Università degli Studi di Milano

PhD Course in Veterinary and Animal Science

In vitro toxicological effects of Fumonisin B_1 alone and combined with other mycotoxins

Marco ALBONICO
University ID: R10526

Tutor: Prof. Francesca CALONI
Coordinator: Prof. Fulvio GANDOLFI

Academic Year: 2015-2016

"I am ignorant of absolute truth.
But I am humble before my ignorance
and therein lies my honor and my reward."

Khalil Gibran

Index

1. Abstract

Mycotoxins, secondary metabolites produced by moulds, mainly *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp., are common contaminants of food and feed. The aim of this project was to evaluate: (i) the potential endocrine disruptor effects of fumonisin B_1 (FB_1), beauvericin (BEA), deoxynivalenol (DON) and zearalenone (ZEA) metabolites α-zearalenol (α-ZEA) and β-zearalenol (β-ZEA), alone and combined, using a bovine granulosa cell (GC) *in vitro* model and (ii) the individual and combined effects of FB_1 and BEA on the intestinal barrier using Caco-2 cells cultured *in vitro* on semipermeable inserts.

The results obtained indicated that FB_1 alone at all tested doses (0; 0.5; 1; 1.5; 3; 6 μM) had no effects on GC proliferation and progesterone (P4) production. In the presence of β-ZEA at30 ng/mL (0.094 μM), FB_1 at 30 ng/mL (0.042 μM) showed a stimulatory effect on GC numbers. Cell proliferation decreased after exposure to β-ZEA alone at 5.0 mg/mL (15.6 μM) and FB_1 with α-ZEA and β-ZEA at the same concentration. Regarding steroid production, FB_1 at 30 ng/mL (0.042 μM) and 100 ng/mL (0.13 μM amplified the inhibitory effect of β-ZEA at 30 ng/mL (0.094 μM) on estradiol (E2) production, while FB_1 alone increased ($P<0.05$) IGF1-induced E2 production. FB_1 in combination with β-ZEA decreased ($P < 0.05$) E2 production. FB_1 at 1, 1.5 and 3 μM slightly inhibited ($P < 0.05$) E2 production. BEA at concentrations ≥ 3 μM was found to strongly decrease ($P < 0.05$) both steroid production and FB_1 did not influence the effects of BEA. At 10 μM both mycotoxins decreased ($P < 0.001$) serum-induced GC proliferation. At 30 μM, BEA showed inhibitory effects on FSH plus IGF-1-induced CYP11A1 and CYP19A1 mRNA abundance ($P < 0.05$), whereas FB_1 at 30 μM had no effect on CYP11A1 and CYP19A1 gene expression.

As regards the effects of FB_1 and BEA, alone and combined, on the Caco-2 intestinal barrier model data showed a TEER decrease after 1 h and 2 h of Bl exposure to BEA at 0.5 and 1.5 μM and after 24 h of Bl exposure to BEA at 0.5 μM, whereas after 24 h of Bl exposure, BEA at 3 and 6 μM was found to significantly ($P < 0.05$) increase TEER.

FB_1 had no effect on the intestinal barrier integrity and when combined with BEA the TEER increase induced by BEA was no longer observed.

Cytokine release was observed only after exposure to BEA alone, and not in combination with FB_1, with an increase of IL-6 and IL8 release after apical exposure to 3 and 6 μM and after basolateral exposure to 1.5, 3, 6 μM for IL-6 and only to 6 μM for IL-8. TNF- α release was induced by Ap (0.5 -1.5 μM) and Bl (1.5 μM) exposure to BEA.

Overall, these results provide information on *in vitro* toxicological effects of *Fusarium* mycotoxins.

2. General introduction

2.1 Mycotoxins

Mycotoxins are secondary metabolites produced by molds that contaminate food and feed worldwide (Schollenberger et al., 2007). 25% of the world's crop production is contaminated by mycotoxins and their global occurrence pose a serious risk to human and animal health (Pinton and Oswald, 2014). Mycotoxins produced by *Aspergillus, Fusarium,* and *Penicillium* genera are particularly significant for their ubiquity and toxicity (Osweiler, 2000).

There are several factors that influence the presence of mycotoxins in foods or feeds: extrinsic and intrinsic factors (Hussein and Brasel, 2001). Extrinsic factors include environmental conditions such as storage, temperature and humidity (Hussein and Brasel, 2001) while intrinsic ones include the strain specificity and the strain variation (Hussein and Brasel, 2001). Mycotoxins have a wide spectrum of toxic effects and the nature and the intensity of these effects are not only related to the time and dose of exposure, but also to the possible co-occurrence of mycotoxins that can result in additive, synergic or antagonistic toxicological effects (Fink-Gremmels, 1999). Chronic exposure to mycotoxins (Hussein and Brasel, 2001; Fink-Gremmels and Malekinejad, 2007) results in reduced feed intake, loss of body weight, immune-suppression with a subsequent increased susceptibility to infections, and possible effects on reproductive function (Fink-Gremmels and Malekinejad, 2007; Pestka, 2007; Voss et al., 2007).

2.2 Fusarium mycotoxins

Fusarium molds are commonly found on commodities in Europe, America and Asia and are considered the most prevalent species in the northern hemisphere (Creppy, 2002). *Fusarium* spp. synthesize a wide range of mycotoxins (Flannigan, 1991; Glenn, 2007) and the most important in terms of impact to animal health and production are fumonisins, trichothecenes and zearalenone (D'Mello et al., 1999; Uhlig et al., 2007; Jestoi, 2008).

Fusarium species require a moisture content of 25% (Newman and Raymond, 2005) and toxinogenesis is strongly influenced by this factor (Sweeney and Dobson, 1998). Consumption of a mycotoxin-contaminated diet may induce acute and chronic effects resulting in a broad variety of toxicological effects in animals (D'Mello et al., 1999; Binder et al., 2007; Smith, 2012; Cortinovis et al., 2014)

2.2.1 Worldwide contamination of Fusarium mycotoxins

The presence of mycotoxins depends on several factors such as their interactions with other organisms on the substrate where they coexist, the production site, humidity, temperature and the agricultural and post-harvest practices (Hussein and Brasel, 2001; Ferre, 2016). Several surveys reported the occurrence of mycotoxins relevant to the feed industry in different regions particularly with respect to fumonisins (Boutigny et al., 2012; Garrido et al., 2012) and deoxynivalenol (DON) (Boutigny et al., 2012; Grajewski et al., 2012). Rodrigues and Naehrer (2012) reported that fumonisins, DON and zearalenone (ZEA) were present in 64%, 59% and 45% of analyzed samples, respectively while, in a study conducted by Yoshinari et al. (2016) beuvericin (BEA) was found in 34% of the samples. As reported by Binder et al. (2007), more than half of the samples collected in Europe were contaminated as well as one third of the Asian-Pacific ones. The European Commission (EC) has established guidance levels for the presence of some *Fusarium* mycotoxins in animal feed (EC, 2006). For fumonisins (fumonisin B_1 and B_2) the guidance values are 60 mg/kg for maize and maize products, 5 mg/kg for complementary and complete feedingstuffs for pigs, horses, rabbits and pet animals, 10 mg/kg for fish, 20 mg/kg for poultry, calves (<4 months), lambs and goat kids and 50 mg/kg for adult ruminants (>4 months) and mink (EC, 2006). Regarding DON the EC guidance values are 8 mg/kg for cereals and cereal products with the exception of corn by-products (12 mg/kg) and 5 mg/kg for complementary and complete feedingstuffs with the exception of feedingstuffs for pigs (0.9 mg/kg) and for calves (<4 months), lambs and goat kids (2 mg/kg) (EC, 2006; Pinton and Oswald, 2014). The ZEA EC guidance values recommended for complementary and complete feedingstuffs for piglets/gilts, sows/fattening pigs, and calves/dairy cattle/sheep/goats are 0.1, 0.25 and 0.5 mg/kg, respectively (EC, 2006; Streit et al., 2012). The ZEA EC guidance values for cereals/cereal products and corn by-products are 2 and 3 mg/kg, respectively (EC, 2006).

2.2.2 Fumonisins

Fumonisins are a family of mycotoxins mainly produced by *Fusarium verticillioides*, *Fusarium proliferatum, Fusarium napiforme* and *Fusarium nygamai* that have been shown to occur worldwide at significant levels in corn and corn by-products (Voss et al., 2007; Glenn, 2007). Fumonisins have been found to commonly occur in combination with deoxynivalenol (DON), zearalenone (ZEA) and beauvericin (BEA) in cereal grains and animal feed (Jestoi, 2008). Fumonisins are divided into four groups known as A, B, C and P (Marasas et al., 1984; Alberts et al., 1990; Yazar and Omurtag, 2008). The most

important is the B group that includes fumonisin B_1 (FB_1), B_2 (FB_2) and B_3 (FB_3) with FB_1 (Fig. 2.1) being the most significant in terms of toxicity and occurrence (EFSA, 2005). FB_1 induces different species-specific effects in animals including leukoencephalomalacia in horses and pulmonary edema in pigs (Marasas et al., 1988; Ross et al., 1993; Voss et al., 2007). Cattle are considerably less sensitive to FB_1 than horses and pigs (Fink-Gremmels, 2008) and signs of liver and kidney injury have been reported in cattle only after exposure to very high concentrations of FB_1 (Osweiler et al., 1993; Baker and Rottinghaus, 1999; Mathur et al., 2001). In ruminants FB_1 has a very low bioavailability , is poorly degraded in the rumen (Caloni et al., 2000) and is found unmetabolized in feces (Cavret and Lacoeur, 2006). The mechanisms of toxicity for FB_1 are complex and may involve several molecular sites (Voss et al., 2007). FB_1 bears a remarkable structural resemblance to the long-chain (sphingoid) base backbones of sphingolipids and thus impairs sphingolipid biosynthesis (Wang et al., 1991; Merrill et al., 2001; Voss et al., 2002; Voss et al., 2007). The inhibition of ceramide synthase leads to the accumulation of sphingoid bases and to the depletion of complex sphingolipids, which interfere with the function of some membrane proteins (Wang et al., 1991; Merrill et al., 2001; Voss et al., 2002; Voss et al., 2007). Thus, the mechanism of toxicity of FB_1 is linked to its ability to disrupt the sphingolipid metabolism and the subsequent adverse effects on cell regulation (Wang et al., 1992; Marasas et al., 2004; Smith., 2012).
The International Agency for Research on Cancer (IARC) classified FB_1 as a possible carcinogen (class 2B) for humans (IARC, 2003).

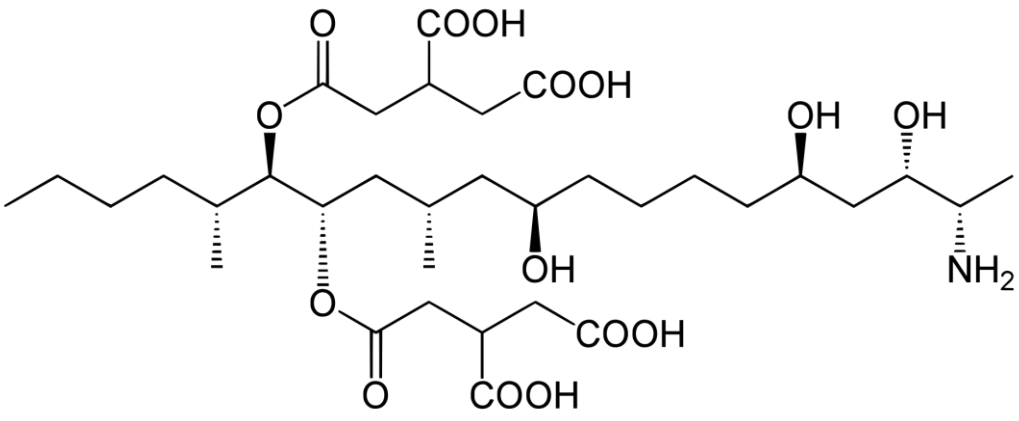

Figure 2.1: Chemical structure of fumonisin B_1 (FB_1).

2.2.3 Trichothecenes

Trichothecenes are a family of *Fusarium* mycotoxins commonly found in cereal grains such as corn, wheat, rye, barley and oats. The family is subdivided into four groups (A, B, C and D) according to their chemical structure. Type A trichothecenes including T-2, HT-2 toxin, neosolaniol and diacetoxyscirpetol and type B including DON and its 3- acetyl and 15-acetyl derivates, nivalenol and fusarenon X are the most important (Escriva et al., 2015).

Trichothecenes inhibit the synthesis of RNA and DNA interfering with cellular metabolic activities and causing cell death (Thompson and Wannemacher, 1990) and are able to readily bind to eukaryotic ribosomes, in particular to the 60S ribosomal subunits, inhibiting protein synthesis (Pestka, 2010).

The general clinical signs of trichothecene toxicosis commonly include loss of appetite, vomiting, diarrhea, leukocytosis and gastrointestinal hemorrhage (Pestka and Smolinski, 2005; Maresca, 2013; Blajet-Kosicka et al., 2014; Pinton and Oswald, 2014). Ruminants are less sensitive to trichothecene toxicity whereas pigs and poultry are the most susceptible species to DON and T-2 toxin respectively (Pestka and Smolinski, 2005; Maresca, 2013; Pizzo et al., 2016).

2.2.4 Deoxynivalenol

Deoxynivalenol (DON), also known as vomitoxin, belongs to the type B group of trichothecenes and occurs commonly in grains such as maize and wheat but also in rice, oats and sorghum (Pinton and Oswald, 2014). DON has been implicated in farm animal disease outbreaks in many areas of the world and is considered one of the most hazardous food-associated mycotoxin for human health (Maresca, 2013; Pinton and Oswald, 2014).

Structurally, DON (Fig. 2.2) is a polar organic compound containing 3 free hydroxy groups (-OH), which are associated with its toxicity (Sobrova et al., 2010). The mechanism of action of DON is known to be related to its ability to bind eukaryotic ribosomes and inhibit protein synthesis by blocking translation and inhibiting the elongation of peptide chains (Larsen et al., 2004; Pestka, 2010). Moreover, DON can also induce cell apoptosis activating the mitogen-activated protein kinases (MAPKs) (Larsen et al., 2004; Pestka et al., 2010; Li et al., 2014). After ingestion of highly DON contaminated food common clinical signs include abdominal pain, vomit, diarrhea, leukocytosis and blood in stool. Loss of weight and altered immune function have been frequently observed after chronic exposure (Pestka and Smolinski, 2005).

DON was found to be able to cross the intestinal barrier modifying cellular functions and causing cell death in pigs (Maresca et al., 2013). *In vitro* studies carried out with the cell lines Caco-2, IPEC-1 and IPEC-J2 demonstrated that DON is able to impair the intestinal barrier function and may have serious consequences for human and animal health (Van de Walle et al., 2010; Awad et al., 2011; Vandenbroucke et al., 2011).
Ruminants are considered less susceptible to the adverse effects of DON because DON is rapidly converted in the rumen into de-epoxy DON (DOM1) that is less toxic compared to the parent compound (Cotè et al., 1986; Fink-Gremmels, 2008).

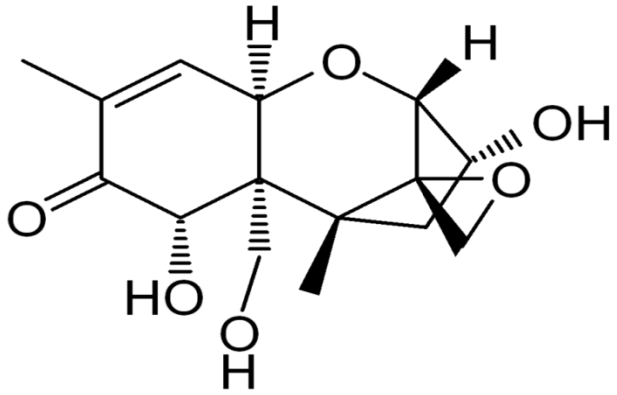

Figure 2.2: Chemical structure of deoxynivalenol (DON).

2.2.5 Zearalenone

Zearalenone (ZEA) (Fig. 2.3) is a widespread mycotoxin that is of great interest due to its toxic effects on animals (Fink-Gremmels, 1999; Fink-Gremmels and Malekinejad, 2007; Zinedine et al., 2007). ZEA is an estrogenic mycotoxin produced by several *Fusarium* species including *F. culmorum*, *F. graminearum*, *F. equiseti* and *F. crookwellense*. This mycotoxin commonly contaminates wheat, rice, maize, barley and other crops (Fink-Gremmels and Malekinejad, 2007). ZEA is a resorcyclic acid lactone, chemically described as 6-[10-hydroxy-6-oxo-trans-1-undecenyl]-B-resorcyclic acid lactone (Zinedine et al., 2007). This particular structure resembles several characteristics of steroid hormones and consents ZEA to bind estrogen receptor α (ESR1) and estrogen receptor β (ESR2) (Zinedine et al., 2007). In this way ZEA can act as an agonist and partial antagonist of estradiol (Malekinejad et al., 2007), thus inducing estrogenic effects such as hyperestrogenism in all animal species (Böhm and Razzai-Fazeli, 2005).
In pigs, which are more sensitive than other species, ZEA is mainly biotransformed in the liver in α-Zearalenol (α-ZEA), which showed higher affinity to bind to estrogen receptors than ZEA and β-Zearalenol (β-ZEA) (Malekinejad et al., 2007). Common

clinical signs of ZEA intoxication are related to the hyperstimulation of estrogen-dependent tissues (Malekinejad et al., 2007). In gilts clinical signs include reddening and swelling of the vulva and an enlarged uterus, whereas in cycling sows fertility is impaired (Malekinejad et al., 2007; Minervini and Dell'Aquila, 2008).
In ruminants, ZEA is converted in the rumen mainly into α-ZEA (Fig. 2.4) and, to a less extent, in β-ZEA (Fig. 2.5) (Abidin and Khatoon, 2012; Winkler et al., 2014) but the rate of absorption of the more polar α-ZEA is poor.
In cow, infertility, reduced milk production and hyperestrogenism have been reported (Minervini and Dell'Aquila, 2008).

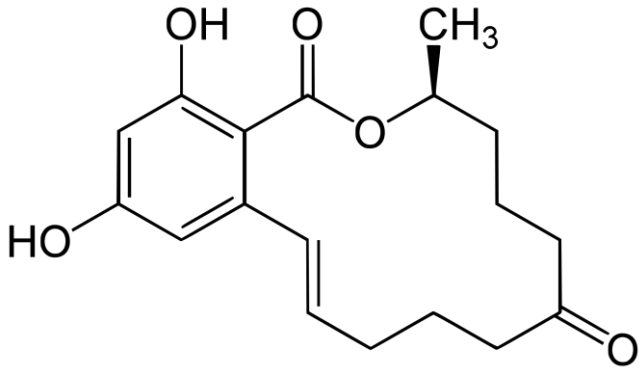

Figure 2.3: Chemical structure of zearalenone (ZEA).

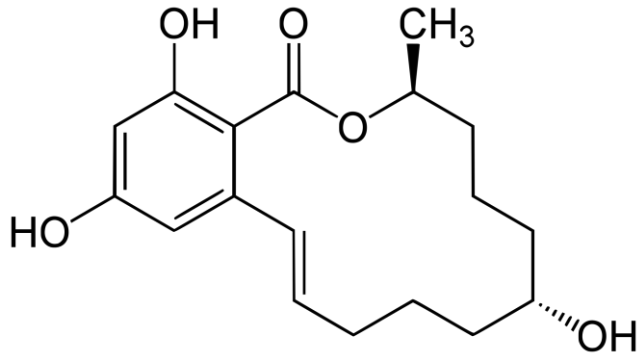

Figure 2.4: Chemical structure of α-zearalenol (α-ZEA).

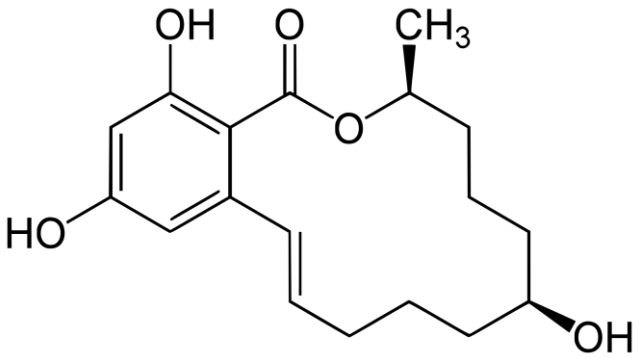

Figure 2.5: Chemical structure of β-zearalenol (β-ZEA).

2.2.6 Beauvericin

Beauvericin (BEA) (Fig. 2.6) is a mycotoxin firstly identified in the culture of the soil-borne entomopathogenic fungus *Beauveria bassiana* but also synthesized by several *Fusarium* spp. parasitic to important cereal grains such as corn, wheat, rice and barley (Leslie and Summerell, 2006). High contamination levels, up to 500 mg/kg of BEA, have been reported in commodities raising serious concerns about the potential impact of this fusariotoxin on human and animal health (Uhlig et al., 2007; Jestoi, 2008). BEA is a ionophoric molecule that can form stable and lipophilic complexes with cations and transport them into the lipophilic phase (Hilgenfeld and Saenger, 1982; Jestoi, 2008; Schoevers et al., 2016). According to this, the primary toxic action of BEA is considered to be related to its ionophoric properties. BEA is thus able to promote the transport of several cations through membranes disturbing the cell homeostasis (Hilgenfeld and Saenger, 1982; Jestoi, 2008; EFSA, 2014; Schoevers et al., 2016). It is well-established that an increase in the intracellular concentration of cations such as calcium and the subsequent activation of calcium-dependent endonucleases lead to DNA fragmentation which is related to several chronic diseases in humans and in animals with many different adverse health effects (Speijers and Speijers, 2004; Kouadio et al., 2007). In recent studies BEA was found to exert potent cytotoxicity against different cell lines (Jestoi, 2008; Ruiz et al., 2011; Prosperini et al., 2012; Mallebrera et al., 2016).

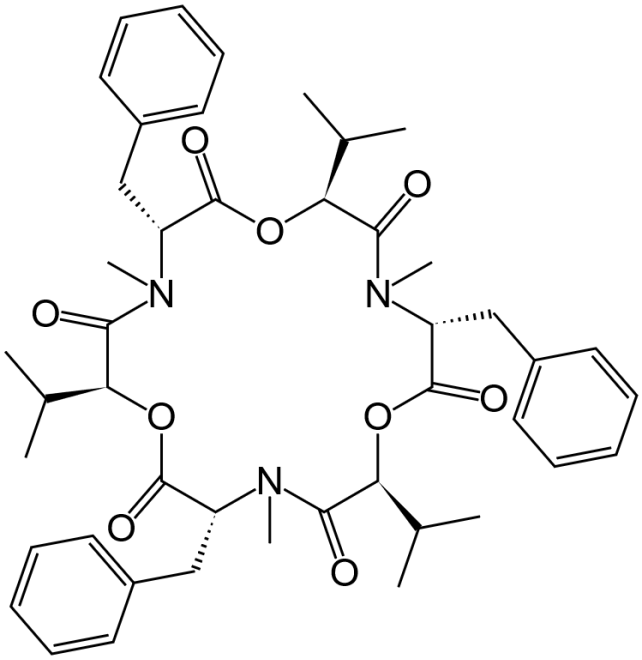

Figure 2.6: Chemical structure of beauvericin (BEA).

2.3 Fusarium mycotoxins and effects on reproductive function

2.3.1 Fumonisins

The potential reproductive toxicity of fumonisins has been only recently investigated. Studies demonstrated that fumonisin B_1 (FB_1) has the potential to impair fertility in pigs and rabbits delaying puberty and impairing semen quality and spermatogenesis (Ogunlade et al., 2006; Gbore and Egbunike, 2008; Ewuola and Egbunike, 2010). Moreover, *in vitro* studies showed that FB_1 affects some functional parameters of equine spermatozoa, such as sperm chromatin stability and motility (Minervini et al., 2010). Recently, FB_1 was found to directly affect *in vitro* porcine granulosa cell (GC) proliferation, steroid production and gene expression (Cortinovis et al., 2014). FB_1 at 10 μM decreased cell proliferation and strongly increased progesterone (P4) production thereby suggesting that this *Fusarium* mycotoxin may affect the normal follicle growth and oocyte survival and interfere with the endocrine regulation of the developing follicle in swine (Cortinovis et al., 2014).

Regarding a possible interaction between FB_1 and other *Fusarium* mycotoxins, a significant interaction between FB_1 at 10 μM and α-ZEA at 9.4 μM was found on P4 production (Cortinovis et al. 2014). On the contrary, no significant interaction was

observed when GC were treated with FB_1 at 10 μM combined with DON at 3.4 μM (Cortinovis et al. 2014).

2.3.2 Deoxynivalenol

The potential of deoxynivalenol (DON) to act as endocrine disruptor has been investigated in previous studied and is the object of increasing interest (Medvedova et al., 2011; Han et al., 2016).
In previous studies, DON at 10 μM was found to affect the process of follicular maturation with a decrease of the reserve pool of follicles resulting in a significant decrease in the number of normal follicles (Gerez et al., 2016). These results are in agreement with previous studies, where porcine cumulus-oocyte complexes exposed to DON at 0.02, 0.2, or 2 μM showed an increase in cumulus cell death and degeneration, with a consequent significant reduction in the proportion of oocytes that reached metaphase II (Schoevers et al., 2010). Concerning the effect of DON on GC proliferation and steroidogenesis in pigs, Ranzenigo et al. (2008) reported DON increasing GC number at 0.034 μM and 0.34 μM and drastically reducing it at 3.4 μM. Differently, Pizzo et al. (2016) found that DON did not alter cell proliferation of bovine GC at concentrations ranging from 0.1 to 3.3 μM.
The effects of DON on steroidogenesis have been also investigated with the bovine GC model (Pizzo et al., 2015, 2016). DON, in presence of FSH and IGF1, was found to inhibit E2 release at concentrations ranging from 0.1 to 3.3 μM and P4 production at 0.33 and 3.3 μM (Pizzo et al., 2015). In absence of IGF1, DON at 3.3 μM significantly up-regulated CYP19A1 mRNA abundance, but had no effect on CYP11A1 mRNA abundance in bovine GC (Pizzo et al., 2016). These results seem to support the theory that DON promotes stability of several mRNAs interfering with post-transcriptional processes and avoiding their rapid degradation with several adverse effects on steroidogenesis in cattle (Pizzo et al., 2015; Pizzo et al., 2016).

2.3.3 Zearalenone

It is already well established that zearalenone (ZEA) and its metabolites have strong estrogenic activities, being able to cause alteration in the reproductive tract (Fink-Gremmels, 1999; Malekinejad et al., 2007; Denli et al., 2017). Particularly, ZEA can induce estrogenic effects such as hyperestrogenism, anoestrus, ovarian atrophy and changes in the endometrium (Böhm and Razzai-Fazeli, 2005; Malekinejad et al., 2007; Minervini and Dell'Aquila, 2008). The effect of ZEA depends on several factors

including the reproductive status (prepuberal, cycling or pregnant) of the affected animal and the administration time and dose (Price et al., 1993; Tiemann and Dänicke, 2007; Döll and Dänicke, 2011; Holda and Glogowski, 2014). A correlation between the level of ZEA in mg/kg and the length of anestrus in days was found by Young and King (1986) who observed an increase of the weaning-to-estrus interval when increased ZEA was fed.

Döll et al. (2004) reported that after 5 weeks of feeding piglets in prepuberal stuatus with feed contaminated by ZEA up to 0.42 mg/kg, the mean weight of the uteri was significantly increased. ZEA and its metabolite adverse effects on estrus were also reported in a recent study (Daia et al., 2016). This *in vivo* study provided evidence that ZEA at 1.04 mg/kg accelerated the development of the ovaries in post-weaning piglets confirming that a diet contaminated by ZEA can accelerate the development of ovarian follicles in post-weaning piglets possibly leading to subsequent reproductive disorders (Daia et al., 2016). In another study, Minervini et al. (2006) studied the effects of ZEA and its metabolites with an *in vitro* culture system of equine GC. The results of this study showed GC apoptosis after exposure to ZEA at 0.1 μM. A recent study confirmed that ZEA induces necrosis and GC death in a dose-dependent manner via a caspase-3- and caspase-9-dependent mitochondrial pathway (Zhu et al. 2012).

The effects of α-ZEA and β-ZEA, ZEA metabolites, on pig oocytes have been investigated (Alm et al., 2002). Oocyte maturation rate resulted in a significant decrease when oocytes were exposed for 48 h to α-ZEA at concentrations up to 7.5 μM (Alm et al., 2002). Differently, β-ZEA showed a significant effect only at 30 μM (Alm et al., 2002). In a subsequent study, Tiemann et al. (2003) found α-ZEA and β-ZEA at concentrations of 15 and 30 μM to inhibit *in vitro* the FSH stimulated P4 synthesis in porcine GC (Tiemann et al., 2003). The ability of ZEA metabolites to affect steroid production in pig GC has also been reported by Ranzenigo et al. (2008) and Cortinovis et al. (2014). In both studies α-ZEA primarily increased progesterone (P4) production induced by FSH and IGF1, whereas estradiol (E2) production exhibited a biphasic dose–response to α-ZEA in the study conducted by Ranzenigo et al. (2008) and was not affected in the study of Cortinovis et al. (2014). Specifically, α-ZEA at 0.094 μM and 9.4 μM increased and decreased E2 production, respectively (Ranzenigo et al., 2008), whereas no effects on E2 production was reported after exposure to α-ZEA at 9.4 μM (Cortinovis et al., 2014). In comparison to monogastric, ruminants seem less susceptible to ZEA toxicity (Upadhaya et al., 2010; Pizzo et al., 2016). In rumen ZEA is converted into α-ZEA and β-ZEA, even if ZEA also undergoes hepatic biotransformation (Seeling et al., 2006). In cows the poor rate of absorption of the ZEA metabolites explains why clinical signs of hyperestrogenism are observed scarcely (Fink Gremmels, 2008). Estrogenic effects of ZEA in cows were found only after the ingestion of highly contaminated feed or after long-term exposure to contaminated feed materials (Fink

Gremmels, 2008). In a recent study Pizzo et al. (2016) determined the impact of α-ZEA and β-ZEA on GC function evaluating cell proliferation, steroid production and gene expression using a bovine GC model. Based on the results reported, in absence of IGF1, α-ZEA at 3.1 μM had inhibitory effects on cell proliferation, whereas it was found to inhibit both E2 and P4 production in GC at concentration ranging from 0.09 to 3.1 μM in presence of IGF1 (Pizzo et al., 2016). Regarding β-ZEA, an inhibitory effect on cell numbers was found at 31 μM both in presence and absence of IGF1, while E2 and P4 production was increased in the absence IGF1. The results obtained by Pizzo et al. (2016) on cell proliferation are in agreement with previous studies on pig GC conducted by Tiemann et al. (2003) and Ranzenigo et al. (2008) which demonstrated the adverse effects of β-ZEA. Regarding steroidogenesis previous studies on pigs (Ranzenigo et al., 2008) showed an increase in E2 production at 0.09 μM whereas in cattle no results were obtained at the same concentration (Pizzo et al., 2016), suggesting a species-specific effect.

In relation to the possible interaction between ZEA metabolites and gene expression Pizzo et al. (2016) demonstrated that α-ZEA in the presence of IGF1 did not affect CYP11A1 and CYP19A1 mRNA abundance. However, in previous studies on pigs Tienmann et al. (2003) showed that α-ZEA at 5 μM was able to increase CYP11A1 protein expression, whereas Ranzenigo et al. (2008) showed that α-ZEA at 9.4 μM decreased CYP11A1 mRNA abundance in porcine GC.

2.3.4 Beauvericin

Beauvericin (BEA) is considered an emerging mycotoxin (Uhlig et al., 2007; Jestoi, 2008; EFSA, 2014). The ability of BEA to increase the cytoplasmic cation concentration may play an important role in the induction of cell apoptosis (Jow et al., 2004). Contaminations, up to 500 mg/kg of BEA, have been reported in commodities raising serious concerns about the potential impact of this mycotoxin (Jestoi, 2008). In previous studies BEA was found to exert potent cytotoxicity on pig, rodent and human cell lines (Klaric et al., 2006; Ruiz et al., 2011; Prosperini, et al., 2012). Recent studies, with estrogen, androgen, progestagen and glucocorticoid reporter gene assays (RGAs), demonstrated that BEA has the potential to modulate the endocrine system by antagonism of nuclear receptor transcriptional activity (Fernández-Blanco et al., 2016).

Information on possible reproductive effects of BEA in domestic animals is lacking.

In a recent study Schoevers et al. (2016) showed that BEA reduced the developmental competence of both the maturing oocyte and the two-four cell stage embryo in pigs, and that BEA only affected the rate of developing embryos. The authors exposed the cumulus-oocyte-complexes and developing embryos to BEA at concentrations ranging

from 0.31 to 10 μM and they studied the effects of this mycotoxins on viability, progesterone synthesis and apoptosis (Schoevers et al., 2016). As reported in this study BEA was toxic to embryos, oocytes and cumulus cells at concentrations exceeding 0.5 μM.

2.3.4.1 Granulosa cells *in vitro* model

The function of granulosa cells (GC) is essential in the process of folliculogenesis and oocyte growth and development (Petro et al., 2012). In fact GC are crucial in the delivery of nutrients to the oocyte and they play an important role in the ovarian steroidogenesis (Scaramuzzi et al., 2011). The use of the GC model to investigate the molecular mechanisms of several compounds, their adverse effects and the mechanisms underlying the process of ovarian follicular atresia has been already reported (Jolly et al., 1994; Kwintkiewicz et al., 2010). For its characteristics the GC model provides the opportunity to examine the factors that influence oocyte competence in a way not previously accessible (Dias et al., 2014). As reported by several authors the bovine GC model is not only able to reproduce the *in vivo* situation, but also allows comparable studies with humans (Anderiesz et al., 2000). Anderiesz et al. (2000) reported the response of bovine and human oocytes to pure recombinant preparations of human follicle stimulating hormone (FSH) and luteinizing hormone (LH) for meiotic maturation and subsequent developmental competence *in vitro*. Specifically, no significant difference was observed in terms of maturation of oocyte to metaphase II and embryonic development between bovines and humans (Anderiesz et al., 2000). These results are in agreement with subsequent studies where similarities in terms of embryonal genome activation and duration of preimplantation development were found, confirming that the bovine GC model represents a good *in vitro* model to evaluate the effects of contaminants in both bovines and humans (Petro et al., 2012).

2.4 *Fusarium* mycotoxins and *in vitro* intestinal models

2.4.1 In vitro intestinal models

The intestinal tract represents the first interface between food and the internal body and is the primary target of dietary compounds, thus *in vitro* intestinal models are of great interest for several toxicological studies (Meca et al., 2011). The intestinal epithelial monolayer consists of several subsets of epithelial cells that constitute a physical and

biochemical network for the maintenance of the homeostasis in the gastrointestinal tract (Goto and Kiyono, 2012). The main functions of the intestinal epithelium include the protection of the body against potentially toxic compounds or microorganisms, and the prevention of the loss of important compounds such as water and solutes (Gordon et al., 2015). There are several *in vitro* intestinal models that can mimic oral toxicity with different advantages, limitations, issues and needs (Table 1).

Tabel 1: Models for intestinal absorption (Gordon et al., 2015)

Models for intestinal absorption					
	Examples	*Advantages*	*Limitations*	*Issues and needs*	*Memo*
Cell-based systems	Caco-2 monolayer (e.g. CacoReady, Advancell)	- easily accessible - regulatory acceptance for BCS classification - reproducible - metabolically competent - information on active transport - Papp useful PBPK or bioavailability screening (e.g. BCS class)	- poor correlation to *in vivo* rat absorption data (since, e.g. no available human absorption data for pesticides) - technical limitations (sufficient water solubility needed, dependence on shipment)	- definition of acceptance criteria (e.g. applicability domains, reference datasets, integrity tests,..?) - higher standardization of the method including standardized integrity tests (SOP?) - think about new approaches (new *in vitro* models? *in silico* models? combination of both?) that cover all pathways - simple system, applicable for screening purposes - applicable for mixtures	- no routine in the regulatory context of chemicals and food, data from intestinal *in vitro* models can be treated as mechanistic add-on information - clarify transporter activity and extent of activity in Caco-2 model
	T84 polarized cells	- depicts apical and basal surface - reflects villi like structures - well defined cellular junctions - easy to assess TEER for permeability measurements	- 7-10 days for polarization - technical limitations		
Artificial surrogates	GiT-PAMPA (Pion)	- easily accessible - standardized - reproducible - Papp useful for PBPK	- only passive diffusion - no metabolism - poor correlation to *in vivo* rat absorption data - technical limitations (sufficient water-solubility, UV-activity needed)		

Among the intestinal models the most common are represented by immortalized human adenocarcinoma cell lines such as Caco-2 or T84 (Raffatellu et al., 2005; Khare et al., 2009; Tran et al., 2010). Caco-2 and T84 models are common to many studies, however each epithelial barrier model has specific characteristics for application (Ward and Tse, 1999). For example, it is well known that there are some significant differences between polarized Caco-2 and T84 cells. Caco-2 cell monolayers exhibit lower Trans-Epithelial Electrical Resistance (TEER) values on confluence than T84 cells (Gordon et al., 2015). An increase in the permeability of intestinal epithelial cells is correlate to a decrease in TEER values and this parameter has been used as an indicator of early sub-lethal epithelial toxicity (McCall et al., 2009; Gordon et al., 2015). Moreover, T84 cells differ from Caco-2 cells in Na^+-dependent nucleoside transport systems (Ward and Tse, 1999). Other differences between these cell lines have been described before by several authors (McCool et al., 1990; Ward and Tse, 1999; Gordon et al., 2015). For example, compared to Caco-2 cells, T84 cells produce mucin in culture and are able to respond to external stimuli to exert innate immune responses thus mimicking more closely the intestinal surface conditions (McCool et al., 1990; Ward and Tse, 1999; Ou et al., 2009; Gordon et al., 2015). Culture-related conditions were shown to influence the expression of Caco-2 cell activity (Delie and Rubas, 1997; Sambuy et al., 2005; Turco et al., 2011). In order to reduce the variability, the TC7 clone was obtained from a late passage of the parental Caco-2 line (Turco et al., 2011). TC7 cells have demonstrated to consist of a more homogeneous population with more developed intercellular junctions, however Turco et al. (2011) reported the non-suitability of TC7 cells to predict intestinal absorption of highly lipophilic or poorly absorbed compounds (Turco et al., 2011).
Previous studies reported that IPEC-1 and IPEC-J2 cell lines are considered for their suitability good *in vitro* intestinal models (Lu et al., 2002; Schierack et al., 2006). Both cell lines are undifferentiated and derived from small intestine of piglets, however, while IPEC-1 derived from the small intestine, IPEC-J2 derived from mid-jejunum (Berschneider, 1989). IPEC-J2 cells grown in monolayers were first employed in transepithelial ion transport and cellular proliferation studies (Kandil et al., 1995; Rhoads et al., 1997). Neither of these cell lines is immortalized and therefore they are considered better models of normal porcine intestinal epithelium than transformed cell lines (Koh et al., 2007).

2.4.2 Caco-2 in vitro intestinal model

Caco-2 cells, originally isolated from a human colorectal adenocarcinoma, represent a very well characterized *in vitro* model of epithelial barrier for intestinal absorption and metabolism studies (Fogh et al., 1977; Gilman and Cashman, 2006; Meca

et al., 2011; Prosperini et al., 2012). This cell line represents a good cell culture model for the study of absorption and metabolism in the small intestine, as well as the most used model for pharmacological and toxicological studies (Delie and Rubas, 1997; Le Ferrec et al., 2001; Sambuy et al., 2005). To better mimic the *in vivo* conditions of the intestine, Caco-2 cells are cultured on permeable filter supports which allow access of ions and nutrients to both sides of the cell monolayer (Turco et al., 2011). Caco-2 cells demonstrated to be highly dependent on culture conditions including medium composition and pH, seeding density and substrate nature (Ranaldi et al., 2003). However, this cell line is considered a suitable physiological model for studies of toxicity and transport of nutrients, cations and contaminants (Hidalgo et al., 1989; Artursson et al., 2001; Martel et al., 2001; Caloni et al., 2006; Caloni et al., 2012) (Fig. 2.7). When grown on a permeable filter support for 21 days Caco-2 cells are able to polarize and differentiate according to some typical enterocytic pathway, with apical microvilli and a basolateral surface, similar to the cellular surface in contact with intestinal vascular and lymphatic circulation (Pinto et al., 1983; Artursson et al., 2001; Caloni et al., 2012; Ferruzza et al., 2012). The Caco-2 cell full differentiation and polarization process has been associated with Trans-Epithelial Electrical Resistance (TEER) values exceeding 300 Ω cm^2 (Van Breemen and Li, 2005). In fact after confluence, TEER and permeability of marker molecules are usually used to investigate the integrity of the epithelial barrier tight junctions (Pinton et al., 2009). Specifically, TEER quantifies ion movement across cellular barriers and is considered a good indicator of the integrity of the epithelial barrier (De Angelis and Turco, 2011).

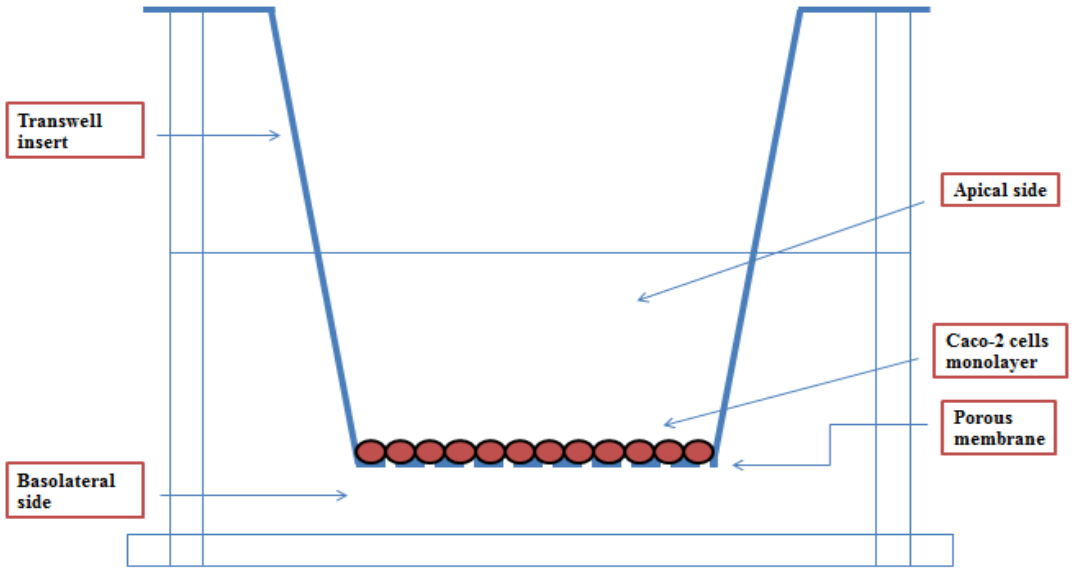

Figure 2.7: Transwell insert which separate the Apical (Ap) compartment from the basolateral (Bl) compartment.

Caco-2 cells also express some transporters and efflux proteins as well as metabolic enzymes that are normally expressed in the gut (Sun et al., 2002; Sambuy et al., 2005). *In vitro* methods based on chemical transport across Caco-2 monolayers are at present the most frequently and successfully exploited procedures to investigate intestinal permeability in humans (Yee, 1997; Yamascita et al., 2000; Van Breemen and Li., 2005; Nigsch et al., 2007), giving good correlation with the fraction absorbed in humans (Van Breemen and Li., 2005; Nigsch et al., 2007).

Passive diffusion is not the only absorption mechanism of xenobiotics, the active transport and efflux systems play also an important role (Yang, 2013). There are many intestinal transporters expressed on the small intestine and among these there are two main groups: the efflux (ABC family) and uptake (SLC family) transporters (Liu et al., 2013). Specifically, ABC transporters are composed by P-glycoprotein (P-gp), multidrug resistance 1 (MDR1), multidrug resistance-associated protein 2 (MRP2) and breast cancer resistance protein (BCRP) (Liu et al., 2013), whereas among SLC transporters we can found the organic cation transporters (OCTs), novel organic cation transporters (OCTNs) organic anion-transporting polypeptides (OATPs) and H+/peptide cotransporter (PEPT1) (Liu et al., 2013).

2.4.3 Fusarium mycotoxins and in vitro studies with intestinal barrier

Different *in vitro* models have been used to evaluate the effects of *Fusarium* mycotoxins on the intestinal barrier (Table 2). Table 2 summarizes the cell models used to assess the impact of the principal Fusariotoxins on the intestinal epithelium.

Tabel 2: *Fusarium* mycotoxins and *in vitro* studies with intestinal models

Mycotoxin	*Model*	*References*
FB_1	Caco-2 cells	Stevens et al., 1997; Caloni et al., 2002; De Angelis et al., 2005; Kouadio et al., 2005; Kouadio et al., 2007; Fernández-Blanco et al., 2016; Romero et al., 2016.
	Ipec-1	Bouhet et al., 2004; Bouhet and Oswald, 2007; Loiseau et al., 2007
	Ipec-J2	Goossens et al., 2012; Wang et al., 2013
	HT-29	Minervini et al., 2014
	SW742	Mahamoodi et al., 2012
BEA	Caco-2 cells	Fernández-Blanco et al., 2016
	Ipec-1	Springler et al., 2016
DON	Caco-2 cells	Kasuga et al., 1998; Kouadio et al., 2005; Sergent et al., 2005; Manda et al., 2015; Vejdovszkya et al., 2016;
	Ipec-1	Pinton et al., 2009; Pinton et al., 2010; Diesing et al., 2011; Alassane-Kpembi et al., 2015
	Ipec-J2	Gu et al 2014; Broekaert et al, 2016; Gu et al 2016
ZEA	Caco-2 cells	Kouadio et al 2005; Kouadio et al, 2007; Videmann et al 2008; Gao et al., 2016
	Ipec-1	Wan et al, 2013b; Taranu et al, 2014
	Ipec-J2	Goossens et al, 2012; Wan et al., 2013b

2.5 References

Abidin, Z., Khatoon, A. (2012). Ruminal microflora, mycotoxin inactivation by ruminal microflora and conditions favouring mycotoxicosis in ruminants: a review. *International Journal of Veterinary Science, 1: 37-44.*

Alassane-Kpembi, I., Paul, O., Oswald, I.P. (2015). Toxicological interactions between the mycotoxins deoxynivalenol, nivalenol and their acetylated derivatives in intestinal epithelial cells. *Archives of Toxicology, 89: 1337–1346.*

Alberts, J. F., W. C. A. Gelderblom, P. G. Thiel, W. F. O. Marasas, D. J. Van Schalkwyk, and Y. Behrend. (1990). Effects of temperature and incubation period on the production of fumonisin B_1 by *Fusarium* moniliforme. *Applied and Environmental Microbiology. 56:1729–1733.*

Alm, H., Greising, T., Brussow, K.P., Torner, H., Tiemann, U. (2002). The influence of the mycotoxins deoxynivalenol and zearalenol on in vitro maturation of pig oocytes and in vitro culture of pig zygotes. *Toxicology in Vitro, 16: 643–648.*

Anderiesz, C., Ferraretti, A., Magli, C., Fiorentino, A., Fortini, D., Gianaroli, L., Jones, G.M., Trounson, A.O. (2000). Effect of recombinant human gonadotrophins on human, bovine and murine oocyte meiosis, fertilization and embryonic development in vitro. *Human Reproduction, 15: 1140-8.*

Antonissen, G., Martel, A., Pasmans, F., Ducatelle, R., Verbrugghe, E., Vandenbroucke, V., Li, S., Haesebrouck, F., Van Immerseel, F., and Croubels, S. (2014). The Impact of Fusarium Mycotoxins on Human and Animal Host Susceptibility to Infectious Diseases. *Toxins, 6: 430-452.*

Artursson, P., Palm, K. and Luthman. K. (2001). Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Advanced Drug Delivery Reviews, 46:27-43.*

Awad, W.A., Aschenbach, J.R., Zentek. J. (2011). Cytotoxicity and metabolic stress induced by deoxynivalenol in the porcine intestinal IPEC-J2 cell line. *Journal of Animal Physiology and Animal Nutrition, 96: 709–716.*

Baker, D.C., G.E. Rottinghaus. (1999). Chronic experimental fumonisin intoxication of calves. *Journal of Veterinary Diagnostic Investigation, 11:289–292.*

Berschneider, H.M. (1989). Development of normal cultured small intestinal epithelial cell lines which transport Na and Cl. *Gastroenterology, 96: 41.*

Binder, E.M., Tanb, L.M., Chinb, L.J., Handla, J., Richardc, J. (2007). Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. *Animal Feed Science and Technology, 137:265–282.*

Błajet-Kosicka, A., Kosicki, R., Twarużek, M., and Grajewski, J. (2014). Determination of moulds and mycotoxins in dry dog and cat food using liquid chromatography with mass spectrometry and fluorescence detection. *Food Additive and Contaminant Part B, 4: 302–308.*

Böhm, J., Razzazi-Fazeli, E. (2005). Effects of mycotoxins on domestic pet species. *In The Mycotoxin Blue Book; Diaz, D., Ed. Nottingham University Press: Nottingham, U.K., pp 77-91.*

Bouhet, S., Hourcade, E., Loiseau, N., Fikry, A., Martinez, S., Roselli, M., Galtier, P., Mengheri, E., Oswald, I.P. (2004) The mycotoxin fumonisin B1 alters the proliferation and the barrier function of porcine intestinal epithelial cells. *Toxicological Sciences, 77:165–171.*

Bouhet, S., Oswald, I.P. (2007). The intestine as a possible target for fumonisin toxicity. *Molecular nutrition & Food Research, 51: 925–931.*

Boutigny, A.L., Beukes, I., Small, I., Zuhlke, S., Spiteller, M., Van Rensburg, B.J., Flett, B., Viljoen, A. (2012). Quantitative detection of *Fusarium* pathogens and their mycotoxins in South African maize. *Plant pathology, 61: 522-531.*

Broekaert, N., Devreese, M., Demeyere, K., Berthiller, F., Michlmayr, H., Varga, E., Adam, G., Meyera, E., Croubels, S. (2016). Comparative in vitro cytotoxicity of modified deoxynivalenol on porcine intestinal epithelial cells. *Food and Chemical Toxicology, 95: 103–109.*

Caloni, F., Cortinovis, C., Pizzo, F., De Angelis, I. (2012) Transport of Aflatoxin M(1) in Human Intestinal Caco-2/TC7 Cells. *Frontiers in Pharmacology, 3:111.*

Caloni, F., Spotti, M., Pompa, G., Zucco, F., Stammati, A., De Angelis, I. (2002). Evaluation of Fumonisin B1 and its metabolites absorption and toxicity on intestinal cells line Caco-2. *Toxicon, 40: 1181-1188.*

Caloni, F., Stammati, A., Friggè, G., and De Angelis, I. (2006). Aflatoxin M1 absorption and cytotoxicity on human intestinal in vitro model. *Toxicon, 47: 409–415.*

Caloni, F., Spotti, M., Auerbach, H., Op den Camp, H., Fink-Gremmels, J., Pompa, G. (2000). *In vitro* metabolism of fumonisin B_1 by ruminal microflora. *Veterinary Research Communication, 24: 379–387.*

Cavret, S., Lecoeur, S. (2006). Fusariotoxin transfer in animal. *Food and Chemical Toxicology, 44: 444–453.*

Cortinovis, C., Caloni, F., Schreiber, N. B., Spicer. L.J. (2014). Effects of fumonisin B1 alone and combined with deoxynivalenol or zearalenone on porcine granulosa cell proliferation and steroid production. *Theriogenology 81:1042-1049.*

Cotè, L.M., Nicoletti, J., Swanson, S.P., & Buck, W.B. (1986). Production of deepoxydeoxynivalenol (DOM-1), a metabolite of deoxynivalenol, by in vitro rumen incubation. *Journal of agricultural and food chemistry, 34: 458-460.*

Creppy, E.E. (2002). Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicology Letters, 127: 19-28.*

Daia, M., Jianga, S., Yuanb, X., Yanga, W., Yanga, Z., Huanga, L. (2016). Effects of zearalenone-diet on expression of ghrelin and PCNA genes in ovaries of post-weaning piglets. *Animal Reproduction Science, 168: 126–137.*

D'Mello, J.P.F., Placinta, C.M., MacDonald, A.M.C. (1999). *Fusarium* mycotoxins: A review of global implications for animal health, welfare and productivity. *Animal Feed and Science Technology, 80: 183–205.*

De Angelis, I., Frigge, G., Raimondi, F., Stammati, A., Zucco, F., Caloni, F. (2005). Absorption of Fumonisin B1 and aminopentol on an *in vitro* model of intestinal epithelium; the role of P-glycoprotein. *Toxicon, 45: 285–291.*

De Angelis, I., Turco, L. (2011). Caco-2 Cells as a Model for Intestinal Absorption. Curr. Protoc. *Toxicol., 47: 20.6.1-20.6.15.*

Delie, F., Rubas, W. (1997). A human colonic cell line sharing similarities with enterocytes as a model to examine oral absorption: advantages and limitations of the Caco-2 model. *Critical Reviews in Therapeutic Drug Carrier Systems, 14: 221–286.*

Denli, M., Blandon, J.C., Salado, S., Guynot, M.E. and Pérez, J.F. (2017) Effect of dietary zearalenone on the performance, reproduction tract and serum biochemistry in young rats. *Journal of Applied Animal Research, 45: 619-622.*

De Walle, J.V., Sergent, T., Piront, N., Toussaint, O., Schneider, Y.J., Larondelle, Y. (2010). Deoxynivalenol affects in vitro intestinal epithelial cell barrier integrity through inhibition of protein synthesis. *Toxicology and Applied Pharmacology, 245:291–298.*

Dias, F.C.F., Khana, M.I.R., Adams, G.P., Sirard, M.A., Singha, J. (2014). Granulosa cell function and oocyte competence: Super-follicles, super-moms and super-stimulation in cattle. Animal Reproduction Science, 149:80–89.

Diesing, A.K., Nossol, C., Panther, P., Walk, N., Post, A., Kluess, J., Kreutzmann, P., Danicke, S., Rothkotter, H.J., Kahlert, S. (2011) Mycotoxin deoxynivalenol (DON) mediates biphasic cellular response in intestinal porcine epithelial cell lines IPEC-1 and IPEC-J2. *Toxicology Letters, 200:8–18.*

Döll, S., Dãnicke, S., Schnurrbusch, U. (2004). The effect of increasing concentrations of *Fusarium* toxins in piglet diets on histological parameters of the uterus and vagina. *Archive of Animal Nutrition 58:413–417.*

Döll, S., Dänicke, S. (2011). The *Fusarium* toxins deoxynivalenol (DON) and zearalenone (ZON) in animal feeding. *Preventive Veterinary Medicine, 102:132–145.*

EFSA (European Food Safety Authority). (2005). Opinion of the Scientific Panel on Contaminants in Food Chain on a request from the Commission related to fumonisins as undesirable substances in animal feed. *EFSA Journal, 235: 1-32.*

EFSA (European Food Safety Authority). (2014). Scientific Opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed. *EFSA Journal 12:3802.*

Escriva, L., Font, G., Manyes, L. (2015). *In vivo* toxicity studies of fusarium mycotoxins in the last decade: a review. *Food Chemistry and Toxicology, 78:185–206.*

European Commission. (2006). Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding (2006/576/EC). *Official Journal of the European Union L, 229: 7-9.*

Ewuola, E.O., Egbunike, G.N. (2010). Effects of dietary fumonisin B1 on the onset of puberty, semen quality, fertility rates and testicular morphology in male rabbits. *Reproduction, 13: 439-445.*

Ferre, F. S. (2016). Worldwide occurrence of mycotoxins in rice. *Food Control, 62:291–298.*

Fernández-Blanco, C., Frizzell, C., Shannon, M., Ruiz, M., Connolly, L. (2016). An *in vitro* investigation on the cytotoxic and nuclear receptor transcriptional activity of the mycotoxins fumonisin B_1 and beauvericin. *Toxicology Letters 257:1–10.*

Ferruzza, S. , Rossi, C., Scarino, M.-L., Sambuy, Y. (2012). A protocol for in situ enzyme assays to assess the differentiation of human intestinal Caco-2 cells. *Toxicology in Vitro, 26: 1247–1251.*

Fink-Gremmels, J. (1999). Mycotoxins: Their implications for human and animal health. Veterinary Quarterly, *21: 115-120.*

Fink-Gremmels, J. (2008). The role of mycotoxins in the health and performance of dairy cows. *The Veterinary Journal, 176:84–92.*

Fink-Gremmels, J., Malekinejad, H. (2007). Biochemical mechanisms and clinical effects associated with exposure to the mycoestrogen zearalenone. Animal Feed Science and Technology, *137: 326-341.*

Flannigan, B. (1991). Mycotoxins. In: D'Mello, J.P.F., Duffus, C.M., Duffus, J.H. (Eds.), Toxic Substances in Crop Plants. The Royal Society of Chemistry, Cambridge, *pp. 226–257.*

Fogh, J., Fogh, J.M., Orfeo, T. (1977). One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *Journal of Natural Cancer Institutte, 59: 221– 226.*

Gao, Y.N., Wang, J.Q., Lia, S.L., Zhang, Y.D., Zheng, N. (2016). Aflatoxin M1 cytotoxicity against human intestinal Caco-2 cells is enhanced in the presence of other mycotoxins. *Food and Chemical Toxicology, 96: 79–89.*

Garrido, C.E., Pezzani, C.H., Pacin A. (2012). Mycotoxins occurrence in Argentina's maize (Zea mays L.), from 1999 to 2010. *Food Control, 2: 660-665.*

Gbore, F.A., Egbunike, G.N. (2008). Testicular and epididymal sperm reserves and sperm production of pubertal boars fed dietary fumonisin B1. *Animal Reproduction Science, 105: 392-397.*

Gerez, J.R., Desto, S.S., Frederico, A.P., Bracarense, R.L. (2016). Deoxynivalenol induces toxic effects in the ovaries of pigs: an *ex vivo* approach. *Theriogenology, in press. doi.org/10.1016/j.theriogenology.2016.10.023.*

Gilman, J., and Cashman, K.D. (2006). The Effect of Probiotic Bacteria on Transepithelial Calcium Transport and Calcium Uptake in Human Intestinal-like Caco-2 Cells. *Current Issues in Intestinal Microbiology, 7: 1–6.*

Glenn, A.E. (2007). Mycotoxigenic Fusarium species in animal feed. Animal Feed Science and Technology, *137: 213-240.*

Gordon, S., Daneshian, M., Bouwstra, J., Caloni, F., Constant, S., Davies, D.E., Dandekar, G., Guzman, C.A., Fabian, E., Haltner, E., Hartung, T., Hasiwa, N., Hayden, P., Kandarova, H., Khare, S., Krug, H.F., Kneuer, C., Leist, M., Lian, G., Marx, U., Metzger, M., Ott, K., Prieto, P., Roberts, M.S., Roggen, E.L., Tralau, T., Van den Braak, C., Walles, H. and Lehr, C.-M. (2015). Non-Animal Models of Epithelial Barriers (Skin, Intestine and Lung) in Research, Industrial Applications and Regulatory Toxicology. *Altex, 32: 327-378.*

Goossens, J., Pasmans, F., Verbrugghe, E., Vandenbroucke, V., De Baere, S., Meyer, E., Haesebrouck, F., De Backer P., and Croubels, S. (2012). Porcine intestinal epithelial barrier disruption by the Fusariummycotoxins deoxynivalenol and T-2 toxin promotes transepithelial passage of doxycycline and paromomycin. *BMC Veterinary Research, 8:245.*

Goto, Y., & Kiyono, H. (2012) Epithelial barrier: an interface for the cross-communication between gut flora and immune system. *Immunology Reviews, 245:147-163.*

Grajewski, J., Błajet-Kosicka, A., Twarużek, M., Kosicki, R. (2012). Occurrence of mycotoxins in Polish animal feed in years 2006-2009. *Journal of Animal Physiology and Animal Nutrition, 96: 870-877.*

Gu, M.J., Song, S.K., Park, S.M., Lee, I.K., Yun, C.H. (2014). Bacillus subtilis protects porcine intestinal barrier from deoxynivalenol via improved zonula occludens-1 expression. *Asian-Australasian Journal of Animal Sciences, 27:580–586.*

Gu, M.J., Song, S.K., Lee, I.K., Ko, S., Han, S.E., Bae, S., Ji, S.Y., Park, B., Song, K., Lee, H., Han, S.H., and Yun, C. (2016). Barrier protection via Toll-like receptor 2 signaling in porcine intestinal epithelial cells damaged by deoxynivalnol. *Veterinary Research, 47:25.*

Han, J., Wang, Q.C., Zhu, C.-C., Liu, J., Zhang, Y., Ci, X.-S., Kim, N.-H., Sun, S.-C. (2016). Deoxynivalenol exposure induces autophagy/apoptosis and epigenetic modification changes during porcine oocyte maturation. Toxicology and Applied Pharmacology, 300:70–76.

Hidalgo, I.J., Raub, T.J., Borchardt, R.T. (1989). Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology, 96: 736-49.*

Hilgenfeld, R., and Saenger, W. (1982). Structural chemistry of natural and synthetic ionophores and their complexes with cations. *In:Topics in Current Chemistry 101. pp. 1–82. Boschke, F.L., Ed., Springer-Verlag, Berlin.*

Holda, K., Glogowski, R. (2014). A survey of Deoxynivalenol and Zearalenone content in commercial dry foods for growing dogs. *Animal Science, 53:11–117.*

Hussein, H.S., Brasel J.M. (2001).Toxicity, metabolism, and impact of mycotoxins on humans and animals. Toxicology, *167: 101-134.*

IARC. (2002). IARC monographs on the evaluation of carcinogenic risks to humans: some traditional herbal medicines, some mycotoxins, napthalene and styrene. *International Agency for Research on Cancer Press, Lyon, p 82.*

IARC. (2003). Toxins derived from Fusarium moniliforme: Fumonisin B1 and B2 and fusarin C. *Monograph and Evaluation of Carcinogenic Risks to Human, Lyon, France, pp. 445-456.*

Jestoi, M. (2008). Emerging *Fusarium*-mycotoxins fusaproliferin, beauvericin, enniatins, and moniliformin: a review. Critical Reviews in Food and Science Nutrition, *48:21-49.*

Jolly, P.D., Tisdall, D.J., Heath, D.A., Lun, S., and McNatty, K.P. (1994). Apoptosis in Bovine Granulosa Cells in Relation to Steroid Synthesis, Cyclic Adenosine 3',5'-Monophosphate Response to Follicle-Stimulating Hormone and Luteinizing Hormone, and Follicular Atresia. *Biology of reproduction, 51: 934-944.*

Jow, G-M., Chou, C-J., Chen, B-F., Tsai, J-H. (2004) Beauvericin induces cytotoxic eVects in human acute lymphoblastic leukemia cells through cytochrome c release, caspase 3 activation: the causative role of calcium. *Cancer Letter, 216:165–173.*

Kandil, H.H., Argenzio, R.A., Chen, W., Berschneider, H.M., Stiles, A.D., Westwick, J.K., Rippe, R.A., Brenner, D.A., Rhoads, J.M. (1995). l-Glutamine and l-asparagine stimulate ODC activity and proliferation in a porcine jejunal enterocyte line. *American Journal of Physiology, 269: 91–599.*

Khare, S., Tranm Q., Adamsm G. L. (2009). Mycobacterium avium ssp. paratuberculosis infection causes change in the epithelial cells permeability: Correlation with the cell adhesion and tight junction molecule expression. *10th International Colloquium on Paratuberculosis, Minneapolis, Minnesota. 147.*

Kasuga, F., Hara-Kudo, Y., Saito, N., Kumagai, S., Sugita-Konishi, Y. (1998). *In vitro* effect of deoxynivalenol on the differentiation of human colonic cell lines Caco-2 and T84. *Mycopathologia 142:161–167.*

Klaric, M.K., Rumora, L., Ljubanovic, D. (2006). Cytotoxicity and apoptosis induced by fumonisin B(1), beauvericin and ochratoxin A in porcine kidney PK15 cells: effects of individual and combined treatment. *Archives of Toxicology, 82: 247–255.*

Koh, S.Y., George, S., Brözel, V., Moxley, R., Francis, D., and Kaushik, R.D. (2007). Porcine intestinal epithelial cell lines as a new *in vitro* model for studying adherence and pathogenesis of enterotoxigenic Escherichia coli. *Veterinary Microbiology, doi 10.1016/j.vetmic.2007.12.018.*

Kouadio, J.H., Mobio, T.A., Baudrimont, I., Moukha, S., Danoc, S.D., Creppy, E.E. (2005). Comparative study of cytotoxicity and oxidative stress induced by deoxynivalenol, zearalenone or fumonisin B1 in human intestinal cell line Caco-2. *Toxicology, 213: 56–65.*

Kouadio, J.H., Dano, S.D., Moukha, S., Mobio, T.A., Creppy, E.E. (2007). Effects of combinations of Fusarium mycotoxins on the inhibition of macromolecular synthesis, malondialdehyde levels, DNA methylation and fragmentation, and viability in Caco-2 cells. *Toxicon 49:306–317.*

Kwintkiewicz, J., Nishi, Y., Yanase, T., Giudice, L.C. (2010). Peroxisome proliferator-activated receptor-gamma mediates bisphenol A inhibition of FSH-

stimulated IGF-1, aromatase, and estradiol in human granulosa cells. Environmental and Health Perspectives, 118:400–6.

Larsen, J.C., Hunt, J., Perrin, I., Ruckenbauer, P. (2004). Workshop on trichothecenes with a focus on DON: summary report. *Toxicology Letters, 153: 1-22.*

Le Ferrec, E., Chesne, C., Artusson, P. (2001). *In vitro* models of the intestinal barrier. The report and recommendations of ECVAM Workshop 46. European Centre for the Validation of Alternative methods. *Alternatives to Laboratoy Animals, 2: 649-668.*

Leslie, J. F., and Summerell B. A. (2006). The *Fusarium* Laboratory Manual. *Blackwell Publishing, Ames, IA, USA. p 388.*

Li D., Ye Y., Lin S., Deng L., Fan X., Zhang Y., Deng X., Li Y., Yan H., Ma Y. (2014). Evaluation of deoxynivalenol-induced toxic effects on DF-1 cells *in vitro*: Cell-cycle arrest, oxidative stress, and apoptosis. *Environmental Toxicology and Pharmacology, 37: 141-149.*

Liu, M.; Gao, R.; Meng, Q.; Zhang, Y.; Bi, C.; Shan, A. (2013). Toxic effects of maternal zearalenone exposure on intestinal oxidative stress, barrier function, immunological and morphological changes in rats. *PLoS One, 9:106412.*

Liu, Y., Wu, F. (2010) Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. *Environmental Health Perspectives, 118:818–824.*

Loiseau, N., Debrauwer, L., Sambou, T., Bouhet, S., Miller, J.D., Martin, P.G., Viadere, J.L., Pinton, P., Puel, O., Pineau, T., Tulliez, J., Galtier, P., Oswald, I.P. (2007) Fumonisin B1 exposure and its selective effect on porcine jejunal segment: sphingolipids, glycolipids and trans-epithelial passage disturbance. *Biochemistry and Pharmacology, 74:144–152.*

Lu, S., Yao, Y., Meng, S., Cheng, X., Black, D.D. (2002). Overexpression of apolipoprotein A-IV enhances lipid transport in newborn swine intestinal epithelial cells, *The Journal of Biological Chemistry, 277: 31929–31937.*

Mahmoodi, M., Alizadeh, A.M., Sohanaki, H., Rezaei, N., Amini-Najafi, F., Khosravi, A.R., Hosseini, S., Safari, Z., Hydarnasa, D., and Khori, V. (2012). Impact of Fumonisin B1 on the Production of Inflammatory Cytokines by Gastric and Colon Cell Lines. *Iran Journal of Allergy Asthma Immunology, 11: 165-173.*

Malekinejad, H., Schoevers, E.J., Daemen, I.J.J.M., Zijstra, C., Colenbrander, B., Fink-Gremmels, J., Roelen, B.A.J. (2007). Exposure of oocytes to the *Fusarium* toxins zearalenone and deoxynivalenol causes aneuploidy and abnormal embryo development in pigs. *Biology of Reproduction, 77: 840-847.*

Mallebrera, B., Juan-Garcia, A., Font, G., Ruiz, M. (2016). Mechanisms of beauvericin toxicity and antioxidant cellular defense. *Toxicology Letters 246:28–34.*

Manda, G., Mocanu, M.A., Marin, D.E., Taranu, I. (2015). Dual effects exerted in vitro by micromolar concentrations of deoxynivalenol on undifferentiated caco-2 cells. *Toxins (Basel) 7:593–603.*

Marasas, W. F. O., Nelson, P. E., and Toussoun, T. A. (1984). Toxigenic Fusarium species: identity and mycotoxicology. *The Pennsylvania State University Press, University Park.*

Marasas, W.F.O., Kellerman, T.S., Gelederblom, W.C.A., Coetzer, J. A. W., Thievi, P.G., and Vander Lugt, J.J. (1988). Leukoencephalomacia in a horse induced by fumonisin B1 isolated from *Fusarium* Monoliforme. *Onderstepoort Journal of Veterinary Research, 55: 197-203.*

Marasas, W.F.O., Riley, R.T., Hendricks, K.A., Stevens, V.L., Sadler, T.W., Gelineau-van Waes, J., Missmer, S.A., Cabrera, J., Torres, O., Gelderblom, W.C.A., Allegood, J., Martìnez, C., Maddox, J., Miller, J.D., Starr, L., Sullards, M., Roman, A.V., Voss, K.A., Wang, E., Merrill, A.H. (2004). Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and *in vivo*: a potential risk factor for human neural tube defects among populations consuming fumonisin- contaminated maize. *Journal of Nutrition, 134:711-716.*

Maresca, M. (2013). From the gut to the brain: journey and pathophysiological effects of the food-associated trichothecene mycotoxin deoxynivalenol. *Toxins 5:784e820.*

Marin, D.E., Motiu, M., Taranu, I. (2015). Food Contaminant Zearalenone and Its Metabolites Affect Cytokine Synthesis and Intestinal Epithelial Integrity of Porcine Cells. *Toxins, 7: 1979-1988.*

Martel, F., Grundemann, D., Calhau, C., Schomig, E. (2001). Apical uptake of organic cations by human intestinal Caco-2 cells: putative involvement of ASF transporters. *N-S Archives Pharmacology, 363: 40–49.*

Mathur, S., P.D. Constable, R.M. Eppley, A.L. Waggoner, M.E. Tumbleson, W.M. Haschek. (2001). Fumonisin B(1) is hepatotoxic and nephrotoxic in milk-fed calves. *Toxicological Sciences, 60:385–396.*

McCall, I. C., Betanzos, A., Weber, D. A. et al. (2009). Effects of phenol on barrier function of a human intestinal epithelial cell line correlate with altered tight junction protein localization. *Toxicology and Applied Pharmacology, 241: 61-70.*

McCool, D. J., Marcon, M. A., Forstner, J. F. et al. (1990). The T84 human colonic adenocarcinoma cell line produces mucin in culture and releases it in response to various secretagogues. *Biochemistry Journal, 267: 491-500.*

Meca, G., Font, G., Ruiz, M.J. (2011). Comparative cytotoxicity study of enniatins A, A1, A2, B, B1, B4 and J3 on Caco-2 cells, Hep-G2 and HT-29. *Food Chemistry and Toxicology 49:2464–2469.*

Medvedova, M. Kolesarova, A. Capcarova, M. Labuda, R. Sirotkin, A.V. Kovacik, J. Bulla J. (2011). The effect of deoxynivalenol on the secretion activity, proliferation and apoptosis of porcine ovarian granulosa cells *in vitro. Journal of Environmental Science and Health, Part B, 46:213–219.*

Merrill, Jr, A.H., Sullards, M.C., Wang, E., Voss, K.A., Riley, R.T. (2001). Sphingolipid metabolism: roles in signal transduction and disruption by fumonisins. *Environmental Health Perspectives, 109:283-289.*

Minervini, F., Dell'Aquila, M.E. (2008). Zearalenone and Reproductive Function in Farm Animals. *International Journal of Molecular Sciences, 9: 2570-2584.*

Minervini, F., Giannoccaro, A., Fornelli, F., Dell' Aquila, M.E., Minoia, P., Visconti, A. (2006). Influence of mycotoxin zearalenone and its derivatives (alpha and beta zearalenol) on apoptosis and proliferation of cultured granulosa cells from equine ovaries. *Reproduction Biology and Endocrinology, 4:62.*

Minervini, F., Lacalandra, G.M., Filannino, A., Garbetta, A., Nicassio, M., Dell'Aquila, M.E., Visconti, A. (2010). Toxic effects induced by mycotoxin fumonisin B1 on equine spermatozoa: assessment of viability, sperm chromatin structure stability, ROS production and motility. *Toxicology in Vitro, 24: 2072-2078.*

Minervini, F. Garbetta, A., D'Antuono, I., Cardinali, A., Antonio, N., Lucantonio, M., Debellis, Visconti, A. (2014). Toxic Mechanisms Induced by Fumonisin B1 Mycotoxin on Human Intestinal Cell Line. *Archives of Environmental Contamination and Toxicology, 67: 115–123.*

Moliné, A., Faucet, V., Castegnaro, M., Pfohl-Leszkowicz, A. (2005) Analysis of some breakfast cereals on the French market for their contents of ochratoxin A, citrinin and fumonisin B1: development of a method for simultaneous extraction of ochratoxin A and citrinin. *Food Chemistry, 92: 391–400.*

Newman, K.E., Raymond, S.L. (2005). Effects of mycotoxins in horses. In: Diaz D.E. (Ed.), *The Mycotoxin Blue Book, Nottingham University Press, Nottingham, UK, pp. 57-76.*

Nigsch, F., Klaffe, W., and Miret. S. (2007). *In vitro* models for processes involved in intestinal absorption. *Expert Opinion on Drugs Metabolism Toxicology, 3:545-556.*

Ogunlade, J.T., Ewuola, E.O., Gbore, F.A., Bandyopadhyay, R., Niezen, J., Egbunike, G.N. (2006). Testicular and epididymal sperm reserves of rabbits fed fumonisin contaminated diets. *World Applied Sciences Journal, 1:35–8.*

Osweiler, G.D. (2000). Mycotoxins-contemporary issues of food animal health and productivity. Veterinary Clinic of North America, *16:511e530.*

Osweiler, G.D., M.E. Kehrli, J.R. Stabel, J.R. Thurston, P.F. Ross and T.M. Wilson. (1993). Eject of fumonisin-contaminated corn screenings on growth and health of feeder calves. *Journal of Animal Science, 71:459-466.*

Ou, G., Baranov, V., Lundmark, E. et al. (2009). Contribution of intestinal epithelial cells to innate immunity of the human gut – studies on polarized monolayers of colon carcinoma cells. *Scandinavian Journal of Immunology, 69: 150-161.*

Pestka, J.J. (2007). Deoxynivalenol: Toxicity, mechanisms and animal health risks. *Animal Feed Science and Technology, 137: 283-298.*

Pestka, J.J. (2010). Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. *Archives of Toxicology, 84: 663-679.*

Pestka, J.J., Smolinski, A.T. (2005). Deoxynivalenol: Toxicology and Potential Effects on Humans. Journal of Toxicology and Environmental Health, Part B, *8: 39- 69.*

Petro, E.M.L., Leroy J.L.M.R., Van Cruchten S.J.M., Covaci A., Jorssen E.P.A., Bols P.E.J. (2012). Endocrine disruptors and female fertility: Focus on (bovine) ovarian follicular physiology. *Theriogenology 78: 1887-1900.*

Pinto, M., Robine-Leon, S., Appay, M.D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, P., Haffen, K.J., Fogh, J., and Zweibaum, A. (1983). Enterocytelike differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biology Cell, 47:323-330.*

Pinton, P., Nougayrède, J.P., Del Rio, J.C., Moreno, C., Marin, D.E., Ferrier, L., Bracarense, A.P., Kolf-Clauw, M., Oswald, I.P. (2009). The food contaminant deoxynivalenol, decreases intestinal barrier permeability and reduces claudin expression. *Toxicology and Applied Pharmacology, 237: 41–48.*

Pinton, P., Braicu, C., Nougayrede, J.P., Laffitte, J., Taranu, I., Oswald, I.P. (2010). Deoxynivalenol impairs porcine intestinal barrier function and decreases the protein expression of claudin-4 through a mitogen-activated protein kinase-dependent mechanism. *Journal of Nutrition 140:1956–1962.*

Pinton, P., Oswald, I.P. (2014). Effect of Deoxynivalenol and Other Type B Trichothecenes on the Intestine: A Review. Toxins, *6: 1615-1643.*

Pizzo, F., Caloni, F., Schutz, L.F., Totty, M.L., Spicer, L.J. (2015). Individual and combined effects of deoxynivalenol and a-zearalenol on cell proliferation and steroidogenesis of granulosa cells in cattle. *Environmental Toxicology and Pharmacology 40:722e728.*

Pizzo, F., Caloni, F., Schreiber, N.B., Cortinovis, C., Spicer, L.J. (2016). *In vitro* effects of deoxynivalenol and zearalenone major metabolites alone and combined, on cell proliferation, steroid production and gene expression in bovine small-follicle granulosa cells. *Toxicon 109:70e83.*

Price, W.D., Lowell, R.A., McChesney, D.G. (1993). Naturally occurring toxins in feedstuffs. *Journal of Animal Science, 71: 2556–2562.*

Prosperini, A., Meca, G., Font, G., Ruiz, M.J. (2012). Study of the cytotoxic activity of beauvericin and fusaproliferin and bioavailability in vitro on Caco-2 cells. *Food Chemical and Toxicology 50:2356-2361.*

Raffatellu, M., Wilson, R.P., Chessa, D. (2005). SipA, SopA, SopB, SopD, and SopE2 contribute to Salmonella enterica serotype Typhimurium invasion of epithelial cells. *Infection and Immunity, 73: 146-154.*

Ranaldi, G., Consalvo, R., Sambuy, Y., Scarino, M.L. (2003). Permeability characteristics of parental and clonal human intestinal Caco-2 cell lines differentiated in serum-supplemented and serum-free media. *Toxicology in Vitro, 17: 761–767.*

Ranzenigo, G., Caloni, F., Cremonesi, F., Aad, P.Y., Spicer, L.J. (2008). Effects of *Fusarium* mycotoxins on steroid production by porcine granulosa cells. *Animal Reproduction Science, 107:115–130.*

Rhoads, J.M., Argenzio, R.A., Chen, W., Rippe, R.A., Westwick, J.K., Cox, A.D., Berschneider, H.M., Brenner, D.A. (1997). l-Glutamine stimulates intestinal cell proliferation and activates mitogen-activated protein kinases. *American Journal of Physiology, 272:943–953.*

Rodrigues, I., Naehrer, K. (2012). A three-years survey on the worldwide occurrences of mycotoxins in feedstuffs and feed. *Toxins, 4: 663-675.*

Romero, A., Ares, I., Ramos, E., Castellano, V., Martinez, M., Martinez-Larranaga, M.R., Anadon, A., Martinez, M.A. (2016) Mycotoxins modify the barrier function of Caco-2 cells through differential gene expression of specific claudin isoforms: protective effect of illite mineral clay. *Toxicology 353–354:21–33.*

Ross, P.F., Ledet, A.E., Owens, D.L., Rice, L.G., Nelson, H.A., Osweiler, G.D., Wilson, T.M. (1993). Experimental equine leukoencephalomalacia, toxic hepatosis, and encephalopathy caused by corn naturally contaminated with fumonisins. *Journal of Veterinary Diagnostic Investigation, 5:69-74.*

Ruiz, M.J., Macáková, P. Juan García, A., Font, G. (2011). Cytotoxic effects of mycotoxin combinations in mammalian kidney cells. *Food and Chemical Toxicology 49:2718–2724.*

Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M.L., Stammati, A., and Zucco, F. (2005). The Caco-2 cell line as a model of the intestinal barrier: Influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biololgy and Toxicology, 21:1-26.*

Scaramuzzi, R.J., Baird, D.T., Campbell, B.K., Driancourt, M.A., Dupont, J., Fortune, J.E., et al. (2011). Regulation of folliculogenesis and the determination of ovulation rate in ruminants. *Reproduction Fertility and Development, 23:444–67*.

Schierack, P., Nordhoff, M., Pollmann, M., Weyrauch, K. D., Amasheh, S., Lodemann, U., Jores, J., Tachu, B., Kleta, S., Blikslager, A., Tedin, K., and Wieler, L.H. (2006). Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine, Histochem. *The Journal of Cell Biology, 125: 293–305*.

Schoevers, E.J., Fink-Gremmel, J., Colenbrander, B., Roelen, B.A.J. (2010). Porcine oocytes are most vulnerable to the mycotoxin deoxynivalenol during formation of the meiotic spindle. *Theriogenology, 74:968–978*.

Schoevers, E.J., Santos, R.R., Fink-Gremmels, J., Roelen, B.A.J. (2016). Toxicity of beauvericin on porcine oocyte maturation and preimplantation embryo development. *Reproductive Toxicology, 65: 159–169*.

Schollenberger, M., Muller, H.M., Rufle, M., Terry Jara, H., Suchy, S., Plank, S., Drochner,W. (2007). Natural occurrence of *Fusarium* toxins in soy food marketed in Germany. International Journal of Food and Microbiology, *113:142-146*.

Seeling, K., Danicke, S., Valenta, H., Van Egmond, H.P., Schothorst, R.C., Jekel, A.A., Lebzien, P., Schollenberger, M., Razzazi Fazeli, E., Flachowsky, G. (2006). Effects of *Fusarium* toxin contaminated wheat and feed intake level on the biotrasformation and carryover of deoxynivalenol in dairy cows. *Food Addititives and Contaminants 23:1008e1020*.

Sergent, T., Parys, M., Garsou, S., Pussemier, L., Schneider, Y., Larondelle, Y. (2005). Deoxynivalenol transport across human intestinal Caco-2 cells and its effects on cellular metabolism at realistic intestinal concentrations. *Toxicology Letters, 164: 167–176*.

Smith, G.W. (2012). Fumonisins. In: Gupta R.C. (Ed.), Veterinary Toxicology: Basic and Clinical Principles (second edition), *Elsevier Inc., pp. 1205-1219*.

Sobrova, P., Adam, V., Vasatkova, A., Beklova, M., Zeman, L., Kizek, R. (2010). Deoxynivalenol and its toxicity. *Interdisciplinar Toxicology, 3: 94-99*.

Speijers, G.J.A., Speijers, M.H.M. (2004). Combined toxic effects of mycotoxins. *Toxicology Letters 153:91–98.*

Springler, A., Vrubel, G., Mayer, E., Schatzmayr, G., and Novak, B. (2016). Effect of Fusarium-Derived Metabolites on the Barrier Integrity of Differentiated Intestinal Porcine Epithelial Cells (IPEC-J2). *Toxins, 8: 345.*

Stevens, V.L., and Tang, J. (1997). Fumonisin B1-induced Sphingolipid Depletion Inhibits Vitamin Uptake via the Glycosylphosphatidylinositol-anchored Folate Receptor. *The Journal of Biological Chemistry, 272: 18020–18025.*

Streit, E., Schatzmayr, G., Tassis, P., Tzika, E., Marin, D., Taranu, I., Tabuc, C., Nicolau, A., Aprodu, I., Puel, O., Oswald, I.P. (2012). Current Situation of Mycotoxin Contamination and Co-occurrence in Animal Feed—Focus on Europe. *Toxins 2012, 4:, 788-809.*

Sun, D., Hans Lennernas, H., Welage, L.S., Barnett, J.L., Landowski, C.P., Foster, D., Fleisher, D., Lee, K.D., and Amidon, G.L. (2002). Comparison of human duodenum and Caco-2 gene expression profiles for 12,000 gene sequences tags and correlation with permeability of 26 drugs. *Pharmaceutical Research, 19:1400-1416.*

Sydenham, E.W., Thiel, P.G., Marasas, W.F.O., Shephard, G.S., Van Schalkwyk, D.J., Koch, K.R. (1990) Natural occurrence of some Fusarium mycotoxins in corn from low and high esophageal cancer prevalence areas of the Transkei, Southern Africa. *Journal of Agricultural and Food Chemistry 38:1900–1903.*

Sweeney, M.J., Dobson, A.D.W. (1998). Mycotoxin production by *Aspergillus*, *Fusarium* and *Penicillium* species. International Journal of Food Microbiology, *43: 141-158.*

Taranu, I., Braicu, C., Marin, D.E., Pistol, G.C., Motiu, M., Balacescu, L., Beridan Neagoe, I.,Burlacu, R. (2014). Exposure to zearalenone mycotoxin alters in vitro porcine intestinal epithelial cells by differential gene expression. *Toxicology Letters, 232: 310–325.*

Thompson, W.L., Wannemacher, R.W. Jr. (1990). *In vivo* effects of T-2 toxin on synthesis of proteins and DNA in rat tissues. *Toxicology Application and Pharmacology 105, 482–491.*

Tiemann, U., Tomek, W., Schneider, F., Vanselow, J. (2003). Effects of the mycotoxins α- and β-zearalenol on regulation of progesterone synthesis in cultured granulosa cells from porcine ovaries. *Reproductive Toxicology, 17: 673–681.*

Tiemann, U., Dänicke, S. (2007). *In vivo* and *in vitro* effects of the mycotoxins zearalenone and deoxynivalenol on different non-reproductive and reproductive organs in female pigs: a review. *Food Additives and Contaminants, 24: 306-314.*

Tran, Q.T., Gomez, G., Khare, S. (2010). The Salmonella enterica serotype Typhi Vi capsular antigen is expressed after the bacterium enters the ileal mucosa. *Infection and Immunology, 78: 527-535.*

Turco, L., Catone, T., Caloni, F. (2011). Caco-2/TC7 cell line characterization for intestinal absorption: How reliable is this in vitro model for the prediction of the oral dose fraction absorbed in human?. *Toxicology In Vitro, 25: 13-20.*

Turner, N.W., Subrahmanyamb, S., Piletskyb, S.A. (2009). Analytical methods for determination of mycotoxins: A review. *Analytica Chimica Acta, 632: 168-180.*

Uhlig, S., M. Jestoi, and Parikka, P. (2007). *Fusarium avenaceum*—the North European situation. International Journal of Food and Microbiology, *119:17–24.*

Upadhaya, S.D., Park, M.A., Ha, J.K. (2010). Mycotoxins and their biotrasformation in the rumen: a review Asian-Aust. *Journal of Animal Science, 23: 1250–1260.*

Van Breemen, R.B., and Li., Y. (2005). Caco-2 cell permeability assays to measure drug absorption. *Expert Opinion on Drugs Metabolism Toxicology 1:175-185.*

Vandenbroucke, V., Croubels, S., Martel, A., Verbrugghe, E., Goossens, J., Van Deun, K., Boyen, F., Thompson, A., Shearer, N., De Backer, P., Haesebrouck, F., Pasmans, F. (2011). The Mycotoxin Deoxynivalenol Potentiates Intestinal Inflammation by Salmonella Typhimurium in Porcine Ileal Loops, *Plosone, doi.org/10.1371/journal.pone.0023871.*

Van de Walle J., Sergent T., Piront N., Toussaint O., Schneider Y.-J., Larondelle Y. (2010). Deoxynivalenol affects in vitro intestinal epithelial cell barrier integrity through inhibition of protein synthesis. *Toxicology Application and Pharmacology*, *245: 291–298*.

Vejdovszkya, K., Warth, B., Sulyok, M., Marko, D. (2016). Non-synergistic cytotoxic effects of Fusarium and Alternaria toxin combinations in Caco-2 cells. *Toxicology Letters, 241: 1–8.*

Videmann, B., Mazallon, M., Tep, J., Lecoeur, S. (2008). Metabolism and transfer of the mycotoxin zearalenone in human intestinal Caco-2 cells. *Food and Chemical Toxicology, 46: 3279–3286.*

Voss, K.A., Howard, P.C., Riley, R.T., Sharma, R.P., Bucci, T.J., Lorentzen, R.J. (2002). Carcinogenicity and mechanism of action of fumonisin B1: a mycotoxin produced by Fusarium moniliforme (= F. verticillioides). *Cancer Detection and Prevention, 26: 1–9.*

Voss, K.A., Smith, G.W., Haschek, W.M. (2007). Fumonisins: Toxicokinetics, mechanism of action and toxicity. *Animal Feed Science and Technology, 137: 299-325.*

Wan, L.Y.M., Turner, P.C., El-Nezami, H. (2013a). Individual and combined cytotoxic effects of Fusarium toxins (deoxynivalenol, nivalenol, zearalenone and fumonisins B1) on swine jejunal epithelial cells. *Food and Chemical Toxicology, 57: 276–283.*

Wan, L.Y.M., Woo, C.J., Allen, K.J., Turner, P.C., and El-Nezami, H. (2013b). Modulation of Porcine β-Defensins 1 and 2 upon Individual and Combined *Fusarium* Toxin Exposure in a Swine Jejunal Epithelial Cell Line. *Applied and Environmental Microbiology, 79: 2225–2232.*

Wang, E., W.P. Norred, C.W. Bacon, R.T. Riley, A.H. Merrill Jr. (1991). Inhibition of sphingolipid biosynthesis by fumonisins. Implications for diseases associated with Fusarium moniliforme. *The Journal of Biological Chemistry, 266: 14486-14490.*

Wang, E., Ross, P.F., Wilson, T.M., Riley, R.T., Merrill, A.H. (1992). Increases in serum sphingosine and sphinganine and decreases in complex sphingolipids in ponies given feed containing fumonisins, mycotoxins produced by *Fusarium moniliforme*. *Journal of Nutrition, 122:1706-1716.*

Ward, J. L., Tse, C. M. (1999). Nucleoside transport in hu¬man colonic epithelial cell lines: Evidence for two Na+-inde¬pendent transport systems in T84 and Caco-2 cells. *Biochimica et Biophysica Acta, 1419: 15-22.*

Winkler, J., Kersten, S., Meyer, U., Engelhardt, U., D€anicke, S. (2014). Residues of zearalenone (ZEN), deoxynivalenol (DON) and their metabolites in plasma of dairy cows fed Fusarium contaminated maize and their relationships to performance parameters. *Food Chemistry and Toxicology, 65: 196e204.*

Yang, Z. (2013). The roles of membrane transporters on the oral drug absorption. *Journal of Molecular Pharmaceutics & Organic Process Research, 1:102.*

Yamascita, S., Furubayashi, T., Kataoka, M., Sakane, T., Sezaki, H., and Tokuda, H. (2000). Optimized conditions for prediction of intestinal drug permeability using Caco-2 cells. *European Journal of Pharmacology Science, 10:195-204.*

Yazar, S., Omurtag, G.Z. (2008). Fumonisins, Trichothecenes and Zearalenone in Cereals. International Journal of Molecular Sciences, *9: 2062-2090.*

Yee, S. (1997). *In vitro* permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man: Fact or myth. *Pharmacology Research, 14:763-766.*

Yoshinari, T., Suzuki, Y., Sugita-Konishi, Y., Ohnishi, T., Terajima, J. (2016). Occurrence of beauvericin and enniatins in wheat flour and corn grits on the Japanese market, and their co-contamination with type B trichothecene mycotoxins. *Food Additives & Contaminants: Part A Vol. 33 , Iss. 10,2016*

Young, L.G., King, G.J. (1986). Low concentrations of zearalenone in diets of mature gilts. *Journal of Animal Sciences, 63: 1191-6.*

Zhu, L., Yuan, H., Guo, C., Lu, Y., Deng, S., Yang, Y., Wei, Q., Wen, H. (2012). Zearalenone induces apoptosis and necrosis in porcine granulosa cells via a caspase-3- and caspase-9-dependent mitochondrial signaling pathway. *Journal of Cell Physiology, 227: 1814–1820.*

Zinedine, A, Soriano, J.M., Moltò, J.C., Manes, J. (2007). Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: An oestrogenic mycotoxin. *Food and Chemical Toxicology, 45: 1-18.*

3. *In vitro* effects of fumonisin B_1 alone and combined with deoxynivalenol, α-zearalenone, β-zearalenone and beauvericin on bovine granulosa

3.1 Introduction

Mycotoxins, secondary metabolites produced by moulds, frequently occur in food and feed (Oswald et al 2005; Schoelleberger et al., 2007) and a worldwide contamination is related to *Fusarium* mycotoxins (Scott, 1997; Placinta et al., 1999; Schollenberger et al., 2007; Lombaert et al., 2003).

Fusarium mycotoxins can be classified in two groups: i) the "traditional" mycotoxins including fumonisins, trichothecenes and zearalenone (ZEA) and ii) the so-called "emerging" mycotoxins such as beauvericin (BEA), fusaproliferin, enniatins (ENNs) and moniliformin (Jestoi, 2008).

Among the fumonisins, fumonisin B_1 (FB_1) is considered the main toxin in terms of toxicity (Rodrigues and Naehrer, 2012) and its co-occurrence with other *Fusarium* mycotoxins is well established (EFSA, 2005; Voss et al., 2007). Leukoencephalomalacia in horses (Kellerman et al., 1990; Raymond et al., 2005), pulmonary edema in pigs (Harrison et al., 1990; Tiemann and Danick, 2007) and nephropathy in rabbits (Gumprecht et al., 1995; Wangikara et al., 2005) are species-specific effects related to the exposure to FB_1. Cattle seem less sensitive than other animals such as horses or pigs to FB_1 (Osweiler et al., 1993; Mathur et al., 2001; Tiemann and Danick, 2007; Fink-Gremmels, 2008).

The complex mechanism of action of FB_1 (Wang et al., 1991; Voss et al., 2002; Voss et al., 2007; Gbore et al., 2012) is based on the inhibition of ceramide synthase (Wang et al., 1991; Voss et al., 2007; Luongo et al. 2008) with the alteration of the sphingolipid metabolism (Wang et al., 1991; Wang et al; 1992; Merrill et al., 2001; Marasas et al., 2004; Voss et al., 2007; Smith, 2012).

In relation to reproductive effects, no data are available on cattle, while *in vitro* studies demonstrated that FB_1 also combined with DON or α-ZEA, affects porcine granulosa cells (GC) (Cortinovis et al., 2014).

Among trichothecenes, DON, also called vomitoxin for its emetic effects, is one of the most important and widespread in cereal grains and animal feed (Rotter et al., 1996). The co-occurrence of DON with other mycotoxins such as ZEA is a common feature of several studies (Richard, 2007; Pinton and Oswald, 2014).

Feed refusal was observed in pigs and in cattle (Trenholm et al., 1984; Dersjant-Li et al., 2003; Akande et al., 2006; Rodrigues and Naehrer, 2012) after exposure to DON.

Reproductive disorders have also been reported in livestock (Diekman and Green, 1992), but little is known about DON effects on ruminant reproduction (Seeling et al., 2006).

ZEA is a non-steroidal estrogenic *Fusarium* mycotoxin known to contaminate various crops, especially corn, wheat, barley and oats (Cheeke, 1998; Zinedine et al., 2007;

Ferrigo et al., 2016). ZEA is able to bind to cytosolic estrogen receptors in target cells (Riley and Norred, 1996) and acts like endocrine disruptor with estrogenic effects (Frizzell et al., 2011). Its activity is related to the transcription of estrogen-responsive genes and consequently to the modulation of the translation of new proteins and the expression of estrogenic effects upon the target cells (Parveen et al., 2009).
The hyperstimulation of estrogen-dependent tissues is the main effect of ZEA exposure (Böhm and Razzai-Fazeli, 2005; Malekinejad et al., 2007). The α-ZEA metabolite seems to have an higher binding affinity to estrogen receptors compared to ZEA and β-ZEA metabolite (Fink-Gremmels, 2008).
As reported for FB_1, cattle are considered to be less susceptible than other domestic animals to the adverse effects of ZEA (Upadhaya et al., 2010). In rumen ZEA is converted into α-ZEA and β-ZEA (Abidin and Khatoon, 2012; Winkler et al., 2014) and the rate of absorption of the more polar α-ZEA is poor and hyprestrogenism is infrequently observed in cows. Study conducted on liver microsomes demonstrated that the ratio between α-ZEA and β-ZEA formation varies among animal species and that in ruminants, because ZEA undergoes hepatic metabolism, β-ZEA seems to prevail as results of hepatic metabolism, whereas α-ZEA is the most frequent metabolite in pigs (Malekinejad et al., 2007; Fink-Gremmels, 2008). ZEA and its metabolites exert their toxicity by binding to estrogens receptors and the clinical signs of hyperestrogenism in ruminants are generally observed after long-term exposure to ZEA and after ingestion of highly contaminated feed (Fink-Gremmels, 2008; Upadhaya et al., 2010). ZEA exposure has also been linked to infertility, reduction in milk production, inhibition of oocytes maturation to metaphase II and an alteration of steroidogenesis in cattle (Minervini and Dell'Aquila, 2008; Pizzo et al., 2016).
BEA is a cyclic hexadepsipeptide that was first isolated from the culture of the soil-borne entomopathogenic fungus *Beauveria bassiana* (Leslie and Summerell, 2006). BEA is also synthesized by several *Fusarium* spp. parasitic to important cereal grains including corn, wheat, rice and barley (Leslie and Summerell, 2006). High contamination levels, up to 500 mg/kg of BEA, detected in surveys carried out across Europe are raising serious concerns about the potential impact of BEA on animal and human health (Uhlig et al., 2007; Jestoi, 2008). Few data are available on BEA toxicity so far (EFSA, 2014). BEA proved to exert potent cytotoxicity against several mammalian cell lines (Jestoi, 2008; Klarić et al, 2008; Ferrer et al, 2009; Ruiz et al., 2011; Prosperini et al., 2012; Mallebrera et al., 2016) and this effect seems to be related to its ionophoric properties (EFSA, 2014). Accordingly, BEA is able to promote the transport of cations, such as calcium, through the membranes disturbing their normal physiological concentrations in the cell and thus affecting ionic homeostasis (Jestoi, 2008).
To date information on the effects of FB_1 alone or combined with other *Fusarium* mycotoxins on reproduction in cattle is lacking.

In the present study primary bovine GC, which are considered a reliable *in vitro* model for reproductive toxicological research (Petro et al., 2012), were used to evaluate the individual and combined effects of FB_1 with DON, α-ZEA, β-ZEA and BEA on cell proliferation, steroid production and gene expression.

3.2 Materials and methods

3.2.1 Reagents and hormones

Reagents were: Dulbecco's Modified Eagle Medium (DMEM), Ham's F12, Fumonisin B_1 (FB_1), Deoxynivalenol (DON), α-Zearalenol (α-ZEA) β-Zearalenol (β-ZEA) and Beauvericin (BEA) obtained from Sigma Chemical Co. (St. Louis, MO); fetal calf serum (FCS) obtained from Atlanta Biologicals (Flowery Branch, GA); purified ovine follicle stimulating hormone (FSH; FSH activity, 15 NIH-FSH-S1 U/mg) obtained from Dr. A. F. Parlow, National Hormone and Pituitary Program (Torrance, CA); recombinant human insulin-like growth factor 1 (IGF1) obtained from R&D Systems (Minneapolis, MN); and testosterone obtained from Steraloids (Wilton, NH).

3.2.2.Cell culture

Ovaries from non-pregnant beef cows were collected from a slaughterhouse as previously described (Langhout et al., 1991; Lagaly et al., 2008). Based on surface diameter small follicles (1-5 mm) were aspirated (Fig. 3.1) and GC were recovered from follicular fluid by centrifugation (291 x *g* for 10 min). GC were washed three times with 7 mL of serum-free medium and resuspended in 2 mL of enzyme containing medium (0.5 mg/mL of DNase and 1.25 mg/mL of collagenase) to prevent clumping of cells as previously described (Spicer et al., 2002; Lagaly et al., 2008). Numbers of viable cells were determined using the trypan blue exclusion method (Langhout et al., 1991; Spicer et al., 1993; Tiemann et al., 2003). Viable cells (2.5×10^5 in 20-80 μL of medium) were plated in 24-well Falcon multiwell plates (Becton Dickinson, Lincoln Park, NJ, USA) in 1 mL of basal medium composed of a mixture of 1:1 DMEM and Ham's F-12 containing glutamine (2 mM), gentamicin (0.12 mM) and sodium bicarbonate (38.5 mM). Cultures were kept at 38.5 °C in a humidified 95% air and 5% CO_2 environment and medium was changed every 24 h. To obtain an optimal attachment, cells were maintained in the presence of 10% FCS for the first 48 h of culture. After this time, GC were washed twice with serum-free medium (0.5 mL) and the various treatments applied

in serum-free medium containing 500 ng/mL of testosterone (as an estradiol precursor) for 48 h with a medium change after 24 h.

Figure 3.1: Collection of granulosa cells from small bovine follicles (1-5 mm) via needle aspiration.

3.2.3 Determination of GC numbers

Medium was collected from individual wells and frozen at -20 °C for subsequent steroid analyses. Numbers of GC, in the same wells from which medium was collected, were determined by a Coulter counter (model Z2; Beckman Coulter, Inc., Hialeah, FL) (Fig. 3.2) as previously described (Lagaly et al., 2008), and used to calculate steroid production on ng or pg per 10^5 cell basis. Briefly, cells were gently washed twice with 0.9% saline solution (500 mL), exposed to 500 mL of trypsin (0.25% wt/vol; 2.5 mg/mL) for 20 min at room temperature, and then scraped from each well and enumerated as previously described (Langhout et al., 1991; Lagaly et al., 2008).

Figure 3.2: Determination of granulosa cell numbers using a Z2 Coulter® Particle Count and Size Analyzer.

3.2.4 Determination of steroid concentrations

Concentrations of progesterone (P4) and estradiol (E2) in culture medium were determined by radioimmunoassay (RIA) as previously described (Spicer and Chamberlain, 1998; Lagaly et al., 2008). The intra- and inter-assay coefficients of variation were 7% and 13%, respectively for the P4 RIA, and 8% and 17%, respectively for the E2 RIA. P4 production was evaluated because it, like E2 production, increases as GC undergo differentiation (Hsueh et al., 1984; Ainsworth et al., 1990).

3.2.5 Progesterone RIA

Progesterone RIA were conducted using rabbit antiserum (X-16), which serves as the first antibody (diluted 1:3000 with assay buffer: PBS, EDTA, NaN3, and gelatin), raised against BSA-11 glutamate derivative as described by Baraño and Hammond (1985). Goat anti-rabbit antibody (diluted 1:15 with assay buffer) was used as the second antibody (Linco Research, Inc., St. Charles, MO). [125I]Iodo-progesterone (ICN Biomedicals, Costa Mesa, CA) was used as the tracer. A progesterone standard curve was prepared from a stock concentration of 80.0 ng/mL that was serially diluted with assay buffer to concentrations of 40.0, 20.0, 10.0, 5.0, 2.5, 1.25, 0.625, 0.31, and 0.16 ng/mL. In duplicate, 20 μL to 100 μL of medium samples were combined with the appropriate volume of assay buffer to make a total volume of 100 μL. One hundred μL

of tracer and first antibody were added and all samples were mixed and allowed to incubate at 37 °C for 1 h. Following incubation, 200 μL of second antibody were added and all samples were incubated overnight at 4 °C. The following day, 50 μL of normal rabbit serum (NRS) (diluted 1:5 with assay buffer from a 15% NRS stock) were added to all samples. Samples were centrifuged at 4 °C in a Sorvall Model RC-3 (Thermo Fisher Scientific, Inc., Miami, OK) at 1800 x *g* for 25 min. Supernatant was aspirated and precipitates were counted for 1 min using a Cobra AII Auto-Gamma counter (Packard Instrument Co., Downers Grove, IL). The intra- and interassay coefficients of variation were 7 and 13%, respectively, for the progesterone RIA.

3.2.6 Estradiol RIA

Estradiol RIA were conducted using anti-estradiol rabbit antibody (diluted 1:12 with assay buffer), which serves as the first antibody (Lilly Research Laboratories, Indianapolis, IN), and goat anti-rabbit antibody (diluted 1:15 with assay buffer) which serves as the second antibody (Linco Research, Inc., St. Charles, MO). Radiolabeled estradiol (125I-estradiol) was used as the tracer (ICN Biomedicals, Costa Mesa, CA). The assay buffer was the same as the progesterone RIA buffer described above. An estradiol dose response curve was prepared from a stock concentration of 256 pg/100 μL that was serially diluted to 128, 64, 32, 16, 8, 4, 2, 1, and 0.5 pg/100 μL using assay buffer. In duplicate, sample media was added at either 50 or 100 μL and (if needed) combined with assay buffer to make a total volume of 100 μL. Two hundred μL of tracer were added to all samples, along with 100 μL of first antibody. All tubes were mixed and allowed to incubate for 1 h at 37 °C. Following this incubation, 200 μL of second antibody were added and the assay allowed to incubate at 4 °C overnight. The following day, assay tubes were centrifuged, supernatant aspirated, and precipitate counted as described for the progesterone RIA. The intra- and interassay coefficients of variation were 8% and 17%, respectively, for the estradiol RIA.

3.2.7 RNA extraction

At the end of the treatment period, cells from two replicate wells were lysed in 500 μL of TRIzol® reagent and RNA was extracted as previously described (Voge et al., 2004; Aad et al., 2006). Briefly, 250 μL TRIzol® reagent was added to all wells and cells were lysed by repeated pipetting and then combined with their respective replicates. Combined wells were then transferred to 1.5 mL eppendorf tubes. Each treatment containing 4 wells generated 2 replicate samples of RNA. Cell lysates were incubated in

TRIzol® reagent for approximately 5 min at room temperature, then 100 μL of chloroform was added to each sample followed by a 15 s vortex. After approximately a 2 min incubation at room temperature, samples were centrifuged at 3500 x *g* for 30 min at 4 °C using eppendorf centrifuge 5417C (Brinkmann Instruments, Westbury, NY). The upper aqueous phase of each sample was then transferred to a fresh eppendorf tube and RNA was precipitated using 250 μL isopropanol. Samples were incubated at room temperature for 10 min and then centrifuged at 3500 x *g* for 10 min at 4 °C. The RNA pellets were washed after discarding the supernatant with 500 μL of 70% ethanol and allowed to dry at room temperature. The RNA pellets were suspended in 16.5 μL of DEPC-treated water. RNA was quantitated by spectrophotmetry at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Aliquots of 1.5 μL of RNA were used to determine the concentration in ng/μL as well as the purity given as a ratio of 260/280 nm where values between 1.8 and 2.2 were acceptable. RNA was then diluted to 10 ng/μL in DEPC-treated water and stored at -80 °C until used for quantification of target gene expression. Just prior to use, an RNA aliquot was thawed on ice for 3-5 min.

3.2.8 Real-time PCR

The target gene primers (forward, reverse) and probe sequences for bovine aromatase enzyme (CYP19A1; Accession NM_174305) were TGCCAAGAATGTTCCTTACAGGTA, CAGAGTGACCTTCATCATGACCAT and CATTTGGCTTTGGGCCCCGG, respectively; and for bovine P450 side-chain cleavage enzyme (CYP11A1; Accession NM_176644) were CTCCGTGACCCTGCAGAGATAC, ATAGACGGCCACTTGTACCAATG and TTGGTTCTTCGAGATTACATGATTCCTGCC, respectively (Lagaly et al., 2008; Spicer and Aad, 2007). The differential expression of target gene mRNA in GC was quantified using the one-step multiplex real-time RT-PCR reaction for Taqman® Gold RT-PCR Kit (Applied Biosystems, Foster City, CA) as previously described (Spicer and Aad, 2007). All samples were run in duplicate. The 18S ribosomal RNA values were used as internal controls to normalize samples for any variation in amounts of RNA loaded, and relative quantification of target gene mRNAs was expressed using the comparative threshold cycle method as previously described (Voge et al., 2004; Aad et al., 2006). Briefly, the ΔCt was determined by subtracting the 18S Ct value from the target unknown value. For each target gene, the ΔΔCt was determined by subtracting the higher ΔCt (the least expressed unknown) from all other ΔCt values. Fold changes in target gene mRNA abundance were calculated as being equal to $2^{-\Delta\Delta Ct}$.

3.2.9 Experimental design

Experiment 1 was performed to evaluate the effects of FB_1 alone and combined with β-ZEA on GC proliferation and steroidogenesis. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium as described earlier, and then treated for 48 h in serum-free medium containing testosterone (500 ng/mL), FSH (30 ng/mL) and IGF1 (30 ng/mL) with or without FB_1 at 0, 30, 100 ng/mL (0; 0.042; 0.13 μM) and β-ZEA at 0 or 30 ng/mL (0; 0.094 μM). After 48 h of treatment, cells were counted and medium was collected for E2 and P4 RIA. Doses of FSH and IGF1 were selected based on previous studies (Spicer et al., 2002; Ranzenigo et al., 2008). Because IGF1 alone has little or no effect on steroid production, FSH was added to all treatments (Spicer et al., 1993; 2002; Ranzenigo et al., 2008).

Experiment 2 was designed to evaluate the effects of interaction between FB_1, DON and DON with β-ZEA. GC were cultured for 48 h in 10% FCS washed twice with serum-free medium as described earlier, and cells treated for 48 h in serum-free medium containing 500 ng/mL of testosterone, 30 ng/mL of FSH and 30 ng/mL of IGF1with FB_1 at 0, 30, 100 ng/mL (0; 0.042; 0.13 μM), DON at 0 or 100 ng/mL (0; 0.33 μM) and β-ZEA at 0 or 30 ng/mL (0; 0.094 μM). After 48 h of treatment, medium was collected for P4 and E2 RIA, and cells were counted.

Experiment 3 was performed to evaluate the effects of FB_1 alone and combined with α-ZEA and β-ZEA in the presence of FSH and with or without IGF1 on GC proliferation and steroidogenesis. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium as described earlier, and cells treated for 48 h in serum-free medium containing testosterone (500 ng/mL), FSH (30 ng/mL) with or without IGF1 (30 ng/mL). FB_1 was tested at 5 μg/mL (6.9 μM) alone and combined with α-ZEA at 5 μg/mL (15.6 μM) and β-ZEA at the same concentration (15.6 μM). After 48 h cells were counted and medium was collected for P4 and E2 RIA.

Experiment 4 was carried out to compare the dose response of FB_1 and BEA on FSH plus IGF1–induced GC proliferation and steroidogenesis. GC were cultured for 48 h in 10% FCS washed twice with serum-free medium as described earlier, and cells treated for 48 h in serum-free medium containing testosterone (500 ng/mL), FSH (30 ng/mL) and IGF1 (30 ng/mL) with the various doses of FB_1 (i.e., 0, 0.3, 1, 3, 10 μM) or BEA (0, 0.3, 1, 3, 10 μM). After 48 h of treatment, medium was collected for determination of progesterone and estradiol concentrations via RIA, and cells were counted. Doses of FSH and IGF1 were selected based on previous studies (Spicer et al., 2002; Ranzenigo et al., 2008). Because IGF1 alone has little or no effect on steroid production, FSH was added to all treatments (Spicer et al., 1993; 2002; Ranzenigo et al., 2008).

Experiment 5 was performed to evaluate the effects of FB_1 and BEA, alone and in combination, on GC proliferation and steroidogenesis. Cells were cultured for 48 h in

10% FCS, washed twice with serum-free medium as described earlier, and cells treated for 48 h in serum-free medium containing testosterone (500 ng/mL), FSH (30 ng/mL) and IGF1 (30 ng/mL) with or without FB_1 (3 μM) and BEA (3 μM). After 48 h of treatment, cells were counted and medium was collected for determination of progesterone and estradiol concentrations via RIA.

Experiment 6 was designed to evaluate the dose response of FB_1 alone and combined with BEA on GC proliferation and steroidogenesis. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium, and then treated for 48 h in serum-free medium containing testosterone (500 ng/mL), FSH (30 ng/mL) and IGF1 (30 ng/mL) with the various doses of FB_1 (i.e., 0, 0.5, 1, 1.5, 3, or 6 μM) with or without BEA (3 μM). After 48 h of treatment, medium was collected for determination of progesterone and estradiol concentrations via RIA, and cells were counted.

Experiment 7 was designed to determine the dose response of BEA alone and combined with FB_1 on GC proliferation and steroidogenesis. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium, and then treated for 48 h in serum-free medium containing FSH (30 ng/mL) and IGF1 (30 ng/mL) with the various doses of BEA (i.e., 0, 0.5, 1, 1.5, 3, or 6 μM) with or without FB_1 (3 μM). After 48 h of treatment, medium was collected for determination of progesterone and estradiol concentrations via RIA, and cells were counted.

Experiment 8 was performed to determine the effects of FB_1 and BEA on *CYP11A1* and *CYP19A1* mRNA abundance. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium as described earlier, and cells treated for 24 h in serum-free medium containing testosterone (500 ng/mL), FSH (30 ng/mL), IGF1 (0 or 30 ng/mL) and FB_1 (30 μM) or BEA (30 μM). After 24 h of treatment, medium was aspirated and cells were lysed for RNA extraction.

Experiment 9 was carried out to determine the effect of FB_1 and BEA on serum-stimulated GC proliferation. Cells were cultured for 4 days in 10% FCS. During the last 2 days of culture, cells were treated as follows: control (no additions), FB_1 (10 μM) or BEA (10 μM). At the end of treatment, cells were counted.

3.2.10 Statistical analysis

Experimental data are presented as the least squares means ± SEM of measurements from replicated experiments (n=3) with each treatment applied in triplicate or duplicate wells for each replicate experiment. Each replicated experiment was derived from a different pool of GC generated from a total volume of 10 mL follicular fluid obtained from twenty to thirty ovaries. For mRNA experiments, treatments were applied in quadruplicate culture wells with each mRNA sample being

obtained from two wells. Treatment effects were assessed by factorial ANOVA designs in the GLM procedure of the Statistical Analysis System (SAS) using SAS for Windows (version 9.2, SAS Institute Inc., Cary, NC). Steroid production was expressed as ng or pg/10^5 cells per 24 h, and GC numbers determined at the end of the experiment were used for this calculation. A P-value of less than 0.05 was considered statistically significant. Mean differences in steroid production, cell numbers and mRNA abundance between treatments were determined using the Fisher's protected least significant difference (LSD) procedure (Ott, 1977).

3.3 Results

3.3.1 Experiment 1: Dose response of FB_1 alone and combined with β-ZEA on GC numbers and steroid production in the presence of FSH with IGF1

The results revealed that FB_1 alone at 30 and 100 ng/mL (0; 0.042; 0.13 μM) had no effect (P>0.10) on GC numbers. In the presence of β-ZEA at 30 ng/mL (0.094 μM), FB_1 at 30 ng/mL (0.042 μM) showed a stimulatory effect (P<0.05) on GC numbers (Fig. 3.3), whereas FB_1 at 100 ng/mL (0.013 μM) increased (P<0.05) P4 production (Fig. 3.4). FB_1 at 30 ng/mL (0.042 μM) and 100 ng/mL (0.13 μM) was found to amplify the inhibitory effect of β-ZEA at 30 ng/mL (0.094 μM) on E2 production (Fig. 3.5).

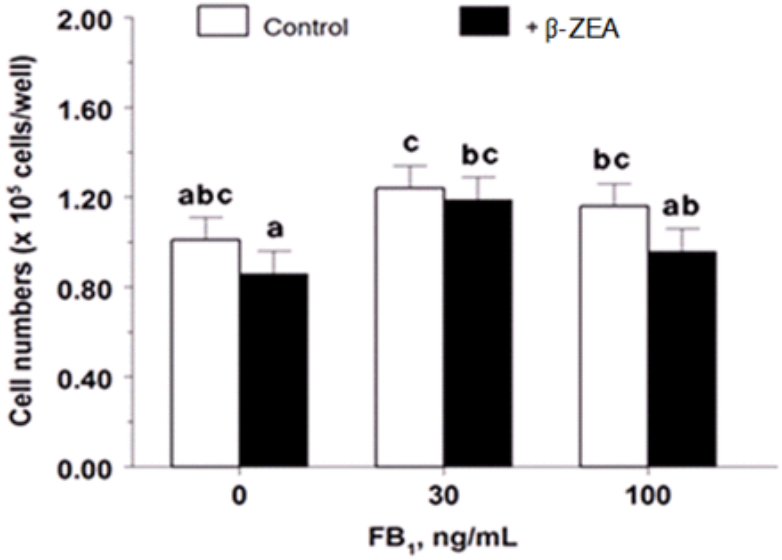

Figure 3.3: Effect of FB_1 on numbers of GC from bovine follicles. Means (± SEM) without a common letter (a–c) differ (P < 0.05). FB_1, fumonisin B_1; β-ZEA, β-zearalenone; SEM, standard error of the mean.

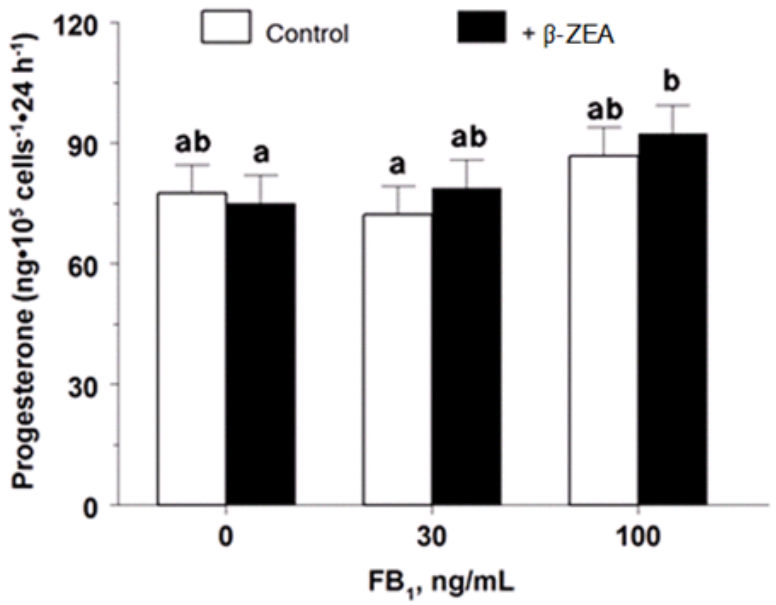

Figure 3.4: Effect of FB_1 on progesterone production of GC from bovine follicles. Means (± SEM) without a common letter (a–b) differ ($P < 0.05$). FB_1, fumonisin B_1; β-ZEA, β-zearalenone; SEM, standard error of the mean.

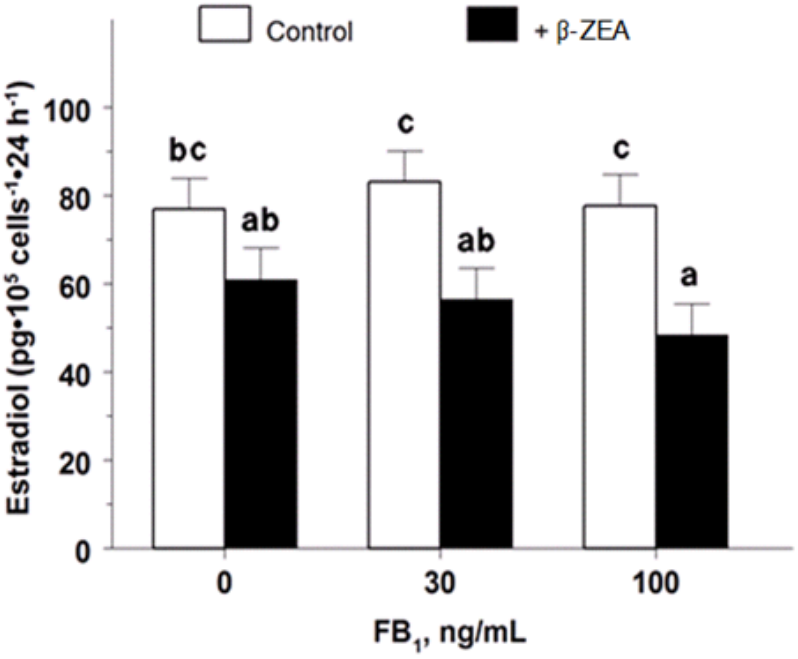

Figure 3.5: Effect of FB_1 on estradiol production of GC from bovine follicles. Means (± SEM) without a common letter (a–c) differ ($P < 0.05$). FB_1, fumonisin B_1; β-ZEA , β-zearalenone;, SEM, standard error of the mean.

3.3.2 Experiment 2: Dose response of FB_1 alone and combined with DON and β-ZEA on GC numbers and steroid production in the presence of FSH with IGF1

No significant interaction (P>0.10) existed between FB_1 at 30 ng/mL and 100 ng/mL (0; 0.042; 0.13 μM) and DON at 100 ng/mL (0.33 μM) or between FB_1 at 30 ng/mL and 100 ng/mL (0; 0.042; 0.13 μM) and the combination of β-ZEA at 30 ng/mL (0.094 μM) and DON at100 ng/mL (0.33 μM) on cell numbers (Fig. 3.6).
Regarding P4 and E2 production, no significant interaction (P>0.10) was observed between FB_1 at 30 ng/mL and 100 ng/mL (0; 0.042; 0.13 μM) and DON at 100 ng/mL (0.33 μM) alone or with β-ZEA at 30 ng/mL (0.094 μM) on P4 (Fig. 3.7) or E2 (Fig. 3.8) production, but β-ZEA inhibited (P<0.05) P4 production in DON treated GC (Fig. 3.7).

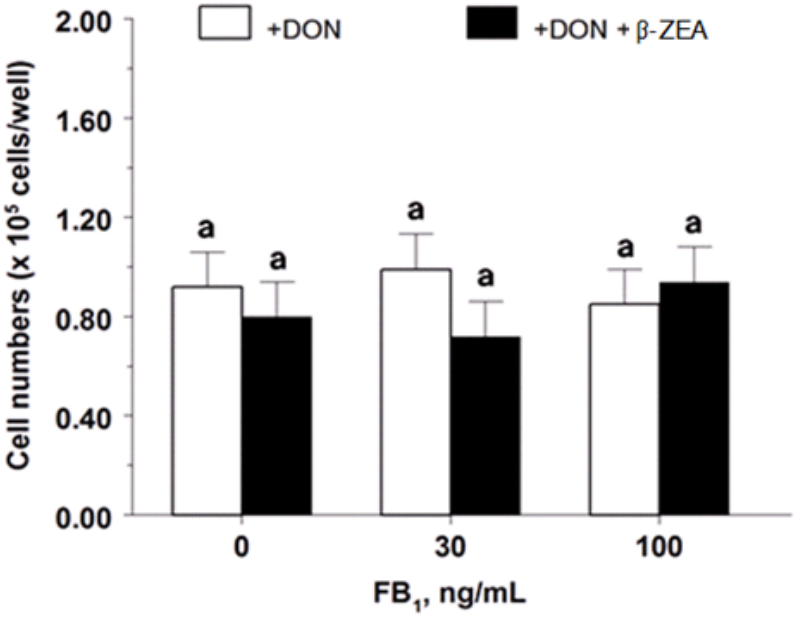

Figure 3.6: Interaction between FB_1 and DON or β-ZEA on proliferation of bovine GC. Values are means ± SEM from three separate experiments. Means without a common letter (a) differ (P < 0.05). FB_1, fumonisin B_1; DON, deoxynivalenol; β-ZEA, β-zearalenone; SEM, standard error of the mean.

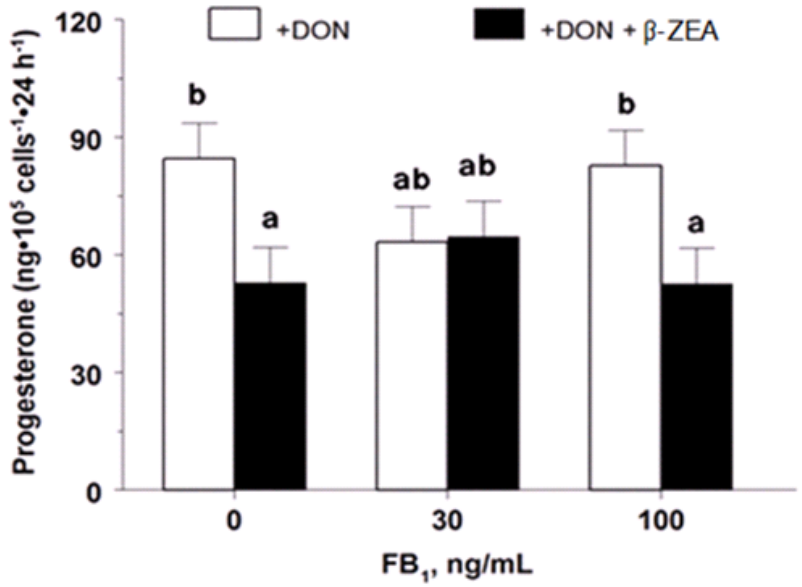

Figure 3.7: Interaction between FB_1 and DON or β-ZEA on progesterone production by GC from bovine follicles. Values are means ±SEM from three separate experiments. Means without a common letter (a–b) differ ($P < 0.05$). DON, deoxynivalenol; FB_1, fumonisin B_1; β-ZEA , β-zearalenone; SEM, standard error of the mean.

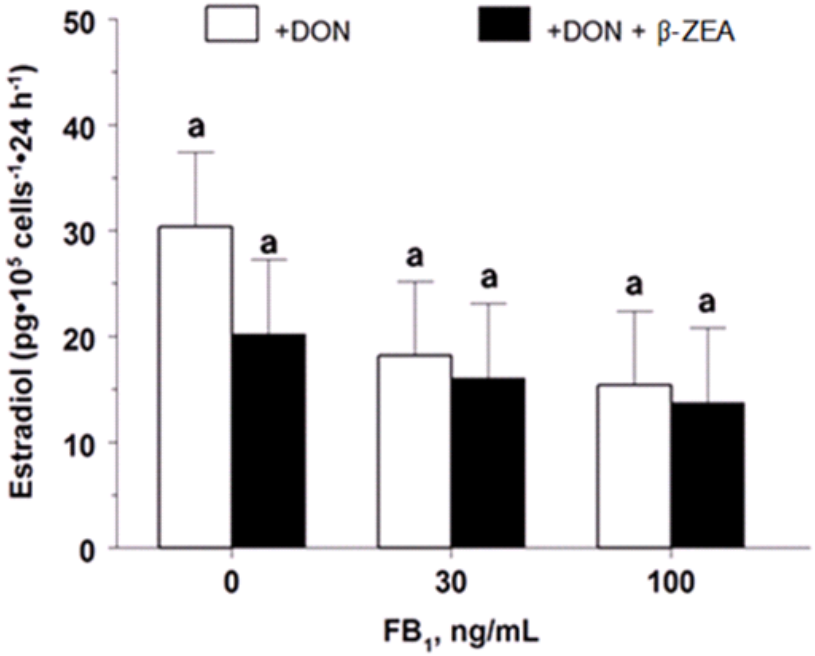

Figure 3.8: interaction between FB_1 and DON or β-ZEA on estradiol production by GC from bovine follicles. Values are means ±SEM from three separate experiments. Means without a common letter (a) differ ($P < 0.05$). DON, deoxynivalenol; FB_1, fumonisin B_1; β-ZEA , β-zearalenone; SEM, standard error of the mean.

3.3.3 Experiment 3: Effect of FB_1 alone and combined with either α-ZEA or β-ZEA on GC numbers and steroid production

Experiment 3 was conducted to assess the effects of FB_1 alone and combined with α-ZEA and β-ZEA on cell proliferation and steroidogenesis with and without IGF1. In the absence of IGF1, GC numbers (Fig. 3.9), P4 production (Fig. 3.10), and E2 production (Fig. 3.11) were not affected by any of the mycotoxins or their combinations. IGF1 induced ($P<0.05$) GC proliferation and this increase was blocked ($P < 0.05$) after exposure to β-ZEA alone and FB_1 with either α-ZEA or β-ZEA (Fig. 3.9). In contrast, IGF1-induced P4 production was not affected ($P>0.10$) by FB_1, α-ZEA , β-ZEA or their combinations (Fig. 3.10). However, IGF1-induced E2 production was increased ($P<0.05$) with FB_1 alone (Fig. 3.11). In contrast, α-ZEA alone, β-ZEA alone, or their combination with FB_1 decreased ($P<0.05$) IGF1-induced E2 production (Fig. 3.11).

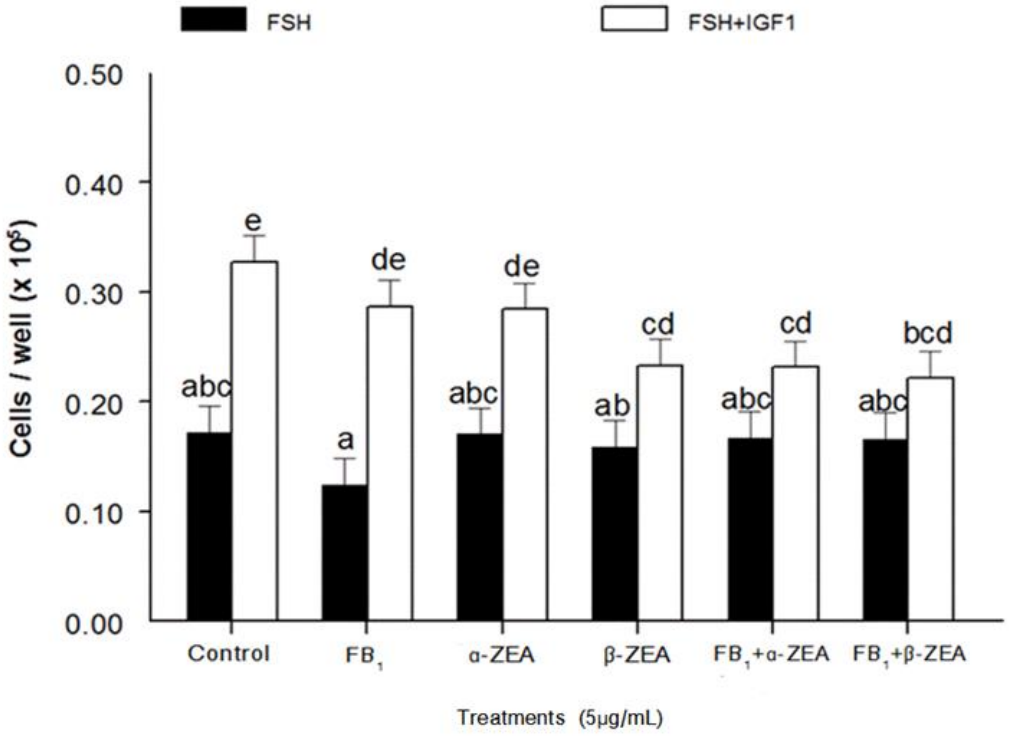

Figure 3.9: Effect of FB_1 on numbers of GC from bovine follicles. Values are means ± SEM from three separate experiments. Means without a common letter (a–e) differ ($P < 0.05$). FB_1, fumonisin B_1; α-ZEA, α–zearalenone; β-ZEA , β-zearalenone; SEM, standard error of the mean.

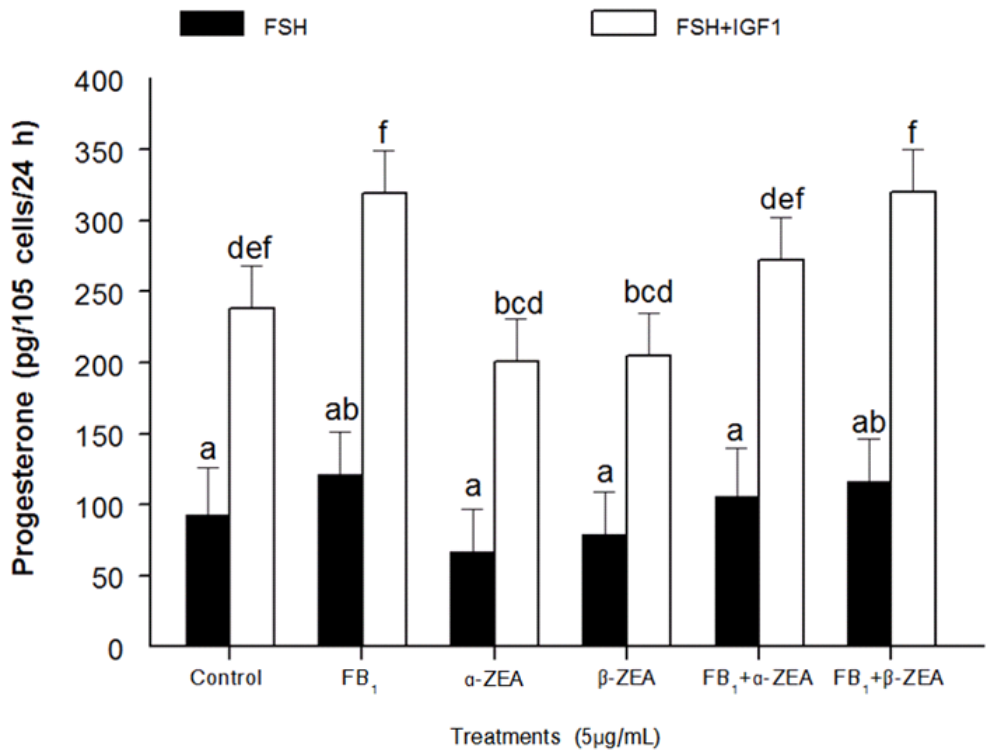

Figure 3.10: Interaction between FB_1, α-ZEA or β-ZEA on progesterone production of bovine GC. Values are means ± SEM from three separate experiments. Means without a common letter (a-f) differ ($P < 0.05$). FB_1, fumonisin B_1; α-ZEA, α-Zearalenone;, β-ZEA, β-Zearalenone, SEM, standard error of the mean.

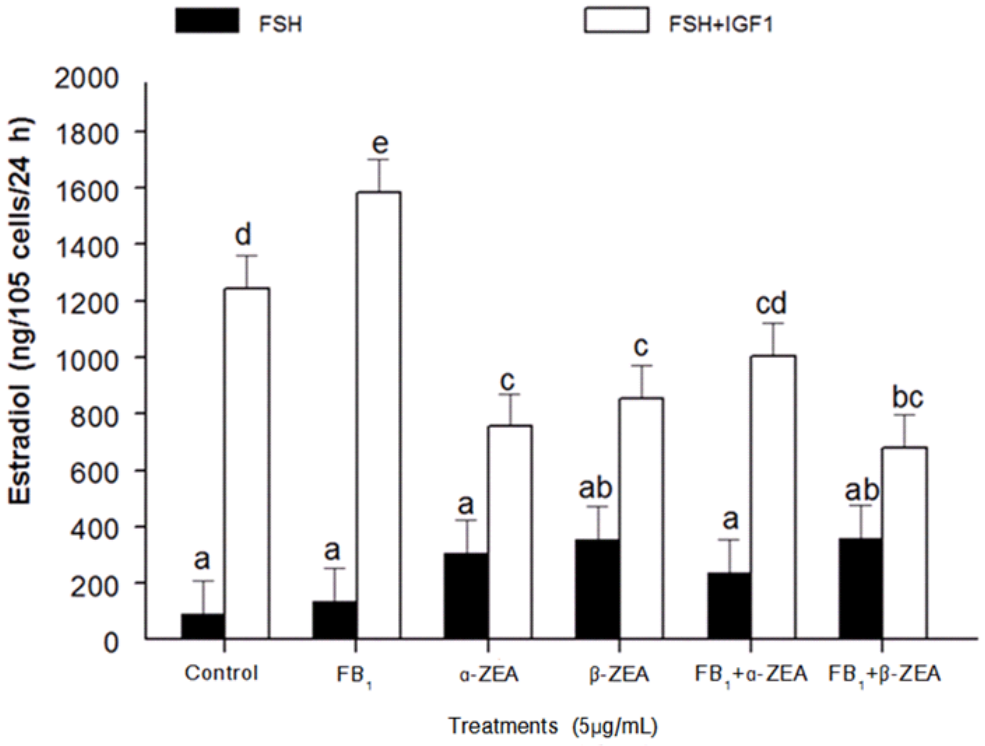

Figure 3.11: Interaction between FB_1, α-ZEA or β-ZEA on estradiol production of bovine GC. Values are means ± SEM from three separate experiments. Means without a common letter (a-e) differ ($P < 0.05$). FB_1, fumonisin B_1; α-ZEA, α-Zearalenone;, β-ZEA, β-Zearalenone, SEM, standard error of the mean.

3.3.4 Experiment 4: Dose response of FB_1 and BEA on GC numbers and steroid production in the presence of FSH plus IGF1

Cell proliferation was not affected ($P \geq 0.05$) by any dose of FB_1 (i.e., 0.3, 1, 3, 10 µM), whereas BEA at the highest dose tested (10 µM) decreased (72%; $P < 0.01$) cell numbers (Fig. 3.12). FB_1 at all doses did not significantly affect progesterone production, whereas BEA at 3 and 10 µM strongly inhibited ($P < 0.0001$) progesterone production (Fig. 3.13). Estradiol production was inhibited ($P < 0.05$) by 25% and by 57% after exposure to FB_1 at 10 µM and BEA at 3 µM, respectively (Fig. 3.14).

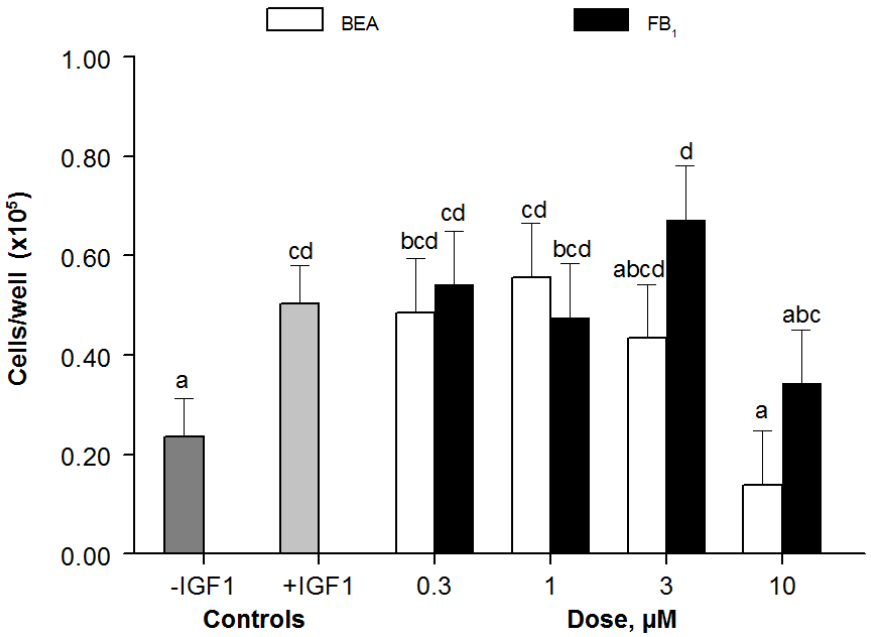

Figure 3.12: Effect of FB_1 and BEA on FSH plus IGF1–induced proliferation of bovine GC. Values are means ± SEM from three separate experiments. Means without a common letter (a–d) differ ($P < 0.05$). BEA, beauvericin; FB_1, fumonisin B_1; GC, granulosa cells.

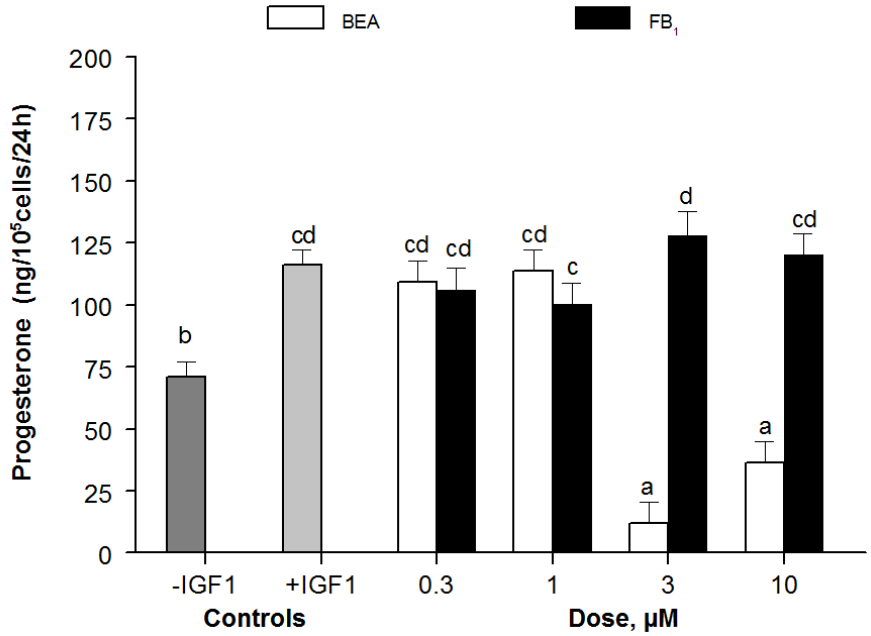

Figure 3.13: Effect of FB_1 and BEA on FSH plus IGF1-induced progesterone production by GC from bovine follicles. Values are means ± SEM from three separate experiments. Means without a common letter (a–d) differ ($P < 0.05$). BEA, beauvericin; FB_1, fumonisin B_1; GC, granulosa cells.

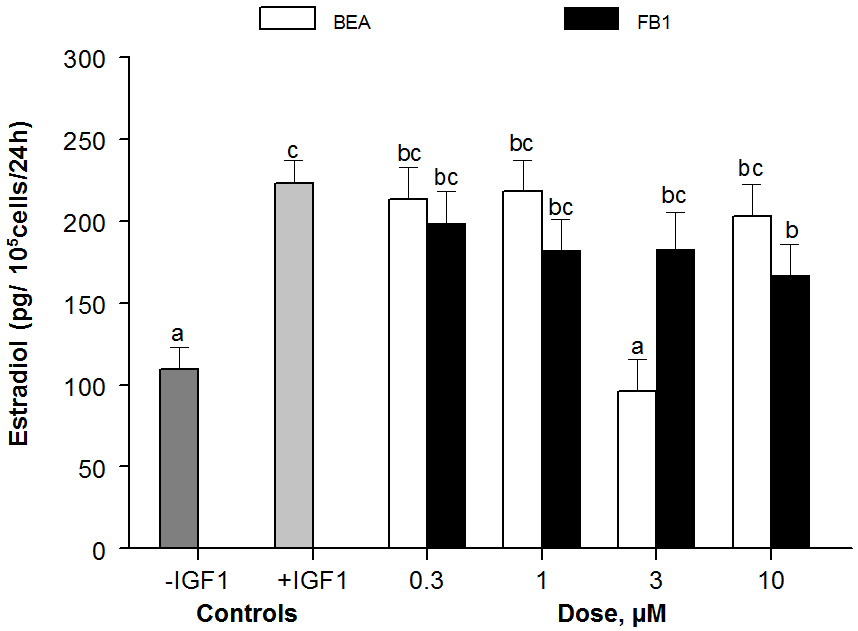

Figure 3.14: Effect of FB_1 and BEA on FSH plus IGF1-induced estradiol production by GC from bovine follicles. Values are means ± SEM from three separate experiments. Means without a common letter (a–d) differ ($P < 0.05$). BEA, beauvericin; FB_1, fumonisin B_1; GC, granulosa cells.

3.3.5 Experiment 5: Individual and combined effects of FB_1 and BEA on GC numbers and steroid production in the presence of FSH plus IGF1

BEA (3 µM) significantly inhibited ($P< 0.0001$) cell proliferation and progesterone and estradiol production by 54%, 97% and 80%, respectively (Fig. 3.15, 3.16, 3.17). FB_1 (3 µM) did not affect ($P > 0.05$) any of the variables measured and did not influence the effect of BEA on cell proliferation (Fig. 3.15)or steroid production (Fig. 3.16, 3.17).

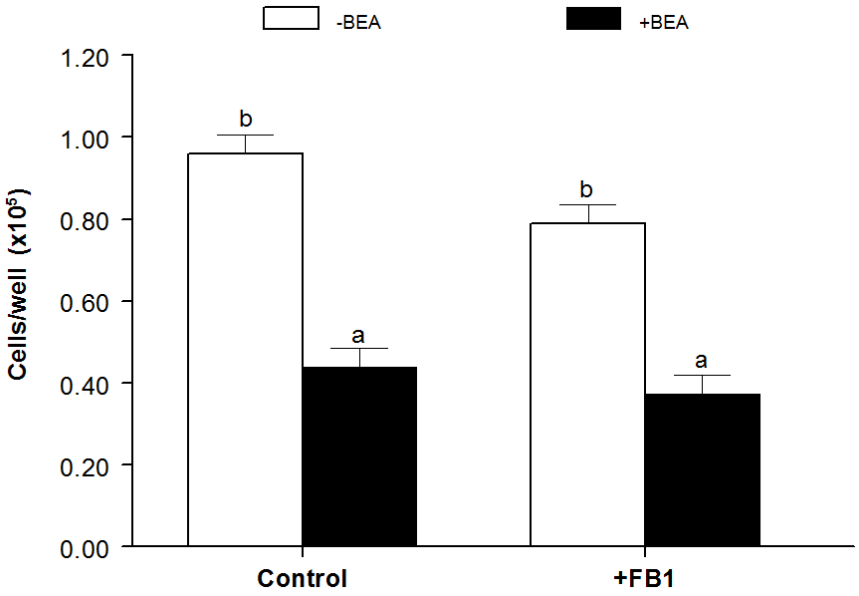

Figure 3.15: Interaction between FB_1 and BEA on FSH plus IGF1–induced proliferation of bovine GC. Values are means ± SEM from three separate experiments. Means without a common letter (a–b) differ ($P < 0.05$). BEA, beauvericin; FB_1, fumonisin B_1; GC, granulosa cells.

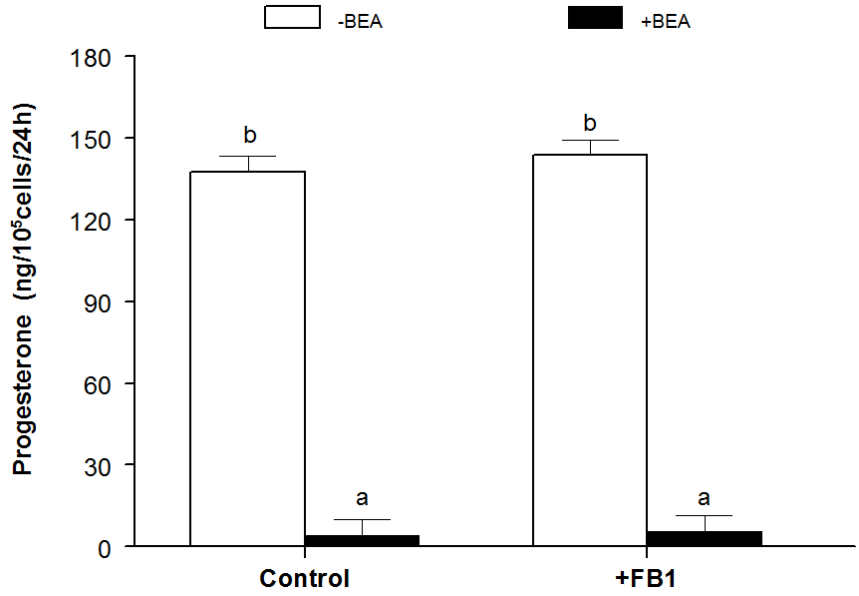

Figure 3.16: Interaction between FB_1 and BEA on FSH plus IGF1-induced progesterone production by GC from bovine follicles. Values are means ± SEM from three separate experiments. Means without a common letter (a–b) differ ($P < 0.05$). BEA, beauvericin; FB_1, fumonisin B_1; GC, granulosa cells.

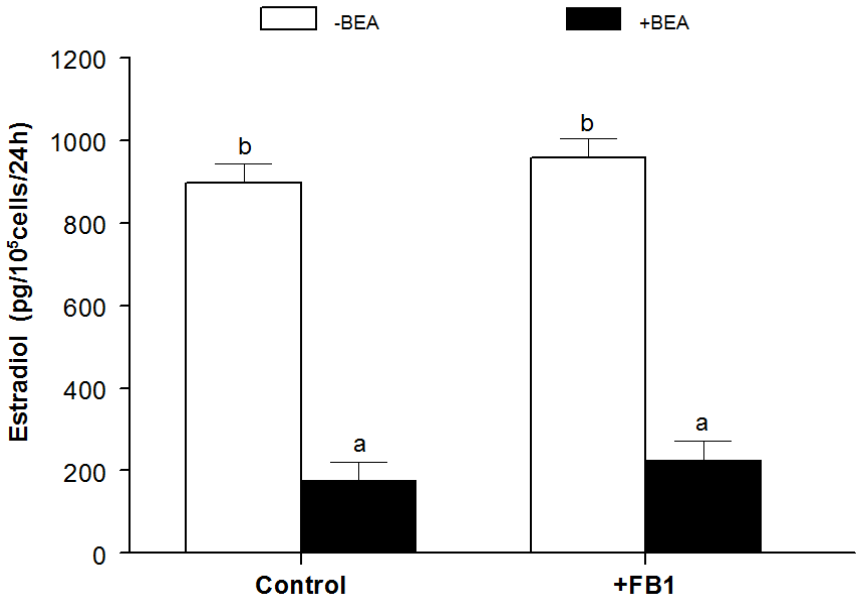

Figure 3.17: Interaction between FB_1 and BEA on FSH plus IGF1-induced estradiol production by GC from bovine follicles. Values are means ± SEM from three separate experiments. Means without a common letter (a–b) differ ($P < 0.05$). BEA, beauvericin; FB_1, fumonisin B_1; GC, granulosa cells.

3.3.6 Experiment 6: Dose response of FB_1 with or without BEA on FSH plus IGF1-induced GC proliferation and steroid production

Cell proliferation was not affected ($P \geq 0.05$) after exposure to FB_1 at 0.5, 1, 1.5 and 3 μM alone or in combination with BEA (3 μM), whereas at the highest dose tested (6 μM) FB_1 in combination with BEA decreased ($P < 0.05$) cell numbers (Fig. 3.18). FB_1 alone had no significant effect ($P > 0.05$) on progesterone production (Fig. 3.19) whereas at 1 and 1.5 μM FB_1 decreased ($P < 0.05$) estradiol production (Fig. 3.20). BEA at 3 μM drastically inhibited both progesterone (88%; $P < 0.001$) and estradiol (96%; $P < 0.0001$) production, and the various doses of FB_1 did not influence these inhibitory effects (Fig. 3.19, 3.20).

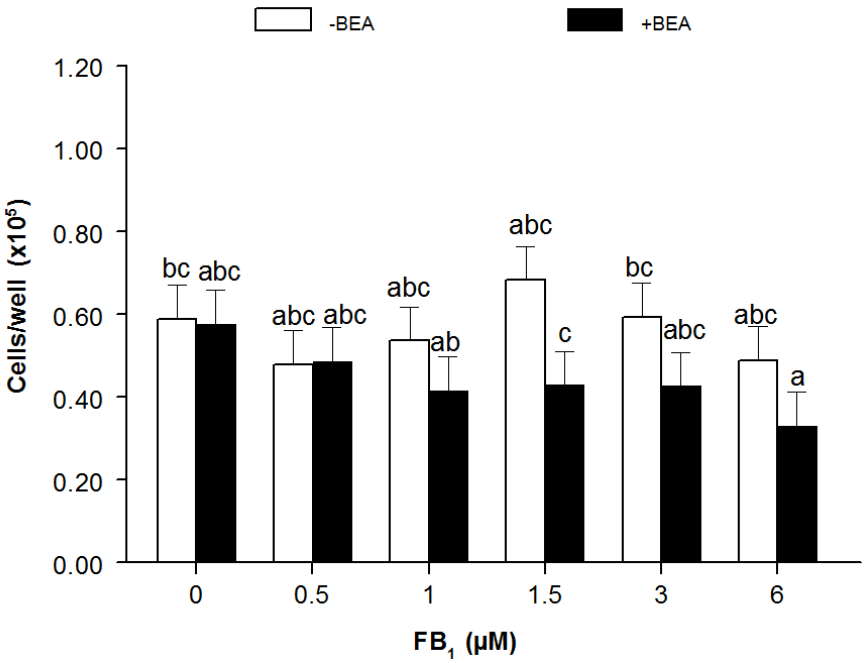

Figure 3.18: Effect of various doses of FB_1 with or without BEA on FSH plus IGF1-induced proliferation of bovine GC. Values are means ± SEM from three separate experiments. Means without a common letter (a–c) differ ($P < 0.05$). BEA, beauvericin; FB_1, fumonisin B_1; GC, granulosa cells.

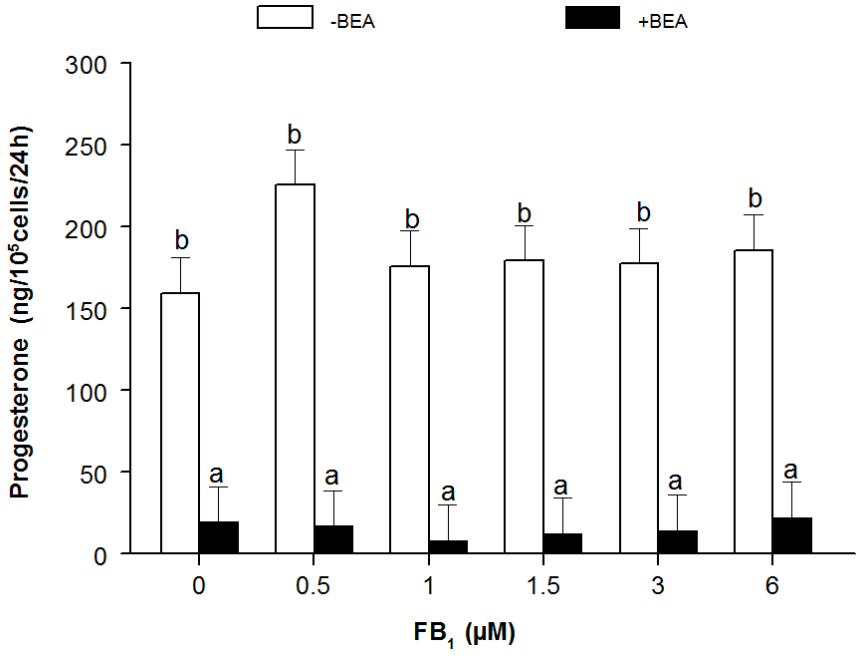

Figure 3.19: Effect of various doses of FB_1 with or without BEA on FSH plus IGF1-induced progesterone production by GC from bovine follicles. Values are means ± SEM from three separate experiments. Means without a common letter (a–b) differ ($P < 0.05$). BEA, beauvericin; FB_1, fumonisin B_1; GC, granulosa cells.

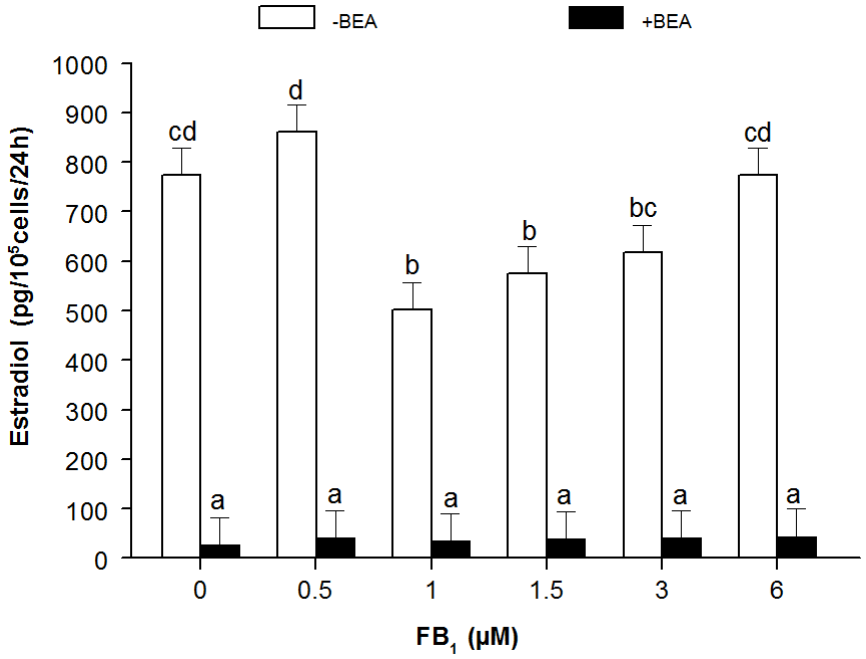

Figure 3.20: Effect of various doses of FB_1 with or without BEA on FSH plus IGF1-induced estradiol production by GC from bovine follicles. Values are means ± SEM from three separate experiments. Means without a common letter (a–b) differ ($P < 0.05$). BEA, beauvericin; FB_1, fumonisin B_1; GC, granulosa cells

3.3.7 Experiment 7: Dose response of BEA with or without FB_1 on FSH plus IGF1-induced GC proliferation and steroid production

At the highest dose tested (6 μM), BEA was found to inhibit (50%; $P < 0.05$) cell proliferation and no significant difference was detected in combination with FB_1 (3 μM) (Fig. 3.21). BEA at 3 and 6 μM was found to strongly decrease ($P < 0.05$) both progesterone and estradiol production, and FB_1 had little effect on these responses (Fig. 3.22, 3.23). FB_1 had no effect ($P > 0.05$) on progesterone production (Fig. 3.22) but at 3 μM FB_1 decreased ($P < 0.05$) estradiol production alone and in combination with 1 μM BEA (Fig. 3.23).

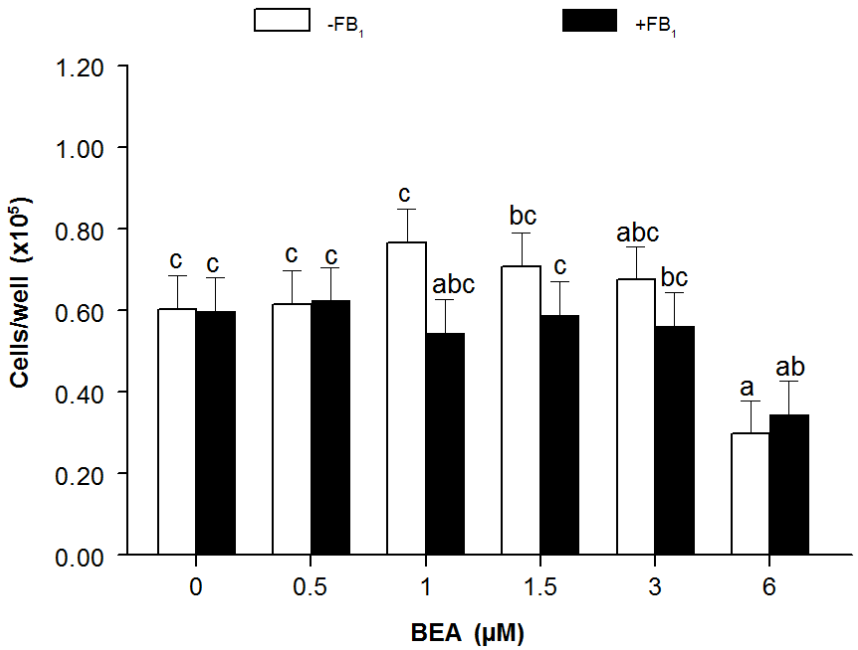

Figure 3.21: Effect of various doses of BEA with or without FB_1 on FSH plus IGF1–induced proliferation of bovine GC. Values are means ± SEM from three separate experiments. Means without a common letter (a–c) differ ($P < 0.05$). BEA, beauvericin; FB_1, fumonisin B_1; GC, granulosa cells.

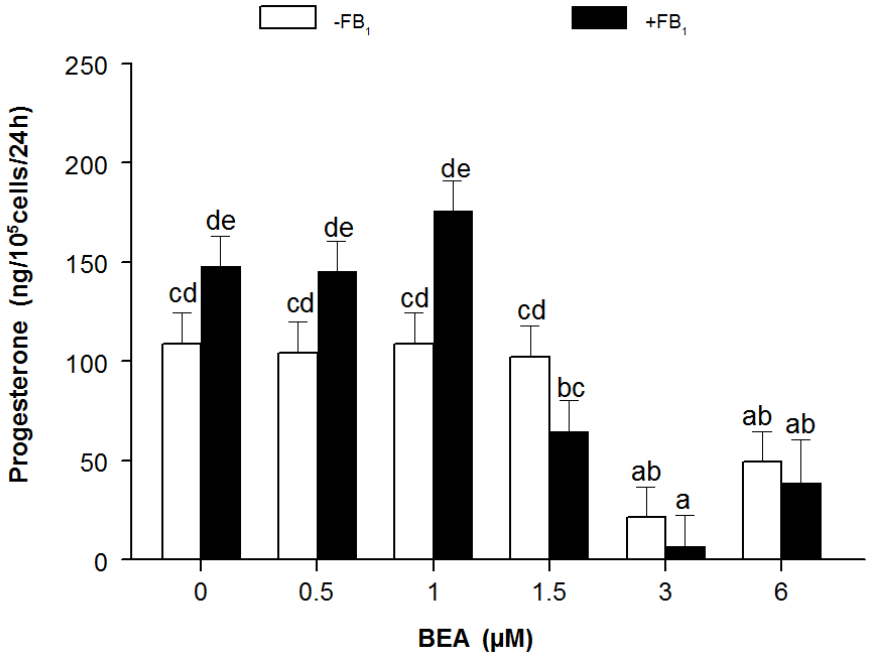

Figure 3.22: Effect of various doses of BEA with or without FB_1 on FSH plus IGF1-induced progesterone production by GC from bovine follicles. Values are means ± SEM from three separate experiments. Means without a common letter (a–e) differ ($P < 0.05$). BEA, beauvericin; FB_1, fumonisin B_1; GC, granulosa cells.

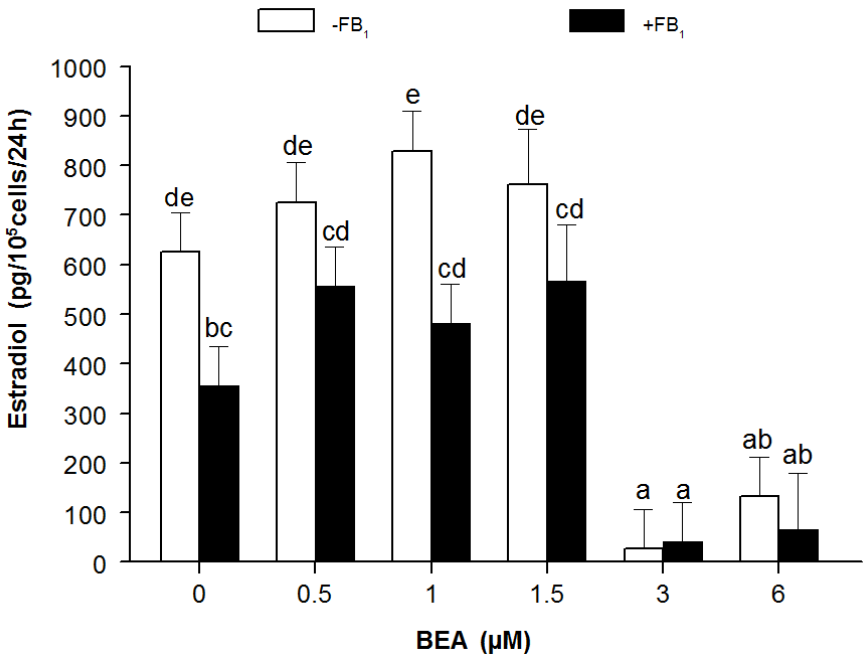

Figure 3.23: Effect of various doses of BEA with or without FB_1 on FSH plus IGF1-induced estradiol production by GC from bovine follicles. Values are means ± SEM from three separate experiments. Means without a common letter (a–e) differ ($P < 0.05$). BEA, beauvericin; FB_1, fumonisin B_1; GC, granulosa cells.

3.3.8 Experiment 8: Effect of FB_1 and BEA on GC CYP11A1 and CYP19A1 mRNA

BEA (30 µM) showed an inhibitory effect on FSH plus IGF1-induced *CYP11A1* and *CYP19A1* mRNA abundances ($P < 0.05$), whereas FB_1 (30 µM) had no effect on gene expression (Fig. 3.24, 3.25).

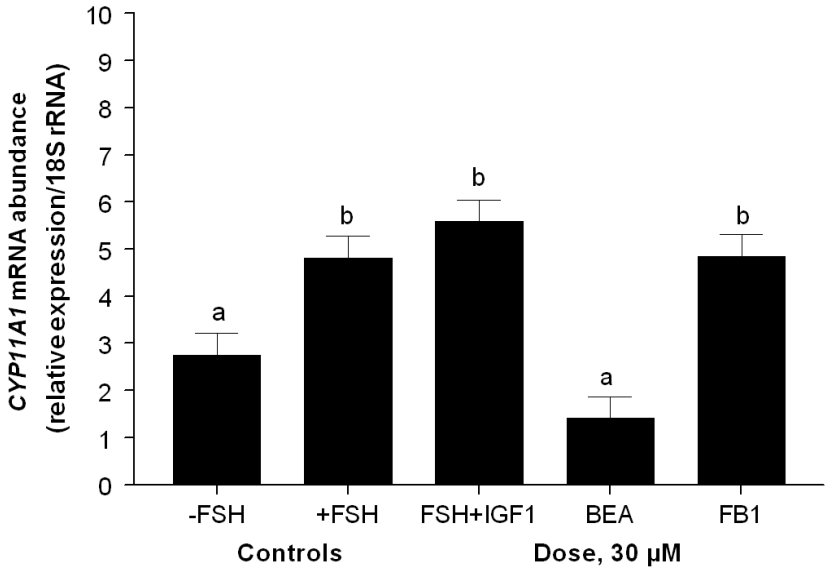

Figure 3.24: Effect of BEA and FB_1 on FSH plus IGF1-induced *CYP11A1* mRNA abundance in GC from bovine follicles. Values are means ± SEM from three separate experiments. Means without a common letter (a–b) differ ($P < 0.05$). BEA, beauvericin; FB_1, fumonisin B_1; GC, granulosa cells.

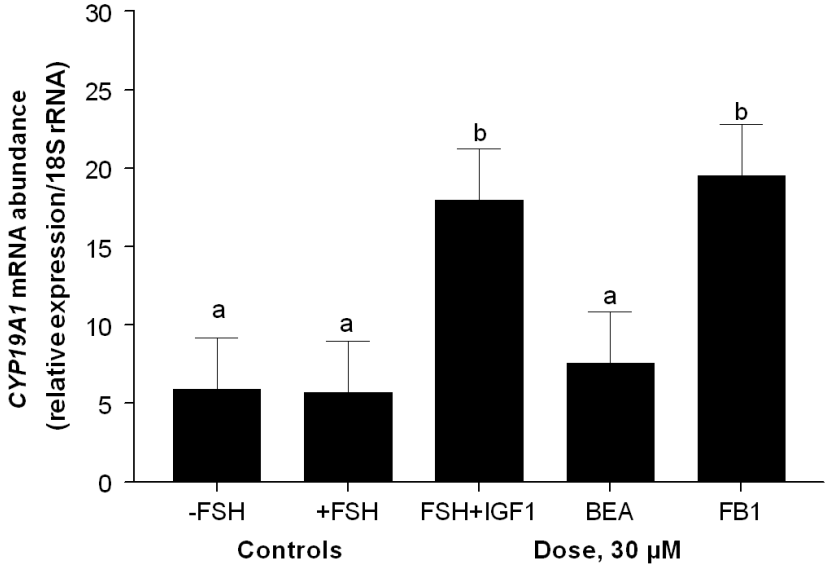

Figure 3.25: Effect of BEA and FB_1 on FSH plus IGF1-induced *CYP19A1* mRNA abundance in GC from bovine follicles. Values are means ± SEM from three separate experiments. Means without a common letter (a–b) differ ($P < 0.05$). BEA, beauvericin; FB_1, fumonisin B_1; GC, granulosa cells.

3.3.9 Experiment 9: Effect of FB_1 and BEA on serum-induced GC proliferation

Both FB_1 (10 μM) and BEA (10 μM) decreased ($P < 0.001$) GC proliferation induced by 10% FCS (Fig. 3.26). Cell numbers were decreased ($P < 0.001$) by 31% after 1 day of BEA treatment and by 88% and 15% after 2 days of BEA and FB_1 treatment, respectively (Fig. 3.26).

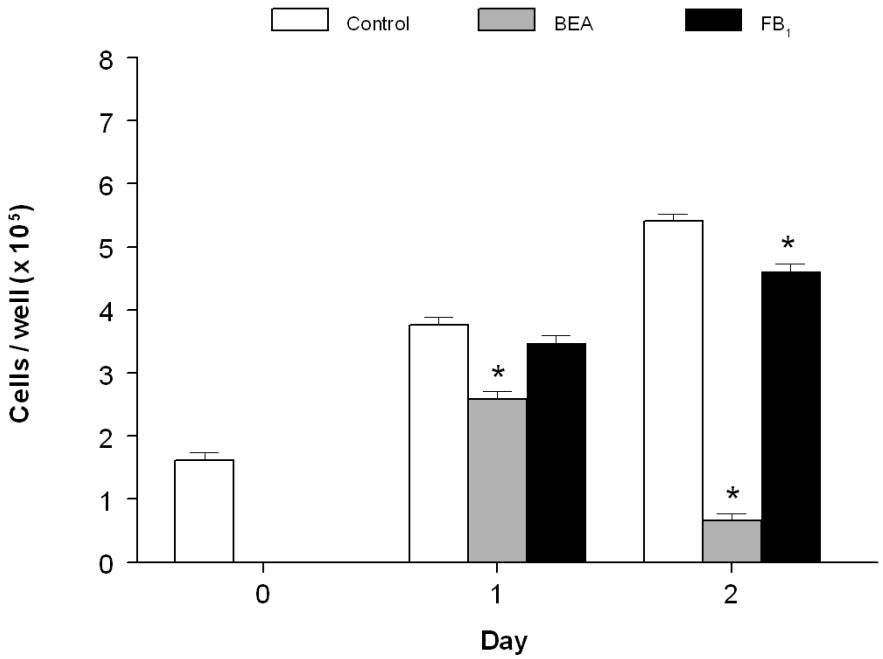

Figure 3.26: Effect of FB_1 and BEA on serum-induced proliferation of bovine GC. Within day of treatment, * indicates mean differs ($P < 0.001$) from control value. Values are means ± SEM from three separate experiments. BEA, beauvericin; FB_1, fumonisin B_1.

3.4 Discussion

GC function is fundamental for normal folliculogenesis and oocyte growth and development (Spicer et al., 2001; Petro et al., 2012; Rawan et al., 2015). Previous *in vitro* studies with *Fusarium* mycotoxins such as DON and ZEA demonstrated that these mycotoxins are able to directly affect GC proliferation and steroid production and consequently the follicle development and oocyte function in different species, such as pigs (Ranzenigo et al., 2008; Caloni et al., 2009; Cortinovis et al., 2014) and cattle (Pizzo et al., 2016). Ruminants are considered more resistant to FB_1 than other species such as pigs and horses (Fink-Gremmels, 2008), but information on reproductive effects is lacking. The purpose of this study was to evaluate the *in vitro* toxicity of FB_1 using a bovine GC model.

Because the co-occurrence of *Fusarium* mycotoxins in commodities and their toxicological interaction have been demonstrated (Boutigny et al., 2012; Rodrigues and Naehrer, 2012), the combination of FB_1 with DON, α-ZEA, β-ZEA and BEA was also evaluated. In this study the results show that FB_1 alone at all the concentrations tested (0.042-10 μM) did not affect IGF1-induced GC proliferation. Differently, FB_1 at 30 ng/mL (0.042 μM) in combination with β-ZEA at 30 ng/mL (0.094 μM) (Fig. 3.3), had a stimulatory effect on GC numbers in the presence of IGF1, while β-ZEA alone at 30 ng/mL (0.094 μM) had no effect on cell proliferation (Fig. 3.3). FB_1 at 5 μg/mL (6.9 μM) in combination with β-ZEA at 5 μg/mL (15.6 μM) inhibited IGF1-induced cell proliferation (Fig. 3.9), but this effect was also observed with β-ZEA alone (15.6 μM) (Fig. 3.9), and is in agreement with a previous study conducted on porcine GC (Tiemann et al., 2003). In accordance with previous studies (Ranzenigo et al., 2008; Pizzo et al., 2016), α-ZEA and FB_1 alone had not effect on GC proliferation, while co-exposure of FB_1 (6.9 μM) and α-ZEA (15.6 μM) decreased IGF1-induced cell proliferation (Fig. 3.9), indicating that these two mycotoxins may interact inducing an inhibition of cell proliferation.

Exposure of bovine GC to DON alone or combined with FB_1 had no effect on cell proliferation, whereas previous studies conducted on porcine GC reported conflicting results on DON activity, showing either an inhibitory effect (Ranzenigo et al., 2008) or a stimulatory effect (Medvedova et al., 2011) depending on the dose of DON and the presence of α-ZEA. All these results suggest that the effects of FB_1, DON, β-ZEA and α-ZEA on GC proliferation may be species-specific and influenced by their interaction.

In the present study, the effect of BEA on GC proliferation was investigated for the first time. At concentrations ≥ 6 μM (Fig. 3.21), BEA was found to inhibit GC proliferation confirming results previously reported for other cell types (Jestoi, 2008). According to previous studies (Klarić et al., 2008; Ferrer et al., 2009; Ruiz et al., 2011; Prosperini et

al., 2012; Mallebrera et al., 2016) BEA exhibits potent cytotoxicity against several mammalian cell lines. In details, BEA was found to reduce cell viability in a time and concentration-dependent manner in Vero cells, CHO-K1 cells and Caco-2 cells at 6.25 to 10.02 (Ruiz et al., 2011), 5 μM (Mallebrera et al., 2016) and at 3.125 to 25 μM (Prosperini et al., 2012), respectively.

The present study evaluated also the effects of *Fusarium* mycotoxins on GC steroidogenesis. FB_1 alone at all the concentrations tested did not affect GC P4 production. On the contrary, when combined with β-ZEA at 30 ng/mL (0.094 μM) and DON at 100 ng/mL (0.33 μM) plus β-ZEA at 30 ng/mL (0.094 μM), FB_1 at 100 ng/mL (0.13 μM) stimulated and inhibited P4 production, respectively.

The lack of an effect of FB_1 on P4 production is also supported by the results showing no effect of FB_1 on *CYP11A1* mRNA abundance in GC. Differently BEA was found to inhibit P4 production and this effect was associated with a significant suppression of *CYP11A1* mRNA abundance, suggesting that BEA may alter P4 production via a change in *CYP11A1* mRNA abundance.

As regards E2 production, in this study FB_1 at 30 (0.042 μM) and 100 ng/mL (0.13 μM) did not affect E2 production (Fig. 3.5), but at 5 μg/mL (6.9 μM) increased E2 production (Fig. 3.11), whereas, at the same concentration (i.e., 5 μg/mL corresponding to 6.9 μM) both α-ZEA and β-ZEA alone inhibited E2 production (Fig. 3.11). The combination of FB_1 at 5 μg/mL (6.9 μM) with α-ZEA at 5 μg/mL (15.6 μM) and β-ZEA at 5 μg/mL (15.6 μM) confirms the inhibition of E2 production induced by these two ZEA metabolites (Fig. 3.11). Indeed, FB_1 combined with α-ZEA at 5 μg/mL (15.6 μM) had no effect on E2 production, whereas in combination with β-ZEA at 5 μg/mL (15.6 μM) an inhibitory effect on E2 release was observed. It is well established that estrogenic α-ZEA and β-ZEA effects are mediated by binding to intracellular estrogen receptors (Parveen et al., 2009). The inhibition on E2 production of ZEA major metabolites, clearly highlighted as a result of co-exposure with FB_1, suggests an interference on aromatase activity. FB_1 at 30 ng/ml (0.042 μM) and 100 ng/mL (0.13 μM) either with or without β-ZEA had no effect on the inhibition of E2 production induced by DON at 100 ng/mL (0.33 μM) (Fig. 3.8), an effect previously reported (Pizzo et al., 2016). The BEA-induced marked inhibition of E2 production by GC was associated with a significant decrease in *CYP19A1* mRNA abundance, suggesting that BEA may alter E2 production via suppression of *CYP19A1* mRNA abundance. Previous studies demonstrated a role of CART (cocaine and amphetamine regulated transcript system) system on the regulation of bovine GC E2 production (Kobayashi et al., 2006; Lv et al., 2009). This effect was linked to the inhibition induced by CART on several components of the FSH signal transduction pathway (Lv et al., 2009) that, since FSH stimulates E2 production, may explain the negative effects of CART observed on bovine GC (Lv et al., 2009). This mechanism of action could be involved for the inhibitory effects of BEA on

E2 production and GC *CYP19A1* mRNA levels observed in the present study. BEA was found to reversibly inhibit L-type calcium channels in a dose-dependent manner in a neuronal cell line (Wu, 2002) and FSH-induced increase in GC calcium uptake is mediated via inhibition of L-type calcium channel activity (Peters et al., 2004; Kobayashi et al., 2006), but whether blockade of calcium channels is involved in BEA-induced inhibition of E2 production will require further study.

The present study has been carried out with FB_1 alone and in co-exposure firstly with DON and ZEA metabolites (α-ZEA and β-ZEA) and secondly with BEA, an emerging mycotoxin, demonstrating effects on cell proliferation and steroidogenesis using a bovine GC *in vitro* model, suggesting possible reproductive effects of these mycotoxins in cattle.

3.5 References

Aad, P.Y., Voge, J.L., Santiago, C.A., Malayer, J.R., Spicer, L.J. (2006). Real-Time RT-PCR Quantification of pregnancy- associated plasma protein-A mRNA abundance in bovine granulosa and theca cells: effects of hormones *in vitro*. *Domestic Animal Endocrinology, 31: 357-372.*

Abidin, Z., Khatoon, A. (2012). Ruminal microflora, mycotoxin inactivation by ruminal microflora and conditions favouring mycotoxicosis in ruminants: a review. International *Journal of Veterinary Science, 1: 37-44.*

Ainsworth, L., Tsang, B.K., Downey, B.R., Marcus, G.J. (1990). The synthesis and actions of steroids and prostaglandins during follicular maturation in the pig. *Journal of Reproduction and Fertility, 40: 137-150.*

Allegood, W.C.A., Martìnez, J., Maddox, C., Miller, J., Starr, J.D., Sullards, L., Roman, M., Voss, A.V., Wang, K.A., Merrill, E. (2004). Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and *in vivo*: a potential risk factor for human neural tube defects among populations consuming fumonisin- contaminated maize. *Journal of Nutrition, 134: 711-716*.

Akande, K.E., Abubakar, M.M., Adegbola, T.A., Bogoro, S.E. (2006). Nutritional and Health Implication of Mycotoxins in Animal Feed: a Review. *Pakistan Journal of Nutrition, 5:398-403.*

Baraño, J.L.S., Hammond, J.M. (1985). Serum-free medium enhances growth and differentiation of cultured pig granulosa cells. *Endocrinology, 116: 51-58.*

Biehl, M., Prelusky, D., Koritz, G., Hartin, K., Buck, W., Trenholm, H. (1993). Bilary excretion and enterohepatic cycling of zearalenone in immature pigs. *Toxicological Application and Pharmacology, 121: 152-159.*

Böhm, J., Razzazi-Fazeli, E. (2005). Effects of mycotoxins on domestic pet species. *In The Mycotoxin Blue Book; Diaz, D., Ed. Nottingham University Press: Nottingham, U.K., pp 77-91.*

Boutigny, A.L., Beukes, .I, Small, I., Zuhlke, S., Spiteller, M., Van Rensburg, B.J., et al. (2012). Quantitative detection of *Fusarium* pathogens and their mycotoxins in South African maize. *Plant Pathology, 61: 522–31.*

Caloni, F., Spotti, M., Pompa, G., Zucco, F., Stammati, A., De Angelis, I. (2002). Evaluation of Fumonisin B_1 and its metabolites absorption and toxicity on intestinal cells line Caco-2. *Toxicon, 40: 1181–1188.*

Caloni, F., Ranzenigo, G., Cremonesi, F., Spicer, L.J. (2009). Effects of a trichothecene, T-2 toxin, on proliferation and steroid production by porcine granulosa cells. *Toxicon, 54: 337-44.*

Cheeke, P.R. (1998). Natural Toxicants in Feeds and Poisonous Plants, *2nd Ed. Interstate Publishers, Danville, United States, pp. 116–122.*

Cortinovis, C., Caloni, F., Schreiber, N. B., Spicer. L.J. (2014). Effects of fumonisin B1 alone and combined with deoxynivalenol or zearalenone on porcine granulosa cell proliferation and steroid production. *Theriogenology 81: 1042-1049.*

Dersjant-Li, Y., Verstegen, M.W.A., Gerrits, W.J.J. (2003). The impact of low concentrations of aflatoxin, deoxynivalenol or fumonisin in diets on growing pigs and poultry. *Nutrition Research Reviews, 16: 223-239.*

Diekman, M.A., Green, M.L. (1992). Mycotoxins and reproduction in domestic livestock. *Journal of Animal Science, 70: 1615-1627.*

EFSA (European Food Safety Authority). (2005). Opinion of the Scientific Panel on Contaminants in Food Chain on a request from the Commission related to fumonisins as undesirable substances in animal feed. *EFSA Journal, 235: 1-32.*

EFSA (European Food Safety Authority). (2014). Scientific Opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed. *EFSA Journal, 12: 3802.*

Fernández-Blanco, C., Frizzell, C., Shannon, M., Ruiz, M., Connolly, L. (2016). An *in vitro* investigation on the cytotoxic and nuclear receptor transcriptional activity of the mycotoxins fumonisin B_1 and beauvericin. *Toxicology Letters, 257: 1–10.*

Ferrer, E., A. Juan-García, G. Font, and M. J. Ruiz. (2009). Reactive oxygen species induced by beauvericin, patulin and zearalenone in CHO-K1 cells. Toxicology *in Vitro*, *23: 1504–1509.*

Ferrigo, D., Raiola, A., and Causin, R. (2016). *Fusarium* Toxins in Cereals: Occurrence, Legislation, Factors Promoting the Appearance and Their Management. *Molecules, 21: 627.*

Fink-Gremmels, J., (2008). The role of mycotoxins in the health and performance of dairy cows. *The Veterinary Journal, 176: 84–92.*

Frizzell, C., Ndossi, D., Verhargen, S., Dahl, E., Eriksen, G., Sorlie, M., Ropstad, E., Muller, M., Elliott, C.T., Connolly, L. (2011). Endocrine disruption effects of zearalenone, alpha and beta zearalenol at the level of nuclear receptor binding and steroidogenesis. *Toxicological Letter, 206: 210-217.*

Gbore, F.A., Owolawi, T.J., Erhunwunsee, M., Akele, O., Gabriel-Ajobiewe, R.A.O. (2012). Evaluation of the reproductive toxicity of dietary fumonisin B1 in rats Jordan. *Journal of Biological Science, 5: 183–90.*

Gumprecht, L.A., Marcucci, A., Weigel, R.M., Vesonder, R.F., Riley, R.T., Showker, J.L et al. (1995). Effects of intravenous fumonisin B1 in rabbits: nephrotoxicity and sphingolipid alterations. *Natural Toxins, 3: 395–403.*

Harrison, L.R., Colvin, B.M., Greene, J.T., Newman, L.E., Cole, Jr. J.R. (1990). Pulmonary edema and hydrothorax in swine produced by fumonisin B1, a toxic metabolite of *Fusarium* moniliforme. *Journal of Veterinary Diagnostic Investigation, 2:217–21.*

Hsueh, A.J., Adashi, E.Y., Jones, P.B., Welsh Jr., T.H. (1984). Hormonal regulation of the differentiation of cultured ovarian granulosa cells. *Endocrinology Reviews, 5: 76-127.*

Jestoi, M., (2008). Emerging Fusarium-mycotoxins fusaproliferin, beauvericin, enniatins, and moniliformin: a review. Critical Reviews in Food and Science Nutrition, *48:21-49.*

Kellerman, T.S., Marasas, W.F.O., Thiel, P.G., Gelderblom, W.C., Cawood, M., Coetzer, J.A. (1990). Leukoencephalomalacia in two horses induced by oral dosing of fumonisin B1. *Onderstepoort Journal of Veterinary Research, 57: 269-275.*

Klarić, M. K., Rumora, L., Ljubanović, D., and Pepeljnjak, S. (2008). Cytotoxicity and apoptosis induced by fumonisin B_1, beauvericin and ochratoxin A in porcine kidney PK15 cells: effects of individual and combined treatment. *Archives of Toxicology, 82: 247–255.*

Kobayashi, Y., F. Jimenez-Krassel, J. J. Ireland and G. W. Smith. (2006). Evidence of a local negative role for cocaine and amphetamine regulated transcript (CART), inhibins and low molecular weight insulin like growth factor binding proteins in regulation of granulosa cell estradiol production during follicular waves in cattle. *Reproduction Biology and Endocrinology, 4:22.*

Lagaly, D.V., Aad, P.Y., Grado-Ahuir, J.A., Hulsey, L.B., Spicer, L.J. (2008). Role of adiponectin in regulating ovarian theca and granulosa cell function. *Molecular and Cellular Endocrinology, 284: 38-45.*

Langhout, D.J., Spicer, L.J., Geisert, R.D. (1991). Development of a culture system for bovine granulosa cells: effects of growth hormone, estradiol, and gonadotropins on cell proliferation, steroidogenesis, and protein synthesis. *Journal of Animal Science, 69: 3321-3334.*

Leslie, J. F., and Summerell B. A. (2006). The *Fusarium* Laboratory Manual. *Blackwell Publishing, Ames, IA, USA. p 388.*

Lombaert, G.A., Pellaers, P., Roscoe, V., Mankotia, M. Neil, R., Scott, P.M. (2003). Mycotoxins in infant cereal foods from the Canadian retail market. *Food Additives and Contaminants, 20: 494–504.*

Luongo, D., De Luna, R. Russo, R., Severino, L. (2008). Effects of four *Fusarium* toxins (fumonisin B_1, α-zearalenol, nivalenol and deoxynivalenol) on porcine whole-blood cellular proliferation. *Toxicon, 52: 156–162.*

Lv, L., Jimenez-Krassel, F., Sen, A., Bettegowd, A., Mondal, M., Folger, J.K., Lee, K.B., Ireland, J.J., Smith, G.W. (2009). Evidence supporting a role for cocaine- and amphetamine-regulated tran Biology of Reproduction script (CARTPT) in control of granulosa cell estradiol production associated with dominant follicle selection in cattle. *Biology of Reproduction, 81: 580-6.*

Malekinejad H., Schoevers E.J., Daemen I.J.J.M., Zijstra C., Colenbrander B., Fink-Gremmels J., Roelen B.A.J. (2007). Exposure of oocytes to the *Fusarium* toxins

zearalenone and deoxynivalenol causes aneuploidy and abnormal embryo development in pigs. *Biology of Reproduction, 77: 840-847.*

Mallebrera, B., Juan-Garcia, A., Font, G., Ruiz, M. (2016). Mechanisms of beauvericin toxicity and antioxidant cellular defense. *Toxicology Letters, 246: 28–34.*

Marasas, W.F.O., Riley, R.T., Hendricks, K.A., Stevens, V.L., Sadler, T.W., Gelineau-van Waes, J., Missmer, S.A., Cabrera, J., Torres, O., Gelderblom,

Mathur, S., P.D. Constable, R.M. Eppley, A.L. Waggoner, M.E. Tumbleson, W.M. Haschek. (2001). Fumonisin B(1) is hepatotoxic and nephrotoxic in milk-fed calves. *Toxicological Sciences, 60: 385–396.*

Medvedova, M., Kolesarova, A., Capcarova, M., Labuda, R., Sirotkin, A.V., Kovacik, J. et al. (2011). The effect of deoxynivalenol on the secretion activity, proliferation and apoptosis of porcine ovarian granulosa cells in *vitro. Journal of Environmental Science and Health Part B Pesticides Food Contaminants and Agricultural Wastes, 46: 213–9.*

Merrill Jr, A.H., M.C. Sullards, E. Wang, K.A. Voss, R.T. Riley. (2001). Sphingolipid metabolism: roles in signal transduction and disruption by fumonisins. *Environmental Health Perspectives, 109:283-289.*

Mirocha, C.J., Pathre, S.V., Christensen, C.M. (1977). Zearalenone. *In: Rodricks JV, Hesseltine CW, Mehlman MA, editors. Mycotoxins in human and animal health. Park Forest South, IL, USA: Pathotox Publisher Inc. p. 346–64.*

Minervini, F., Dell'Aquila, M.E. (2008). Zearalenone and reproductive function in farm animals. *International Journal of Molecular Science, 9: 2570–84.*

Ndossi, D.G., Frizzell, C., Tremoen, N.H., Fæste, C.K., Verhaegen, S., Dahl, E et al. (2012). An in vitro investigation of endocrine disrupting effects of trichothecenes deoxynivalenol (DON), T-2 and HT-2 toxins. *Toxicology Letters, 214: 268–78.*

Olsen, M. (1989). Metabolism of zearalenone in farm animals. *In: Chelkowski, J. (Ed.), Fusarium: Mycotoxins, Taxonomy and Pathogenicity. Elsevier, Amsterdam, 167-177.*

Oswald, I.P., Marin, D.E., Bouhet, S., Pinton, P., Taranu, I., Accensi, F. (2005). Immunotoxicological risk of mycotoxins for domestic animals. *Food Additives and Contaminants, 22: 354–360.*

Osweiler, G.D. (2000). Mycotoxins-contemporary issues of food animal health and productivity. *Veterinary Clinic of North America, 16: 511e530.*

Osweiler, G.D., M.E. Kehrli, J.R. Stabel, J.R. Thurston, P.F. Ross and T.M. Wilson. (1993). Eject of fumonisin-contaminated corn screenings on growth and health of feeder calves. *Journal of Animal Science, 71: 459-466.*

Parveen, M., Zhu, Y., Kiyama, R. (2009). Expression profiling of the genes responding to zearalenone and its analogues using estrogen responsive genes. *FEBS Letters, 583: 2377-2384.*

Peters, M. W., J. R. Pursley, and G.W. Smith. (2004). Inhibition of intrafollicular PGE2 synthesis and ovulation following ultrasound-mediated intrafollicular injection of the selective cyclooxygenase-2 inhibitor NS-398 in cattle. *Journal of Animal Science, 82: 1656-1662.*

Petro, E.M., Leroy, J.L., Van Cruchten, S.J., Covaci, A., Jorssen, E.P., Bols, P.E. (2012). Endocrine disruptors and female fertility: focus on (bovine) ovarian follicular physiology. *Theriogenology, 78: 1887–900.*

Pinton, P., Oswald, I.P. (2014). Effect of Deoxynivalenol and Other Type B Trichothecenes on the Intestine: A Review. *Toxins, 6: 1615-1643.*

Pizzo, F., Caloni, F., Schreiber, N.B., Cortinovis, C., Spicer, L.J. (2016). *In vitro* effects of deoxynivalenol and zearalenone major metabolites alone and combined, on cell proliferation, steroid production and gene expression in bovine small-follicle granulosa cells. *Toxicon, 109:70-83.*

Placinta, C.M., D'Mello, J.P.F., Macdonald., A.M.C.A. (1999). Review of worldwide contamination of cereal grains and animal feed with *Fusarium* mycotoxins. *Animal Feed and Science Technology. 78: 21–37.*

Prosperini, A., Meca, G., Font, G., Ruiz, M.J. (2012). Study of the cytotoxic activity of beauvericin and fusaproliferin and bioavailability *in vitro* on Caco-2 cells. *Food Chemical and Toxicology, 50: 2356-2361.*

Ranzenigo, G., Caloni, F., Cremonesi, F., Aad, P.Y., Spicer, L.J. (2008). Effects of *Fusarium* mycotoxins on steroid production by porcine granulosa cells. *Animal Reproduction Science, 107: 115–30.*

Rawan, A.F., Yoshioka, S., Abe, H., Acosta, T.J. (2015). Insulin-like growth factor-1 regulates the expression of luteinizing hormone receptor and steroid production in bovine granulosa cells. *Reproduction of Domestic Animals, 50: 283-91.*

Raymond, S.L., Smith, T.K., Swamy, H. V. L. N. (2005). Effects of feeding a blend of grains naturally contaminated with *Fusarium* mycotoxins on feed intake, metabolism, and indices of athletic performance of exercised horses. *Journal of Animal Science, 83: 1267-1273.*

Richard, J.L. (2007). Some major mycotoxins and their mycotoxicosis-an overview. *International Journal of Food and Microbiology, 119: 3-10.*

Riley, R.T., Norred,W.P. (1996). Mechanism of mycotoxicity. *In: Howard, D.H., Miller, J.D. Eds. The Mycota, vol. VI. Springer-Verlag, New York, NY, USA, p. 193.*

Rodrigues, I., Naehrer, K. (2012). A three-year survey on the worldwide occurrence of mycotoxins in feedstuffs and feed. *Toxins, 4: 663–75.*

Rotter, B.A., Prelusky, D.B., Pestka, J.J. (1996). Toxicology of deoxynivalenol (vomitoxin). *Journal of Toxicology and Environmental Health Science, 48: 1–34.*

Ruiz, M.J., Macáková, P. Juan García, A., Font, G. (2011). Cytotoxic effects of mycotoxin combinations in mammalian kidney cells. *Food and Chemical Toxicology, 49: 2718–2724.*

Schollenberger, M., Muller, H.M., Rufle, M., Terry Jara, H., Suchy, S., Plank, S., Drochner,W. (2007). Natural occurrence of *Fusarium* toxins in soy food marketed in Germany. *International Journal of Food and Microbiology, 113: 142e146.*

Scott, P.M. (1997). Multi-year monitoring of Canadian grains and grain-based foods for trichothecenes and zearalenone. *Food Additives and Contaminants, 14: 333–339.*

Seeling, K., Danicke, S., Valenta, H., Van Egmond, H.P., Schothorst, R.C., Jekel, A.A., Lebzien, P., Schollenberger, M., Razzazi Fazeli, E., Flachowsky, G. (2006). Effects of *Fusarium* toxin contaminated wheat and feed intake level on the

biotrasformation and carryover of deoxynivalenol in dairy cows. *Food Additives and Contaminants, 23: 1008-1020.*

Smith, G.W. (2012). Fumonisins. In: Gupta R.C. (Ed.), Veterinary Toxicology: Basic and Clinical Principles (second edition), *Elsevier Inc., pp. 1205-1219.*

Spicer, L.J., Aad, P.Y. (2007). Insulin-like growth gactor (IGF) 2 stimulates steroidogenesis and mitosis of bovine granulosa cells through the IGF1 receptor: role of follicle-stimulating hormone and IGF2 receptor. *Biology of Reproduction, 77: 18-27.*

Spicer, L.J., Alonso, J., Chamberlain, C.S., (2001). Effects of thyroid hormones on bovine granulosa and thecal cell function in vitro: dependence on insulin and gonadotropins. *Journal of Dairy Science, 83: 1069-1076.*

Spicer, L.J., Alpizar, E., Echternkamp, S.E. (1993). Effects of insulin, insulin like growth factor I, and gonadotropins on bovine granulosa cell proliferation, progesterone production, estradiol production, and (or) insulin like growth factor I production in vitro. *Journal of Animal Science, 71: 1232-1241.*

Spicer, L.J., Chamberlain, C.S., Maciel, S.M. (2002). Influence of gonadotropins on insulin and insulin like growth factor 1 (IGF1) induced steroid production by bovine granulosa cells. *Domestic Animal Endocrinology, 22: 237-254.*

Spicer, L.J., Chamberlain, C.S. (1998). Influence of cortisol on insulin- and insulin-like growth factor 1 (IGF1)-induced steroid production and on IGF1 receptors in cultured bovine granulosa cells and thecal cells. *Endocrine, 9: 153-161.*

Tiemann, U., Dänicke, S. (2007). *In vivo* and *in vitro* effects of the mycotoxins zearalenone and deoxynivalenol on different nonreproductive and reproductive organs in female pigs: A review. *Food Additives and Contaminants, 24: 306–314.*

Tiemann, U., Tomek, W., Schneider, F., Vanselow, J. (2003). Effects of the mycotoxins α and β-zearalenol on regulation of progesterone synthesis in cultured granulosa cells from porcine ovaries. *Reproductive Toxicology, 17: 673-681.*

Trenholm, H.L., Hamilton, R.M., Friend, D.W., Thompson, B.K., Hartin, K.E. (1984). Feeding trials with vomitoxin (deoxynivalenol)-contaminated wheat: effects on swine, poultry, and dairy cattle. *Journal of the American Veterinary Medical Association, 85: 527-531.*

Uhlig, S., M. Jestoi, and Parikka, P. (2007). *Fusarium avenaceum*—the North European situation. *International Journal of Food and Microbiology, 119: 17–24.*

Upadhaya, S.D., Park, M.A., Ha, J.K. (2010). Mycotoxins and their biotrasformation in the rumen: a review. *Asian-Australasian Journal of Animal Sciences, 23: 1250-1260.*

Voge, J.L., Santiago, C.A., Aad, P.Y., Goad, D.W., Malayer, J.R., Spicer, L.J. (2004). Effect of insulin like growth factors (IGF), FSH, and leptin on IGF-binding-protein mRNA expression in bovine granulosa and theca cells: quantitative detection by real-time PCR. *Peptides, 25: 2195-2203.*

Voss, K.A., Howard, P.C., Riley, R.T., Sharma, R.P., Bucci, T.J., Lorentzen, R.J. (2002). Carcinogenicity and mechanism of action of fumonisin B1: a mycotoxin produced by Fusarium moniliforme (= F. verticillioides). *Cancer Detection and Prevention, 26: 1–9.*

Voss, K.A., Smith, G.W., Haschek, W.M. (2007). Fumonisins: Toxicokinetics, mechanism of action and toxicity. *Animal Feed Science and Technology, 137: 299-325.*

Wang, E., W.P. Norred, C.W. Bacon, R.T. Riley, A.H. Merrill Jr. (1991). Inhibition of sphingolipid biosynthesis by fumonisins. Implications for diseases associated with Fusarium moniliforme. *The Journal of Biological Chemistry, 266: 14486-14490.*

Wang, E., Ross, P.F., Wilson, T.M., Riley, R.T., Merrill, A.H. (1992). Increases in serum sphingosine and sphinganine and decreases in complex sphingolipids in ponies given feed containing fumonisins, mycotoxins produced by *Fusarium moniliforme*. *Journal of Nutrition, 122: 1706-1716.*

Wangikara, P.B., Dwivedia. P., Neeraj Sinhab, Sharmaa, A.K. Telanga, A.G. (2005). Teratogenic effects in rabbits of simultaneous exposure to ochratoxin A and aflatoxin B_1 with special reference to microscopic effects. *Toxicology, 215: 37–47.*

Winkler, J., Kersten, S., Meyer, U., Engelhardt, U., D€anicke, S., (2014). Residues of zearalenone (ZEN), deoxynivalenol (DON) and their metabolites in plasma of dairy cows fed Fusarium contaminated maize and their relationships to performance parameters. *Food Chemistry and Toxicology, 65: 196e204.*

Wu, S.N., C. Hsinyo, Y.C. Liu, and H.T. Chiang. (2002). Block of L-type Ca2+ current by beauvericin, a toxic cyclopeptide, in the NG108-15 neuronal cell line. *Chemical Research and Toxicology, 15: 854–860.*

Zinedine, A., Soriano, J.M., Molto, J.C., Manes, J. (2007). Review on the toxicity, occurence, metabolism, detoxification, regulation and intake of zearalenone: an estrogenic mycotoxin. *Food and Chemical Toxicology, 45: 1-18.*

4. Effects of fumonisin B_1 alone and combined with beauvericin on Caco-2 *in vitro* intestinal model

4.1 Introduction

Mycotoxins are secondary metabolites produced by molds, such as *Aspergillus*, *Fusarium* and *Penicillium* (Osweiler, 2000). *Fusarium* mycotoxins are world-spread mycotoxins naturally occurring in cereals (Jurjevic, 2002; Logrieco et al., 2003; Domijan et al., 2005; Molinié et al., 2005; Ferrigo et al., 2016) and animal and human exposure is well established (Diekman and Green 1992; Binder et al., 2007; Fink-Gremmels, 1999; Prosperini, et al., 2012; Wu et al., 2014; EFSA 2014).

Fumonisins are a group of mycotoxins mainly produced by *Fusarium verticillioides*, *Fusarium proliferatum*, *Fusarium napiforme* and *Fusarium nygamai* that have been shown to occur worldwide primarily in corn (Glenn, 2007; Voss et al., 2007).

The most important group of fumonisins is the B series including fumonisin B_1 (FB_1), B_2 (FB_2) and B_3 (FB_3) (EFSA 2005). In 2002 the IARC classified FB_1 as possibly carcinogenic to humans (group 2B) (IARC, 2002; Santini et al., 2015; Hove et al., 2016) and several studies reported that FB_1 is associated with an increased prevalence of esophageal cancer in humans (Marasas et al., 1988; Sydenham et al. 1990; Rheder et al., 1992; Hove et al., 2016).

The complex mechanism of action of FB_1 is based on the inhibition of sphingosine (sphinganine) N-acetyltransferase (ceramide synthase) (Wang et al., 1991; Voss et al., 2002, 2007; Luongo et al. 2008; Gbore et al., 2012) with the consequent alteration of the sphingolipid metabolism (Wang et al., 1991; Wang et al; 1992; Merrill Jr. et al., 2001; Marasas et al., 2004; Voss et al., 2007; Smith, 2012). Specifically FB_1, structurally similar to sphingoid bases, inhibits ceramide synthase and thus disrupts the *de novo* biosynthesis of ceramide and sphingolipid metabolism (Wang et al., 1991). FB_1 is a competitive inhibitor with respect to both substrates of ceramide synthase (Merril Jr. et al., 2001; Marasas et al., 2004; Voss et al., 2007). This leads to the blockage of complex sphingolipid biosynthesis, essential for cell regulation, and to the accumulation of sphinganine, and, to a lesser degree, sphingosine (Wang et al., 1992; Merril Jr. et al., 2001; Marasas et al., 2004; Smith, 2012).

Fumonisins have been found to commonly occur in cereal grains and animal feed in combination with other *Fusarium* mycotoxins including beauvericin (BEA), a so-called emerging mycotoxin (Jestoi, 2008; EFSA, 2014).

BEA is a ionophoric molecule that can form lipophilic complexes with cations and transport them into biological membranes altering cell homeostasis (Hilgenfeld and Saenger, 1982; Jestoi et al., 2008; Prosperini et al., 2012; Fernandez-Blanco et al., 2016). In fact, it is well-known that an increase in the intracellular concentration of cations such as calcium and the subsequent activation of calcium-dependent endonucleases lead to DNA fragmentation which is involved in apoptosis (Speijers and Speijers, 2004; Kouadio et al., 2007).

Recent studies reported the *in vitro* adverse effects of BEA on human, porcine and rodent cell lines (Jestoi, 2008; Prosperini et al., 2012; Fernandez Blanco et al., 2016; Mallebrera et al., 2016). Schoevers et al. (2016) studied the effects of BEA on porcine cumulus cells, oocytes and embryos. In this study the cumulus-oocyte-complexes and developing embryos were exposed to BEA at concentrations ranging from 0.31 to 10 μM and the effects of this mycotoxin on viability, progesterone synthesis and apoptosis were evaluated (Schoevers et al., 2016). Schoevers et al. (2016) demonstrated that BEA reduced the developmental competence of both the maturing oocytes and the two-four cell stage embryos in pigs, and that BEA only affected the rate of developing embryos at concentrations exceeding 0.5 μM. BEA toxic effects on human cell lines were investigated by Prosperini et al. (2012). The authors evaluated the cytotoxicity of BEA on human Caco-2 cells testing concentrations ranging from 0.6 to 30 μM. The IC_{50} obtained for BEA was 20.62 ± 6.9 μM at 24 h of exposure and 12.75 ± 4.8 at 48 h (Prosperini et al., 2012).

Recently, exposure of Caco-2 cells to 0.001–10 μM BEA showed that BEA at 1 μM and 10 μM had cytotoxic effects on Caco-2 cells (Fernandez-Blanco et al., 2016).

The *in vitro* absorption and toxicity of FB_1 have been investigated with different intestinal models (Caloni et al., 2002; Bouhet et al., 2004; De Angelis et al., 2005; Loiseau et al., 2007; Ulluwishewa et al., 2011; Minervini et al., 2014; Romero et al., 2016; Wentzel et al., 2016). *In vitro* studies with Caco-2 cell monolayers (Caloni et al., 2002) demonstrated low toxicity of FB_1 and its metabolites after 48 h of exposure to concentrations ranging from 1 to 138 μM, even though FB_1 appeared to be the most active. The results of a subsequent study carried out with Caco-2 cells cultured on inserts (Caloni et al., 2005) found that FB_1 was not absorbed by Caco-2 cells and did not affect the barrier integrity. Recently, exposure of Caco-2 cells to 0.001–10 μM FB_1 revealed no cytotoxic effect of FB_1 at all the concentrations tested (Fernandez-Blanco et al., 2016).

In IPEC-1, a porcine cell line, a significant TEER decrease was reported after 13 days of exposure to FB_1 (50 μM) (Bouhet et al., 2004).

The aim of this study was to clarify the *in vitro* effects of FB_1 and BEA, alone and combined, on human intestinal Caco-2 cells cultured on inserts through the evaluation of TEER and cytokine release.

4.2 Materials and methods

4.2.1 Chemicals

Dulbecco's Modified Eagles Medium (DMEM) high glucose, heat inactivated fetal bovine serum (FBS), glutamine, non-essential amino acids (NEAA), N-2-hydroxyethylpiperazine-N-2'-ethanesulfonic acid (HEPES), penicillin/streptomycin were all purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA).
Fumonisin B_1, beauvericin and methanol were all obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

4.2.2 Cell culture

Caco-2 cells were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) and routinely grown in an humidified atmosphere of 5% carbon dioxide at 37°C in DMEM high glucose standard medium supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin, 4 mmol/l glutamine, 1% NEAA, 10 mM HEPES and 10% heat inactivated FBS. Starting from a cell suspension in culture medium (3×10^5 cells/ml), the cells were seeded at a density of 1.5×10^5 cells/filter on 4 μm pore size 12-well plate polyethylene terephthalate (PET) transparent membrane inserts (Millicell®, Millipore Corporation) (Fig. 4.1); 0.5 ml of cellular suspension were added in the apical (Ap) compartment and 1.5 ml of supplemented DMEM in the basolateral (Bl) compartment of each insert. The plate was then shaken gently to avoid non uniform cell distribution.
The plates were transferred to a 37°C, 5% CO_2 incubator. Cells were allowed to differentiate for 21 days with regular medium changes three times per week.
The following procedure was used to change the medium on the inserts: first the medium from the Bl side of a single well was removed, then the medium from the Ap side of the same well was slowly removed avoiding to touch the cellular monolayer with the pipet tip; the medium in the Ap compartment was replaced (0.5 ml) first, then in the Bl compartment (1.5 ml). This procedure was repeated for each well. The culture was regularly checked with an inverted microscope to identify contaminations and/or morphological variations. Treatments were applied at the end of the differentiation process.

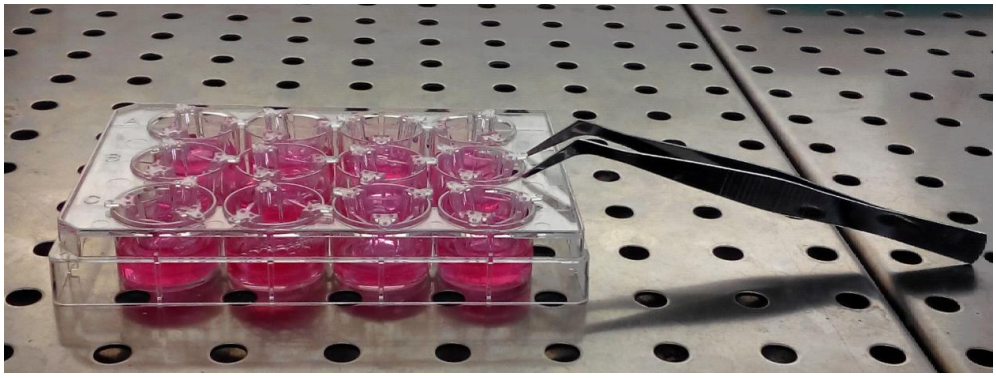

Figure 4.1: Caco-2 cells cultured on 12 well plate inserts

4.2.3 Barrier Integrity Assessment (Trans Epithelial Electrical Resistance Evaluation)

Barrier integrity was assessed by measuring the trans-epithelial electrical resistance (TEER) which quantifies the movement of ions across the cell barrier. TEER values were recorded in the culture medium at 37°C using an epithelial volthommeter (Millicell-ERS, Millipore; Fig: 24) just before (0 h) and 1, 2 and 24 h after treatment.
Before the measurement, the chop-stick electrodes were sterilized. After sterilization, the electrodes were placed in the culture medium, both in the Ap and Bl compartments. Three separate measurements were quickly performed for each filter and the TEER values were calculated with the following equation:

TEER = (Ω cell monolayer – Ω filter cell-free) x filter area

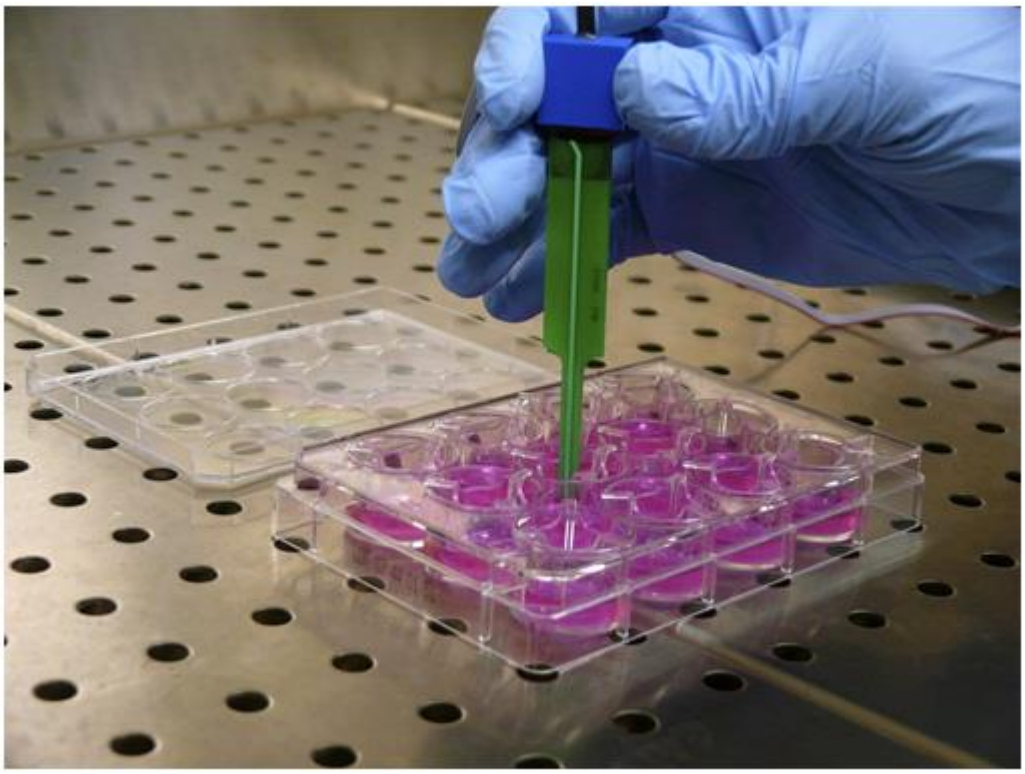

Figure 4.2: measurement of TEER

4.2.4 Measurement of pro-inflammatory mediator release

After 24 hours of treatment, culture medium was collected from individual wells and frozen at -80°C for subsequent pro-inflammatory cytokine release determination.
In all experiments, the culture medium was analysed for the presence of interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor-α (TNF-α) using commercially available quantitative ELISA assay kits (Sigma-Aldrich Chemical Company, St. Louis, MO, USA).

4.2.4.1 IL-6 determination

The ELISA procedure was performed according to the manufacturer's instructions of the Human IL-6 ELISA Kit (Sigma-Aldrich Chemical Company, St. Louis, MO, USA).
Briefly, all reagents and samples were taken to room temperature (18-25°C) before use. 100 µl of each standard and sample were placed into appropriate wells, covered and incubated for 2.5 h at room temperature. All standards were performed in duplicate.
After incubation, the solution was discarded and the wells were washed 4 times with 1x Wash Buffer (300 µl). After the washing step, 100 µl of 1x prepared Biotinylated

Detection Antibody were introduced in each well and the plate was incubated for 1 h at room temperature. The solution was then discarded and the washing step repeated. Successively, 100 µl of prepared HRP-Streptavidin Solution were added to each well and incubation was carried out for 45 minutes. The solution was then discarded and after the washing step, 100 µl of ELISA Colorimetric TMB Reagent were added to each well. After 30 minutes of incubation, 50 µl of Stop Solution were introduced in each well and the optical density (OD) was immediately measured at 450 nm by an ELISA reader (Multiskan GO microplate spectrophotometer, Thermo Scientific, Waltham, MA, USA).
A calibration curve was constructed with eight standard concentrations: 0 pg/ml – 1.37 pg/ml – 4.12 pg/ml – 12.35 pg/ml – 37.04 pg/ml – 111.1 pg/ml – 333.3 pg/ml – 1000 pg/ml. After the subtraction of the average zero standard OD, IL-6 sample concentration was measured by interpolation from the calibration curve.

4.2.4.2 IL-8 determination

The ELISA procedure was performed according to the manufacturer's instructions of the Human IL-8 / CXCL8 ELISA Kit (Sigma-Aldrich Chemical Company, St. Louis, MO, USA).
Briefly, all reagents and samples were taken to room temperature (18-25°C) before use. 100 µl of each standard and sample were placed into appropriate wells, covered and incubated for 2.5 h at room temperature.
All standards were performed in duplicate and samples were diluted (1:3).
After incubation, the solution was discarded and the wells were washed 4 times with 1x Wash Buffer (300 µl). After the washing step, 100 µl of 1x prepared Biotinylated Detection Antibody were introduced in each well and the plate was incubated for 1 h at room temperature. The solution was then discarded and the washing step repeated. Successively, 100 µl of prepared HRP-Streptavidin Solution were added to each well and incubation was carried out for 45 minutes. The solution was then discarded and after the washing step, 100 µl of ELISA Colorimetric TMB Reagent were added to each well. After 30 minutes of incubation, 50 µl of Stop Solution were introduced in each well and the optical density (OD) was immediately measured at 450 nm by an ELISA reader (Multiskan GO microplate spectrophotometer, Thermo Scientific, Waltham, MA, USA).
A calibration curve was constructed with eight standard concentrations: 0 pg/ml – 0.8 pg/ml – 2.5 pg/ml – 7.4 pg/ml – 22.2 pg/ml – 66.7 pg/ml – 200 pg/ml – 600 pg/ml. After the subtraction of the average zero standard OD, IL-8 sample concentration was measured by interpolation from the calibration curve.

4.2.4.3 Tumor Necrosis Factor α determination

The ELISA procedure was performed according to the manufacturer's instructions of the Human Tumor Necrosis Factor α ELISA Kit (Sigma-Aldrich Chemical Company, St. Louis, MO, USA).
Briefly, all reagents and samples were taken to room temperature (18-25°C) before use. 100 µl of each standard and sample were placed into appropriate wells, covered and incubated for 2.5 h at room temperature. All standards were performed in duplicate. After incubation, the solution was discarded and the wells were washed 4 times with 1x Wash Buffer (300 µl). After the washing step, 100 µl of 1x prepared Biotinylated Detection Antibody were introduced in each well and the plate was incubated for 1 h at room temperature. The solution was then discarded and the washing step repeated. Successively, 100 µl of prepared HRP-Streptavidin Solution were added to each well and incubation was carried out for 45 minutes. The solution was then discarded and after the washing step, 100 µl of ELISA Colorimetric TMB Reagent were added to each well. After 30 minutes of incubation, 50 µl of Stop Solution were introduced in each well and the optical density (OD) was immediately measured at 450 nm by an ELISA reader (Multiskan GO microplate spectrophotometer, Thermo Scientific, Waltham, MA, USA).
A calibration curve was constructed with eight standard concentrations: 0 pg/ml – 24.58 pg/ml – 61.44 pg/ml – 153.6 pg/ml – 384 g/ml – 960 pg/ml – 2400 pg/ml – 6000 pg/ml. After the subtraction of the average zero standard OD, TNF-α sample concentration was measured by interpolation from the calibration curve.

4.2.5 Experimental Design

Experiment 1 was performed to evaluate the dose response of fumonisin B_1 (FB_1) on Caco-2 barrier integrity and the release of the pro-inflammatory cytokines IL-6, IL-8 and TNF-α. Caco-2 cells were cultured as previously described and, after differentiation, cells were treated with different concentrations of FB_1 (0, 0.5, 1.5, 3 µM) in triplicate from both Ap and Bl sides. The barrier integrity was evaluated after 1, 2 and 24 h of exposure to FB_1 by measuring TEER. After 24 h of treatment, medium was collected for IL-6, IL-8 and TNF-α determination.
Experiment 2 was performed to evaluate the dose response of beauvericin (BEA) on Caco-2 barrier integrity and the release of the pro-inflammatory cytokines IL-6, IL-8 and TNF-α . Caco-2 cells were cultured as previously described and, after differentiation, cells were treated with different concentrations of BEA (0, 0.5, 1.5, 3, 6 µM) in triplicate from both Ap and Bl sides. The barrier integrity was evaluated after 1, 2 and 24 h of

exposure to BEA by measuring TEER. After 24 h of treatment, medium was collected for IL-6, IL-8 and TNF-α determination.
Experiment 3 was designed to evaluate the effects of FB_1 (1.5 μM) and BEA (3 μM), alone and combined, on Caco-2 barrier integrity and the release of the pro-inflammatory cytokines IL-6, IL-8 and TNF-α. Caco-2 cells were cultured as previously described and, after differentiation, cells were treated with FB_1 (0 or 1.5 μM) and BEA (0 or 3 μM) in triplicate from both Ap and Bl sides. The barrier integrity was evaluated after 1, 2 and 24 h of exposure to treatments by measuring TEER. After 24 h of treatment, medium was collected for IL-6, IL-8 and TNF-α determination.

4.2.6 Statistical analysis

Each experiment was performed in triplicate. Results are expressed as mean ± standard deviations (SD). Statistical evaluation was performed by two tailed Student's t-test. The level of significance was established at $P < 0.05$.

4.3 Results

4.3.1 Experiment 1: effect of fumonisin B_1 (FB_1) on Caco-2 barrier integrity and cytokine release

Caco-2 cells were treated with different concentrations of FB_1 (0, 0.5, 1.5, 3 µM) from both Ap and Bl sides. The barrier integrity was evaluated after 1, 2 and 24 h of exposure to FB_1 by measuring TEER. After 24 h of treatment, medium was collected for IL-6, IL-8 and TNF-α determination.

No significant ($P \geq 0.05$) effect on TEER was observed after 24 h of both Ap and Bl exposure to all doses (0.5, 1.5, 3 µM) of FB_1 (Fig. 4.3 and 4.4). On one hand, no significant release of the inflammatory mediators IL-6 and TNF-α was observed after Ap and Bl exposure to FB_1 (Fig. 4.5 and 4.7) at all doses (0.5, 1.5, 3 µM). On the other hand, a significant ($P < 0.05$) increase of IL-8 release was induced by Bl exposure to FB_1 at 3 µM (Fig. 4.6).

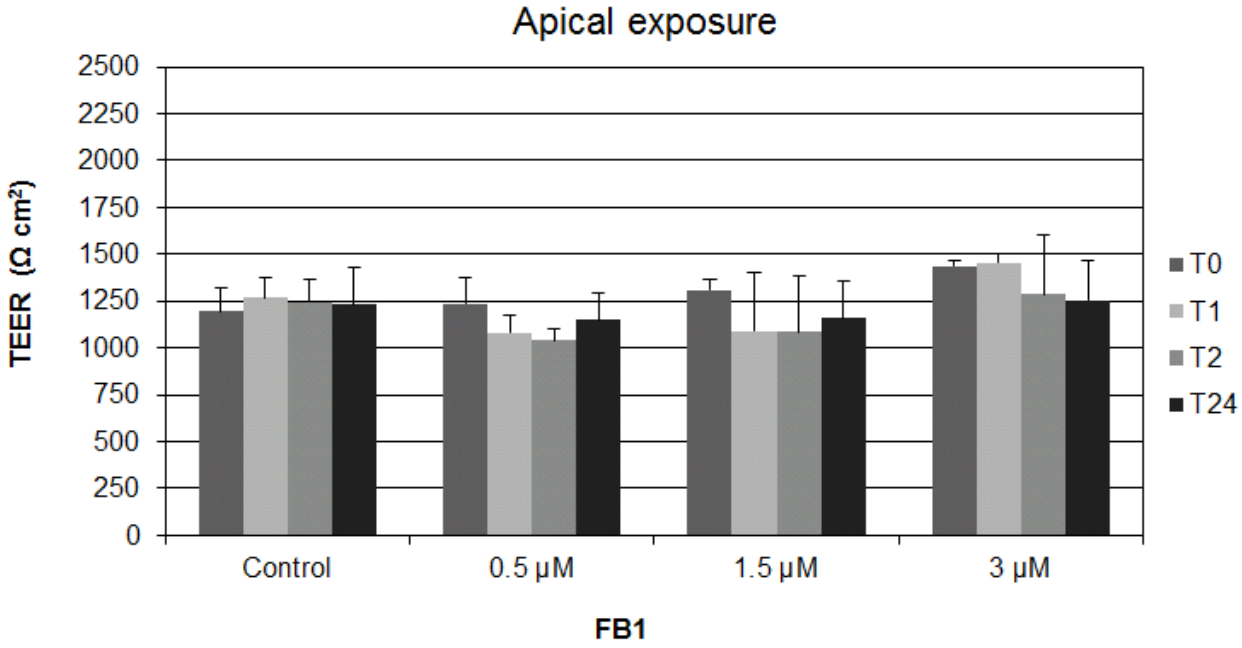

Figure 4.3: Effect of FB_1 on Caco-2 TEER values after apical exposure. Graph shows the mean values and standard deviations (n=3). (*) $P < 0.05$.

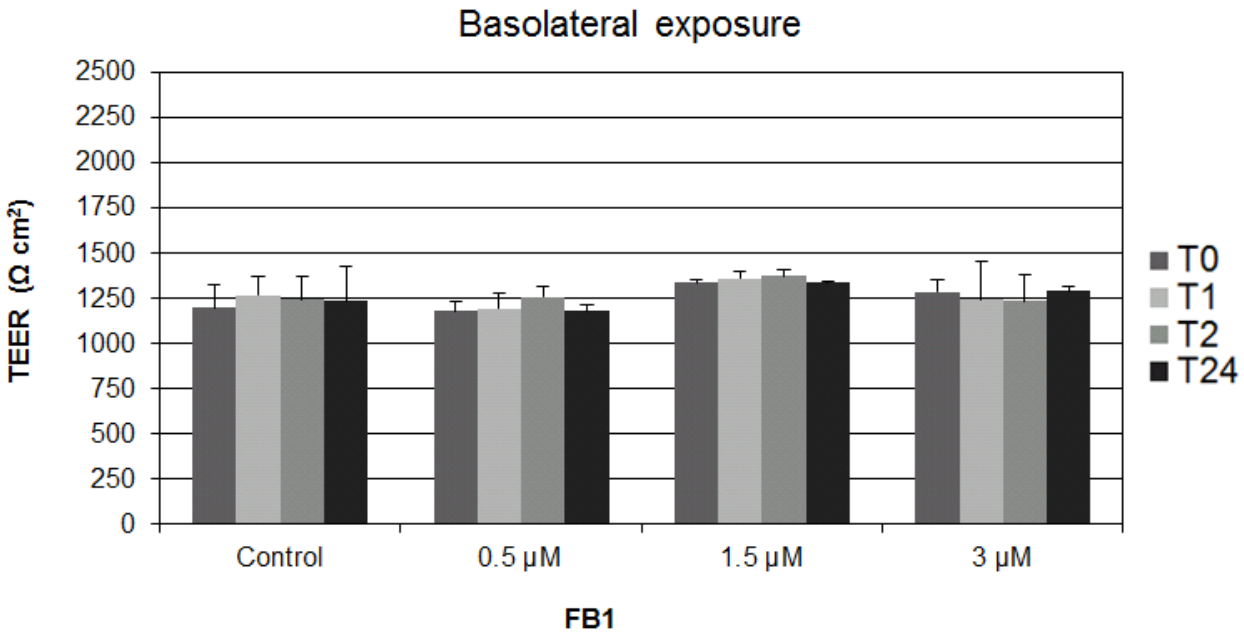

Figure 4.4: Effect of FB_1 on Caco-2 TEER values after basolateral exposure. Graph shows the mean values and standard deviations (n=3). (*) $P < 0.05$.

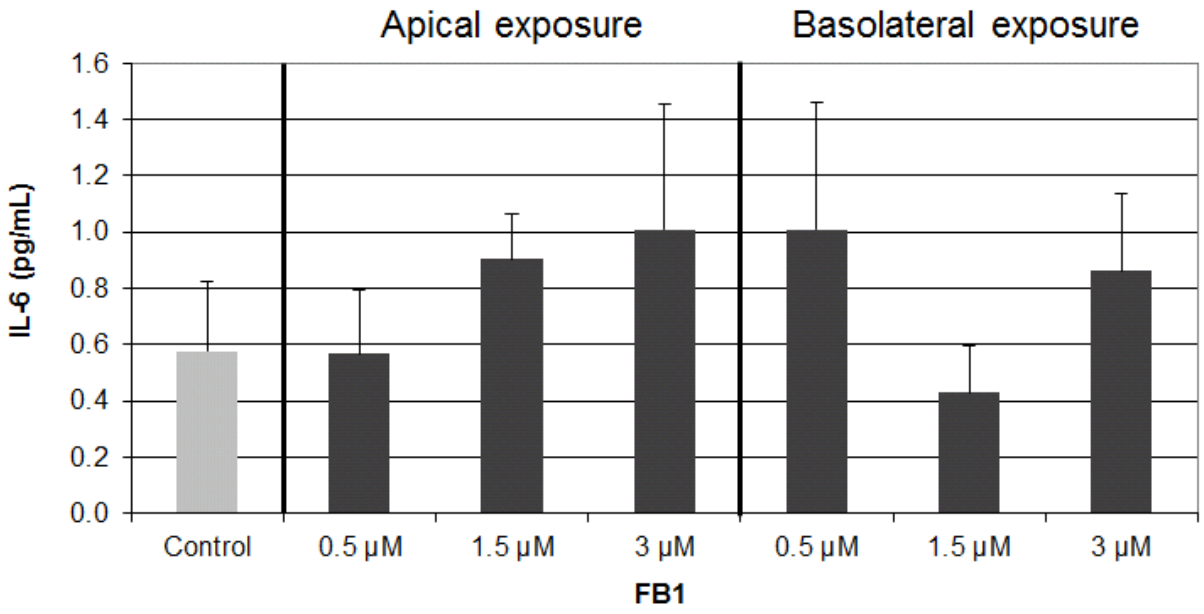

Figure 4.5: Effect of FB_1 on Caco-2 IL-6 release after apical and basolateral exposure. Graph shows the mean values and standard deviations (n=3). (*) $P < 0.05$.

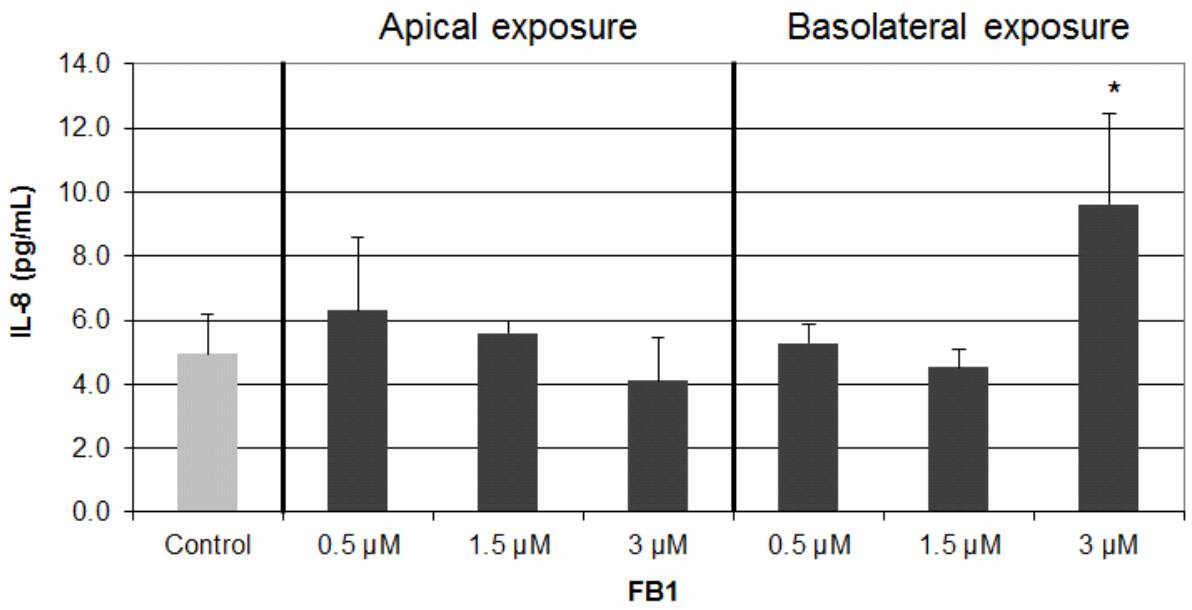

Figure 4.6: Effect of FB_1 on Caco-2 IL-8 release after apical and basolateral exposure. Graph shows the mean values and standard deviations (n=3). (*) $P < 0.05$.

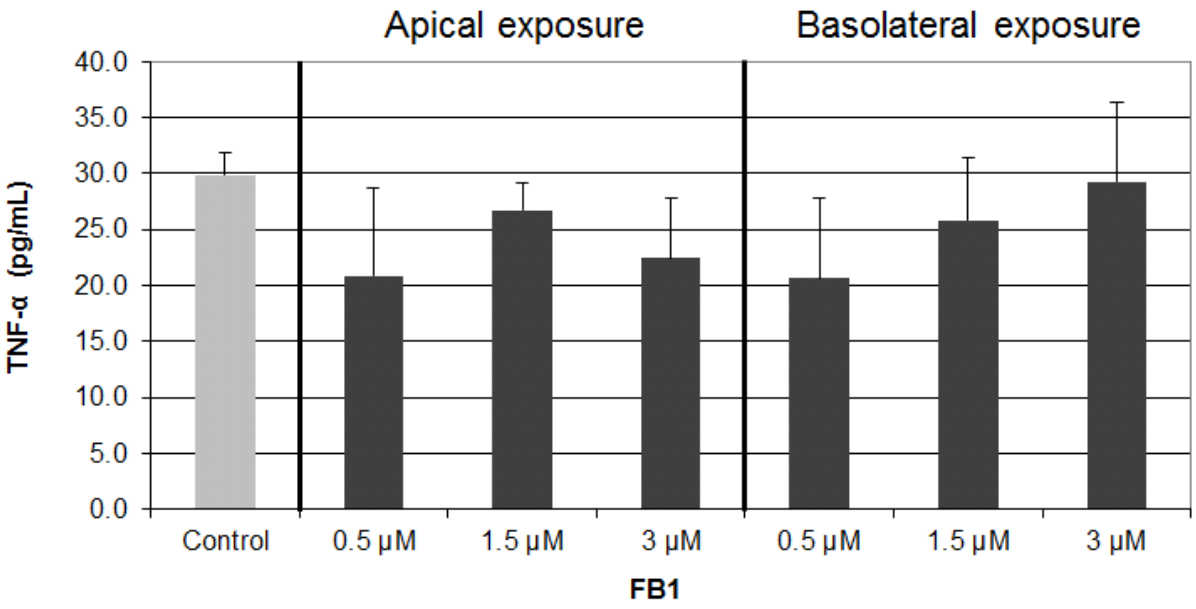

Figure 4.7: Effect of FB_1 on Caco-2 TNF-α release after apical and basolateral exposure. Graph shows the mean values and standard deviations (n=3). (*) $P < 0.05$.

4.3.2 Experiment 2: effect of beauvericin (BEA) on Caco-2 barrier integrity and cytokine release

Caco-2 cells were treated with different concentrations of BEA (0, 0.5, 1.5, 3, 6 μM) from both Ap and Bl sides. The barrier integrity was evaluated after 1, 2 and 24 h of exposure to BEA by measuring TEER. After 24 h of treatment, medium was collected for IL-6, IL-8 and TNF-α determination.

TEER was not significantly affected ($P \geq 0.05$) by Ap exposure to all doses of BEA (Fig. 4.8). On the contrary, a significant ($P < 0.05$) TEER decrease was observed after Bl exposure to BEA at 0.5 and 1.5 μM starting from the first hour of treatment (Fig. 4.9). This decrease in TEER was no more observed after 24 h of Bl exposure to BEA at 1.5 μM. Differently, at higher concentrations (3 and 6 μM), BEA was found to significantly ($P < 0.05$) increase TEER after 24 h of Bl exposure (Fig. 4.9).

A significant release of the inflammatory mediator IL-6 was observed after Ap exposure to BEA at 3 and 6 μM and after Bl exposure to BEA at 1.5, 3 and 6 μM (Fig. 4.10). Regarding IL-8, a significant ($P < 0.05$) release was induced by Ap exposure to BEA at 3 and 6 μM and by Bl exposure to BEA only at 6 μM (Fig. 4.11).

Concerning TNF-α, a significant release was observed after Ap exposure to 0.5 and 1.5 μM BEA and after Bl exposure to 1.5 μM BEA (Fig. 4.12).

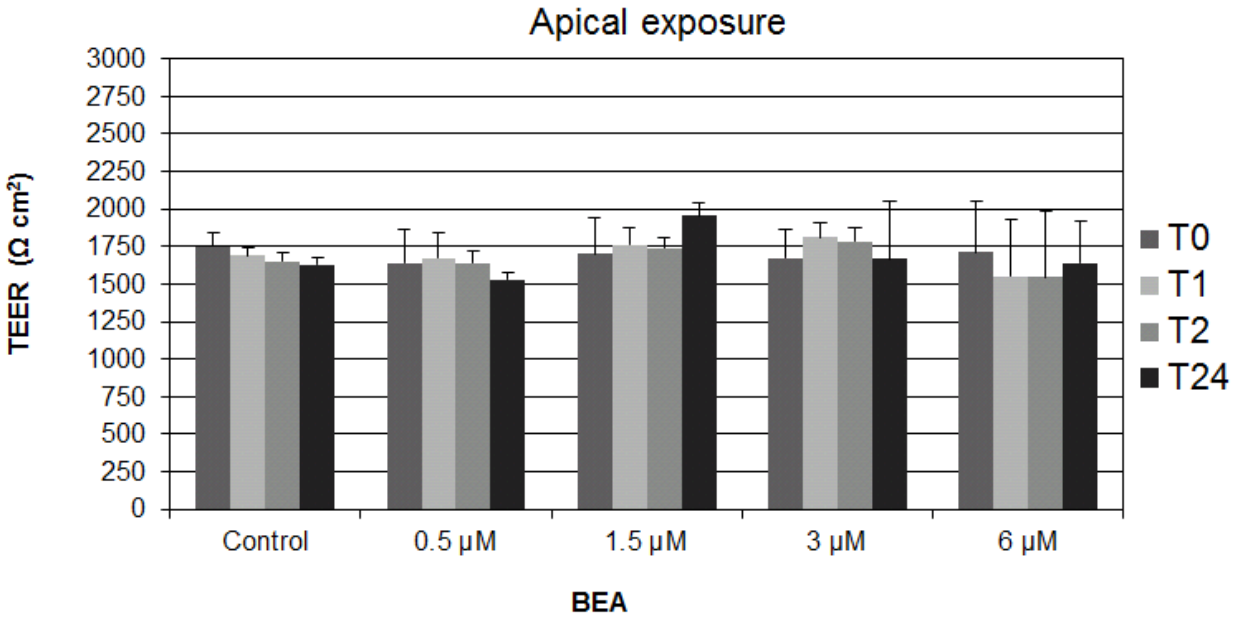

Figure 4.8: Effect of BEA on Caco-2 TEER values after apical exposure. Graph shows the mean values and standard deviations (n=3). (*) $P < 0.05$.

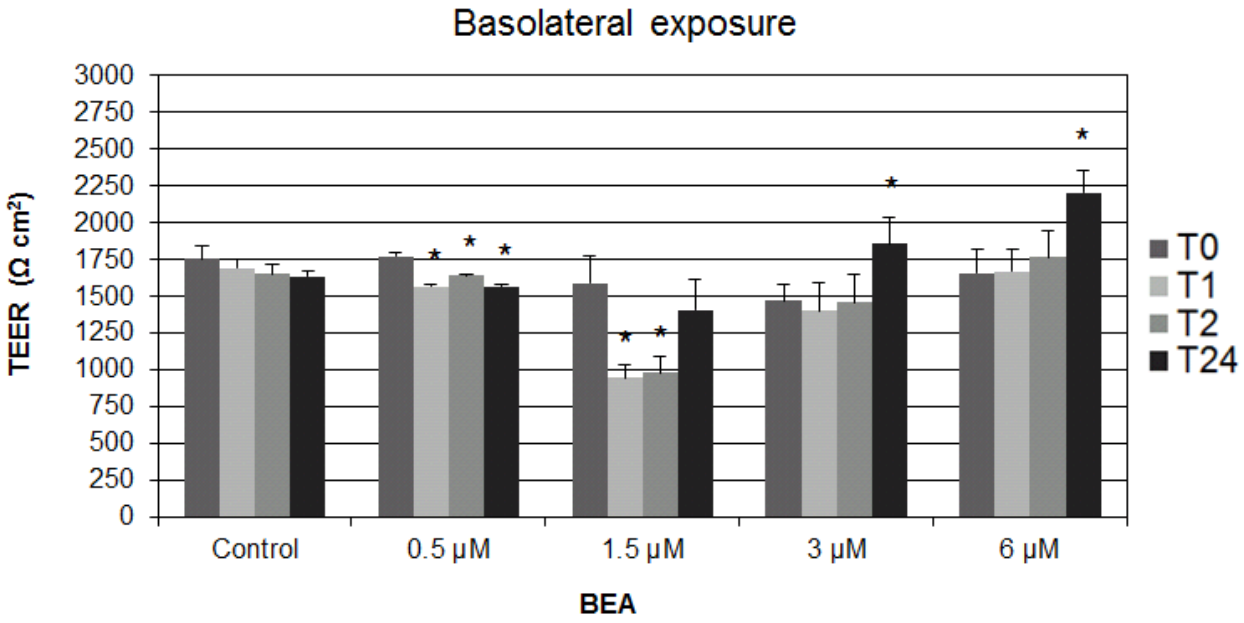

Figure 4.9: Effect of BEA on Caco-2 TEER values after basolateral exposure. Graph shows the mean values and standard deviations (n=3). (*) $P < 0.05$.

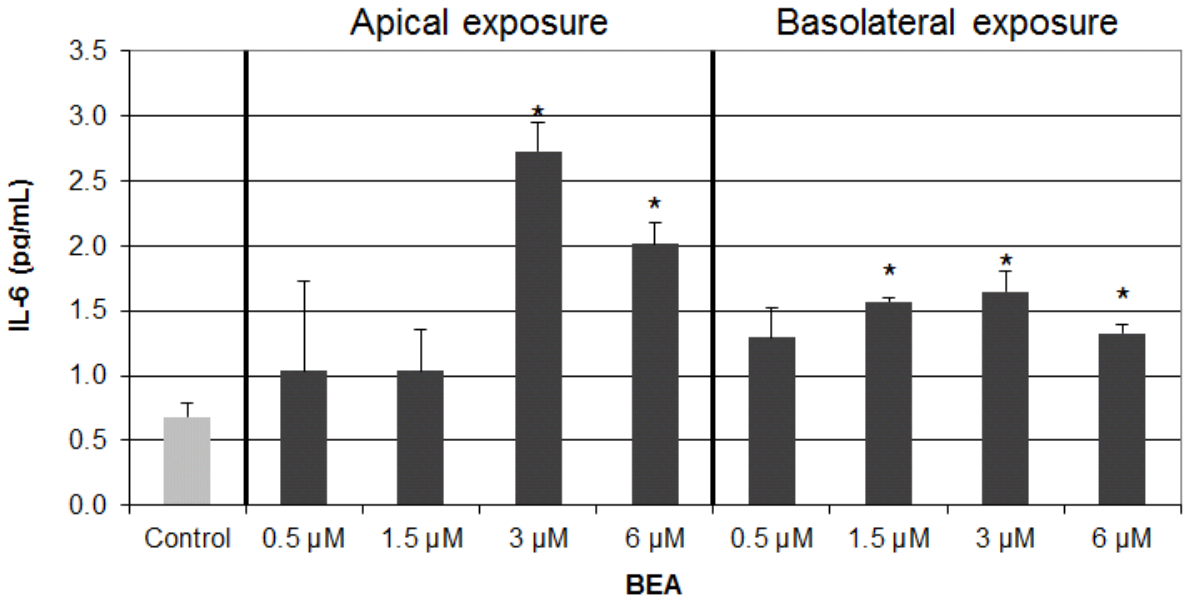

Figure 4.10: Effect of BEA on Caco-2 IL-6 release after apical and basolateral exposure. Graph shows the mean values and standard deviations (n=3). (*) $P < 0.05$.

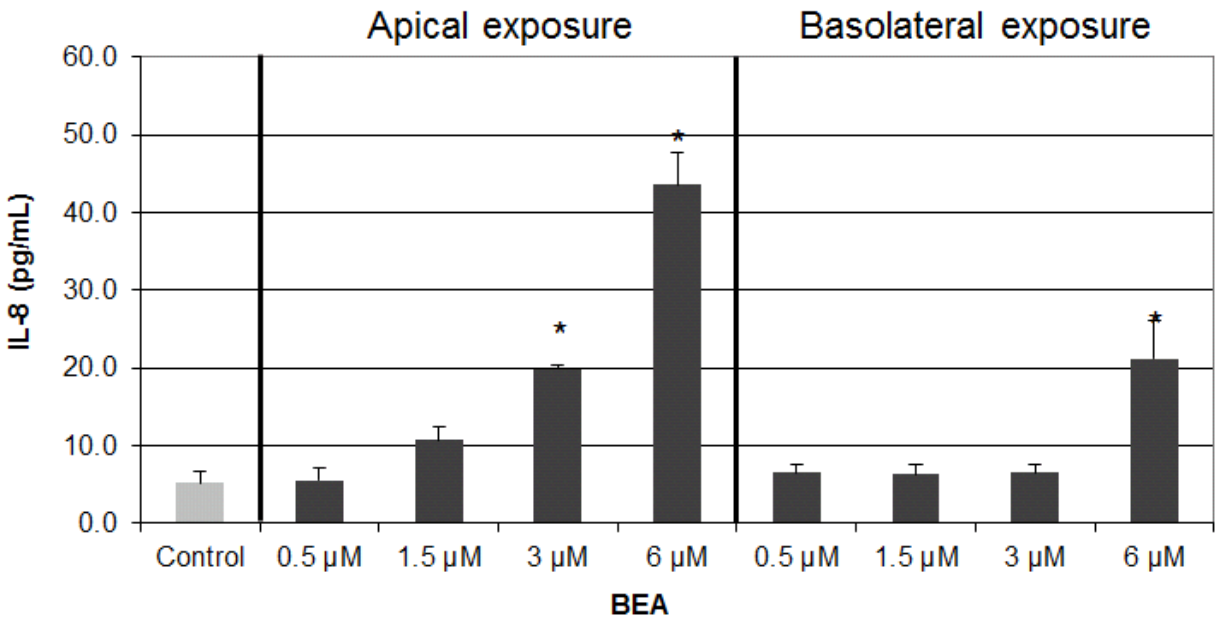

Figure 4.11: Effect of BEA on Caco-2 IL-8 release after apical and basolateral exposure. Graph shows the mean values and standard deviations (n=3). (*) $P < 0.05$.

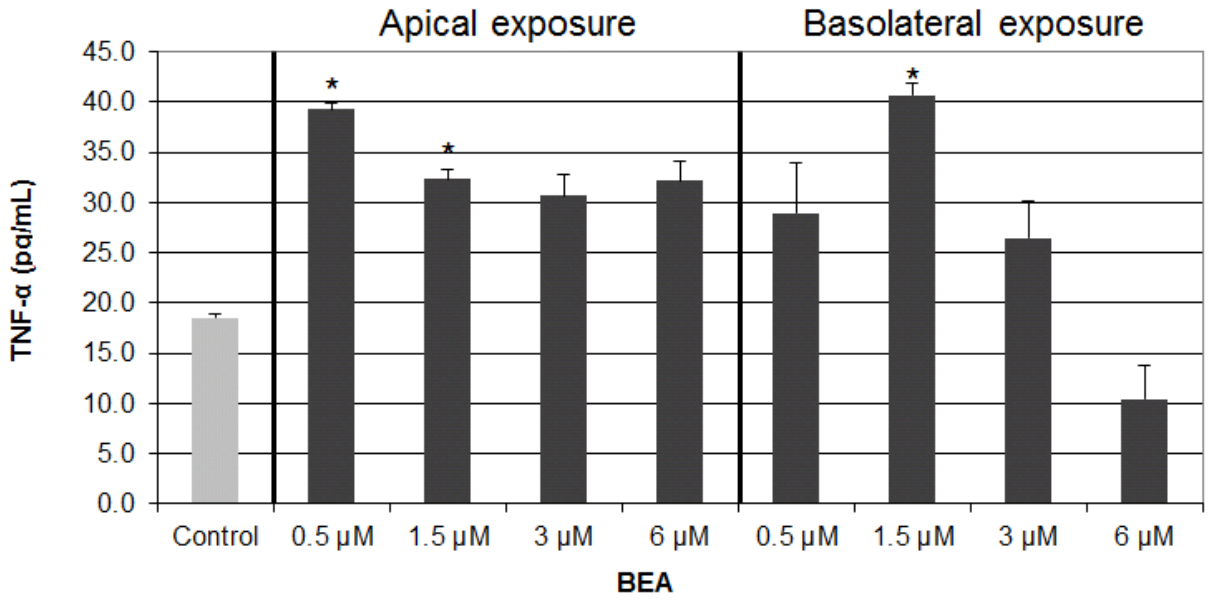

Figure 4.12: Effect of BEA on Caco-2 TNF-α release after apical and basolateral exposure. Graph shows the mean values and standard deviations (n=3). (*) $P < 0.05$.

4.3.3 Experiment 3: individual and combined effects of fumonisin B_1 and beauvericin (BEA) on Caco-2 barrier integrity and cytokine release

Caco-2 cells were treated with FB_1 (0 or 1.5 µM) and BEA (0 or 3 µM) from both Ap and Bl sides. The barrier integrity was evaluated after 1, 2 and 24 h of exposure to treatments by measuring TEER. After 24 h of treatment, medium was collected for IL-6, IL-8 and TNF-α determination.

No significant ($P \geq 0.05$) effect was observed on TEER after 24 h of Ap exposure to FB_1 at 1.5 µM, BEA at 3 µM and the combination of these mycotoxins (Fig. 4.13). Only after 24 h of Bl exposure to BEA at 3 µM a significant ($P < 0.05$) increase of TEER was observed (Fig. 4.14). This TEER increase was not observed after Bl exposure to BEA at 3 µM in combination with FB_1 at 1.5 µM (Fig. 4.14)

FB_1 and BEA, alone and combined, had no significant effects on cytokine release (Fig. 4.15, 4.16, 4.17).

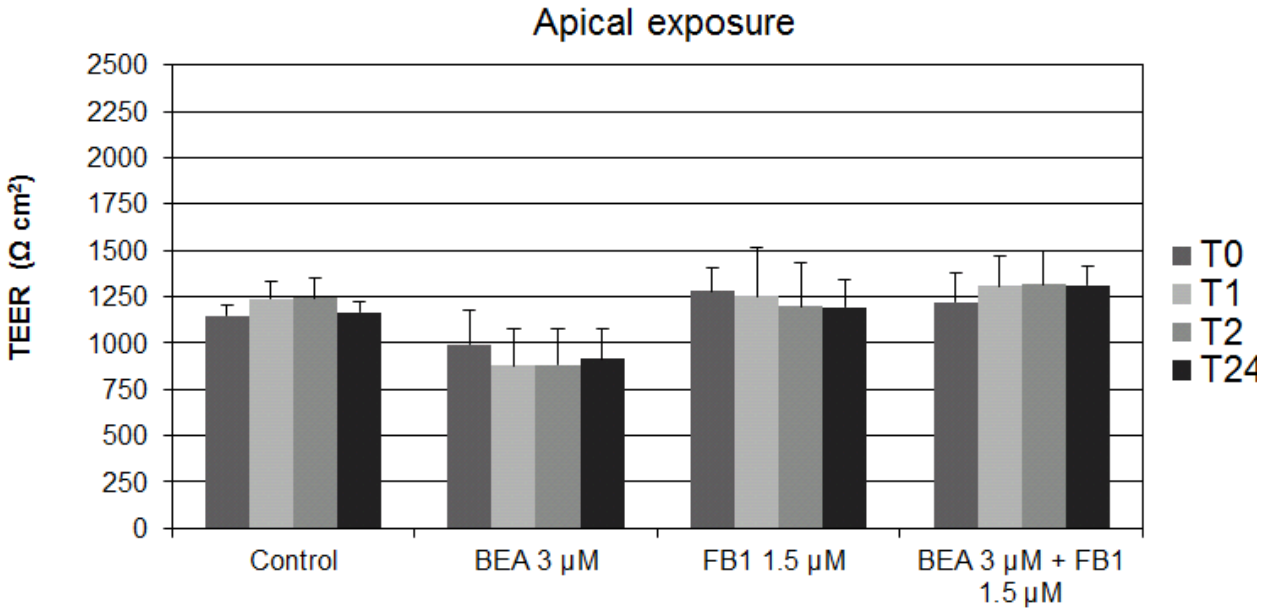

Figure 4.13: Effect of FB_1 with or without BEA on Caco-2 TEER values after apical exposure. Graph shows the mean values and standard deviations (n=3). (*) $P < 0.05$.

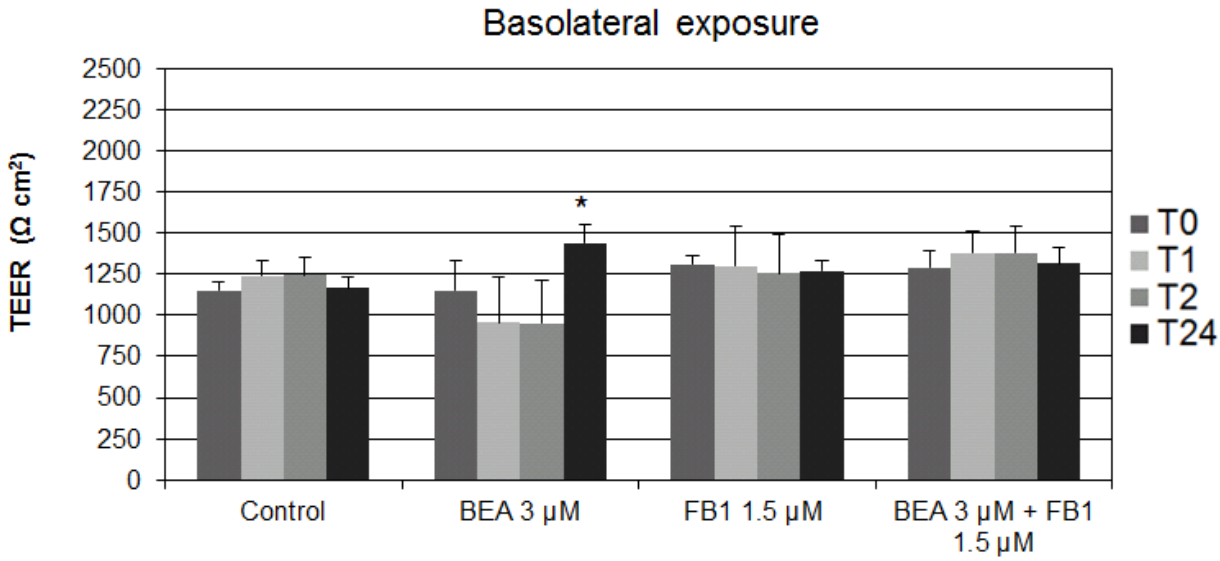

Figure 4.14: Effect of FB_1 with or without BEA on Caco-2 TEER values after basolateral exposure. Graph shows the mean values and standard deviations (n=3). (*) $P < 0.05$.

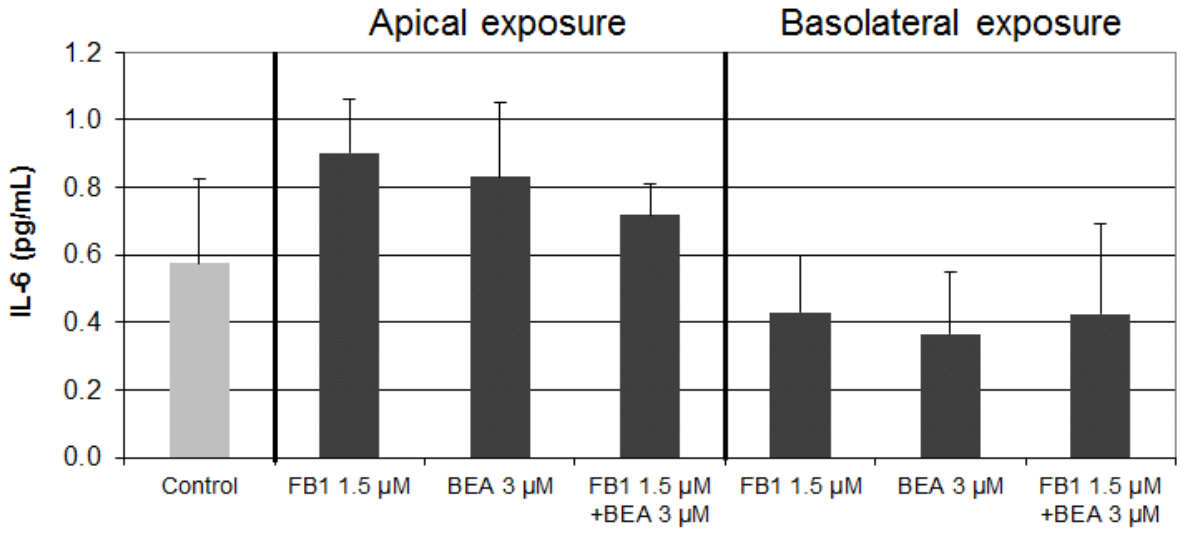

Figure 4.15: Effect of FB_1 with or without BEA on Caco-2 IL-6 release after apical and basolateral exposure. Graph shows the mean values and standard deviations (n=3). (*) $P < 0.05$.

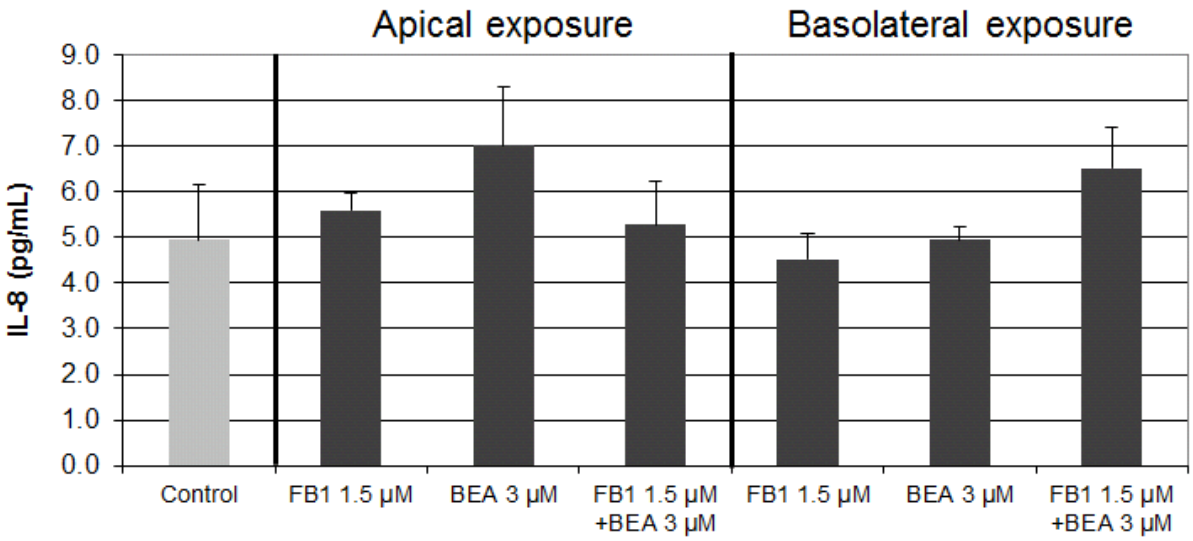

Figure 4.16: Effect of FB_1 with or without BEA on Caco-2 IL-8 release after apical and basolateral exposure. Graph shows the mean values and standard deviations (n=3). (*) $P < 0.05$.

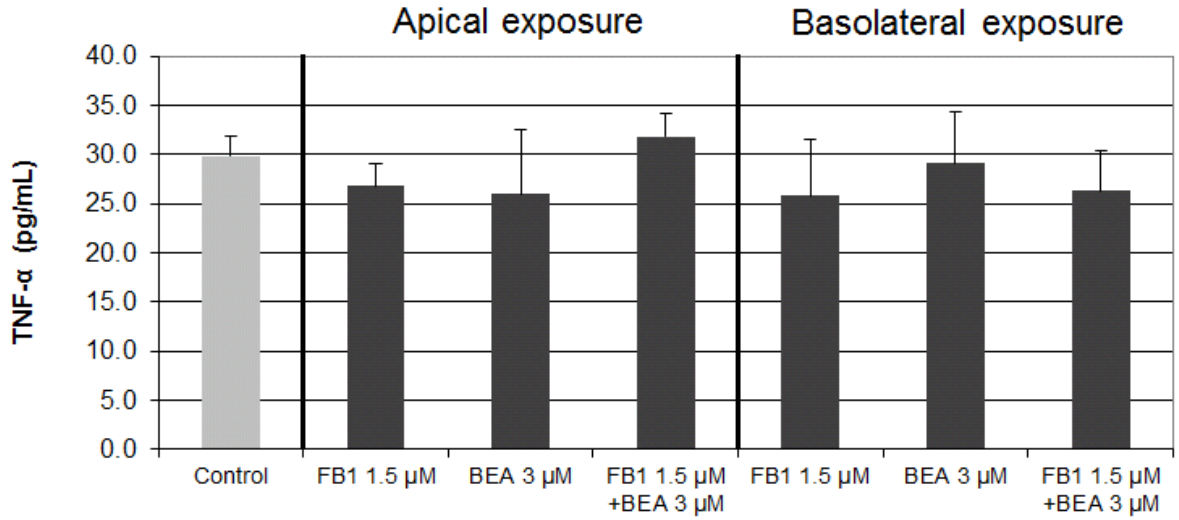

Figure 4.17: Effect of FB_1 with or without BEA on Caco-2 TNF-α release after apical and basolateral exposure. Graph shows the mean values and standard deviations (n=3). (*) $P < 0.05$.

4.4 Discussion

The gastrointestinal tract is an important environment responsible for absorbing nutrients, protecting the body against pathogens entering with food and preventing the loss of important compounds such as water and solutes (Gordon et al., 2015). The integrity of the intestinal barrier is crucial for the maintenance of the homeostasis (Goto and Kiyono, 2012). An impairment of the intestinal barrier function may cause autoimmune responses, inflammation and atopic diseases (Antonissen et al., 2014).
The trans-epithelial electrical resistance (TEER) is a parameter that gives information on the barrier integrity and its decrease corresponds to an impairment (Marin et al., 2015).
TEER is considered a good indicator of the organization of the TJ proteins (Pinton et al., 2009). TJ proteins seal the intercellular space between adherent epithelial cells, thus preventing paracelluar transport of luminal antigens (Schneeberger and Lynch, 1992; Liu et al., 2000; Tsukita et al., 2001; Maresca and Fantini, 2010). TJ proteins are therefore involved in barrier function and their damage leads to an increase of the intestinal permeability and thus to an increased transepithelial passage of bacteria and antigens (Maresca and Fantini, 2010). The number of TJ strands and their ramification depend on the cell type, producing marked variation in the morphology of TJ strand networks. The main TJ integral membrane proteins are occludins (OCLN), claudins (CLDN) and junctional adhesion molecules (JAM) (Tsukita et al., 2001; Maresca and Fantini, 2010).
The ability of FB_1 to cause toxicity on intestinal epithelial cell lines have been already reported. Bouhet et al. (2004) showed that FB_1 at concentrations ranging from 2 to 700 μM decreased in a time- and dose-dependent manner TEER of IPEC-1 cells (Bouhet et al., 2004). The authors reported a significant TEER decrease after 13-day exposure to FB_1 at 50 μM and a drastic impairment of the intestinal barrier integrity after 15 to 18 days of exposure to high concentrations of FB_1 (200 and 500 μM) (Bouhet et al., 2004).
FB_1 (1 to 138 μM) was previously reported to not exert cytotoxic effects on Caco-2 cells (Caloni et al., 2002). Only a transient decrease of TEER was observed after 6-h exposure to the highest concentration tested, without FB_1 epithelial passage (Caloni et al., 2005; De Angelis et al., 2005). Recently, after treatments with FB_1 at 1, 3, 10, 30 μM a reduction in mRNA levels of CLDN3 and CLDN4 transmembrane proteins was observed in FB_1-exposed Caco-2 cells showing the ability of FB_1 to impair the intestinal barrier integrity (Romero et al., 2016).
In our study no significant effect ($P \geq 0.05$) was observed on TEER after 1, 2 or 24 h of Apical (Ap) or Basolateral (Bl) exposure to all the doses of FB_1 (0.5, 1.5, 3 μM) (Fig. 4.3; 4.4). Our results are in agreement with previous studies (Wentzel et al., 2016) where Caco-2 cells were exposed for 24 h to different concentrations (0.5–45 μM) of FB_1. The results reported by Wentzel et al. (2016) showed that even at concentrations up to 45

μM, FB_1 did not affect cells. Similarly, exposure of Caco-2 cells to 0.001–10 μM FB_1 revealed no cytotoxic effect of FB_1 at all the concentrations tested (Fernandez-Blanco et al., 2016).

Based on our findings FB_1 induced IL-8 release in Caco-2 cells even if only after Bl exposure to FB_1 at 3 μM (Fig. 4.6), while no significant release of IL-6 or TNF-α was observed after Ap and Bl exposure to FB_1 at all doses (0.5, 1.5, 3 μM) (Fig. 4.5).

The significant ($P < 0.05$) increase of IL-8 release induced by Bl exposure to FB_1 (3 μM) (Fig. 4.6) was previously observed but after exposure to a higher concentration of FB_1 (17.2 μM) (Minervini et al., 2014).

Regarding BEA its cytotoxicity was observed on Caco-2 cells at 20.6 and 12.7 μM after 24 and 48 h exposure, respectively (Prosperini et al. 2012) and at 1 and 10 μM (Fernandez-Blanco et al., 2016).

In our study a significant ($P < 0.05$) TEER decrease was observed after 1 h and 2 h of Bl exposure to BEA at 0.5 and 1.5 μM and after 24 h of Bl exposure to BEA at 0.5 μM, whereas after 24 h of Bl exposure, BEA at 3 and 6 μM was found to significantly ($P < 0.05$) increase TEER (Fig. 4.9). When forced by an inducible promoter, an over-expression of OCLN leaded to a TEER increase of MDCK cells in a reversible manner, paradoxically increasing the paracellular flow of uncharged solutions (McCarthy et al., 1996). This paradoxical behavior still needs to be clarified (Barrett and Donowitz et al., 2001). Phosphorylation of TJ proteins, in particular CLDN, has also been shown to affect the epithelial barrier function (Stevenson et al. 1989; Findley et al., 2009). Moreover, protein kinase C (PKC) is involved in TJ regulation and has been demonstrated to act through an increase or decrease of TEER (Farhadi et al. 2006; Plotnikov et al., 2010).

The increase of TEER induced by BEA may be related to the modulation of TJ proteins, like claudin-2 and OCLN, with the effect of improving overall epithelial barrier function.

A significant ($P < 0.05$) release of IL-8 was induced by Ap exposure to BEA at 3 and 6 μM and by Bl exposure to BEA only at 6 μM (Fig. 4.11).

Concerning TNF-α, a significant release was observed after Ap exposure to 0.5 and 1.5 μM BEA and after Bl exposure to 1.5 μM BEA (Fig. 4.12).

In the present study the combined effects of FB_1 and BEA on Caco-2 cells were also evaluated.

After 24 h of Bl exposure, BEA alone at 3 μM increased TEER ($P < 0.05$), but this effect was no longer observed when combined with FB_1 (Fig. 4.14), which shows no effect at 1.5 μM after 1, 2 and 24 h of Ap or Bl exposure ($P \geq 0.05$) (Fig. 4.13). These results suggest a possible interaction between these mycotoxins.

As previously discussed BEA alone was found to increase IL-6 and IL-8 release (Fig. 4.10; 4.11), but these increased releases were not observed when BEA was combined

with FB_1 (Fig. 4.15; 4.16), which induces IL-8 release at 3 μM. Ap (0.5 -1.5 μM) and Bl (1.5 μM) exposure to BEA increased TNF-α release (Fig. 4.12), but not in the presence of FB_1 (Fig. 4.17).

TNF-α seems to play a key role in intestinal inflammation exerting a protective effect on the intestinal barrier function (Corridoni et al., 2012; Naito et al., 2003; Noti et al., 2010). The mechanisms of FB_1 toxicity include inhibition of ceramide synthase and accumulation of sphingoid bases, repression of protein kinase C and its down-stream effector TNF-α and mobilization of cellular calcium (Kim et al., 2006; Gopee et al., 2003). On the other hand BEA increases intracellular calcium and activates the calcium-dependent endonucleases and causes subsequent DNA fragmentation (Jestoi, 2008).

Only few studies so far have investigated the *in vitro* interactions between FB_1 and BEA, however synergic effects of these mycotoxins have been already reported (Klaric et al., 2007), even if the exact mechanism of the interaction needs to be clarified.

In this study FB_1 did not exert significant adverse effects on intestinal Caco-2 cells cultured on inserts, while BEA alone affected TEER and induced cytokine release but, in combination with FB_1, these effects were no more observed. The disappearance of IL-6 and IL-8 release induced by BEA, may suggest a possible protective action of FB_1 (Cuzzocrea et al., 2008).

Our results suggest an interaction between FB_1 and BEA when combined on TEER and cytokine release of *in vitro* intestinal Caco-2 cells. Other studies are in progress to better understand these aspects.

4.5 References

Antonissen, G., Martel, A., Pasmans, F., Ducatelle, R., Verbrugghe, E., Vandenbroucke, V., Li, S., Haesebrouck, F., Van Immerseel, F., and Croubels, S. (2014). The Impact of Fusarium Mycotoxins on Human and Animal Host Susceptibility to Infectious Diseases. *Toxins, 6: 430-452.*

Barrett, K.E., Donowitz, M. (2001). Gastrointestinal Transport. *Molecular Physiology, 4; 50.*

Baker, D.C., G.E. Rottinghaus. (1999). Chronic experimental fumonisin intoxication of calves. *Journal of Veterinary Diagnostic Investigation, 11:289–292.*

Bhandari, N., Sharma, R.P. (2002) Fumonisin B(1)-induced alterations in cytokine expression and apoptosis signaling genes in mouse liver and kidney after an acute exposure. *Toxicology 172(2):81–92.*

Binder, E.M., Tanb, L.M., Chinb, L.J., Handla, J., Richardc, J. (2007). Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. *Animal Feed Science and Technology, 137:265–282.*

Bouhet, S., Hourcade, E., Loiseau, N., Fikry, A., Martinez, S., Roselli, M., Galtier, P., Mengheri, E., Oswald, I.P. (2004) The mycotoxin fumonisin B1 alters the proliferation and the barrier function of porcine intestinal epithelial cells. *Toxicological Sciences, 77:165–171.*

Caloni, F., Cortinovis, C., Pizzo, F., De Angelis, I. (2012) Transport of Aflatoxin M(1) in Human Intestinal Caco-2/TC7 Cells. *Frontiers in Pharmacology, 3:111.*

Caloni, F., Spotti, M., Pompa, G., Zucco, F., Stammati, A., De Angelis, I. (2002). Evaluation of Fumonisin B1 and its metabolites absorption and toxicity on intestinal cells line Caco-2. *Toxicon, 40: 1181-1188.*

Caloni, F., Stammati, A.L., Raimondi, F., and De Angelis, I. (2005). *In vitro* Study With Caco-2 Cells on Fumonisin B1: Aminopentol Intestinal Passage and Role of P-Glycoprotein. *Veterinary Research Communications, 29: 285–287.*

Caloni F., Stammati A., Friggè G., and De Angelis I. (2006). Aflatoxin M1 absorption and cytotoxicity on human intestinal in vitro model. *Toxicon, 47: 409–415.*

Caloni, F., Spotti, M., Auerbach, H., Op den Camp, H., Fink-Gremmels, J., Pompa, G. (2000). *In vitro* metabolism of fumonisin B_1 by ruminal microflora. *Veterinary Research Communication, 24: 379–387.*

Corridoni, D., Pastorelli, L., Mattioli, B., Locovei, S., Ishikawa, D., Arseneau, K.O., Chieppa, M., Cominelli, F., Pizarro, T.T. (2012). Probiotic Bacteria Regulate Intestinal Epithelial Permeability in Experimental Ileitis by a TNF-Dependent Mechanism. *PLOSONE http://dx.doi.org/10.1371/journal.pone.0042067.*

Cuzzocrea, S., Di Paola, R., Genovese, T., Mazzon, E., Esposito, E., Crisafulli, C., Bramanti, P., Salvemini, D. (2008). Anti-inflammatory and anti-apoptotic effects of fumonisin B1, an inhibitor of ceramide synthase, in a rodent model of splanchnic ischemia and reperfusion injury. *Journal of Pharmacology and Experimental Therapeutics, 327: 45-57.*

De Angelis, I., Frigge, G., Raimondi, F., Stammati, A., Zucco, F., Caloni, F. (2005). Absorption of Fumonisin B1 and aminopentol on an *in vitro* model of intestinal epithelium; the role of P-glycoprotein. *Toxicon, 45: 285–291.*

Diekman, M.A., Green, M.L. (1992). Mycotoxins and reproduction in domestic livestock. *Journal of Animal Science, 70:1615e1627.*

Domijan, A-M., Peraica, M., Jurjevi, L., Ivi, D., Cvjetkovi, B. (2005) Fumonisin B1, fumonisin B2, zearalenone and ochratoxin A contamination of maize in Croatia. *Food Add Contam 22:677–680.*

EFSA (European Food Safety Authority) (2005). Opinion of the Scientific Panel on Contaminants in Food Chain on a request from the Commission related to fumonisins as undesirable substances in animal feed. *EFSA Journal, 235: 1-32.*

EFSA (European Food Safety Authority). (2014). Scientific Opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed. *EFSA Journal 12:3802.*

Farhadi, A., Keshavarzian, A., Ranjbaran, Z., Fields, J.Z., Banan, A. (2006). The role of protein kinase C isoforms in modulating injury and repair of the intestinal barrier. *Journal of Pharmacology and Experimental Therapeutics, 316: 1–7.*

Ferrigo, D., Raiola, A., and Causin, R. (2016). *Fusarium* Toxins in Cereals: Occurrence, Legislation, Factors Promoting the Appearance and Their Management. *Molecules, 21: 627.*

Fernández-Blanco, C., Frizzell, C., Shannon, M., Ruiz, M., Connolly, L. (2016). An *in vitro* investigation on the cytotoxic and nuclear receptor transcriptional activity of the mycotoxins fumonisin B_1 and beauvericin. *Toxicology Letters 257:1–10.*

Findley, M.K., Koval, M. (2009). Regulation and roles for claudin-family tight junction proteins. *IUBMB Life, 61: 431–437.*

Fink-Gremmels J. (1999). Mycotoxins: Their implications for human and animal health. Veterinary Quarterly, *21: 115-120.*

Fogh, J., Fogh, J.M., Orfeo, T., (1977). One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *Journal of Natural Cancer Institutte, 59: 221– 226.*

Gbore, F.A., Owolawi, T.J., Erhunwunsee, M., Akele, O., Gabriel-Ajobiewe, R.A.O. (2012). Evaluation of the reproductive toxicity of dietary fumonisin B1 in rats Jordan. *Journal of Biological Science, 5: 183–90.*

Glenn A.E., (2007). Mycotoxigenic Fusarium species in animal feed. Animal Feed Science and Technology, *137: 213-240.*

Gopee, N.V., He, Q., Sharma, R.P. (2003). Fumonisin B1-induced apoptosis is associated with delayed inhibition of protein kinase C, nuclear factor-κB and tumor necrosis factor α in LLC-PK1 cells. *Chemico-Biological Interactions, 146: 131–145.*

Gordon, S., Daneshian, M., Bouwstra, J., Caloni, F., Constant, S., Davies, D.E., Dandekar, G., Guzman, C.A., Fabian, E., Haltner, E., Hartung, T., Hasiwa, N., Hayden, P., Kandarova, H., Khare, S., Krug, H.F., Kneuer, C., Leist, M., Lian, G., Marx, U., Metzger, M., Ott, K., Prieto, P., Roberts, M.S., Roggen, E.L., Tralau, T., Van den Braak, C., Walles, H. and Lehr, C.-M. (2015). Non-Animal Models of Epithelial Barriers (Skin, Intestine and Lung) in Research, Industrial Applications and Regulatory Toxicology. *Altex, 32: 327-378.*

Goto, Y., & Kiyono, H. (2012) Epithelial barrier: an interface for the cross-communication between gut flora and immune system. *Immunology Reviews, 245:147-163.*

Hilgenfeld, R., and Saenger, W. (1982). Structural chemistry of natural and synthetic ionophores and their complexes with cations. *In:Topics in Current Chemistry 101. pp. 1–82. Boschke, F.L., Ed., Springer-Verlag, Berlin.*

Hove, M., Van Poucke, C., Njumbe-Ediagea, E., Nyanga, L.K., De Saeger, S. (2016). Review on the natural co-occurrence of AFB_1 and FB_1 in maize and the combined toxicity of AFB1 and FB_1. *Food Control, 59: 675–682.*

IARC (2002). IARC monographs on the evaluation of carcinogenic risks to humans: some traditional herbal medicines, some mycotoxins, napthalene and styrene. *International Agency for Research on Cancer Press, Lyon, p 82.*

Jestoi, M. (2008). Emerging Fusarium-mycotoxins fusaproliferin, beauvericin, enniatins, and moniliformin: a review. Critical Reviews in Food and Science Nutrition, *48:21-49.*

Jurjevic, Z. (2002). Occurrence of beauvericin in corn from Croatia. *Food Technology and Biotechnology, 40: 91.*

Hilgenfeld, R., and Saenger. W. (1982). Structural chemistry of natural and synthetic ionophores and their complexes with cations. *In:Topics in Current Chemistry 101. pp. 1–82.*

Kim, D., Yoo, H., Lee, Y., Kie, J., Jang, S., Oh, S. (2006). Elevation of Sphinganine 1-Phosphate as a Predictive Biomarker for Fumonisin Exposure and Toxicity in Mice. *Journal of Toxicology and Environmental Health, 69: 2071-2082.*

Klaric, M.K., Rumora, L., Ljubanovic, D., Pepeljnjak, S. (2007). Cytotoxicity and apoptosis induced by fumonisin B1, beauvericin and ochratoxin A in porcine kidney PK15 cells: eVects of individual and combined treatment. *Archives of Toxicology, 82: 247–255.*

Kouadio, J.H., Dano, S.D., Moukha, S., Mobio, T.A., Creppy, E.E. (2007). Effects of combinations of Fusarium mycotoxins on the inhibition of macromolecular synthesis, malondialdehyde levels, DNA methylation and fragmentation, and viability in Caco-2 cells. *Toxicon 49:306–317.*

Liu, Y. et al. (2000). Human junction adhesion molecule regulates tight junction resealing in epithelia. *Journal of Science, 113: 2363–2374.*

Logrieco, A., Bottalico, A., Mulé, G., Moretti, A., Perrone, G. (2003). Epidemiology of Toxigenic Fungi and their Associated Mycotoxins for Some Mediterranean Crops. *European Journal of Plant Pathology, 109: 645–667.*

Loiseau, N., Debrauwer, L., Sambou, T., Bouhet, S., Miller, J.D., Martin, P.G., Viadere, J.L., Pinton, P., Puel, O., Pineau, T., Tulliez, J., Galtier, P., Oswald, I.P. (2007) Fumonisin B1 exposure and its selective effect on porcine jejunal segment: sphingolipids, glycolipids and trans-epithelial passage disturbance. *Biochemistry and Pharmacology, 74:144–152.*

Luongo, D., De Luna, R. Russo, R., Severino, L. (2008). Effects of four *Fusarium* toxins (fumonisin B_1, α-zearalenol, nivalenol and deoxynivalenol) on porcine whole-blood cellular proliferation. *Toxicon, 52: 156–162.*

Mallebrera, B., Juan-Garcia, A., Font, G., Ruiz, M. (2016). Mechanisms of beauvericin toxicity and antioxidant cellular defense. *Toxicology Letters 246:28–34.*

Marasas, W.F.O., Kellerman, T.S., Gelederblom, W.C.A., Coetzer, J. A. W., Thievi, P.G., and Vander Lugt, J.J. (1988). Leukoencephalomacia in a horse induced by fumonisin B1 isolated from *Fusarium* Monoliforme. *Onderstepoort Journal of Veterinary Research, 55: 197-203.*

Marasas, W.F.O., Riley, R.T., Hendricks, K.A., Stevens, V.L., Sadler, T.W., Gelineau-van Waes, J., Missmer, S.A., Cabrera, J., Torres, O., Gelderblom, W.C.A., Allegood, J., Martìnez, C., Maddox, J., Miller, J.D., Starr, L., Sullards, M., Roman, A.V., Voss, K.A., Wang, E., Merrill, A.H. (2004). Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and *in vivo*: a potential risk factor for human neural tube defects among populations consuming fumonisin- contaminated maize. *Journal of Nutrition, 134:711-716.*

Maresca, M., Fantini, J. (2010). Some food-associated mycotoxins as potential risk factors in humans predisposed to chronic intestinal inflammatory diseases. *Toxicon, 56: 282–294.*

Marin, D.E., Motiu, M., Taranu, I. (2015). Food Contaminant Zearalenone and Its Metabolites Affect Cytokine Synthesis and Intestinal Epithelial Integrity of Porcine Cells. *Toxins, 7: 1979-1988.*

McCarthy, K.M., Skare, I.B., Stankewich, M.C., Furuse, M., Tsukita, S., Rogers, R.A., Lynch, R.D., Schneeberger, E.E. (1996). Occludin is a functional component of the tight junction. *Journal of Cell Science, 109: 2287-2298.*

Meca, G., Font, G., Ruiz, M.J., (2011). Comparative cytotoxicity study of enniatins A, A1, A2, B, B1, B4 and J3 on Caco-2 cells, Hep-G2 and HT-29. *Food Chemistry and Toxicology 49:2464–2469.*

Merrill Jr, A.H., M.C. Sullards, E. Wang, K.A. Voss, R.T. Riley. (2001). Sphingolipid metabolism: roles in signal transduction and disruption by fumonisins. *Environmental Health Perspectives, 109:283-289.*

Minervini, F., Garbetta, A., D'Antuono, I et al. (2014) Toxic mechanisms induced by fumonisin b1 mycotoxin on human intestinal cell line. *Archives of Environmental Contamination and Toxicology, 67: 115–123.*

Molinié, A., Faucet, V., Castegnaro, M., Pfohl-Leszkowicz, A. (2005) Analysis of some breakfast cereals on the French market for their contents of ochratoxin A, citrinin and fumonisin B1: development of a method for simultaneous extraction of ochratoxin A and citrinin. *Food Chemistry, 92: 391–400.*

Naito, Y., Takagi, T., Handa, O., Ishikawa, T., Nakagawa, S., et al. (2003). Enhancedintestinal inflammation induced by dextran sulfate sodium in tumor necrosisfactor-alpha deficient mice. *Journal of Gastroenterology and Hepatology, 18: 560–9.*

Noti, M., Corazza, N., Mueller, C., Berger, B., Brunner, T. (2010). TNF suppressesacute intestinal inflammation by inducing local glucocorticoid synthesis. *The Journal of Experimental Medicine, 207: 1057–66.*

Osweiler, G.D. (2000). Mycotoxins-contemporary issues of food animal health and productivity. Veterinary Clinic of North America, *16:511e530.*

Ou, G., Baranov, V., Lundmark, E. et al. (2009). Contribution of intestinal epithelial cells to innate immunity of the human gut – studies on polarized monolayers of colon carcinoma cells. *Scandinavian Journal of Immunology, 69: 150-161.*

Pinton P., Nougayrède J.P., Del Rio J.C., Moreno C., Marin D.E., Ferrier L., Bracarense A.P., Kolf-Clauw M., Oswald I.P. (2009). The food contaminant

deoxynivalenol, decreases intestinal barrier permeability and reduces claudin expression. *Toxicology and Applied Pharmacology, 237: 41–48.*

Plotnikov, A., Zehorai, E., Procaccia, S., Seger, R. (2010). The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation. *Biochimica et Biophysica, 1813: 1619-1633.*

Prosperini, A., Meca, G., Font, G., Ruiz, M.J. (2012). Study of the cytotoxic activity of beauvericin and fusaproliferin and bioavailability in vitro on Caco-2 cells. *Food Chemical and Toxicology 50:2356-2361.*

Rheder, J.P., Marasas, W.F.O., Thiel, P.G., Sydenham, E.W., Shephard, G.S., van Schalkwyk, D.J. (1992) *Fusarium* moniliforme and fumonisins in corn in relation to human esophageal cancer in Transkei. *Phytopathology 82:353–357.*

Romero, A., Ares, I., Ramos, E., Castellano, V., Martinez, M., Martinez-Larranaga, M.R., Anadon, A., Martinez, M.A. (2016). Mycotoxins modify the barrier function of Caco-2 cells through differential gene expression of specific claudin isoforms: protective effect of illite mineral clay. *Toxicology 353–354:21–33.*

Santini, A., Raiola, A., Meca, G., Ritieni, A. (2015) Aflatoxins, Ochratoxins, Trichotecenes, Patulin, Fumonisins and Beauvericin in Finished Products for Human Consumption. *Journal of Clinical Toxicology, 5:265.*

Schneeberger, E.E., and Lynch, R.D. (1992). Structure, function, and regulation of cellular tight junctions. *American Journal of Physiology, 262: L647–L661.*

Schoevers, E.J., Santos, R.R., Fink-Gremmels, J., Roelen, B.A.J. (2016). Toxicity of beauvericin on porcine oocyte maturation and preimplantation embryo development. *Reproductive Toxicology, 65: 159–169.*

Smith, G.W., (2012). Fumonisins. In: Gupta R.C. (Ed.), Veterinary Toxicology: Basic and Clinical Principles (second edition), *Elsevier Inc., pp. 1205-1219.*

Speijers, G.J.A., Speijers, M.H.M. (2004). Combined toxic effects of mycotoxins. *Toxicology Letters 153:91–98.*

Stevenson, B.R., Anderson, J.M., Braun, I.D., Mooseker, M.S. (1989). Phosphorylation of the tight-junction protein ZO-1 in two strains of Madin-Darby

canine kidney cells which differ in trans epithelial resistance. *Biochemical Journal, 263: 597–599.*

Sydenham, E.W., Thiel, P.G., Marasas, W.F.O., Shephard, G.S., Van Schalkwyk, D.J., Koch, K.R. (1990) Natural occurrence of some Fusarium mycotoxins in corn from low and high esophageal cancer prevalence areas of the Transkei, Southern Africa. *Journal of Agricultural and Food Chemistry 38:1900–1903.*

Tsukita, S., Furuse, M., and Itoh, M. (2001). Multifunctional strands in tight junctions. *Nature Reviews Molecular Cell Biology, 2: 285-293. doi:10.1038/35067088.*

Ulluwishewa, D., Anderson, R.C., McNabb, W.C., Moughan, P.J., Wells, J.M., and Roy, N.C., (2011). Regulation of Tight Junction Permeability by Intestinal Bacteria and Dietary Components. *The Journal of Nutrition, 141: 769-776.*

Voss, K.A., Howard, P.C., Riley, R.T., Sharma, R.P., Bucci, T.J., Lorentzen, R.J. (2002). Carcinogenicity and mechanism of action of fumonisin B1: a mycotoxin produced by Fusarium moniliforme (= F. verticillioides). *Cancer Detection and Prevention, 26: 1–9.*

Voss K.A., Smith G.W., Haschek W.M. (2007). Fumonisins: Toxicokinetics, mechanism of action and toxicity. Animal Feed Science and Technology, *137: 299-325.*

Wang, E., W.P. Norred, C.W. Bacon, R.T. Riley, A.H. Merrill Jr. (1991). Inhibition of sphingolipid biosynthesis by fumonisins. Implications for diseases associated with Fusarium moniliforme. *The Journal of Biological Chemistry, 266: 14486-14490.*

Wang, E., Ross, P.F., Wilson, T.M., Riley, R.T., Merrill, A.H. (1992). Increases in serum sphingosine and sphinganine and decreases in complex sphingolipids in ponies given feed containing fumonisins, mycotoxins produced by *Fusarium moniliforme*. *Journal of Nutrition, 122:1706-1716.*

Wentzel, J.F., Lombard, M.J., Du Plessis, L.H., Zandberg, L. (2016). Evaluation of the cytotoxic properties, gene expression profiles and secondary signalling responses of cultured cells exposed to fumonisin B1, deoxynivalenol and zearalenone mycotoxins. *Archives of Toxicology, DOI 10.1007/s00204-016-1872-y.*

Wu, F., Groopman, J.D., Pestka, J.J. (2014). Public health impacts of foodborne mycotoxins. *Annual Review of Food Science and Technology, 5:351–372.*

5. Aknowledgements

I would like to express my deep gratitude to my supervisor, Prof.ssa Francesca Caloni, for her valuable and constructive suggestions during the planning and development of this research work. Special thanks should be given to Dr.ssa Cristina Cortinovis, for her patient guidance, enthusiastic encouragement and valuable support. I would also like to extend my thanks to Dr. Leon J. Spicer for his help doing on this project, for his willingness to give his time so generously has been very much appreciated.
The Animal Science Department's group of the Oklahoma State University that has contributed immensely to my personal and professional time during the months that I spent in USA. The group has been a source of friendships and good advices: Luis Fernando Schütz, Lingna Zhang, Cheyenne Robinson, Morgan Totty and Jacqueline Ervin…thank you.
Finally, I wish to thank my family for their support and encouragement throughout my study and my life.