Nutritional strategies
to control mycotoxin damages in swine

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Chapter 1. Introduction

1.1 Mycotoxins

The term mycotoxin was created in 1962 as a consequence of a veterinary crisis near London, during which approximately 100,000 turkey died (Blout, 1961).

There are over 300 mycotoxins that have been isolated and chemically characterized (Betina, 1984), but research has focused on those forms causing significant injuries to humans and farm or companion animals.

Mycotoxins are secondary metabolites which are produced by several fungi belonging to the genera: *Aspergillus, Penicillium, Fusarium,* and *Alternaria* (Moss, 1992; Overy et al., 2003; Placinta et al., 1999; Sweeney and Dobson, 1998). During processes of food drying and storage, *Aspergillus* and *Penicillium* species, are generally found as contaminants while, before or after harvesting, *Fusarium* and *Alternaria spp*. mycotoxins are produced (Sweeney and Dobson, 1999). Secondary metabolites are synthesized during the end of the exponential phase of mould growth and seem to have no biochemical and/or biological significance in mould growth and/or development or competitiveness (Jay, 1992; Hussein and Brasel, 2001). Mycotoxins primarily occur in the mycelium of toxigenic moulds but could also be found in spores indicating that moulds are not toxigenic *per se* and while some mycotoxins are produced by only a limited number of moulds species, others may be produced by several genera (Deacon, 2001; D'Mello and Macdonald, 1997).

1.1.1 Ochratoxin A

Ochratoxin A (OTA) molecule was discovered as a metabolite of *Aspergillus ochraceus* in 1965 (Van der Merwe et al., 1965), is the most toxic molecule in ochratoxins group. Ochratoxins are a group of secondary metabolites produced by *Aspergillus and Penicillium spp.* They consist of OTA-ethyl ester known as ochratoxin C (OTC), ochratoxin B (OTB) and its methyl and ethyl esters, and other molecules as reported in Figure 1.1. OTA is mainly produced by *Penicillium verrucosum* or *P. nordicum* in cool and temperate regions, (Castella et al., 2002; Larsen et al., 2001; Pitt and Hocking, 1997) while in tropical and semitropical regions, it is mainly produced by *Aspergillus ochraceus* (Kozakiewicz, 1989; Pardo et al., 2005; WHO/FAO, 2001).

Ochratoxin A has been found in barley, oats, rye, wheat, coffee beans, and other plant products, with barley having a particularly high likelihood of contamination (Bennet and Klich, 2003). There is also concern that ochratoxin may be present in certain wines, especially

those from grapes contaminated with *Aspergillus carbonarius* (Marquardt and Frohlich, 1992; Pitt, 2000; Van Egmond and Speijers, 1994).

Ochratoxin A is nephrotoxic in both human and animal species studied. Kidney is the primary target organ, but animal studies indicate that ochratoxin A may cause liver toxicity, mutagenicity, teratogenicity, neurotoxicity, and immunotoxicity (Beardall and Miller, 1994; Kuiper-Goodman and Scott, 1989). OTA has been classified as a class 2B carcinogen (possibly carcinogenic to humans) by the IARC (International Agency for Research on Cancer) based on animal studies and epidemiological studies in human populations (IARC, 1993).

There has been speculation that ochratoxins are involved in a human disease called Balkan Endemic Nephropathy (Krogh, 1987; Hult et al., 1982). This condition is a progressive chronic nephritis that occurs in populations who live in areas bordering the Danube River in some areas of Romania, Bulgaria, and the former Yugoslavia. In one Bulgarian study, ochratoxin contamination of food and presence of ochratoxin in human serum were more common in people with Balkan Endemic Nephropathy and urinary tract tumors than in unaffected people (Castegnaro et al., 1987).
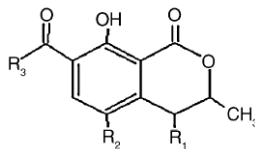
Ochratoxins	R1	R2	R3
OTA	H	Cl	-NH-CH(COOH)-CH$_2$- Phenyl
OTB	H	H	-NH-CH(COOH)-CH$_2$- Phenyl
OTC	H	Cl	-NH-CH(COOC$_2$H$_5$)-CH$_2$-Phenyl
4-hydroxyochratoxin A	OH	Cl	-NH-CH(COOH)-C$_2$H- Phenyl
OTα	H	Cl	-OH

Figure 1.1 Structure of ochratoxins (from Ringot et al., 2006)

Toxicokinetic of Ochratoxin A

After oral ingestion, OTA is rapidly absorbed and reaches the systemic circulation, where it is extensively bound to plasma proteins: the extent of absorption varies from 40% in chickens to 66% in pigs (Galtier et al., 1981). Following absorption, OTA is bound readily to serum albumin and other macromolecules (Hult and Fuchs, 1986). The unbound fraction is lower than 0.02% in humans, indicating an extent of protein binding of 99.98% (Hagelberg et al., 1989). In most animal species, the kinetic behaviour of OTA has been described as a two

compartments open model, although recent data on the accumulation in kidneys suggest that these models are too simple and data should be re-analysed using multi-compartment models. In monkeys and humans the major route for plasma clearance is urinary elimination, whereas in rodents biliary excretion seems to prevail. In blood, liver, and kidneys, OTA is accumulated; in females of mammalian species, OTA is excreted via milk (Marquardt and Frohlich, 1992). Biliary excretion and entero-hepatic re-circulation of OTA-glucuronides may account for the inter-individual and interspecies variability of kinetic parameters observed in kinetic studies. The elimination half-lives of OTA in Wistar rats and pigs were reported to be 5 and 6 days, respectively (Dietrich et al., 2005). In human, OTA has longer half-life for its elimination than in any of the species examined: data indicate that elimination half-lives in humans is 35 days (based on one individual) and in non-human primates is 19 to 21 days (Creppy, 1999). These species differences seem to be due to largely to differences in the degree of serum protein binding and its effect on renal clearance, as well as the rate of conjugation and extent of entero-hepatic re-circulation. Bacterial metabolism in the gastrointestinal tract produces the cleavage product, ochratoxin α, which can be absorbed from the lower gastrointestinal tract. The cellular effects of OTA include inhibition of enzyme involved in phenylalanine-tRNA complex synthesis (Bunge et al., 1979; Marquardt and Frohlich, 1992), mitochondrial ATP production inhibition (Meisner and Meisner, 1981) lipid peroxidation stimulation (Rahimtula et al., 1988).

Ochratoxin A in pigs

Pig is one of the most sensitive species to the adverse effects of OTA. Pigs are much more sensitive than most laboratory animal species excepting the dog. The oral LD_{50} in the pig is about 1 mg/kg b.w. (Harwig et al., 1983).

In a series of experiments, groups of three to six sows were given feed containing OTA at a concentration of 0, 0.2, 1, or 5 mg/kg, equivalent to 0, 8, 40, and 200 μg/kg b.w. per day, for periods of 5 days, 5 or 12-16 weeks, respectively, or up to 2 years. In female pigs, dietary levels of 0.2 mg/kg diet (equivalent to 8 μg/kg b.w. per day) for 90 days caused a reduction in renal activity of cytosolic phosphoenolpyruvate carboxykinase and gamma-glutamyl transpeptidase together with a decreased kidney function as indicated by reduced tubular excretion of p-aminohippurate and increased glucosuria. Cytosolic, and not mitochondrial, phosphoenol-pyruvate carboxykinase activity was reduced. Lowest Observed Effect Level (LOEL) for effects on the kidneys (effects on enzymes and function) was established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) at 8 μg/kg b.w. per day in a

90-day feeding study (FAO/WHO, 2001). Ochratoxin A is believed to be responsible for a porcine nephropathy that has been studied intensively in the Scandinavian countries. The disease is endemic in Denmark, where rates of porcine nephropathy and OTA contamination in pig feed are highly correlated (Krogh, 1987).

Ochratoxin A has been described to affect humoral and cellular immunity in several species: in pigs, after subcutaneous injection of pure or crude OTA were found reduction in relative lymphocyte counts and an increase in total leukocyte, relative neutrophil and eosinophil counts (Müller et al., 1999).

Ochratoxin A in rats

In rats, the effects of OTA on renal function and morphology are indicated by increased relative kidney weight, urine volume, blood urea nitrogen, urinary glucose, proteinuria, and impaired urinary transport of organic substances. Renal lesions were histologically characterised by karyomegaly, necrosis of tubular cells, and thickening of tubular basement membranes. The target site is specific, being the straight segment of the proximal tubule S3 in the outer stripe of the outer medulla. The most comprehensive studies on OTA toxicity in rats have been performed within the US National Toxicology Program (US-NTP, 1989). In these studies, groups of 80 male and female Fischer 344/N rats were given OTA by gavage in maize oil at a dose of 0, 21, 70, or 210 μg/kg b.w. per day, 5 days/week for up to 103 weeks. The studies confirmed the specific site of renal injury and the sex differences in susceptibility. Renal lesions consisted in contraction and disorganization of the normal linear pattern of the S3 tubules due to marked development of karyomegaly and cytomegaly. The overall Non Observed Adverse Effect Level (NOAEL) derived from these studies was 21 μg/kg b.w. per day for 5 days/week, equivalent to 15 μg/kg b.w. per day (US-NTP, 1989; FAO/WHO, 2001). Petrik et al. (2003) showed that administration of 120 μg OTA/kg b.w. per day to Wistar rats, for 10, 30 or 60 days, produced oxidative stress and dose/time related apoptosis in both, proximal and distal epithelial kidney cells. Studies in rats have shown a preventive effect of pre- or co-treatment with melatonin (10-20 mg/kg b.w. per day) on parameters of OTA-induced liver and kidney toxicity (Aydin et al., 2003) and on OTA-induced oxidative stress, such as changes in liver and kidney glutathione peroxidase, superoxide dismutase and malondialdehyde (Meki and Hussein, 2001; Abdel-Wahhab et al., 2005). Bertelli et al. (2005) have reported that flavonoids in red wine may exert a protective effect against OTA nephrotoxicity in rats by limiting oxidative damage as measured by renal lipohydroperoxides, reduced and oxidized glutathione, and renal superoxide dismutase activity.

1.1.2 Fumonisin B1

Fumonisins were first isolated in 1988 from a culture of *Fusarium verticillioides* (earlier *Fusarium moniliforme*) grown on maize and their structures were also determined (Bezuidenhout et al., 1988; Gelderblom et al., 1988). Fumonisin B analogues, including toxicologically important fumonisin B1, B2 and B3, are the most abundant naturally occurring fumonisins (Marasas, 1996).

The fumonisin B1 (FB1) molecule includes a long chain aminopentol backbone with two ester-linked tricarballylic acids. Aminopentol originates from FB1 by hydrolysis of the tricarballylic acid side chains at carbon 14 and 15, which are then replaced by hydroxyl groups.
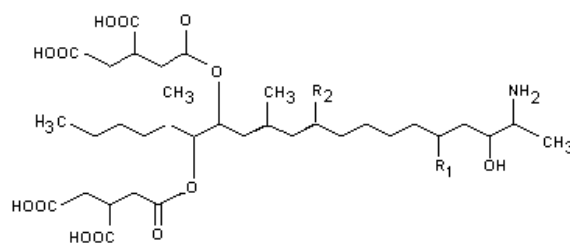
	R_1	R_2	Formula	CAS Number	Molecular mass
Fumonisin B_1	OH	OH	$C_{34}H_{59}NO_{15}$	116355-83-0	721.838
Fumonisin B_2	OH	H	$C_{34}H_{59}NO_{14}$	116355-84-1	705.839
Fumonisin B_3	H	OH	$C_{34}H_{59}NO_{14}$	136379-59-4	705.839
Fumonisin B_4	H	H	$C_{34}H_{59}NO_{13}$	136379-60-7	689.840

Figure 1.2 Chemical structure of fumonisins
(www.inchem.org, last access March, 7th 2008)

The exact mechanism of action of FB1 is still unclear. Shier (2000) refers to the low degree of absorption of FB1 as the "fumonisin paradox" (how can a toxin cause agriculturally significant diseases and possibly human cancers if it is not effectively absorbed after oral administration?). Little is known about the possible endogenous hydrolysis of FB1 by the mammalian metabolism, even if some studies performed on primates (Shephard et al., 1994a) and ruminants (Rice and Ross, 1994) revealed that the ester moiety of FB1 was hydrolysed in the intestine. Absorption of FB1 by enterocytes has also been studied in vitro with Caco-2 cells that are representative of human small intestine enterocytes: Caloni et al. (2002) showed no absorption of FB1 and no absorption of partially hydrolyzed metabolites by both undifferentiated or differentiated cells. By contrast, absorption of totally hydrolyzed FB1 (aminopentol or HFB1) was evident in differentiated Caco-2 cells, which expressed

enzymatic and metabolic characteristics of mature enterocytes (De Angelis et al., 2005). Molecular structures of fumonisins are very similar to sphingolipids (Shier, 1992); thus, they interfere in the metabolism of the latter and disturb the processes mediated by these molecules (Kim et al., 1991). It has been postulated that FB1 exerts its toxicological effects inhibiting ceramide synthase enzymes. This lead to an increased concentration of sphingoid bases (sphinganine and sphingosine and their phosphorylated derivatives) as well as the depletion of ceramide and complex sphingolipids (Merrill et al., 2001; Riley and Voss, 2006; Suzuky et al., 2007). The elimination route of FB1 is via bile and the excreted toxin is still biologically active (Enongene et al., 2000) either unchanged or depleted of one ester-linked tricarballylic acid (at the C14-position). Traces amounts of aminopentol, the fully hydrolyzed FB1, were found in feces (Shephard et al., 1994a). As no hydrolyzed product has been found in the urine or bile, it is assumed that the hydrolysis occurs in the gut, probably performed by microorganisms (Shephard et al., 1995; Fodor et al., 2006). Caloni et al. (2000) observed a poor rate of hydroxylation in the rumen fluid. Fumonisin B1 may be hydroxylated, but this limited hydroxylation seems to take place pre-systemically, as studies with bovine liver microsomes did not show any significant transformation of FB1 (Spotti et al., 2001). Although the biotransformation of FB1 is very limited, it has been shown to inhibit certain P450 enzymes, both *in vivo* and *in vitro* (Spotti et al., 2000).

Toxicokinetic of Fumonisin

Fumonisins are poorly absorbed and the oral bioavailability remains generally below 5% for FB1, and seems to be even lower for FB2. The absorbed fractions are rapidly distributed and eliminated. In rats and most other animals, the absorption kinetic of FB1 indicates a rapid distribution and elimination that is adequately described by a two- or three-compartments model (Martinez-Larranaga et al., 1999). Only a low level of FB1 is detected in plasma and tissues after oral administration, indicating that the absorption is negligible. Indeed, in cows and laying hens, systemic absorption of orally given FB1 is less than 1% (Martinez-Larranaga et al., 1999; Vudathala et al., 1994) In pigs, the bioavailability of FB1 following intragastric administration is estimated to be 3–6% (Prelusky et al., 1995).

In rats, FB1 shows a rapid distribution and renal elimination rates ($t_{\frac{1}{2}el}$ = 40 minutes after intravenous injection). After intragastric administration of the toxin, up to 80% of the radiolabel is recovered in feces and up to 3% in urine. Tissue levels were found to be the highest in kidneys, followed by liver (Norred et al., 1993) while no detectable quantity is reported in other organs (Shier, 2000). Fumonisin B1 is glucuronidated and excreted with bile

fluid (approximately 1.4% of the dose), which may result in entero-hepatic re-circulation. The entero-hepatic re-circulation probably increases the exposure of the intestine to the mycotoxin, at least for rats and pigs (Dantzer et al., 1999; Norred et al., 1993; Prelusky et al., 1996a; Shephard et al., 1994b). Intestinal cells are exposed to a substantial portion of the ingested FB1. Intestinal epithelial cells of nonhuman primates 24 h after administration of radiolabeled FB1, contained 25% of the dose.

Carry-over studies showed that in pigs fed 2-3 mg/kg feed ^{14}C-FB1 for a period of 24 days, concentrations of 160 and 65 ng/g of tissue FB1 were found in liver and kidney, respectively. Muscle and fat tissue did not contain residues of FB1 (Prelusky et al., 1996b). A more recent study on residue formation of FB1 in porcine tissues showed that after oral administration of a very high (experimental) dose (100 mg FB1 per animal per day) for 5-11 days, residues of FB1 can be detected in kidneys (833 ng/g), liver (231 ng/g), lung (170 ng/g), spleen (854 ng/g), muscle (26 ng/g) and fat (2 ng/g). All the animal studies carried out to determine the potential carry-over of fumonisins from animal feed into animal products indicated that levels of FB1 can be found in various tissues, but the low carry-over rate suggests that these low residue levels do not to contribute substantially to human exposure.

Fumonisin B1 in pigs

Fumonisin toxicosis in pigs is characterized by pulmonary, cardiovascular and hepatic symptoms. Moreover, hyperplastic oesophagitis, gastric ulceration, hearth hypertrophy and pulmonary arteries hypertrophy have been described (Casteel et al., 1994; Gumprecht et al., 1998, 2001; Smith et al., 1996, 1999). Lethal pulmonary oedema and hydrothorax has been observed in pigs exposed to feed containing > 12 mg FB1/kg feed (corresponding to 0.6 mg/kg b.w. per day) (Haschek et al., 2001). Gross pathology reveals a severe pulmonary oedema (heavy, wet lungs) with widened interlobular septa, but without further signs of inflammation. When exposure was consistent over a period of 8 weeks, levels as low as 1 mg FB1/kg feed produced proliferation of the connective tissue, primarily around the lymphatic vessels and in the subpleural and interlobular connective tissue, extending into the peribronchial and peribronchiolar area. However, these alterations were not accompanied by clinical signs (Zomborszky-Kovács et al., 2002a,b). It has been suggested that the pulmonary injury is preceded by cardiovascular abnormalities and haemodynamic changes with pulmonary hypertension and left heart insufficiency (Smith et al., 1999). When pigs are exposed to fumonisins they develop also hepatic injury with necrosis and cholestasis. Affected animals become anorexic; they show signs of encephalopathy, loss of body weight,

and hepatic nodular hyperplasia. These changes are associated to alterations in serum biochemical parameters, including an increase in circulating bile acids, elevated bilirubin concentrations, and increased values for serum aspartate aminotransferase, alkaline phosphatase, gamma-glutamyl transferase and lactate dehydrogenase activity (Zomborsky-Kovács et al., 2002a,b).

A dose- and time-dependent increase in the concentration of free sphinganine in serum and tissues is also observed early resulting in an increase in the sphinganine to sphingosine (Sa:So) ratio. In lung, liver, and kidneys, Sa:So ratio is increased and significant changes have been observed after exposure to 5 mg FB1 per kg feed (corresponding to an exposure of 0.2 mg/kg b.w. per day) for a period of 2 weeks. Riley et al. (1993) found high free sphinganine level in liver, lung, and kidney tissues from pigs that were exposed to concentration ≥23 mg total FB1 + FB2 per kg feed, and an increased Sa:So ratio in serum from pigs occurred when concentrations were ≥ 5 mg/kg feed.

Rotter et al. (1996) and Zomborszky-Kovács et al. (2002a) found that the Sa:So ratio was not increased in pigs fed 1 mg pure FB1/kg feed. Increased serum Sa:So ratios were found by these studies in pigs fed diets contaminated with 10 mg/kg feed or with ≥5 mg/kg feed, respectively.

In practical conditions, the increase in the Sa:So ratio can be used to establish a diagnosis, as this phenomenon is unique for a fumonisin toxicosis. Alterations in the Sa:So ratio in organs are a sensitive biomarker, monitoring of the Sa:So ratio (for example in serum or urine samples) has a double function in monitoring exposure and assessing the onset of adverse effects due to fumonisin toxicosis. Considering the Sa:So ratio as the most sensitive parameter in the assessment of adverse effect exerted by fumonisins, the Lowest Observed Adverse Effect Level (LOAEL) was found to occure when pigs were exposed to feed containing 5 mg of fumonisins per kg feed. Lung lesions in pigs were observed at a dose of 0.4 mg/kg b.w. per day (Riley et al., 1993; Zomborszky-Kovács et al., 2002a).

Fumonisin in feeds and foods

Natural occurrence of fumonisins on maize and maize-based products has been reported in different parts of the world (Dutton, 1996). The natural occurrence of FB1 has also been reported in sorghum (Shetty and Bhat, 1997). Ingestion of feed contaminated with fumonisins cause syndromes like equine leukoencephalomalacia (Marasas et al., 1988), porcine pulmonary edema (Harrison et al., 1990), and induces hepatotoxic effects in rats (Gelderblom et al., 1994). Several human epidemiological studies have shown a strong correlation between

the natural occurrence of dietary fumonisins with high incidences of human esophageal cancer. Fumonisins are implicated in the pathogenesis of oesophageal cancer in humans (Marasas et al., 2000) in different parts of the world (South Africa, China, Italy, and South Carolina). Studies in the Texas-Mexico border suggest that intake of fumonisin-contaminated corn (in the form of tortillas) induce an increased neural tube defects among Mexican-American women (Missmer et al., 2006). Fumonisin B1 has been classified by the International Agency for Research on Cancer (IARC, 1993) as "possibly carcinogenic to humans" (class 2B). Food of animal origin could be contaminated with FB1 after the toxin has been absorbed from the digestive tract, entered the bloodstream and reached other tissues.

1.1.3 Deossinivalenol

Deoxynivalenol (DON), also known as vomitoxin, is mainly produced by *Fusarium graminearum (Gibberella zea)*, in some areas by *F. culmorum* (Richard, 2000). The geographical distribution of the two species appears to be related with temperature, since *F. graminearum* predominantly is found in warmer climates.
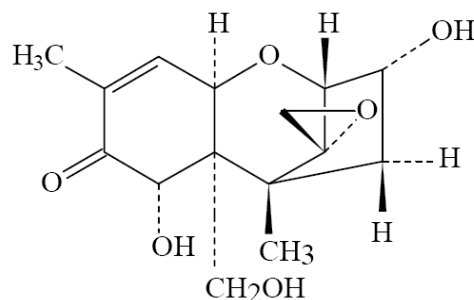
Figure 1.3 Chemical structure of deoxynivalenol (EFSA, 2004).

Deoxynivalenol is the most common thricotecene, a class of mycotoxins, worldwide found in feedstuffs, particularly cereals (wheat, maize, barley, oat, rye and less often in rice, sorghum and triticale). Corn and wheat are the major crops affected, while small grains such as oat, rye, and barley has low incidence of DON contamination (CAST, 2003). Fungi survive on residue leaves on the field from the previous season, providing an inoculum source for the new crop. These fungi find the best conditions for development in cool, moist conditions with contamination of the crop occurring when conidia of the organism are windblown to the corn silks or in small grains to the anthers which emerge outside the floret during anthesis.

The fungus penetrates the host ear or floret and produces the disease which may be ear rot in corn or head blight in small grains. A direct relationship between the incidence of *Fusarium* head blight and contamination of wheat with DON has been established. The incidence of *Fusarium* head blight is most affected by moisture during flowering season (WHO, 2001). The time of rainfall is the most critical period for toxin production. Certain environmental conditions may allow for late growing season development of DON in crops. In corn, the ear rot produced by *F. graminearum* may appear. (Richard, 2007). Deoxynivalenol occurrence is almost exclusively associated with cereals, and the levels of occurrence are in the order of hundreds of µg/kg upwards. Contamination occurs mainly in field pre-harvest. As seasonal variations significantly influence the extent of Fusarium infections, levels of DON tend to vary from year-to-year. Wheat may appear prematurely ripe and at harvest the kernels will have a blanched appearance and pink staining. In cereals where there is pink staining the disease may be referred to as pink scab. Storage under good conditions (<14% moisture) will minimize further elaboration of the toxin and DON does not further accumulate in storage.

There is no experimental or epidemiological evidence for mutagenic and/or carcinogenic properties of DON and it was classified in 1993 by the International Agency for Research on Cancer (IARC) in Group 3 (not classifiable as to its carcinogenicity to humans). A Temporary Tolerable daily Intake (TDI) of 1 µg/kg b. w. was established by the EU Scientific Committee on Food (SCF, 2002), and the Provisional Maximum Tolerable Daily Intake (PMTDI) established by JECFA (WHO, 2001).

Mechanism of action of Deoxynivalenol
The first toxic effect associated with trichothecenes including DON was the inhibition of protein synthesis. Trichothecenes bind to the 60S subunit of eukaryotic ribosomes and interfere with the activity of peptidyltransferase. Based on the induction of emesis, suppose a possible interaction with serotinergic and dopaminergic receptors (Fioramonti et al., 1993). Loss of appetite, and subsequently reduced feed intake resulted in low weight gain in growing animals due to this effect. DON is also an immunosuppressor (reduction of immunoglobulins production and depletion of lymphocytes from spleen, Peyers patches and thymus). Clinical studies revealed cell depletion in thymus, spleen or bursa Fabricius in exposed animals, and *in vitro*, the sensitivity of B- and T- cells, isolated from spleen, thymus and Peyer's patches towards DON has been described in many studies. *In vivo* cell apoptosis in thymus, spleen, Peyer's patches, bone marrow, and liver has been also demonstrated after administration of other trichothecenes (Poapolathep et al., 2002, 2003; Shinozuka et al., 1997a,b). Recently,

DON involvement in upregulation of pro-inflammatory cytokines has been studied: evidence in rodent species that DON increases the expression of proinflammatory cytokines, might provide the explanation for a various effects observed in pigs, including:

- feed refusal, as IL-6 is known to induce anorexia,

- inhibition of protein-synthesis and reduced weight gain,

- IL-6 dependant deregulation of IgA production: elevated levels of circulatory IgA might impair glomerular function, resulting in a renal wasting syndrome (Pestka and Zhou, 2000).

Concomitant factors (lipopolysaccharide of Gram-negative bacteria, viral infections) that modulate transcription of cytokines and chemokines, could explain the differences in clinical responses to low levels of DON in individual herds, due to the strong influence of these factors on DON response. It is important to note that these mechanistic studies have been almost entirely conducted in mice, or rodent and human cell lines. However, the described mechanisms are highly preserved in mammals, including pigs where the response may be even more pronounced due to the renowned susceptibility of pigs to bacterial and viral agents modulating cytokine response.

Toxicokinetic of Deoxynivalenol

Deoxynivalenol is rapidly absorbed in pigs and oral bioavailability is estimated to be 55% (Rotter et al, 1996). After intragastric dosing of radiolabeled DON, absorption half time was less than 30 minutes (Prelusky et al., 1988). After feeding a diet containing naturally contaminated wheat (4.2 mg/kg feed), the maximum serum DON concentration was found after 4.1 h (Dänicke et al., 2004a). Organ distribution was measured in pigs only following a single intravenous injection of DON (1 mg/kg b.w.) and revealed high initial concentrations in plasma, kidney and liver. Measurable concentrations were detected also in the abdominal fat, back fat, lung, adrenals, spleen, testis, heart, brain, muscle tissue, intestines and pancreas, indicating a large volume of distribution (Prelusky and Trenholm, 1991). The plasma eliminination half-life was found to vary between 1.2 and 3.9 hours in pigs depending on study (Eriksen et al. 2003; Prelusky and Trenholm, 1991) and reach 7.14 hours when radio-labelled DON was given by gavage (Prelusky et al., 1988). Excretion of DON occurs predominantly via urine. Deoxynivalenol may be de-epoxidated by the microbial flora of the intestinal tract with an increasing capacity from the small to the large intestine (Dänicke et al., 2004a). De-epoxy DON has not been detected in blood, although it was excreted in the urine (Eriksen et al., 2003; Dänicke et al., 2004b-d). Moreover, the glucuronidated DON is found in

blood and urine. Acetyl-DON is rapidly deacetylated in the upper intestinal tract and absorbed exclusively as de-acetylated DON (Eriksen et al., 2003). Deoxynivalenol is not significantly carried over into tissues or fluids of animals consuming toxic levels (Prelusky, 1994). Residue analyses of DON in tissues from pigs fed DON concentration ranging between 0.7–7.6 ppm revealed in most cases low (<20 ppb) or undetectable DON concentrations (Cote et al., 1985; Prelusky and Trenholm, 1992).

Deoxynivalenol in pigs

In swine, DON induces reduction of feed intake or even feed refusal, and, at high dosages pigs may vomit. Differences are reported for *in vivo* trials when DON contamination of the diets is obtained adding crystalline DON or naturally/artificially contaminated raw material: naturally/artificially contaminated feed had a stronger effect on the feed intake and weight gain reduction than pure toxin (Forsyth et al., 1977; Foster et al., 1986; Rotter et al., 1994; Trenholm et al., 1994). In pigs, when crystalline DON was applied, complete feed refusal was observed at 12 mg/kg feed and vomiting at 20 mg/kg feed (Young et al., 1983; Forsyth et al., 1977). When naturally or artificially DON contaminate raw materials are mixed into the diets, the decrease of feed consumption, and consequently weight gain, was observed at 0.6 and 2 mg/kg feed for naturally and artificially contamination, respectively (Bergsjö et al., 1993b; Friend et al., 1982; Overnes et al., 1997; Young et al., 1983). The difference is not clearly explained, but hypotheses include the presence of other toxins in the raw materials, the presence of other compounds (for example bacterial polysaccharides) increasing the toxicity of trichothecenes and inducing taste aversion (Rotter et al., 1996). The observed reduction in feed intake at the lowest doses of contamination was temporary, but the loss in weight gain during the first period was not completely compensated later and the animals reach slaughter weight at a higher age.

1.1.4 Mycotoxins legislation in European Union

Regulations for ochratoxin A, fumonisins and deoxynivalenol are present in the European Union (Table 1.1). No regulations have been established in United States for ochratoxin A. Guidelines and advisory levels are proposed by FDA for fumonisins and deoxynivalenol, respectively (Table 1.2).

Recently, the Scientific Panel on Contaminants in food chain of European Food Safety Agency (EFSA) has published an opinion where OTA is considered an undesirable substance in food (EFSA, 2006) after a previous opinion about its presence in animal feed (EFSA,

2004a). Human exposition to OTA seems to be associated primarily to consumption of contaminated vegetable products (cereals, coffee, wine) and, partially to ingestion of animal products derived from animals fed contaminated feeds. Data on human exposition to OTA revealed that weekly exposure in adults ranges from 15 to 60 ng/kg b. w. including high consumers of foods containing ochratoxin. This rate of exposure is below the Tolerable Weekly Intake value of 120 ng/kg body weight as derived by the Panel. However, as current EFSA consumption databases do not include infants and children, they concluded that more data would be needed to assess exposure rates of this segment of consumers, taking into account their dietary preferences (EFSA, 2006).

DON and fumonisins were classified as undesirable substances in animal feeds, in 2004 and 2005 respectively (EFSA, 2004b; EFSA 2005). No opinions on DON and fumonisins in food are published at the moment.

Table 1.1. European Union regulations for ochratoxin A, fumonisins (including FB1, FB2, and FB3), and deoxynivalenol

Mycotoxin/Product	Concentration (µg/kg)
Ochratoxin A	
Raw cereals grains	5
All products derived from cereals intended for direct human consumption	3
Dried vine fruit (currants, raisins, and sultanas)	10
Fumonisins (including FB1, FB2, and FB3)	
Unprocessed maize	2000
Maize grits, meal, and flour	1000
Maize-based food for direct consumption except maize grits, meal, and flour and processed maize based foods for infants and young children and baby food	400
Processed maize-based foods for infants and young children and baby foods	200
Deoxynivalenol	
Cereal products as consumed and other products at retail stage	500
Flour used as raw material in food products	750

Table 1.2. United States regulations for fumonisins (including FB1, FB2, and FB3), and deoxynivalenol

Mycotoxin/Product	Concentration (µg/kg)
Fumonisins (including FB1, FB2, and FB3)	
Human foods	
Degermed dry milled corn products	2
Whole/partially degermed dry milled corn product	4
Dry milled corn bran	4
Cleaned corn intended for mass production	4
Cleaned corn intended for popcorn	3
Corn and corn byproducts for animals	
Equids and rabbits	5<20% diet
Swine and catfish	5<20% diet
Breeding ruminants, poultry, mink, dairy cattle, laying hens	30<50% diet
Ruminants >3 mos. before slaughter and mink for pelts	60<50% diet
Poultry for slaughter	100<50% diet
All other livestock and pet animals species	10<50% diet
Deoxynivalenol	
Finished wheat products for human consumption	1
Grain and grain by products destined for swine and other animals (except cattle and chickens), not to exceed 20% of diet for swine (40% for other species)	5
Grain and grain byproducts for beef, feedlot cattle older than 4 months and chickens; not to exceed 50% of the diet.	10

1.2 Prevention strategies

Mycotoxicoses are the toxic responses caused by mycotoxins ingestion by humans and animals and are examples of "poisoning by natural means" analogous to the pathologies caused by exposure to pesticides or heavy metal residues (Bennett and Klich, 2003). Toxic effects depend on different factors including type of mycotoxin and its mechanism of action, amount and duration of the exposure, age, health, sex, and metabolism of the exposed individual (Galvano et al., 2001; Hussein and Brasel, 2001). Synergistic effects involving genetics, dietary status, and interactions with other toxic insults could occur and are not well understood. Toxic effects of mycotoxin ingestion get worse if other nutritional injuries such as vitamin deficiency, caloric deprivation, alcohol abuse, malnutrition, and infectious disease status occur (Bennett and Klich, 2003).

Consumption of mycotoxin contaminated foods lead to the induction of teratogenic, cancerogenic, oestrogenic, neurotoxic, and immunosuppresive effect in humans and/or animals (Atroshi et al., 2002). To control mycotoxins induced damages, different strategies have been developed to reduce the growth of mycotoxigenic fungi as well as to decontaminate and/or detoxify mycotoxin contaminated foods and animal feeds. Critical points, target for these strategies, are: prevention of mycotoxin contamination, detoxification of mycotoxins already present in food and feed, inhibition of mycotoxin absorption in the gastrointestinal tract, reduce mycotoxin induced damages when absorption occurs.

1.2.1 Prevention of contamination

Mycotoxin contamination of agricultural product can occur in the field as well as during storage: phytopathogenic fungi such as *Fusarium spp* can produce mycotoxins before or immediately post harvesting. Strategies to prevent mycotoxins contamination can be divided regard the use of different products, such as cereals, nuts, fruits, coffee, wine, etc. for the prevention and reduction of various mycotoxins.

Main mycotoxin hazards associated with wheat pre-harvest in Europe are the toxins that are produced by fungi belonging to the genus *Fusarium* in the growing crop: mycotoxins produced by these fungi include trichothecenes (deoxynivalenol, nivalenol, and T-2 toxin) as well as zearalenone. Although *Fusarium* infection is generally considered to be a pre-harvest problem, if poor drying practices are made, can increase the susceptibility for in storage

mycotoxin contamination (Aldred and Magan, 2004). There are inherent differences in the susceptibility of various cereal species to mycotoxin contamination and these differences between crop species appear to differ between countries. This is due to differences in the genetic pool within each country's breeding program and the different environmental and agronomic conditions in which crops are cultivated (Edwards, 2004). For these reasons particular seed varieties are recommended for the use in specific geographical areas (Codex Alimentarius, 2002). Mesterhazy et al. (2002) suggested, based on a study about DON contamination in wheat, that an increased availability of resistant varieties, coupled with the use of appropriate fungicides, was the key in an integrated approach to mycotoxin control associated with *Fusarium*.

In maize, hybrids genetically engineered for insect resistance (by insertion of *Bacillus thuringiensis* genes encoding the endotoxin CryA), had kernels that consistently had less fumonisins than kernels from normal plants. Other maize hybrids genetically engineered for fumonisin degradation (by insertion of a gene encoding a fumonisin esterase enzyme, from a Gram-positive bacterium) have been produced. These transgenic maize plants can degrade FB1 to aminopentol, its hydrolysis product, a compound that however retains most of the FB1 toxicity (EMAN, 2008a).

Another factor which is known to increase the susceptibility of cereals to toxigenic mould invasion is injury due to insect, bird, or rodent damage (Smith et al., 1994). Insect damage and fungal infection must be controlled by proper use of insecticides, fungicides, and other appropriate practices. Benefits of fungicide is due to their efficacy in preventing or reducing toxin synthesis in naturally-infected fields (Ioos et al., 2005). The use of fungicides introduces a complication: there is evidence that under certain conditions, fungicide use may actually stimulate toxin production. This is an important finding as it indicates that the impact of the fungicide is not directly related to mycotoxin production.

A limited number of biocompetitive microorganisms have been found for the management of *Fusarium* infections. Antagonistic bacteria and yeasts may also lead to reductions in pre-harvest mycotoxin contamination. For instance, *Bacillus subtilis* has been shown to reduce mycotoxin contamination by *F. verticilloides* during the endophytic growth phase. Similarly antagonistic yeasts such as *Cryptococcus nodaensis* have also been shown to inhibit various *Fusarium* species (Cleveland et al., 2003).

Moisture management is the critical point: the main control measure in preventing mycotoxin contamination from harvest until the end of the production chain. An appropriate sampling procedure to control moisture content of the harvested grain during the harvesting operation is

very important because moisture vary considerably within the same batch of grains. Another strategy to reduce mycotoxin contamination is the separation of damaged grains from healthy grains. Fungal infection is minimized by avoiding the mechanical damage to grains and contact with soil (Codex Alimentarius, 2002; EMAN, 2008b).

Post harvest strategies are important in the prevention of mycotoxin contamination including improved drying and storage conditions, and eventually, the use of natural and chemical agents, as well as irradiation. Mycotoxigenic fungal growth can arise in storage as a result of moisture variability within the bulk or as a result of condensation of moisture in cooled grains located closed to the interface with the wall of the storage silo.

The moisture levels in stored crops is one of the most critical factors in the growth of mycotoxigenic moulds and in mycotoxin production: aflatoxins can be produced at a_w values ranging from 0.95 to 0.99 with a minimum a_w value of 0.82 for *A. flavus*, while the minimum a_w for OTA production is 0.80 (Sweeney and Dobson, 1998). Aflatoxins are produced by *A. parasiticus* at minimum relative humidity of 14% in wheat grains while for OTA production the moisture content needs to be higher, approximately 17-18% (Codex Alimentarius, 2002).

Storage temperature is another critical factor influencing mould growth and mycotoxin production: OTA production by *P. verrucosum* occurs between 10 to 25°C, while aflatoxins produced by *A. flavus* can growth in the temperature range from 10-43°C, with an optimum in the 32 to 33°C range (ICMSF, 1996; Olsen et al., 2003). Monitoring of stored grain temperature several times during storage may be important in determining mould growth: temperature increase of 2-3°C may indicate mould growth or insect infestation. To control and reduce mould growth and mycotoxins production, Codex Alimentarius (2002) suggests that storage facilities must be dry, provide protection from rain, ground water, rodents and birds, with minimum temperature fluctuations.

Various natural and chemical molecules are known to prevent both mycotoxigenic mould growth and mycotoxin formation. Fungicides have effect on mould growth and mycotoxins biosynthesis regarding their chemical type, dose of application, cereal type, fungal species, and storage conditions. Other approaches and possible alternatives to fungicides are the use of organic acids that inhibit growth and mycotoxins production (sorbic acid and its salts, sodium benzoate, propionic and citric acids) and the potential use of antagonistic bacteria, fungi, and yeast. Growth and OTA production were inhibited in many strains of *Aspergillus* tested by vanillic acid but no inhibition nor reduction in OTA production was detected in *Aspergillus ochraceus* strain. (Palumbo et al., 2007). It is well established that antagonistic yeast can

reduce the growth of spoilage moulds both *in vitro* and under simulated full-scale storage conditions.

Pichia anomala and *Saccharomyces cerevisiae* and their activity against *P. verrucosum* induce a decrease in OTA production to non detectable levels (Petersson et al., 1998). Lactic Acid Bacteria (LAB) or their antifungal metabolites have been studied as natural preservatives to inhibit mycotoxigenic mould growth and mycotoxin production in recent years (Magnusson et al., 2003; Stiles et al., 2002). Lactic acid bacteria showed reduction or prevention of aflatoxins production by *Aspergillus* species (Gourama and Bullerman, 1997; Luchese et al., 1992). Special interest to LAB is due to their activity as preservatives in foods and the classification of some of them as GRAS "Generally Recognized As Safe". In some cases the antifungal and antimycotoxigenic potential of LAB are still unknown, it is widely believed that inhibition of mycotoxin synthesis is due to microbial competition, the depletion of nutrients, low pH, and also due to the production of heat-stable low-molecular weight of metabolites which are produced by LAB (Batish et al., 1997; Laitila et al., 2002). Natural plant extracts and spices are also known to prevent mould growth and mycotoxin production (Dobson, 2002; Yin and Cheng, 1998). Juglal et al., (2002) suggested that the inhibitory effects exerted by spices and herbs may rely at least in part on phenolic compounds such as coumarins and flavonoids.

With respect to OTA production, spice essential oils of oregano, mint, basil, sage, and coriander have been shown to be effective against ochratoxin-producing fungi, with oregano and mint oils completely inhibiting the growth of a strain of *A. ochraceus* and OTA production after 21 days at the concentrations of 1000 ppm (Basìlico and Basìlico, 1999).

If fungal contaminaton is already present in feed and foods, different strategies can be applied to remove them and reduce mycotoxins production. Radiation is typically categorized as either ionizing or non-ionizing, with ionizing rays involving X-rays, and gamma rays and non-ionizing rays involving UV rays, microwaves, infrared rays, and radio waves. In relation to mycotoxin prevention irradiation has been used to inhibit mycotoxin biosynthesis during storage period, and many studies have been conducted to access the use of γ-irradiation in particular to prevent mould growth and mycotoxin formation (Aziz et al., 2002). Refai et al. (1996) showed that radiation dose of 4 kGy both inhibit growth of *A. ochraceus* and subsequent ochratoxin production in poultry feed concentrates. Not all fungi however respond to irradiation in the same way as is evidenced by the report of Aziz and Smyk (2002) where they showed that exposure to near UV radiation induced the synthesis of both AFB1 (200 ppm) and OTA (210 ppm), in non-toxigenic fungal strains of *A. flavus* EP-63 and *A.*

ochraceus P-153, respectively. The effectiveness of irradiation in the inhibition of mould growth and mycotoxin biosynthesis is both strain and dose dependant, as well as being influenced by humidity and storage conditions.

1.2.2 Detoxification of contaminated food and feed

The prevention of mycotoxin contamination prior to harvest or during post-harvest and storage is not always possible therefore various detoxification processes play an important role in helping prevent exposure to the toxic and carcinogenic effect of mycotoxins. Detoxification of mycotoxins is typically achieved by removal or elimination of the contaminated commodities or by inactivation of the toxins present in these commodities by physical, chemical, or biological methods but chemical treatments to detoxify foods and feeds is not allowed in European Union. The dilution of contaminated products with good quality products is also prohibited (Reg. CE 466/2001).

Decontamination processes, as indicate by FAO, needs the following requisites to reduce toxic and economic impact of mycotoxins (Bata and Lasztity, 1999; Galvano et al., 2001; Piva G. et al., 1995):

- it must destroy, inactivate, or remove mycotoxins;

- it must not produce or leave toxic and/or carcinogenic/mutagenic residues in the final products or in food products obtained from animals fed decontaminated feed;

- it must be capable of destroying fungal spores and mycelium in order to avoiding mycotoxin formation under favorable conditions;

- it should not adversely affect desirable physical and sensory properties of the feedstuff;

- it has to be technically and economically feasible.

Physical segregation and removal of contaminated grains from cereals is an important option since chemical detoxification is not acceptable.

The removal of seeds and kernels visible contaminated by moulds can lead to the removal of significant quantities of mycotoxins since the mycotoxins contamination occurs in small amounts of grains (Scott, 1998). Sorting can be based on damaged and discolored grains with manual, mechanical, and electronic methods. Manual selection is based on the fact that damaged kernels will vary in size, shape, and color and that visible mould growth will be present on the affected kernels. This method is the simplest way for the physical removal of contaminated grains but it is a very time-consuming procedure and it is not applicable in

cereals. Manual sorting is very useful for the removal of mouldy apples to reduce the level of patulin contamination (FAO, 2001).

In mais and cotton seeds fluorescent sorting is useful to remove aflatoxin contaminated grains (Scott, 1998) but in this case electronic sorting is unlikely to be used on large scale due to economic factors.

The use of floating and density segregation is another method to decrease the mycotoxin content of crops: aflatoxin levels in toxic maize kernels can be decreased in this way (Huff and Hagler, 1985).

It has been reported that gravity separation can be useful in reducing DON levels in grains. It has also been shown that the use of specific gravity tables which allow the removal of the least dense fractions containing the tombstone kernels can reduce DON contamination by 68 to 85% in wheat containing 4–7 mg/kg toxin (EMAN, 2008c).

During milling process it is possible to remove certain grain components to reduce levels of contamination. In standard milling practice to obtain white flour, OTA can be reduced by 66% from hard wheat inoculated with *P. verrucosum* contaminated at 618 μg/kg OTA but only a 40% reduction can be obtained for soft wheat (EMAN, 2008c).

Physical methods for mycotoxin decontamination include thermal and irradiation inactivation of mycotoxins.

Most mycotoxins are heat-stable within the range of conventional food processing temperatures (80–121°C), so little or no destruction occurs under normal cooking conditions such as boiling and frying, or even following pasteurization (Scott, 1984; Smith et al., 1994). The sensitivity of mycotoxins to heat treatment is affected by many factors including moisture, pH, and ionic strength of food (Samarajeewa et al., 1990). Degradation by heat treatment depends on type of mycotoxin and its concentration, the extent of binding between the mycotoxin and the food constituents, the degree of heat penetration, as well as the heating temperature and the processing time (Rustom, 1997). Deoxynivalenol levels were unaffected by heat treatments of 100–120°C at pH 4.0 and 7.0, but at pH 10.0 heat treatments of 120°C for 30 min or 170°C for 15 min resulted in the complete degradation of DON (Wolf and Bullerman, 1998). FB1 is completely removed from corn meal if it is roasted at 218°C for 15 min (Castelo et al., 1998).

High temperatures reached during extrusion has been reported to be a useful technique in the destruction of some naturally occuring food toxins. Cazzaniga et al. (2001) reported a greater than 95% reduction of DON in maize flour following extrusion cooking (140–200°C).

Irradiation has proven useful in the post harvest control of mycotoxins in storage: irradiation is a non-thermal treatment and is increasingly being referred to as "cold pasteurization" given that it can eliminate food borne pathogens without increasing in product temperature, and that overall average doses of 10 kGy presents no toxicological hazard (Molins et al., 2001; Rustom, 1997).

A wide range of chemicals have been shown to reduce, destroy, or inactivate mycotoxins (Piva G. et al., 1995; Scott, 1998; Sinha, 1998). These chemicals include acids (hydrochloric acid), bases (ammonia, sodium hyroxide), oxidizing agents (hydrogen peroxide, ozone), reducing agents (sodium bisulfite), chlorinating agents (sodium hypochlorite, chlorine dioxide and gaseous chlorine), and miscellaneous reagents such as formaldehyde. Many chemical treatments may destroy mycotoxins present in many foods and feeds, but in many cases they significantly decrease the nutritional value of foods or produce toxic products or other products with undesirable effects; these side-effects limit their widespread use.

The use of many of the available physical and chemical methods for the detoxification of products contaminated with mycotoxins is limited due to problems concerning safety issues, possible losses in the nutritional quality of treated commodities, limited efficacy and economic implications (Bata, and Lasztit, 1999). This has led to the search for alternative strategies such as biological agents.

Various fermentation processes have also been shown to result in reducing the toxic effects of mycotoxins. Varga et al. (2000) reported the ability of various *Aspergillus* strains (*A. fumigatus*, *A. japonicus*, and *A. niger*) to degrade OTA in liquid YES media. It as been suggested that mycotoxins present in agricultural products may be removed by ethanol fermentation as occurs for OTA in beers, patulin in apple juice and OTA in barley after ethanol fermentation (Bennet and Richard, 1996; Karlovsky, 1999). By contrast, it is a well-established that OTA has been found in a wide variety of wines, indicating that it does in fact survive the fermentation process (Cabanes et al., 2002; Lopez de Cerain et al., 2002).

1.2.3 Inhibition of gastrointestinal tract absorption

One approach to the prevention of mycotoxicosis in livestock is the addition in the diets of the non-nutritionally adsorbents that bind mycotoxins preventing the absorption in the gastrointestinal tract. The addition of adsorbents to feeds is the most widely applied way of protecting animals against mycotoxin damage. Activated carbons, hydrated sodium calcium aluminosilicate (HSCAS), zeolites, bentonites, and certain clays, are the most studied adsorbent and they possess a high affinity for mycotoxins (Huwig et al., 2001; Nageswara

Rao and Chopra, 2001; Ramos et al., 1996). Esterified glucomannan derived from yeast cell wall have been tested for the adsorption of mycotoxins. The cell walls polysaccharides (glucan and mannan), proteins, and lipids have been reported to exhibit numerous different adsorption mechanisms, e.g. hydrogen bonding, ionic, or hydrophobic interaction (Diaz et al., 2002).

The adsorption efficiency of these compounds depends on the chemical structure of both the adsorbent and the mycotoxin. The physico-chemical structure of the adsorbent including dose, total charge and charge distribution, size of the pores and surface area, as well as physico-chemical properties of the mycotoxins play a significant role in the achievement of mycotoxin binding by adsorbent materials (Huwig et al., 2001). Mycotoxins that contain polar functional groups such as aflatoxins can be adsorbed by many effective adsorbents including certain clays e.g. montmorillonite and zeolite-clinoptolite. Non-polar mycotoxins such as OTA and zearalenone are not effectively adsorbed on the hydrophilic negatively charged surfaces of unmodified clays.

Activated carbons are known as one of the most effective and non-toxic group of sorbents with a high surface to mass ratio (500–3500 m^2/g). Proposed mechanism for activated carbon is the adsorption of mycotoxins by hydrogen bonding. According to Lemke et al. (2001), binding of mycotoxins to HSCAS and activated carbons was nearly 100% available in both water and simulated gastrointestinal fluid. In another study, Galvano et al. (2001) showed that the adsorption abilities of the activated carbon ranging from 0.80 to 99.8% and 1.83 to 98.93% for OTA and DON, respectively.

Similarly, Avantaggiato et al. (2003) investigated the effectiveness of activated and cholestyramine in reducing the intestinal absorption of zearalenone. They observed that, the use of 0.25 to 2% activated carbon or cholestyramine resulted in a reduction of zearalenone absorption of 43 to 84% or 19 to 52%, respectively. HSCAS from natural zeolite has been the most extensively investigated adsorbent because of possessing high affinity for aflatoxins demonstrated by several *in vivo* and *in vitro* experiments.

Zeolites are crystalline, HSCAS of alkali and alkaline-hearth cations characterized by infinite three-dimensional structure. Dakovie et al. (2003) indicated that the degree of hydrophobicity plays a role in OTA adsorption on organo-zeolites and reported that the toxic effects of OTA in the gastrointestinal tract can be prevented by the use of organo-zeolite in animal feed.

Bentonites are another category of adsorbents commonly used in the adsorption of mycotoxins. Bentonites have been extensively used in the clarification of beverages and

decoloration of oils (Ramos et al., 1996). The adsorption ability of bentonites mainly depends on the interchangeable cations (Na^+, K^+, Ca^{2+}, and Mg^{2+}) present (Galvano et al., 2001).

In recent years, there has been increasing interest on the hypothesis that the absorption in consumed food can be inhibited by microorganisms in the gastrointestinal tract (Kankaanpää et al., 2000) Numerous investigators showed that some dairy strains of LAB and bifidobacteria were able to bind aflatoxins effectively (El-Nezami et al., 1998a; Oatley et al., 2000). The mechanisms of aflatoxin binding by specific LAB and bifidobacteria are unclear: cell wall peptidoglycans and polysaccharides have been suggested to be the two most important elements responsible for the binding by LAB and the absorption of mutagens/carcinogens in the small intestine (El-Nezami et al., 2000; Haskard et al., 2000). This suggestion is supported by the study of El-Nezami et al. (1998b) where all the Gram-positive strains tested were more efficient than *Escherichia coli*, suggesting that the bacterial ability to remove AFB1 is dependent on the cell wall structure.

A *Eubacterium* (BBSH 797) strain is able to inactivate trichothecenes by reduction of the epoxide ring (Binder et al., 1996; CAST, 2003). This strain was isolated out of bovine rumen fluid and the mode of action was proven *in vitro* and also *in vivo*. A novel yeast strain, capable of degrading OTA and zearalenone was isolated and characterized (Bruinink et al., 1998; Schatzmayr et al., 2003).

The ability of *Lactobacillus rhamnosus* strains GG and LC-705 to remove AFB1 and OTA, were studied by Turbic et al (2002); they reported removal of high amounts (77-95%) of AFB1 and moderate amounts (36-76%) of OTA and not for other substrates (caffeine, vitamin B12, folic acid) suggesting that these strains may be useful for dietary detoxification. This suggestion is also reported by Fuchs et al (2008) who studied the ability of LAB strains to degrade OTA *in vitro*.

Piotrowska and Zakowska (2005) reported a good ability by three *Lactobacillus* strains to remove OTA from a contaminated liquid medium during first 5 hours of incubation, reaching 90% of initial OTA concentration reduction at 15 hours.

1.2.4 Reduction of damages when absorption occurs

In addition to special care to prevent the growth of moulds, detoxification measures, reduction of gastrointestinal absorption, there is a need for prevention of the mycotoxin-induced toxic effects once the toxin is ingested. Nutritional approaches, such as supplementation of nutrients, food components, or additives with protective effects against mycotoxin toxicity are assuming increasing interest. Since some mycotoxins (i.e., OTA, FB1, AFB1, DON, and T2

toxin) have been known to produce damages by increasing oxidative stress (Atroshi et al., 1995; Baudrimont et al., 1997a; Creppy et al., 1998; Hoehler and Marquardt, 1996; Hoult and Payà, 1996), the protective properties of antioxidant substances have been extensively investigated. Selenium, some vitamins, and their precursors have marked antioxidant properties that act as superoxide anion scavengers. For these reasons, these substances have been investigated as protecting agents against toxic effects of mycotoxins. Selenium protects spleen and brain against membrane damage caused by DON and T2 toxin (Atroshi et al., 1995); vitamins A, C, and E reduced DNA adducts in kidney and liver of mice exposed to OTA and zearalenone from 70 to 90% (Grosse et al., 1997); vitamin C in mice exposed to OTA showed reduction of abnormalities in both mitotic and meiotic chromosomes and morphologies of the sperm head (Bose and Sinha, 1994). Similar protective actions have also been attributed to vitamin E (Ibeh and Saxena, 1998) and vitamin A against exposure of animals to OTA (Kumari and Sinha, 1994).

Other nutritional components with antioxidant properties were investigated: phenolic compounds were found to induce cytosolic glutathione S-transferase activity that stimulated the formation of specific AFB1-glutathione conjugate. Rompelberg et al. (1996) found that eugenol does not modify the unscheduled DNA synthesis in hepatocytes exposed to AFB1.

Aspartame has a wide protective action against OTA-induced subchronic effects (Baudrimont et al., 1997b; Creppy et al., 1996). When given after intoxication of animals with OTA, aspartame eliminated the toxin efficiently from the body. The protective action should be due to its structural similarity to OTA and phenylalanine. Authors suggest that aspartame is the best candidate for preventing OTA-induced subchronic effects, also considering the absence of adverse effects in humans and animals.

Cyproheptadine, a serotonine antagonist with appetite-stimulant properties, has been tested to reduce feed refusal due to the presence of DON (Prelusky et al., 1997). Cyproheptadine offset the reduction of feed intake. It was concluded that, although serotoninergic mechanism is involved in reducing DON-induced feed refusal, further investigations are needed to better understand the reasons of anorectic effect. Carnosol and carnosic acid, two natural polyphenols found in *Rosmarinus officinalis*, are potent inhibitors of *in vitro* AFB1-induced DNA adduct formation (Offord et al., 1997).

Anthocyanin contained in oranges, blackberries, strawberries, and cranberries, had shown protective effect against cytotoxicity induced by OTA and AFB1 in a human hepatoma-derived cell line (Hep G2) and a human colonic adenocarcinoma cell line (CaCo-2). Cyanidin-3-O-beta-glucopyranoside attenuated ROS production induced by the two toxins in

both cell lines and inhibition of DNA and protein synthesis induced by the mycotoxins was counteracted by pretreatment with the antioxidant. Similarly, apoptotic cell death was prevented as demonstrated by a reduction of DNA fragmentation and inhibition of caspase-3 activation. (Guerra et al., 2005). Similar preventing effects on Hep G2 cells, were observed by Renzulli et al. (2004) by rosmarinic acid and its properties to prevent and reduce ROS production, DNA fragmentation, protein synthesis inhibition, and apoptosis cell death.

Role of melatonin as antioxidant and free radicals scavenger related to OTA induced damages were evaluated in rats (liver and kidneys) where melatonin showed a protective effect against OTA toxicity through the inhibition of oxidative damage and stimulation of GST activities (Meki and Hussein, 2001). In rats, OTA (289 μg/kg b.w. per day) induced histopathological damages in heart and lung and treatment with melatonin (10 mg/kg b.w. per day) significantly reduced the degree of damages (Okutan et al., 2004); in kidneys of treated rats, OTA induce oxidative stress and melatonin somministration did not change significantly these parameters in comparison to control rats (Ozçelik et al., 2004). Melatonin showed positively effects in rats fed fumonisin contaminated diets, reducing kidneys damage (Morsy et al., 2006). Against fumonisin induced damages, royal jelly was evaluated in rats. In liver and kidney of rats fed contaminated diet (200 mg/kg b.w. per day of fumonisins) supplemented with royal jelly (100 and 150 mg/kg b.w. per day) there was a significant improvement in growth performance, oxidative stress parameters, and histological and histochemical parameters. These improvements were pronounced in animals fed FB1 contaminated diet plus the high dose of royal jelly. Authors conclude that royal jelly have a protective effects against fumonisin toxicity and this protection was dose dependent (El-Nekeety et al., 2007).

Chapter 2. Objectives

Mycotoxins are a worldwide food and feed safety threat. They are genotoxic carcinogens that, independently from the dosage ingested by animals and human, cause health and economic problems. Ochratoxin A and fumonisin B1 have been classified by the International Agency for Research on Cancer (IARC, 1993) as "possibly carcinogenic to humans" (class 2B). Studies were performed to investigate strategies that could act on the critical point in order to limit mycotoxin contamination and absorption.

Purpose of the present study was to investigate *in vitro* and *in vivo*, strategies to counteract mycotoxin threat particularly in swine husbandry.

Nutritional strategies to prevent mycotoxins absorption and their adverse effects need an improved knowledge about intestinal absorption kinetics of these contaminants. Due to the lack of studies on the intestinal absorption kinetic of OTA and FB1, Ussing chambers technique was applied on rat intestinal mucosa.

Lactic Acid Bacteria strains were tested *in vitro* for their efficacy to reduce, by binding and/or degradation, OTA, FB1, and DON levels in bacterial medium.

An *in vivo* study in rats were performed to evaluate the effects of in-feed supplementation of a LAB strain, *Pediococcus pentosaceus* FBB61, to counteract the toxic effects induced by exposure to OTA contaminated diets.

Two separated *in vivo* trials on weaning piglets were performed to evaluate the efficacy of two commercial products preventing mycotoxin absorption. Growth performance of piglets fed FB1 and DON contaminated diets were monitored.

Chapter 3. *In vitro* studies

3.1 Ussing diffusion chambers technique applied to assess rat small intestinal permeability of ochratoxin A and fumonisin B1

Nutritional strategies to prevent mycotoxins absorption and their adverse effects need an improved knowledge about intestinal absorption kinetics of these contaminants. The kinetics of OTA absorption was studied *in vitro* using cell cultures (Berger et al., 2003; Heussner et al., 2002; O'Brien et al., 2001) and monolayer of cultured cells supported on membranes (Caloni et al., 2005; McLaughlin et al., 2004); as well as *in vivo* in humans and animals (Dietrich et al., 2005). CaCo-2 cells were used to study *in vitro* toxicity of FB1 (Caloni et al., 2002). The Ussing chambers technique used in the present study for the first time to study the intestinal absorption of OTA and FB1. Ussing chambers technique allows to study the passage of compounds across live intestinal tissue in 2 contiguous chambers (mucosal and serosal, respectively) for a 2 hours in a controlled environment similar to the physiological conditions (Söderholm et al., 1998). Tissue metabolism after mounting of mucosa in the Ussing chambers is maintained fully functional for during the absorption study (Berggren et al., 2003). The absorption of different kind of molecules (ions, drugs, nutrients, macromolecules) could be monitored by adding the compound to be studied in the mucosal chamber and by retrieving it from the serosal chamber.

The present study investigated the absorption of OTA and FB1 through viable tissues excised from small intestine of rats with Ussing chambers technique (Grass and Sweetana, 1988), a method to predict *in vivo* intestinal absorption in humans (Fagerholm et al., 1996; Lennernäs et al., 1997).

3.1.1 Materials and methods

Animals

The Ethics Committee of Lund University had previously granted approval for the animal study. Male rats (n = 10), Sprague-Dawley strain (Möllegaard, Skensved, Denmark), weighing 379 ± 45 g, were kept on chopped wood bedding in polycarbonate cages (12 h day-night photoperiod, at $20 \pm 2°C$, and a relative humidity of 50 ± 10 %). Rats had free access to a pelleted rat standard diet (B & K Universal, Sollentuna, Sweden) and water prior to sacrifice. Under diethyl ether anaesthesia, jejunum from each rat (10 cm from the ligament of Treitz) was removed, immediately immersed in a modified oxigenated Krebs-Ringer buffer

(KR) at room temperature (Pantzar et al., 1993). The jejunal segment from each rat was cut in 12 pieces (2.5 cm), opened along the mesenteric border, rinsed with the same buffer and mounted in Ussing chambers.

Experimental procedure

Both serosal and mucosal reservoirs of the Ussing diffusion chambers with 1.78 cm^2 of exchange tissue area (Navicyte, San Diego, CA, USA) were filled with 5 ml KR which was continuously bubbled with carbogen (95% O_2 and 5% CO_2) and circulated by gas at 37°C (Grass and Sweetana, 1988). The experiment started (t = 0) within 30 min from the anaesthesia, by replacing the buffer in the 12 mucosal reservoirs with 5 ml KR containing mycotoxins and/or marker molecules to assess mucosal integrity. Treatment arrangement had been designed to reduce possible functional and/or anatomical differences among sections excised along the cut intestinal tract (Figure 3.1). At the onset of experiments, substance concentrations in mucosal reservoir were OTA, 0.50 µmol/l (Sigma Chemical Co., St. Louis, MO, USA); FB1, 41.6 µmol/l (Sigma Chemical Co., St. Louis, MO, USA); ^{14}C-Mannitol, 11.5 kBq/ml (DuPont, Dreieich, Germany, Mw: 182); FITC-Dextran 4400, 1 mg/ml (Sigma, St Louis, MO, USA, Mw: 4.400); ovalbumin, 25 mg/ml (A-7641 Sigma, Mw 45000). Experiments were continued for 2 h and every 20 min a 1 ml sample was taken from all the serosal reservoirs for the subsequent analysis and replaced with 1 ml of fresh KR. At 2 h, a 1 ml sample was withdrawn from mucosal reservoirs of chambers 1, 4, 7, and 10 for mycotoxins analysis.
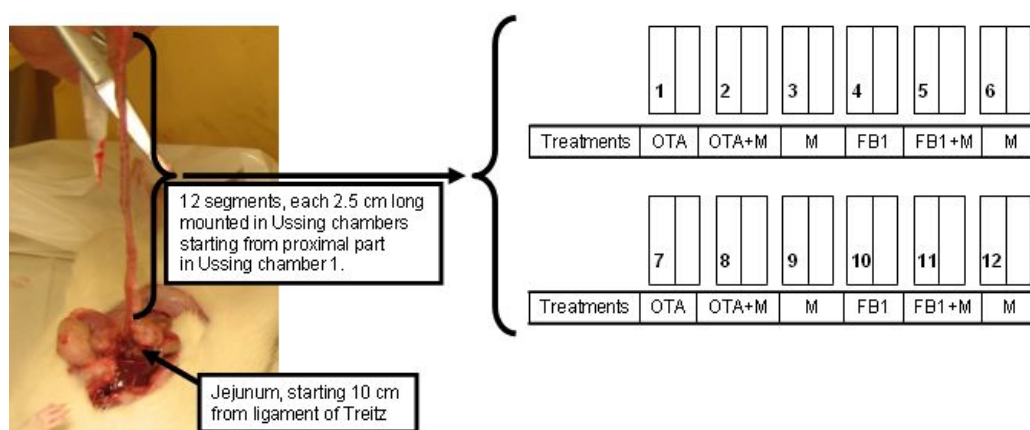

Fig. 1. Treatments arrangement along the 12 jejunal segments from each of the sacrificed rats (n=10). OTA, ochratoxin A = 0.50 µmol/l; FB1, fumonisin B1 = 41.6 µmol/l; M, markers solution composed by ^{14}C-Mannitol = 11.5 kBq/ml, FITC-Dextran 4400 = 1 mg/ml, and ovalbumin = 25 mg/ml.

Analysis

Marker molecules analysis were performed according to Nejdfors et al. (2000). The amount of radiolabeled mannitol in 0.5 ml samples was directly measured by using a beta counter (LKB, Bromma, Sweden) after mixing 5 ml of a liquid scintillation cocktail (Ready Safe®, Beckman, Fullerton, CA, USA). FITC-dextran was measured by spectrophotofluorometer (CytoFluor 2300, Millipore, Bedford, MA, USA) at an excitation wavelength of 480 nm and an emission wavelength of 520 nm, with the appropriate FITC-dextran dissolved in KR as a standard. Quantification of ovalbumin was carried out by electroimmunoassay (Laurell, 1972), using specific antiserum (Department of Animal Physiology, Lund University), in samples that had been kept at -20°C.

The apparent permeability coefficients (P_{app}) for the markers across the intestinal mucosa were calculated from the equation: P_{app} (cm s^{-1}*10^{-6})=dc/dt * [V/(A*C$_0$)], where dc/dt is the variation of the serosal concentration over 60-120 minutes (mol/l/sec), V is the volume of the serosal side reservoir (cm^3), C_0 is the initial marker concentration in the mucosal reservoir (mol/l) and A is the exposed intestinal area in the chamber (cm^2).

All the solvents used for mycotoxins analysis were of analytical or HPLC gradient grade and were obtained from Merck (Darmstadt, Germany). The solution of o-phthalaldehyde, purchased from Sigma Chemical Co. (St. Louis, MO, USA), was weekly prepared according to Solfrizzo et al. (2001) and stored at +4°C.

FB1 analysis were carried out using a Kontron Instruments 325 ternary liquid chromatograph equipped with a 10 µl loop, combined with a Spectra System FL3000 (Thermo Separation Products) fluorescence detector with excitation and emission wavelength of 360 and 434 nm, respectively. A Luna Phenylhexyl column (5 µm; 250 mm x 4.6 mm I.D.) (Phenomenex, Torrance, CA, USA) was used. Prior to injection 70 µl of sample were derivatized with 70 µl of o-phthalaldehyde for 30 seconds and injected within 1 min. As mobile phase two solvent systems were employed: 3.4 pH aqueous buffer with 2% of glacial acetic acid and 0.1% of triethylamine (A) and acetonitrile (B). The separation was operated at a flow rate of 1.2 ml/min with the following isocratic gradient A:B (60:40) in 30 min. The fluorescence chromatogram was recorded starting from the 13th minute because the glutamic acid, present in the buffer, reacts with o-phthalaldehyde causing the detector overload. The limit of quantification was 0.69 µmol/l.

OTA analysis were carried out using a liquid chromatograph (P4000, ThermoFinnigan, California, USA) equipped with a 20 µl loop, combined with a fluorescence detector (FL300, ThermoFinnigan, California, USA) with excitation and emission wavelength of 332 and

470nm, respectively. A Supersphere 100 RP-C18 column (4 μm; 125mm×4.0mm I.D.) (Merck & Co., Whitehouse Station, NJ, USA) was used. As mobile phase two solvent systems were employed: aqueous buffer with 2 % of glacial acetic acid and acetonitrile in isocratic gradient elution (59:41) at a flow rate of 1 ml/min in 13 min. The limit of quantification was 0.0037 μg/l.

Statistical analyses

Data were reported as mean ± SE. The difference was considered significant when $P < 0.05$. For statistical evaluations means of the P_{app} from the individual intestinal segments from each animal were calculated. The experimental unit, n, represents the number of animals (n = 10 rats) and not the number of observations (each animal had 2, 2 or 4 observations, for mycotoxins, mycotoxins plus marker molecules, and marker molecules treatments, respectively). Means were compared by one-way ANOVA and Newman Keuls post test. Data were analyzed using the program GraphPad Prism (GraphPad Software 4.00, San Diego, CA).

3.1.2 Results and discussion

[14]C-mannitol, FITC-dextran, and ovalbumin analysis in chambers containing only marker molecules (3, 6, 9, and 12) showed no significant differences ($P>0.05$) in the calculated P_{app} for any of the different markers, indicating no differences of absorption kinetic along the excised portion of small intestine.

As reported in Table 3.1, marker analysis revealed no significant differences ($P>0.05$) in calculated P_{app} coefficient of marker molecules between chambers containing marker molecules plus OTA (chambers 2 and 8), and chambers containing only marker molecules (chambers 3, 6, 9, and 12); no significant differences ($P>0.05$) were detected in calculated P_{app} coefficient of marker molecules between chambers containing marker molecules plus FB1 (chambers 5 and 11), and chambers containing only marker molecules (chambers 3, 6, 9, and 12), suggesting no damages of rat small intestine mucosa permeability induced by mycotoxins.

Concentrations of OTA (chambers 1 and 7) and FB1 (chambers 4 and 10) in serosal side reservoirs of Ussing chambers were below the limit of quantification throughout the 2 h experiment (data not shown).

Table 3.1. Calculated apparent permeability coefficient (P_{app} * 10^{-6}) for ^{14}C-mannitol, FITC-dextran, and ovalbumin, in Ussing diffusion chambers mounting pieces of intestinal mucosa excised from rat small intestine (n=10) exposed to ochratoxin A or fumonisin B1.

Treatments[*]	OTA + M	M	FB1 + M		
	P_{app} (cm s^{-1} * 10^{-6})			Pooled SEM	*P* of the model
^{14}C-Mannitol	4,53	5,18	4,95	0,55	0,52
FITC-Dextran	0,76	0,82	0,88	0,12	0,61
Ovalbumin	0,05	0,09	0,09	0,02	0,12

[*]*OTA, ochratoxin A = 0.50 µmol/l; FB1, fumonisin B1 = 41.6 µmol/l; M, markers solution composed by ^{14}C-Mannitol = 11.5 kBq/ml, FITC-Dextran 4400 = 1 mg/ml, and ovalbumin = 25 mg/ml.*

Ussing chamber technique has been applied to kinetic and intestinal permeability studies on drugs, nutrients, proteins, prions, and macromolecules using pieces of mucosa from various animal species (rat, pig, ovine, cattle, rabbit) as models for animal and human absorption (Keljo and Hamilton, 1983; McKie et al., 1999). Moreover, this technique has been applied to study the permeability properties of human intestinal mucosa (ileal and colonic segments) used to reconstruct the urinary tract (Nejdfors et al., 2000). The application of Ussing chambers on piglet intestinal mucosa to study alternatives to in-feed antibiotic for young pigs (Boudry, 2005) represent a useful reference for the application of this technique in animal nutrition studies.

In the present study, marker analysis results from Ussing chambers containing marker molecules alone (chambers 3, 6, 9, and 12) revealed no differences in P_{app} for any of the different marker molecules suggesting that marker molecules absorption kinetics was not affected by the different position along the excised mucosa. These latter P_{app} values suggested that KR solution had not damaged the functional integrity of rat intestinal mucosa during the 2 h exposure.

Ochratoxin A

The absorption of solutes along the gastrointestinal tract is regulated by several factors, among which the pH value of intestinal content that is highly relevant and affects the protonated to non-protonated ratio of solutes in relation to their individual pKa. The OTA pKa values are in the range 4.2-4.4 and 7.0-7.3 respectively for the carboxyl group of

phenylalanine mojety and for the phenolic-hydroxyl group of the isocumarin part. OTA is rapidly absorbed from stomach and, even against a concentration gradient, from proximal jejunum (Kumagai and Aibara, 1982) in rats and mice. Walker and Larsen (2005) reviewed the OTA percentage absorbed from small intestine of rats was approximately 56%. Berger et al. (2003) reported that OTA was absorbed from apical to basolateral compartment of CaCo 2-cell monolayer cultivated for 21 days, mimicking the in vivo situation when apical and basolateral compartment pH were 6.0 and 7.4, respectively. Same authors reported absorption even when the pH value was the same (7.4) in apical and basolateral compartments. In the present study, pH values of buffer solutions in mucosal and serosal side of rat small intestinal mucosa, were 7.4. Over the 120 min of experiment, OTA was not detected in serosal side reservoirs of Ussing chambers when OTA (chambers 1 and 7) or OTA plus marker molecules (chambers 2 and 8) were present in the mucosal side. Such time interval is comparable to the physiological residence time of feed in the gut during digestion. Moreover, OTA analysis from samples withdrawn from mucosal side at the end of 120 min of experiment, revealed that OTA concentrations were not different from those contained in KR daily fresh prepared buffer at the beginning of the experiment (t = 0). These results confirmed that under our *in vitro* conditions no-absorption occurred from mucosal to serosal side of rat small intestine mucosa. In an *in vivo* experiment with bile salt-depleted rats, Kerkadi et al. (1999) showed that OTA absorption was significantly decreased. The absence of bile salts in the OTA containing mucosal solutions could be a possible explanation for the non-absorption of OTA throughout the rat mucosa observed in the present study. We decided to avoid the addition of bile salts to KR solution in mucosal side reservoirs of Ussing chambers according to the study of Patel et al. (2006). They suggested a progressive loss of viability of excised rat mucosa in presence of simulated intestinal fluids containing bile salts. Kinetics of OTA distribution and OTA binding to plasma proteins has been studied by Kumagai (1985) concluding that albumin-deficient rats were able to clear OTA from systemic circulation faster than normal rats. In our study the presence of ovalbumin as marker for endocytotic transport has not affected the OTA absorption as suggested from results obtained in Ussing chambers without marker molecules (chambers 1 and 7), where OTA was not detected in serosal side reservoirs and persist in mucosal side reservoirs over the 120 min experiment.

Fumonisin B1

The zero-absorption of FB1 reported by Caloni et al. (2005) through CaCo-2 cells cultured on semi-permeable membrane, was confirmed by our study in excised rat intestinal mucosa: FB1 was not detected in serosal side samples throughout the 120 min experiment and, moreover, as reported for OTA, the FB1 concentrations detected in samples from mucosal side reservoirs at the end of the experiment were not different from FB1 concentrations in KR daily fresh buffer at the beginning of the experiment (t = 0). Such findings are in accordance with Voss et al. (2001) that reported low absorption of FB1 in rats and rapid elimination in feces, however no clear indications were found about the *in vivo* site of absorption of FB1 (Shier, 2000) but previous swine study, feeding piglets with 30 ppm of FB1 (similar to the dose used in the present study) strongly impaired animal health and growth (Piva et al., 2005). Pagliuca et al. (2005) in these piglets found both FB1 and aminopentol in liver suggesting that aminopentol was originated from dietary FB1.

The present study firstly develops the Ussing chamber technique to investigate *in vitro* the intestinal permeability of OTA or FB1: results showed that OTA and FB1 were not absorbed from rat small intestine mucosa. Since *in vivo* absorption of both the mycotoxins normally occurs, it is evident that in these experimental conditions Ussing diffusion chambers were not able to assess the intestinal permeability of OTA and FB1. Whether the lack of absorption is due to the Ussing diffusion chambers *per se* or to the overall experimental conditions deserves further investigations actually in progress.

3.3 Do Lactic Acid Bacteria detoxify mycotoxins contaminated medium?

Also with other fungal toxins such as zearalenone, trichothecenes and fumonisins, binding effects have been observed in chemical analytical investigations (El-Nezami et al., 2002ab; Kabak et al., 2006). The first hypothesi on the efficacy of LAB to detoxify bacterial broth contaminated by mycotoxins is reported by El-Nezami et al (1998a). Strains of LAB and bifidobacteria were able to bind aflatoxins. Gram-positive strains were more efficient than Gram-negative strains (El-Nezami et al., 1998b) suggesting that the mechanism of aflatoxin binding involves the cell wall peptidoglicans and polysaccharides as the two most important elements responsible for the binding and the absorption of mutagens and carcinogens in small intestine (El-Nezami et al., 2000; Haskard et al., 2000). Lactic Acid Bacteria recently has been evaluated for the ability to reduce OTA (Fuchs et al., 2008; Turbic et al., 2002). Piotrowska and Zakowska (2005) showed the ability to remove OTA from a contaminated liquid medium by LAB (*Lactobacillus rhamnosus* GG, *L. acidophilus* CH-5, *L. plantarum* LOCK0862).

In these preliminary *in vitro* studies, LAB strains were tested for their efficacy to reduce OTA, FB1, and DON levels in bacterial contaminated medium.

3.3.1 Materials and methods

Rogosa Biosbroth was obtained by Biolife Italiana S.r.l., Milan, Italy; ochratoxin A, fumonisin B1, and deoxynivalenol, were purchased from Sigma-Aldrich (Milan, Italy). Inorganic salts, acetonitrile, methanol, water HPLC grade were purchased from Merck (Darmstadt, Germany). Phosphate buffer solution (PBS) was prepared by mixing 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4, final pH 7.2.

Lactic Acid Bacteria strains
Intestinal LAB were isolated from different gastrointestinal tract regions of pigs and poultry by subsequently isolation on Rogosa Bios broth + agar 1.5%. Culture media was prepared, for this and the following studies, as reported by the supplier but without addition of Tween 80. A total of 240 bacteria strains from swine and 200 from poultry were sampled, isolated and stored at -20°C with culture media and glycerol 20% until revitalization for screening.

Ochratoxin A study
Sixty-four LAB strains isolated from feces of newborn piglets, sows, weaning piglets, growing pigs, and duodenal, jejunum, ileum, cecum content were tested.

Strains stored at -20°C were revitalized in 10 ml of Rogosa broth, incubated at 39°C for 18 hours. Fresh culture were used to inoculate (1% v/v) 10 ml of Rogosa broth contaminated with 200 ppb of OTA. After 20 hours of incubation at 39°C, samples were centrifuged (6500 rpm, 20 min, 20°C) and 4 ml of broth were diluted with 4 ml of PBS. pH value was adjusted between 7 and 8 prior to OTA purification. OTA was purified with immunoaffinity column (Ochraprep, R-Biopharm, Rhone LTD, Glasgow, Scotland). The sample was applied and allowed to pass the column and flow rate was maintained at 1-2 ml/min. Then the column was washed with 10 ml of PBS. For the eluition of bound OTA, 2 ml of methanol and 2 ml of water HPLC grade were used. The eluate was dried down and the residue re-dissolved in 1 ml of methanol. OTA analysis were carried out using a liquid chromatograph (P4000, ThermoFinnigan, California, USA) equipped with a 20 μl loop, combined with a fluorescence detector (FL300, ThermoFinnigan, California, USA) with excitation and emission wavelength of 332 and 470 nm, respectively. A Supersphere 100 RP-C18 column (4 μm; 125mm×4.0mm I.D.) (Merck & Co., Whitehouse Station, NJ, USA) was used. A mobile phase with two solvent systems were used: aqueous buffer with 2 % of glacial acetic acid and acetonitrile in isocratic gradient elution (59:41) at a flow rate of 1 ml/min in 13 min. The limit of quantification was 0.07 μg/ml.

Lactic Acid Bacteria that showed good efficacy to reduce OTA contamination were evaluated for their ability to reduce OTA contamination in *in vitro* cecal fermentation system (batch-culture techniques), which simulate the intestinal environment and microflora. A two step procedure was applied: first the digestion of the feed through enzymatic reactions followed by the fermentation of the digested diet with intestinal content and LAB strains under study.

In vitro enzymatic digestion of the feed

A standard diet for weaner pigs was digested *in vitro* to simulate ileal digestion as described by Verveake et al. (1989). This was a stepwise procedure with an incubation of feed (25 g; particle size < 1 mm) in 500 ml of pepsin solution (0.2% pepsin w/v, HCl 0.075 N; P7000 from porcine gastric mucosa; Sigma Chemical, St. Louis, MO, USA) in a shaking waterbath at 37°C for 4 h. At the end of the 4h incubation, the solution was adjusted to pH 7.5 with NaOH 0.1 N. In the second step 500 ml of a pancreatin-NaHCO$_3$ mixture solution (10 g/l pancreatin of 1M NaHCO$_3$; P1500, from porcine pancreas; Sigma Chemical, St. Louis, MO, USA) was added and the mixture was reincubated for 4 h at 37°C to simulate pancreatic digestion. Composition of the phosphate buffer solution was as follows: 26.2 mM Na$_2$HPO$_4$, 46.7 mM NaHCO$_3$, 3.3 mM NaCl, 3.1 mM KCl, 1.3 mM MgCl$_2$, 0.7 mM CaCl$_2$.

After enzymatic digestion, the preparation was centrifuged (6500 rpm, 10 min, 4°C), washed twice with distilled water, re-centrifuged (6500 rpm, 5 min., 4°C), and dried at 60°C overnight. Chemical analyses of the diet after digestion are (% of dry matter): crude protein, 8.06; ether extract, 11.80; crude fiber, 12.47; ash, 6.69; NDF, 38.01; ADF, 17.60; ADL, 3.70; starch, 18.45. The digested diet was used as the substrate in the *in vitro* fermentation study.

In vitro cecal fermentation

Cecal contents from 6 pigs (approx. 120 kg BW) were collected within 20 min after slaughter and kept in a sealed nylon bag at 37°C during transfer to the laboratory. Cecal contents were then diluted with buffer (ratio 1:3) and filtered through 6 layers of cheese cloth. The filtered liquid was used as inoculum. The buffer composition (McDougall, 1948) was as follows: 9.8 g $NaHCO_3$; 0.57 g KCl; 0.079 g $CaCl_2.6H_2O$; 9.3 g $Na_2HPO_4.12H_2O$; 0.67 g NaCl; 0.12 g $MgSO_4.7H_2O$ in 1 l of distilled water. Buffer pH was then adjusted to pH 6.7 by adding 3 M HCl. The buffer solution was kept at 37°C and flushed with CO_2 for 20 minutes before use. The inoculum was dispensed into five 10 ml glass syringes (5 ml of inoculum in each syringe) and five 50 ml vessels (previously flushed with CO_2, 15 ml of inoculum in each vessel) per treatment, containing 20 and 100 mg of predigested diet, respectively (Piva et al., 1996). Syringes and vessels were sealed and incubated at 37°C for 24 h.

Six treatments were investigated: negative control (predigested diet and cecal inoculum); positive control (predigested diet, cecal inoculum) contaminated with OTA at 251.8 ± 29.8 ppb; treatment 1, as positive control treatment inoculated with strain A at $2.5x10^{11}$ cfu/ml; treatment 2, as positive control treatment inoculated with strain A at $1.3x10^{11}$ cfu/ml; treatment 3, as positive control treatment inoculated with strain B at $1.3x10^9$ cfu/ml; treatment 4, as positive control treatment inoculated with strain B at $4.5x10^9$ cfu/ml. Lactic Acid Bacteria strains stored at -20°C were revitalized in 10 ml of Rogosa broth, incubated at 39°C for 20 hours. Fresh culture were used to inoculate the fermentation system.

Gas production was measured as described by Menke et al. (1979), using 10 ml glass syringes and recording the cumulative volume of gas produced every 30 min. Samples of fermentation fluid were collected from each vessel at time 0, 4, 8 and 24 h after incubation in a shaking water bath for ammonia analysis; OTA concentration were determined in the starting cecal inoculum and at the end of the fermentation as follow: 3 ml of fermentation fluid were diluted with 3 ml of PBS. pH value was adjusted between 7 and 8 prior to OTA purification by immunoaffinity clean-up as previous described.

Chemical analyses of feed and fermentation fluid

Analyses of the diets (crude protein, crude fiber, ether extract, ash, and starch) were performed according to AOAC standard methods (AOAC, 2000; Method 954.01 for crude protein, Method 962.09 for crude fiber, Method 920.39 for ether extract, Method 942.05 for ash, Method 920.40 for starch). Ammonia in fermentation fluid and intestinal chymus was measured as described by Searcy et al. (1967).

Statistical analyses

A modified Gompertz bacterial growth model (Zwietering et al., 1992) was used to fit gas production data. This model assumes that substrate levels limit growth in a logarithmic relationship as follows:

$$V = V_F \exp \{ - \exp [1 + (\mu_m e/V_F)(\lambda - t)]\}$$

where symbols have the meanings assigned by Zwietering et al. (1990): V = volume of gas produced at time t, t = fermentation time, V_F = maximum volume of gas produced, μ_m = maximum rate of gas production, which occurs at the point of inflection of the gas curve and λ = the lag time, as the time-axis intercept of a tangent line at the point of inflection.

The duration of the exponential phase was calculated from the parameters of the modified Gompertz equation, as suggested by Zwietering et al. (1992) with the following:

$$\text{exponential phase (h)} = V_F/(\mu_m e)\{1 - \ln[(3 - \sqrt{5})/2]\}.$$

Curve fitting was performed using the program GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA). Total gas production, maximum rate of gas production, duration of the exponential phase, ammonia data were analyzed by ANOVA using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA) in a completely randomized design. Each syringe and vessel formed the experimental unit. The differences among means of groups were analyzed using the Newmann-Keuls test. Differences were considered statistically significant at *P*<0.05.

Fumonisin B1 study

Seventy-nine LAB strains isolated from feces of newborn piglets, sows, weaning piglets, growing pigs, and duodenal, jejunum, ileum, cecum content of pigs and poultry at slaughter were tested.

Strains stored at -20°C were revitalized in 10 ml of Rogosa broth, incubated at 39°C for 18 hours. Fresh culture were used to inoculate (1% v/v) 10 ml of Rogosa broth contaminated

with 5 ppm of FB1. After 20 hours of incubation at 39°C, samples were centrifuged (6500 rpm, 20 min, 20°C) and 3 ml of broth were diluted with 6 ml of distilled water. pH value was adjusted between 7 and 8 prior to FB1 purification. FB1 was purified with sax columns (Isolute, International Sorbent Technology LTD, Mid Glamorgan, UK) as reported by the supplier. Diluted broth sample was applied and allowed to pass the column and flow rate was maintained at 1-2 ml/min. Then the column was washed with 8 ml of methanol:water (60:40, v/v). For the eluition of bound FB1, 2 ml of methanol + 1% acetic acid was used. The eluate was dried down and the residue re-dissolved in 1 ml of methanol. FB1 analysis were carried out on derivatized sample: 70 μl of sampled were added to 70 μl of derivatization agent o-phthaldialdialdehyde, before HPLC analysis. The solution of o-phthalaldehyde, purchased from Sigma Chemical Co. (St. Louis, MO, USA), was weekly prepared according to Solfrizzo et al. (2001) and stored at +4°C. Detection of FB1 were performed with a Varian chromatographer (Mod. 9012) equipped with a 20 μl loop combined with Varian fluorimetric detector (Mod. 9070). Samples were injected in a Phenomenex C18 column (3 μm; 150 x 4.6 mm) with excitation and emission wavelength of 340 and 440 nm, respectively. As mobile phase two solvent systems were employed: an aqueous buffer (pH 3.4) with 2% of glacial acetic acid and 0.1% of triethylamine and HPLC-grade acetonitrile. The separation was operated at a flow rate of 1.2 ml/min with the following isocratic gradient of acqueous buffer:acetonitrile (60:40, v/v) in 30 min.

Deoxynivalenol study

One-hundred and twelve LAB strains isolated from duodenum, jejunum, ileum, cecum contents, and feces of weaning piglets were tested.

Strains stored at -20°C were revitalized in 10 ml of Rogosa broth, incubated at 39°C for 18 hours. Fresh culture were used to inoculate (1% v/v) 10 ml of Rogosa broth contaminated with 2.5 ppm of DON. After 20 hours of incubation at 39°C, samples were centrifuged (6500 rpm, 20 min, 20°C) and 4 ml of broth were diluted with 4 ml of PBS. pH value was adjusted between 7 and 8 before DON purification. DON was purified with immunoaffinity column (Donprep, R-Biopharm, Rhone LTD, Glasgow, Scotland). Diluted broth sample (1 ml) was applied and allowed to pass the column and flow rate was maintained at 1-2 ml/min. Then the column was washed with 10 ml of HPLC-grade water. For the eluition of bound DON, 1 ml of methanol was used. The eluate was dried down and the residue re-dissolved in 1 ml of mobile phase. DON analysis were carried out using a liquid chromatograph (P4000, ThermoFinnigan, California, USA) equipped with a 20 μl loop, combined with UV detector

(UV2000, ThermoFinnigan, California, USA) at 218 nm. As mobile phase water:acetonitrile (90:10, v/v) were employed at a flow rate of 0.8 ml/min in 6 min.

3.3.2 Results and discussion

Ochratoxin A study results and discussion

Sixty-four LAB strains were evaluated for their efficacy to reduce OTA contamination in bacterial broth. Four strains showed a reduction of initial OTA contamination (200 ppb) of 10% (Figure 3.2) while other strains showed no reduction or reduction lower than 10%. Two strain with major degradation properties, named strain A and strain B, were tested for their ability to degraded OTA in *in vitro* cecal fermentation. No OTA was detected in cecal content sampled at slaughter and used to prepare the fermentation inoculum. After 4 and 8 hours, fermentation parameters were not statistically different (data not shown); after 24 hours of fermentation (Table 3.2), treatments 3 and 6, inoculated with strain A e B, respectively, showed a higher gas production than positive and negative control treatments ($P<0.05$). Lactic Acid Bacterial inoculum in treatments 3, 4, 5, and 6, increase significantly ($P<0.05$) the rate of gas production than positive and negative control treatment. No statistically differences were reported of ammonia production at 4, 8, and 24 hours of fermentation (data not shown).

After 24 hours of fermentation OTA was not detected in treatment 6 whereas in treatment 2, 3, 4, and 5, was decreased by 95% of the initial concentration of 251.8 ± 29.8 ppb.

Based on these data it is not clearly if LAB strain inoculated are able to decrease OTA contamination per se in cecal *in vitro* fermentation system. Further studies are needed to better understand *in vitro* mechanism by which LAB may reduce OTA contamination and if other intestinal bacteria species can do the same.

Figure 3.2. Ochratoxin A percentual reduction by Lactic Acid Bacterial strains.
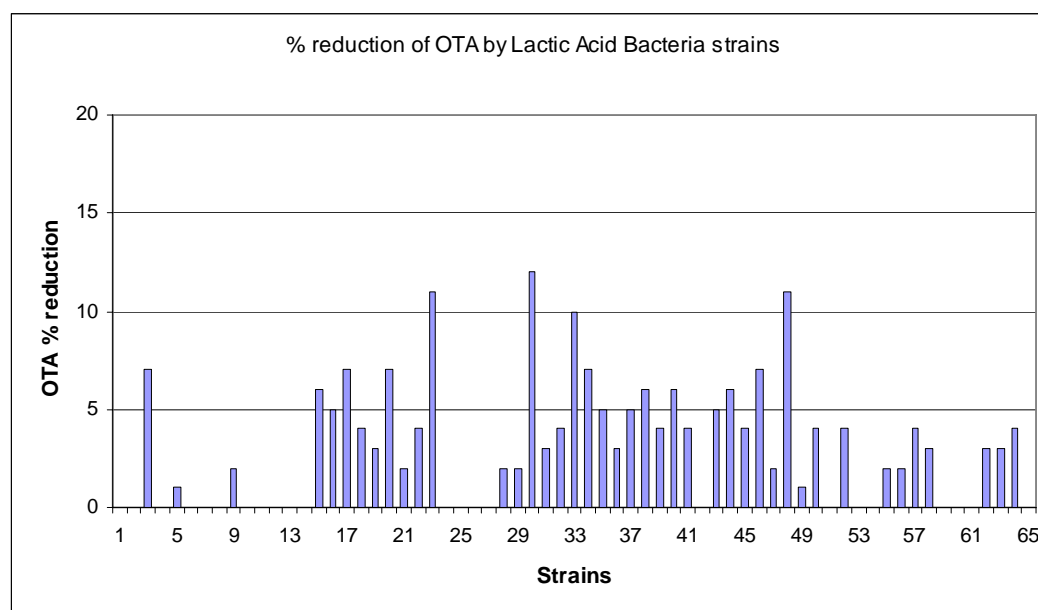
Table 3.2. Fermentation parameters and ochratoxin A concentration at 24 hours. Within a column, means without a common superscript letter differ (P<0.05; n=5)

|	Bacterial strain	Ochratoxin A concentration		R^2	Ymax		Rate max		Log phase	
	cfu/ml	ppb			(ml)		(ml/h)		(h)	
		Mean	SD		Mean	SD	Mean	SD	Mean	SD
Treatment 1	-	-	-	0,920	7,14[a]	0,95	0,64[a]	0,05	8,16	1,44
Treatment 2	-	12.2	3.1	0,956	8,61[b]	0,23	0,69[a]	0,07	9,15	0,97
Treatment 3	A: (2.5×10^{11})	12.0	2.5	0,957	9,74[bc]	0,77	0,83[b]	0,05	8,48	0,20
Treatment 4	A: (1.3×10^{11})	9.6	0.8	0,931	9,18[b]	0,50	0,79[b]	0,05	8,44	0,36
Treatment 5	B: (1.3×10^9)	14.4	2.1	0,964	8,98[b]	0,13	0,82[b]	0,04	7,94	0,45
Treatment 6	B: (4.5×10^9)	-	-	0,926	10,72[c]	1,02	0,87[b]	0,05	8,90	0,36

Fumonisin B1 study results and discussion

Results of LAB evaluation for degradation of FB1 in Rogosa broth were reported in Figure 3.3. FB1 initial concentration (5 ppm) was reduced above 50% by 12 LAB strains.

Studies of Mokoena et al. (2005) on fermented mais to investigate the potential of LAB fermentation in reducing FB1 contamination showed a decrease of FB1 and zearalenone (56% and 67%, respectively) concentration after 3 days of incubation. Reduction of FB1 showed in the present study need further investigations to better understand the mechanisms by which

LAB remove the mycotoxin (absorption and/or degradation) and the effect of FB1 on LAB viability.

Figure 3.3. Fumonisin B1 percentual reduction by Lactic Acid Bacterial strains.

% reduction of FB1 by Lactic Acid Bacteria strains
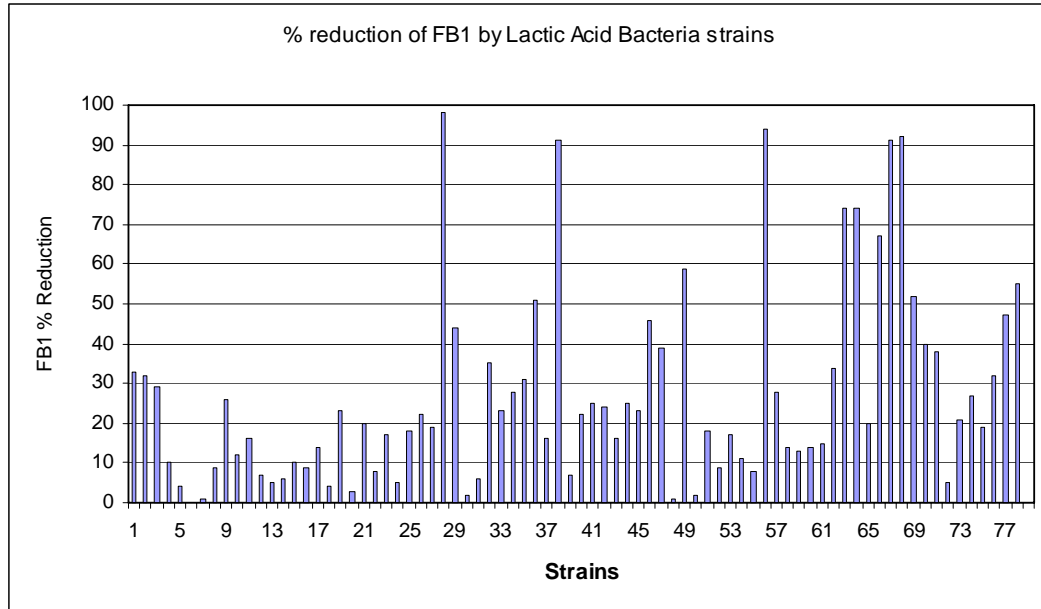
Deoxynivalenol study results and discussion

Results of LAB evaluation for degradation of DON in Rogosa broth were reported in Figure 3.4. No strains were individuated with percentual reduction higher than 5% of the initial concentration in Rogosa broth (2.5 ppm). Microbes in the digestive tract of pigs are capable of causing detoxifing reactions as demonstrated by the formation of de-epoxy-DON and the concomitant decrease of DON after incubation of digesta and feces from pigs in the presence of DON (Kollarcik et al., 1994; Lauber et al., 2000; Eriksen et al., 2002). These studies tested DON degradation by intestinal contents that include different microbial species. The present study tested only LAB and recorder no degradation of DON, further studies need to better understand intestinal and fecal microbial species that could detoxify DON.

Figure 3.4. Deoxynivalenol percentual reduction by Lactic Acid Bacterial strains.
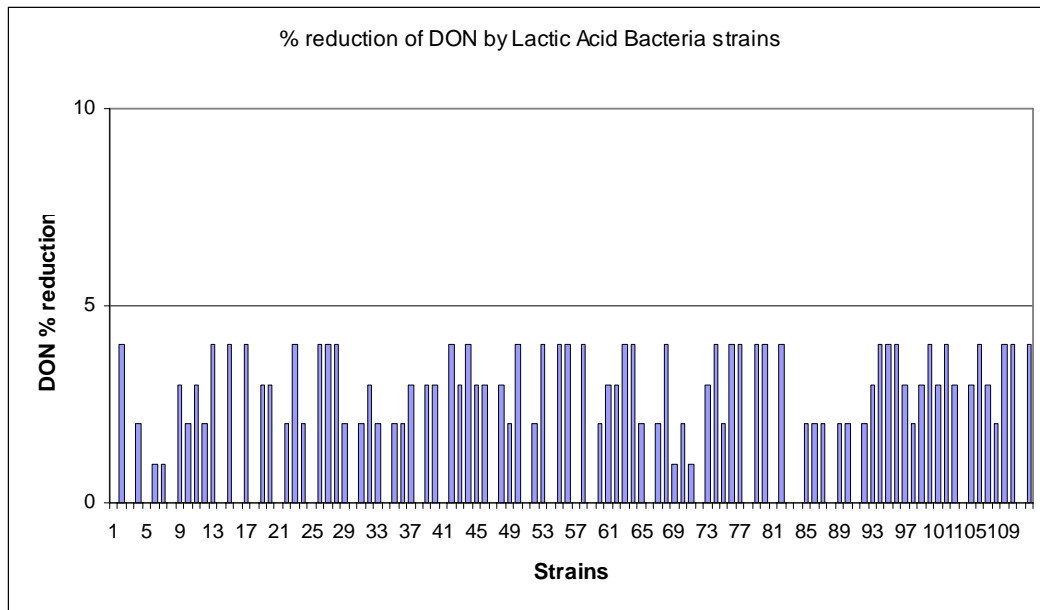
This part of the studies allow to conclude that isolated LAB strains from pigs and poultry feces and intestinal contents, showed low efficacy to reduce OTA, FB1, and DON contamination from Rosoga broth. Piotrowska and Zakowska (2005) monitored the ability to remove OTA from a contaminated liquid medium by LAB (*Lactobacillus rhamnosus* GG, *L. acidophilus* CH-5, *L. plantarum* LOCK0862) during 40 hours. They observed that OTA concentration dropped significantly during the first 5 hours of incubation, and the lowest OTA concentration (90% reduction of initial concentration) was reached after 15 hours of incubation. From 15 to 40 hour of incubation, 9% of OTA previously removed, come back to the medium. This pathway was present in all tested strains. The OTA binding to bacterial biomass is the possible explanation for OTA removal from the medium but other unknown mechanisms (for example bacterial enzymatic detoxification) should not be omitted.

In the present study only final mycotoxin concentration was recorded therefore it is not possible to hypothesize the evolution of mycotoxins removal from the medium even if good removal ability was recorded by 12 LAB strains regarding FB1. Further studies are needed to better understand the evolution of mycotoxin removal from liquid medium and the mechanism followed by each mycotoxin.

Chapter 4. *In vivo* studies

4.1 Feeding *Pediococcus pentosaceus* FBB61 reduces oxidative stress induced by ochratoxin A in rats

In vitro studies of Piotrowska and Zakowska (2005) which confirmed OTA elimination from culture media due to OTA binding to LAB cells are not confirmed, in the literature available, by *in vivo* studies with OTA and LAB in any animal species. We selected for this study rats as animal model, and *Pediococcus pentosaceus* FBB61 (ATCC43200) as a probiotic strain with a specific bactericidal activity against Gram positive bacteria (Piva and Headon, 1994), and with ability to positively modulate *in vitro* cecal fermentation in swine (Piva A. et al., 1995). Ochratoxin A-mediated carcinogenesis can be divided into direct (formation of covalent DNA adducts) and indirect (DNA damage by oxidative metabolism) modes of action (Pfohl-Leszkowicz et al., 2007).

The purpose of the present study was to investigate whether microbial balance and induced gut metabolic modifications associated to the use of *P. pentosaceus* FBB61, could result in the reduction of oxidative stress associated to OTA consumption in rats.

4.1.1 Materials and methods

Chemicals

OTA from *Aspergillus ochraceus* was purchased from Sigma-Aldrich (Milan, Italy) and *Pediococcus pentosaceus* FBB61 (ATCC 43200) was purchased from LGC Promochem (Milan, Italy). M17 broth was purchased from Oxoid Ltd (Dansingstoke, UK). All other chemicals were purchased from Merck (Frankfurt, Germany).

Animals and treatments

The experiments reported in the present paper complied with current Italian law and met the guidelines of the Institutional Animal Care and Use Committee of Sacred Heart Catholic University of Piacenza (Italy). The experiments were performed with male 40 Sprague–Dawley albino rats (83.2 ± 4.2 g body weight at the beginning of experiments). They had free access to water and were kept at 20 ± 2°C, with a natural photo-period (12 h light − 12 h dark cycle). Rats were subdivided into four groups (10 rats/group), individually caged and received the substances under study orally, via their meal feed, for 4 weeks: control group (CTRL) received a commercial balanced standard diet; group PP received CTRL diet containg 100 g/kg of freeze-dried corn meal with *P. pentosaceus* FBB61 (replacing equal amount of corn

meal without PP); group OTA received the CTRL diet contaminated with OTA; group OTA+PP received CTRL diet supplemented with both the previous treatments (OTA and *P. pentosaceus* FBB61). To obtain freeze-dried corn meal with *P. pentosaceus* FBB61, strain was cultured for 22 h at 37°C in M17 broth; 1 l of the cultured broth was centrifuged 1100xg for 10 min and the pellet obtained was mixed with 200 ml of glycerol and 800 g of corn meal prior to freeze-drying, in order to obtain 10^9 Colony Forming Unit (cfu) of *P. pentosaceus* FBB61 per 100 g of corn meal. The corn meal containing freeze-dried *P. pentosaceus* FBB61 was stored at –20°C until feed supplementation. High contaminated corn meal (280 ppm) was added to diets in order to obtain final concentration in experimental diets as follows: 179 ppb in OTA diet; 174 ppb in PP+OTA diet. Diets not added with concentrated contaminated corn meal was analysed for basal OTA concentration and 2 ppb in CTRL diet, 3 ppb in PP diet was recorded.

After 3 weeks of daily treatment, animals were sacrificed by cervical dislocation and the liver, kidneys and brain of each rat were rapidly removed in a cold room. All samples were immediately frozen (−80°C), and were processed within 1 week of collection. Tissues sampled were homogenized in 9 volumes of cold PBS. Samples of homogenate were used to evaluate non-proteic thiol groups (RSH), lipid hydroperoxide (LOOH) levels as indicators of oxidative status of tissues.

Non-proteic thiol groups

Levels of RSH were measured, in 200 μl homogenate, using Hu's (1996) method partially modified. This spectrophotometric assay is based on the reaction of thiol groups with 2,2-dithio-bis-nitrobenzoic acid at $\lambda = 412$ nm in absolute ethanol ($\varepsilon M = 13600$). Results are expressed as nmol/mg proteins.

Lipid peroxidation

Levels of LOOH were measured in tissue homogenates following the oxidation of Fe^{+2} to Fe^{+3} in the presence of xylenol orange at $\lambda = 560$ nm. The assay mixture contained in a total volume of 1 ml: 10% homogenate, 90% methanol containing reagents at the following concentrations: 100 μm-xylenol orange, 250 μm-ammonium ferrous sulfate, 4 mm-butylated hydroxytoluene, and 25 mm-H2SO4. After 30 min incubation at room temperature, the absorbance was measured at $\lambda = 560$ nm using a U2000 Hitachi spectrophotometer (Hitachi, Ibaraki, Japan). Calibration was obtained using H_2O_2 (0.2–20 μm). Results are expressed as μmol/mg proteins.

Statistical evaluation

Data are reported as means and standard deviation, and the level of significance was P<0.05. Data were analysed for statistical significance using one-way ANOVA. (Prism 4.00, GraphPad Software, San Diego, CA).

4.1.2 Results and discussion

Gut microbial modulation induced by LAB in the gastrointestinal tract is a crucial feature of probiotics to maintain a healthy intestinal microflora. Moreover, the ability of some LAB to bind aflatoxins to their cell wall or cell membrane (El-Nezami et al., 1998a; 2000), and the hypothesis that OTA could be eliminated by this mechanism (Piotrowska and Zakowska, 2005), make probiotics potential candidate to counteract mycotoxin threat in human and animals.

During the present study, general clinical exam was performed every day. Body weight, feed and water intake measured weekly and no significant differences were observed in body weight, average daily feed and water intake (Table 4.1).

Table 4.1. Growth performance of rats fed a commercial standard pellet diet (control, CTRL) supplemented with *P. pentosaceus* FBB61 (10^6 cfu/g of feed, PP) or/and 200 ppb of ochratoxin A (OTA and PP+OTA, respectively). Rat, individually caged, is the experimental unit (n=10).

Item			CTRL	PP	OTA	PP+OTA	SEM
Final weight (21 d)	g		182.4	187.4	182.2	184.4	4.94
ADFI (0-21 d)	g		14.23	14.77	14.24	14.22	0.40
ADWI (0-21 d)	g		25.01	26.37	25.04	26.27	1.13

Abbreviations: ADFI, Average Daily Feed Intake; ADWI, Average Daily Water Intake

Sulfhydryl groups are important elements of the anti-oxidant defence in the organism limiting the production of oxygen-derived free radicals: high levels of non proteic thiol groups suggest a good capacity to limit the ROS formation. Free radicals react with unsaturated fatty acids of cell membrane and cause oxidative damage. Lipid hydroperoxidation is indicated by LOOH levels: low levels of LOOH suggest low damage to cell membranes.

Involvement of OTA in oxidative pathway (Baudrimont et al., 1997a) is confirmed by results of the present study: in liver (Figure 4.1) of OTA fed rats where higher RSH levels and lower LOOH levels than CTRL rats were detected (P<0.001). The same pattern was detected in kidney (Figure 4.2) and brain (Figure 4.3).

In liver, PP fed rats showed higher RSH and lower LOOH levels than CTRL fed rats. This indicate PP in-feed supplementation ameliorate oxidative status in liver. In kidney and brain, PP fed rats showed RSH and LOOH levels not different from CTRL rats indicating that no oxidative stress is induced by PP in-feed supplementation.

Figure 4.1. Non proteic thiol group and ipid peroxidase levels in liver from rats fed a commercial standard pellet diet (control, CTRL) supplemented with *P. pentosaceus* FBB61 (10^6 cfu/g of feed, PP) or/and 200 ppb of ochratoxin A (OTA and PP+OTA, respectively). Each value represents mean ± SD (n = 10). Different letters in the same tissue means P<0.001.
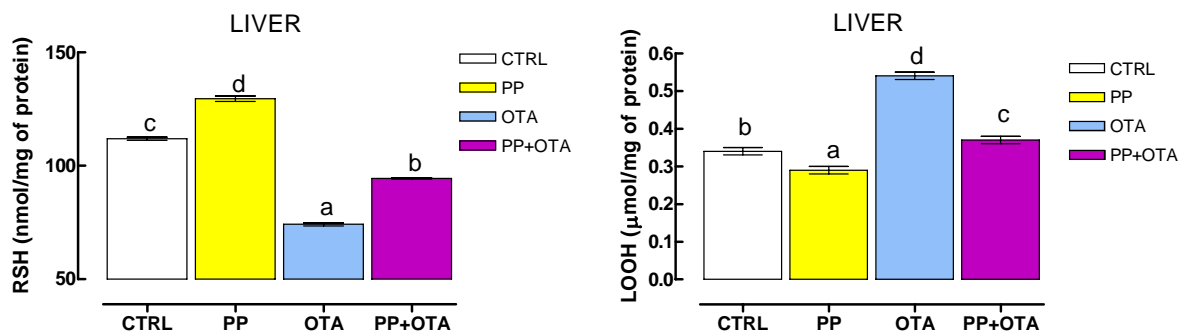
Figure 4.2. Non proteic thiol group and ipid peroxidase levels in kidney from rats fed a commercial standard pellet diet (control, CTRL) supplemented with *P. pentosaceus* FBB61 (10^6 cfu/g of feed, PP) or/and 200 ppb of ochratoxin A (OTA and PP+OTA, respectively). Each value represents mean ± SD (n = 10). Different letters in the same tissue means P<0.001.

Figure 4.3. Non proteic thiol group and ipid peroxidase levels in brain from rats fed a commercial standard pellet diet (control, CTRL) supplemented with *P. pentosaceus* FBB61 (10^6 cfu/g of feed, PP) or/and 200 ppb of ochratoxin A (OTA and PP+OTA, respectively). Each value represents mean $\pm$ SD (n = 10). Different letters in the same tissue means P<0.001.
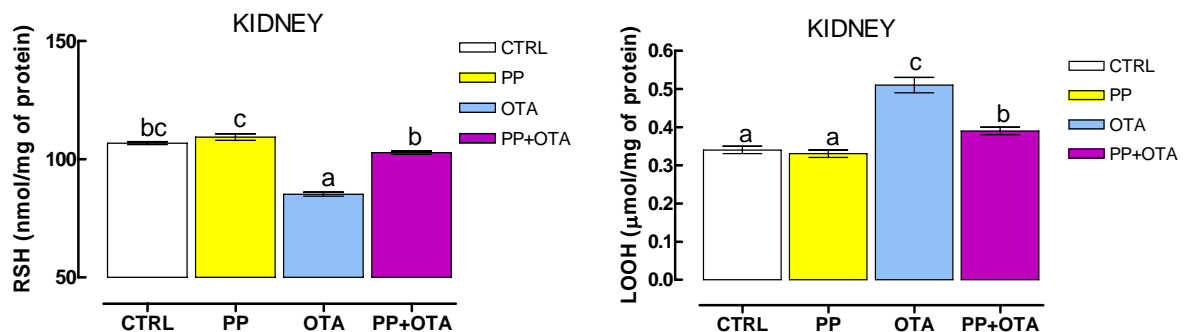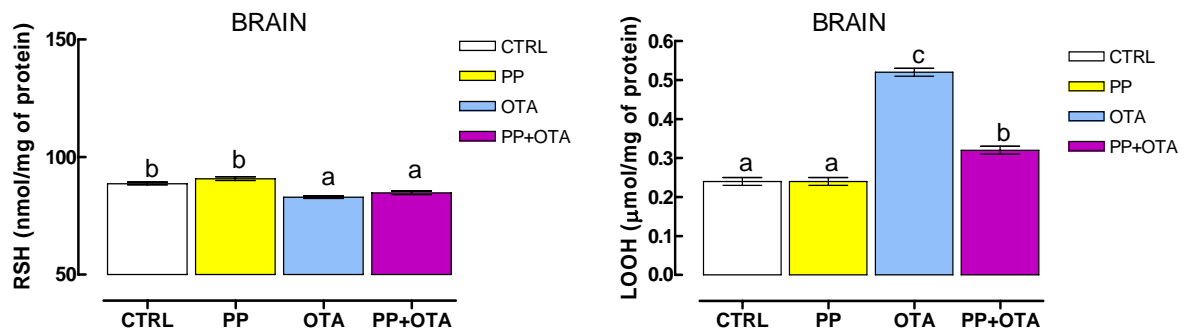

In liver and kidney, data from PP+OTA fed rats demonstrated that supplementation of *P. pentosaceus* FBB61 to a ochratoxin A contaminated diet is able to increase RSH levels and decrease LOOH levels (P<0.001). Moreover, in kidney RSH level of PP+OTA fed rats is restored to levels in CTRL rats. PP+OTA fed rats showed reduction of LOOH levels in liver, kidney and brain (P<0.001) than rats receiving OTA contaminated diet without supplementation of *P. pentosaceus* FBB61. The OTA induced oxidative stress is confirmed by increased RSH values and reduced LOOH levels in all tissues examined than CTRL tissues. Results on tissues from PP+OTA fed rats suggest that contaminated OTA diet supplemented with *P. pentosaceus* FBB61 reduce OTA induced oxidative damages. However, additional experiments are necessary in order to improve knowledge of toxicokinetics studies to know the concentration of OTA and genotoxicity studies related to beneficial effect and dosage of probiotic administration.

The present study allows to conclude that feed supplementation with *P. pentosaceus* FBB61 in rats ameliorates the oxidative status in liver, and lowers OTA induced oxidative damage in liver and kidney if diet was contaminated by OTA. This *P. pentosaceus* FBB61 feature joined to its bactericidal activity against Gram positive bacteria and its ability to modulate gut microflora balance in pigs, encourage additional *in vivo* experiments in order to better understand the potential role of *P. pentosaceus* FBB61 as probiotic for farm animals and humans. However, data drive to perform in depth studies i) to elucidate, *in vitro* and *in vivo*,

the effect of possible interactions among different bacteria strains and mycotoxins or their metabolites able to reducing toxicants absorption, ii) to investigate different pathways, related to DNA damage/repair, influenced by PP and or OTA ingestion.

4.2 Effects of fumonisins contaminated diet on weaning piglet growth performance

The fumonisin B analogues, including toxicologically important FB1, FB2 and FB3, are the most abundant naturally occurring fumonisins (Marasas, 1996). Fumonisin B1 causes pulmonary oedema in swine (Harrison et al., 1990). The results of surveys indicate that fumonisins contaminate maize kernels in all corn-growing countries of the world and can cause fumonisin toxicosis (Dutton 1996). Food materials of animal origin may become contaminated with FB1 after the toxin has been absorbed from the digestive tract, entered the bloodstream and reached the peripheral tissues.

Aim of the study was to evaluate the effect of ingestion of FB1 contaminated diet on growth performance of weaning piglets. The effects were measured on the production results daily gain, feed intake and feed conversion.

4.2.1 Materials and methods

Animals

The Ethics Committee of University of Bologna had previously granted approval for the animal study. Seventy male weaning piglets (initial body weight 7.03 ± 0.13 kg) were house in 14 cages (5 pigs/pen; 7 pens/treatment) homogeneous by weight. Piglets were divided into two groups: control group (CTRL) receiving a standard piglets diet at 50% corn meal; group receiving standard piglets diet where corn meal was contaminated by fumonisin B1 and B2 (FB). Two diets were fed in each group: a phase 1 diet from 4 to 6 weeks of age and a phase 2 diet from 6 to 10 weeks of age (Table 4.2). Piglets had been weighed at the beginning of the trial, when switching from diet 1 to diet 2 at day 14 of the trial, and at the end of the trial after 42 days from the beginning. Feed intake and daily gain were recorded. Clinical findings and mortality were daily recorded.

Statistical analyses

Data were analyzed for normal distribution and prevalence of outliers, and were subjected to test t-Student in GraphPad Prism. Data are shown as means $\pm$ SEM (n = 7). Significant differences are stated at $P<0.05$.

Table 4.2. Ingredient of experimental diet.

Group	Phase 1 diets		Phase 2 diets	
	CTRL	FB	CTRL	FB
Ingredient	*% as-feed basis*			
Corn	50.00	-	50.00	-
Contaminated corn	-	50.00	-	50.00
Soy-bean meal (48)	15.83	15.83	19.75	19.75
Milk whey sweet dried	12.00	12.00	5.00	5.00
Barley	5.00	5.00	16.94	16.94
Soy bean extruded	5.00	5.00	-	-
Soy oil	1.71	1.71	1.97	1.97
Potato protein	4.00	4.00	3.00	3.00
Wheat bran	3.55	3.55	-	-
Calcium carbonate ($CaCO_3$)	-	-	0.80	0.80
Monocalcium phosphate	0.72	0.72	1.16	1.16
Calcium formate	0.70	0.70	-	-
Sodium chloride	0.37	0.37	0.47	0.47
L-Lysine HCl	0.33	0.33	0.24	0.24
DL-Methionine	0.15	0.15	0.08	0.08
L-Threonine	0.09	0.09	0.50	0.50
L-Tryptophan	0.06	0.06	0.03	0.03
Vitamins+Oligo premix[1]	0.50	0.50	0.50	0.50
Feed analysis			-	-
Fumonisin B1+B2 (ppm)	0.91	7.32	2.34	7.56

[1] *Standard vitamin+oligominerals for piglets without selenium*

4.2.2 Results and discussion

As reported in Table 4.3, not statistically significant differences were observed in growth performance of piglets fed fumonisin contaminated diets for 6 weeks after weaning (7.32 ppm during first 2 weeks, then 7.56 ppm for 4 weeks). These results confirmed literature data, where no response to growth rate was observed at FB1 concentrations below 40 mg/kg of feed (Rotter et al., 1997) and no effects were reported on body weight and feed consumption on weaned piglets fed 10, 20, and 40 mg/kg FB1 contaminated feed (Zomborszky-Kovács et al., 2004a). Fumonisin toxicosis induce the increase in the Sa:So ratio that can be used to establish a diagnosis (Riley et al., 1993). Alterations in the Sa:So ratio in organs are a sensitive biomarker of the onset of adverse effects. Considering the Sa:So ratio as the most sensitive parameter in the assessment of adverse effect exerted by fumonisins the lowest observed adverse effect level (LOAEL) was found when pigs were exposed to feed containing 5 mg of fumonisins per kg feed. Monitoring of the Sa:So ratio (for example in serum or urine samples) has a dual function in monitoring exposure and assessing the onset of adverse effects. Analysis of Sa:So ratio on blood samples collected at day 14 and 42 of the study are in progress.

Table 4.3. Growth performance of piglets fed a control diet (CTRL) added with fumonisins (FB). Pen is the experimental unit (7 pens/treatment), each pen house 5 piglets. Data are shown as means ± SEM (n = 7).

Item		CTRL	FB	*P*
Initial weight	kg	7.05 ± 0.22	7.01 ± 0.16	0.91
Phase 1		*0 – 14 Days*		
ADFI	kg/d	0.36 ± 0.02	0.37 ± 0.03	0.65
Weight (14d)	kg	10.14 ± 0.29	10.09 ± 0.43	0.92
ADG	kg/d	0.22 ± 0.01	0.21 ± 0.02	0.65
Feed:Gain		1.59 ± 0.10	1.83 ± 0.11	0.13
Phase 2		*15-42 Days*		
ADFI	kg/d	0.92 ± 0.009	0.95 ± 0.02	0.28
Weight (42d)	kg	23.93 ± 0.50	24.09 ± 0.62	0.85
ADG	kg/d	0.51 ± 0.01	0.52 ± 0.02	0.77
Feed:Gain		1.79 ± 0.03	1.83 ± 0.04	0.49
Overall period		*0 – 42 Days*		
ADFI	kg/d	0.71 ± 0.008	0.72 ± 0.01	0.10
ADG	kg/d	0.41 ± 0.01	0.39 ± 0.005	0.22
Feed:Gain		1.75 ± 0.04	1.82 ± 0.03	0.22
Mortality		1 (at day 6)	1 (at day 7)	

Abbreviations: ADFI, Average Daily Feed Intake; ADG, Average Daily Gain

4.3 Effects of a mycotoxin detoxifying commercial product on growth performance of piglets fed deoxynivalenol contaminated diet

Swine are very susceptible to DON (Rotter et al., 1996) and could therefore serve as a model for human sensitivity to this mycotoxin. Study of Dänicke et al. (2004a) evidenced that the majority of the ingested DON is quickly and nearly completely absorbed in the proximal part of the small intestine. To avoid absorption of DON through gastrointestinal mucosa, detoxifying agents studied to adsorb or degrade DON in the digestive tract, must be effective under the physiological conditions in the stomach and the duodenum within a very limited period of time.

Aim of the present study was to evaluate of the effect on growth performance of weaned piglets fed DON contaminated diets, supplemented with a commercial detoxifying agent for mycotoxins.

4.3.1 Materials and methods

Animals

Ninety-six piglets (half males half females, Landrace x Large White), weaned at 28 days of age and weighing 7.7 ± 1.1 kg (mean $\pm$ SD), were divided into four groups, homogenous for weight and gender, of 24 animals each. Animals were housed in cages (six animals per cage, four cages per treatment) in an environmentally controlled room for a 40 days feeding trial. The first 4 days of the trial, piglets received the same base diet for an adaptation period. After the adaptation period, piglets received experimental diets: group CTRL continue to receive the base diet; group PROD, received base diet with the addition of a commercial product for mycotoxin detoxification at 2.5 g/kg; group DON, received base diet contaminated with DON at 2.5 ppm; group PROD+DON, received base diet contaminated with DON at 2.5 ppm and added with commercial product (2.5 g/kg). Feed and water were provided *ad libitum*. Composition of base diet is reported in Table 4.4. Chemical composition of the experimental diets is reported in Table 4.5.

Animals were individually weighed and feed consumption was recorded weekly.

Analyses of the diets (crude protein, crude fiber, ether extract, ash and starch) were performed according to AOAC standard methods (AOAC, 2000) and Van Soest et al. (1991) for neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) determinations.

Table 4.4. Ingredient of experimental diet.

Phase	Days 0-21	Days 22-40
Ingredient	*% as-feed basis*	
Corn meal	27.81	35.48
Barley meal	15.00	20.00
Barley flakes	12.00	6.00
Soy-bean meal 44%	15.00	17.50
Wheat bran	6.00	6.00
Dried milk whey	11.00	5.00
Potato protein concentrate	6.00	3.50
Soy-bean oil	3.30	2.80
Vitamin/Mineral Mix[1]	0.50	0.50
Calcium carbonate	1.40	1.30
Dicalcium phosphate	1.35	1.25
NaCl	0.30	0.40
L-Lysine-HCl	0.22	0.20
DL-Methionine	0.11	0.06

[1]*Vitamin/mineral premix containing per kg: vitamin A, 2,500,000 IU; vitamin D3, 400,000 IU; vitamin E, 10 mg; vitamin K, 400 mg; vitamin B1, 300 mg; vitamin B2, 1,000 mg; vitamin B6, 600 mg; vitamin B12, 8 mg; biotin, 30 mg; niacin, 5,000 mg; pantothenic acid, 3,000 mg; folic acid, 200 mg; choline, 100,000 mg; Fe (as iron carbonate), 20,000 mg; copper (as copper sulphate), 4,000 mg; manganese (as manganese chelated with amino acids), 2,000 mg; manganese (as manganese oxide), 18,000 mg; zinc (as zinc oxide), 25,000 mg; cobalt (as cobalt carbonate), 100 mg; iodine (as potassium iodine), 300 mg; selenium (as sodium selenite), 50 mg.*

Table 4.5. Chemical composition of the experimental diets (% D.M.)

	Days 0-21		Days 22-40	
	CTRL	PROD	CTRL	PROD
Dry Matter (%)	91.29	91.28	90.70	90.69
Crude protein	19.94	19.94	20.28	19.69
Ether extract	5.38	5.32	4.71	4.71
Crude fiber	3.58	3.76	4.19	4.84
Ash	6.75	6.75	6.32	6.31
NDF	12.16	12.54	13.80	15.18
ADF	5.22	5.20	6.11	6.46
ADL	0.68	0.78	0.94	0.90
Starch	36.27	34.73	40.14	42.75

Statistical analyses

Data were analyzed according to a complete two by two factorial design of ANOVA with DON and commercial product as the factors (the effect of gender was not considered). The differences among means of groups were analyzed using the Student-Newman-Keuls test. Each cage formed the experimental unit. Differences were considered statistically significant at $P<0.05$.

4.2.2 Results and discussion

Growth performance of piglets are reported in Table 4.6 The addition of DON to the diets significantly reduced ($P<0.05$) average daily gain (ADG) between day 0 and 21 as well as between day 0 and 40. Piglets fed DON contaminated diets reported body weight significantly lower than CTRL piglets ($P<0.05$). Feed to gain ratio was significantly increased by DON throughout the trial ($P<0.05$). Commercial product tested did not influence piglet growth performance and the interaction between commercial product and DON was not significant for any of the parameters tested.

Feeding DON caused higher incidence of diarrhea during the third and fourth week of the experiment.

Table 4.6 Growth performances of weaned piglets fed a diet added or not with deoxynivalenol (DON) and/or a commercial product (PROD).

		CTRL	PROD	DON	PROD +DON	ANOVA P values		
						PROD	DON	PRODxDON
Initial weight	kg	7.72	7.78	7.70	7.77	-	-	-
Phase 1		*Day 0 - 21*						
Weight (21d)	kg	14.0	14.2	12.4	13.1	0.528	0.095	0.724
ADG	kg/d	0.297[b]	0.304[b]	0.224[a]	0.255[a]	0.413	**0.019**	0.599
ADFI	kg/d	0.466	0.445	0.421	0.477	0.510	0.793	0.160
Feed:Gain		1.58[A]	1.49[A]	1.90[B]	1.89[B]	0.568	**0.002**	0.686
Phase 2		*Day 22 - 40*						
Weight (40d)	kg	24.8[b]	24.4[b]	22.1[a]	23.3[a]	0.652	**0.046**	0.354
ADG	kg/d	0.573	0.541	0.511	0.537	0.875	0.121	0.161
ADFI	kg/d	1.048	1.010	1.026	1.038	0.701	0.930	0.466
Feed:Gain		1.83[a]	1.87[a]	2.01[b]	1.94[b]	0.707	**0.038**	0.300
Overall period		*Day 0 - 40*						
ADG	kg/d	0.428[B]	0.417[B]	0.361[A]	0.389[AB]	0.543	**0.006**	0.191
ADFI	kg/d	0.733	0.699	0.693	0.734	0.891	0.918	0.140
Feed:Gain		1.71[A]	1.67[A]	1.92[B]	1.89[B]	0.351	**< 0.001**	0.995

* Values are means of 4 cages per treatment; ADG = Average Daily Gain; ADFI = Average Daily Feed Intake; different letters within the same row mean significant difference ($P<0.05$).

Growth results show that the presence of DON at 2.5 ppm in piglet diets significantly reduced average daily gain of animals. Because daily feed intake was not significantly affected by DON, the poor growth performances of DON-fed piglets seem to be caused mainly by the significantly higher feed to gain ratio that was induced by DON throughout the trial.

Despite the fact that the interaction between DON and PROD did not reach the significance level for any of the parameters tested, piglet growth performances were numerically improved when PROD was added to DON containing diet. In fact, PROD+DON-fed piglets showed

higher final live weight (23.3 vs 22.1 kg), average daily gain (0.389 vs 0.361 kg/d), and daily feed intake (0.734 vs 0.693 kg/d) than DON-fed animals. Conversely, PROD showed no positive effect on feed to gain ratio and diarrhea incidence. When added to the basal diet, PROD did not affect animal growth, suggesting that there is no detrimental effect of PROD on the absorption of nutrients.

The present trial confirmed that pigs are very sensitive to DON and that dietary contamination with DON strongly affects piglet growth performances.

Chapter 5. Conclusions

Mycotoxins are contaminants of agricultural products both in the field and during storage and can enter the food chain through contaminated cereals and foods (milk, meat, and eggs) obtained from animals fed mycotoxin contaminated feeds. Mycotoxins are genotoxic carcinogens that cause health and economic problems. Ochratoxin A and fumonisin B1 have been classified by the International Agency for Research on Cancer (IARC, 1993) as "possibly carcinogenic to humans" (class 2B).

Different strategies can be applied separately or in synergy to reduce mycotoxin damage in human and animals:

The first step is the reduction of fungi contamination, and consequently mycotoxin production, in cereals and vegetal products. Moisture and temperature during storage are critical factors influencing mould growth and mycotoxin production: proper storage conditions and the use of fungicides or their alternatives (organic acids and essential oils) can be applied to reduce mycotoxin contamination.

The second step is the detoxification of contaminated feeds and foods. The use of many of the available physical (sorting and segregation) and chemical (HCl, NH_3, H_2O_2, O_3) methods is limited due to problems concerning safety issues, possible losses in the nutritional quality of treated commodities, limited efficacy and economic implications. This has led to the search for alternative strategies such as biological agents. Biological agents, such as LAB, belong to this second step and to the third step of intervention.

The third step of intervention is the inhibition of mycotoxins gastrointestinal absorption. Lactic Acid Bacteria were able to bind mycotoxins reducing their concentration in *in vitro* systems. This step of intervention is investigated in the present study evaluating *in vitro* the efficacy of LAB strains to reduce, by binding and/or degradation, OTA, FB1, and DON levels in bacterial medium. A large number of LAB strains isolated from feces and different gastrointestinal tract regions of pigs and poultry were screened for their ability to remove OTA, FB1, and DON from bacterial medium. Results of this *in vitro* study showed low efficacy of isolated LAB strains to reduce OTA, FB1, and DON from bacterial medium. Consequently, an *in vivo* trial in rats was performed to evaluate the effects of in-feed supplementation of a LAB strain, *Pediococcus pentosaceus* FBB61, to counteract the toxic effects induced by exposure to OTA contaminated diets. The study allows to conclude that feed supplementation with *P. pentosaceus* FBB61 ameliorates the oxidative status in liver, and lowers OTA induced oxidative damage in liver and kidney if diet was contaminated by

OTA. This *P. pentosaceus* FBB61 feature joined to its bactericidal activity against Gram positive bacteria and its ability to modulate gut microflora balance in pigs, encourage additional *in vivo* experiments in order to better understand the potential role of *P. pentosaceus* FBB61 as probiotic for farm animals and humans. The inhibition of mycotoxins gastrointestinal absorption can be reached also by the addition in the diets of the non-nutritionally adsorbents that bind mycotoxins. The most studied non nutritional adsorbents are activated carbons, hydrated sodium calcium aluminosilicate, clays and yeast cell walls polysaccharides (glucan, mannan, esterified glucomannan). To better understand the intestinal absorption of mycotoxins, the Ussing chambers technique was applied in the present study that for the first time to investigate *in vitro* the permeability of OTA and FB1 through rat intestinal mucosa. Results showed that OTA and FB1 were not absorbed from rat small intestine mucosa. Since *in vivo* absorption of both mycotoxins normally occurs, it is evident that in these experimental conditions Ussing diffusion chambers were not able to assess the intestinal permeability of OTA and FB1.

The fourth step of intervention is the reduction of damages when mycotoxins absorption occurs. Nutritional approaches, such as supplementation of nutrients, food components, or additives with protective effects against mycotoxin toxicity are assuming increasing interest. Evidence of the feasibility of this approach are reported by the present study on rats were the in-feed supplementation of a probiotic strain reduce the oxidative damage in liver and kidneys of rats fed OTA contaminated diets.

In the present study, *in vivo* trial on weaned piglets fed FB1 allow to conclude that feeding of 7.32 ppm of FB1 for 6 weeks did not impair growth performance.

Deoxynivalenol contamination of feeds was evaluated in an *in vivo* trial on weaned piglets. The comparison between growth parameters of piglets fed DON contaminated diet and contaminated diet supplemented with the commercial product did not reach the significance level but piglet growth performances were numerically improved when the commercial product was added to DON contaminated diet.

Further studies are needed to improve knowledge on mycotoxins intestinal absorption, mechanism for their detoxification in feeds and foods, and nutritional strategies to reduce mycotoxins induced damages in animals and humans. The multifactorial approach acting on each of the various steps could be a promising strategy to counteract mycotoxins damages.

Chapter 6. References

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