INTERACTIONS OF THE *FUSARIUM* MYCOTOXINS, FUMONISIN B1, DEOXYNIVALENOL AND ZEARALENONE. A REVIEW

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SUMMARY

Multi-mycotoxin exposure is quite frequent since farm animals consume a mixture of commodities potentially contaminated with different types of mycotoxins. Combined toxicity has gained increased attention the last 15 years. In this review, the *in vitro* and *in vivo* combined toxicity studies of fumonisin B1 (FB1), deoxynivalenol (DON) and zearalenone (ZEN) have been summarised. The interactions revealed, varied from antagonism to synergism which can be attributed to different animals/cell lines, mycotoxin concentrations and mathematical designs used. However, addition and synergism occurred more often suggesting that animals are more adversely affected by the combined toxins than the single exposure. *Fusarium* mycotoxins are the most studied mycotoxins, yet there are few studies regarding their ternary mixtures. Thus more *in vitro* studies on ternary mixtures are needed with further confirmation of the effects by *in vivo* studies. In this fashion time and resources can be saved and animal welfare is respected as well.

ÖSSZEFOGLALÁS

Kachlek M. – Szabó-Fodor J. – Kovács M.: A FUMONIZIN B1, A DEOXINIVALENOL ÉS A ZEARALENON *FUSARIUM* MIKOTOXINOK KÖLCSÖNHATÁSA. IRODALMI ÁTTEKINTÉS

A multi-mikotoxin expozíció meglehetősen gyakori, minthogy a gazdasági állatok olyan keverék-takarmányokat fogyasztanak, amelyek különböző típusú mikotoxinokkal lehetnek szennyezettek. A kombinált toxicitás az utóbbi 15 éveben kapott nagyobb figyelmet. Tanulmányunkban a fumonizin B1 (FB_1), a deoxinivalenol (DON) és a zearalenon (ZEN) kombinált toxicitására irányuló kutatásokat összegezzük. A megfigyelt interakciók, amelyek az antagonizmustól a szinergizmusig terjednek, a vizsgálatokban szereplő különböző állatfajoknak/sejtvonalaknak, a mikotoxinok koncentrációjának és a statisztikai elemzéshez használt matematikai modelleknek tulajdoníthatók. Jóllehet, az additív és szinergista hatás sokkal gyakrabban előfordul, ami arra utal, hogy az állati szervezetet fokozottan károsítja a kombinált, mint az önálló toxinnal történő expozíció. A *Fusarium* mikotoxinok a leginkább vizsgált mikotoxinok közé tartoznak, azonban hármas kombinációban történő vizsgálatuk kevésbé kutatott. Ennélfogva a háromszoros kombinációra vonatkozóan több *in vitro* kísérlet tűnik szükségesnek, hogy alátámaszthatók legyenek az *in vivo* kísérletek során nyert tapasztalatok. Ilyen módon nem csak az állati jóléttel szemben támasztott követelmények vehetők figyelembe, de idő és erőforrás is megtakarítható.

INTRODUCTION

Mycotoxins are secondary metabolites of filamentous fungi. The most important genera are *Fusarium, Aspergillus, Penicillium, Claviceps* and *Alternaria.* Around 300 to 400 mycotoxins are known so far (*Bennet and Klich*, 2003). The most important mycotoxins' groups the most thoroughly studied are fumonisins, trichothecenes, zearalenone, aflatoxins and ochratoxins. The occurrence of mycotoxins worldwide is a very important issue in the aspect of both food and feed safety. The *European Commission* has set guidance levels for several mycotoxins (2006/576/EC).

Many fungal species can produce more than one mycotoxin simultaneously. In addition to that crops can be infected by different genera of fungi at the same time and last but not least the complete feed is prepared by various commodities. All these factors (can) lead to frequent co-contamination of grains and feed, which is supported by several reviews and surveys (*Speijers and Speijers*, 2004; *Monbaliu et al.*, 2010; *Rodrigues and Naehrer*, 2012). Legislation is enacted based on risk assessment studies which depend on the toxicity and exposure data of single mycotoxins. However when mycotoxins interact with each other, they may exert synergistic, antagonistic or additive effects. Thus determination of the interactive effects of mycotoxins and especially in low concentrations is important for the revision/reconsideration of tolerable daily intake (TDI; *Speijers and Speijers*, 2004) and/or maximum/guidance levels (*Verstraete*, 2006). In a survey by *Streit et al.* (2013) that lasted from 2004 to 2012 all the samples where co-contaminated with 7 to 69 metabolites with 28 being the most frequent number of metabolites per sample.

Three *Fusarium* toxins (deoxynivalenol, fumonisins and zearalenone) are of particular interest since they are frequent contaminants of cereal grains such wheat and corn and they co-occur very frequently. In a survey conducted over a period of 4.5 years in countries of Southern Europe (Portugal, Spain, Italy, Greece and Cyprus) the *Fusarium* mycotoxins were found to be the major contaminants (fumonisins, type B trichothecenes and zearalenone) of feedstuffs and compound feed samples (*Griessler et al.*, 2010). *BIOMIN* (2015) analysed 8271 feed commodities from 75 countries. Deoxynivalenol (DON) had a prevalence of 73%, fumonisin B1 (FB1) 61% and zearalenone (ZEN) 56%. DON poses the most frequent threat to farm animals on the grounds that 56% exceeded the risk thresholds although levels of fumonisins and ZEN indicated a cause of concern too. In their most recently conducted survey 94% of the samples were contaminated with more than ten mycotoxins with DON, ZEN and FUMs being present in over 50% of the samples tested worldwide (*BIOMIN*, 2016).

Fumonisin B1

Fumonisins consist of four series (A, B, C and P), fumonisin A (FA), fumonisin B (FB), fumonisin C (FC), and fumonisin P (FP) respectively (*Rheeder*, 2002). The most important analogues in toxicology are the FB analogues. Fumonisin B group comprises of six compounds; fumonisins B1, B2, B3, B4, B5 and B6 (FB1, FB2, FB3, FB4, FB5 and FB6) with FB1 being the major metabolite (*Månsson et al.,* 2010; *Bartók et al.,* 2013) which chemically is a diester (*Marasas*, 2001).

The commodity that is mostly affected by FB1 is maize as well as maize-based products. FB1 is mainly produced by *Fusarium verticillioides (syn. F. moniliforme*) and *F. proliferatum,* which mostly occur in corn.

The main mechanism of action of FB1 is a result of its similarity (long-chain hydrocarbon unit) to sphingosine and sphinganine thus disturbing their ratio (Sa/So). FB1 inhibits the ceramide synthase which essentially leads to impairment of cell membrane (*Riley et al.*, 2001). Apart from disrupting the ceramide synthase, FB1 is suggested to stimulate apoptosis in cells but the mechanism is not clear. Some possible explanations could be the induction of lipid peroxidation and the decreased concentrations of antioxidants such as glutathione (GSH) (*Surai and Dvorska*, 2005).

Regarding animals, FB1 is the causative factor of equine leucoencephalomalacia (*Marasas et al.,* 1988; *Kellerman et al.,* 1990; *Ross et al.,* 1993), porcine pulmonary oedema syndrome (*Colvin and Harisson,* 1992) and hepatocellular carcinoma in rats (*Gelderblom et al.,* 1994). FB1 is possibly carcinogenic for humans; it has been classified as a possible carcinogen (group 2B) by *IARC* (2002). FB1 has been associated with esophageal cancer in rural regions of Southern Africa and China. FB1 can also induce hepatotoxicity and elevate the serum cholesterol concentration (*Haschek et al.,* 2001).

Deoxynivalenol

DON is a member of trichothecene family, which is consisted of approximately 200 compounds (*Grove*, 2000; *Pestka*, 2010). Trichothecenes are structurally related (sesquiterpenes) include four basic types, from which the most important representatives belong to types A, B and D (*Pestka*, 2007). Although DON is the least toxic among them it has been thoroughly studied because it is one of the most common contaminants of grains (*Pestka*, 2010).

Like the rest trichothecenes, DON inhibits the protein synthesis (the initiation and/or elongation of the polypeptide chain is inhibited) because of its ability to bind to eukaryotic ribosomes (*Ueno* et al., 1968; *Ueno*, 1984).

In swine DON is causing feed refusal (threshold concentration is 1 mg/kg) and emesis (vomiting; minimum oral dose is 100μg/kg of body weight) thus given the trivial name vomitoxin (*Vesonder et al.*, 1973). DON (as well as other trichothecenes) has immunomodulatory abilities. It was suggested by *Rotter et al.* (1996) that the immunosuppression induced by trichothecenes is due to the inhibition of translation (*Bamburg*, 1983) whereas the mechanism of immunostimulation is not so clear. DON is not classified as carcinogenic (Group 3) (*IARC*, 1993).

Zearalenone

Chemically ZEN is a resocyclic acid lactone (6-[10-hydroxy-6-oxo-trans-1-undecenyl]-β-resocyclic acid lactone (*Urry et al.,* 1966). Zearalenone was isolated from Christensen *et al.* (1965) which gave it the name F-2 toxin. The next year *Urry et al.* (1966) characterised its chemical structure and gave its present name Zearalenone. ZEN is produced by various species of *Fusarium* genus, namely *Fusarium graminearum* (*Giberella zeae)*, *F. culmorum*, *F. equiseti*, *F. sambucinum*

and *F.crokwellense* (*Placinta et al.,* 1999; *Bennet and Klich,* 2003). ZEN very often co-occurs with DON due to their common i.e. *F. graminearum* and *F. culmorum*.

ZEN is considered by many not a real toxin but rather a mycoestrogen due to its similarity to oestrogens. This similarity leads to an antagonistic binding to estrogenic receptors which affects the reproduction system of both male and female animals (*Abdelhamid et al.,* 1992; *Nakaido et al.,* 2004). Apart from its pronounced ability to affect the reproductive system ZEN may cause effects in non-reproductive systems of the organisms (*in vitro* and *in vivo*), i.e. it has been proven to be cytotoxic, genotoxic and inducing immune response (*Ouanes et al.,* 2003; *Abid-Essefi et al.,* 2004; *Vlata et al.,* 2006; *Marin et al.,* 2010; *Gao et al.,* 2013). In the case of ZEN swine is the most sensitive species as well (*Tiemann and Dänicke,* 2007). ZEN has not yet been classified as a carcinogen (Group 3) for humans by IARC (1993).

Taking the aforementioned into account this review focuses on the interaction studies of FB1, DON and ZEN in binary and ternary mixtures *in vivo* and *in vitro.* In the present paper the reviews on combined mycotoxins studies' are also presented briefly as well as the mathematical designs used for the interpretation of the observed interactions in separate sections.

REVIEWS ABOUT COMBINED EFFECTS OF MYCOTOXINS

The first studies on mycotoxin interactions were performed in the 1980's but a special focus was given to studies of combined mycotoxins in the last years. As mentioned in the introduction, the chromatographic methods that have been developed recently have allowed for multi-mycotoxin analysis, revealing the fact that multi-mycotoxin occurrence is not the exception but the rule. Although there are a few reviews on the interactions of mycotoxins, their focus is not specific. Reviews about *in vitro* studies were published recently (*Alassane-Kpembi et al.,* 2016; *Smith et al.,* 2016). *Alassane-Kpembi et al.* (2016) analysed the methological aspects and the biological relevance of interactive studies. *Smith et al.* (2016) apart from the analysis of combined toxicity studies made an extent reference to the legislation regarding mycotoxin exposure as well as the natural occurrence of mycotoxins worldwide.

A very robust and extensive meta-analysis of combined toxicity *in vivo* studies was published a few years ago (*Grenier and Oswald,* 2011). Although a lot of different classes are mentioned in the review the most of the studies published concern aflatoxins and their respective mixtures with other mycotoxins (especially fumonisins, trichothecenes, ochratoxin A (OTA) etc.).

The review of *Escrivá et al.* (2015) focuses on the *in vivo* studies on *Fusarium* mycotoxins during the last decade have been summarized but only 15% of the studies mentioned regarded interactions.

STUDIES ON COMBINED TOXICITY OF FB1, DON AND ZEN

There are several studies about mycotoxins' interaction (*Grenier and Oswald,* 2011; *Alassane-Kpembi et al.,* 2016; *Smith et al.,* 2016) but fewer report the combined

Table 1.

The in vivo studies on combined effects of fumonisin B1 (FB1), deoxynivalenol (DON) and zearalenone (ZEN)

Animal (1)	Mycotoxin (2)	Interaction (3)	Exposure period (4)	Parameters examined (5)	References (6)
B6C3F1 mice (7)	DON + ZEN	No interaction (8)	56 days	Productive traits and blood parameters, organ weights, histopathology (9)	Forsell *et al.*, 1986
B6C3F1 mice (7)	DON + ZEN	Synergism (10)	14-21 or 56 days	Immune response (11)	Pestka *et al.*, 1987
Growing barrows (12)	FB1 + DON	Additive and greater-than additive (13)	28 days	Productive traits and blood parameters, immune response, histopathology (14)	Harvey *et al.*, 1996
Broiler chickens (15)	FB1 + DON/ T-2	Additive and less than additive (16)	19 or 21 days	Productive traits and blood parameters (17)	Kubena *et al.,* 1997
Piglets (18)	DON + FB1	Synergism (10)	35 days	Productive traits and blood parameters, histopathology, immune response (19)	Grenier *et al.,* 2011
Piglets (18)	FB1 + DON	Antagonistic to synergistic (20)	35 days	Morphology, histology, cytokines' expression (intestine) (21)	Bracarense *et al.,* 2012
Swiss mice (7)	FB1 + DON	Additive or more than additive, synergistic (22)	7 days	Serum and urine chemistry, renal DNA methylation (23)	Kouadio *et al*., 2013
Rabbit bucks (24)	FB1, DON + ZEN	Antagonistic to synergistic (20)	65 days	Reproductive parameters (25)	Szabó-Fodor *et al*., 2015
Kunming mice (7)	DON+ZEN	Sub-additive (26)	4 days	Serum chemistry, antioxidant status of kidney, cell apoptosis (27)	Liang *et al*., 2015
Kunming mice (7)	DON+ZEN	Additive and synergistic (28)	4 days	Antioxidant status of spleen, interferon levels, T-cell subsets (29)	Ren *et al.,* 2016

1. táblázat Fumonizin B_1 (FB_1), deoxinivalenol (DON) és zearalenon (ZEN) kombinált hatására vonatkozó in vivo kísérletek

állat (1); mikotoxin (2); kölcsönhatás (3); expozíció hossza (napokban megadva) (4); a vizsgált hatás (5); hivatkozás (6); egér (7); nincs kölcsönhatás (8); termelési és vér paraméterek, szervek súlya, kórszövettan (9); szinergizmus (10); immunválasz (11); növendék ártány (12); additív, vagy erősebb, mint additív hatás (13); termelési és vér paraméterek, immunválasz, kórszövettan (14); broiler csirke (15); additív, vagy kisebb, mint additív hatás (16); termelési és vér paraméterek (17); malac (18); termelési és vér paraméterek, kórszövettan, immunválasz (19); antagonizmustól a szinergizmusig (20); alaki elváltozások, szövettan, citokin expresszió (bél) (21); additív, vagy erősebb, mint additív hatás, illetve szinergizmus (22); vér és vizelet kémia, vese DNS-metiláció (23); baknyúl (24); szaporodási paraméterek (25); kisebb mint additív hatás (26); szérum kémia, a vese antioxidáns státusza, apoptózis (27); additív és szinergista (28); a lép antioxidáns státusza, interferon szint, T-sejt típusok (29)

Table 2.

***In vitro* studies on combined effects of fumonisin (FB1), deoxynivalenol (DON) and zearalenone (ZEN)**

Cell line (1)	Mycotoxin (2)	Interaction (3)	Exposure period (4)	Parameters examined (5)	References (6)
Mouse fibroblasts (L929) (7)	FB1, DON, ZEN, NIV[1], T2	Addition and in few exceptions synergism (8)	Not specified (9)	DNA synthesis (10)	Groten *et al.*, 1998
Mouse fibroblasts (L929) (7)	FB1, DON, ZEN, NIV, T2	Less than addition to synergism (11)	24h	DNA synthesis (10)	Tajima *et al.*, 2002
Caco-2	FB1, DON, ZEN	Antagonism, less than addition, synergism (12)	24/72h	Cell viability, protein synthesis, MDA levels, DNA synthesis, methylation and fragmentation (13)	Kouadio *et al.*, 2007
Mononuclear cells (from cord blood from placenta) (14)	FB1, DON, ZEN, BEA, ENB[2], T2	Antagonism and addition (15)	14 days	Myelotoxicity (16)	Ficheux *et al.*, 2012
Intestinal porcine enterocytes (IPEC-J2) (17)	FB1, DON, ZEN, NIV	Addition and synergism (18)	48h	Cell viability, cytokine expression (19)	Wan *et al.*, 2013a, b
Porcine granulosa cells (20)	FB1 + DON/ZEN	No interaction, addition and synergism (21)	24 and 48h	Cell proliferation, steroid production, gene expression (22)	Cortinovis *et al.*, 2014
Human colorectal carcinoma (HCT116) (23)	DON + ZEN	Sub-addition (24)	24h/48h	Cell cycle and viability, mitochondrial transmembrane potential, PTP[3] opening (25)	Bensassi *et al.*, 2014
Bovine granulosa cells (26)	FB1, DON, α-ZEL[4], β- ZEL	No interaction (27)	48h	Cell proliferation, steroid production (28)	Albonico et al., 2016

2. táblázat Fumonizin B_1 (FB_1), deoxinivalenol (DON) és zearalenon (ZEN) kombinált hatására vonatkozó in vitro kísérletek

sejtvonal (1); mikotoxin (2); kölcsönhatás (3); expozíció hossza (órákban vagy napokban megadva) (4); a vizsgált hatás (5); hivatkozás (6); egér fibroblaszt (7); additív hatás, néhány esetben szinergizmus (8); nem meghatározott (9); DNS szintézis (10); gyengébb mint additív hatástól a szinergizmusig (11); antagonizmus, gyengébb mint additív, szinergista (12); sejt életképesség, fehérjeszintézis, MDA szint, DNS szintézis, DNS-metiláció és fragmentáció (13); mononukleáris sejtek (köldökérből) (14); antagonista és additív (15); mielotoxicitás (16); sertés bélhámsejtek (17); additív és szinergista (18); sejt életképesség, citokin expresszió (19); sertés granulóza sejtek (20); nincs interakció, additív és szinergista (21); sejtosztódás, szteroid termelés, génexpresszió (22); humán colorectalis carcinoma sejt (23); gyengébb mint additív hatás (24); sejtciklus és életképesség, mitokondriális transzmembrán potenciál, PTP nyitás (25); szarvasmarha granulóza sejt (26); nincs interakció (27); sejtosztódás, szteroid termelés (28)

effects of three specific *Fusarium* mycotoxins which are more likely to co-occur; i.e. FB1, DON and ZEN (*Tables 1, 2*). Most of the studies about the interactions of FB1, DON and ZEN regard binary mixtures although there are some ternary mixtures studies (*Forsell et al.,* 1986; *Pestka et al.,* 1987; *Harvey et al.,* 1996; *Kubena et al.,* 1997; *Grenier et al.,* 2011; *Bracarense et al.,* 2012; *Ficheux et al.,* 2012; *Bensassi et al.,* 2014; *Cortinovis et al.,* 2014; *Kouadio et al.,* 2007; *Wan et al.,* 2013a, b; *Szabó-Fodor et al.,* 2015; *Albonico et al.,* 2016; *Tables 1, 2*).

IN VIVO STUDIES

Animal experiments with combined *Fusarium* toxins have been performed on swine, poultry, mice and rabbits. On the following paragraphs an overview of these studies will be provided.

Mice were used in the early studies (*Forsell et al.,* 1986; *Pestka et al.,* 1987; *Table 1*). In the study of *Forsell et al.* (1986) weanling female mice (B6C3F1) were exposed to dietary DON and ZEN (5 and 10mg/kg of feed respectively). No interaction was observed for any of the endpoints (production traits and blood parameters, organ weights, histopathology and immune parameters).

Pestka et al. (1987) studied the interactions of DON and ZEN on the immune function of B6C3F1 mice that were infected with the bacteria *Listeria monocytogenes*. The mycotoxin combination resulted in a decreased resistance to the bacteria suggesting synergism. *Kouadio et al.* (2013) performed a study on Swiss mice which superseded their *in vitro* study about FB1, DON and ZEN on Caco-2 cell line (*Kouadio et al.,* 2007). Differences among male and female mice were also investigated. The combination of DON (45 μg/kg bw/day) and FB1 (110 μg/kg bw/day) for 7 days, exerted an additive or more than additive effect on kidney of female mice regarding DNA methylation. Moreover for both male and female mice the renal clearances of creatinine were higher when the two toxins were combined (synergistic effect). The most recent study on mice regarding mycotoxins' interactions was performed by *Ren et al.* (2016). Female Kunming mice were used for the investigation of the combined effects of DON and ZEN (intraperitoneal injection in both studies) by *Liang et al.* (2015) and *Ren et al.* (2016). In the study of *Liang et al.* (2015) the target organ was the kidney. The endpoints used were serum chemistry, antioxidant capacity of kidneys and cell apoptosis. The combination of DON and ZEN resulted in sub-additive nephrotoxic effect. *Ren et al.* (2016) had as a target organ the spleen. The endpoints used were antioxidant status of the spleen, interferon levels and T-cell subsets. The combined effects were additive and synergistic.

Swine is an animal species quite suitable for mycotoxin research because of its confirmed sensitivity to *Fusarium* mycotoxins (FB1, DON and ZEN) (*Colvin and Harisson,* 1992; *Pestka*, 2007; *Marin et al.,* 2010; *Cortinovis et al.,* 2013). The first study was conducted by *Harvey et al.,* (1996) on growing barrows (*Table 1*). The barrows were fed FB1 (56mg/kg of feed) and DON (3.6 mg/kg of feed). The interactions were additive and greater-than-additive for most of the investigated endpoints. Ingestion of subclinical doses of DON (3.1 mg/kg of feed or 130 μg/kg of body weight) and FBs (6.5 mg/kg of feed- 4.5 mg/kg FB1 and 2.0 mg/kg of FB2 or 260 μg/kg of body weight) respectively for 5 weeks by pigs, induced greater

histological damages and higher immunosuppression when these two toxins were consumed simultaneously than the single toxins (*Grenier et al.,* 2011). From the same research group a similar study (using the same toxin concentrations) revealed interactions which varied from synergistic to antagonistic depending on the parameters examined (*Bracarense et al.,* 2012). For example in jejunum the villi height was significantly lower for the combined mycotoxins than FB1 alone (synergism), whereas in the ileum there was no significance difference among the groups (less than addition). The lymphocytic infiltration was decreased in the ileum of the animals consuming DON+FB1 (antagonism). The authors' conclusion was that chronic ingestion of mycotoxins in low doses could predispose farm animals to infections caused by enteric pathogens due to alterations in the intestine.

Poultry is another species commonly used in mycotoxins research, because poultry is frequently exposed to mycotoxins due to the nature of their feeds. Although they are not as sensitive as swine, stress and potential high concentrations of mycotoxins due to special weather conditions could lead to hazardous health effects (*Kubena et al.,* 1997). There are several studies concerning combined effects of mycotoxins on poultry (*Grenier and Oswald,* 2011) but regarding FB1, DON and ZEN there is only one (*Kubena et al.,* 1997). In this trial the combination of FB1 and DON (300 mg/kg and 15 mg/kg of feed respectively) was studied. The interactions observed were synergistic (serum chemistry), less than additive (body weight gain) and antagonistic (relative heart weight).

Rabbits are widely used as model animals in toxicological studies due to their high reproduction rate and the facilitation of measurement of various physiological parameters (*Kachlek et al.,* 2016). Thus they have also been used in mycotoxin research although mycotoxicoses occur less frequently than in other animal species. The only study about the combination of FB1, DON and ZEN was performed by *Szabó-Fodor et al.* (2015). Rabbit bucks were exposed (subchronically) to dietary FB1, DON+ZEN and their ternary mixture (FB1+DON+ZEN). The toxin concentrations used were chosen according to the lowest limits as set (for piglets in the lack of limits for rabbit feed) by the *European Commission* (2006/576/EC). The combined toxins DON+ZEN (1 and 0.25 mg/kg respectively) and FB1+DON+ZEN (5, 1 and 0.25 mg/kg respectively) were fed to the bucks for a period of 65 days. The study was focused on the combined effects on the reproductive system. An additive or less than additive effect was observed regarding spermatogenesis and sperm cell morphology; a synergistic effect was observed concerning testosterone production whereas antagonism of FB1 against DON and ZEN was observed in the case of genotoxicity.

In vivo studies are important to determine the effect on complex systems as living organisms. Still unravelling the mechanisms of actions in *in vitro* level is equally important. In addition, spearing animal experiments is in agreement with the Three Rs (replacement, reduction and refinement; *Fenwick et al.,* 2009).

In some of the studies other *fusariotoxins* like trichothecenes (T-2 and nivalenol-NIV), metabolites of ZEN (α-ZEN) and emerging mycotoxins [Beauvericin (BEA), enniatin B (ENB)] were used. The toxins are only referred in the tables (*Tables 1, 2*) for the convenience of the readers but they will not be analysed since they are not in the scope of this review.

IN VITRO STUDIES

Bensassi et al. (2014) studied the interaction of DON and ZEN in human colon carcinoma (HCT116) cell line. The endpoints used were cell viability, cycle analysis, mitochondrial apoptosis (mitochondrial membrane potential and permeability transition pore (PTP) opening). The combination of the toxins increased the cell proliferation as compared to the individual toxins thus showing an antagonistic effect on cytotoxicity whereas a subadditive effect was observed in the mitochondrial apoptosis.

Kouadio et al. (2007) studied the combined effects of FB1, DON and ZEN in binary and ternary mixtures using the human intestinal cell line Caco-2. In this study a lot of endpoints were investigated: cytotoxicity, protein synthesis, malondialdehyde (MDA) levels and DNA synthesis, methylation and fragmentation. The least cytotoxic mixture was the FB1+ZEN (far less than additive) and the most cytotoxic was FB1+DON+ZEN (synergism). The binary mixtures of DON with ZEN or FB1 increased lipid peroxidation in a synergistic manner. The binary mixtures acted synergistically since they induced greater DNA damage than that of the individual toxins. On the other hand the percentage of DNA synthesis of the ternary mixture was lower (25%) than of any of the three mycotoxins individually (45, 70 and 43% for ZEN, DON and FB1 respectively) indicating antagonism.

Two studies by the same research group have been performed in swine jejunal epithelial cells on cytotoxicity and the expression of pro-inflammatory cytokines respectively (*Wan et al.,* 2013a, b). The study about cytotoxicity revealed that even at non-cytotoxic concentrations the different combinations of FB1, DON, and ZEN were cytotoxic. Particularly, the greatest loss (synergism) of viability was induced by the ternary mixture DON-ZEN-FB1 (*Wan et al.,* 2013a). The second study investigated the mRNA expression of pro-inflammatory cytokines genes (IL1α, IL1β, IL6, IL8, TNFα and MCP-1). Non-cytotoxic and cytotoxic concentrations of the toxins were used. Surprisingly the mixtures in non-cytotoxic concentrations acted in a synergistic manner causing a significant up-regulation of pro-inflammatory cytokine mRNA which can lead to immunostimulation (*Wan et al.,* 2013b).

A recent study on porcine granulosa cells exposed to FB1 alone and in combination with DON or α-ZEN was performed by *Cortinovis et al.* (2014). DON inhibited cell proliferation, but when it was combined with FB1 no significant difference was detected (no interaction). The same (no interaction) was observed regarding steroid (progesterone and oestradiol) production. Bovine granulosa cells were studied for the first time regarding *Fusarium* mycotoxins interactions. The combination of FB1 (30 ng/ml and 100 ng/ml) and DON (100 ng/ml) did not result in interaction for neither cell proliferation nor steroid (progesterone and oestradiol) production (*Albonico et al.,* 2016).

Despite the classic cell lines, there are studies using less common cell lines to assess the adverse effects of mycotoxins. In the study of *Ficheux et al.* (2012) human hematopoietic progenitors were used for the assessment of *in vitro* myelotoxicity (by CFU-GM clonogenic assay) after co-exposure to six *Fusarium* mycotoxins (BEA, ENB, T-2, FB1, DON and ZEN) in binary mixtures. DON+ZEN and DON+FB1 showed additive and antagonistic myelotoxic (as measured by CFU-GM colorimetric assay) effects respectively.

The aforementioned studies focused either on binary or ternary mixtures. In the studies of the same research group i.e. *Groten et al.* (1998) and *Tajima et al.* (2002), the interactions of five *Fusarium* toxins (FB1, DON, NIV, T-2 and ZEN) were investigated using DNA synthesis as only endpoint on mouse fibroblast (L929) cells. The observed effects were mostly addition and in a lesser extent synergism (*Groten et al.,* 1998) and in the other study five of the mixtures produced less than additive effect and other four mixtures revealed significant synergistic interactions (*Tajima et al.,* 2002). These studies were mainly performed to prove the importance of the use of a robust mathematical design (further details in the following chapter) so the details of the interactions are not mentioned here.

MATHEMATICAL AND STATISTICAL ANALYSIS OF THE INTERACTIONS

A quite crucial issue regarding toxicological interactions is the experimental design in order to be able to characterize the nature of interaction(s) (*Chou*, 2006; Šegvić Klarić, 2012). The selection of the right experimental design is essential for accurate mathematical and statistical analysis.

The simplest and most commonly used mathematical model is the arithmetic definition of additivity. The expected combined effect is the sum of the effects of the individual toxins [cytotoxic effect (mycotoxin 1+ mycotoxin 2) = cytotoxic effect (mycotoxin 1) + cytotoxic effect (mycotoxin 2)]. Half of the analysed studies employed this model (*Kouadio et al.,* 2007; *Ficheux et al.,* 2012, *Bensassi et al.,* 2014; *Cortinovis et al.,* 2014). Although arithmetic definition of additivity is widely used it is a disputed model and it is not considered as the most reliable (*Alassane-Kpembi et al.,* 2016).

On the other hand factorial (fractional, full, central composite) designs are considered more robust and reliable. In this review the other 50% of the studies discussed used factorial designs (*Groten et al.,* 1998; *Tajima et al.,* 2002; *Wan et al.,* 2013a, b). The use of factorial designs is very useful when a lot of mycotoxins are tested simultaneously and in various concentrations each. It thus dramatically decreasing time and costs since the combinations tested are far less than the originally calculated (*Groten et al.,* 1988; *Tajima et al.,* 2002).

CONCLUSION

Legislation for mycotoxins' levels or maximum limits has been enacted in many countries worldwide but it regards only single toxin exposure. However, in nature mycotoxins are most likely to co-occur. The first experiments on mycotoxin interaction were performed quite early (in the 1980's) a systematic investigation on mycotoxins' combinations mostly occurred the last 15 years. FB1, DON and ZEN are not highly toxic or confirmed carcinogens, nevertheless their occurrence is ubiquitous. Hence the investigation of their combined effects is crucial for animal and human health.

From the studies analysed in this review it is concluded that interactions vary from antagonism to synergism even within the same trial. Despite that addition and synergism occur more frequently that the antagonism which is a fact highlighting

the need of multi-mycotoxin investigations. The observed variation is due to the use of different animals/cell lines, mycotoxins' concentrations, endpoints and mathematical designs.

In the future more studies on combined effects of FB1, DON and ZEN (especially in ternary mixtures) should be performed. Furthermore the results of in *vitro* studies could be confirmed *in vivo*. This practice can save resources, time and as it was aforementioned and it respects animal welfare as well.

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IN MEMORIAM

Dr. Salamon István (1918-2017)

Simon De Graaf-tól a Sydney Egyetem professzorától (Animal Reproduction, School of Life and Environmental Sciences) érkezett, a hír, hogy egykori elődje Professzor Steven Salamon, az MTA Agrártudományok Osztálya külső tagja (1993-tól), 2017. szeptember 17-én, 99 éves korában Sydney-ben elhunyt. Salamon professzor 1918.10.08.-án született Marosvásárhelyen, és 1957-ben emigrált Ausztráliába. 1958-tól dolgozott a Sydney egyetemen, egészen 1983-as nyugdíjba vonulásáig. Nemzetközileg is kiemelkedő kutatómunkáját a szaporodásbiológia szakterületén végezte és számos (a mai napig sokat idézett) tudományos közlemény szerzője, társszerzője volt. Elsősorban a spermakonzerválás és a mesterséges termékenyítés volt a szakterülete, ezen belül pedig a juhok szaporításában végzett úttörő munkát. Salamon professzor idős korában is aktív volt, több tudományos rendezvényen is előadott és társszerzője volt meghatározó közleményeknek. 2008-ban részt vett a Budapesten rendezett szaporodásbiológiai világkongresszuson (ICAR 2008), ahol a magyar Szaporodásbiológiai Társaság Hetzel Henrik díjával is kitüntették. Temetése 2017. szeptember 11-én Sydney-ben volt.