# Effect of the grape seed meal administration on inflammation and oxidative stress in the spleen of piglets fed aflatoxin B1

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SUMMARY

Aflatoxin B1 is considered to be one of the most potent natural toxins and exposure to high concentrations of aflatoxin results in liver necrosis, which subsequently develops into cirrhosis or hepatocarcinoma. Grape seed meal (GSM) represents a potentially valuable source of phenolic antioxidants, which can have technological applications as feed additives and possible nutritional and health benefits. Due to the potential ability of grape pomace to bind different mycotoxin in vitro experiments and its well-known health benefits, the objective of the present work was to explore the benefits of the use of grape pomace seed as a low-cost bio-sorbent for AFB1 decontamination. Twenty-four cross-bred TOPIGS-40 weaned piglets were distributed to one of four groups: control group, AFB1 group (diet contaminated with 320 μg AFB1/kg feed), GSM group (diet with 8% GSM), AFB1+GSM group (320 μg AFB1/kg feed + 8% GSM). Our study has demonstrated the capacity of AFB1 to induce inflammation and oxidative stress in spleen when administered for 30 days at a level of 320 μg/kg feed. GSM administration has a moderate effect in reducing the inflammation and oxidative stress induced by the toxin, suggesting that higher level of GSM inclusion is needed for a positive effect.

**Keywords**: aflatoxin B1, piglets, inflammation, oxidative stress, grape seed meal

## INTRODUCTION

Aflatoxins (AF) are the most widespread mycotoxins in food, considered by the Food and Drug Administration (FDA) to be unavoidable contaminants (Williams et al., 2004). Aflatoxins generally contaminate foods from hot and humid regions (South America, Africa, Asia). However, during the last years, cereals contamination with aflatoxin was increased in Europe due to climate change (Battilani et al., 2016).

Aflatoxins can contaminate cereals (corn, wheat, barley, oats, rice), cereal products, oilseeds (soy), peanuts and derivatives (peanuts, peanut butter, pistachios), vegetables (potatoes, lentils, peppers), fruits dehydrated (figs). Aflatoxin B1 is the most toxic and is considered to be one of the most potent natural toxins being classified by the International Agency for Research on Cancer (IARC, 1993) in group 1B of human carcinogenic substances.

Exposure to high concentrations of aflatoxin results in liver necrosis, which subsequently develops into cirrhosis or hepatocarcinoma (Schoental, 1967). Acute hepatic crisis is associated with hemorrhage, edema, impaired digestion, changes in nutrient uptake and/or metabolism, neuropathy and / or coma.

AFB1 is immunotoxic to animals and a suspected immunosuppressant in humans. Recent studies, (Zhu et al., 2017) have shown that AFB1 caused slight congestion and lymphocytic depletion in the spleen. Moreover, quantitative real-time PCR analysis revealed that AFB1 induced the elevated mRNA expression of genes involved in apoptosis and inflammation (Fas, FasL, TNF-α, TNF-R1, Caspase-3, Caspase-8, Caspase-10, Grp78 and Grp94) in the spleen of chicken.

Physical, chemical and biological methods were used in the attempt to reduce the mycotoxins negative effect. Except for activated carbon, most substances (clays) used as mycotoxin binders to decontaminate mycotoxin-contaminated feeds fail in sequestering structurally different mycotoxins (Kolosova and Stroka, 2011). Activated carbon completely prevented the absorption of deoxynivalenolin pigs received deoxynivalenol alone or in combination with active carbon (Devreese et al., 2014).

They appear to bind to only a limited group of mycotoxins (mainly aflatoxins) while showing very little or no binding to others. Therefore, feed additives acting as multi-mycotoxin binders are sought.

Food and agricultural waste is a growing problem that, if not properly addressed, has negative effects on the economy, environment, and human health (Lucarini et al., 2018). Grape pomace represents a potentially valuable source of phenolic antioxidants, which can have technological applications as feed additives and possible nutritional and health benefits (Pinelo et al., 2006). In vitro adsorption experiments using grape pomace showed that this pomace is able to sequester rapidly and simultaneously different mycotoxins, aflatoxin B1 (AFB1) being the most adsorbed mycotoxin (Avantaggiato et al., 2014).

Due to the potential ability of grape pomace to bind different mycotoxin in vitro experiments and its well-known health benefits, the objective of the present work was to explore the benefits of the use of grape pomace seed as a low-cost bio-sorbent for AFB1 decontamination and the effect on the immune response in the spleen as the principal immune organ.

## MATERIAL AND METHODS

### *Animal and experimental treatments*

Twenty four cross-bred TOPIGS-40 weaned piglets with an average body weight of 9.13 ± 0.03 were distributed to one of four groups: control group (normal compound feed for starter piglets), AFB1 group (diet contaminated with 320 ppb pure AFB1), GSM group (diet with 8% of grape seed meal), AFB1+GSM group (diet with 8% of grape seed meal and 320 ppb AFB1). Animals were cared for in accordance with the Romanian Law 206/ 2004 and the EU Council Directive 98/58/EC for handling and protection of animals used for experimental purposes.

The grape seed meal was provided by a local commercial S.C. OLEOMET-S.R.L., Bucuresti, Romania and included in the GSM and GSM+AFB1 diets. Identification of different classes of polyphenols and polyunsaturated fatty acids (PUFA) of the diets was measured by HPLC-DAD-MS and gas chromatography.

High purity AFB1 (FERMENTEC, Jerusalem, Israel) was mixed into basal diet in order to provide a concentration of 320 ppb AFB1 in the diets (AFB1 and AFB1+GSM groups). The animals were individually identified by ear tag, housed in pens and fed with experimental diets for 30 days. They had free access to feed and water every day of the experimental period. All diets were formulated to meet specific requirements for weaning feed as indicated by NRC (2012) and the diet composition was presented in a previous study (Taranu et al., 2019). To prepare the AFB1 contaminated diet 50 mg toxin was dissolved in DMSO (dimethyl sulfoxide) and mixed into basal diet to provide a feed diet containing 320 μg/kg feed. At the end of the experiment pigs were killed and spleen samples were collected; organs aliquots were perfused with ice-cold saline solution to remove blood and then stored at−80 °C until analyzed for inflammatory as well as for antioxidant indices.

### *Antioxidant status*

Total antioxidant capacity (TCA) and the activity of antioxidant enzymes, superoxide dismutase (SOD), gluthathione peroxidase (GPx) and catalase (CAT) in plasma and organs were assessed by using Cayman kits according to the manufacturers' instructions as described by Taranu et al., (2019). The level of lipid peroxidation in hepatic tissue was evaluated by measuring thiobarbituric acid-reactive substances (TBARS) as already described by Taranu et al., (2018).

*Inflammatory cytokine synthesis*

Supernatants from tissue lysate prepared as described by Pistol et al., (2014) were used to perform ELISA measurement by using IL-1β, IL-6, IL-8, TNF-α, IFN-γ cytokines kits (R&D Systems, Minneapolis, USA) according to manufacturer's instructions as described by Taranu et al., (2014). Data were analyzed against the linear portion of the generated standard curve. Recombinant swine IL-1β, IL-6, IL-8, TNF-α and IFN-γ protein were used as standards. Data were analyzed against the linear portion of the generated standard curve.

*Gene expression level of pro-inflammatory cytokines*

Frozen spleen tissue samples (50mg) were disrupted and homogenized in TRI Reagent (Sigma-Aldrich, Germany) using an Ultra-Turrax homogenizer (IKA, Germany). The total RNA was extracted in chloroform-isopropanol and cDNA was generated using total cell RNA and M-MuLV Reverse Transcriptase kit, as already described by Marin et al. (2017). To evaluate the gene expression of inflammatory cytokines (IL-1beta and TNF-alpha) the qPCR reaction was performed in Rotor-Gene-Q (QIAGEN GmbH, Germany) machine using 25ng cDNA, 10µl SYBR Green qPCR Master Mix (Applied Biosystems, USA) and 0.3µM each of gene-specific primer using the PCR cycling conditions were described in Taranu et al., (2015). The nucleotide sequences of the primers used in these experiments were published by Marin et al (2019); Taranu et al., (2018). The relative product levels were quantified using the 2(−ΔΔ CT) method (Bustin et al., 2009). Two reference genes, RPL32 and HGPRT (selected from a panel of four references genes, using NormFinder software) were used for data normalization. The results were expressed as relative fold change (Fc) compared with control animals.

*Statistical analyses*

The statistical differences between treatments for all parameters analyzed was assessed by using OneWay ANOVA & Student's t-test (SAS Analytics, USA). Further differences between means were determined by Fisher's procedure of the least square difference. Values of P lower than 0.05 were considered significant, and P values between 0.051-0.09 were considered as tendency.

## RESULTS AND DISCUSSION

Grape seeds are a by-product of the pressing of grapes (Vitis vinifera L.) for making wine or grape juice. Grape seed oil meal is the by-product of oil extraction from grape seeds. Grape seeds and grape seed oil meal are fibrous,

tannin-rich by-products and lately, these products have received renewed interest due to their potential as sources of polyunsaturated fatty acids and beneficial antioxidants. Indeed, inclusion of GSM meal increased the crude fat and crude fiber of GSM experimental diet as already described by Taranu et al., (2019). Also, the concentration of polyphenols and the antioxidant activity of GSM diet were also higher than that of control diet as well as the concentration of the omega-6 polyunsaturated fatty acids.

In pigs, dietary supplementation of a polyphenol rich GSGME suppresses the activity of NF-κB in the duodenal mucosa of pigs and thus might provide a useful dietary strategy to inhibit inflammation in the gut frequently occurring in pigs (Gessner et al., 2013).

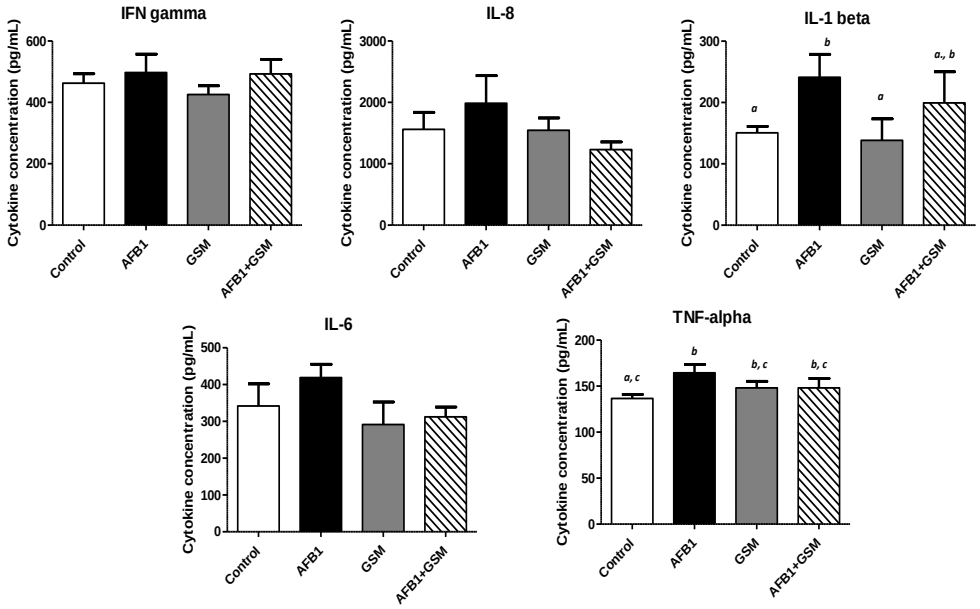


**Figure 1.** Effect of AFB1 and GSM exposure on cytokine synthesis

As, IL-1 beta and TNF alpha synthesis was significantly increased by the exposure to AFB1 as markers of inflammations, we have further investigated the effect of administration of a diet contaminated with AFB1 in the presence or not of GSM.

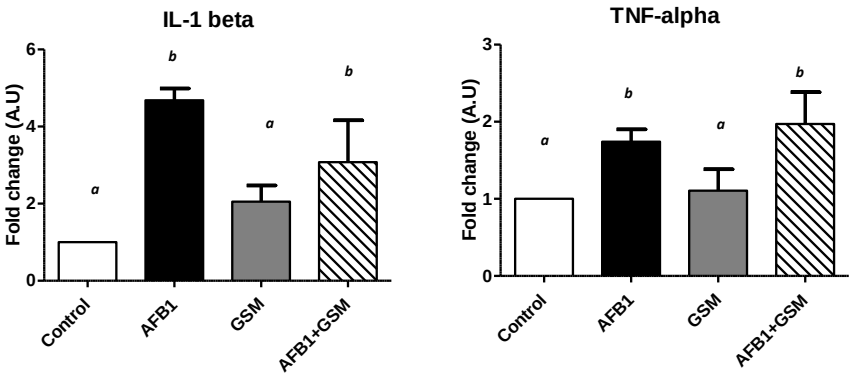


**Figure 2.** Effect of AFB1 and GSM exposure on the expression of pro-inflammatory cytokines

As expected, both expression of IL-1 beta and TNF-alpha was increased by AFB1 by 4.69 and 1.71 times respectively as compared with the control. However, GSM administration was not able to reduce the AFB1 induced inflammation, as no significant effect was observed for the expression of both cytokines when comparing the AFB1 group with AFB1+GSM group.

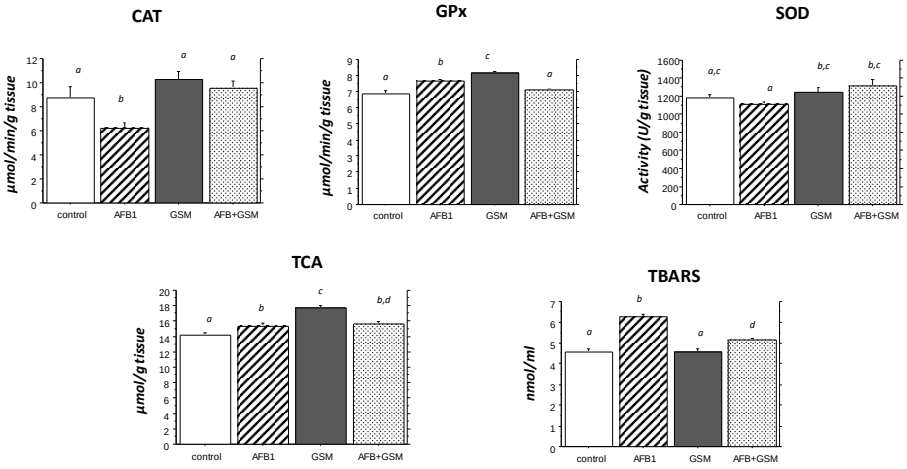


**Figure 3.** Effect of AFB1 and GSM exposure on oxidative stress

Many studies have shown the pro-inflammatory effect of AFB1 especially at hepatic level. Thus, AFB1 increased the secretion of IL-1β in rat serum, and was responsible for hepatic hemorrhage and inflammatory

infiltration (Akinrinmade et al., 2016). Also, in the liver tissue of zebrafish, AFB1 up-regulated the expression of cyclo-oxygenase-2 (COX-2) and interleukin-6 (IL-6) via activating nuclear factor-κB (NF-κB) signaling pathway (Lu et al., 2013).

In our study, AFB1 induces a significant increase of pro-inflammatory cytokines IL-1 beta and TNF-alpha by 60.1% and 20.26% respectively. AFB1 seems to increase the synthesis of the other investigated cytokines: IFN gamma, IL-8 and IL-6, but this increase was not significant ($p=0.57$; $p=0.30$; $p=0.27$). As shown in the Fig. 1, GSM induced little or no effect on the synthesis of pro-inflammatory cytokines. The concomitant administration of GSM and AFB1 induced a little decrease of the inflammation induced by AFB1 for all investigated inflammatory cytokines, but this tended to be significant only in the case of IL-6 ($p=0.077$).

Oxidative stress is a disturbance in the balance between the production of reactive oxygen species (ROS) and the antioxidant defense, and is often associated with toxin exposure (Nita and Grzybowski 2016). ROS can stimulate proliferation, invasiveness, angiogenesis and metastasis and inhibit apoptosis being considered as a pro-neoplastic factor (Halliwell 2007). In our study, AFB1 has significantly decreased the activity of antioxidant enzymes: catalase, while increased the lipid peroxidation as resulted from the assessment of substances reactive to thiobarbituric acid.

However, other indicators of oxidative stress, GPx and total antioxidant status are significantly increased by AFB1, as an attempt of the organism to better respond to the toxic effect of AFB1. Administration of GSM to the piglets intoxicated with AFB1, was able to significantly increase the catalase and superoxide dismutase activity and to decrease the lipid oxidation and GPx activity, while GSM has no effect on total antioxidant status. Similarly, resveratrol, a type of natural phenol found in grapes was shown to inhibit oxidative stress induced with aflatoxin B1 in bovine mammary epithelial cells (Zhou et al., 2019).

## Conclusions

Our study has demonstrated the capacity of AFB1 to induce inflammation and oxidative stress in spleen when administered for 30 days at a level of 320 μg/kg feed. GSM administration has a moderate effect in reducing the inflammation and oxidative stress induced by the toxin, suggesting that higher level of GSM inclusion is needed for a positive effect.

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