Effect of *Lactobacillus* feed supplement in deoxynivalenol intoxicated piglets

Daniela Eliza Marin¹, Ionelia Ţăranu¹, Monica Moţiu¹, G. Manda²

¹National Institute for Research and Development in Biology and Animal Nutrition, Baloteşti, Romania
²National Institute “Victor Babes”, Bucureşti, Romania

**SUMMARY**

A feeding trial was conducted to evaluate the effect of a *Lactobacillus* polyculture (LB) on growth and immunological parameters of piglets fed a deoxynivalenol (DON) contaminated diet. A dose of DON (1.8 ppm) was included in a corn-soybean diet provided for *ad libitum* consumption to twelve weanling piglets for a period of 24 days. The piglets were randomly allotted to one of the following groups: control group (C), Lactobacillus group (LB), deoxynivalenol group (DON), deoxynivalenol and *Lactobacillus* group (DON + LB). During the experiment, 50 mL of the Lactobacillus polyculture (Suinlact® INCDBNA Balotesti, Romania) was given daily to the pigs from the LB and DON+LB groups. No effect of the treatment was observed concerning body and organ weight of the animals. Comparing with the control, DON induces an increase of: lymphocyte proliferation, liver cytokine synthesis (TNF-α and IL-8), intracellular oxidative activity of the granulocytes and IgM synthesis. DON doesn’t affect the proportion of the lymphocytes subsets in the blood of piglets from the DON group. The LB supplement, significantly alleviate the toxic effects of DON concerning the: lymphocyte proliferation, liver cytokine synthesis (TNF-α and IL-8), intracellular oxidative activity of the granulocytes. In conclusion, these results show that the LB polyculture could counteract some toxic effects of DON administration.

Keywords: piglets, deoxynivalenol, *Lactobacillus*, toxic effects

**INTRODUCTION**

Trichothecenes are mycotoxins produced by several species of *Fusarium* genera: *Trichotecium*, *Myroccotrichum*, *Trichoderma*, *Stachybotrys*, etc. *Fusarium* fungi are considered responsible for most of mycotoxins contaminations (Curtui et al., 1998). Under the climacteric conditions of Romania, wheat is a substrate potentially favourable for the development of *Fusarium* moulds. The most frequently found trichotenes is deoxynivalenol (DON), which was traced in raw materials in concentrations generally lower than 1000 ppb (Curtui et al., 1998), but which can get as high as 4000 – 5000 ppb. Pigs are exposed to
contamination with DON due to their diets rich in cereal grains. The pigs are more sensitive than poultry or ruminants to the action of this toxin (Rotter et al., 1996). At high concentrations (15-20 000mg/kg), DON results in feed refusal and vomiting (Bakan, 2001). Several studies conducted on piglets or growing pigs showed that lower concentrations of DON can induce modifications in the immune system (Rotter et al., 1996; Swamy et al., 2004).

The potential exposure to a mycotoxins contaminated feed of swine could draw negative effects and important economical loses. Recent studies (Charmley et al., 1995; Garcia et al., 1997) showed that economic losses occur at all levels of food and feed production, processing and distribution. For this reason, prevention, decontamination and detoxification of mycotoxins are issues of great importance.

A wide range of chemical, physical and biological approaches has been experienced in the attempt to reduce the toxicity of the mycotoxins. Although some chemical detoxification methods (ammonia, sodium bisulphite and calcium hydroxide treatments) are effective, they do not fulfil all the requirements, especially those concerning the safety of end products and the safeguarding of the nutritional characteristics of the treated foods and feeds (Devegowda et al., 1998; Piva et al., 1995). In present the nutritional approaches, such as supplementation of nutrients, food components, or additives with protective properties are assuming increasing interest. Lactobacilli take part from the additives group and they are Gram-positive facultative anaerobic probiotics. Probiotics have been shown to be effective in varied clinical conditions ranging from infantile diarrhoea, necrotizing enterocolitis, antibiotic-associated diarrhoea, inflammatory bowel disease to cancer, female urogenital infection and surgical infection (Gupta and Garg, 2009).

The transformation of some mycotoxins (DON, ZEN, OTA) on fermentation by some strains of yogurt bacteria and bifidobacteria has been repeatedly reported (Kollarczik et al., 1994; Skrinjar et al., 1996, Niderkorn et al., 2007). Deoxynivalenol, the best known trichotecenes is enzymatically reduced by an epoxidase of Eubacterium BBSH 797 to the nontoxic metabolite deepoxy-deoxynivalenol (DOM-1). The mode of action was proven in vitro and also in vivo by applying trichotecenes, and the detoxifying strain Eubacterium BBSH 797 has been the first microbe used in a mycotoxin deactivating in feed additive (Binder et al., 2000).

Few in vivo studies investigated the capacity of probiotics to alleviate the negative effects of mycotoxins and no study investigates the in vivo interaction between DON and LB.

For this purpose, the present study investigates the role of a Lactobacillus supplement in counteracting the DON effects in intoxicated weanling piglets.
MATERIAL AND METHODS

Experimental design

Twelve, 4-week-old, crossbred weanling piglets were studied for 24 days. They were given ad libitum access to water and feed and randomly allotted to one of the following groups: control group (C), Lactobacillus group (LB), deoxynivalenol group (DON), deoxynivalenol and Lactobacillus group (DON + LB). They were fed a maize-soybean-meal-based diet (Marin et al., 2002). Naturally contaminated DON maize was included in the diet in order to obtain the 1.8 ppm DON final concentration. The final concentration was verified by an ELISA assay using a Veratox kit (Neogen). 50 mL of the Lactobacillus product (Suinlact® INCDBNA Balotesti, Romania) was given daily to the pigs from the LB and DON+LB groups. Body weights and food consumption were recorded twice throughout the experiment (day 0 and day 24). Blood samples were aseptically collected in the day 24, by jugular vein puncture for immunological and biochemical analyses. At the end of the experiment, animals were slaughtered and organs were weighted and samples of liver were taken for the cytokine analysis.

Measurement of Total Immunoglobulin Subsets (IgG, IgM, IgA)

The blood was collected in vacutainer with Li-heparin tubes and centrifuged at 2500 rpm in order to obtain the plasma. The samples were frozen at -100°C until analyzed for biochemical parameters, immunoglobulins and ROS concentration. Total concentration of the immunoglobulin subsets was measured in plasma by ELISA (Bethyl). The plasma were diluted 1/60000, 1/6000, 1/4000 in Tris–buffered saline to detect IgG, IgM and IgA respectively, and processed according to the manufacturer’s instructions. Absorbance was read at 450 nm using a TECAN Sunrise microplate reader.

Intracellular oxidative activity of the granulocytes (PMN)

It was determined in blood collected from the jugular vein into heparinized Vacutainer tubes (Becton Dickinson, Plymouth, UK) by flow cytometry, using dihydrorhodamine 123 as maker according to the manufacturer’s instructions (BurstTest kit; OrpenGen Pharma). Briefly, 100µL blood/sample was stimulated with a bacterial antigen (contained in the kit) and incubated at 37°C for 10 min. Then, the substrate (dihydrorhodamine 123) was added and the samples were incubated for another 10 min at 37°C. The erythrocytes were then lysed using the kit buffer at room temperature for 20 min. The samples were centrifuged for 5 min at 250g and 40°C and the sediment was washed with the special solution of the kit, cooled on ice. In order to evaluate the cell viability, the samples were treated with propidium iodide for 10 min. The samples were read using a FACScan flow cytometer (Becton Dickinson), using the CellQuest soft.
Isolation and culture of porcine peripheral mononuclear cells

Blood was mixed with an equal volume of Dulbecco’s phosphate buffered saline – PBS (Sigma-Aldrich Chemical Co., St Quentin Fallavier, France), layer over Ficoll-Hypaque (density 1.077, Eurobio, Les Ulis, France) and centrifuged at 800g, 20 minutes at room temperature as already described (Dozois et al., 1997). Peripheral mononuclear cells PBMC were collected, washed twice in PBS and resuspended in RPMI-1640 (Eurobio) supplemented with 10% foetal calf serum (Hyclone, Perbio, Brebières, France), 2mM L-glutamine, 100 U/mL penicillin, 50 µg/mL streptomycin (Eurobio). Cells were counted and viability assessed using tripan blue (Eurobio).

Measurement of proliferation

Cell proliferation was evaluated by the incorporation of the tritiated thymidine (3H-Td) assay. PBMC, cultured at a density of 1 x 10^6 cells/well in 96-well flat-bottomed tissue plates (Falcon, Franklin Lakes, N.J.) were stimulated with 10 µg/mL concanavalin A (ConA) (Sigma). PBMC were incubated for 72 hours at 37°C and 5% CO₂. 6 hours before the end of the cultivation period, the cells were marked with 1µCi 3H-Td/sample. Cell suspension was filtered (Skatron filters), and the radioactivity was measured in scintillation liquid (4g PPO, 50mg POPOP/1L toluene) using a β-counter (Packard). Experimental results were expressed as count per minutes (cpm).

Fenotyping of T lymphocytes

The lymphocytes were phenotype by flow cytometry, using anti-pig mouse monoclonal antibodies anti-CD3ε marked with fluoresceine FITC and anti-CD3δ, anti-CD4α, anti-CD8α marked with fluoresceine PE (Becton Dickinson). Mouse antibody with isotype IgG2a,K or IgG2b,K marked with FITC and PE respectively, represented the isotype controls. The samples were analyzed using a FACScan flow cytometer (Becton Dickinson), using the soft CellQuest and the percentage of the lymphocytes TCD3+, TCD4+, TCD8+ were determined.

Measurement of cytokine synthesis in liver

5 g of frozen liver were homogenised in cold RIPA buffer (Igepal 1%, sodium deoxycholate 0.5%, SDS 0.1%) supplemented with antiproteases (Protease Inhibitor Cocktail, LaRoche). The liver extract was centrifuged at 1000g and the supernatant were frozen at -20°C, until analyzed for cytokine content by ELISA. Briefly, a purified fraction of anti-swine cytokines (R&D Systems): TNF-α(MAB6902) and IL-1 β(MAB6811) were used as capture antibody in conjunction with biotinylated anti-swine cytokines: TNF-α (BAF 690) and IL-1 β(BAF 681). Streptavidin-HRP (Biosource) and TMB were used for detection. Absorbance was read at 450 nm using the microplate reader.
Recombinant swine TNF-α and IL-1β were used as standards and results were expressed as picograms of cytokine/mL.

**Statistical analysis**
Student's t-tests were used to analyze the differences in term of body and organs weight, biochemical parameters, lymphocyte proliferation and phenotyping, cytokine and antibody synthesis between control and treated animals. The p values of 0.05 were considered significant.

**RESULTS AND DISCUSSION**
Lactic acid bacteria or their antifungal metabolites have been studied as natural preservatives to inhibit mycotoxigenic mould growth and mycotoxin production in recent years (Kebak et al., 2006). Lactic acid bacteria are of special interest as preservation organisms since they have a long history of use in food and they may be particularly useful in the preservation of foods from mould spoilage and mycotoxin contamination (Kebak et al., 2006). Also, consumption of lactic acid bacteria has been suggested to confer a range of health benefits including stimulation of the immune system and increased resistance to malignancy and infectious illness (Gilla et al., 2000). In this study we investigated the ability of LB probiotic to alleviate the toxic effects induced in piglets by DON.

**Effect of LB supplementation on the body and on the organs weight of the intoxicated animals**
We first investigated the effect of dietary treatment on clinical signs and animal performance. Piglets were fed with control or DON-contaminated diets (1.8 ppm) supplemented or not with LB product for a period of 4 wk. Control animals as well as piglets fed LB, DON, DON+LB appeared clinically normal during the whole experiment and no deaths resulted from the treatments. The treatments animal weight gains are reported in Fig. 1. Even that no effect of the treatments was observed in the treated animals, a light decrease of the body weight after DON administration could be observed in the DON group. The effect of the treatments on the organs weight is presented in Fig. 2. No significant difference between groups resulted after 24 days of treatment. Our results concerning DON intoxication with 1.8 ppm are similar with those obtained by Accensi et al. in 1996. In this experiment, piglets intoxicated with 0.28 – 0.84 ppm DON, did not present any alteration of the animal performances (feed intake and BW gain). Also, higher doses of DON (2,038 ppm) don’t affect the young swine performances (Pinton et al., 2004).
Effect of LB supplementation on the antibody synthesis of the intoxicated animals

To investigate the effect of LB probiotic on the humoral immune response in DON intoxicated piglets, total serum antibody levels (IgA, IgM and IgG) were measured by ELISA. As expected, the injections increased (P < 0.05) the antibody levels (data not shown). This increase was observed in the control piglets as well as in the treated animals (Table 1). When comparing with the control, LB induced an increase of the IgM (144.9%) and IgG synthesis (149.2%), while DON induces only an increase of the IgM (169.7%) synthesis. In the case of IgG synthesis, for the B+DON group the increase was of 169%, suggesting that the LB and DON treatment have a synergic effect. The IgA synthesis was not affected by the LB supplement or DON intoxication. Lower
doses than that used in our experiment (0.28 – 0.84 ppm DON) doesn’t affect the immunoglobulin subset concentration (Accensi et al., 2006). It seems that the IgM increase is due to the higher dose used in our experiment.

Table 1. Effect of dietary *Fusarium* toxins exposure and LB treatment on cytokines synthesis in liver

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>DON concentration (ppm)</th>
<th>0</th>
<th>0</th>
<th>1.4</th>
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<tbody>
<tr>
<td></td>
<td>LB probiotic (g)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>TNF-α (pg)</td>
<td>131.25 ± 20.6</td>
<td>102.94b ± 6.85</td>
<td>373.05c ± 79.1</td>
<td>114.30b ± 35.2</td>
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<tr>
<td>IL-8 (pg)</td>
<td>44.42a ± 8.9</td>
<td>68.97b ± 10.5</td>
<td>143.1c ± 22.6</td>
<td>72.43b ± 12.4</td>
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</tbody>
</table>

Results show means ± SEM for five different experiments.

Table 2. Effect of *Fusarium* toxins and LB supplementation on the blood immunoglobulin concentrations

<table>
<thead>
<tr>
<th>Antibody</th>
<th>DON concentration (ppm)</th>
<th>0</th>
<th>0</th>
<th>1.4</th>
<th>1.4</th>
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<tr>
<td></td>
<td>LB probiotic (g)</td>
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<td></td>
<td></td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>50</td>
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<tr>
<td>IgA (mg)</td>
<td>1.36 ± 0.6</td>
<td>1.26 ± 0.5</td>
<td>1.42 ± 0.8</td>
<td>1.09 ± 0.4</td>
<td></td>
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<tr>
<td>IgM (mg)</td>
<td>2.05 ± 0.4</td>
<td>2.97a ± 0.5</td>
<td>2.43a ± 0.8</td>
<td>2.63a ± 0.4</td>
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</tr>
<tr>
<td>IgG (mg)</td>
<td>6.36a ± 0.8</td>
<td>9.49b ± 0.5</td>
<td>7.80a ± 0.6</td>
<td>10.79b ± 6.2</td>
<td></td>
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</table>

Results show means ± SEM for five different experiments.

Effect of LB supplementation on the cytokine synthesis in the liver of the intoxicated animals

The ability of LB probiotic to alleviate the effect of DON on cytokine expression was investigated in liver samples (d 24) by ELISA. The results are presented in the Table 2. DON induce an increase of TNF-α (373 ± 79 pg) and IL-8 (143 ± 22 pg) synthesis comparing with the control (131 ± 20 pg for TNF-α and 44 ± 8 pg for IL-8). The addition of LB supplement induce a significant decrease of synthesis of the investigated cytokines in the liver of the weanling piglets, with values more closed to those observed in the control. Thus, the LB probiotic could alleviate the negative effect of DON on the cytokine synthesis. Similar results were obtained by Li et al. (2009) in an experiment realized in gastrostomy-fed rat. They showed that a strain of *Lactobacillus rhamnosus* decreased LPS-induced IL-8 production in liver.
Effect of LB supplementation on the lymphocyte proliferation of the intoxicated animals

Porcine lymphocyte proliferation after ConA stimulation in control and treated animals was then evaluated. We first determined the basal proliferation of the cells by measuring the tritiated thymidine assay (Fig. 3, bars graph). Then we analyzed the proliferation after the Con A stimulation. As expected, ConA stimulation significantly increased tritiated thymidine incorporation, reflecting an increased PBMC proliferation (Fig.3, line graph). As shown in the Fig.1, DON significantly increases the basal proliferation as well as the proliferation of the stimulated cells. Also, the lymphocytes from the animals that ingested a DON contaminated feed (2.038 ppm) during the growing phase had a higher proliferation capacity (Pinton et al., 2004). In our experiment, the LB administration decrease significantly the PBMC proliferation, and the values obtained for the DON+LB group are similar with that obtained for the control.

![Fig.3. Ex vivo measurement of PBMC proliferation](image)

Effect of LB supplementation on the fenotyping of T lymphocytes of the intoxicated animals

In order to better understand the effect of DON and LB supplementation on lymphocyte proliferation, the different lymphocyte subsets was determined by flow cytometry analysis. The effect of LB supplementation on the fenotyping of T lymphocytes of the intoxicated animals is presented in the Fig. 4. Intoxication with DON doesn’t affect the lymphocytes subsets in the blood of piglets from the DON group. By contrast, in mice, a single dose of 12.5 mg/kg body of DON induced a significant decrease of the CD4⁺CD8⁺ thymus T cells after 12 or 24 h of exposure, when compared with the control (Islam et al., 2002). The supplementation with LB induce an increase of the CD4⁺ and CD8⁺ subpopulation in the LB group, and of the CD3⁺ and CD8⁺ in the DON+LB group.
Fig.4. Change in the percentage of T-lymphocyte subsets

Effect of LB supplementation on the intracellular oxidative activity of the blood polymorphonuclear cells

Intracellular oxidative activity of the granulocytes in control and treated animals was determined in blood by flow cytometry assay. The results are presented in the Fig. 5. No effect was observed in the absence of *E. coli* stimulation (control bars). Stimulation with *E. coli* determined an increase of the intracellular oxidative activity of the granulocytes in the DON group (70.9%), comparative with 22.6% in control group. The LB treatment alleviates the DON effect, decreasing the intracellular oxidative activity to 36.3%.

Fig.5. Intracellular respiratory burst developed by blood PMN under basal and *E. coli* activated conditions
CONCLUSIONS

This study focused on the effect of a Lactobacillus supplement on the negative effects induced by DON in weanling piglets. Our results showed that LB probiotic has a beneficial effect on some parameters of the immune response (lymphocyte proliferation, cytokine synthesis in liver or intracellular oxidative activity) that have been affected after 24 days of intoxication with DON. This study was supported by National Program for Research Development and Innovation, (contract CEEX 25/2005).

REFERENCES


vivo following coexposure to lipopolysaccharide and vomitoxin (deoxynivalenol) Toxicology and Applied Pharmacology 187, 69–79.