08
p- Value (Orthogonal contrasts)														
0 *vs* DON (T1 *vs* T2)			**0.01**	**0.008**	***0.08***	0.82	0.42	0.84	0.94	0.35	0.26	***0.09***	0.44	0.31
DON *vs* DON + MFA (T2 *vs*T4)			0.44	0.32	0.33	0.90	0.99	0.75	0.43	0.88	0.69	0.31	0.50	0.29
DON *vs* DON + IMP (T2 *vs* T6)			***0.08***	0.22	0.43	0.19	0.25	0.46	0.88	0.59	0.44	0.27	0.51	0.64
DON *vs* DON + MDE (T2*vs*T8)			0.32	0.33	0.17	0.63	0.27	0.96	0.76	0.70	0.75	**0.04**	0.10	0.34

[1]DON: deoxynivalenol; MFA: microbial feed additive; IMP: immuno-protector additive; MDE: mycotoxin degrading enzyme; [2]HCT: hematocrit; HGB: hemoglobin; RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean cell hemoglobin; MCHC: mean corpuscular hemoglobin concentration.

[3]RMSE: root mean square error; n: 12.

[a,b] Means within a column with different superscripts differ significantly; Significant *p* values ($p \leq 0.05$) and significant tendency($p \leq 0.10$) are italicized in bold.

Table 13. Effect of experimental diets on leukogram.

Dietary treatments[1]/Item			10 d						42 d					
Treatment	**DON (mg/kg)**	**Feed additive**	**LEUK[2] (× 10^3 /µl)**	**EOS[2] (%)**	**BAS[2] (%)**	**LYM[2] (%)**	**MON[2] (%)**	**TOT HET[2] (%)**	**LEUK (×10^3 /µl)**	**EOS (%)**	**BAS (%)**	**LYM (%)**	**MON (%)**	**TOT HET (%)**
1	0	0	15.5	0.33	7.66	54.7	4.00	33.2	14.8	1.90	6.90	57.0^{A}	1.40	35.5
2	5	0	18.1	0.41	5.41	53.3	1.10	37.5	19.5	3.09	6.81	47.2^{AB}	1.00	39.5
3	0	MFA	19.6	0.33	8.41	46.4	3.72	40.5	18.6	3.00	7.70	49.5^{AB}	3.10	35.3
4	5	MFA	16.1	0.41	7.91	52.4	2.50	36.7	17.6	3.08	8.16	52.3^{A}	2.41	32.1
5	0	IMP	17.3	0.72	4.45	45.0	4.63	45.1	18.1	1.20	7.60	54.1^{A}	2.50	34.6
6	5	IMP	17.2	0.60	7.30	49.6	4.30	35.2	19.2	1.54	6.80	40.5^{B}	3.18	46.7
7	0	MDE	16.7	0.08	8.91	50.5	2.58	37.9	19.7	1.25	6.58	55.1^{A}	1.91	35.1
8	5	MDE	17.3	0.70	6.45	44.8	2.30	43.6	19.5	1.09	08.0	47.4^{AB}	3.40	37.8
p- Value			0.41	0.49	0.32	0.18	0.13	0.35	0.51	0.28	0.95	***0.07***	0.13	0.16
RMSE[3]			4.28	0.93	5.28	10.6	3.08	12.8	5.77	2.30	4.33	12.6	2.23	11.8
p- Value (Orthogonal contrasts)														
0 *vs* DON (T1 *vs* T2)			0.14	0.70	0.28	0.74	***0.07***	0.41	**0.05**	0.46	0.86	***0.07***	0.88	0.43
DON *vs* DON + MFA (T2 *vs*T4)			0.24	0.80	0.20	0.83	0.33	0.87	0.43	0.99	0.44	0.33	0.16	0.14
DON *vs* DON + IMP (T2 *vs* T6)			0.61	0.24	0.77	0.43	**0.009**	0.66	0.90	0.42	0.83	0.22	**0.05**	0.15
DON *vs* DON + MDE (T2*vs*T8)			0.65	0.20	0.75	**0.05**	0.26	0.26	0.98	***0.06***	0.43	0.97	**0.01**	0.73

[1]DON: deoxynivalenol; MFA: microbial feed additive; IMP: immuno-protector additive; MDE: mycotoxin degrading enzyme; [2]LEUK: leukocytes; EOS: eosinophils; BAS: basophils; LYM: lymphocytes; MON: monocytes; TOT HET: total heterophils.

[3]RMSE: root mean square error; n: 12.

Significant *p* values ($p \leq 0.05$). ^{A,B} Means within a column with different superscripts tended to be differ significantly different; Significant tendency ($p \leq 0.10$) are italicized in bold.

3.9. Proinflammatory cytokines production

The addition of feed additives to the basal diet, with or without DON, resulted in higher concentrations of IL-8 in plasma of 10-d-broiler chicken old (Figure 1). Regarding of IL-8 in jejunum tissue, the mRNA expression was very low in all experimental treatments (Figure 2).

At the end of the experiment, DON contaminated feed (T2) resulted in a down-regulation of TNF- α in jejunum tissue of broilers (p = 0.03) (Figure 3).

IL-6 mRNA relative expression was significantly upregulated in jejunum tissue of broilers fed DON and IMP regarding those fed DON contaminated feed (T2) (p = 0.02) (Figure 4). The dietary treatments did not affect the mRNA relative expression of IFN-γ and IL-1β in jejunum of broiler chickens at 42 d (Figure 4).

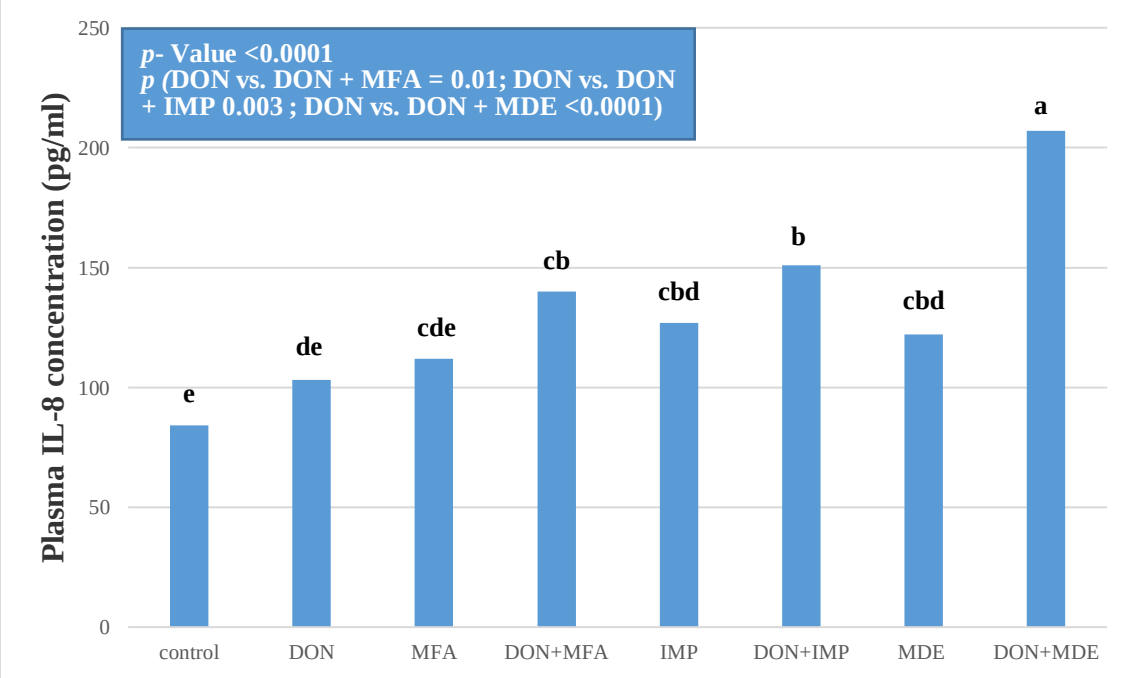

Figure 1. Effect of experimental diets on plasma IL-8 concentration in 10-d-old broiler chickens. [a,b,c,d] Means within a column with different superscripts differ significantly ($p \leq 0.05$).

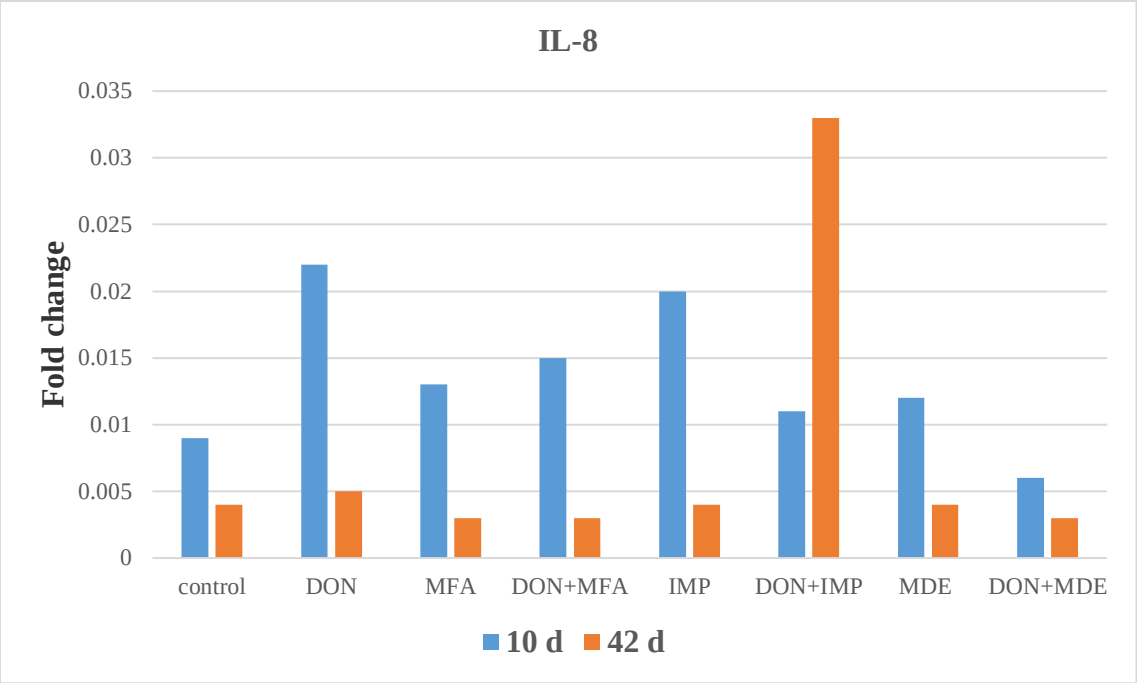

Figure 2. Effect of experimental diets on IL-8 mRNA expression in jejunum tissue of broiler chickens.

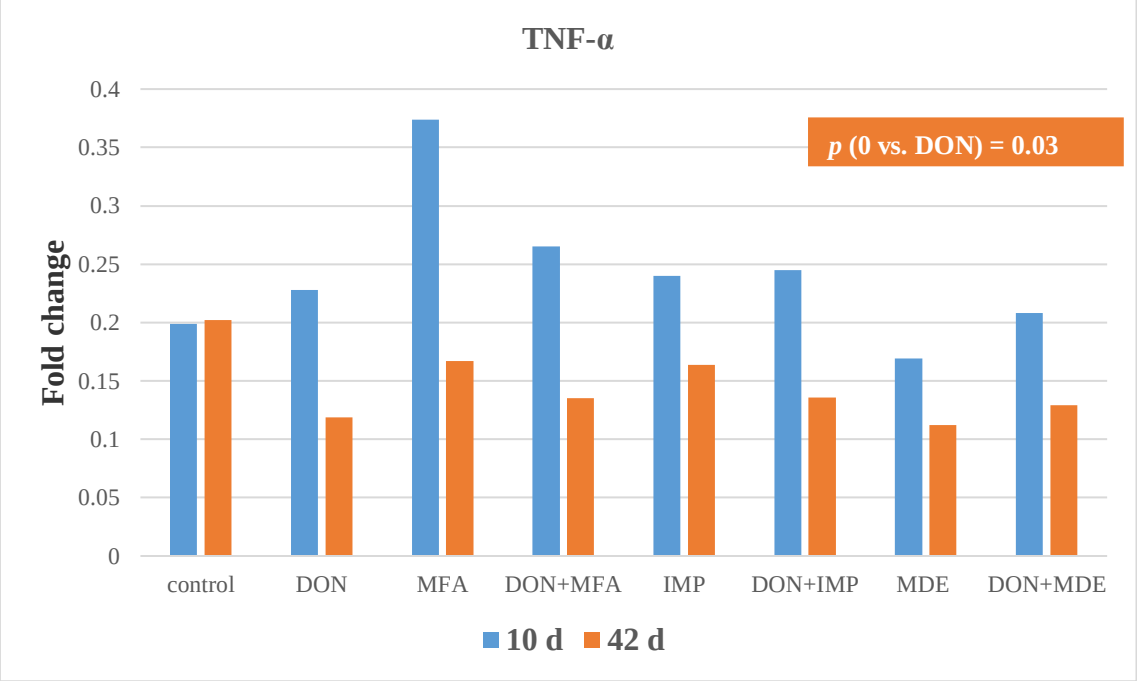

Figure 3. Effect of experimental diets on TNF-α mRNA expression in jejunum tissue of broiler chickens.

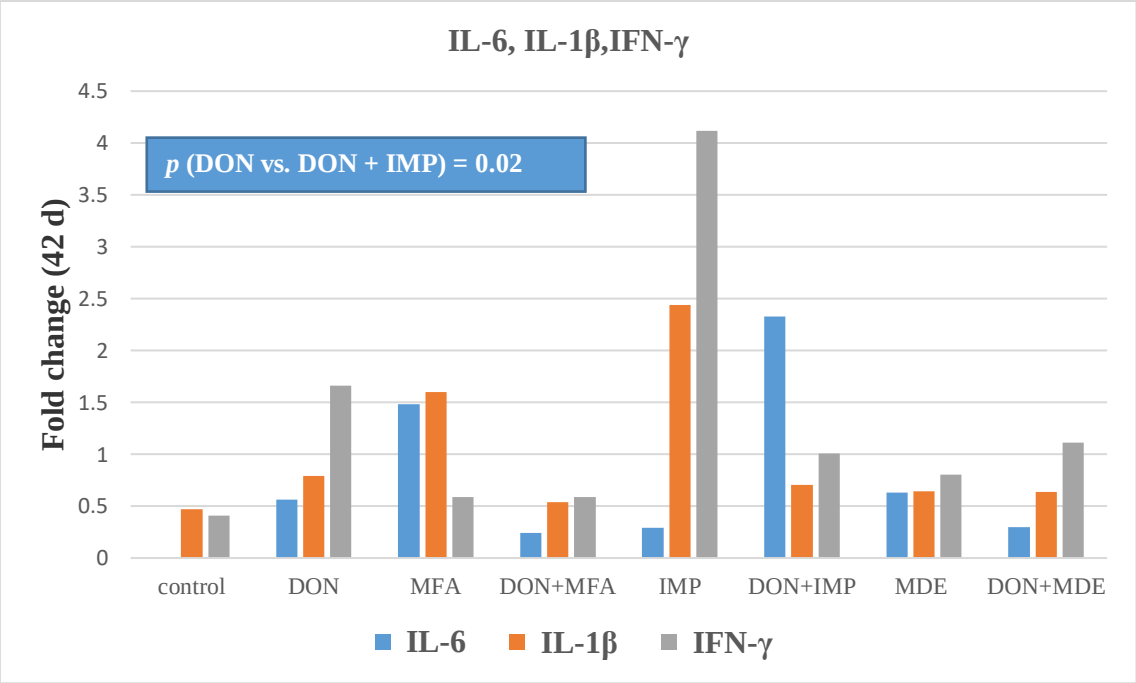

Figure 4. Effect of experimental diets on mRNA expression IL-6, IL-1β and IFN- γ in jejunum tissue of broiler chickens.

3.10. Welfare-based parameters

The results of the effects of DON and IMP, individually and in combination, on the FCORT are shown in Table 14. The FCORT (pg/mg) was significantly higher in broiler chickens fed DON contaminated diet compared to birds fed a control diet ($p = 0.03$).

Table 14. Effect of deoxynivalenol (DON) and immune-portector addtitive (IMP) on feather corticosterone (FCORT) in broiler chickens at 42 d.

Dietary treatments[1]/Item			**42 d**
Treatment	**DON (mg/kg)**	**Feed additive**	**FCORT [2](pg/mg)**
1	0	0	15.9
2	5	0	26.7
5	0	IMP	23.6
6	5	IMP	21.3
p- Value (ANOVA)			0.17
RMSE[3]			7.24
p- Value (Orthogonal contrasts)			
0 *vs* DON			**0.03**
DON *vs* DON + IMP			0.28

[1] DON: deoxynivalenol; IMP: immuno-protector additive; [2] FCORT: Feather corticosterone.

[3]RMSE: root mean square error; n: 6.

Significant *p* values ($p \leq 0.05$).

The results of the stress index (H/L ratio) are indicated in Table 15.

At 10 d, the H/L ratio was higher in birds receiving IMP than in birds receiving control diet or IMP and DON (a tendency, $p = 0.08$). At 42 d, DON contaminated feed tended to increase the H/L ratio compared to control ($p = 0.07$). However, the addition of MFA to DON contaminated feed tended to counteract this effect ($p = 0.08$). The H/L ratio was lower in birds exposed to IMP than birds receiving IMP and DON diet ($p = 0.06$) at 42 d.

Table 15. Effect of experimental diets on the stress index (heterophil to lymphocyte ratio (H/L)).

Dietary treatments[1]/Item[2]			**10 d**	**42 d**
Treatment	**DON (mg/kg)**	**Feed additive**	**H/L**	
1	0	0	0.64^{C}	0.61^{B}
2	5	0	0.74^{BC}	0.92^{AB}
3	0	MFA	0.96^{ABC}	0.82^{AB}
4	5	MFA	0.78^{BC}	0.63^{B}
5	0	IMP	1.30^{A}	0.73^{B}
6	5	IMP	0.78^{BC}	1.13^{A}
7	0	MDE	0.96^{BC}	0.72^{B}
8	5	MDE	0.78^{AB}	0.67^{B}
p- Value			***0.08***	***0.06***
RMSE[3]			0.53	0.39
P- Value (Orthogonal contrasts)				
0 *vs* DON			0.64	***0.07***
DON *vs* DON + MFA			0.84	***0.08***
DON *vs* DON + IMP			0.73	0.24
DON *vs* DON + MDE			0.10	0.15

[1]DON: deoxynivalenol; MFA: microbial feed additive; IMP: immuno-protector additive; MDE: mycotoxin degrading enzyme; [2]H/L: heterophil to lymphocyte ratio.

[3]RMSE: root mean square error; n: 12.

[A,B] Means within a column with different superscripts tended to be differ significantly different; Significant tendency ($p \leq 0.10$) are italicized in bold.

Table 16 summarizes the DON effects on the different parameters evaluated as well as the effects of the feed additives tested on counteracting the adverse effects observed.

Table 16. Summary of the effects of deoxynivalenol (DON) and 3 detoxifying agents in broiler chickens.

Parameter	**DON[1] effect**		**Feed additive effect**		
	10 d (7.42 mg/kg)	**42 d (7.42 mg/kg)**	**MFA[2]**	**IMP[3]**	**MDE[4]**
DON / metabolites					
DON in excreta					
DON-3S[5] in excreta					
DOM-3S[6] in excreta					
Productive parameters					
BWG[7]					
Feed intake					
Feed conversion ratio					
Relative weights of organ					
Gizzard					
Liver					
Kidneys					
Small intestine					
Colon					
Spleen					
Bursa of Fabricius					
Thymus					
Morphology of small intestine					
Length					
Density					
Villus height					
Crypt depth					
Villus height to crypt depth					

Blood biochemistry					
Total protein					
Cholesterol					
Triglycerides					
Blood hematology					
Hematocrit					
Hemoglobin					
Red blood cells					
MCV[8]					
MCH[9]					
MCHC[10]					
White blood cells					
Eosinophils					
Basophils					
Monocytes					
Lymphocytes					
Heterophils					
Proinflammatory cytokines					
IL[11]-8 in plasma					
IL-8 in jejunum					
IL-6 in jejunum					
IL-1 β					
IFN-γ[12]					
TNF-α[13]					
Welfare related parameters					
H/L[14] ratio					
FCORT[15]					

Negative effect; positive effect; no significant effect; not determined.

[1]DON: deoxynivalenol; [2]MFA: microbial feed additive; [3]IMP: immuno-protector additive; [4]MDE: mycotoxin degrading enzyme; [5]DON-3S: DON-3 sulphate; [6]DOM-3S: deepoxyDON-3 sulphate; [7]BWG: body weight gain; [8]MCV: mean corpuscular volume; [9]MCH: mean corpuscular hemoglobin; [10]MCHC: mean corpuscular hemoglobin concentration; [11]IL: interleukin; [12]IFN-γ: interferon-gamma; [13]TNF-α: tumor necrosis factor; [14]H/L ratio: heterophil to lymphocyte ratio; [15]FCORT: feather corticoste

4. Discussion

4.1. Dietary mycotoxin concentrations

In general, and despite the efforts made by the feed industry, the presence of mycotoxins in feed is unavoidable, especially considering that its composition includes ingredients of vegetal origin, in which fungal contamination can be developed. The case of poultry feed is not different, so these feeds are usually contaminated with mycotoxins, frequently with more than one, at varying levels. Regarding DON, and according to the EU recommendations, complete poultry feed should not contain more than 5 mg DON/kg feed (European Commission, 2006). In the current study, the mycotoxin analysis of the feed used revealed contamination by DON and FB1. However, taking into account the recommended levels for each of these two toxins, the amount of FB1 could be considered negligible, as only represents the 0.13-0.49% of the maximum FB1 level recommended, so we considered that all the negative effects observed on the evaluated parameters were due to DON.

4.2. Zootechnical parameters

Due to their relative resistance to the effects of DON in terms of performance parameters, high DON concentrations are needed to cause adverse effects on poultry (Awad et al., 2012). It should be noted that contaminated diets with DON cultured material may be less toxic than naturally contaminated diets due to the presence of masked mycotoxins, such as DON-3-glucoside, which add to the toxicity of the diet but are not detected by routine feed analyses (Sasanya et al., 2008). It has been reported that broiler chickens could tolerate up to 15 mg DON/kg without alteration on productive parameters (Awad et al., 2006a). The exposure to diets contaminated with up to 10 mg DON/kg, also, did not affect broiler chickens performances (Awad et al., 2004; Awad et al., 2006a; Ghareeb et al., 2014, 2016). Other studies, moreover, did not show impaired poultry performance when fed DON contaminated diets (Antonissen et al., 2017; Liu et al., 2020). However, Lucke et al. (2017) found significant reduction in BWG and feed consumption of broiler chickens fed different concentrations of DON contaminated feed, including lower dosis (2.5, 5 and 10 mg/kg feed).

On the other hand, broiler chickens could be sensitive to DON but only during specific periods of their growth cycle, as reported by Wang & Hogan (2019). In the present study, feed conversion ratio was impaired in the DON contaminated group only

at 10 d. These findings suggest that broilers are more susceptible to contaminated diets with DON during early stage of growth, and detrimental effects on productive parameters are not necessarily cumulative with chronic exposure to DON-contaminated diets. This temporary inhibitory effect of DON in growth performance of chickens was observed in several studies using the guidance levels of DON contamination (Awad et al. 2006b; Swamy et al., 2004) at early ages (Awad et al. 2006b; Awad et al., 2011; Kubena et al., 1989). A recent meta-analysis reported by Andretta et al. (2011) supported our findings, and also concluded that the effect of mycotoxins on broiler BWG was not constant along all growth phases and that this effect was greater in young broilers. At older ages, broiler chickens may developed different metabolic, physiological and hormonal compensatory mechanisms (Osselaere et al., 2012). Interestingly, the addition of IMP counteracted this DON-induced effect at 10 d, while the addition of MDE impaired the FCR independently of the presence or not of DON.

The FCR was improved in the DON-contaminated group from 21 d to the end of the experimental feeding trial. A growth stimulation by moderate DON levels (near or equal to 5 mg/kg feed) has already been reported in broiler chickens (Chen et al., 2017; Hamilton et al., 1985; Swamy et al., 2002).This could be associated to an effect of hormesis. Hormesis is defined as low levels of potentially toxic substance causing a stimulatory effect (Stebbing, 1982). This could explain the transient improvement in feed conversion ratio showed in the current study.

4.3. Relative weight of organs

The effects of dietary DON mycotoxin on organ weights in chickens in the literature are contradictory (Dänicke et al., 2003; Swamy et al., 2004; Yunus et al., 2012). In the present study, feeding the DON-contaminated diet during different growth stages had no effect on relative organ weights, either evaluated at 10 d or at the end of the trial (42 d).

The only exception was a decrease in relative liver weight at 42 d in birds exposed to DON contaminated feed during 42 d. This result was in agreement with Luke et al. (2017) which observed a decreased of RW of livers in DON fed-chickens (2, 5 or 10 mg/kg) for 21 d compared with control birds. Awad et al. (2006b) reported a significantly reduced liver absolute weight and numerically reduced liver relative weight in broilers fed a 5 mg/kg DON-contaminated diet at 21-day-old. Furthermore, Kubena et al. (1985) observed a decreased of RW of liver in growing chicks aged 35 d fed DON-contaminated grains (9 or 18 mg/kg feed). The addition of the feed additive IMP in the current study

counteract this change. Liver could be the site of the formation of DON-3S (Schwartz-Zimmermann et al., 2015). Decreased relative liver weight may be due to the hepatic detoxification activity.

4.4. Intestinal morphological parameters

The gastrointestinal tract is the site of nutrient digestion and absorption, and represents the first barrier against contaminated food and feed (Ghareeb et al., 2015). Small intestine enterocytes are exposed to relatively high levels of mycotoxins (Broom, 2015). The effects of DON on intestinal morphology of broiler chickens have been described before (Antonissen et al., 2014; Riahi et al., 2020; Wang & Hogan, 2019; Yu et al., 2018). Feeding diets naturally contaminated with DON (6.62 to 7.90 mg/kg) decreased the density of the small intestine, jejunum and ileum villus height, and ileum crypt depth of broiler chickens. However, in the current study, we have used a similar concentration of DON as the authors mentioned before, and no significant differences were observed on length and density of the small intestine at 10 and 42 d, neither on villus height, nor crypt depth and ratio villus height to crypt depth of broiler chickens at 42 d old. The differences between results may be due to the source of contamination, as DON purified contaminated feed could be less toxic than DON naturally contaminated feed. These findings agree with Chen et al. (2017), who did not observe any significant effect of feeding "purified" DON (2 or 5 or 10 mg/kg) on villus height of Taiwan country chickens. No intestinal morphological alterations were observed indicating no reduction of nutrient digestion and absorption.

4.5. Blood biochemistry and hematology

4.5.1. Blood biochemical parameters

One of the aim of this study was to observe parameters of clinical biochemistry and hematology to gather information about possible influences of long-term exposure to DON and the tested feed additives on the health of broiler chickens. It has been previously suggested that DON contaminated diets affects protein and lipid metabolism in broiler chickens (Ghareeb et al., 2014). Therefore, serum cholesterol, serum triglycerides and serum total protein could be good indicators of DON toxicity in broiler chickens. In a study of Ghareeb et al. (2016), serum cholesterol concentration increased in broilers fed DON-contaminated diet. These findings are comparable with the results of the present study, where DON significantly increased cholesterol concentration in serum of broilers

at 10 d. Probably, this effect can be linked to liver damage and changes in concentration of circulating cholesterol to the liver in broiler chickens fed the DON contaminated feed (Ghareeb et al., 2016). In contrast, Kubena et al. (1989) found a decreased cholesterol concentration in broilers fed a diet containing a DON and T-2 contaminated wheat. The authors also suspected liver damage as a possible reason for this reduced cholesterol concentration. This DON-induced effect was shifted by the addition of MFA ($p = 0.09$) or IMP ($p = 0.05$). In the present study, higher level of triglycerides was observed also in broiler chickens fed with DON-contaminated feed. Serum triglycerides elevation might be linked to biliary obstruction (Ghareeb et al., 2016). Furthermore, the increased level of cholesterol and triglycerides might indicate the stressful status of the birds due to DON feeding.

4.5.2. Blood hematological parameters

4.5.2.1. Blood count

In farm animals and rodents, long-term ingestion of DON causes a decrease in circulating blood cells, frequently associated with bone marrow dysfunction (Parent-Massin, 2004). Therefore, hematological parameters were selected as health status and immune system markers. The effects of feeding artificially-contaminated diet containing DON were previously shown to decrease hemoglobin, hematocrit and red blood cells in broiler chickens (Riahi et al., 2020). Feeding diets naturally contaminated with 6.8 mg DON/kg lowered hematocrit and hemoglobin concentrations in turkeys at early growth stage (Chowdhury et al., 2005). Broiler studies conducted supplying 9 to 18 mg of DON/kg feed for 3 to 12 weeks showed that DON lowered the hematocrit, hemoglobin and red blood cells level concentrations (Harvey et al., 1991; Huff et al., 1986; Kubena et al., 1985). In agreement with these previous studies Andretta et al. (2012) reported that mycotoxins reduced ($p < 0.05$) the concentration of hematocrit by 5% and hemoglobin by 15% in broiler chickens. The current results also demonstrate a significant reduction of hematocrit, hemoglobin and red blood cell at 10 d. Reduced hematocrit within 10 d observed in DON treated group implyed that hematopoietic tissue may be affected by DON mycotoxin (Chattopadhyay et al., 2013). However, no hemorrhage or hemolysis was observed in broilers at 10 d. The minor change induced by DON mycotoxin on hematocrit blood level was overcome by the addition of the IMP.

4.5.2.2. Leukogram

DON as other trichothecenes, target the 60S ribosomal unit, where this toxin inhibit the elongation-termination step during protein synthesis (Ueno, 1984). Mitogen-activated protein kinases (MAPKs) are activated and induced ribotoxic stress response (Pestka, 2007). Leukocytes were considered as sensitive cell types to DON mycotoxins due to its high turnover rate. The effects of DON, like other trichothecenes, can be immunostimulatory or immunosuppressive depending on dose, exposure frequency and timing of functional immune assay (Bondy & Pestka, 2000). DON at low concentration is immunostimulatory and upregulates expression of cytokines, chemokines and inflammatory genes, while, DON at high concentration is immunosupressive and induces leukocyte apoptosis (Pestka et al., 2004). The present results showed that DON contaminated feed resulted in a high level of white blood cells indicating that DON at the concentration used was not enough to depress-white blood cells of broiler chickens.

Mycotoxins, mainly DON, are known to be able to decrease the amounts of blood lymphocytes (Chowdhury et al., 2005). The B cells and T cells counts were reduced in broilers exposed to DON from 5.9 to 9.5 mg/kg feed (Swamy et al., 2004). Moreover, DON contaminated-feed (10 mg/kg feed) decreased blood lymphocytes count in 35-old-d broiler chickens (Ghareeb et al., 2012). Sharma (1993) indicated that lymphocyte cytotoxicity is a result of DON immunotoxic effect. In the current study, DON contaminated feed tended to slightly reduce blood lymphocytes level at 42d. The observed changes on lymphocytes in peripheral blood in mycotoxicosis conditions can be attributed to the immune system being sensitive to DON (Escrivá et al., 2015).

Blood monocytes decreased linearly in birds fed contaminated grains (Swamy et al., 2004). Monocytes are originated in the bone marrow and are released into the peripheral blood where they circulate for several days before entering the tissues to become the tissue macrophages (McDevitt, 2010). The present results show reduced number of blood monocytes in birds fed DON contaminated diet at 10 d. Decrease on blood monocytes might be attributed to their synthesis reduction in bone marrow or removal increase from the blood (Swamy et al., 2004). This effect was reversed by addition of IMP to the contaminated diet. Moreover, the results found on hematological parameters implies possible DON activity at the level of the bone marrow (Prelusky et al., 1994).

In summary, it should be noted that blood biochemical and hematological parameters were slightly affected by DON contaminated feed in the present study, although these

parameters were still within their physiological reference range (Al-Nedawi, 2018; Meluzzi et al., 1992; Talebi et al., 2005), and therefore, of limited physiological relevance.

4.6. Proinflammatory cytokines

Proinflammatory cytokines such as IL-1β, IL-6, IL-8, TNF-α, and IFN-γ are released predominantly by cells of the innate immune system or macrophages activated by molecular components of the pathogens. These cytokines are involved in the coordination of a local and systemic inflammation and the regulation of immune responses leading to deep physiological and behavioural changes (Bruno et al., 2015). Results reported from *in vitro* and *in vivo* studies indicated that DON ingestion at low to moderate dose induced proinflammatory gene expression with consequent induction of proinflammatory cytokines and chemokines in different tissues, and, at very high doses, apoptosis (Pestka, 2010). Among the cytokines induced by DON, TNF-α, IL-1β, IL-6 and IL-8 are well-known to significantly affect feed intake, growth and immune function (Pestka & Amuzie, 2008). On the other hand, the intestine is a considerable immunologic organ due to its capability to mount innate and specific challenges to antigens associated with microorganisms and ingested feed (Hughes,2005). Proinflammatory interleukins have been shown to participate in the gastrointestinal inflammatory process (Hardin et al., 2000). The intestine cells are sensitive to DON mycotoxicosis due to its high turnover rate. The impact of DON on the relative expression levels of immune genes, including cytokines and transcription factors, in the intestines of broiler chickens have been described (Lucke et al., 2018). Proinflammatory cytokines might serve as sensitive biomarkers of DON's adverse effects. However, for IL-8 we found a low response in all the birds form the different experimental treatments. Present results also demonstrated that the presence of DON in broilers feed did not affect the relative expression of IL-6, IL-1β and IFN-γ, but down-regulated the relative expression of TNF-α at 42d. Lower TNF-α expression was shown also in domestic pigs exposed to DON contaminated feed, suggesting the inhibition of immune system (Becker et al., 2011). Ghareeb et al. (2013) also observed that DON (10 mg/kg) decreased plasma TNF-α in broilers chickens at 35 d which indicates the ability of DON to inhibit the protein synthesis. The authors suggested that the TNF-α down-regulation is a significant indicator that DON can impair immune function and increase susceptibility to infectious diseases (Awad et al., 2013; Ghareeb et

al., 2013). However, from our results we consider that the decrease of relative expression of TNF-α was not sufficient to cause a distinct physiological response between birds.

On the other hand, the addition of IMP to DON contaminated diet induced the release of cytokines, probably suggesting the immune stimulating properties of these feed additives.

4.7. Welfare based parameters

Based on our knowledge, this is the first time that broiler feathers are used as a target to evaluate the corticosterone level after exposure to DON contaminated feed. In this context, some previous studies have used plasma corticosterone level as a biomarker of the stress response in broilers fed DON-contaminated diets. Thus, and as a stress indicator, it has been seen that feeding broiler chickens with 4.6 mg DON/kg for 15 d increases the plasma corticosterone level (Antonissen et al., 2017). Same findings were reported when broiler chickens were fed 10 mg DON/kg feed for 35 d (Ghareeb et al., 2014) affecting negatively animal welfare. However, in a recent work, it has been found that plasma corticosterone level was reduced in broilers fed up 15 mg/kg DON during 42 d (chapter 3). Corticosterone in plasma is an acute timeframes (seconds to days) measurement, and such point-in-time measurements can be subject to handling-induced release of corticosterone (Freeman & Newman, 2018). Corticosterone in feathers is a long-term, integrated measure of avian stress physiology (Bortolotti et al., 2008). Then, we have suggested that corticosterone in feather could be a more relevant biomarker for chronic toxicity of DON in chickens. In the current study, DON increased the feather corticosterone content in 42 d old broiler chickens. The addition of IMP did not significantly cover this deleterious effect.

Another physiological parameter that could reflect the stress of chickens is the ratio between the circulating total heterophils and lymphocytes (Gross & Siegel, 1983). It has been reported that the presence of DON in chickens feed increases the total heterophils and reduced the lymphocytes and consequently increases the stress index (H/L ratio).In the actual study, this parameter tended to be higher in birds fed DON contaminated feed for 42 d, reinforcing the results observed on feather corticosterone and leading to the conclusion that DON induced stress responses in birds. Ghareeb et al. (2012, 2014) also observed an increase in H/L ratio in broilers fed DON at 10 mg/kg for 35 d. MFA addition to the DON-contaminated diet tended to counteract the toxic effects of DON on this stress

index. These results indicate that addition of MFA tend to overcome the stress of broilers due to exposure to DON, suggesting that supplementing the broiler diet with MFA could modulate the physiological stress responses.

4.8. Analysis of DON and its metabolites in excreta

A direct approach to evaluate the efficacy of a mycotoxin detoxifying agent is measuring DON and its metabolites in a biological matrix (blood, urine, milk, organs), determined by accurate methods of analysis (EFSA, 2017). Recently, DON-3S has been proposed as a major DON metabolite in poultry. Wan et al. (2014) found DON-3S in excreta of chickens (89±7%) after an administered dose of DON (2.5 mg/kg BW), using radioisotope counting radio-HPLC for quantification. In the same way, and after chronic feeding of 1.7 mg DON/kg to broiler chickens, DON-3S was the most abundant metabolite found in broiler chicken excreta (Schwartz-Zimmermann et al., 2015). It has been previously described that DON is rapidly absorbed between crop and upper jejunum, metabolized rapidly after absorption and excreted into urine (Lun et al., 1988). Sulfation of DON could occur in enterocytes of the intestinal mucosa and liver (Schwartz-Zimmermann et al., 2015). The elimination of DON-3S into excreta may be achieved via bile and via urine (Schwartz-Zimmermann et al., 2015). The same authors found DOM-3-S in excreta of pullets and roosters after feeding a DON-contaminated diet. It has been supposed that the presence of this metabolite in excreta was due to the formation of DOM-1 by intestinal microbes, absorption of DOM-1 from the gastrointestinal tract, sulfation in the intestinal mucosa, liver and/or kidney, and excretion into the cloaca via urine or back into the gastrointestinal tract via bile. Another explanation argues that the formation of DOM-3S could be the result of the microbial biotransformation of DON-3S in the caecum or the sulfation of DOM-1 by intestinal microbes (Schwartz-Zimmermann et al., 2015). In the current study, DON, DON-3S, and DOM-3S were found as specific metabolites of exposure of DON in broiler chickens after chronic feeding, confirming that sulfation is the most important pathway of metabolization of DON in this target specie. The biotransformation of DON to DON-3S in chickens was considered rapid and extensive (Broekaert et al., 2017). Furthermore, we observed that the additives MFA (at 21 and 42 d) increased significantly the excretion of DON in broiler chickens but no increase was observed for the metabolites DON-3S and DOM-3S. These results imply the efficacy of this product in counteracting DON adverse effect (Rychen et al., 2018). Whilst, MFA is a product claiming its ability to biotransform DON into a non-toxic

metabolite, however, in this study we have not observed the formation of DOM-1 or the formation of other specific DON metabolites, as metabolites detected in excreta were the same for the groups DON+MFA or DON+MDE regarding the control group only containing DON.

5. Conclusions

Summarizing, it can be concluded from the present results that specific biomarkers of exposure of DON in broilers are DON, DON-3S and DOM-3S in excreta. On the other hand, feeding a DON-contaminated diet made using cultured contaminated material showed a temporally negative impact on performance of broiler chickens, which can be seen at 10 d, but this negative effect reverted afterwards. Moreover, feeding DON contaminated diet to broiler chickens slightly affected some nonspecific parameters such as biochemical, hematological and welfare related parameters. Regarding the assayed additives, MFA increased the excretion of DON suggesting the ability of this additive to reduce the animal exposition to DON, this could be explained by a capacity of the product to adsorb DON, but contrary to what we expected the product was not able to biotransform DON in less toxic metabolite. Moreover, to confirm the reduction of animal exposure to DON, MFA counteracted DON-induced effect on cholesterol level and stress index. The supplementation of IMP showed to reverse DON- effects on feed conversion ratio, relative weight of liver, serum cholesterol level, blood hematocrit, and monocytes level. For MDE, no new metabolites compared to DON treatment were detected, so the activity of the product expected could not be demonstrated. Moreover, MDE increased slightly the DON excretion but concomitantly decreased the DON-3S and DOM-3S excretion suggesting a shift of the DON metabolism (DON detoxification pathway) in broilers. To confirm this, no effect of the MDE product was observed on nonspecific induced parameters. Nevertheless, it should be taken into account that the nature and the mode of action of each feed additive could be the responsible for the different effects observed. Furthermore, other results might be obtained when assessing different feed additive inclusion rates and/or mycotoxin contamination source and levels. Therefore, it would be convenient to carry out more *in vivo* trials in order to evaluate the effectiveness of these additives. In the same way, it would be very interesting to evaluate their protective effect in other animal species.

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GENERAL DISCUSSION

Mycotoxins occurrence in feed is really a significant problem worldwide because of their adverse effects on health and animal performance, which results in great economic loss. The trichothecene deoxynivalenol (DON) is a mycotoxin mainly produced by *Fusarium graminearum* and *Fusarium culmorum*. DON is the mycotoxin most commonly detected in cereals and grains, particularly in wheat, barley, maize and their by-products. In poultry, chronic exposure to low to moderate doses of DON, the most common situation, can lead to reduced feed consumption, reduced weight gain, nutrients malabsorption, metabolic and physiological changes and immunological alterations. Due to these toxic effects, it is essential to reduce or minimize the contamination by this mycotoxin in poultry feed and avoid its presence in the food chain.

Besides prevention and the different biological, chemical and physical approaches that are used for mycotoxin detoxification, the most commonly used method to counteract the negative impact of mycotoxins on animals is adding mycotoxin detoxifying agents (mycotoxin detoxifiers) to feed. These feed additives, so-called mycotoxin binders or mycotoxin modifiers, either adsorb or biotransform mycotoxins in the gastrointestinal tract of the animal. Depending on their mode of action, these feed additives may act by reducing the bioavailability of the mycotoxins (adsorbing agents) or by degrading them into less toxic metabolites (biotransforming agents). For DON, adsorbing agents have shown poor efficiency while biotransforming agents seem to be the most promising products to reduce the mycotoxin toxicity. In all cases, these products must be tested on their ability to detoxify mycotoxins *in vivo* (efficacy). At the time being, no reliable *in vivo* models in chicken are available to evaluate the efficacy of DON detoxifiers based on specific biomarkers.

The aim of this thesis was to investigate the efficacy of three DON mycotoxin detoxifying agents. To achieve this aim, firstly a suitable *in vivo* model was developed in broiler chicken to evaluate the specific and nonspecific parameters of toxicity of DON. In this *in vivo* "toxicity trial":

i) Specific parameters related to DON were estimated in biological fluids after DON exposure of broiler chickens.

ii) Nonspecific parameters related to the indirect effects of DON on various physiological functions in broiler chickens were evaluated.

Secondly, *in vivo* trials, based on the parameters identified in the first part of the thesis, were performed to study the efficacy of three detoxifying agents to reduce the toxicity of DON. An overview of the main results obtained are discussed in this section. Table 25 summarizes the results obtained in this doctoral thesis.

Table 25. Overview of the main results of this doctoral thesis.

	In vivo "toxicity trial", 42 d (chapter 2 and 3)				*In vivo* "additive efficacy trial", DON at 5 mg/kg (chapter 4)				
Biomarkers	**DON at 5 mg/kg**	**DON at 15 mg/kg**	**Biomarkers selected**	**Biomarkers added**	**10 d**	**42 d**	**MFA[1]**	**IMP[2]**	**MDE[3]**
DON[4] and its metabolites									
DON in plasma									
DON in liver									
DON in excreta							+++		+++
DON-3S[5] in plasma									
DON-3S in liver									
DON-3S in excreta									
DOM-3S[6] in excreta									
DOM-1[7] in excreta									
Productive parameters									
BWG[8]									
Feed intake									
Feed conversion ratio		+++			+++	---			
Relative weight of organs									
Heart									
Proventriculus									
Pancreas									
Cecum									
Gizzard	+++	+++							
Liver						---			

Table 25. Overview of the main results of this doctoral thesis.

	***In vivo* "toxicity trial", 42 d (chapter 2 and 3)**				***In vivo* "additive efficacy trial", DON at 5 mg/kg (chapter 4)**				
Biomarkers	**DON at 5 mg/kg**	**DON at 15 mg/kg**	**Biomarkers selected**	**Biomarkers added**	**10 d**	**42 d**	**MFA[1]**	**IMP[2]**	**MDE[3]**
Kidneys									
Small intestine	---	---							
Colon	---	---							
Spleen									
Bursa of Fabricius	---	---							
Thymus	+++	+++							
Morphology of small intestine									
Length	+++	+++							
Density	---	---							
Villus height									
Crypt depth									
Villus height to Crypt depth									
Blood biochemistry									
Total protein									
Cholesterol		---			+++				
Triglycerides					+++				
Creatine kinase	---								
Albumin									
AST[9]									
ALT[10]									
ALP[11]									
GGT[12]									
LDH[13]									

Table 25. Overview of the main results of this doctoral thesis.

	In vivo "toxicity trial", 42 d (chapter 2 and 3)				*In vivo* "additive efficacy trial", DON at 5 mg/kg (chapter 4)				
Biomarkers	**DON at 5 mg/kg**	**DON at 15 mg/kg**	**Biomarkers selected**	**Biomarkers added**	**10 d**	**42 d**	**MFA[1]**	**IMP[2]**	**MDE[3]**
Uric acid									
Glucose									
Blood hematology									
Hematocrit					---				
Hemoglobin	---	---			---				
Red blood cell		---			---				
MCV[14]		+++							
MCH[15]									
MCHC[16]		---							
White blood cell						+++			
Eosinophils									
Basophils									
Monocytes					---				
Lymphocytes						---			
Heterophils									
Response to common vaccines									
NDV[17]									
IBV[18]									
Cytokines									
IL[19]-8 in plasma	+++	+++							
IL-8 in jejunum									
IL-1β in jejunum	+++								
IFN-γ[20] in jejunum	+++								
TNF-α[21] in jejunum						---			

Table 25. Overview of the main results of this doctoral thesis.

	***In vivo* "toxicity trial", 42 d (chapter 2 and 3)**				***In vivo* "additive efficacy trial", DON at 5 mg/kg (chapter 4)**				
Biomarkers	**DON at 5 mg/kg**	**DON at 15 mg/kg**	**Biomarkers selected**	**Biomarkers added**	**10 d**	**42 d**	**MFA[1]**	**IMP[2]**	**MDE[3]**
IL-10 in jejunum									
IL-6 in jejunum	+++								
Welfare									
H/L[22] ratio						+++			
Plasma corticosterone	---	---							
FCORT[23]						+++			
Fear behaviour									
Leg color									

Not detected; detected or quantified; adverse effect of DON; no significant effect; biomarker selected or added for the *in vivo* efficacy study; positive effect of DON; detoxifying agent counteracting effect; unfilled cells: not determined; +++: increase; ---: decrease; [1]MFA: microbial feed additive; [2]IMP: immuno-protector additive; [3]MDE: mycotoxin degrading enzyme; [4]DON: deoxynivalenol; [5]DON-3S: DON-3 sulphate; [6]DOM-3S: deepoxyDON-3 sulphate; [7]DOM-1: deepoxyDON; [8]BWG: body weight gain; [9]AST: aminotransferase; [10]ALT: alanine transferase; [11]ALP; alkaline phosphatase; [12]GGT; gamma-glutamyltransferase; [13]LDH: lactate dehydrogenase; [14]MCV: mean corpuscular volume; [15]MCH: mean corpuscular hemoglobin; [16]MCHC: mean corpuscular hemoglobin concentration; [17]NDV: Newcastle disease virus; [18]IBV: infectious bronquitis; [19]IL: interleukin; [20]IFN-γ: interferon-gamma; [21]TNF-α: tumor necrosis factor; [22]H/L ratio: heterophil to lymphocyte ratio; [23]FCORT: feather corticosterone

1. *In vivo* toxicity trial

An *in vivo* trial was performed based on specific and nonspecific biomarkers related to toxicity. Specific biomarkers, also named biomarkers of exposure, were studied in a toxicokinetic study, and nonspecific biomarkers were studied in a long-term study to see the chronic effects of DON exposure.

1.1. Specific biomarkers

In a toxicity study, the primary objective of toxicokinetics is to describe the systemic exposure achieved in animals and its relationship to dose level and time course. Toxicokinetic is largely reflective of the ADME (absorption, distribution, metabolism and elimination) of a molecule as it moves through the body of an organism.

1.1.1. ADME process

1.1.1.1. Absorption

Absorption of mycotoxins, including DON, in target animals depends on specie, age and even sex (Payros et al., 2016). The absorption rate of DON in broiler chickens showed to be low (Broekaert et al., 2015; Osselaere et al., 2013), probably explaining the low susceptibility of broiler chickens to DON mycotoxicosis. Our toxicokinetic study **(chapter 1)** indicated that DON could not be detected in plasma after a single oral bolus dose of the mycotoxin (0.75 or 2.25 mg DON/kg BW, equivalent to 5 or 15 mg DON/kg in feed). This result confirms the low absolute oral bioavailability of DON in broilers. It is important to note that administration of a higher dose than the guidance value did not necessary lead to acute mycotoxicosis. In conclusion, plasma or blood are not suitable matrices to test the efficacy of mycotoxin detoxifiers in broilers when DON at maximum recommended level (0.75 mg/kg BW equivalent to 5 mg/kg feed) is given to broilers, as it is not also when the triple DON concentration is used.

1.1.1.2. Distribution

Distribution of mycotoxins in a target species refers to the deposition of this mycotoxin in specific parts of the body of the tested animal. It is measured by the volume of distribution (Vd). As shown in the literature, the analysis of DON after oral administration of tritium-labelled DON at the dose of 2.5 mg/kg BW, indicated that DON was rapidly and transiently distributed to all tissues in broiler chickens (gastrointestinal tract, plasma, bile, brain, heart, liver, kidney, lung, spleen, pancreas, urinary bladder, ovary/testis, adipose, muscle, and skin) (Wan et al., 2014). Limited distribution frequently implies transient tissue persistence which is probably related to the rapid elimination of DON (Guerre, 2015).

1.1.1.3. Metabolism

Xenobiotic (as mycotoxin) metabolism can be defined as chemical transformation by a biological system, which typically converts relatively lipophilic compounds into more readily excreted hydrophilic metabolites. This metabolism is divided in three phases and achieved usually through specialized enzymatic systems. Phase II reactions are catalyzed by transferase enzymes which conjugate directly the parent molecule (the toxin itself) or its metabolites to polar compounds as glutathione, glucuronic acid or sulfate. Sulfation has shown to be the most important pathway of DON metabolization in broiler chickens **(chapter 1, 3 and 4)**, by the formation of DON-3S, probably in liver or in enterocytes cells (Schwartz-Zimmermann et al., 2015). Moreover, mycotoxins can also be degraded in the digestive tract of the animal by the microbiota (Dänicke & Brezina, 2013; Guerre, 2015).

The detection of DON specific metabolite (DON-3S) in liver of birds fed 15 mg/kg feed but not in birds fed the guidance level (5 mg/kg feed) **(chapter 3)** may be explained by the difference of the volume of distribution between the two concentrations: we found 22.7 *vs* 13.1 L/kg for 0.75 and 2.25 mg/kg BW, respectively **(chapter 1)**. The limited accumulation into organs could be due to rapid metabolization and excretion.

1.1.1.4. Elimination

The disappearance of the toxin from the body is the last step of ADME process. Elimination can be the result of metabolism or direct excretion of the toxin in body fluids (bile, urine). It should be noted that the excretion in poultry is a mixed white pasty urine poured into the cloaca with solid excrements from the gastrointestinal tract, which could not help to know the origin of excreted DON-3-sulphate and DOM-3-sulphate (Schwartz-Zimmermann et al., 2015). Terminal elimination half-life (T1/2elim) and clearance are representative parameters of elimination. Rapid clearance and elimination of DON in broiler chickens have been showed in **chapter 1**. The toxin clearance from plasma of broilers intravenously exposed to DON (0.75 or 2.25 mg/kg BW) were 0.16 or 0.33 L/min kg). The $T_{½elimination}$ were 57.1 or 47.7 min after intravenous injection for both doses.

1.1.2. Biomarkers of exposure.

To evaluate the efficacy of mycotoxin detoxifiers, measuring specific biomarkers in tissues or body fluids is the appropriate method according to the EFSA (EFSA, 2010). On the other hand, the improvement of chromatographic methods made it possible to reduce LOD and detect phase II metabolites of parent toxins (Guerre, 2015). For DON, the parent compound in excreta has demonstrated to be a sensitive biomarker for exposure and for additive efficacy testing in broiler chickens **(chapter 2 and 4)**. Its phase I metabolite DOM-1 was not suitable as a biomarker in broiler

chickens **(chapter 4)**. However, it has been shown that the main suitable metabolite in this species is DON-3S in plasma **(chapter 1 and 3)** as well as in excreta **(chapter 3 and 4)**, indicating that this phase II metabolite is suited as biomarker for DON. Interestingly, DOM-3 S was also found in excreta **(chapter 4)**, indicating that, in case of broiler chickens, the sulfation is the main pathway of metabolization.

In conclusion, DON is highly metabolized, leading to the formation of sulfate(s), which are a detoxified form of the toxin (Yi et al., 2006). The analysis of DON and its metabolites in plasma is not a relevant end-point for the efficacy testing of mycotoxin detoxifying agents *in vivo* with broilers when fed in a continuous design, as EFSA pointed out (EFSA, 2010). DON and its metabolites should be quantified in excreta instead of in plasma.

1.2. Nonspecific biomarkers

In the present thesis, broiler chickens raised as long as 42 d at marketing age, and it was considered to be a suitable animal model for evaluating the chronic effects of DON in concentrations that normally occur in nature. The nonspecific effects of toxicity are mediated through the effects via various physiological functions (Becker et al., 2011). The selection of nonspecific parameters for the *in vivo* toxicity trial **(chapter 2 and 3)** was based on previous reports (Ghareeb et al., 2012, 2013, 2014, 2016). The selection of nonspecific parameters for the *in vivo* additive efficacy trial was based principally on the significant results found in our studies **(chapter 2 and 3)** and on previous reports.

1.2.1. Experimental conditions

1.2.1.1. Contaminated feeds

The source of mycotoxin contamination has to be taken into account when an *in vivo* animal model is developed. Indeed, mycotoxin can be added to the feed by different ways, either by a natural contamination of a selected raw material, either by the addition of a cultured mycotoxin material or even by the addition of pure mycotoxin.

Diets containing cultured DON material may be less toxic than naturally contaminated diets, because the possible presence of masked mycotoxins in the last case, such as DON-3-glucoside, which adds toxicity to the diet but are not detected by routine feed analyses (Sasanya et al., 2008). Moreover, in natural contamination multi-mycotoxin contamination is very frequent, as most of fungi are able to produce several mycotoxins simultaneously, and feed can be contaminated by several fungi species at the same time. Thus, mycotoxin multi-contamination of raw materials is more likely to occur than a single mycotoxin contamination (Escrivá et al., 2015; Grenier & Oswald, 2011).

As DON and ZEN can be both produced by the *Fusarium* species under similar conditions, their co-occurrence is highly possible, but toxicological synergism between DON and ZEN was rarely noticed in poultry studies (Swamy et al., 2002; Yegani et al., 2006). Moreover, presence of ZEN in feeds was far lesser to induce synergism at DON concentrations that adversely affect broiler chickens (Chen et al., 2017).

In this research, as DON was added to the diet through a cultured DON material, all effects observed in studies were attributed to DON only, and no risk of effect of mycotoxins multi-contamination was considered **(chapter 2, 3 and 4).**

1.2.1.2. Mycotoxin concentration levels

To alleviate the effect of mycotoxins on human and animal health, as well as due to the economic consequences, regulations or recommendations have been established in EU and other parts of the world, especially for mycotoxins which often contaminate agricultural commodities and with potential to induce toxic effects in farm animal (European Commission, 2006). A guidance or recommended value of 5 mg/kg was set for DON in poultry feed (European Commission, 2006). For this reason, 5 mg/kg feed have been tested in the *in vivo* toxicity trial **(chapter 2, 3 and 4)** to better understand the effect of this guidance value on broiler chickens. However, these maximum levels are 'safe' values, established at a level that should not impair animal performance.

To be sure of the adverse effect of DON in broiler chickens we have used a higher concentration than the recommended value. Concentration of 15 mg DON/kg feed was used in the *in vivo* toxicity trial **(chapter 2 and 3)**, because artificially contaminated diets with cultured DON material are less toxic than naturally DON-contaminated diets (Awad et al., 2004; Ghareeb et al., 2014) and to verify if broiler chickens could tolerate up 15 mg/kg feed in terms of growth, as mentioned in previous studies (Harvey et al., 1991; Kubena et al., 1997). In the *in vivo* trials, high feed contamination levels are used to evoke negative effects on animal performance (Kolosova & Stroka, 2011).

To test the efficacy of detoxifying agents, as recommended by EFSA, studies have to be performed with the maximum guidance level (EFSA, 2010). For this reason, we opted to use this concentration in the *in vivo* efficacy trial **(chapter 4)**. However, mycotoxin analysis in feed revealed some different DON concentrations in the different treatments, with a mean concentration of 7.42 mg/kg feed.

1.2.1.3. Duration of exposure (and sampling time)

It should be noted that DON acute exposure poultry trials have been rarely described in literature (Surai et al., 1999). Our *in vivo* model was based on chronic feeding for 42 d (the marketing age). Broiler chickens at old age consume significantly more feed than during other periods of age, and

also feed conversion ratio increases with age in broilers (Aviagen, 2014). In fact, it has been supposed that a sampling time at 42 d in the *in vivo* toxicity trial **(chapter 2 and 3)** will be suitable to evaluate toxic DON effect on broilers, due to the fact that older birds consume more total mycotoxins, which is estimated by feed intake (kg) x dietary mycotoxin levels (mg/kg). The differences in ADME could be age related and then could influence animal's response to mycotoxins (Payros et al., 2016).

In literature, the effect of DON in poultry has been observed only during specific parts of their growth cycle, and in most of the cases, in early ages of chicken's development (Awad et al., 2006; Awad et al., 2011; Kubena et al., 1989; Yunus et al., 2012). Furthermore, based on the meta-analysis conducted by Andretta et al. (2011), the effect of mycotoxins on broiler's growth was greater in young animals. For this reason, 10 d was added as a sampling time for the *in vivo* efficacy trial **(chapter 4)**.

1.2.2. Biomarkers of effects

1.2.2.1. Productive parameters

Zootechnical parameters should be reported, but cannot be used for the demonstration of mycotoxin detoxifiers additives efficacy (EFSA, 2010).

Previous studies indicated that the impact of DON on poultry performance was highly variable. This effect could be dependent on the concentration of DON in feed, the duration of exposure, the risk of feed multi-contamination as well as the bird's initial health status. In fact, in some studies it has been reported that, even at lower concentration than the recommended value in feed, DON could have adverse effect on growth performance of chickens (Awad et al., 2011). However, other studies have indicated that even at greater level than the guidance, DON did not show any negative effect regarding bird's growth (Harvey et al., 1997). In the current research, in the *in vivo* toxicity trial **(chapter 2)**, the guidance level (5 mg/kg feed) did not affect the productive parameters. This result was in agreement with recent studies (Antonissen et al., 2017; Liu et al., 2020; Yu et al., 2018). Usually, the maximum EU permissible level for DON in poultry feed represents the concentration at or below which no growth depression is observed (Antonissen et al., 2015; Awad et al., 2008). Accordingly, no clinical signs of mycotoxicosis were observed in the animal trial. However, feeding 15 mg/kg to broiler chickens impairedthe BWG and feed conversion ratio. Similar results were obtained in other studies using 14, 15 or 16 mg/kg feed (Dänicke et al., 2003; Kubena et al., 1988, 1989, 1997). The inhibition of protein synthesis by DON could be the contributing factor for this result (Feinberg & Mclaughlin, 1989).

In the *in vivo* additive efficacy trial **(chapter 4)**, the presence of DON at approximately 7.42 mg/kg feed impaired the feed conversion ratio at 10 d. The impairment of performance of broiler chickens upon feeding DON at guidance level was observed in several studies (Awad et al., 2006;

Awad et al., 2011, 2019; Lucke et al., 2017; Swamy et al., 2002, 2004). However, DON at this level at 42 d **(chapter 4)** seems to slightly stimulate growth of male chickens. Some studies have also shown growth promotion of DON. Hamilton et al. (1985) reported that Leghorn and broiler chicks consuming 4.6 mg/kg of DON for four weeks had higher BWG than controls, which was in accordance with our findings. In addition, Swamy et al. (2002) reported an increase in feed intake and weight gain of broilers fed 4.7 mg/kg of DON for eight weeks. A recent study conducted by Chen et al. (2017) indicated a stimulating growth effect on chickens receiving 5 mg/kg of DON in feed during 16 wk, compared with chickens exposed to 2 mg DON/kg feed. These findings suggested that DON at concentration near or equal to 5 mg/kg could have growth promoting effects in broilers. This response may be attributed to hormesis, defined as a toxic agent characterized by low-dose stimulation (Swamy et al., 2002).

1.2.2.2. Relative weight of organs

The reported effect of DON on relative weight of internal organs is very contradictory. From the *in vivo* toxicity trial **(chapter 2)**, relative weights of heart, proventriculus, pancreas, caecum, liver, kidneys, and spleen were not affected by DON diets. Awad et al. (2011) did not also observe changes of the relative weight of these organs in broiler fed DON contaminated diets. In the **chapter 2**, an increase of RW of gizzard of broilers fed DON contaminated diet was shown, indicative of upper gastrointestinal irritation, as described in literature (Awad et al., 2014; Kubena et al., 1985, 1988; Kubena & Harvey, 1988). RW of small intestine, colon and bursa of Fabricius decreased and thymus RW increased **(chapter 2),** as also reported in literature (Awad et al., 2006; Kubena et al., 1997; Yunus et al., 2012). Adverse effects of DON could be seen primarily in organs containing rapidly dividing cells, such as the small intestine, liver and immune organs (IARC, 1993). Therefore, for the *in vivo* additive efficacy study, moreover than the significant RW of organs studied in **chapter 2**, RW of liver, kidneys, and spleen were evaluated. Among all the organs evaluated, only RW of broiler chickens liver decreased **(chapter 4)** after DON feeding, as found also in previous reports (Kubena et al., 1984; Lucke et al., 2017). The liver is a major site of *in vivo* trichothecene toxicity (Fuchs et al., 2002). Lipid metabolism alterations could be an explanation of this decrease. Liver could be the site of the formation of DON-3S (Schwartz-Zimmermann et al., 2015). In other studies, RW of liver increased or remain unaffected in broilers fed contaminated diets with DON (Kubena & Harvey, 1988; Yunus et al., 2012a). RW of organs is not consistent enough across studies to be considered a reliable parameter for efficacy testing of mycotoxin detoxifiers.

1.2.2.3. Immunity and health: blood parameters

Data on biochemical and hematological parameters is an important tool for health assessment of animals under mycotoxicosis conditions.

1.2.2.3.1. Blood biochemistry

Impairment in plasma biochemistry profile is often used as a parameter to indicate the negative impact of mycotoxins. Studies related to the effect of DON on biochemical parameters in broiler chickens showed inconsistent responses across studies. Feeding diets contaminated with DON lowered the blood indicators related to protein metabolism (protein, albumin and uric acid), increased or decreased the blood enzymes related to liver and kidneys functions (ALP, AST, ALT, LDH, and GGT), and changed the blood indicators related to lipid metabolism (cholesterol and triglycerides) (Faixová et al., 2007; Ghareeb et al., 2012; Klapácová et al., 2011). A decrease in liver enzymes in blood during mycotoxicosis is believed to be due to subsequent leakage of enzymes into the circulation because of liver damage (Frankič et al., 2008). Meanwhile, no effect of DON was observed on blood enzymes related to liver and kidneys functions in our study **(chapter 2)**. These results are in accordance with those observed by Harvey et al. (1997), who tested 16 mg DON/kg contamination level for 21 d. However, blood creatine kinase was reduced in broilers fed 5 mg/kg, but this parameter showed a high variability between animals. Thus, blood creatine kinase seems not to be a sensitive indicator for our *in vivo* additive efficacy trial. The reduced protein concentrations indicated in several reports on broiler chickens were linked to the inhibitory effect of DON on protein synthesis (Klapácováet al., 2011; Kubena et al., 1997; Swamy et al., 2002). In the current research, no alterations in total protein **(chapter 2 and 4)**, albumin, globulin, uric acid, or glucose **(chapter 2)** were observed in broilers fed the contaminated diet, which agrees with the findings observed by Dänicke et al. (2003). In the *in vivo* toxicity trial, serum cholesterol decreased **(chapter 2)**. Similarly, Kubena et al. (1989) observed reductions in serum cholesterol concentration in broilers fed with DON and T-2 contaminated wheat. However, in the *in vivo* additive efficacy trial **(chapter 4)** serum cholesterol concentrations were higher in birds fed DON contaminated diet. This is in agreement with Ghareeb et al. (2016), who reported an increase in cholesterol level in broilers fed DON. Cholesterol changes could be a result of liver involvement (Kubena et al., 1989; Ghareeb et al., 2016). Furthermore, it has been suggested the adverse effect of DON on lipid metabolism (Ghareeb et al., 2016). Blood triglycerides was then evaluated in the *in vivo* additive efficacy trial and showed a significant decrease in our study, probably linked to biliary obstruction (Ghareeb et al., 2016). These results, indicative of metabolic outcome of DON in broiler chickens, should be taken into account, even if values are still within their physiological reference range.

1.2.2.3.2. Blood hematology and response to common vaccines (NDV and IBV)

As previously reported, DON disrupts protein synthesis and thus makes dividing and activated cells, such as immune cells, specific targets (Grenier et al., 2013). DON could affect immune functions by depressing leukocytes, suppressing antibody production, and impairing macrophage and

neutrophils functions (Grenier et al., 2013). In line with that, we have proposed that blood parameters such as hematological indices, response to common vaccines and IL-8 production in plasma could be suitable biomarkers related to immunotoxicity of DON. The effect of DON on poultry blood hematology is not extensively documented. Slight decrease on hematological indices was observed in trials presented in **chapter 3 and 4**, as already observed by several authors (Harvey et al., 1991; Huff et al., 1986; Kubena et al., 1985). However, the values observed across some studies and this thesis **(chapter 3 and 4)**, were within the reference ranges and therefore might be with limited physiological effect in birds (Al-Nedawi, 2018). Bone marrow is an organ susceptible to DON mycotoxin because it is an organ with rapidly dividing cells. Depressed hematological variables may be attributed to bone marrow malfunction (Prelusky et al., 1994). In the present research responses to common vaccines (NDV and IBV) were not affected even at 15 mg/kg feed **(chapter 3)**, suggesting that DON under the current experimental conditions did not alter humoral immune response in broiler chickens. Similarly, the incorporation of DON in the feed did not affect antibody titers to NDV (Harvey et al., 1991) or antibody titers to IBV in broiler chickens (Swamy et al., 2002; Yunus, et al., 2012a). Therefore, this parameter was not selected as suitable biomarker for the *in vivo* additive efficacy trial **(chapter 4)**.

1.2.2.3.3. IL-8 in plasma

IL-8 is one of the major mediators of the inflammatory response, considered also as a biomarker of early inflammation process (Sallusto & Baggiolini, 2008) . The capacity of DON to upregulate IL-8 production could be of toxicological significance. This upregulation has been found in various *in vitro* as well as *in vivo* studies in human, rodents and farm animals (Lessard et al., 2015; Maresca et al., 2008). An upregulation of IL-8 in plasma has been observed due to DON exposure **(chapter 3 and 4)**, suggesting that DON could cause inflammation in broiler chickens.

Results obtained in blood parameters through reducing hematological parameters and increasing plasma IL-8 suggested that DON may affect health status of broiler chickens even if no clinical signs were observed.

1.2.2.4. Morphology and digestive immune system

DON is also known to affect the intestinal health of animals. In fact, the gastrointestinal tract is the first organ mycotoxins encounter. Since DON is poorly absorbed (approximately 10%), assuming that a considerable part of DON remains in the gastrointestinal tract, then the intestinal cells could be exposed to high concentrations of the mycotoxin (Broekaert et al., 2015; Ghareeb et al., 2015). This leads to the deterioration of intestinal functions (Grenier et al., 2016). In **chapter 2**, morphometric observations showed a longer length and a lower density of small intestine in birds fed DON, probably

indicating greater absorption of DON and low absorption and utilization of nutrients, as explained by Yunus et al. (2012b). However, in **chapter 4**, no significant differences were observed in these small intestine organometric parameters. Histopathological observations in the jejunum did not exhibit villus height and crypt depth decrease **(chapter 2 and 4)**. Therefore, concentrations of DON tested did not affect the nutrients digestion and absorption of birds raised in healthy conditions.

DON modulates the immune responses depending on the dose and the duration of exposure (Bondy & Pestka, 2000). High concentrations of DON induces immune cells apoptosis leading to immune-suppression (Pestka, 2007). DON at low concentrations, might result in constant inflammatory condition in the intestine, can exacerbate cytokines production or interrupt normal immune responses (Payros et al., 2016). As Pinton & Oswald (2014) demonstrated a greater expression of cytokines involved in inflammation and the recruitment of immune cells, in the *in vivo* toxicity trial (**chapter 3**), we have evaluated IL-1β, IL-6 and IFN-γ as proinflammatory intestine markers, and IL-10 as a marker of immune recruitment. In this toxicity trial, the upregulation of proinflammatory cytokines IL-1β, IL-6 and IFN-γ was observed in jejunum of broiler chickens fed DON at 5 mg/kg but not when they were fed 15 mg/kg **(chapter 3)**, which indicates the stimulatory effect of the DON guidance value in broilers. The induction of IL-6 in duodenum of broilers fed up to 5 mg DON/kg feed has been previously demonstrated (Lucke et al., 2018). However, the expression of IL-1β and IFN-γ was down-regulated in the jejunum of broiler chickens exposed to 10 mg/kg feed (Ghareeb et al., 2013). The induction of proinflammatory cytokines is often associated with DON-induced reduction of feed consumption. From our results, the inflammation is not sufficient to induce the reduction of feed intake **(chapter 3)**.

TNF-α is a cytokine of innate immunity, initiator of immune response (Al-Banna et al., 2018). DON down- regulated this cytokine in the intestine of pigs (Becker et al., 2011) or in plasma of broiler chickens (Ghareeb et al., 2013), indicating the alterations of immune functions. To obtain more evidence on the proinflammatory cytokines on the intestine, in the *in vivo* additive efficacy trial we added TN-α and IL-8 evaluation in the jejunum of DON-exposed broilers **(chapter 4)**. However, no DON-induced release of proinflammatory cytokines was observed, except for TNF-α **(chapter 4)**.

1.2.2.5. Welfare parameters

One of the markers of animal health is welfare status. Few studies pointed out the effect of DON mycotoxin on welfare related indicators in broiler chickens (Antonissen et al., 2017; Ghareeb et al., 2014). Physiological stress in poultry includes changes in circulating heterophils and lymphocytes (Gross & Siegel, 1983). The stress index is defined as the ratio of circulating heterophils and lymphocytes, and could be considered as a reliable indicator of stress (Gross & Siegel, 1983). This parameter increased in broiler chickens fed DON contaminated feed **(chapter 4)**, as previously

reported by Ghareeb et al. (2012, 2014), and Xu et al. (2011). These results may indicate DON-induced physiological stress in broiler chickens.

On the other hand, the hypothalamic–pituitary–adrenal (HPA) axis of vertebrates responds to stress stimulus by releasing circulating glucocorticoids, mainly corticosterone. Corticosterone in plasma was evaluated and showed a significant elevation in broiler chickens exposed to *Fusarium* mycotoxins (Antonissen et al., 2017; Ghareeb et al., 2014). This result also strengthens the conclusion of DON-induced physiological stress in broiler chickens. In contrast, the results of **chapter 3** showed a reduced corticosterone level in the plasma of broilers. We propose that plasma corticosterone could be an adequate marker of stress in a short-term or acute feeding design but not in a chronic one (our case).

During feather growth, corticosterone could be deposited. Therefore, for the *in vivo* additive efficacy trial, feather corticosterone might be an appropriate measurement because it integrates the intensity of the physiological response, how long corticosterone rises in the bloodstream, and the frequency of exposure to stressors (Bortolotti et al., 2008). Interestingly, DON increased the level of corticosterone in feathers **(chapter 4)**, indicative of physiological stress in birds induced by chronic feeding with DON.

As previously mentioned, tonic immobility reaction could be an indicator of bird fear response reflecting their behaviour (Ghareeb et al., 2014). However, this measurement is subject to handling and showed high variability inter-birds **(chapter 2)**. Thus, tonic immobility reaction was not selected as relevant biomarkers to assess the efficacy of DON-detoxifying agents.

The changes obtained on feathers corticosterone, but also on blood parameters and cytokines release can be attributed to the immune system being susceptible to DON.

2. Detoxifying-agents efficacy

In vitro studies cannot be used to demonstrate additive efficacy under practical conditions, because they do not sufficiently mimic the conditions in the digestive tract, the differences between the target animals, and their metabolism (EFSA, 2010). Therefore, *in vivo* studies are needed to confirm the efficacy of detoxifying agents for mycotoxins. However, the results from *in vivo* studies could also vary within the same species, depending on mycotoxin level, detoxifier level, age of animal, nutritional and health status, and sensitivity of the animals, among other factors. In this thesis, three feed additives, MFA, MDE and IMP, with different modes of action were tested, aiming at the detoxification of DON in broiler chickens under the current experimental conditions.

The appropriate endpoints, for the demonstration of the efficacy of technological additives "substances for the reduction of contamination of feed by mycotoxins" that should be based on *in vivo* studies (Rychen et al., 2018), are indicated in Table 2:

Table 2. Demonstration of efficacy for technological additives exerting their effect in the animal.

Functional group	**Demonstration of efficacy**
Substances for the reduction of contamination of feed by mycotoxins	Reduction of the absorption of mycotoxins.
	Increased excretion of mycotoxins.
	Degradation/transformation of mycotoxins.
	Reduced concentration of mycotoxins in food of animal origin.

2.1. Microbial feed additive (MFA)

MFA is a complete anti-mycotoxin solution including a microorganism with the theoretical ability to degrade DON into DOM-1, a less toxic metabolite, but also combined with different adsorbents. Upon MFA supplementation of DON contaminated feed, we did not observe the production or the increase of DOM-1 metabolite as expected. These results indicate that MFA did not show significant abilities on biotransforming DON into less toxic metabolites **(chapter 4),** as hypothesized. On the other hand, the capability of MFA to decrease the bird's exposure to DON was demonstrated, as showed by the increase of excretion of the parent mycotoxin. This increase of DON excretion may indicate that MFA is able to adsorb DON. This result of MFA action on specific biomarker (DON excretion) correlated with some nonspecific biomarkers, such as serum cholesterol level and stress index **(chapter 4)**. *In vivo* reports of the effects of biotransforming agents against DON have shown that microbial based feed additives are capable of counteracting the negative effects induced by DON on blood biochemistry and welfare parameters in broiler chickens (Ghareeb et al., 2012, 2014). However, these reports did not evaluate the presence of DOM-1 in biological matrices.

2.2. Immuno-protector additive (IMP)

IMP is characterized by its ability to enhance immune function of animals. As one of the outcomes of DON is an immunotoxic effect, IMP was used to overcome the possible adverse effects of DON on broilers immune system. Indeed, nonspecific increases in hematocrit, monocytes count, and cytokines release in birds fed DON supplemented with IMP at specific parts of chicken's growth cycle **(chapter 4)** might be indicative of its immunostimulation properties. Moreover, the IMP was effective in preventing mycotoxin-induced growth depression. Some beneficial effects of this type of feed additives based on immunoprotection mode of action in preventing DON-induced alterations were previously reported (Swamy et al., 2002, 2004).

2.3. Mycotoxin-degrading enzyme (MDE)

Application of enzymes responsible for degradation of mycotoxins could be an attractive alternative to the use of live microbes to counteract mycotoxins in animal feed (Kolosova & Stroka, 2011). MDE is one kind of enzyme with biotransforming activities of mycotoxin into its non-toxic metabolites. However, this product showed only slight modification on DON metabolism and was not effective neither on specific biomarkers nor on nonspecific ones. In contrast, it has been reported that a mycotoxin degrading enzyme was very effective in alleviating the adverse effects of DON on blood biochemistry and immune parameters in ducks (Cheng et al., 2004), and on growth performance, blood biochemistry, and immune response of pigs (Cheng et al., 2006). However, these studies did not evaluate the specific metabolites of biotransformation of DON.

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CONCLUSIONS

In this thesis, toxicity of DON mycotoxin and efficacy of three mycotoxin-detoxifying feed additives have been evaluated using broiler chickens as a model, in an *in vivo* chronic feeding design, using DON contaminated feed close to the EU maximum recommended level for this toxin. The first part of the thesis was devoted to establishing the *in vivo* model and identifying biomarkers and the second part to evaluate the feed additives efficacy. From the results presented in this dissertation, the following conclusions can be drawn:

1) It has been confirmed that DON in broiler chickens is characterized by its low absorption, limited deposition and rapid elimination. DON was metabolized quickly and excreted mainly as DON-3S.
2) Sulfation has proven to be the most important pathway of metabolisation of DON in broiler chickens, producing metabolites as DOM-3S and DON-3S in excreta. Besides, DON-3S can be also detected in plasma, proving to be a very useful DON biomarker of exposure in chickens.
3) DON at the tested levels is a relevant end-point to test the efficacy of mycotoxin detoxifying agents in excreta but nor in plasma neither in liver of broiler chickens.
4) The effect of DON on broiler chicken's growth performance depends on toxin concentration and could be inhibitor or promotor of growth performance. In the same way, the effect of DON also depends on the duration of exposure, so it can only be observed during specific moments of the chicken's growth cycle. DON is inhibitor at 15 mg/kg feed (42 d) and at 5 mg/kg feed (10 d), and promotor of growth performance at 5 mg/kg feed (42 d).
5) Relative weight of organs is not a suitable biomarker of effect of DON due to non-consistent results found along the trials, and cannot be used to test the efficacy of DON mycotoxin detoxifiers in broiler chickens.
6) Slight effects of DON have been found on blood parameters (cholesterol, triglycerides, blood count), indicating DON adverse effect on bird's lipid metabolism, health and physiology.
7) Concentration of DON around the guidance value could affect the immune responses through the modulation of proinflammatory cytokines
8) In a chronic toxicity design, corticosterone in plasma is not a relevant biomarker of effect of DON, being much better to measure the corticosterone in feathers.
9) MFA showed to detoxify DON in broiler chickens, as its use increases the excretion of the parent mycotoxin. This effect correlates with the beneficial effect of this product found on some health and welfare parameters. However, this product was not able to biotransform DON into less toxic metabolites, as expected.

10) IMP showed to be feasible on overcoming the effects induced in chickens by DON on productive, physiological and immunological parameters, confirming its immune-stimulant capacities.
11) MDE was not a suitable feed additive in counteracting DON mycotoxin effects in broiler chickens.

FUTURE PROSPECTS

This Doctoral Thesis established a good *in vivo* model to evaluate the efficacy of detoxifying products that allows monitoring the effects of DON in chickens and identifying the most relevant biomarkers.

Specific biomarkers as DON and metabolites measurement in excreta were interesting and a powerful indication. New *in vivo* trials could be done through using:

- Higher number of animals per cage
- Natural contaminated diets
- Multi-contaminated diets
- Under stress conditions

In addition, different poultry species should be targeted, as we know that toxic effects of DON are species specific. Then the model should be applied to pigs with the same nonspecific biomarkers but with different DON level of exposure as pigs are very sensitive to DON.