LEILA GERTRUDIS MAIDANA MORENO

LACTOBACILLUS PLANTARUM MODULATES INTESTINAL TOXICITY:

AN *EX VIVO* APPROACH

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Dissertation submitted to the Animal Health Science Postgraduation Programme (area of concentration Animal Health) of Universidade Estadual de Londrina as requirement for the Master degree.

Supervisor: Profª. Drª. Ana Paula Frederico Rodrigues Loureiro Bracarense.

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“The essence of all beautiful art, all great art, is gratitude”

Friedrich Nietzsche

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RESUMO

Antimicrobianos têm sido amplamente utilizados na dieta de leitões para promover o crescimento e reduzir a incidência de diarreia. A resistência dos patógenos aos antimicrobianos e a possibilidade de resíduos nos produtos de origem animal aumentaram o interesse em alternativas de additivos nas dietas. As bactérias ácido lácticas (LAB) são uma alternativa potencial ao uso de antibióticos. O desoxinivalenol (DON) é um metabólito secundário da família dos tricotecenos produzido pelo *Fusarium graminearum* e *F. culmorum* que afeta a saúde animal e humana. A deterioração de alimentos devido à contaminação por fungos e produção concomitante de micotoxinas é um problema crescente, particularmente quando se considera a possibilidade dos fungos adquirirem resistência a conservantes químicos comumente usados. Portanto, métodos eficazes de detoxificação são estratégias úteis para minimizar esses riscos. A biotransformação de micotoxinas por LAB é uma forma promissora de reduzir os efeitos deletérios destas toxinas. O objetivo deste estudo foi investigar os efeitos de duas cepas de *Lactobacillus plantarum* (LP) inativadas por calor e seus sobrenadantes de cultura (SC) isoladas ou associadas ao DON em explantes de jejuno de suínos. As LAB foram inativadas por esterilização (121°C por 30 min) e posteriormente as suspensões (1,1 x 108 e 2,0 x 109 CFU ml-1 de LAB para a cepa 1 e cepa 2, respectivamente) foram centrifugadas (3000 g, 10 min, 5°C). Os pellets resultantes (LP1 e LP2) e os sobrenadantes (CS1 e CS2) foram separados e armazenados a -20 °C até sua utilização. Os explantes foram incubados a 37°C durante 4 horas sob agitação orbital com os seguintes tratamentos: meio de cultura DMEM (controle), MRS, LP1+DMEM, LP2+DMEM, CS1+DMEM, CS2+DMEM, DON (10 μM), DON+LP1, DON+LP2, DON+CS1 e DON+CS2. Após a incubação, os explantes foram processados para análise histológica, morfométrica e ultraestrutural. Os explantes controle apresentaram atrofia leve das vilosidades e edema da lâmina própria, enquanto que os explantes expostos a LP1, LP2, LP1+DON e LP2+DON apresentaram atrofia difusa das vilosidades, necrose apical e achatamento dos enterócitos. As principais alterações nos explantes tratados com CS1, CS2, CS1+DON e CS2+DON foram atrofia leve das vilosidades e necrose apical leve dos enterócitos. A análise morfométrica mostrou um aumento significativo na altura das vilosidades nos explantes tratados com o sobrenadante da cultura de *L. plantarum* individualmente ou combinado com DON em comparação com o grupo DON e com ambas as cepas das bactérias. A avaliação ultraestructural demonstrou vilosidades do grupo controle bem delineadas com alguns leucócitos na superfície dos enterócitos. Os explantes tratados com DON mostraram uma atrofia severa das vilosidades acompanhada de um elevado número de leucócitos e restos celulares na superfície intestinal. Os explantes tratados com LP+DON mostraram atrofia moderada de vilosidade com necrose apical multifocal. Os explantes expostos aos sobrenadantes de ambas as cepas, individualmente ou associados ao DON apresentaram vilosidades bem delineadas e com deposição de muco na superfície. A capacidade antioxidante do *L. plantarum* e dos sobrenadantes foi avaliada por meio das técnicas ABTS e FRAP. O tratamento com CS2 induziu um aumento significativo na resposta antioxidante em comparação ao grupo tratado com DON. Em conclusão, os sobrenadantes de *L. plantarum* apresentaram efeitos benéficos nos explantes intestinais expostos ao DON caracterizados por incremento na morfologia intestinal e na capacidade antioxidante.

Palavras chave: Desoxinivalenol. Explantes. Toxicidade. *Lactobacillus plantarum*. Sobrenadante.

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ABSTRACT

Antimicrobials have been widely used in the diets of piglets to promote growth performance and reduce the incidence of diarrhea. Nevertheless, the resistance of pathogens to antibimicrobials and the possibility of residues of antimicrobials in animal products resulted in an increasing interest in alternatives for in-feed treatments. Lactic acid bacteria (LAB) in pigs provide a potential alternative to antimicrobials strategies. Deoxynivalenol (DON) is a secondary fungal metabolite of the trichothecene family produced by *Fusarium graminearum* and *F. culmorum* that affects animal and human health. Deterioration of food/feeds stuffs due to fungal colonization and concomitant production of this mycotoxin is an increasing concern; especially considering fungi acquiring resistance to many commonly used chemical preservatives. Therefore, effective detoxification methods would be useful in counteracting this problem. Biotransformation of mycotoxins by LAB is a promising way to minimize the deleterious effects of these toxins. The purpose of this study was to investigate the effects of two heat-inactivated *Lactobacillus plantarum* (LP) strains and the respective culture supernatants (CS) alone or combined with DON on jejunal explants of pigs. LAB were heat inactivated by sterilization (121^{o}C for 30 min). Posteriorly, the cell suspensions (1.1 x 10^{8} and 2.0 x 10^{9} CFU ml^{-1} of LAB for strain 1 and strain 2, respectively) were centrifuged (3000 g, 10 min, 5^{o}C). The resulting pellets (LP1 and LP2) and supernatants (CS1 and CS2) were separated and stored at -20^{o}C until the use. Explants were incubated at 37°C for 4 hours with orbital shaking in the presence of the following treatments: control – only culture media (DMEM), MRS, LP1+DMEM, LP2+DMEM, CS1+DMEM, CS2+DMEM, DON (10 μM), DON+LP1, DON+LP2, DON+CS1 and DON+CS2. After incubation, explants were processed for histological, morphometric and ultraestructural analysis. Control explants presented mild villi atrophy and edema of lamina propria, whereas explants exposed to LP1, LP2, LP1+DON and, LP2+DON showed multifocal to difuse villi atrophy, villi apical necrosis and enterocyte flattening. The main alterations in explants treated with CS1, CS2, CS1+DON and CS2+DON were mild villi atrophy and mild enterocyte apical necrosis. The morphometric analysis showed a significant increase in villi height and antioxidant capacity in explants treated with CS individually or combinated with DON when compared to the DON and LP groups. Ultraestructural assesment showed control explants with well delineated finger-shaped villi with few leukocytes lining the surface of enterocytes. DON-treated explants showed a severe and diffuse villi atrophy accompanied by remarkably number of leukocytes and cell debris in the intestinal surface. LP+DON-treated explants showed moderate villi atrophy, multifocal villi apical necrosis (resulting in exposure of the lamina propria). CS-explants alone or combined with DON presented well delineated villi and mucus surfacing the villi. In addition, the antioxidant capacity of *L. plantarum* and the supernatants were also evaluated using the ABTS and FRAP assays. Treatment with CS2 resulted in a significant increase in the antioxidant response compared to explants exposed to DON. In conclusion, culture supernatants of *L. plantarum* showed a beneficial effect on intestinal explants exposed to DON, characterized by an improvement in tissue morphology and antioxidant capacity.

Key words: Deoxynivalenol. Explants. Toxicity. *Lactobacillus plantarum*. Culture Supernatant.

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Literature Review

LIST OF ABBREVIATIONS

3-acDON	3-acetyl Deoxynivalenol
A	*Aspergillus*
AFL	Aflatoxin
AFB1	Aflatoxin B1
ATP	Adenosine triphosphate
BALB/c	Albino mice
Caco-cells	Colonic adenocarcinoma cells
CCL5	Chemokine ligand 5
CD14	Cluster of differentiation 14
CFS	Culture free supernatant
DAS	Diacetoxyscirpenol
DCs	Dendritic cells
DON	Deoxynivalenol
FAO	Food and Agriculture Organization
FB1	Fumonisin
FUX	Fusarenone-X
GIT	Gastrointestinal tract
hk-Lp	Heat-killed *Lactobacillus plantarum*
hPBMCs	Human peripheral blood mononuclear cells
HPLC	High performance liquid chromatography
Hsps	Heat shock proteins
HUVE	Human umbilical vein endothelial cells
IARC	International Agency for Research on Cancer
IFN-γ	Interferon gamma
IgE	Immunoglobulin E
IL	Interleukin
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
JAMs	Junctional adhesion molecules
LAB	Lactic acid bacteria
L.	*Lactobacillus*
LGG-s	*Lactobacillus rhamnossus* GG

Lp-CS	*Lactobacillus plantarum* culture supernatant
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MDC	Myeloid dendritic cell
MIP1 α/CCL3	Macrophage inflammatory protein 1/ α chemokine ligand 3
MCT1	Monocarboxylate transporter 1
mRNA	Messenger RNA
MRS broth	De Man, Rogosa and Sharpe media, HiMedia
NIV	Nivalenol
NOD2	Nucleotide-binding oligomerization
NO	Nitric oxide
TCT	Trichothecene
TJs	Tight junctions
Th1	T-helper 1
Th2	T-helper 2
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TLR5	Toll-like receptor 5
TLR9	Toll-like receptor 9
TNF-α	Tumor necrosis factor-alpha
OTA	Ochratoxin A
PAT	Patulin
PKC	Protein kinase C
WHO	World Health Organization
ZEA	Zearalenone
ZO-1	Zonula occludens-1

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1 INTRODUCTION

Deoxynivalenol (DON) is a secondary fungal metabolite of the trichothecene family mainly produced by *Fusarium graminearum* and *F. culmorum* that affects human and animal health (PIERRON et al., 2016) due to its co-occurrence in cereal-based food and feed (SMITH et al., 2016). A worldwide surveillance confirms its occurrence in 55% of cereal crops (STREIT et al., 2013), ranging from 80-100% of cereal contamination in some countries (BRYLA et al., 2016; TIMA et al., 2016). DON occurs predominantly in grains such as wheat, barley, oats, rye and maize, and less often in rice, sorghum and triticale; representing the most prevalent mycotoxin in cereals. Considering that wheat is the most important human grain and ranks second in total production as a cereal crop behind maize, this mycotoxin represents a very serious health risk. Deterioration of food/feeds stuffs due to fungal colonization and concomitant production of mycotoxins is an increasing concern, especially considering fungi acquiring resistance to many commonly used chemical preservatives (DEEPTHI et al., 2016). Considering these risks, the European Commission and the United States Department of Agriculture released a guidance value for food ranging from 200 to 1750 µg/kg and a concentration of 9.9 mg/kg of the daily ration for animals. In Brazil, ANVISA had established permitted levels between 200-1000 µg/kg for grains, but no legislation has been established for feed.

The intestine is the major site of DON absorption (GHAREEB et al., 2015). In the pig, DON is rapidly and efficiently absorbed, most probably in the upper part of the small intestine, and is mainly excreted in the urine (GAMBACORTA et al., 2016), with no accumulation in tissues (WACHE et al., 2009). Consumption of DON-contaminated feed in these animals impacts the gastrointestinal tract, affecting intestinal barrier function by modulating the expression of tight junctions proteins (PINTON et al., 2009; PINTON et al., 2010; BASSO et al., 2013; LUCIOLI et al., 2013), altering enterocyte proliferative and apoptosis index (WANG et al., 2014; CHEAT et al., 2016), decreasing the number of goblet cells (GEREZ et al., 2015) and also leading to intestinal inflammatory response (VAN DE WALLE et al., 2010) and *in-vivo* (GAUTHIER et al., 2013). Moreover, authors have investigated the interaction between some intestinal microflora strains and this mycotoxin (KOLLARCZIK et al., 1994). Taken together, DON alters intestinal cell function, inhibiting intestinal nutrient absorption, affecting the intestinal permeability and immune function.

At the cellular level, DON inhibits protein synthesis (at the elongations-termination step of protein translation) through interaction with the peptidyl transferase at the

60S ribosomal subunit (ROCHA et al., 2005). The binding of DON to the ribosome rapidly activates MAPK signaling pathways and induces caspase-mediated apoptosis in a process known as the “ribotoxic stress response” (ROCHA et al., 2005; PESTKA, 2010). Furthermore selected genes were reported to represent altered pathways of the cellular metabolism *in vitro* (DIESING et al., 2012).

Effective detoxification methods would be useful in counteracting this worldwide problem (GRENIER et al., 2013). De-epoxidation and epimerization have been reported in DON-degrading bacteria, showing promising approaches, considering that the 12,13-epoxide group is essential for the toxic activity of trichothecenes (IKUNAGA et al., 2011; PIERRON et al., 2016). Moreover, *in vivo* efficacy of DON binders were described to reduce the gastrointestinal absorption of the mycotoxin (TAMURA et al., 2013; DEVREESE et al., 2014; GAMBACORTA et al., 2016).

This study aimed to compare intestinal histopathological features of DON, alone or in combination with two strains of heat-inactivated *Lactobacillus plantarum* (LP) and their culture supernatant (CS) in jejunal explants of pigs. Furthermore, this *ex vivo* model was used in the context of improving the 3Rs “Replace, Reduce, Refine”.

2 LITERATURE REVIEW

2.1 PROBIOTICS

The Expert Panel commissioned in 2001 by the Food and Agriculture Organization of the United Nations, and supported by the World Health Organization (FAO/WHO) defined for probiotics "live microorganisms that, when administrated in adequate amounts, confer a health benefit on the host". Recently, this FAO/WHO definition has been reevaluated and supported (REID, 2016).

2.1.1 Lactic Acid Bacteria

Lactic acid bacteria (LAB) are classified as substances generally recognized as safe and probiotics (SON et al., 2015). They comprise a group of Gram-positive, acid tolerant, generally non-sporulating, usually catalase-negative, non-respiring rod shaped (bacillus) or spherical (coccus) bacteria, that grow under microaerophilic to strictly anaerobic conditions (KLEIN et al., 1998). LAB are associated by their common metabolic and physiological characteristics, since produce acid lactic as the major metabolic end-product of carbohydrate fermentation. This trait has linked LAB with food fermentation, as acidification inhibits the growth of spoilage agents (CAPLICE and FITZGERALD, 1999).

LAB includes various major genera, including *Lactobacillus* spp., *Bifidobacterium* spp., *Lactococcus* spp., *Lactosphera* spp., *Leuconostoc* spp., *Melissococcus* spp., *Oenococcus* spp., *Pediococcus* spp., *Streptococcus* spp., and *Enterococcus* spp. (KLEIN et al., 1998; CARR et al., 2002).

To act as a probiotic, LAB must first be able to survival passage through the upper gastrointestinal tract (GIT), meaning that LAB must have the characteristics of resistance to increased acidity from inorganic acid production and pancreatic enzymes (HAVENAAR et al., 1992). Secondly, have the potential to adhere to intestinal epithelial cells (HAVENAAR et al., 1992). Adhesion of a probiotic strain to the GIT is important for bacterial colonization, pathogen exclusion and interaction with host cells for the protection of epithelial cells or immune modulation (LEBEER et al., 2008).

Mechanisms of adherence to an epithelial surface involve receptor-specific binding and charge as well as hydrophobic interaction. LAB commonly express cell surface

hydrophobicity, contact angle and adhesion to xylene. This may facilitate adhesion to mucus. Furthermore, cell surface proteins have been also shown to mediate adhesion to mucus by various LAB (KIRJAVAINEN et al., 1998). Interestingly, LAB showed no host specificity in adhesion to intestinal mucus, but differed between the different compartments of the GIT (RINKINEN et al., 2003). Adhesion factors include passive forces, electrostatic interactions, hydrophobic reactions, steric forces, lipoteichoic acids, adhesion-promoting proteins, autoaggregative properties, and proteinaceous structures (TUOMOLA et al., 2000; SERVIN and COCONNIER, 2003; COLLADO et al., 2006).

As mentioned, multiple reports have described the beneficial effects of LAB, such as regulation of the intestinal microbiota, inhibition or prevention of pathogens in the GIT, enhancement of intestinal mucosal immunity and maintaining intestinal barrier function (LINDFORS et al., 2008; YANG et al., 2015), which provides defense by inducing anti-inflammatory cytokines and reducing pro-inflammatory cytokines from intestinal epithelial cells (O'HARA et al., 2006; WALSH et al., 2008), enhancing the gut inflammatory immune response (MATSUGUCHI et al., 2003). Indeed, some authors demonstrated that *Lactobacillus casei* and *Lactobacillus plantarum* were able to increase $CD4^+$ T cells in the lamina propria of the small intestine (PERDIGON et al., 1999).

Immune modulation by probiotics was recognized as an important aspect of host gut health (PAGNINI et al., 2010), suggesting that the health benefits of some probiotics used in functional foods and pharmaceutical preparations are due to the capacity of these microorganisms to stimulate the host immune system (PERDIGON et al., 2002).

Another potential mechanism of the effects of probiotics have been proposed including the upregulation of mucus production (LEE et al., 2003), and the production of antibiotic peptide bacteriocins (CLEVELAND et al., 2001). Indeed, the anti-microbial effects of LAB are mainly related to the production of organic acids such as lactic, acetic, and propionic acid. Acid production lowers the pH of food and helps inhibiting the growth of other microorganisms (BRUL and COOTE, 1999). LAB produces other compounds such as hydrogen peroxide, CO_2, diacetyl and bacteriocin that may also contribute to their preserving properties (STILES, 1996). This bacteriocins produced by LAB have been reported to have antibacterial and antifungal activity (CROWLEY et al., 2013).

But the exact underlying mechanisms of the beneficial effects of probiotics on barrier function are largely unknown.

2.1.2 Heat-inactivated Bacteria

Several studies have reported that live as well as heat-inactivated LAB have stimulatory properties (FUJIWARA et al., 2004; SASHIHARA et al., 2006), suggesting that the surface of some lactobacilli have properties that elicit an immune response, as reflected by the release of IFN-γ and lymphocyte proliferation (LEE et al., 2011).

As mentioned, the most widely studied probiotics are the lactic acid bacteria. Some heat-killed strains, including *Streptococcus thermophilus* and *Lactobacillus acidophilus*, have been showed to play a preventive role in intestinal epithelial barrier dysfunction (RESTA-LENERT and BARRETT, 2003; ZENG et al., 2008). Also, heat-killed *L. acidophilus* strain LB and the fermented culture medium have stabilized the aspirin-induced alterations (expression of ZO-1 and distribution) in human intestinal epithelial HT-29 cells (MONTALTO et al., 2004).

There are increasing reports indicating that LAB inactivated by heating still display adhesive capabilities. *In vitro*, a mix of three strains of *L. acidophilus* heat-killed at 100°C for 30 min was able to inhibit *Salmonella* invasion (PENG et al., 2007; OSTAD et al., 2009). In addition, the adhesive activity of *L. acidophilus* inactivated by heat treatment at 60°C for 30 min is similar to that of live bacteria. On the other hand, Ou et al. (2011) showed that the adhesive ability of LAB decreases as temperatures increase. Some studies have revealed that the ability to adhere to Caco-2 cells or intestinal mucus is strain-species, and genus-dependent (COLLADO et al., 2006). Heat-inactivated *L. salivarius* AP-32 and *E. faecalis* YM-73 treated at 60°C for 30 min or 80°C for 20 min still showing the ability to adhere to Caco-2 cells, and induce hPBMCs to increase the production of IFN-γ and IL-12p70 (Th1- t-helper1) and decrease IL-13 (Th2) (OU et al., 2011).

LAB also induce the expression of IL-12, contributing to the immunomodulatory activity. IL-12 is produced by antigen-presenting cells, such as dendritic cells and macrophages and stimulates both natural killer cells and Th1 cells to produce IFN-γ (AOKI et al., 2011). Living or dead *L rhamnosus*, as well as their products alone, were able to induce the synthesis of TNF-α by macrophages, and the suspensions containing live and dead cells of the microorganism generated the same amount of TNF-α as LPS (JORJAO et al., 2015).

Additionally, heat-inactivated *L. brevis* induced an increased expression of Hsps, activated the p38 MAPK pathway, regulated the secretetion of proinflammatory cytokines, protected intestinal tissues from oxidant stress, and improved the intestinal

integrity (UENO et al., 2011). Heat-inactivated LAB prevented an increase in inducible nitric oxide synthase mRNA (iNOS mRNA) levels (ZENG et al., 2016). iNOS mRNA levels and the subsequent NO production are well recognized as a key factor responsible for cytokine-induced epithelial barrier failure *in vitro*. An increase in iNOS-dependent NO has been shown to exert an adverse effect on the structure and function of epithelial tight junctions that maintain and regulate normal epithelial permeability (HAN et al., 2004). Furthermore, substances that inhibit iNOS or scavenge NO have been proven to increased permeability induced by cytokines.

Moreover, exposure to heat-inactivated bacteria and bacteria remnants leads to increased levels of cellular proliferation (evaluated by PCNA expression) in the intestine, liver and spleen tissues but not in muscle tissues of mice (KOTURBASH et al., 2009). In this study, the increase in the expression of two different proteins could be related to a response to genotoxic stress-induced by damage to p53. Elevated levels of PCNA could be explained by its involvement in DNA repair and may reflect the increased DNA repair activity

Taken together, the results of previous studies indicated that heat-inactivated bacterial agents (pathogenic or not) can lead to distinct molecular and morphological changes in the living organisms. Indeed, heat-killed bacterial lysates, and clear lysate supernantant of *L. sakei* K101 and *L. plantarum* K55-5 induced a greater increase in TNF-α production than live bacteria in Raw 264.7 cells (a mouse macrophage cell line) (LEE et al., 2016). The immune-inducing potential of heat-killed *L. plantarum* K55-5 was higher than that of *L. sakei* 101, suggesting that *L. plantarum* K55-5 could be a powerful candidate for an immune-stimulating LAB probiotic.

Heat-inactivated LAB have been reported to exhibit immunomodulatory properties (MATSUZAKI et al., 1998; MAASSEN et al., 2000; FUJIWARA et al., 2004). However, the effects of conditions such as temperature and time are not consistent among individual strains. For example, oral administration of heat-killed *L. gasseri* OLL2809, treated at 75°C for 60 min, stimulated higher IL-12p70 production and reduced the serum antigen-specific IgE levels in ovalbumin-sensitized BALB/c mice when compared with control mice (SASHIHARA et al., 2006). A heat-killed mix-3 strains of *Lactobacillus acidophilus* treated at 100°C for 60 min-has better ability to prevent bacterial infection in mice than viable monostrain, which may be due to the immunomodulating role of activated macrophages (LIN et al., 2007). Additionally, Segawa et al. (2008) reported that the oral administration of *L. brevis* SBC8803 strain, killed by heating at 120°C for 20 min, inhibits IgE production in

ovoalbumin (OVA)-sensitized BALB/c mice through improvement of the Th1/Th2 balance toward Th1 dominance.

A comparative study with heat-inactivated bacterial strains indicated that Gram-positive and Gram-negative bacteria differ in dose-dependent patterns in their impact on TLR2 (toll-like receptor 2) and TLR4 (toll-like receptor 4) expression and the intensity of cytokine production (BERAN et al., 2011). Heat-killed Gram-negative bacteria induce an activation phenotype in human umbilical vein endothelial cells (HUVE), indicated by HUVE surface expression of both E-selectin and tissue factor activity; in contrast, heat-killed Gram-positive bacteria do not induce any phenotypic changes in HUVE (NOEL et al., 1995).

The immunostimulatory activities of different bacterial species and strains correlate with the variations in cell wall components, for example lipoteichoic acids and peptidoglycan (SASHIHARA et al., 2006).

Although their effectiveness has been attributed to their viability and metabolic activity, viable LAB cannot be added to food, as this would induce fermentation that alter the taste, texture and freshness of the food and are not easy to store. For these reasons, the use of living bacteria has been limited to a very narrow range of products such as yogurt. Non-viable LAB, however, which are easier to store and transport, and have a longer shelf life, have a beneficial effect to the host (SIMAKACHORN et al., 2000; MONTALTO et al., 2004; REID et al., 2004) similar to that of living bacteria.

2.1.3 Bacteria Culture Supernatants

Previous studies have demonstrated that the mechanisms and effects of probiotics vary based on whether viable, inactivated bacteria or cell-free supernatant are used (KLINGBERG et al., 2005; PUTAALA et al., 2008; DONATO et al., 2010). Indeed, both bacterial cells and their metabolites may have a beneficial impact on the intestinal epithelial barrier.

Considering that the secreted factors from probiotic bacterial growth are likely to be major contributors to the beneficial effects of probiotics, some active ingredients in probiotic culture supernatant have been identified, including conjugated linoleic acids (EWASCHUK et al., 2006), short-chain fatty acids (MEIMANDIPOUR et al., 2010), polyamines (MATSUMOTO et al., 2011), peptides (FUJIYA et al., 2007), proteins (YAN et al., 2007) and polyphosphate (SEGAWA et al., 2011). These active compounds have been demonstrated to be effective in the treatment of several intestinal disorders and liver disease

through stimulating Gram-positive bacterial growth, changing the intestinal pH, promoting immune function, and inhibiting intestinal barrier dysfunction.

As an alternative, heat-inactivated bacteria and probiotic-produced nonviable soluble proteins have been demonstrated to be effective in studies. *L. rhamnossus* GG culture supernatant was shown to protect intestinal epithelial cells from apoptosis and promote proliferation (CHEN et al., 2016), whereas another group showed that LGG-s attenuated alcohol induced a decrease in epithelial cell resistance and an increase in Caco-2 permeability (WANG et al., 2012). Moreover, Caco-2 cells exposed to a culture supernatant of *L. acidophilus* presented an increased expression of monocarboxylate transporter-1 (MCT1) on the apical surface of enterocytes (KUMAR et al., 2015).

The culture free supernatant (CFS) of *Bifidobacterium breve* CNCM I-4035 decreased the release of pro-inflammatory cytokines (IL-6 and IL-12p40) and chemokines (RANTES/CCL5 and MIP1 α/CCL3) in human intestinal dendritic cells (DCs) challenged with *Salmonella typhi*. Moreover, DCs interacting with the CFS, in absence of pathogenic bacteria, released low amounts of pro-inflammatory cytokines (IL-6 and IL-12p40) and chemokines (MDC and RANTES). In addition, CFS was a potent inducer of TLR9 expression than live *B. breve* CNCM I-4035 in the presence of *Salmonella typhi*. CFS and live *B. breve* CNCM I-4035 both induced strong and sustained TLR2 transcription. The live bacteria upregulated TLR4, whereas CFS upregulated TLR1 and TLR5 (BERMUDEZ-BRITO et al., 2013). The main finding of this study was that *Bifidobacterium breve* CNCM I-4035 and its supernatant could modify the release of cytokines by DCs in specific and differing manners. The CFS exhibited an anti-inflammatory behavior by decreasing proinflammatory cytokines and chemokines in DCs challenged with *Salmonella typhi* (BERMUDEZ-BRITO et al., 2015).

Treatment of *Clostridium difficile* supernatant with the *Bacillus clausii* supernatant reduced the cytotoxic effects, and neither cell detachment nor loss of mitochondrial activity was observed in Vero cell line. This protective effect was also achieved in Caco-2 cells (RIPERT et al., 2016).

Suo et al. (2012) have demonstrated that the culture supernatant of *Lactobacillus plantarum* ZJ316 (1×10^9 CFU/d) had a strong inhibitory effect on some pathogenic bacteria, such as *Salmonella, Escherichia coli* and *Listeria monocytogenes*. Diarrhea ratios were significantly lower, the height of the villi was greater and the density was thicker in *Lactobacillus plantarum* treated groups comparing with the antibiotic-treated group. Furthermore, the same work demonstrated that the pH and several meat texture evaluation

parameters, such as hardness, stickiness, chewiness, gumminess and restoring force were significantly improved by *Lactobacillus* compared with antibiotic-treated pigs. More interesting was the fact that the pig meat quality had some changes one week after stopping *Lactobacillus* treatment.

A recent study assessed the antibacterial spectrum of the culture supernatant from *L. plantarum* against multiresistant and virulent food-associated pathogens (Al-MADBOLY et al., 2015). In addition, it was reported that both liquid culture and supernatant of *L. casei, L. acidophilus, L. paracasei, L. rhamnosus* and *B. bifidum* exhibited high antifungal activity against *A. niger, A. flavus, A. parasiticus* and *Penicilium chrysogenum* (ABBASZADEH et al., 2015).

2.1.4 *Lactobacillus plantarum*

Within the genus *Lactobacillus, L. plantarum* is a member of the facultative heterofermentative group of lactobacilli. It is a heterogenous and versatile species that is encountered in a variety of environmental niches, including meat, fish, and many vegetable, plant fermentations, and in many cheese varieties. Moreover, some strains showed ability to survive the gastric transit and to colonize the intestinal tract of humans and other mammals (DE VRIES et al., 2006; MATHARA et al., 2008; GEORGIEVA et al., 2009; SUO et al., 2012).

L. plantarum was already used in starter cultures of yogurt and other products, showing resistance to extreme acid and bile salt conditions. These characteristics make it a potential candidate for use as a probiotic (HUANG et al., 2015). Oral administration of *L. plantarum* substantially restored the gut barrier, decreased enterocyte apoptosis, decreased intestinal oxidative stress, promote the activity and expression of protein kinase and particularly enhanced the expression and phosphorylation of TJ (tight junction) proteins in the experimental obstructive jaundice (ZHOU et al., 2012).

Furthermore, this probiotic has been found to inhibit epithelial barrier dysfunction and IL-8 secretion induced by TNF- α tumor necrosis factor-α and prevent cytokine-induced apoptosis in intestinal epithelial cells (YAN and POLK, 2002). Also, in an *in vitro* model *L. plantarum* have stabilized the cellular TJ, thereby preventing enteropathogenic *Escherichia coli*-induced redistribution of integral TJ proteins (QIN et al., 2009). Some strains of *L. plantarum* demonstrated also the ability to adhere to the HT-29 cells (LEE et al., 2011).

Two *L. plantarum* strains with antifungal compounds were isolated from kimchi (a korean spicy pickled). The isolates, *L. plantarum* AF1 (YANG and CHANG, 2010) and *L. plantarum* HD1 (SON et al., 2015) showed strong inhibitory activity against food and feed-borne filamentous fungi and yeasts. On the other hand, Son et al. (2015) demonstrated that acute (up to 5.0g/kg/d for 14 days) and subacute (up to 2.0 g/kg/d over a 4-week period) toxicity of crude antifungal compounds produced by *L. plantarum* HD1 is practically null via oral route in male and female SD rats. In another study in rodents, an acute and repeated oral administration of *L. plantarum* AF1 showed non-toxic effects of the crude antifungal compounds (LEE et al., 2013; SON et al., 2015).

Thirty *Lactobacillus* strains were isolated from animal manure. All the *Lactobacillus* strains were screened for their antifungal activity against *A. fumigatus, P. chrysogenum, P. roqueforti, B. elliptica* and *F. oxysporum*. All the strains showed significant antifungal activity, suggesting that this inhibition may be due to fermentative metabolites produced by the strains. The strains that had the ability to grow in low pH also produced different kind of extracellular enzymes such as ß-galactosidade, α-galactosidade, α-glucosidase, ß-glucosidase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-biphosphohydrolase, trypsin-like serine protease and other enzymes. All the strains showed significant hydrophobicity and aggregative property (ILAVENIL et al., 2015).

2.1.5 Mechanism of Mycotoxin Detoxification by Probiotics

Considering the toxic effects of mycotoxins on human and animal health, strategies involving the reduction or inactivation of these deleterious effects are of increasing interest. Probiotics were reported as a promising way of mycotoxin detoxification. Binding seems to be the main mechanism involved in the mycotoxins´ removal because: i) no differences were observed between incubation of viable and non-viable bacteria and ii) no toxin degradation products were observed on the HPLC chromatograms (NIDERKORN et al., 2006). Previous studies also indicated that binding at the bacterial cell wall is the mechanism of removal of mycotoxins by LAB (HASKARD et al., 2000; EL-NEZAMI et al., 2002).

Bueno et al. (2007) suggested the attachment of aflatoxin B_1 molecules to the surface of LAB takes two processes into consideration: binding (adsorption) and release (desorption) of aflatoxin to and from the binding site on the surface of the microorganisms. It was suggested that carbohydrates and/or protein components of LAB play a major role in aflatoxin B_1 binding since the effect of pronase E and periodate (periodate causes oxidation of

cis OH groups to aldehydes and carbon acid groups) on heat-killed, acid killed and viable LAB strains resulted in a considerable decrease in aflatoxin B_1 binding.

Concerning the mechanisms of action involved in the removal of fumonisins by LAB, Niderkorn et al. (2007) suggested that peptydoglycans were the most plausible fumonisin binding sites. The quenching ability of LAB was increased when bacteria were killed using different physical and chemical treatments.

For all the investigations conducted until current date, use of some strains of LAB could be beneficial for humans and animals constantly exposed to mycotoxins by reducing the absorption of these toxins and increasing the excretion of mycotoxin bound to bacterial cells from the body (GRATZ et al., 2004).

2.2 MYCOTOXINS

Mycotoxins are secondary toxic fungal metabolites that contaminate a wide range of food and feed (BINDER, 2007), and this contamination has become a serious worldwide problem. All mycotoxins are low-molecular-weight natural products (i.e. small molecules), that have no biochemical significance in fungal growth and development (DINIS et al., 2007).

The Food and Agricultural Organization (FAO) estimates that as much as 25% of the world´s animal feedstuff is contaminated by mycotoxins (FERRER et al., 2015). Several steps of the food production are susceptible by mold and mycotoxins. The contamination may occur before harvesting, between harvesting and drying, and during storage. An important fact is that these bioactive compounds are persistent in the final products alone or in co-occurrence with other toxic compounds (BULLERMAN and BIANCHINI, 2007; SANTINI et al., 2009).

That is why, mycotoxin-producing fungi can be classified into either field or storage fungi. Field fungi, such as the *Fusarium* species, produce mycotoxins on the crops in the field, whereas storage fungi, such as the *Aspergillus* and *Penicillium* species, produce mycotoxins on the crops after harvesting (FILTENBORG et al., 1996). *Fusarium* fungi have traditionally been associated with temperate climatic conditions, since they require somewhat lower temperature for growth and mycotoxins production compared, for example, to the *Aspergillus* species (PLACINTA et al., 1999).

The actual colonization and proliferation of fungi is not clear cut, but depends on the environmental and ecological circumstances, and the resulting toxins will differ

accordingly. Moisture and temperature have a major influence on mold growth and mycotoxin production. Pathogenic fungi that invade crops prior to harvest usually require higher moisture levels (200-250 g/kg) for infection than fungi that can proliferate during storage (130-180 g/kg) (BRYDEN, 2012).

Mycotoxins are challenging to classify. Due to their diverse chemical structures and byosinthetic origins, their myriad of biological effects, and their production by a wide number of different fungal species, a usual clinical classification is related to the affected organ. Thus, mycotoxins can be classified as hepatotoxins, nephrotoxins, neurotoxins and immunotoxins. Cell biologists put them into generic groups such as teratogens, mutagens, carcinogens, and allergens. Organic chemists have attempted to classified them by their chemical structure (e.g., lactones, coumarins); biochemists according to their byosinthetic origins (polyketides, amino acid-derived); physicians by the illnesses they cause (e.g., St. Anthony´s fire, stachybotryotoxicosis), and mycologists by the fungi that produce them (e.g., *Aspergillus* toxins, *Penicillium* toxins) (BENNETT and KLICH, 2003).

Exposure to molds and their toxins could take place mainly through contact, inhalation or ingestion (MAZUR and KIM, 2006). Among the large number of existing mycotoxins, i.e. more than 350 (MATTSSON, 2007), some have attracted special attention, justifying that their amount in food is regulated in relation to their high prevalence in commodities (cereals, fruits, beverages, coffee, animal products and flesh) and to their toxicity in human/animals. These mycotoxins include: i) aflatoxins (AFL), mainly AFB1, ii) ochratoxins, mainly ochratoxin A (OTA), iii) trichothecenes, such as T-2 toxin and deoxynivalenol (DON), iv) fumonisins, such as FB1, v) patulin (PAT) and vi) zearalenone (ZEA) (OSWEILER, 2000; PINEIRO, 2003; BRYDEN, 2007). Indeed, intoxication by these toxins leads to general cytotoxicity usually related to macromolecule synthesis inhibition at high doses of toxins (CALVERT et al., 2005; BOUAZIZ et al., 2006) but also, at lower doses, to subtle alterations of the functions of various tissues and organs such as the intestinal, hepatic, renal epithelium, nervous, reproductive and immune systems (BONDY and PESTKA, 2000; CAMPBELL et al., 2004; FUNG and CLARK, 2004; BOUHET and OSWALD, 2005; MALIR et al., 2016) resulting in altered host pathogen interactions and thus a different outcome of infection (ANTONISSEN et al., 2014). Furthermore, ingestion of some of these mycotoxins has been linked to carcinogenic (OSTRY et al., 2016) and teratogenic effects (BRYDEN, 2007) and as potential players on the development of inflammatory bowel disease (MARESCA and FANTINI, 2010; MARESCA, 2013).

These toxins account for millions of dollars annually in losses worldwide in human health, animal health, and condemned agricultural products. The economic impact of mycotoxins on animal production is generally considered to be mainly due to losses related to direct effects on animal health and trade losses related to grain rejection (WU, 2007). It is clear, however, that the indirect influence of mycotoxins on animal health, by enhancing infectious diseases, should also be taken into account.

Human exposure to mycotoxins may result from consumption of plant-derived foods that are contaminated with toxins, they carry-over of mycotoxins and their metabolites in animal products such as meat and eggs (SUDAKIN, 2003) or exposure to air and dust containing toxins (JARVIS, 2002).

Interestingly, the presence of mycotoxin-producing fungi in a plant is not always conducive to contamination with mycotoxins. In order for fungi to produce these secondary metabolites, they have to be stressed by some factor, such as nutritional imbalance, drought or water excess (DUTTON, 2009). A considering fact is that some of these toxigenic molds are known to produce one or more of these toxic secondary metabolites. It is well established that not all molds are toxigenic and not all secondary metabolites from molds are toxic.

2.2.1 Factors Affecting Production, Contamination of Foods and Feeds, and Toxicity of Mycotoxins

A main difficulty in assessing the risk of mycotoxins to human and animal health is the multiplicity of factors affecting the production or presence of mycotoxins in foods or feeds. Upon development of accurate and sensitive techniques for qualitative and quantitative analysis of mycotoxins, researchers have found that various factors operate interdependently to affect fungal colonization and/or production of the mycotoxins. Some authors categorized the factors as physical, chemical and biological (D'MELLO and MACDONALD, 1997).

Physical factors include the environmental conditions conducive to fungal colonization and mycotoxin production. Stresses such as a drought, an increase in temperature, an increase in relative humidity may selectively alter colonization and metabolism of mycotoxigenic fungi and thus alter mycotoxin production (RUSSELL et al., 1991). Chemical factors include the use of fungicides and/or fertilizers. The biological factors are based on the interactions between the colonizing toxigenic fungal species and substrate. The biological factors have been further sub-categorized (MOSS, 1991) into intrinsic factors

including fungal species, strain specificity, strain variation, and instability of toxigenic properties.

2.2.2 Co-occurrence of Mycotoxins

Some molds are capable of producing more than one mycotoxin and some mycotoxins are produced by more than one fungal species. Often more than one mycotoxin is found on a contaminated substrate. Mycotoxins occur more frequently in areas with a hot and humid climate, favorable for the growth of molds. They can also be found in temperate zones (ZAIN, 2011).

The effect of multi-mycotoxin contamination and of less well-known or emerging mycotoxins on the human or animal susceptibility to infectious diseases is rather unknown. Multi-mycotoxin contamination of feed is frequently occurring, raising the question on the impact on animal toxicity (PLACINTA et al., 1999). Several *in vitro* and *in vivo* studies demonstrated an enhanced toxicity and more severe immune suppression compared to single mycotoxin contamination (GRENIER et al., 2011; GRENIER and OSWALD, 2011; DE BOEVRE et al., 2012; WAN et al., 2013). In addition, plant metabolites of mycotoxins may also be present in feed and are known as masked mycotoxins (DE BOEVRE et al., 2012). *Fusarium* fungi and infected plants may produce conjugated forms of, for instance, DON, such as 3-AcDON (3-acetylDON), 15-AcDON and DON-3G (DON-3-glucoside). Furthermore, mycotoxins can also be conjugated by certain food-processing techniques. These conjugated forms could have a direct toxic effect, or may be hydrolyzed to their precursor mycotoxin in the digestive tract of animals, resulting in higher exposure levels (NAGL et al., 2012; DALL'ERTA et al., 2013; BROEKAERT et al., 2014). The influence of mycotoxin co-occurrence and masked mycotoxin on human and animal susceptibility to infectious diseases is already an important research question.

As just mentioned above, the simultaneous exposure of animals and poultry to more than one toxin is of concern (SPEIJERS and SPEIJERS, 2004). Synergistic effects may explain why animals sometimes respond negatively to mycotoxin levels much lower than those reported in scientific studies as able to cause mycotoxicosis.

When co-occurrence is evaluated in finished feed from different regions, differences are obvious, 10% of finished feeds in the Americas tested below the limit of detection for all analyzed mycotoxins; 50% tested positive for the presence of one mycotoxin, and in 40% of the samples, two or more mycotoxins were present. In Europe, 39% of the

finished feed samples analyzed tested positive for two or more mycotoxins, 37% tested positive for one mycotoxin and 24% tested negative (below the limit of detection) for all five mycotoxins. In Asia, multi-mycotoxin contamination seems to be more prevalent, as 82% of the finished feed samples tested positive for the presence of two or more mycotoxins and 12% showed to be contaminated with one mycotoxin. Only 6% of the tested samples were found to be below the limits of detection (RODRIGUES and NAEHRER 2012).

From January 2009 until December 2011, 23.781 mycotoxin analyses were performed in 7049 samples sourced in North and South America (the Americas). Samples were analyzed for some or all mycotoxins: Afla, ZEA, DON, FUM and OTA. Afla, ZEN, DON, FUM and OTA were respectively present in 33%, 45%, 59%, 64% and 28%. Positive samples respectively averaged contamination levels of 63, 233, 1104, 1965 and 11 ppb for these mycotoxins (RODRIGUES et al., 2011; RODRIGUES and NAEHRER, 2012). From the 7049 samples in the study, only 19% of them tested negative for the presence of the five analyzed mycotoxins, 33% showed the presence of them and two or more of the tested mycotoxins were present in 48% of the commodities.

2.2.3 Intestinal Toxicity of Mycotoxins

In the gastrointestinal tract, epithelial cells form a barrier that enables the absorption of dietary nutrients but at the same time provides a defense against intestinal pathogens, allergens, and toxins. These cells are undergoing constant renewal from epithelial stem cells in the crypt with support of the other epithelial and stromal cells in the niche. Progeny from the stem cell differentiate into the four specialized epithelial cell lineages, absorptive enterocytes, with metabolic digestive functions, mucus-secreting goblet cells, digestive-hormone secreting enteroendrocine cells and Paneth cells (absent in pigs) (CUNLIFFE, 2003; CUNLIFFE and MAHIDA, 2004).

The integrity of the intestinal barrier is maintained by intercellular junction complexes, which consist of tight junctions (TJs), adherens junctions, desmosomes, and gap junctions (SUZUKI, 2013). Tight junctions, the most apical complex, are responsible for regulating the paracellular transport of ions, solutes, and water and are composed of multiple proteins, such as claudins (FURUSE et al., 1998), occludin (SAITOU et al., 1997), and junctional adhesion molecules (JAMs) (MARTIN-PADURA et al., 1998). Cytosolic zonula occludens proteins interact with the cytosolic domain of TJ proteins, which form an anchor

between these transmembrane proteins and the cellular actin cytoskeleton (SCHNEEBERGER and LYNCH, 2004).

Maintaining the integrity of the intestinal barrier is essential for human and animal health, and indeed increased intestinal permeability exists in several disorders such as mycotoxicosis. Impairment of the barrier function may increase the risk for metabolic endotoxemia, condition where excess amounts of lipopolysaccharide (LPS), passes through the intestinal wall into blood circulation, resulting in an increased risk for systemic low-grade inflammation (CANI et al., 2008; MOREIRA et al., 2012).

The mammalian intestine encounters many more microorganisms than any other tissue in the body thus making it the largest and most complex component of the immune system (KOBOZIEV et al., 2014). This intestinal immune system has developed a plethora of mechanisms to protect the host from pathogenic infectious (KOBOZIEV et al., 2014) maintaining tolerance to commensal microbial and food-and-water antigens (O'KEEFFE et al., 2015) while limiting the inflammatory tissue damage that accompanies these innate and adaptive immune responses (KOBOZIEV et al., 2014). Taken together, in a healthy gut the synergistic co-existance of intestinal microbiota and the host is secured by an intact mucosal barrier.

Numerous mycotoxins are able to alter the intestinal barrier function. Aflatoxin B1 (AFB1) can compromise the barrier integrity of Caco-2 cells, a model of human enterocytes, with this effect being partially inhibited by the presence of probiotic bacteria (GRATZ et al., 2007). The mechanism involved could be related to the general cytotoxicity of aflatoxins (i.e. inhibition of macromolecules and ATP synthesis, oxidative stress, membrane leakage) (GUERRA et al., 2005; CALONI et al., 2006). DON is also able to increase the intestinal permeability of human enterocytes (KASUGA et al., 1998; SERGENT et al., 2006; PINTON et al., 2009).

Although cytotoxicity and protein synthesis inhibition could participate in the effect of DON on the intestinal barrier (KOUADIO et al., 2007), a specific alteration of the expression of claudins, a major component of the tight junctions, has been also importantly observed both on human enterocytes and on pig intestine (PINTON et al., 2009; BASSO et al., 2013). Treatment of human enterocytes with OTA also reduces the human intestinal barrier function (MARESCA et al., 2001; LAMBERT et al., 2007). Like DON, OTA specifically affects the expression of claudin isoforms and their membrane-association to lipid rafts (LAMBERT et al., 2007) although global cytotoxicity (mainly oxidative stress

and protein synthesis inhibition) could also participate in the effect of OTA on intestinal permeability (MARESCA et al., 2001; KOUADIO et al., 2007). PAT was also found to compromise the permeability of human enterocyte models due to its ability to inhibit protein tyrosine phosphatase, a key regulator of intestinal epithelial barrier function and to cause the proteolytic cleavage and relocalisation of tight junction proteins (claudins, occluding, and ZO-1) (MAHFOUD et al., 2002; McLAUGHLIN et al., 2009).

Although the effect of ZEA on intestinal barrier has not been evaluated, one could speculate, in the basis of its proapoptotic and cytotoxin effects on human enterocytes (CALVERT et al., 2005), that ZEA could also potentially induce intestinal barrier defects.

Intestinal mucus secreted by goblet cells covers the gut epithelium, protecting the epithelium against adhesion/invasion by pathogens and against physical/chemical assaults (MONTAGNE et al., 2004). Mycotoxin effects on mucus production is variable: co-exposure of low doses of DON, T-2, and ZEN reduces the number of goblet cells in pigs (OBREMSKI et al., 2008), but ZEN given alone at higher doses increases the activity of goblet cells (OBREMSKI et al., 2005). Similarly, high dose of FB1 was found to cause goblet cells hyperplasia in chickens (BROWN et al., 1992).

2.3 *FUSARIUM* MYCOTOXINS

Fusarium mycotoxins are capable of inducing both acute and chronic toxic effects. These effects are dependent on the mycotoxin type, the level and duration of exposure, the animal species that is exposed and the age of the animal (D'MELLO et al., 1999). Intake of high doses of mycotoxins may lead to acute mycotoxicosis, which are characterized by well-described clinical signs. For example, exposure to pigs to high concentrations of DON causes abdominal distress, malaise, diarrhea, emesis and even shock or death. Exposure of pigs to fumonisins can lead to pulmonary edema due to cardiac insufficiency. In horses, fumonisins target the brain and can cause equine leukoencephalomalacia (ELEM).

Following oral intake of low to moderate amounts of these mycotoxins, the gastro-intestinal epithelial cell layer will be exposed first (BOUHET and OSWALD, 2005). As described, the intestinal mucosa acts as a barrier, preventing the entry of foreign antigens including food proteins, xenobiotics (such as drugs and toxins), commensal microbiota and pathogens into the underlying tissues (BOUHET and OSWALD, 2005; OSWALD, 2006).

The mucosal immunity, which consists of an innate and adaptive immune system, can be affected by *Fusarium* mycotoxins.

By measuring the transepithelial electrical resistance (TEER), several *in vitro* and *ex vivo* studies indicate that DON and FB1 are able to increase the permeability of the intestinal epithelial layer of human, porcine and avian origin (SERGENT et al., 2006; SCHENK and MUELLER, 2008; PINTON et al., 2009). Also the viability and proliferation of animal and human intestinal epithelial cells can be negatively affected by *Fusarium* mycotoxins (BOUHET et al., 2004; YUNUS et al., 2012; MARESCA, 2013). Several mycotoxins are also able to modulate the production of cytokines *in vitro* and *in vivo* (BONDY and PESTKA, 2000; BOUHET and OSWALD, 2005).

Fusarium mycotoxins can cross the intestinal epithelium and reach the systemic compartment (MARESCA, 2013; OSSELAERE et al., 2013), affecting the immune system. Exposure to these toxins can either result in immunostimulatory or immunosuppressive effects depending on the age of the host and exposure dose and duration (CORRIER, 1991; MARESCA, 2013). Mycotoxin-induced immunomodulation may affect innate and adaptive immunity by impaired function of macrophages and neutrophils, a decreased T-and B-lymphocyte activity and antibody production (CORRIER, 1991; BONDY and PESTKA, 2000; OSWALD et al., 2005). In addition to the effect of *Fusarium* mycotoxins on the animal or human host, these mycotoxins may alter the metabolism of the pathogen, which may alter the outcome of the infectious disease (VANDENBROUCKE et al., 2011; VERBRUGGHE et al., 2012).

In recent years, research investigating the effects of *Fusarium* mycotoxins on the intestinal and immune functions has made substantial progress. However, only limited information is available on the interaction between mycotoxins and infectious diseases. Depending on host, pathogen and mycotoxin characteristics, exposure to *Fusarium* mycotoxins can generally exacerbate infectious diseases. On the other hand, T-2 has been shown to decrease the colonization capacity of *Salmonella* in the pig intestine. *Fusarium* mycotoxins may influence the host-pathogen interaction by negatively affecting the intestinal barrier function and the innate and adaptive immune response (BONDY and PESTKA, 2000; BOUHET and OSWALD, 2005).

Fusarium mycotoxins have also various acute and chronic effects on human health (ZAIN, 2011). It is important to highlight that conditions such as food-associated mycotoxin exposure is even higher in developing countries (WAGACHA and MUTHOMI, 2008). Besides the risk for acute mycotoxicosis in developing countries (WILD and GONG,

2010), results obtained in animals suggest that low to moderate concentrations of these mycotoxins could also influence human susceptibility to infectious diseases.

Global warming and increasing world population of humans are further important issues. Climate changes may affect the global distribution of mycotoxigenic fungi and their mycotoxins (PATERSON and LIMA, 2010), but also the distribution of infectious diseases will be important future topics to produce enough safe food for the entire human population.

2.3.1 Trichothecenes

The trichothecenes mycotoxins (TCT) comprise a group of over 180 sesquiterpenoids with the same basic molecular structure that are produced by *Fusarium*, *Stachybotrys*, *Myrothecium*, and other fungal genera (GROVE, 1993; 1996). However, most of them have been isolated from *Fusarium* spp.

All trichothecenes contain an epoxide at the $C_{12\text{-}13}$ positions, which is responsible for their toxicological activity. At the cellular level, the main toxic effect of TCT mycotoxins appears to be a primary inhibition of protein synthesis (Figure 1). TCT affect actively dividing cells such as those lining the gastrointestinal tract, the skin, lymphoid and erythroid cells (ZAIN, 2011).

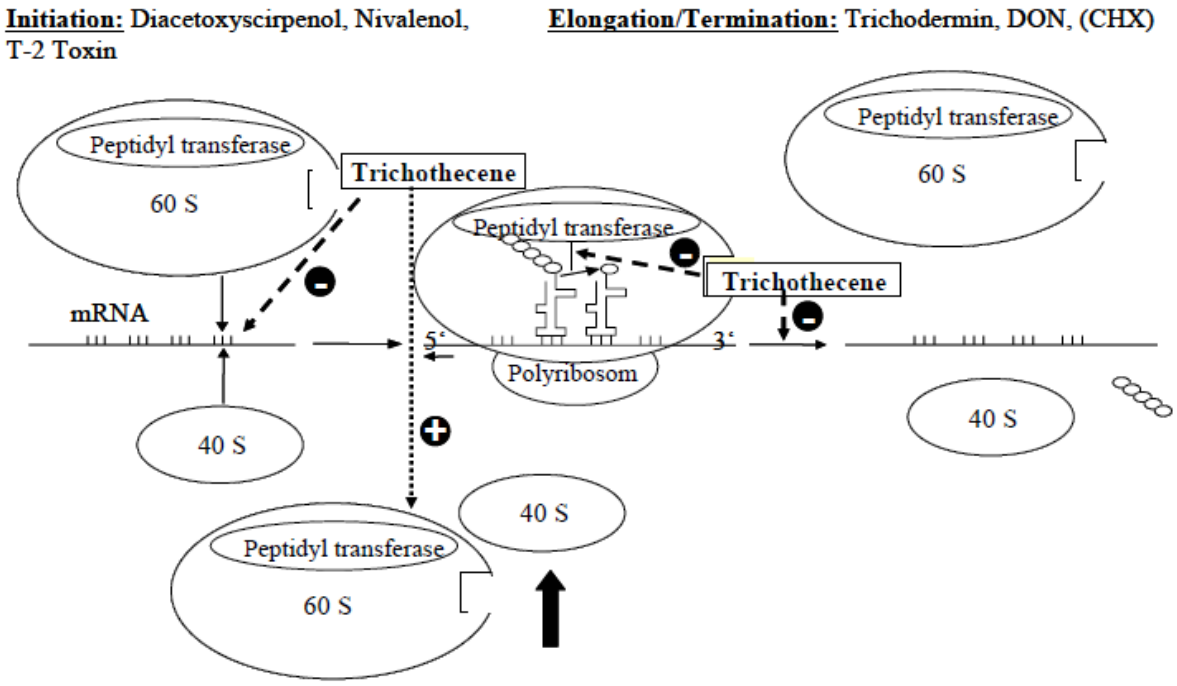

Figure 1. Mechanism of protein synthesis inhibition by trichothecenes according to Danicke et al. (2000) modified. Inhibitors of initiation (I-Type) will accumulate free ribosomes (40S + 60S) as these are not able to bind to mRNA (initiation complex). Elongation and termination inhibitors (E-Type) will increase the amount of polyribosomes (80S) as the uncoupling from mRNA and release of peptide chain is inhibited, inhibitory ⊖ or ⊕ activating effects. Source: Goyarts, 2006.

These mycotoxins occur worldwide in grains and other commodities. Toxin production is greatest with high humidity and temperatures ranging from 6-24°C. Natural occurrence of TCT has been reported in Asia, Africa, South America, Europe, and North America (RICHARD, 2007), contaminating corn, wheat, barley, oats, rice, rye, vegetables, and other crops (WASKIEWICZ et al., 2013; DONG et al., 2015). They are common contaminants of feeds and feedstuffs.

The trichothecenes family is divided into four groups (A through D) (Figure 2) based on modifications to the parent trichothecenes ring system (BONDY and PESTKA, 2000). Examples of type A TCT include T-2 toxin (T-2) and HT-2 toxin (HT-2 Diacetoxyscirpenol (DAS), fusarenone-X (FUX), deoxynivalenol (DON), and nivalenol (NIV) are some of the common naturally occurring type B TCT, and are among the most common trichothecenes detected in cereal grains (ROTTER et al., 1996). Types A and B

trichothecene are distinguished by the presence or absence of a carbonyl group at the C8 position, respectively (SCHWARZER, 2009).

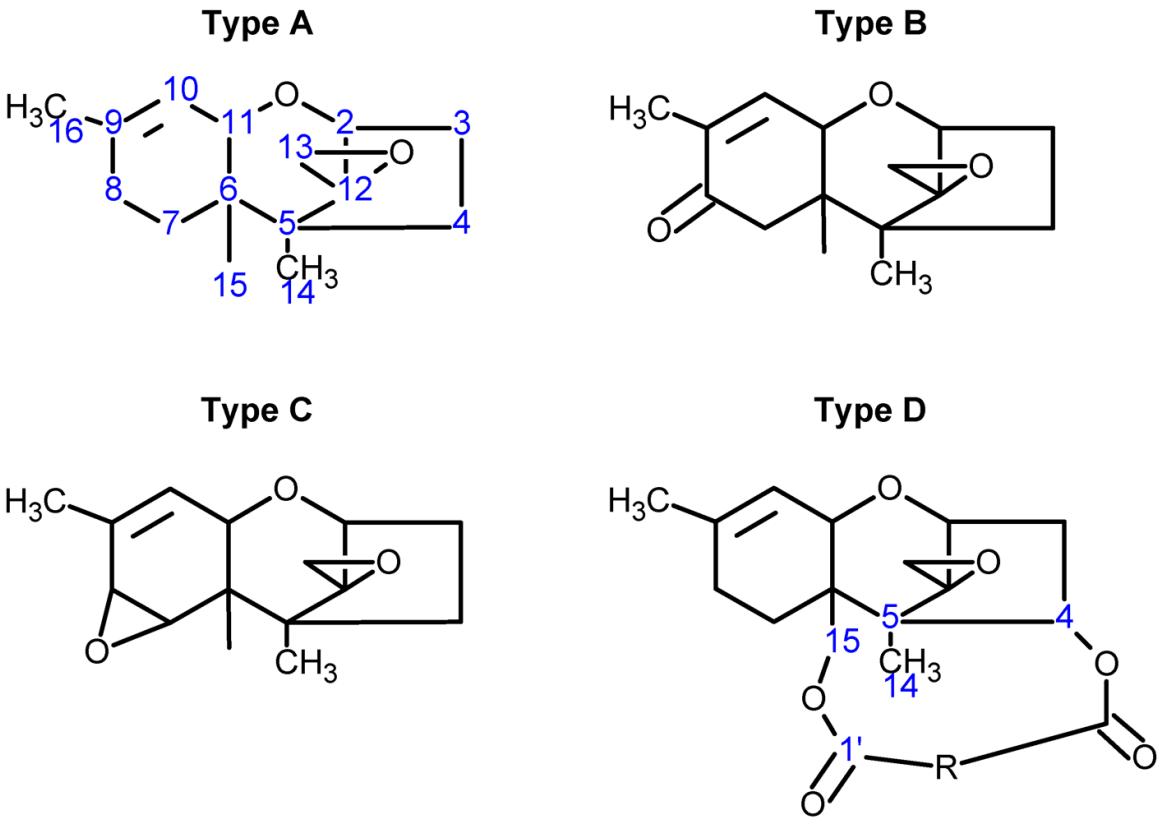

Figure 2. General chemical structure for type A, B, C and D trichothecenes. Source: Shank et al., 2011.

These compounds have been associated with human and animal toxicoses that are sometimes fatal. Experimentally, acute oral exposure to trichothecenes mycotoxins causes severe damage to actively dividing cells in bone marrow, lymph nodes, spleen, thymus, and intestinal mucosa. Thus, leukocytes and the immune system are a primary target for trichothecenes (BONDY and PESTKA, 2000).

Depending on dose and exposure regimen, trichothecenes can be both immunosuppressive and immunostimulatory. Repetitive exposure to trichothecenes increases susceptibility to a diverse array of pathogens that include *Mycobacterium* (KANAI and KONDO, 1984), *Candida* (SALAZAR et al., 1980), *Cryptococcus* (FROMENTIN et al., 1981), *Listeria* (CORRIER et al., 1987), *Salmonella* (TAI and PESTKA, 1990; SUGITA-KONISHI et al., 1998), *Aspergillus* (NIYO et al., 1988), and herpes simplex virus type I (FRIEND et al., 1983).

Also, depending on dose, trichothecenes exposure *in vitro* impairs or enhances mitogen-induced lymphocyte proliferation (ATKINSON and MILLER, 1984; COORAY, 1984; BONDY et al., 1991). The rank order of potency among trichothecenes classes for *in vitro* inhibition of rodent and human lymphocyte proliferation assays is macrocyclicic (for example, roridins and verrucarins) > type A group (for example, T-2 toxin and diacetoxyscirpenol) > type B group (for example, DON and nivalenol) (PESTKA and FORSELL, 1988).

In vitro lymphotoxicity of the type A and type B trichothecenes apparently depends on the degree of acylation in substituent groups. One of the most dramatic effects of DON is a pronounced elevation in serum immunoglobulin A (IgA) and concurrent depression in IgM and IgG that is found in mice orally exposed to DON in feed (PESTKA and FORSELL, 1988). The threshold for this inductive effect is 2 mg/kg, with maximal effects occurring in the 10-25 mg/kg range (PESTKA et al., 1989; DONG et al., 1991). Increased serum IgA appears concomitantly with elevated IgA immune complexes and polymeric IgA. Peyer´s patch lymphocytes and to a lesser extent splenic lymphocytes isolated from mice fed DON produce significantly more IgA than those cultures derived from mice receiving *ad libitum* or restricted control diets (BONDY and PESTKA, 1991). These results suggest that DON enhances differentiation to IgA-secreting cells at the Peyer´s patch level and that this impacts the systemic compartment. Dietary DON can also drive IgE elevation in mice (PESTKA and DONG, 1994).

2.3.1.1 Deoxynivalenol (DON)

Deoxynivalenol (DON) is a secondary fungal metabolite of the trichothecene family mainly produced by *Fusarium graminearum* and *Fusarium culmorum* that affects human and animal health (PIERRON et al., 2016) due to its co-occurrence in cereal-based food and feed (SMITH et al., 2016).

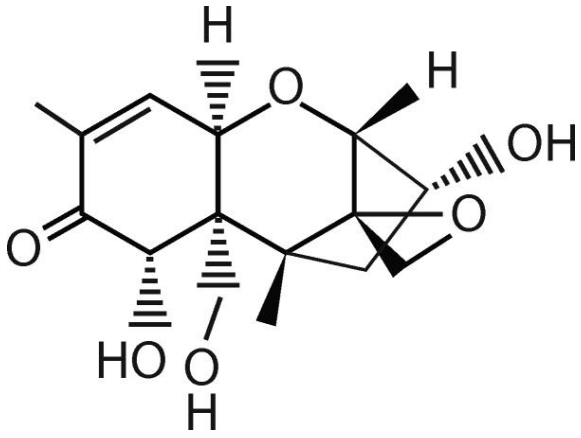

Figure 3. Chemical structure of Deoxynivalenol (DON). Source: Sobrova et al., 2010.

Its worldwide surveillance confirms its occurrence in 55% of cereal crops (STREIT et al., 2013) in occasions, ranging from 80-100 % in some countries (BRYLA et al., 2016; TIMA et al., 2016), occurring predominantly in grains such as wheat, barley, oats, rye and maize, and less often in rice, sorghum and triticale; representing the most prevalent mycotoxin in cereals (PLACINTA et al., 1999). DON is the most frequently occurring type B tricothecene in Europe (SAINT-CYR et al., 2015). A large-scale data survey indicated that DON is present in 43.5% of food and 75.2% of feed samples collected in the European Union (EFSA, 2013). In Brazil, toxic analysis in a recent work showed 99% contamination with deoxynivalenol of freshly harvested wheat grains collected in three different states of Brazil (São Paulo, Parana and Rio Grande do Sul) (TRALAMAZZA et al., 2016).

Considering that, wheat is the most important human grain and ranks second in total production as a cereal crop behind maize. This mycotoxin represents a very serious health risk. Deterioration of food/feeds stuffs due to fungal colonization and concomitant production of mycotoxins is a serious problem, especially in the wake of fungi acquiring resistance to many commonly used chemical preservatives (DEEPTHI et al., 2016).

For this, the European Commission and United States Department of Agriculture (EC 1881/2006 Regulation) released a guidance value for food ranging from 200 to 1750 μg/kg and a concentration of 9.9 mg/kg of the daily ration for animals. The joint WHO/FAO Expert Committee on Food Additives set a provisional maximum tolerable daily intake (PMTD) of DON at 1μg/kg of body weight.

The ingestion of DON-contaminated grains by animals can cause chronic effects such as immune dysregulation, anorexia and symptoms such as emesis and diarrhea (ROCHA et al., 2005; PESTKA, 2010). At the cellular level, even though DON poses no

genotoxic or mutagenic risk (LE HÉGARAT et al., 2014; TAKAKURA et al., 2014), it inhibits protein synthesis (at the elongations-termination step of protein translation) through interaction with the peptidyl transferase at the 60S ribosomal subunit (ROCHA et al., 2005). The binding of DON to the ribosome rapidly activates MAPK signaling pathways and induces caspase-mediated apoptosis in a process known as the "ribotoxic stress response" (ROCHA et al., 2005; PESTKA, 2010) and, furthermore selected genes were reported to represent pathways of the cellular metabolism *in-vitro* (DIESING et al., 2012).

2.3.1.1.1 Absorption kinetic and intestinal toxicity

DON seems to be rapidly and efficiently absorbed in gastrointestinal tract, whatever the animal species. In pigs, it can be detected in blood 30 min after their ingestion. Absorbed rates varies and studies between 10% and 50% (ERIKSEN and PETTERSSON, 2004). Maximal concentration detected in plasma never exceeds 25% of the oral dose; its plasmatic half-life is 4 hours (ERIKSEN et al., 2003). In pig, the kinetics of DON appearance in small intestine and in plasma show similar profiles, suggesting that molecule absorption is linked to DON passage through the small intestine (DANICKE et al., 2004). The greater part of the compound would be absorbed at the level of the first intestinal segment. The use of a model simulating pig gastrointestinal tract confirms the absorption at the small intestinal level, and specifies a jejunum preponderance (AVANTAGGIATO et al., 2004).

Species differences in susceptibility towards DON arise from species-related differences in metabolism (ROTTER et al., 1996). DON was rapidly and nearly completely absorbed in the upper gastrointestinal tract in swine, and maximum serum concentrations appeared 4h after intake. In cattle, ruminal microbes convert DON almost completely to the non-toxic metabolite deepoxy-DON (DOM-1). In swine, however, the de-epoxidation pathway does not significantly contribute to the detoxification of DON (DANICKE et al., 2004; PESTKA, 2007).

This mycotoxin is of major concern in pig feeding because of its frequent occurrence in feedstuffs at concentrations high enough to cause adverse effects on the health and performance of this sensitive species (DANICKE et al., 2010). In pigs, consumption of DON-contaminated feed impacts the gastrointestinal tract, affecting intestinal barrier function by modulating the expression of tight junctions proteins (PINTON et al., 2009; PINTON et al., 2010; BASSO et al., 2013; LUCIOLI et al., 2013), altering enterocyte proliferative and apoptosis index (WANG et al., 2014; CHEAT et al., 2016), decreasing the number of goblet

cells (GEREZ et al., 2015) and also leading to intestinal inflammatory response *in-vitro* (VAN DE WALLE et al., 2010) and *in-vivo* (GAUTHIER et al., 2013). Moreover, authors have investigated the interaction between some intestinal microflora strains and this mycotoxin (KOLLARCZIK et al., 1994; PIERRON et al., 2016).

In vivo and *ex vivo* models (KOLF-CLAW et al., 2009) showed the main intestinal histological changes observed in piglets fed with deoxynivalenol were multifocal atrophy and villi fusion, apical necrosis of villi, cytoplasmatic vacuolation of enterocytes and oedema of lamina propria, cuboid or flattened enterocytes, decreased goblet cells (BRACARENSE et al., 2012; LUCIOLI et al., 2013) and lymphatic vessel dilation (BRACARENSE et al., 2012).

Enterocytes apoptosis and total-cell proliferation at the villus tips were affected by DON *in vivo* and *ex vivo* models (CHEAT et al., 2015). Lucioli et al. (2013) demonstrated the ability of DON to induce significant increase in MAPK phosphorylation of p38 and ERK in piglets jejunal explants exposed for 4 h to 10μM of DON and jejunal samples of piglets fed 2.3 mg of DON/kg for 35 days. Meanwhile, Przybylska-Gornowicz (2015) demonstrated increased number of lymphocytes in the villus epithelium and the lamina propria of pigs treated with DON for six weeks; and increased plasma cells were observed in the lamina propria after one, three and six weeks of exposure.

DON decreases TEER as well as increases paracellular permeability to 4-kDa dextran and to pathogenic *E. coli* in porcine (IPEC-1) and human (Caco-2) intestinal epithelial cell lines (PINTON, 2009). Increased permeability was similarly observed in piglets jejunal explants treated with DON and is especially associated with the reduction in the expression of E-cadherins (BASSO, GOMES, BRACARENSE, 2012).

Taken together, DON alters intestinal cell function, inhibiting intestinal nutrient absorption, comprising the intestinal permeability and immune function.

Therefore, and as already mentioned, the European Commission has recommended a guidance value for critical DON concentrations for feed materials and complete feedstuffs for farm animals. The recommended guidance value for critical concentrations of DON in complete daily diets is 0,9 mg/kg (88% DM) for swine, which should not exceeded to avoid deleterious effects (SCHULZ et al., 2015).

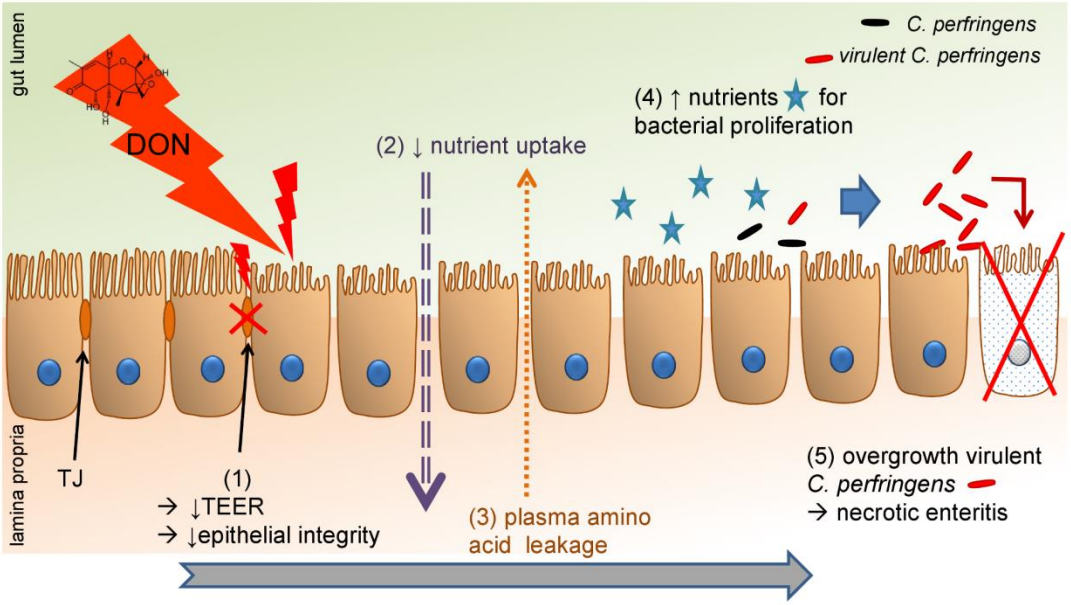

Figure 4. The impact of Deoxynivalenol (DON) on pig´s intestine. DON decreased transepithelial resistance (1), leading to a decreased absorption and digestion of dietary nutrients; and an increased intestinal barrier permeability, respectively. Taken together with an increased intestinal protein level, these results suggest an impaired nutrient uptake (2) and leakage of plasma amino acids (3) into the lumen, providing the necessary growth substrate for *C. perfringens* proliferation (4) inducing necrotic enteritis (5). Source: Antonissen et al., 2014.

2.3.1.1.2 Deoxynivalenol biotransformation

The current methods cannot completely remove DON from feed and grain, resulting in great economic losses for the feed industry, food safety, and animal husbandry (WANG et al., 2016). Physical and chemical methods have been developed to control the occurrence of fungi and their toxins. For example, reduction of DON in animal feed by treatment with sodium bisulfite and sodium metabisulfite has been successfully demonstrated.

There are three different DON sulfonates. DON sulfonate 1 characterized is by loss of the epoxide group. DON sulfonate 2 has a hemiketal formation. DON sulfonate 3 is an equilibrating mixture of two isomers, a ketone and a hemiketal (SCHWARTZ et al., 2013).

The acetylated derivative 3-acetyl-deoxynivalenol (3ADON) has been shown to be equivalent to or lower than DON in toxicity based on 50% lethal dose (LD_{50}). This work showed that *Fusarium graminearum* contains a gene called TRI101 that encodes a trichothecene 3-O-acetyltransferase (KHATIBI et al., 2011). Pinton et al. (2012) proved that 3ADON did not alter the TEER in IPEC-1 cells, resulting therefore less toxic than DON.

Fruhmann et al. (2014) had synthesized, isolated, and characterized all possible DON-sulfates including its acetylated derivatives, which occur as 3-

acetyldeoxynivalenol (3ADON), 15-acetyldeoxynivalenol (15ADON), and 3,15-diacetyldeoxynivalenol (3,15-diADON), this considering the proven unreactivity of the C_7 position to chemical sulfation.

Current publications showed the ability of some probiotics to degrade mycotoxins such as zearalenone (KHALIL et al., 2015), aflatoxina B1 (MONSON et al., 2015), trichothecenes (FRANCO et al., 2011), ochratoxin A (SLIZEWSKA and PIOTROWSKA, 2014), and fumonisins (KHALIL et al., 2015; GRENIER et al., 2013).

An example of this were twenty-nine strains of lactic acid bacteria (LAB) and propionic acid bacteria (PAB) tested for their ability to removed deoxynivalenol (DON) and fumonisins B_1 and B_2 (FB_1 and FB_2) from an acid, pH 4, medium (NIDERKORN et al., 2006). Furthermore, viable cells and cells that were heat inactivated by pasteurization or sterilization reduced DON in liquid medium as was determined by high performance liquid chromatography (HPLC) (FRANCO et al., 2011). Because of their fermentative role, lactic acid bacteria (LAB) and propionic acid bacteria (PAB) could be particularly well adapted for the detoxification of silage (NIDERKORN et al., 2006).

DON microbial transformation in digestive tract is well documented. Few traces of de-epoxy-DON are found in pig stomach and small intestine after ingestion of 4.2 mg/kg feed during 7 days, whereas its quantity increase in large intestine to reach 80% of trichothecenes present in rectum (DANICKE et al., 2004). *In vitro*, DON incubation with caecum, colon or rectum microbiota leads to an important DON biotransformation on contrary to incubation with jejunum or duodenum microbiota (KOLLARCZIK et al., 1994). So DON de-epoxydation takes places essentially in large intestine and would little participate in detoxification, notably in pig. More than 90% of 3-acDON ingested by pigs is de-epoxyded in the large intestine (ERIKSEN et al., 2003).

2.4 OXIDATIVE STRESS AS A CONSEQUENCE OF MYCOTOXIN EXPOSURE

Reactive oxygen species (ROS) are normal products of the cellular metabolism. ROS have beneficial effects on several physiological processes including killing of invading pathogens, wound healing, and tissue repair processes. However, disproportionate ROS generation poses a serious problem to body homeostasis and causes oxidative tissue damage. The gastrointestinal tract (GIT) is a key source of ROS. Despite the protective barrier provided by the epithelial layer, ingested materials and pathogens can cause inflammation by activating the epithelium, neutrophils and macrophages to produce inflammatory cytokines

and other mediators that contribute further to oxidative stress (BHATTACHARYYA et al., 2014).

Recent results show that in many cases membrane-active properties of various mycotoxins determine their toxicity. Indeed, incorporation of mycotoxins into membrane structures causes various detrimental changes (GUILFORD and HOPE, 2014). These changes are associated with alteration of fatty acid composition of the membrane structures and with peroxidation of long chain polyunsaturated fatty acids (PUFAs) onside membranes. This ultimately damages membrane receptors, causing alterations in second messenger systems and inactivation of a range of membrane-binding enzymes responsible for regulation of important pathways. Finally, this causes alterations in membrane permeability, flexibility and other important characteristics determining membrane function. Detrimental effects of mycotoxins on DNA, RNA and protein synthesis together with pro-apoptotic action further compromise important metabolic pathways (Figure 5). Consequently, changes in physiological functions including growth, development and reproduction occur. An importance of oxidative stress and lipid peroxidation in all these processes is confirmed by protective effects of natural antioxidants against mycotoxin toxicity. However, protective effects of antioxidants including selenium (WANG et al., 2016) are of limited value and a combination of mycotoxin binders with natural antioxidants could be the next step in preventing damaging effects of mycotoxins in animal and poultry production (SURAI et al., 2002).

It has been previously demonstrated that DON can induce oxidative stress in various *in vitro* and *in vivo* studies (PESTKA, 2007; WU et al., 2014). Modes of DON action are still to be elucidated but impaired antioxidant status of cell and increased production of ROS induced by DON may induce cell damage leading to apoptosis as observed previously in *in vitro* studies with different types of cells including T cells, B cells and intestinal epithelial cells (PESTKA, 2007; KRISHNASWAMY et al., 2010). Lessard et al. (2015) showed that genes related to oxidative stress were modulated in pigs fed DON diet.

A delicate balance between antioxidants and pro-oxidants in the body in general and specifically in the cell is responsible for regulation of various metabolic pathways leading to maintenance of immunocompetence, growth and development and protection against stress conditions associated with commercial animal/poultry production. This balance can be regulated, as mentioned, by dietary antioxidants, including vitamin E, carotenoids and selenium (ATROSHI et al., 2002). On the other hand, nutritional stress factors have a negative impact on this antioxidant/pro-oxidant balance. In this respect mycotoxins are

considered to be among most important feed-born stress factors. It is not clear at present if mycotoxins stimulate lipid peroxidation directly by enhancing free radical production or the increased tissue susceptibility to lipid peroxidation is a result of compromised antioxidant system. It seems likely that both processes are involved in this stimulation. In most cases lipid peroxidation in tissues caused by mycotoxins was associated with decreased concentrations of natural antioxidants (SURAI et al., 2008).

It has been shown that, OTA, T-2 toxin, DON, aflatoxins, fumonisins and zearalenone impose an oxidative stress and have a stimulating effect on lipid peroxidation (CALVERT et al., 2005; REZAR et al., 2007). In most cases, thiobarbituric acid reactive substances (TBARS) accumulation was used as a measurement of lipid peroxidation. Furthermore, ethane exhalation, EPR registered free radicals, hydroxyl radical formation, single-strand cleavage DNA, DNA adduct formation as well as LDH release were also used including liver microsomes, phospholipid vesicles, primary cell cultures, whole organs and whole body. TBARS accumulation was substantially increased and at the same time vitamin E and GSH and activities of antioxidant enzymes significantly declined as a result of mycotoxicosis (SURAI et al., 2008).

Previous studies have shown that DON can triggers inhibition of protein, DNA, and RNA synthesis (ERIKSEN and PETTERSSON, 2004; KOUADIO et al., 2007). *Fusarium* mycotoxins have also been shown to inhibit macromolecular synthesis, malondialdehyde (MDA) levels, DNA methylations, and apoptosis *in vitro* (LI et al., 2014; REN et al., 2015). It is assumed that DON affects the synthesis of antioxidant enzymes *via* these factors.

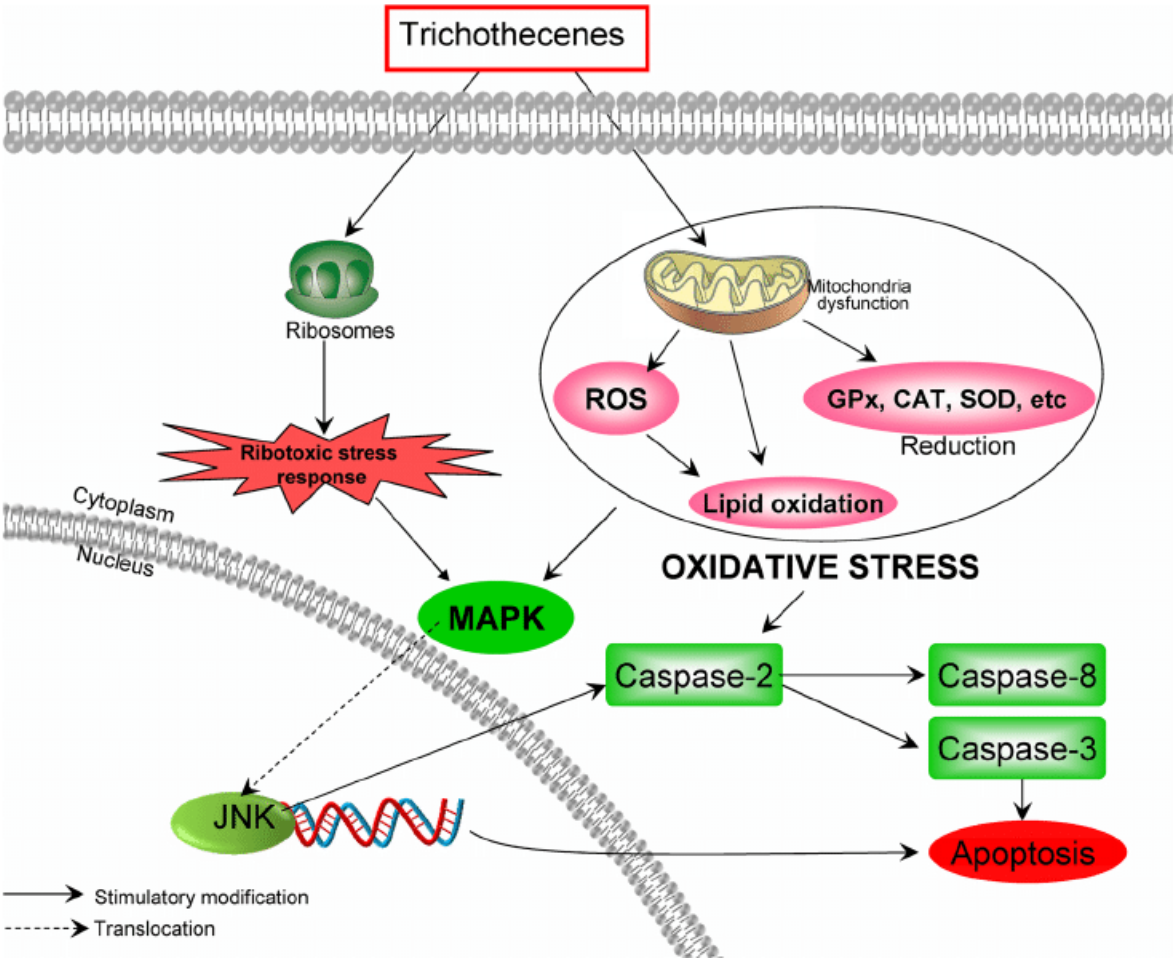

Figure 5 – Speculated mechanism of oxidative stress-mediated toxicity of trichothecene in animals. Source: Wu et al., 2014.

In conclusion, lactic acid bacteria present morphological and biochemical characteristics that have been used in improving intestinal health. In addition, the use of lactobacilli could be a valuable strategy of mycotoxin detoxification. Considering the levels of mycotoxin contamination of cereals worldwide, studies focusing on mycotoxin inactivation are of increasing interest.

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3 OBJECTIVES

3.1 GENERAL OBJECTIVE

- The purpose of this study was to investigate the potential modulation of heat-inactivated *Lactobacillus plantarum* (LP) and the culture supernatants (CS) in deoxynivalenol (DON) toxicity using an *ex vivo* model.

3.2 SPECIFICS OBJECTIVES

- Evaluate histological changes in jejunal explants of piglets exposed to two strains and concentrations of heat-inactivated *L. plantarum* and their culture supernatants alone and in combination with DON.

- Evaluate morphometrical aspects in jejunal explants of piglets exposed to two strains and concentrations of heat-inactivated *L. plantarum* and their culture supernatants alone and in combination with DON.

- Evaluate the goblet cells density in jejunal explants of piglets exposed to two strains and concentrations of heat-inactivated *L. plantarum* and their culture supernatants alone and in combination with DON.

- Evaluate morphological changes using scanning electron microscopy in jejunal explants of piglets exposed to two strains and concentrations of heat-inactivated *L. plantarum* and their culture supernatants alone and in combination with DON.

4 MANUSCRIPTS FOR SUBMISSION

4.1 Article 1. Histopathological and ultrastructural findings induced by heat-inactivated *Lactobacillus plantarum* and the culture supernatants on the intestinal mucosa of piglets: an *ex vivo* approach.

4.2 Article 2. *Lactobacillus plantarum* culture supernatants improve intestinal tissue exposed to deoxynivalenol.

4.3 Article 3. *Lactobacillus plantarum* culture supernatant induces protective effects on jejunal explants of piglets exposed to deoxynivalenol: histological and antioxidant activity aspects.

Article 1

Histopathological and ultrastructural findings induced by heat-inactivated *Lactobacillus plantarum* and the culture supernatants on the intestinal mucosa of piglets: an *ex vivo* approach

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Manuscript prepared accordingly to submission instructions of *Journal of Natural Products*

Histopathological and ultrastructural findings induced by heat-inactivated *Lactobacillus plantarum* and the culture supernatant on the intestinal mucosa of piglets: an *ex vivo* approach

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ABSTRACT

In the present study, histological, morphometrical and ultrastructural analysis were performed to investigated intestinal mucosa changes in piglets jejunal explants exposed to two concentration of heat-inactivated *Lactobacillus plantarum* and their respectively culture supernatants. Jejunal explants were incubated for 4 hours in DMEM culture medium with a) only culture medium (control group), b) heat-inactivated *Lactobacillus plantarum* strain1 – LP1 (1.1×10^8 CFU/ml), c) heat-inactivated *Lactobacillus plantarum* strain2 – LP2 (2.0×10^9 CFU/ml), d) heat-inactivated *Lactobacillus plantarum* strain1 culture supernatant (CS1), and e) heat-inactivated *Lactobacillus plantarum* strain2 culture supernatant (CS2). Explants exposed to heat-inactivated *L. plantarum* strain 1 and 2 showed multifocal to difuse villi atrophy, villi apical necrosis and enterocyte flattening. Morphological assessment revealed similar results with bacterial adhesion to mucus and intestinal epithelial cells and, morphometric analysis showed a decreased villi height compared to the control group. Alterations in explants treated with the culture supernatant of both strains had mild villi atrophy and mild enterocyte apical necrosis. Morphological assesment revelead numerous well delinead villi and, morphometric analysis showed a significant increase in villi height compared to the control group. In general, exposure to the culture supernatants improve the intestinal morphology.

Antimicrobials have been widely used in the diets of piglets to promote growth performance and reduce the incidence of diarrhea.[1,2] Nevertheless, the resistance of pathogens to antimicrobials and the possibility of their residues in animal products resulted in an increasing interest in the use of alternatives to in-feed antibiotics.[3,4] Additionally, as a result of cross-resistance, the European Union has completely banned the use of antimicrobials in animal feed to prevent diseases or promote growth.[5]

Previous studies with lactic acid bacteria (LAB) in pigs suggest that probiotics provide a potential alternative to antimicrobial strategies[6] and that certain strains of bacteria are effective in maintaining intestinal homeostasis. The effects of LAB include effective enhancement of the intestinal barrier function, modulation of the mucosal immune system, production of antimicrobials products, and alteration of the intestinal microbiota.[7]

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host".[8] However, there is evidence that probiotic preparations comprised of inactivated bacteria cells and their metabolites can also exert a biological response, similarly to that seen with live cells. Consequently, probiotics consisting of either live or dead cells or their metabolites may play an important role in health maintenance and disease avoidance in the host.[9]

Stimulation of the immune response has been associated with the oral administration of both live and heat-killed LAB.[10,11] This effect is reflected by an increased lymphocyte proliferation and expression of IFN-γ probably related to specific properties of the lactobacilli membrane.[12] Heat inactivated cells of *Enterococcus faecalis* stimulated the gastrointestinal immune system in chickens[13] and increased neutrophil phagocytosis in healthy dogs.[14] Furthermore, heat inactivated strains of *Lactobacillus* were able to modulate the immune response by stimulating the proliferation of murine splenocytes.[15] This probiotic paradox

comprises a concept where both live and inactivated bacterial cells are capable of generating a biological response.

Bacterial metabolites may also have a favourable impact on the intestinal epithelial barrier function. Active factors produced during the bacterial growth are likely to be major contributors to the beneficial effects of probiotics, and active components in probiotic culture supernatant have been identified, including conjugated linoleic acids[16], short-chain fatty acids[17], polyamines[18], peptides[19], proteins[20] and, polyphosphates.[21]

Most of the previous studies concerning the effects of LAB and their metabolites on intestinal homeostasis were performed in *in-vivo* and *in-vitro* models. No available data were found regarding the interactions between LAB and the *ex vivo* model. Considering the need to broaden knowledge about the results of potential properties of LAB, this study aimed to investigate the effects of the exposure of intestinal explants of pigs to heat inactivated *Lactobacillus plantarum* strains and their culture supernatant. To assay this, we performed histological, morphometrical and ultrastructural assessment using the jejunal explants culture technique. This investigation system allowed us to evaluate the intestinal tissue morphology, maintaining the complex patterns of differentiation seen *in vivo*.

RESULTS AND DISCUSSION

Effects of heat inactivated *L. plantarum* and the culture supernatants on morphological score

After four hours of incubation, untreated explants (control group) presented mild villi atrophy and edema of lamina propria (Figure 1A), whereas explants exposed to LP1 and LP2 showed multifocal to difuse villi atrophy, villi apical necrosis and enterocyte flattening (Figure 1B, Figure 1C). Histological changes were more severe in explants exposed to LP1 compared to LP2. The main alterations in explants treated with CS1 and CS2 were mild villi atrophy and mild enterocyte apical necrosis. In general, the histological aspect of the CS1 and CS2 groups were similar or even better than the control explants.

The mean morphological score was of 35±4.15, 27.75±4.62, 29.17±2.12, 34.78±3.69 and 38±1.19 for the control, LP1, LP2, CS1 and CS2, respectively. A significant decrease in the score was observed in explants exposed to both strains of heat inactivated lactobacilli (p= 0.01 and 0.05 for LP1 and LP2, respectively) when compared to control group. On the other hand, explants submitted to CS1 and CS2 remained statistically similar to the control group and showed a significant increase in the morphological score when compared to explants exposed to LP1 and LP2 (Figure 1F).

Effects of heat inactivated *L. plantarum* and the culture supernatants on villi height and crypt depth

The morphometric analysis showed a significant increase in villi height in explants treated with both *L. plantarum* culture supernatant ($p \leq 0.05$) compared to the control group and to both strains of the heat inactivated bacteria (Fig. 2). The mean villi height of the control group was 124.67±37.15 μm, while the group treated with CS1 and CS2 was 184.26±30.45 and 216.26±25.34 μm, respectively. The mean villi height of explants exposed to LP1 and LP2 was 114.37±21.89 and 108.13±24.54 μm, respectively.

Crypt depth mean in untreated explants was 137.27±5.32, whereas for LP1, LP2, CS1 and CS2 was 140.72±8.35, 138.27±4.60, 150.24±3.75 and 148.35±4.79 μm, respectively. A significant increase in crypt depth was observed in explants submitted CS1 when compared to the control group (Figure 2).

Effects of heat inactivated *L. plantarum* and the culture supernatants on morphological assessment

The scanning electron micrographs of the apical membranes of control jejunal explants showed numerous finger shaped, well delineated villi with goblet cells at surface (Figure 3A-3B), heat inactivated LP1 and LP2 showed mild atrophy (Figure 3C) and bacterial adherence properties to the villi (Figure 3D). CSs groups showed well defined villi in intestinal surface (Figure 3E) and mucin covering villi surface (Figure 3F).

LAB have been suggested to be an alternative strategy to antibiotic growth promoters and, many species of these bacteria are promising natural alternatives to chemical preservatives in food and feed.[22] Nevertheless, additional research still to be performed on the effects of LAB on intestinal morphology, since few studies have focused on this aspect. Most of the available data have reported the effects of LAB on performance parameters or in the interaction of intestinal pathogens.[6] In addition, it remains to be investigated if cell-free supernatants of LAB are toxic to animals and humans. In the present study, we have used two strains of *L. plantarum* heat inactivated and their culture supernatant on piglets jejunal explants in order to determine if they have an effect on jejunal morphology. We have observed that the exposure of jejunal explants to heat inactivated *L. plantarum* strains induced moderate toxic effects, whereas the exposure to their culture supernatants improved the intestinal morphology. Nevertheless, a morphological improvement trent was achieved in explants treated with LP2 when comparing to LP1 treated explants. Since LP2 was a wild strain this difference could be implicated to the ecological niche and environmental condition of the bacteria.

The toxic effects were verified by a significant decrease in the morphological score (20% for LP1 and 17% for LP2) represented by changes such as villi atrophy, enterocyte flattening and necrosis compared to the control explants. Interestingly, the mean villi height and crypt depth of these groups remained similar to untreated explants. Both strains of heat inactivated *L. plantarum* retained the ability to adhere to enterocytes as demonstrated by scanning electron microscopy analysis. Enterocyte adherence and competition binding for mannosylated receptors are described as strategies of *L. plantarum* to reduce the number of pathogenic bacteria in the intestine. [23] In the present study bacteria adherence showed no positive effect, since villi atrophy is clearly evident by the SEM assay. By contrast, the majority of the *in vivo* or *in vitro* studies report beneficial effects following the ingestion or

exposure to different strains of *L. plantarum*. These effects include intestinal immune stimulation (modulating cytokines expression)[24], reduction in oxidative stress and elimination of pathogens.[25] To the best of authors' knowledge it is the first study using an *ex vivo* model that have focused on the histological aspects of healthy intestinal samples exposed to *L. plantarum*.

In this study, the histological aspects of the explants untreated and exposed to the culture supernatants were similar. These mild histological changes are expected to occur in the *ex vivo* model as a consequence of a relative status of hypoxia.[26] However, a significant increase in villi height was observed in explants exposed to both culture supernatants (1.5-fold for strain 1 and 1.7 for strain 2) when compared to the control group. Also, a significant increase (1.1-fold) in crypt depth was verified in explants treated with strain 2 supernatant. When compared to explants exposed to heat inactivated *L. plantarum* an improvement in the histological parameters were verified. This improvement is mainly associated with a reduction in the villi atrophy and apical necrosis. In the ultrastructural analysis, besides these aspects we also observed an increase in the amount of mucus covering enterocytes. Similar aspects were reported in HT-29 cells treated with cell-free supernatant of *L. plantarum* 299v. The treated cells presented an increased expression of the MUC2 and MUC3 genes and the stimulation of mucin production.[27]

A possible mechanism for the improvement induced by the culture supernatants could involve the production of soluble bioactive factors released by *L.plantarum* during culture process[2], which are capable of eliciting responses in epithelial cells, triggering activation of various cell signaling pathways that led to intestinal homeostasis.[28] Culture supernatant of *L. plantarum* 2142 downregulated significantly the expression of proinflammatory cytokines IL-8 and TNF-α and simultaneously increased the Hsp70 level in IPEC cells. The authors hypothesized that these effects are associated with the production of small peptides by this

strain of lactobacillus.[25] Taken together these results indicate that culture supernatants from *L. plantarum* promote enhancement in intestinal morphology through bioactive metabolites.

EXPERIMENTAL SECTION

Animals

Six 24 days-old Landrace piglets were used to sample the explants. All animals were weaned at 21 days of age and then subjected to a standard diet in separate bays. Piglets were euthanized with intravenous injection of 1,3-diisopropilfenol (Propofol). The experimental procedures were conducted in accordance with the institutional (Universidade Estadual de Londrina, Brazil) Ethics Committee for Animal experimentation (number 11361.2014.30).

Lactobacillus plantarum strains and growth conditions

Two strains of *L. plantarum* gently ceded by the Food Science and Technology Laboratory (Universidade Estadual de Londrina, Brazil) were used in this study. Strain 1 was a *L. plantarum* American Type Culture Collection (ATCC 14917) and strain 2 was isolated from a sample of wheat grain from Paraná State, south Brazil. The isolation, identification and storage of LAB were described elsewhere [29]. Briefly, strains of LAB were grown in *Lactobacillus* MRS broth (De Man, Rogosa and Sharpe media, HiMedia) and incubated at 37^{o}C for 24 h. Subsequently, 2 ml of each culture was transferred to a flask containing 200 ml of sterile MRS broth and incubated at 37^{o}C for 24 h. Microrganisms were counted by the double layer inoculation method in MRS agar plates after incubation at 37^{o}C for 48 h.

Lactic acid bacteria were heat inactivated by sterilization (121^{o}C for 30 min).[30] After this procedure, the cell suspensions (1.1 x 10^{8} and 2.0 x 10^{9} CFU ml^{-1} of LAB for strain 1 and strain 2, respectively) were centrifuged (3000 g, 10 min, 5^{o}C). The resulting pellets and supernatant were separated and stored in microtubes tubes at -20^{o}C until the use. The inactivation of bacteria was confirmed by inoculation in MRS plates.

Jejunum explants culture and bioassay

The procedures performed to obtain jejunal explants from piglets were previously described. [31] The explants were collected with a biopsy punch (8 mm) and placed in six-well cell culture plates (3 explants/well) (Cellstar- Grenier bio-one, São Paulo-Brazil) filled with 3 ml of agar and containing Dulbecco's modified Eagle's medium (DMEM, Gibco- BRL Life Technologies, Carlsbad, CA) plus fetal bovine serum (10%), glutamine (0.2ml/L), gentamicin (0.5mg/ml) and penicillin/streptomycin (10ml/L). From each animal six explants were collected for each treatment.

The explants were incubated at 37°C for 4 hours with orbital shaking in the presence of the following treatments: control – only culture media (DMEM), culture media plus heat inactivated *L. plantarum* strain 1 (LP1), culture media plus heat inactivated *L. plantarum* strain 2 (LP2), culture media plus culture supernatant of strain 1 (CS1) and culture media plus culture supernatant of strain 2 (CS2). All the experimental procedures were performed in duplicated. Stored pellets were resuspended in the culture medium.

Histological and morphometrical analysis

After the incubation period, explants were fixed in 10% buffered formalin solution, dehydrated in increasing alcohols and embedded in paraffin for histological analysis. Explants were sectioned of 5 μm thickness parallel to the villi axis and stained with hematoxylin and eosin (HE), and mounted with coverslips. The histological changes were evaluated and a tissue morphological score was performed based on the intensity and severity of lesions. The criteria included in the tissue score were previously described. [31] The maximum score (39) indicates the overall integrity of the intestine.

The villi height was measured as the distance between the crypt mouth and the top of the villi randomly on ten villi. Ten intestinal crypts depth were also randomly measured. The

morphometric analysis was performed from images taken with a Motic Image Plus 2.0 software (Motic Instruments, Richmond, Canada).

Electron microscopy evaluation

The explants exposed to the different treatments were submitted to scanning electron microscopy (SEM). Samples were fixed with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature for 20 h. The samples were then washed with sodium cacodylate buffer (0.1 M, pH 7.2) and treated with 1% osmium tetroxide in sodium cacodylate buffer for 1 h, subjected to gradual dehydration in ethanol (70, 80, 90 and 100%), and dried to the critical point (CPD 030 Critical Point BALTEC Dryer, Leica Microsystems, Liechtenstein). After drying, the samples were glued on stubs using carbon tape and coated with gold (Sputter Coater BALTEC SDC 050, Leica Microsystems, Liechtenstein). The explants were analyzed using a scanning electron microscope (FEI Quanta 200).

Statistical analysis

The data used for statistical analysis was represented as means with their standard deviation. The experimental design used in the present study was entirely randomized with 30 repetitions for each treatment (each explant representing one repetition). Oneway analysis of variance (ANOVA) followed by multiple comparison procedure (Tukey test) was used for statistical analysis. Normality and homogenity of total score and morphometric means were attended. Differences were considered statistically significant at $p \leq 0.05$. Statistical analysis were performed with free software Action 2.3 (Campinas, SP, Brazil).

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Conflict of interest

The authors declare no conflict of interest.

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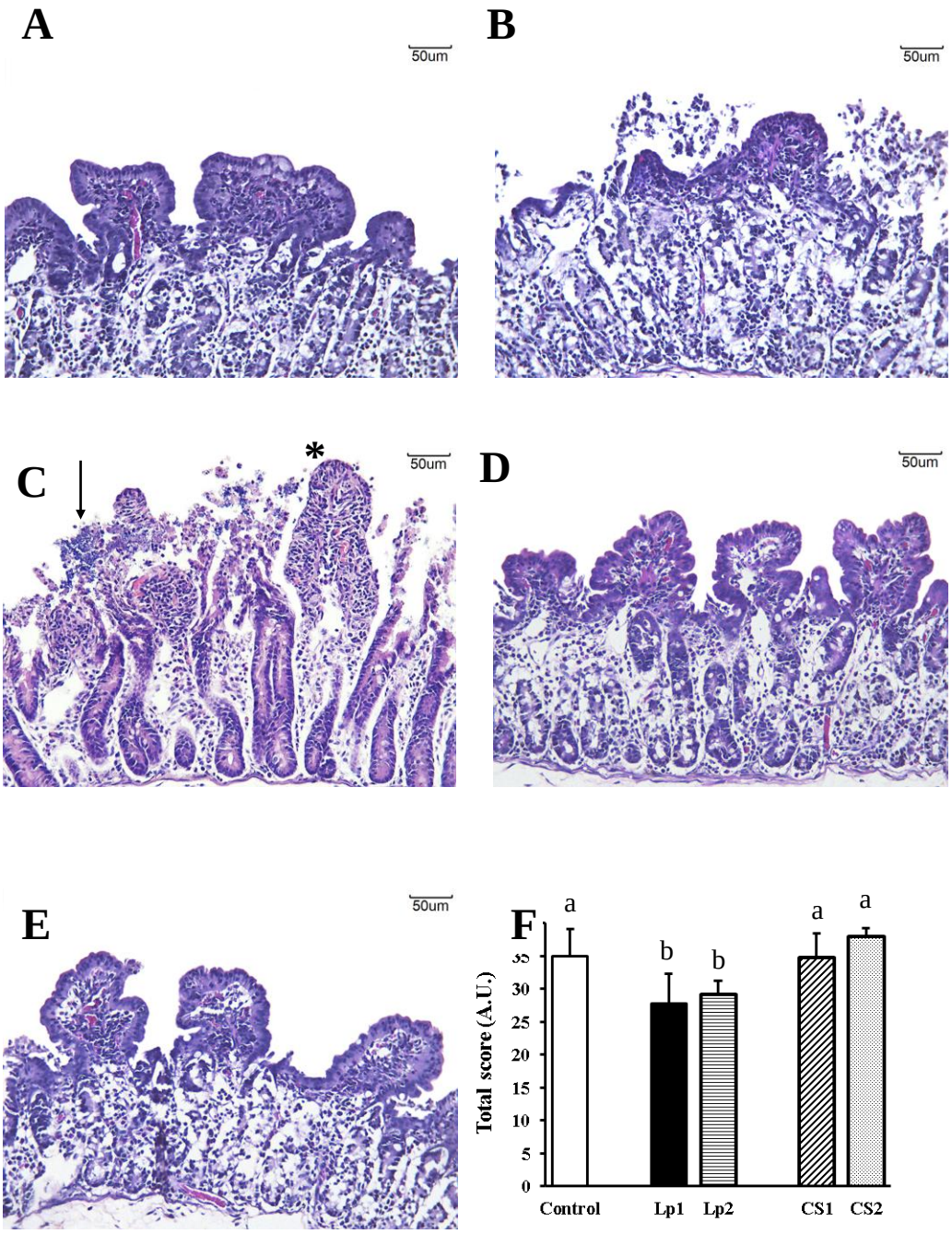

Figure 1. Effects of heat-inactivated *L. plantarum* and the culture supernatant exposure on jejunal explants of pigs. (A) Control explant showing mild edema of the lamina propria. Bar 50 µm (B) Explant exposed to LP1. Villi apical necrosis, cell debris, enterocyte flattening. Bar 50 µm (C) Explant exposed to LP2. Villi atrophy and diffuse enterocyte flattening (*), bacteria adhesion (arrow). Bar 50 µm (D and E) Explants exposed to culture supernatant of strain 1 (D) and strain 2 (E). Mild edema of the lamina propria and normal columnar enterocytes. Bar 50 µm (F) Morphological score of the explants exposed to different treatments. LP1- heat-inactivated *L. plantarum* strain 1, LP2- heat-inactivated *L. plantarum* strain 2, CS1- culture supernatant of heat-inactivated *L. plantarum* strain 1, CS2- culture supernatant of heat-inactivated *L. plantarum* strain 2.

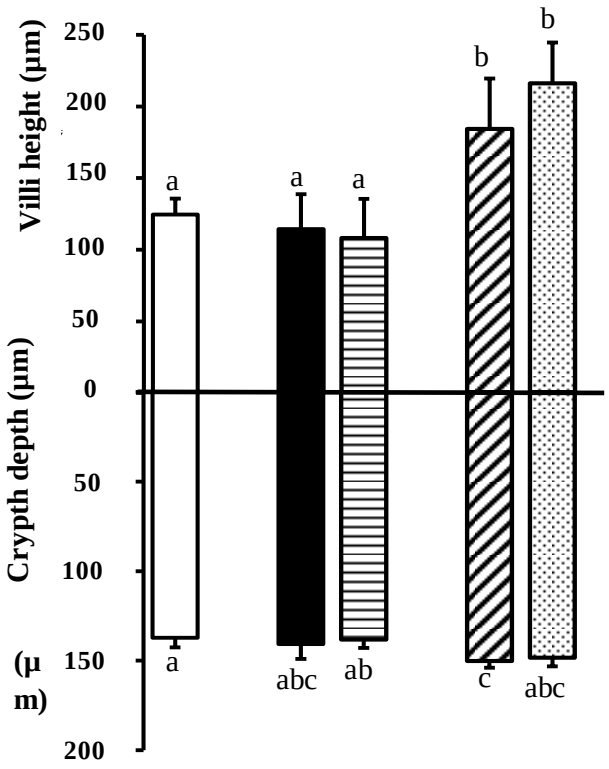

Figure 2. Effects of heat-inactivated *L. plantarum* and the culture supernatant exposure on villi height and crypt depth of jejunal explants of pigs. Values are mean height and depth (µm). Values are means with standard deviation of the mean represented by vertical bars (n 6 animals). Mean values with unlike letters were significantly different ($p<0.05$). Tukey test. Control(□). LP1-heat-inactivated *L. plantarum* strain 1 (■), LP2-heat-inactivated *L. plantarum*strain 2 (▤), CS1- culture supernatant of heat-inactivated *L. plantarum* strain 1 (▨), CS2- culture supernatant of heat-inactivated *L. plantarum* strain 2 (▦).

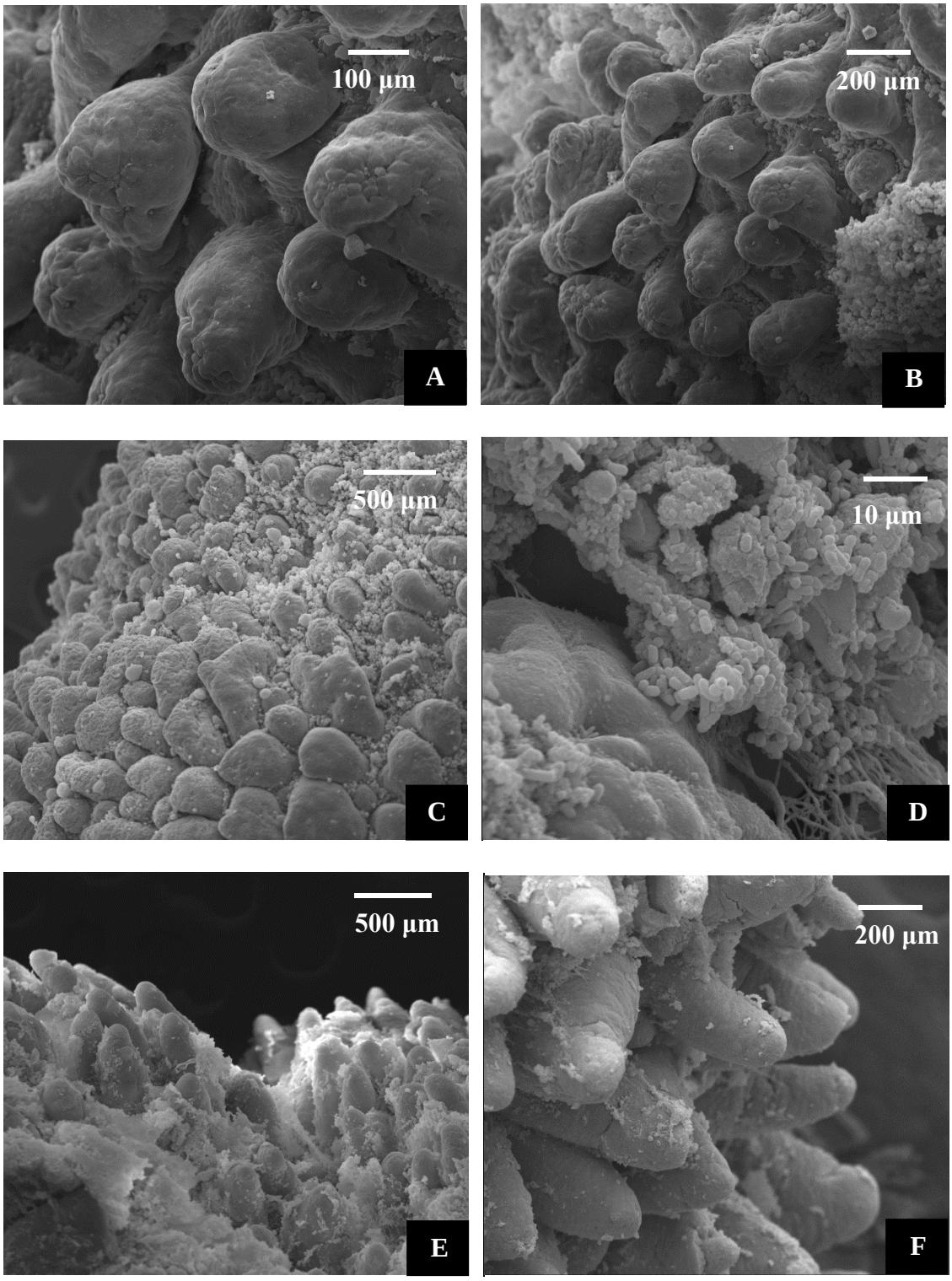

Figure 3. Morphological effects of heat-inactivated *L. plantarum* and the culture supernatant exposure on pigs jejunal explants in scanning electron microscopy assessment. Explants of jejunum of a 24 days old piglet. Normal jejunum as seen by scanning electron microscopy from a piglet of 24 days old. x 800. Bar 100 µm (A). A low magnification survey picture of the tissue shows numerous leaf and finger shaped villi of normal sizes and shapes. x 200. Bar 200 µm (B). Villi of explants treated with LP1 with mild atrophy. x 200. Bar 500 µm (C). LP2 showed adherence properties to the villi. x 6000. Bar 10 µm (D). Explants treated with CS1. x 200. Bar 500 µm (E) and CS2. x 400. Bar 200 µm (F) showed well delineated villi.

Article 2
***Lactobacillus plantarum* culture supernatants improve intestinal tissue exposed to deoxynivalenol**

Manuscript prepared accordingly to submission instructions of *Food and Chemical Toxicology*

Lactobacillus plantarum culture supernatants improve intestinal tissue exposed to deoxynivalenol

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ABSTRACT

In the present study, histological, morphometrical and ultrastructural analysis were performed to investigate intestinal mucosa changes in piglets exposed to deoxynivalenol alone or associated with two strains of *Lactobacillus plantarum* and the respective culture supernatants. Jejunal explants were incubated for 4 hours in culture medium with a) only culture medium (DMEM, control group), b) deoxynivalenol (DON, 10µM), c) heat-inactivated *Lactobacillus plantarum* strain1 - LP1 (1.1×10^8 CFU/ml) plus DON, d) heat-inactivated *Lactobacillus plantarum* strain2 – LP2 (2.0×10^9 CFU/ml) plus DON, e) heat-inactivated *Lactobacillus plantarum* strain1 culture supernatant (CS1) plus DON, and f) heat-inactivated *Lactobacillus plantarum* strain1 culture supernatant (CS1) plus DON. Explants exposed to DON and DON plus LP1 and LP2 showed a significant increase in histological changes (mainly villi atrophy and apical necrosis) and a significant decrease in villi height when compared to unexposed explants. However, explants treated with CS1+DON and CS2+DON remained similar to the control group both in histological and morphometrical aspects. DON also induced a significant decrease in goblet cell density compared to control whereas CS1+DON treatment induced an increase in the number of goblet cells in comparison to DON explants. In addition, ultrastructural assessment showed control, CS1+DON and CS2+DON explants with well delineated finger shape villi, meanwhile DON-treated, LP1+DON and LP2+DON explants showed a severe villi atrophy with leukocytes exudation on the intestinal surface. Taken together, our results indicate that the culture supernatant treatment reduced the toxic effects induced by DON on intestinal tissue and may contribute as an alternative strategy to reduce mycotoxin toxicity.

1. Introduction

Deoxynivalenol (DON) is a secondary fungal metabolite of the trichothecene family mainly produced by *Fusarium graminearum* and *Fusarium culmorum* that affects human and animal health (Pierron et al., 2016). DON contaminates cereal-based food and feed worldwide, affecting 55% of the cereal crops (Smith et al., 2016; Streit et al., 2013).

Toxicological effects of DON include increase in intestinal permeability (Wache et al., 2009), decrease in transepithelial electrical resistance (TEER) (Ling et al., 2016; Romero et al., 2016), tight junction protein expression (Bracarense et al., 2012) and goblet cells density (Gerez et al., 2015), alteration in the enterocyte proliferative and apoptotic index (Cheat et al., 2016; Wang et al., 2014). In addition, DON exposure has been associated as a possible cause of allergic (Bol-Schoenmakers et al., 2016) and celiac diseases in humans (Cano et al., 2013)

Considering these harmful effects, strategies to reduce fungi contamination and minimize mycotoxin production are of increasing interest (Hepworth et al., 2012; Turner et al., 2011; Wang et al., 2014; Warth et al., 2012). Furthermore, it is necessary to amplify the knowledge about effective detoxification methods, attenuation in toxicity and enhancement of the intestinal tissue response to the damage (Karlovsky, 2011; Pierron et al., 2016; Suzuki and Iwahashi, 2015, 2016). In this context, cell walls of viable and non-viable lactic acid bacteria (LAB) has been suggested as an accessible strategy in inactivating mycotoxins (El-Nezami et al., 2002; Haskard et al., 2000; Niderkorn et al., 2006), suggesting that carbohydrates and/or protein components of LAB play a major role in mycotoxin binding (Niderkorn et al., 2006). The beneficial effects of LAB for humans and animals constantly exposed to mycotoxins are attributed to a reduction in the absorption of the toxins (Gratz et al., 2004), production of antimicrobial peptides (Anyogu et al., 2014; Habil et al., 2014) and a general improvement in the intestinal barrier function (Anderson et al., 2010a; Anderson et al., 2010b). Interestingly,

previous studies have indicated that even LAB inactivated by heating still displaying protective competences (Ostad et al., 2009; Peng et al., 2007).

Our group is interested in evaluating the effects of mycotoxins in animal and human health (Bracarense et al., 2012, Lucioli et al., 2013, Maidana et al., 2016), but also in developing strategies to minimize the toxic effects of mycotoxins. In previous studies using *in vitro* and *ex vivo* models we have demonstrated that phytic acid reduced significantly the intestinal toxicity of DON (Pacheco et al., 2012; da Silva et al., 2014). Furthermore, *Lactobacillus* strains isolated by another research group of our institution reduced the levels of DON in an *in vitro* model (Franco et al., 2011). Considering the need to amplify the knowledge related to mycotoxin inactivation, the aim of the present study was to investigate the effects of heat-inactivated *Lactobacillus plantarum* (LP) and their culture supernatant (CS) on intestinal toxicity induced by deoxynivalenol using the model of jejunal explants of pigs. Recognizing, this *ex-vivo* model (Maidana et al., 2016) as a suitable model for toxicological tests and used in the context of implementing the 3Rs "Replace, Reduce, Refine" (Cheat et al., 2015).

2. Material and methods

2.1 Animals

Six 24 day-old Landrace piglets were used to sample the explants. All animals were weaned at 21 days of age and then subjected to a standard diet in separate bays. All piglets were euthanized with intravenous injection of 1,3-diisopropilfenol (Propofol). The experimental procedures were conducted in accordance with the institutional (Universidade Estadual de Londrina, Brazil) Ethics Committee for Animal experimentation (number 11361.2014.30).

2.2 *Lactobacillus plantarum* strains and growth conditions

Two strains of *L. plantarum* gently ceded by the Food Science and Technology Laboratory (Universidade Estadual de Londrina, Brazil) were used in this study. Strain 1 was a *L. plantarum* American Type Culture Collection (ATCC 14917) and strain 2 was isolated from a sample of wheat grain from Paraná State, south Brazil. The isolation, identification and storage of LAB were described elsewhere (Franco et al., 2011). Briefly, strains of LAB were grown in *Lactobacillus* MRS broth (De Man, Rogosa and Sharpe media, HiMedia) and incubated at 37°C for 24 h. Subsequently, 2 ml of each culture was transferred to a flask containing 200 ml of sterile MRS broth and incubated at 37°C for 24 h. Micro-organisms were counted by the double layer inoculation method in MRS agar plates after incubation at 37°C for 48 h.

Lactic acid bacteria were heat inactivated by sterilization (121°C for 30 min) (Shahin, 2007). After this procedure, the cell suspensions (1.1×10^8 and 2.0×10^9 CFU ml^{-1} of LAB for strain 1 and strain 2, respectively) were centrifuged (3000 g, 10 min, 5°C). The resulting pellets and supernatant were separated and stored in microcentrifuge tubes at -20°C until the use. The inactivation of bacteria was confirmed by inoculation in MRS plates.

2.3 Deoxynivalenol

The purified DON (MW: 296.32) mycotoxin was purchased from Sigma-Aldrich (St. Louis, MO, USA). The mycotoxin was dissolved in ultrapure water at final dilution of 10 μM for DON and stored at -20°C. The concentration used was equivalent to an ingestion of 3mg of DON/kg. The dose used in this experiment was previously used to induce toxic effects on intestinal tissue (Basso et al., 2013; da Silva et al., 2014).

2.4 Jejunum explants culture and bioassay

Sampling of jejunal explants were described elsewhere (Maidana et al., 2016). The explants were sampled using a punch (8 mm) and placed in six-well cell culture plates (3

explants/well) (Cellstar®- Grenier bio-one, São Paulo-Brazil) filled with 3 ml of agar and containing Dulbecco's modified Eagle's medium (DMEM, Gibco- BRL Life Technologies, Carlsbad, CA) plus fetal bovine serum (10%), glutamine (0.2ml/L), gentamicin (0.5mg/ml) and penicillin/streptomycin (10ml/L). From each animal six explants were collected for each treatment.

The explants were incubated at 37°C for 4 hours with orbital shaking in the presence of the following treatments: control – only culture media (DMEM), deoxynivalenol (10 mM), deoxynivalenol plus heat inactivated *L. plantarum* strain 1 (LP1), deoxynivalenol plus heat inactivated *L. plantarum* strain 2 (LP2), deoxynivalenol plus culture supernatant of strain 1 (CS1) and deoxynivalenol plus culture supernatant of strain 2 (CS2). All the experimental procedures were performed in duplicated. Stored pellets were suspended in the culture medium.

2.5 Histological and morphometrical analysis

After 4 hours, explants were fixed in 10% buffered formalin solution, dehydrated in increasing alcohols and embedded in paraffin for histological analysis. Explants were sectioned of 5 µm thickness parallel to the villi axis and stained with hematoxylin and eosin (HE), and mounted with coverslips. The histological changes were evaluated and a tissue morphological score was performed based on the intensity and severity of lesions. The criteria included in the tissue score were previously described (Maidana et al., 2016). The maximum score (39) indicates the overall integrity of the intestine. Density of goblet cells in intestinal explants was evaluated by the Periodic acid Schiff (PAS) staining. The cells were counted throughout villus and crypt axis in histological sections of jejunal explants. Positively stained goblet cells were counted randomly in five fields per slide at 40x magnification, and the means were subjected to statistical analysis.

The villi height was measured as the distance between the crypt mouth and the top of the villi randomly on ten villi. Ten intestinal crypts depth were also randomly measured. The morphometric analysis was performed from images taken with a Motic Image Plus 2.0 software (Motic Instruments, Richmond, Canada).

2.6 Scanning electron microscopy evaluation

The explants exposed to the different treatments were submitted to scanning electron microscopy. Samples were fixed with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature for 20 h. The samples were then washed with sodium cacodylate buffer (0.1 M, pH 7.2) and treated with 1% osmium tetroxide in sodium cacodylate buffer for 1 h, subjected to gradual dehydration in ethanol (70, 80, 90 and 100%), and dried to the critical point (CPD 030 Critical Point BALTEC Dryer, Leica Microsystems, Liechtenstein). After drying, the samples were glued on stubs using carbon tape and coated with gold (Sputter Coater BALTEC SDC 050, Leica Microsystems, Liechtenstein). The explants were analyzed using a FEI Quanta 200 scanning electron microscope.

2.7 Statistical analysis

The experimental design used was entirely randomized and consisted of six repetitions per treatment resulting in six replicates from each animal. A total of 36 explants were analyzed for each treatment. Data used for statistical analysis were represented as means ± standard deviation of the mean and analyzed using the free software R 3.2.2. The response variables were compared by ANOVA analysis. If significantly different ($p \leq 0.05$), the means were compared by Tukey's test for multiple comparisons ($p \leq 0.05$).

3 Results

3.1 Effects of DON alone and associated with heat inactivated *L. plantarum* and the culture supernatants on the morphological score

After four hours of incubation, untreated explants (control group) presented mild histological changes as villi atrophy and edema of lamina propria (Figure 1A), whereas explants exposed to DON showed severe intestinal lesions consisting in diffuse apical necrosis and flattened or cuboid enterocytes, villi fusion and atrophy (Figure 1B). Histological changes in explants exposed to LP1+DON (Figure 1C) and LP2+DON (Figure 1D) were characterized by mild villi atrophy and enterocyte apical necrosis. In contrast, explants treated with CS1+DON (Figure 1E) and CS2+DON (Figure 1F) remained similar to the control group.

Control explants showed a mean histological score of 37.33±2.61, whereas a significant decrease in the score (24.36±4.80, $p < 0.01$) was observed in explants exposed to DON when compared to the untreated group. The mean morphological score for LP1+DON, LP2+DON, CS1+DON and CS2+DON was 26.82±3.40, 26.58±4.54, 33.21±5.73 and 37.83±0.93, respectively. A significant increase ($p<0.001$) in the morphological score was observed in the explants treated with the culture supernatant when compared to explants exposed to DON (Figure 1G).

In addition, the number of goblet cells in explants treated with DON was significantly lower in villus and crypts ($p<0.001$) compared to control group. On the other hand, CS1+DON–treated explants showed a significant increase in the mean number of the goblet cells when compared to the DON group ($p<0.005$) (Figure 2).

3.2 Effects of DON alone and combined with heat inactivated *L. plantarum* and the culture supernatants on villi height and crypt depth

The mean villi height and crypt depth were assessed in all groups and the values achieved were as follow: control (145.03±33.31 μm and, 137.27±5.37 μm), DON

(93.332±23.73, 139.4±15.208 μm), LP1+DON (89.60±9.27, 142.404±6.42 μm), LP2+DON (88.98±5.20, 138.6675±4.27 μm), CS1+DON (194.08±39.88, 149.056±10.77 μm) and, CS2+DON (227.95±17.32, 148.475±7.55 μm). DON-treated and DON plus LP1 and LP2 explants showed a significant decrease in villi height compared to the control group ($p<0.05$). On the other hand, a significant increase in explants exposed to CS+DON groups was observed when compared to DON group ($p<0.001$) (Figure 1H).

3.3 Effects of heat inactivated *L. plantarum* and the culture supernatants on villi morphology by scanning electron microscopy assessment

Control explants showed well delineated finger-shaped villi with few leukocytes lining the surface of enterocytes (Figure 3A). DON-treated explants showed a severe and diffuse villi atrophy accompanied by remarkably number of leukocytes and cell debris in the intestinal surface (Figure 3B). In fractured villi it was possible to observe an increased number of leukocytes in the lamina propria. Meanwhile, LP1+DON-treated explants showed moderate villi atrophy, multifocal villi apical necrosis (resulting in exposure of the lamina propria) (Figure 3C) and a moderate number of leukocytes in the lumen. LP2 bacteria plus DON showed adhered to villi surface showing a conserved wall structure (Figure 3D). Explants exposed to DON plus culture supernatants of both strains presented well delineated villi, showing no atrophy or apical necrosis. Also, enterocytes were coated by mucus especially in the bottom of the villi (Figure 3E and 3F).

4. Discussion

Intestinal toxicity induced by deoxynivalenol has been previously reported (Basso et al., 2013; Gerez et al., 2015). Considering this fact and the frequency that DON is found on feed and food worldwide (Streit, 2013; Lee et al., 2017), strategies to alleviate the harmful effects represent an issue of increasing interest. *Lactobacillus* of different strains have been widely used to promote a preventive role in the intestinal epithelial barrier dysfunction, maintaining

cell monolayer integrity (Resta-Lenert and Barrett, 2003; Zeng et al., 2016). In the present study, we present evidence that cell free extracts of heat inactivated *L. plantarum* induced a protective effect on intestinal explants exposed to DON.

Similarly to previous studies (Lucioli et al., 2013), jejunal explants exposed to DON showed severe histological lesions, mainly affecting enterocytes and goblet cells, resulting in villi atrophy. This change was also confirmed by ultrastructural analysis, where the reduction in villi height was evident. The addition of heat inactivated *L. plantarum* extracts to intestinal explants induced similar histological changes, showing no favorable effect. Meanwhile, explants exposure to DON plus CS promoted a significant increase in the morphological score (1.4 fold) compared to DON group, with no significant difference between the strains. A significant increase in villi height was also verified in explants treated with culture supernatants, however, in this case LP2 showed a 2.4 fold-increase, whereas LP1 induced a 2.08 fold-increase. The protective effect of the culture supernatants against DON toxicity was also confirmed by scanning electron microscopy where villi showed ultrastructural aspects similar to control explants.

Detoxification of mycotoxins by LAB was previously reported in aflatoxins and fumonisins exposure (Bueno et al., 2007; Niderkorn et al., 2007). In these cases the mechanism is through mycotoxin binding (Bueno et al., 2007). In a previous *in vitro* study using viable and inactivated LAB, including *L. plantarum* strains, a reduction in the concentration of DON was observed, ranging from 16 to 71% (Franco et al., 2011). The authors hypothesized that the reduction is achieved by mycotoxin adsorption or metabolic degradation. However, in the present study, *L. plantarum* combined with deoxynivalenol showed no positive modulatory activity on intestinal histology. In all parameters evaluated the aspects were similar to explants exposed to DON. These contradictory results may be explained by the different methods used (*in vitro* and *ex vivo*) and the different concentrations

of bacteria. On the other hand, previous studies comparing various binders compounds showed a lower effectiveness in adsorbing DON when compared to other mycotoxins as aflatoxins and zearalenone (Kong et al., 2014; Sabater-Vilar et al., 2007). These differences are related to the chemical structure of trichothecenes, characterized by a lower polarity, resulting in a lower binding affinity (Huwig et al., 2001).

DON induces toxic effects through protein synthesis inhibition (Foroud et al., 2016), oxidative stress and cell apoptosis (Springler et al. 2016). The inhibition of these intestinal toxic effects by culture supernatants was probably related to bioactive and thermo-resistant factors secreted by bacteria during their growth. Soluble factors recovered from *L. rhamnosus* (Yan et al., 2002; Yan et al. 2007) and *L. acidophilus* strains (Resta-Lenert and Barret, 2003) have been shown to regulate intestinal epithelial signaling pathways and biologic functions, such as cell survival signaling and inhibition of cytokine-induced apoptosis in intestinal epithelial cells. However, the specific bacterial factors that elicit tissue responses remain unclear. It was hypothesized that the first signal transduction pathway that LAB exert is an Akt initial (Yan and Polk, 2002), leading to cell proliferation. Taken this, we could hypothesize that the factors released by *L. plantarum* in the culture supernatants protected intestinal epithelial cells from DON-induced apoptosis through an activation of Akt signaling. Interestingly, low-molecular-weight factors secreted by *L. rhamnossus* GG (<10 kilodaltons) have been reported to stimulated Hsp25 and Hsp72 production by intestinal epithelial cells, resulting in a blockage of cell apoptosis (Tao et al., 2006).

In addition, bacteriocins released by certain *Lactobacillus* in culture supernatants have been reported to promote intestinal epithelial integrity *in vitro* (Zhao et al., 2015b) and *in vivo* (Sha et al., 2016), having inhibitory effects on pathogens (Aryantini et al., 2016; Therdtatha et al., 2016; Vahedi Shahandashti et al., 2016). Results obtained by our study group with intestinal explants incubated with the LP metabolites demonstrated that bioactive compounds

released during bacteria culture modulate the improvement in intestinal structure (data not shown).

Mucin synthesis and secretion play an important role as a physical barrier (highly glycosylated proteins) on intestinal mucosa (Pelaseyed et al., 2014). In the present study, as previously reported, explants exposed to DON showed a significant decrease in goblet cells density (Basso et al., 2013) compared to the control group. This decrease could be associated to cell apoptosis in intestinal crypts (da Silva et al., 2014) or a decrease in the expression of mucin genes (Pinton et al., 2015). Also, the treatment with heat inactivated *L. plantarum* showed similar effect. On the other hand, a significant increase in cell goblet density was observed in explants treated with culture supernatant of strain 1 compared to the DON group. This increase could be related to an induction of the expression of MUC2 and MUC3 genes as has been reported in HT-29 cells treated with *L. plantarum* 299v (Mack et al., 1999), resulting in mucin production. Interestingly, in the ultrastructural analysis the culture supernatants groups presented an increased layer of mucin lining the villi surface, reinforcing this hypothesis.

In conclusion, we have showed that culture supernatants of *L. plantarum* reduced the toxic effects induced by DON in the intestinal tissue. The improvement in intestinal health and reduced toxicity of DON are probably related to soluble factors released in *L. plantarum* supernatant culture. These soluble factors should be further confirmed and identified to support a potential application for preventing mycotoxin intestinal injury.

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Conflict of interest

The authors declare no conflict of interest.

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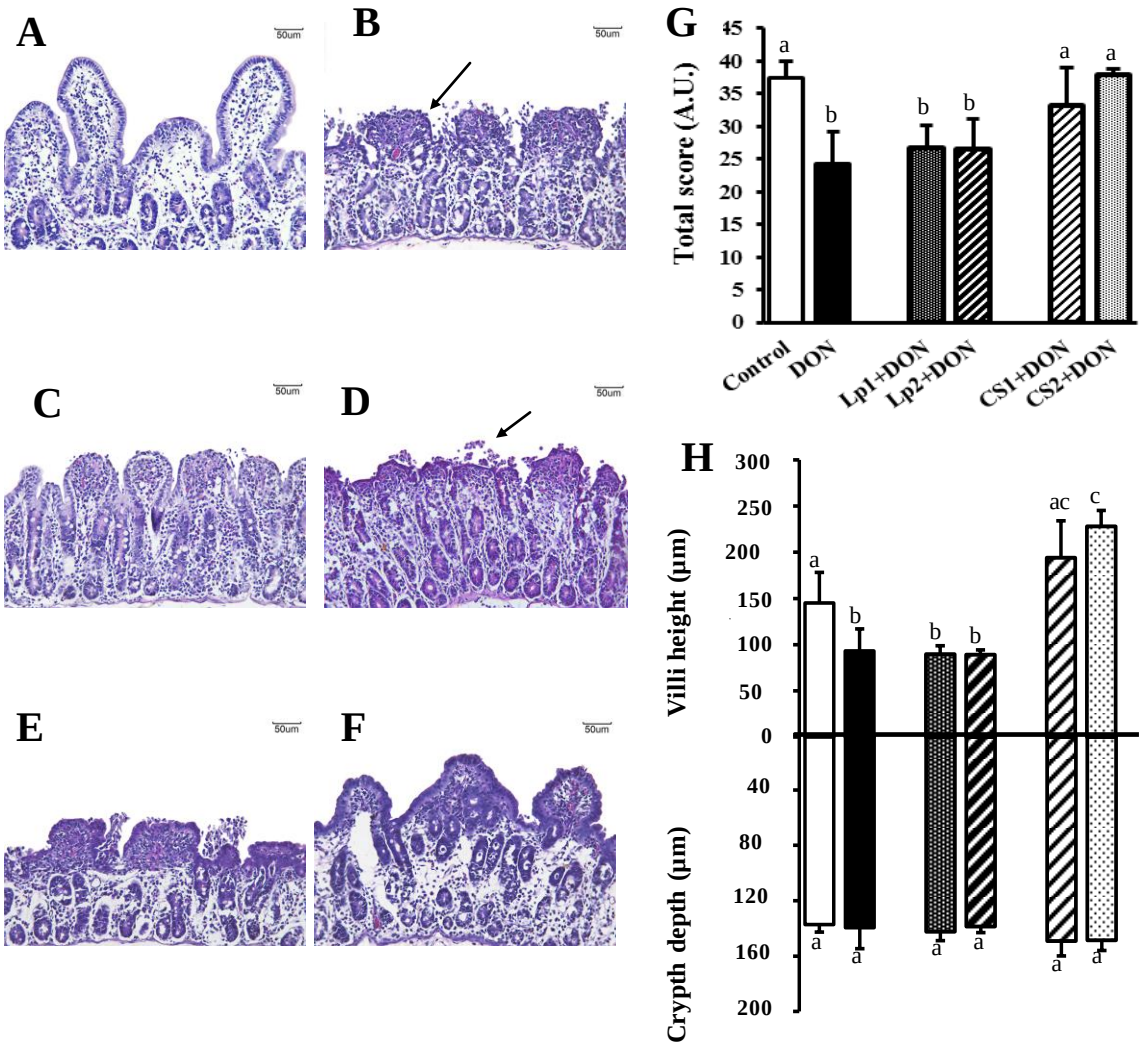

Figure 1. Effect of DON alone and combined with heat-inactivated *Lactobacillus plantarum* (LP) and the culture supernatants (CS) exposure on histology. Explants were exposed to culture medium (□) or culture medium with deoxynivalenol (DON) (■), or DON+LP1 (▣), or DON+LP2 (◪), or DON+CS1 (◪) or DON+CS2 (⊡). A) Control explants; B) DON-exposed explant. Severe apical necrosis, villi atrophy and enterocyte flattening (arrow); C) DON+LP1-exposed explant; D) DON+LP2-exposed explant; Severe loss of apical enterocytes and cellular debris (arrow); E) DON+CS1-exposed explant; F) DON+CS2-exposed explant. Villi with normal morphology. HE. Bar 50 µm; G) Tissue scores of pig intestinal explants exposed to DON, DON+LP1, DON+LP2, DON+CS1 and DON+CS2; H) Villi height and crypt depth in pig intestinal explants treated with DON, DON+LP1, DON+LP2, DON+CS1 and DON+CS2. Values are means with the standard deviation of the mean represented by vertical bars (*n* 36 explants/treatment). [a,b,c] mean values with unlike letters were significantly different by Tukey's test ($p \leq 0.05$). LP= *L. plantarum* strains. (LP1, strain 1 ; LP2, strain 2). CS= culture supernatants (CS1, strain 1; CS2 strain 2).AU=Arbitrary units.

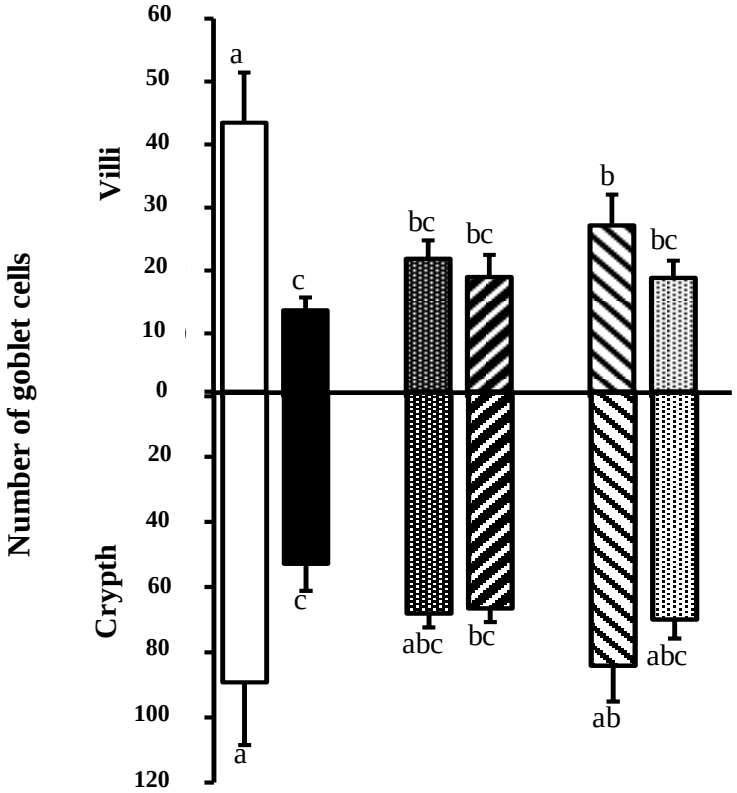

Figure 2. Effects of DON individual and combinated with heat inactivated *Lactobacillus plantarum* (LP) and its culture supernatantd (CS) exposure on number of goblet cells. Explants exposed to DMEM for 4 hours (control) (□), deoxynivalenol (DON) (■), DON+LP1 (◙), DON+LP2) (◩), DON+CS1(◪) and DON+CS2 (⊡).Values are means number of goblet cells with their standard deviation of the mean represented by vertical bars (*n* 36 explants/treatment). [a,b,c] Mean values with unlike letters were significantly different by Tukey's test ($p \leq 0.05$). LP= *L. plantarum* strains (LP1, strain 1 ; LP2, strain 2). CS= culture supernatants (CS1, strain 1; CS2 strain 2).

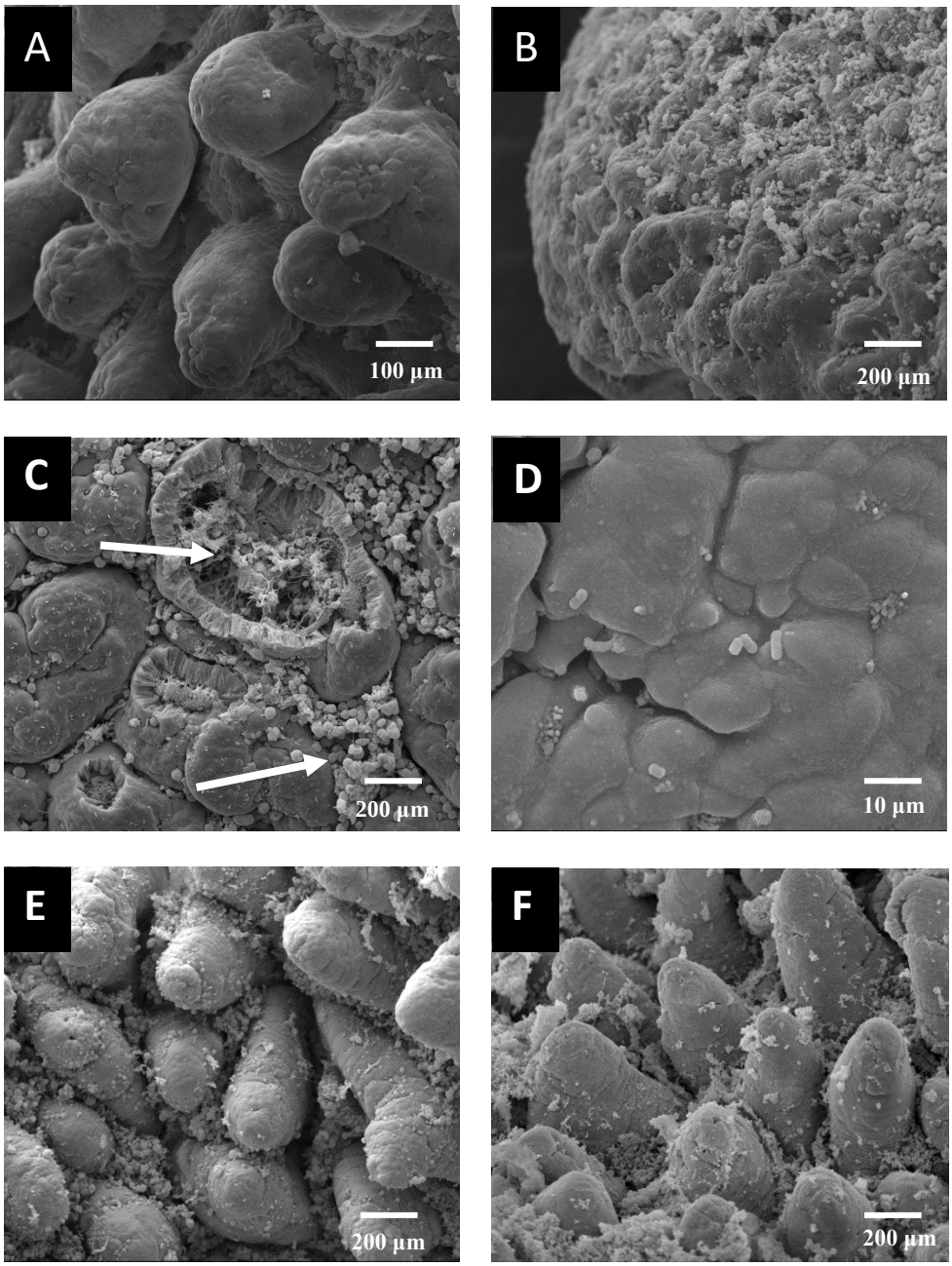

Figure 3. The scanning electron micrographs of mucosal surface of jejunal explants exposed to heat inactivated *Lactobacillus plantarum* (LP) and its culture supernatant (CS). A) Explants exposed to DMEM for 4 hours (control). Normal aspect of villi. Bar 100 µm; B) deoxynivalenol (DON). Severe atrophy of villi. Bar 200 µm; C) LP strain1+DON (LP1+DON). Increased number of leukocytes surfacing the villi and in the lamina propria (arrows). Bar 100 µm; D) LP strain2+DON (LP2+DON). Villi atrophy and adherence of LAB to villi surface. Bar 10 µm; E) CS1+DON and F) CS2+DON. Normal aspect of villi and increased mucin layer. Bar 200 µm.

Article 3

***Lactobacillus plantarum* culture supernatant induces protective effects on jejunal explants of piglets exposed to deoxynivalenol: histological and antioxidant activity aspects**

Manuscript prepared accordingly to submission instructions of *Food and Chemical Toxicology*

Lactobacillus plantarum culture supernatant induces protective effects on jejunal explants of piglets exposed to deoxynivalenol: histological and antioxidant activity aspects

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ABSTRACT

In the present study, histological, morphometrical and antioxidant activity analysis were performed to investigate intestinal mucosa changes in piglets exposed to deoxynivalenol alone or associated with two strains and concentrations of *Lactobacillus plantarum* and the respective culture supernatants. Jejunal explants were incubated for 4 hours in DMEM culture medium with a) only culture medium (control group), b) deoxynivalenol (DON), c) MRS broth, d) heat-inactivated *Lactobacillus plantarum* strain1 - LP1 (1.1×10^8 CFU/ml) plus DON, e) heat-inactivated *Lactobacillus plantarum* strain2 – LP2 (2.0×10^9 CFU/ml) plus DON, f) heat-inactivated *Lactobacillus plantarum* strain1 culture supernatant (CS1) plus DON, and g) heat-inactivated *Lactobacillus plantarum* strain1 culture supernatant (CS1) plus DON. LP1, LP2, CS1 and CS2 were incubated one hour previously to three hours of DON exposure. Histological assessment showed DON, MRS broth, LP1+DON, and LP2+DON-treated explants with villi fusion and atrophy, multifocal apical necrosis and cuboid or flattened enterocytes; CSs+DON groups remained similar to control. Villi and crypt goblet cells were reduced in DON explants comparing to control group; on the other hand a significant increase in the number was achieved in CSs+DON. Morphometric assessment showed a significant villi height decreased in explants incubated with DON, LP1+DON and LP2+DON, whereas CSs+DON groups presented a significant increase. Antioxidant activity assessments (FRAP and ABTS assays) showed an increase antioxidant activity in CS2+DON. In general, DON associated with both culture supernatant presented intestinal improvements at histological, morphometrical and antioxidant evaluations.

1. Introduction

Deoxynivalenol (DON) is a type B trichothecene predominantly produced by *Fusarium graminearum* and *F. culmorum* that contaminates grains such as wheat, maize, barley, and oats. DON is mainly produced in field conditions, however secondary contamination may occur during storage (Mishra et al., 2014). Considering its global occurrence, DON seems to be one of the most important trichothecenes mycotoxins in cereal contamination, and becomes a serious human (Pestka and Smolinski, 2005) and animal health (Gerez et al., 2015) concern.

At the molecular level, the capacity of DON to inhibit protein synthesis upon its binding to ribosomal RNA is pivotal for its effects (Pierron et al., 2016a). This binding to ribosome rapidly activates mitogen-activated kinases (MAPKs) via a so-called ribotoxic stress response (Iordanov et al., 1997; Zhou et al., 2003). DON also induces transactivation of a number of pro-inflammatory cytokines (Pestka, 2010).

Oxidative stress is characterized by an imbalance favoring prooxidants and/or disfavoring antioxidants, potentially leading to damage of biomolecules. Increased quantities of reactive oxygen species initiate lipid peroxidation in the cellular, mitochondrial and nuclear membranes, along with the degradation of cytosolic proteins and damage to DNA (Hassan et al., 2017; Sies, 2017). Conversely, the function of antioxidant enzymes is to reduce the elevated levels of radical oxygen species (ROS). However, depletion of these defense elements further promotes oxidative stress (Pisoschi et al., 2016).

DON has been reported to impair antioxidant enzymes (Zhang et al., 2009). *In vitro* studies have demonstrated that DON treatment of different cells (HepG2, U937, Hek-293, Caco-2) decreases the activities of intracellular antioxidant enzymes in a dose-and-time-dependent manner (Costa et al., 2009; Dinu et al., 2011; Kouadio et al., 2007; Kouadio et al., 2005).

Methods that effectively detoxify or biotransform DON would be suitable in reducing the harmful effects in animal health (De Zutter et al., 2016; Doll and Danicke, 2004). Probiotics are commonly used in the feed of piglets to stabilize the gut microflora as a preventive measure during the critical period of weaning (Vahjen et al., 2007). In a previous study we have investigated the effects of LAB and the correspondent cell free extracts on DON intestinal toxicity. These previous results indicated a protective effect of the culture supernatants of LAB when they were added concomitantly with DON. In the present study we have evaluated the effects of LAB and the culture supernatants previously added to jejunal explants exposed to DON. The aim was to investigate if a previous exposition of intestinal tissue to probiotics could enhance the beneficial effects. To achieve this we have used histological and morphometrical analysis in an *ex vivo* model. The oxidative response to DON and to LAB was also considered.

2. Material and methods

2.1 Animals

Six 24 days-old Landrace piglets were used to sample the explants. All animals were weaned at 21 days of age and then subjected to a standard diet after weaning in separate bays. All piglets were euthanized with intravenous injection of 1,3-diisopropilfenol (Propofol®). The experimental procedures were conducted in accordance with the institutional (Universidade Estadual de Londrina, Brazil) Ethics Committee for Animal experimentation (number 11361.2014.30).

2.2 *Lactobacillus plantarum* strains and growth conditions

The strains of *L. plantarum* used in this study were a *L. plantarum* American Type Culture Collection (ATCC 14917) (strain 1) and a strain isolated from a sample of wheat grain (strain 2) acquired in Paraná State, south Brazil. The procedures of isolation, identification and storage of LAB were previously described (Franco et al., 2011). Concisely,

the strains were grown in Lactobacillus MRS broth (De Man, Rogosa and Sharpe media, HiMedia) and incubated at 37°C for 24 h. Subsequently, 2 ml of each culture was transferred to a flask containing 200 ml of sterile MRS broth and incubated at 37°C for 24 h. Micro-organisms were counted by the double layer inoculation method in MRS agar plates after incubation at 37°C for 48 h.

Lactic acid bacteria were heat inactivated by sterilization (121°C for 30 min) (Shahin, 2007). After this procedure, the cell suspensions (1.1×10^8 and 2.0×10^9 CFU ml^{-1} of LAB for strain 1 and strain 2, respectively) were centrifuged (3000 g, 10 min, 5°C). The resulting pellets and supernatant were separated and stored in microcentrifuge tubes at -20°C until the use. The inactivation of bacteria was confirmed by inoculation in MRS plates.

2.3 Deoxynivalenol

The purified DON (MW: 296.32) mycotoxin was purchased from Sigma-Aldrich (St. Louis, MO, USA). The mycotoxin was dissolved in ultrapure water at final dilution of 10 µM for DON and stored at -20°C. The concentration used was equivalent to an ingestion of 3mg/kg. The dose used in this experiment was based in previous experiments (Basso et al., 2013; da Silva et al., 2014).

2.4 MRS broth

Lactobacilli MRS broth (55 g) was suspended in 1 L of purified water; solution was autoclaved at 121°C for 15 minutes. Solution prepared appearance was amber, clear to very slightly opalescent with pH 6.5 ± 0.2 at 25 °C.

2.5 Jejunum explants culture and bioassay

The procedures performed to obtain jejunal explants from piglets were previously described (Maidana et al., 2016). Briefly, the explants were collected with a biopsy punch (8 mm) and placed in six-well cell culture plates (3 explants/well) (Cellstar®- Grenier bio-one, São Paulo-Brazil) filled with 3 ml of agar and containing Dulbecco's modified Eagle's

medium (DMEM, Gibco- BRL Life Technologies, Carlsbad, CA) plus fetal bovine serum (10%), glutamine (0.2ml/L), gentamicin (0.5mg/ml) and penicillin/streptomycin (10ml/L). From each animal six explants were collected for each treatment.

The explants were incubated at 37°C for 4 hours with orbital shaking in the presence of the following treatments: control – only culture media (DMEM), deoxynivalenol (10 mM), MRS broth, deoxynivalenol plus heat inactivated *L. plantarum* strain 1 (LP1), deoxynivalenol plus heat inactivated *L. plantarum* strain 2 (LP2), deoxynivalenol plus culture supernatant of strain 1 (CS1) and deoxynivalenol plus culture supernatant of strain 2 (CS2). Deoxynivalenol was added individually or associated with LP1, LP2, CS1 and CS2 to the culture plaques 1 (one) hour after these last four treatments were already incubated. MRS and control (only DMEM) explants were incubated individually for 4 hours. All the experimental procedures were performed in duplicated. Stored pellets were suspended in the culture medium.

2.6 Histological and morphometrical analysis

After the incubation period, explants were fixed in 10% buffered formalin solution, dehydrated in increasing alcohols and embedded in paraffin for histological analysis. Explants were sectioned of 5 µm thickness parallel to the villi axis and stained with hematoxylin and eosin (HE), and mounted with coverslips. The histological changes were evaluated and a tissue morphological score was performed based on the intensity and severity of lesions. The criteria included in the tissue score were previously described (Maidana et al., 2016). The maximum score (39) indicates the overall integrity of the intestine.

The villi height was measured as the distance between the crypt mouth and the top of the villi randomly on ten villi. Ten intestinal crypts depth were also randomly measured. The morphometric analysis was performed from images taken with a Motic Image Plus 2.0 software (Motic Instruments, Richmond, Canada).

In order to evaluate the goblet cells density in intestinal explants the Periodic acid Schiff (PAS) staining was performed. The cells were counted throughout villus and crypt axis in histological sections of jejunal explants. Positively stained goblet cells were counted randomly in five fields per slide at 40x magnification, and the means were subjected to statistical analysis.

2.7 Determination of the antioxidant activity of heat inactivated *L. plantarum* and the culture supernatants by ABTS and FRAP assays

In order to evaluate the antioxidant capacity, the ABTS and FRAP assays were performed. ABTS assay is based on the ability of antioxidant in sequestering the free radical $ABTS^{*}$, whereas FRAP assay measures the ferric reduction of 2,4,4-tripiridil-s-triazin (TPTZ).

ABTS Assay

Jejunal explants of piglets were homogenized in 500 µL of KCl (1.15%) using a tissue-tearor (Biospec) and centrifuged at 1000g for 10 min at 4°C, the supernatant was employed for measurement the antioxidant capacity of the explant. The solution of ABTS was prepared with 7mM of potassium persulfate diluted with phosphate buffer pH 7.4 to an absorbance of 0.7-0.8 in 730 nm. The supernatant was mixed on ABTS solution and after 6 min the absorbance was determined in 730 nm (Helios Alfa, Thermo Spectronic). Previously, a curve of trolox (1-25µM) was prepared and the results are presented as µM trolox equivalent per mg of tissue (intestinal explant) (Ivan et al., 2014).

FRAP assay

The samples of jejunal explants were homogenized in 500 µL of KCl (1.15%) using a Tissue-Tearor (Biospec) and centrifuged at 1000g for 10 min at 4°C, the supernatant was employed for measurement of the antioxidant capacity of the explant. The reaction consists in adding the supernatant to the FRAP reagent prepared with 0.3 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM hydrochloride acid and 20 mM ferric chloride. The FRAP reagent was

warmed to 37°C for 30 min. The absorbance was determined at 595 nm (Helios Alfa, Thermo Spectronic). Previously, a curve of trolox (0.5-20μM) was prepared and the results are presented as μM trolox equivalent per mg of tissue (intestinal explant) (Ivan et al., 2014).

2.8 Statistical analysis

The experimental design used was entirely randomized and consisted of six repetitions per treatment resulting in six replicates from each animal. A total of 42 explants were analyzed for each treatment. Data used for statistical analysis were represented as means ± standard deviation of the mean and analyzed using the free software R 3.2.2. The response variables were compared by ANOVA analysis. If significantly different ($p \leq 0.05$), the means were compared by Tukey's test for multiple comparisons ($p \leq 0.05$).

3. Results

3.1 Effects of MRS broth and DON alone and associated with heat inactivated *L. plantarum* and the culture supernatants on morphological score

Control, MRS broth and DON treated explants showed a mean morphological score of 36.50±2.28, 31.33±2.13 and 26.33±3.47, respectively. Meanwhile, the mean morphological score for LP1+DON, LP2+DON, CS1+DON and, CS2+DON were 29.89±2.90, 28.41±4.29, 36.65±2.12 and 36.83±2.45, respectively. DON, MRS broth, LP1+DON and, LP2+DON treated explants showed a significant reduction in the morphological score when compared to the control group ($p<0.001$). This decrease was associated to lesional changes characterized by villi fusion and atrophy, multifocal apical necrosis of enterocytes and, cuboid or flattened enterocytes. Moreover, explants incubated alone with MRS broth showed apical necrosis, cell debris in the lumen and, and enterocytes were mostly cuboidal (Figure 1).

3.2 Effects of MRS broth, DON alone and associated with heat inactivated *L. plantarum* and the culture supernatants on villi height and crypt depth

Mean values of morphometrical measures of villi height in control, MRS broth, DON, LP1+DON, LP2+DON, CS1+DON and, CS2+DON – treated explants were as follow:

163.29±31.69 μm; 162.51±21.90 μm; 126.27±14.23 μm; 120.36±10.95 μm; 118.34±12.11 μm; 281.66±38.94 μm and 290.80±42.23 μm, respectively. Morphometric analysis of crypth depth values of the same treatments were: 190.88±38.03 μm, 179.65±40.41 μm, 158.14±30.55 μm, 164.92±35.09 μm, 169.92±42.62 μm, 198.44±42.59 μm and 198.68±44.53 μm. Both *L. plantarum* culture supernatant plus DON groups differ statistically from DON treated explants ($p<0.005$) remaining similar to control showing an enhancement in villi height. On the other hand, DON, LP1+DON and LP2+DON remained statistically similar (Figure 2).

3.3 Effects of MRS broth, DON alone and associated with heat inactivated *L. plantarum* and the culture supernatants on goblet cells density

In addition, a significant reduction in the number of goblet cells was observed in explants treated with DON both in villi and crypts ($p<0.05$; $p<0.001$) compared to the control group. On the other hand, CS+DON–treated explants showed a significant increase in the mean number of the goblet cells in villus and crypt when compared to DON group. LP+DON groups remained statistically similar to DON (Figure 3).

3.4 Effects of MRS broth, DON alone and associated with heat inactivated *L. plantarum* and the culture supernatants on antioxidant activity

To evaluate the cellular oxidation-reduction state, we performed FRAP and ABTS assay in all treatments. After 4 hours of incubation, an increased antioxidant activity in CS2+DON group was observed in both assays when compared to the DON group. Nevertheless, both *L. plantarum* plus DON groups showed a trend to antoxidant activity. On the other hand, explants treated only with DON showed a non significant reduction in the antioxidant response when compared to the control group (Figure 4).

4. Discussion

DON toxicity has been an important issue of study during the last decade, since extensive proof of toxicity was achieved *in vivo* (Cheat et al., 2015), *in vitro* (Springler et al.,

2017) and *ex vivo* (Basso et al., 2013), affecting mostly intestinal epithelial (Bracarense et al., 2012) and immunity cells (Sugiyama et al., 2016). It was reported that DON exposure induces an oxidative stress in *in vivo* and *in vitro* conditions (Alizadeh et al., 2015; Van Le Thanh et al., 2016). In the present study we have evaluated the potential effects of *Lactobacillus plantarum* and the respective culture supernatants on reducing the toxic effects induced by DON. Our results demonstrated that the culture supernatant of a wild *Lactobacillus* strain has the capacity to improve intestinal tissue integrity and also the antioxidant response.

In this study, explants exposed to DON showed an increase in histological changes similar to previous studies (Silva et al., 2014). Surprisingly, treatment with both strains of heat-inactivated *L. plantarum* induced histological lesions comparable with explants exposed to DON. In addition, morphometrical aspects were also similar in these groups. Some strains of *L. plantarum* were associated with mycotoxins' binding (Abbes et al., 2016; Jebali et al., 2015; Zoghi et al., 2016) as well as others *Lactobacillus* species (El-Nezami et al., 1998; Haskard et al., 2001; Peltonen et al., 2001). In addition, it was demonstrated that *L. plantarum* was able to adhere to cells/other compounds via pyruvate dehydrogenase E_1 β-sub-unit (PDHB), a component of the pyruvate dehydrogenase complex and a factor contribuiting to fibronectinn-binding (Vastano et al., 2014). This, could explain the binding mechanism itself but clearly systematic studies are still needed to understand precise binding mechanisms to specific mycotoxins, such as deoxynivalenol. The ability to adhere to host intestinal mucosa is considered an important selection criteria for acid lactic bacteria strains intended for probiotic use and/or toxin detoxification (De Angelis et al., 2006; Dunne et al., 2001).

On the other hand, when explants were incubated with both *L. plantarum* culture supernatant for one hour previously to DON challenge, a significant increase in the morphological score (1.4 fold), villi height (2.3 fold), and the goblet cell density (2.5 fold) was observed when compared to DON. This decrease in DON toxicity was probably related to

soluble bioactive factors released by the bacteria into the culture. These factors could be associated with bacteriocins that are biologically active proteins or proteins complexes that improve intestinal barrier function, increase mucus secretion (Kumar et al., 2017) and display a bactericidal mode of action (Todorov, 2009). Small heat-stable bacteriocins membrane-active and heat resistant up to temperatures of 100°C are described (Klaenhammer, 1993) in *L. plantarum*. This could explain the effects observed even considering that both strains of *L. plantarum* used in the present study and their culture supernatant were submitted to high temperature levels. Although numerous bacteriocins produced by *L. plantarum* have been recorded, most cannot be classified for a lack of sufficient information (Todorov, 2009), but it is known that the majority of these bacteriocins are small heat-stable cationic peptides.

Furthermore, ABTS and FRAP antioxidant assays revealed a significant improvement in antioxidant activity in explants treated with *L. plantarum* CS2 plus DON. These results indicate that CS2 was able to mitigate/prevent oxidative stress toxicities levels raising to values comparable to control group. DON-dependent production of reactive oxygen species (ROS) has been reported in several studies (Costa et al., 2009; Ji et al., 1998; Sahu et al., 2008), these fact enhances lipid peroxidation that resulted from free radical-mediated toxicity. Apart from lipids, targets oxidative damage by these radicals also usually include critical biomolecules like proteins. It is hypothetized that DON alters mitochondrial dehydrogenases and destabilizates lysosomes, therefore the production of oxygen reactive species is through damage cells by a mitochondrial and lisosomal pathway (Koaudio et al., 2005).

In conclusion, CS of LP reveals an antioxidant and toxicity reducing capacity induced by deoxynivalenol, as well as an improvement in morphological aspects on piglets intestinal mucosa.

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Conflict of interest
The authors declare no conflict of interest.

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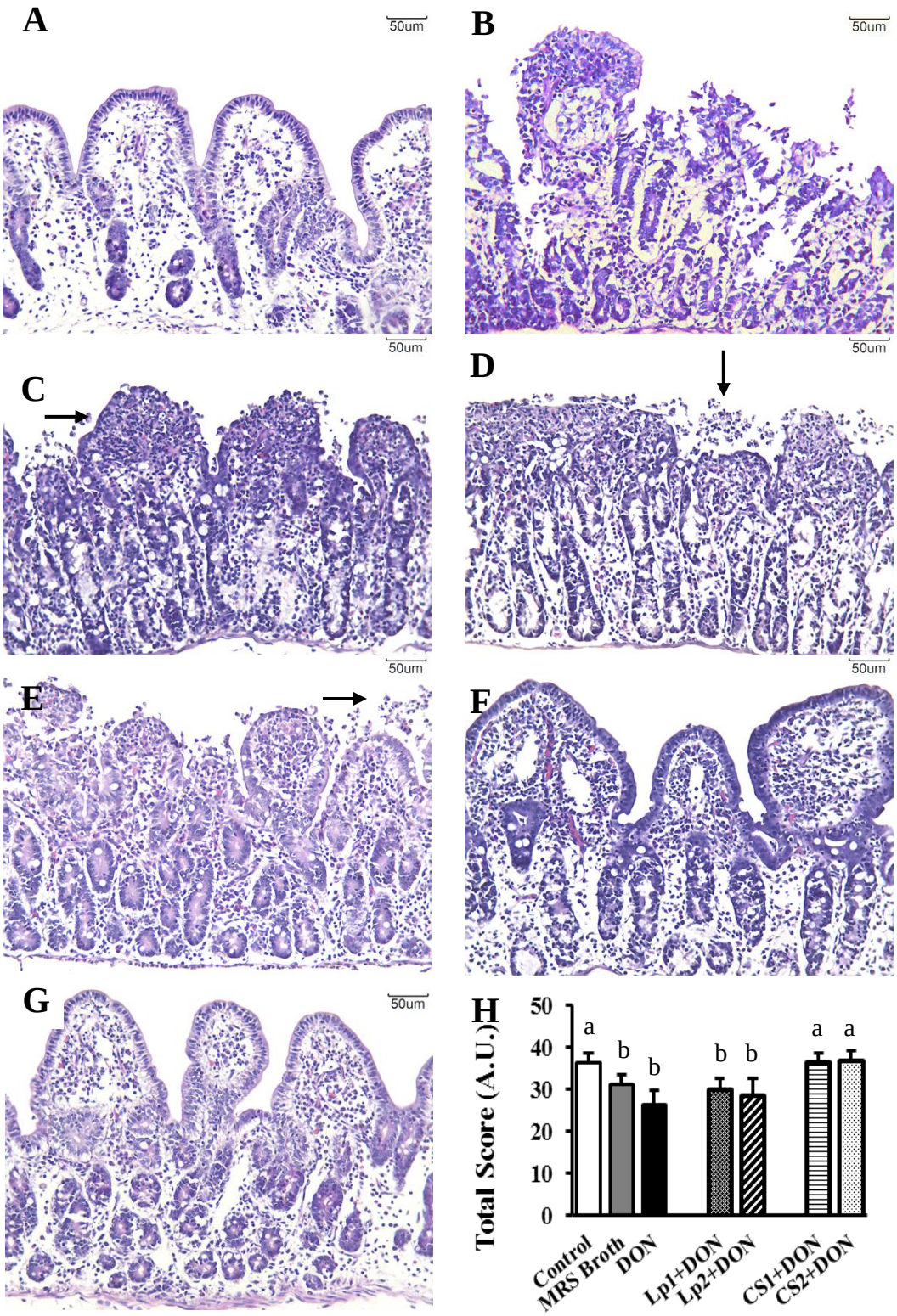

Figure 1. Effect of DON individual and combined with heat-inactivated *Lactobacillus plantarum* (LP) and its culture supernatant (CS) exposure on histology. Explants were exposed to culture medium (□) or culture medium with MRS broth (▤) culture medium with deoxynivalenol (DON) (■), or DON+LP1 (▩), or DON+LP2 (◪), or DON+CS1 (◪) or DON+CS2 (⊡). A) Control explants with normal intestinal morphology; B) MRS broth-exposed explant with cellular debris and areas with columnar epithelium; C) DON-exposed explant. Severe apical necrosis, villi atrophy and enterocyte flattening (arrow); D) DON+LP1-exposed explant and; E) DON+LP2-exposed explant. Severe loss of apical enterocytes and cellular debris (arrow); F) DON+CS1-exposed explant; G) DON+CS2-exposed explant. Villi with preserved morphology. HE. Bar 50 µm; H) Tissue scores of pig intestinal explants exposed to MRS broth, DON, DON+LP1, DON+LP2, DON+CS1 and DON+CS2. Values are means with the standard deviation of the mean represented by vertical bars (*n* 36 explants/treatment). [a,b] mean values with unlike letters were significantly different by Tukey's test ($p \leq 0.05$). AU=Arbitrary units.

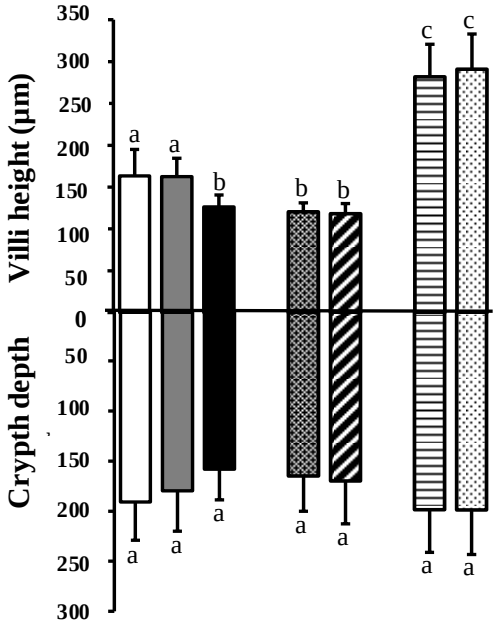

Figure 2. Effects of heat-inactivated *L. plantarum* and the culture supernatant exposure on villi height and crypt depth of jejunal explants of pigs. Values are mean height and depth (µm). Values are means with standard deviation of the mean represented by vertical bars (n 6 animals). Mean values with unlike letters were significantly different ($p<0.05$). Tukey test. Control(□). MRS broth (▣). DON (■), LP1+DON (▩), LP2+DON (▨), CS1+DON (▤),CS2+DON(▦).

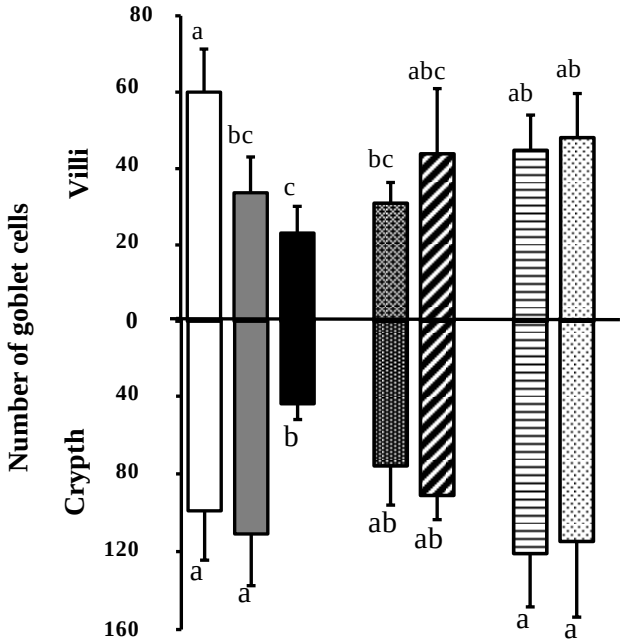

Figure 3. Effects of DON individual and combinated with heat inactivated *Lactobacillus plantarum* (LP) and its culture supernatant (CS) exposure on number of goblet cells. Explants exposed to DMEM for 4 hours (control) (□), MRS broth (▩), DON (■), LP1+DON (▩), LP2+DON (▨), CS1+DON (▤),CS2+DON(▦). Values are means number of goblet cells with their standard deviation of the mean represented by vertical bars (*n* 36 explants/treatment). [a,b,c] Mean values with unlike letters were significantly different by Tukey's test ($p \leq 0.05$).

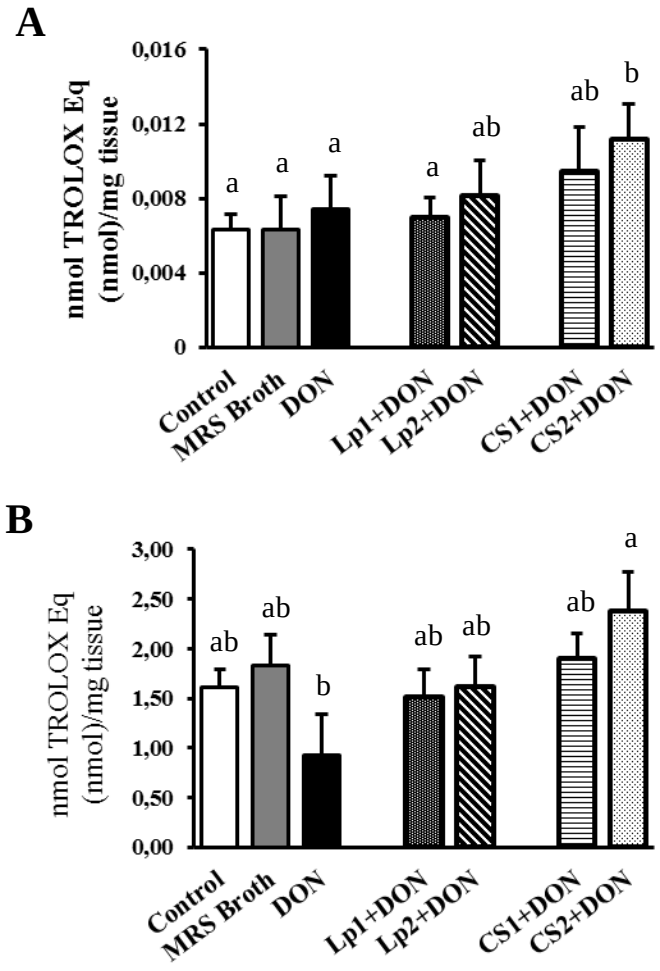

Figure 4. Antioxidant capacity of DON individual and combinated with heat inactivated *Lactobacillus plantarum* (LP) and its culture supernatant (CS) exposure. A) FRAP assay. B) ABTS assay. Explants exposed to DMEM for 4 hours (control) (□), MRS broth (▩), DON (■), LP1+DON (▩), LP2+DON (▨), CS1+DON (▤),CS2+DON(▦). Values are means number of antioxidant activity with their standard deviation of the mean represented by vertical bars (*n* 36 explants/treatment). [a,b,c] Mean values with unlike letters were significantly different by Tukey's test ($p \leq 0.05$).

5 CONCLUSION

- Heat-inactivated *Lactobacillus plantarum* (LP) culture supernatant (CS) improves intestinal epithelial morphology in histological, morphometrical and ultraestructural assessments, alone or in combination with deoxynivalenol (DON) in an *ex vivo* model.

- Heat-inactivated *Lactobacillus plantarum* (LP) and DON caused intestinal epithelial lesions, alone or combined, in histological, morphometrical and ultraestructural assessments, in an *ex vivo* model.

- The use of culture supernatant of lactobacilli is an interesting strategie to minimize the toxic effects of deoxynivalenol on intestinal health.

ANNEXS

ANNEX A

Submission instructions of *Journal of Natural Products*

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JOURNAL OF NATURAL PRODUCTS

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Contents (click on the topic)

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Abstract

The abstract, detailing, in one paragraph, the problem, experimental approach, major findings, and conclusions, should appear on the second page. It should be double spaced and should not exceed 200 words for Full Articles and Reviews or 100 words for Notes and Rapid Communications. Compounds mentioned in the abstract, and given as specific Arabic numerals that are bolded in the text, should also be accompanied in the abstract by the same bolded numerals. The abstract should be on a separate page and should be provided with the bolded and capitalized heading "ABSTRACT".

Introduction

The manuscript should include an untitled introduction stating the purpose of the investigation and relating the manuscript to similar research.

Results and Discussion

The "Results and Discussion" should be presented as a coherent whole section, in which the results are presented concisely. The discussion should interpret the results and relate them to existing knowledge in the field in as clear and brief a fashion as possible. Tables and figures

ANNEX B

Submission instructions of *Food and Chemical Toxicology*

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FOOD AND CHEMICAL TOXICOLOGY

AUTHOR INFORMATION PACK

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DESCRIPTION

Food and Chemical Toxicology (FCT), an internationally renowned journal, that publishes original research articles and reviews on **toxic effects**, in animals and humans, of natural or synthetic chemicals occurring in the human environment with particular emphasis on **food, drugs, and chemicals, including agricultural and industrial safety**, and **consumer product safety**. Areas such as safety evaluation of **novel foods and ingredients, biotechnologically-derived** products, and **nanomaterials** are included in the scope of the journal. FCT also encourages submission of papers on **inter-relationships between nutrition and toxicology** and on *in vitro* techniques, particularly those fostering the **3 Rs**.

The principal aim of the journal is to publish high impact, scholarly work and to serve as a multidisciplinary forum for research in toxicology. Papers submitted will be judged on the basis of scientific originality and contribution to the field, quality and subject matter. **Studies should address at least one of the following**: Adverse physiological/biochemical, or pathological changes induced by **specific defined** substances New techniques for assessing potential toxicity, including molecular biology Mechanisms underlying toxic phenomena Toxicological examinations of specific chemicals or consumer products, both those showing adverse effects and those demonstrating safety, that meet current standards of scientific acceptability

Authors must **clearly and briefly identify what novel toxic effect (s) or toxic mechanism (s)** of the chemical are being reported and what their **significance** is in the abstract. Furthermore, sufficient doses should be included in order to provide information on NOAEL/LOAEL values.

Manuscripts describing research involving the following areas will not be considered: materials/substances of only local interest materials/substances for which the chemical composition is not clearly defined only pharmacological properties, or potentially beneficial effects using in vitro or in vivo systems chemical analyses of toxins in foods without addressing the toxic implication to humans [risk assessment should be included] unrealistic human doses, inappropriate route of exposure, or in vitro experiments that do not reflect serum levels in humans

FCT is committed to the highest standards. Only papers that have not been previously published, that fit in the above mentioned scope, and that have been reviewed by experts in the field prior to publication will be accepted. Cover letters must state that the manuscript is new and original and not under consideration for publication elsewhere. Co-authors should be individuals who have contributed substantially to the content of the papers. All authors must declare any potential conflict of interest and all financial support.

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