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Assessment of the efficacy of a grape seed waste in counteracting the changes induced by aflatoxin B1 contaminated diet on performance, plasma, liver and intestinal tissues of pigs after weaning

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ABSTRACT

The aim of this study was to investigate the potential of a grape seed byproduct to mitigate the harmful damage produced by aflatoxin B1 (AFB1) at systemic level in plasma and liver as well as at local level in the gastrointestinal tract in weaned piglets. Twenty four crossbred pigs (TOPIG) were randomly assigned to one of four experimental diets: 1)- control diet (normal compound feed for starter piglets without mycotoxin), 2)- AFB1 diet (compound feed contaminated with 320 ppb pure AFB1), 3)- GS diet (compound feed including 8% of grape seed meal), 4)- AFB1 + GS diet (compound feed containing 8% of grape seed meal contaminated with 320 ppb AFB1) for 30 days. The results showed that pigs fed AFB1 diet had altered performance (−25.1%), increased the thiobarbituric substances (TBARS) concentration wile reduced total antioxidant capacity and activity of antioxidant enzymes (CAT, SOD and GPx) in plasma and organs. AFB1 produced a dual effect on inflammatory response by increasing the level of pro-inflammatory cytokines in liver and colon and decreasing these cytokines in duodenum. The inclusion of grape seed in the diet of AFB1 intoxicated pigs enhanced the antioxidant enzymes activity, decreased the pro-inflammatory cytokines and TBARS level and ameliorated the growth performance of AFB1-treated animals. These findings suggest that grape waste is a promising feed source in counteracting the harmful effect of aflatoxin B1.

1. Introduction

Mycotoxins are toxic chemical structure produced by moulds/fungi in different certain conditions which contaminate all stages of feed and food chain (Grenier et al., 2012). Their role is not yet elucidated in the physiology of fungi, but it is consider that fungi use them probably as a strategy to improve environment for further proliferation (Fox and Howlett, 2008). Mycotoxins toxicity varies considerable depending on the host sensitivity, metabolism and defence mechanism (Hussein and Brasel, 2001). There are more than 300 known mycotoxins, but the most common considered as a major risk factor for human and animal health are aflatoxins, fumonisins, ochratoxins, zearalenone, thrichotecenes etc. Aflatoxins (AFs) are a group of mycotoxins produced by species of *Aspergilus sp.* mainly *Aspergilus flavus, Aspergilus parasiticus* and *Aspergillus nomius* (Kos et al., 2013). Their toxic potential is very high being mutagenic, teratogenic and carcinogenic. Among the eighteen identified AFs, aflatoxin B1 (AFB1) presents the highest toxic potential being classified as primary carcinogenic compounds by the International Agency for Research on Cancer based on the development of liver cancer (Cancer, 2012; Kos et al., 2013; Nurul Adilah et al., 2018). Indeed, liver is the main target of AFB1 toxicity where the toxin is biotransformed through the Cytochrome p450 (phase-I) enzymes in highly toxic compounds such as AFB1-8-9-epoxide, aflatoxin M1 and B2a (Cancer, 2012; Jager et al., 2016; Muhammad and Wang, 2018). High exposure to AFB1 especially in developing country can increase the incidence of hepatocellular carcinoma (Kucukcakan and Hayrulai-Musliu, 2015). It was reported that exposure to AFB1 led *in vivo* to liver injury, connective tissue proliferation, liver cirrhosis and finally hepatocellular carcinoma (Kucukcakan and Hayrulai-Musliu, 2015; Long XD, 2014; Wang et al., 2019) and had genotoxic effect in hepatocytes (Hep-G2) cell line (Zheng et al., 2018).

As the main site of absorption, intestine is another important organ affected by exposure to AFs toxins which are absorbed through the gastrointestinal epithelium (Akinrinmade et al., 2016). Literature data described different adverse effect on intestine (Grenier et al., 2012; Jiang et al., 2015; Taranu et al., 2015). For example, AFB1 diminished

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the intestinal Caco-2 cells' integrity after AFB1 treatment (Romero et al., 2016) and in pig, one of the most sensitive animals to mycotoxins due to its diet rich in cereals, the cellular proliferation and synthesis of IL-8 were affected in intestinal IPEC-1 cell line (del Rio Garcia et al., 2007) by AFB1. Study of (Clarke et al., 2018) showed also that in finisher pigs offered low quality diets naturally contaminated with high level of zearalenone, aflatoxin and ochratoxin, digestibility was significantly reduced through the reduction of the gene expression involved in intestinal nutrient transport. Enhance inflammation by increasing expression of key markers of inflammation and intestinal integrity like, tumour necrosis factor (TNF) in the duodenum and colon and claudin 2 (CLDN2) in the duodenum (Clarke et al., 2018) was observed.

It was very recently (Jebali et al., 2018) reported that in other species, mice for example, AFB1 or AFM1 increased DNA fragmentation and modulated the gene expression of important molecules with key role in apoptosis and inflammation in intestine of mice treated with 100 μg/kg b.w. toxins (Jebali et al., 2018) and (Jiang et al., 2015) demonstrated that AFB1 (0.6 mg/kg) in the broiler diet affected the immune function of the intestine by reducing the percentages of T-cell subsets and the cytokine gene expression (Jiang et al., 2015).

Taken into consideration the risk for animal and human as well as the economic losses produced by aflatoxin in livestock, detoxification strategies have been developed in time and the procedures can be grouped into physical, chemical and biological methods (Jard et al., 2011; Peng et al., 2014; Zhu et al., 2017). In the last decade there has been an increasing interest in the use of biodegradation agents such as probiotics (bacteria and yeast) and prebiotics (carbohydrates) in aflatoxin mitigation effects. In pigs, it was reported for instance, that prebiotics and probiotics are effective in detoxifying aflatoxins and trichothecenes, but the precise mechanism (transformation, toxicity of transformed products), need to be elucidated. It was also shown that natural phytochemicals, plant extracts and spice oils, antioxidant compounds (selenium, vitamins, provitamins), food components (phenolic compounds, coumarin, chlorophyll and its derivatives, fructose) could be effective in counteracting the toxic effect of aflatoxins (EFSA, 2009; Galvano et al., 2001; Jiang et al., 2015; Peng et al., 2014; Tulayakul et al., 2007).

It is clear that much more work has to be done *in vivo* with the above mentioned mitigating agents, to elucidate their mode of action and their economic and technical ability to counteract the effects of mycotoxins in food and feed.

More recently, waste grape (pomace or seed) has been investigated as a new biosorbent for removing mycotoxins from liquid media (Avantaggiato et al., 2014). Different mycotoxins were successfully sequestered in *vitro* absorption experiments, aflatoxin B1 being the most adsorbed mycotoxin followed by zearalenone, ochratoxin A and fumonisin B1. The advantage of grape waste consists in its complex composition combining antioxidant potency and adsorbent characteristics (Avantaggiato et al., 2014). Thus, beside the adsorbant properties it could manifest protective effects against mycotoxins toxicity due to its antioxidant capacity similar with that of other substances called by EFSA "interacting agents" (EFSA, 2009) which interact with the mechanism of action of mycotoxins. It was reported that bioactive compounds from grape waste can improve the antioxidant system in organs and could prevent drug-induced liver and kidney of mice (Bagchi et al., 2000; Long et al., 2016). Previous studies of (Ali Rajput et al., 2017) showed also that grape seed proanthocyanidin extract mitigated AFB1-induced toxicity in broiler. For pig, beside the study of (Gambacorta et al., 2016) which demonstrates that grape pomace reduced the gastrointestinal absorption of mycotoxins, there are no investigations on the efficacy of grape waste as mitigating agent. Therefore, the objective of this study was to investigate the capacity of a diet including 8% grape seed meal to mitigate the effect of aflatoxin B1 on performance, inflammatory and antioxidant status in plasma and tissue (duodenum, colon and liver) of piglets after weaning exposed to feed contaminated with 320 ppb AFB1. Weaning is a critical period especially for pig during which transitory physiological and immunological disturbances like an increase in pro-inflammatory cytokines and oxidative (Brunton, 2013; Pluske, 2013) stress could be aggravated by a mycotoxin contaminated diet.

2. Material and methods

2.1. Animal and experimental treatments

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 study protocol was approved by the Ethical Committee of the National Research-Development Institute for Animal Nutrition and Biology, Balotesti, Romania (Ethical Committee no. 52/2014). No veterinary drugs were used during the experimental period.

Twenty four cross-bred TOPIGS-40 hybrid [♀ Large White × Hybrid (Large White × Pietrain) × ♂ Talent, mainly Duroc] weaned piglets with an average body weight of 9.13 ± 0.03 were allocated to one of four diets: 1)- control diet (normal compound feed for starter piglets without mycotoxin), 2)- AFB1 diet (compound feed contaminated with 320 ppb pure AFB1), 3)- GS diet (compound feed including 8% of grape seed meal), 4)- AFB1 + GS diet (compound feed containing 8% of grape seed meal contaminated with 320 ppb AFB1). The grape seed meal was provided by a local commercial S.C. OLEOMET-S.R.L., Bucuresti, Romania and AFB1 (> 98% pure by high performance liquid chromatography [HPLC]) used to contaminate the diet 2 and diet 4 (AFB1 and GS + AFB1 respectively) was obtained from FERMENTEC (Jerusalem, Israel) and incorporated into pig feed to provide a diet with 320 ppb AFB1.

The animals individually identified by ear tag were housed in pens (two replicates of 3 pigs per pen per treatment) and fed with experimental diets for 30 days. They had free access to feed and water every day of the experimental period. All diets was formulated to meet specific requirements for weaning feed as indicated by NRC (2012); control diet contained corn (67.47%), soya meal (19%), corn gluten (4%), milk replacer (5%), L-lysine (0.4%), DL-methionine (0.1%), limestone (1.46%), monocalcium phosphate (1.37%), salt (0.1%), choline premixes (0.1%) and 1% vitamin-mineral premixes (10,000 IU vitamin A; 2000 IU vitamin D; 30 IU vitamin E; 2 mg vitamin K; 1.96 mg vitamin B1; 3.84 mg vitamin B2; 14.85 mg pantothenic acid; 19.2 mg nicotinic acid; 2.94 mg vitamin B6; 0.98 mg folic acid; 0.03 mg vitamin B12; 0.06 mg biotin; 24.5 mg vitamin C; 40.3 mg Mn; 100 mg Fe; 100 mg Cu; 100 mg Zn; 0·38 mg I; 0·23 mg Se). The inclusion of grape seed meal was made by replacing corn. To prepare the AFB1 contaminated diet 50 mg toxin was dissolved in DMSO (dymethil sulfoxide) and mixed into basal diet to provide a feed diet containing 320 μg/kg.

The body weight was recorded at day 0 and at day 30 for each animal and feed intake was recorded daily per pen. At the end of the experiment pigs were sacrificed and blood and organ (duodenum, colon, liver) samples were collected; organs aliquots were perfused with ice-cold saline solution to remove blood and then stored at −80 °C until analysed for inflammatory as well as for antioxidant indices. Blood was processed to obtain plasma as described in subchapter liver biomarkers analysis.

2.2. Chemical characterization of the diets

Feed samples of control and experimental diets were analysed for nutrients content, dry matter, crude protein, crude fat, crude fibre and ash according to the International Standard Organization methods (SR ISO 6496/2001, Standardized Bulletin (2010). http://www.asro.ro). Total polyphenol content, identification of different classes of polyphenols and polyunsaturated fatty acids (PUFA) of the diets was measured by Folin-Ciocalteu reaction, HPLC-DAD-MS and gas

chromatography as described by (Taranu et al. 2014, 2018). Diets antioxidant activity was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, DPPH as described previously by (del Rio Garcia et al., 2007).

2.3. Mycotoxin analysis

Experimental feed ingredients and feed compound were screened by ELISA for mycotoxins (AFB1, ZEA, DON, OTA, FBs) content using ELISA kits Veratox (Neogen, Lansing, MI, USA) according to the manufacturer's instructions, and the following quality parameters: limit of detection (LOD) between 0.1 and 100 ppb (0.5ppb-AFB1; 5 ppb-ZEA; 100ppb-DON; 1 ppb ppb-OTA, 0.2ppm-FBs) and limit of quantification (LOQ) between 1 and 500 ppb (1-8ppb-AFB1; 25-500ppb-ZEA; 25-250ppb-DON, 2-25ppb-OTA, 5-6ppm-FBs). The mycotoxin level was under the EU limits for pigs. AFB1 was detected also by UPLC (LOD-0.008 ng/ml and LOQ-0.02 ng/ml, recover rate, 96.7%) after cleaning up with an immune affinity column and the concentration found was 320 ± 10.9 ppb in the aflatoxin B1 contaminated diet and 2.4 ± 0.15 ppb in the control diet, respectively.

2.4. Analysis of liver's function biomarkers in plasma

To assess the effect of AFB1 on liver functions, blood samples from fasted piglets were collected aseptically from jugular vein on day 30 and then centrifuged at 775 g for 25 min to separate plasma and liver biomarkers, AST (aspartate aminotransferase), ALT (alanine aminotransferase), GGT (gamma glutamate transpherase), total protein, total bilirubin, AKL (alkaline phosphatase) and albumin, were estimated by using a Clinical Chemistry benchtop analyser Horiba Medical - ABX Pentra 400, (Irvine, CA, US). Plasma duplicates were stored at −80 °C for further analyses concerning antioxidant status.

2.5. 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 (Giriwono et al., 2010). The absorbance was measured using a Tecan microplate reader (Tecan Infinite M200 PRO, Austria). The level of lipid peroxidation in hepatic tissue was evaluated by measuring thiobarbituric acid-reactive substances (TBARS) as described by (Taranu et al., 2018).

2.6. Inflammatory status

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). Supernatant samples were diluted for IL-8 in duodenum (1:2) and for IL-1β, IL-6, IL-8, TNF-α in liver (1:4). The absorbance at 450 nm was measured using a Tecan microplate reader (Tecan Infinite M200 PRO, Austria). Data were analysed against the linear portion of the generated standard curve. Recombinant swine IL-1β, IL-6, IL-8, TNF-α and IFN-γ protein diluted according to manufacturer's instructions were used as standards. Data were analysed against the linear portion of the generated standard curve.

2.7. Statistical analyses

The statistical differences between treatments for all parameters analysed 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. Each pig was considered an experimental unit. Values of *P* lower than 0.05 were considered significant. Differences were considered as tendencies when *P* value was between 0.051 and 0.10.

Table 1
Diets composition.

Component	Control diet	Grape Seed meal diet
Dry matter (DM)	89.28	89.77
Crude protein (CP)	22.19	20.93
Ether extract (EE)	1.97	4.18
Crude fiber (CF)	3.38	6.13
Ash	6.18	6.30
Calcium	0.88	0.90
phosphorus	0.92	0.92
Metabolisable energy (ME, kcal/kg)	3248	3178
Total polyphenols (mgGAE/100 g)	897.15	382.93
DPPH (μM TRE/g sample)	206.89	966.35

3. Results

3.1. Diet composition

Nutrient content of control and GS diet is presented in Table 1. Inclusion of GS meal increased the crude fat (4.18% *vs* 1.97%) and crude fibre (6.13% *vs* 3.38%) of GS experimental diet. Concentration of polyphenols and the antioxidant activity of GS diet were also higher than that of control diet (Tabel 2) as well as the concentration of the omega-6 polyunsaturated fatty acids (50.56 g vs 45.38 g/100 g total FAME).

3.2. Effect of AFB1 and dietary grape seed meal on body weight

As expected piglets fed for 4 weeks the AFB1 contaminated diet recorded a significantly lower body weight and weight gain ($P < 0.05$) when compare either with control or GS group (Table 3). This effect was counteracted by the inclusion of 8% of GS meal (80 g GS/kg feed) into the diet contaminated with 320 ppb AFB1, the body weight increasing with 13.11% in pig group fed this treatment compared to AFB1 group. By contrast, GS alone had no effect on growth performance of pigs.

3.3. Effect of AFB1 and dietary grape seed meal on liver's function biomarkers

Results for plasma total protein and albumin were statistically analysed and the concentration were within the normal limits ranged

Table 2
Polyphenols composition (μg/ml extract catechin equivalent) of experimental diet.

Components	Control diet	Grape Seed meal diet
Ferulic acid derivat	105.507	116.542
Caffeoylquinic acid	109.908	173.426
Procyanidin trimer	0	23.728
Catechin	0	48.492
Daidzein-glucoside	23.860	26.684
Glycitein-glucoside	26.553	40.413
Epicatechin	0	47.966
Epigalocatechin	0	48.295
Procyanidin dimer	0	89.348
Petunidin 3-O-glucoside	0	82.780
Procyanidin dimer	0	53.287
Genistein-malonylglucoside	34.829	55.323
Cyanidin coumaroyl-glucoside	0	10.854
Malvidin coumaroyl-glucoside	0	94.012
Dicaffeoylquinic acid	59.593	13.481
Dicaffeoylquinic acid	43.106	58.805

Table 3
Effect of AFB1 and GS diet on pig growth performance.

	Control		AFB1		GS		AFB1 + GS	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Body Weight (kg)	22.92[a]	1.5	17.17[c]	1.4	21.10[ab]	0.7	19.42[b]	0.9
Body weight gain (kg)	0.490[a]	0.1	0.286[c]	0.0	0.432[ab]	0.1	0.366[b]	0.0

Table 4
Effect of AFB1 and GS diet on plasma total protein and albumin.

	Control		AFB1		GS		AFB1 + GS	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Total protein (g/dl)	5.743[b]	0.08	5.265[b]	0.35	7.797[a]	0.269	6.532[c]	0.26
Albumin (g/dl)	3.453[b]	0.04	3.293[b]	0.15	3.783[a]	0.09	3.882[a]	0.87

by Merck guidelines (2014–2015). No significant difference was found between the group receiving AFB1 contaminated diet and control group. By contrast, the dietary inclusion of GS led to a significantly increased concentration of total protein and albumin in plasma of piglets receiving GS diet alone or contaminated with 320 ppb AFB1. These results are presented in Table 4.

3.4. Effect of AFB1 and dietary grape seed meal on antioxidant status

Administration of AFB1 contaminated diet lead to a significant decrease in antioxidant status at local level in duodenum, the situs of absorption of both nutrients and contaminants. Activity of catalase, glutathione peroxidase and superoxide dismutase as well as the total antioxidant capacity decreased under AFB1 action by 32.7% (CAT), 13.65% (GPx), 9.50% (SOD) and 48.4% (TCA) respectively (Fig. 1A, B, 1C and Table 5) in comparison with control and GS group. The dietary inclusion of GS meal ameliorated the AFB1-reduced enzyme activity and TCA toward the control level suggesting the potential of this waste to counteract the oxidative damage induced by mycotoxin. GS diet alone enhanced GPx and SOD activity in duodenum.

A lower level of CAT and a higher one for GPx and TCA was found in colon in comparison with duodenum, but the effect of treatments was similar; compared to control AFB1 diet decreased the activity of CAT, GPx, SOD and TCA while the GS diet restore enzyme activity toward that of the control (Fig. 1D, 1E, 1F and Table 5).

At systemic level in plasma, the activity of CAT, GPx and TCA was negatively affected by the AFB1 contaminated diet as compared to control. Like in duodenum and colon, in the plasma of pigs fed AFB1 + GS diet enzyme activity and TCA increased toward the control group. Noticeably, enzymes activity was increased over the control level by GS diet acting alone (Fig. 2A, 2B, 2C and Table 5).

By contrast, in the liver of AFB1-treated pigs TCA, CAT and SOD activity decreased ($P < 0.05$) under the control level (−21.9%, −24% and - respectively) while GPx activity was not modified when compared to control. This effect was counteracted in liver of pigs fed AFB1 + GS meal. Again GS diet alone stimulated TAC (+9.2%) and enzymes activity (CAT +29.7% and GPx 53.9%, Fig. 2D, 2E, 2F and Table 5).

The effect of AFB1 exposure on TBARS concentration as a biomarker of lipid oxidation products in plasma and organs is presented in Table 6. Pigs receiving the AFB1 contaminated diet had a significantly higher level of TBARS compared with control group ($p < 0.05$). The addition of the GS into AFB1 diet counteracted the level of TBARS in both plasma and organs of pigs fed mycotoxin contaminated diet. GS individual treatment decreased alone the TBARS ($p < 0.05$) in plasma, duodenum and liver.

3.5. Effect of AFB1 and dietary grape seed meal on inflammatory status

The inflammatory response evaluated by the pro-inflammatory cytokine production in duodenum, colon and liver is presented in Tables 7–9. The results showed a biphasic effect of the toxin on the two intestinal segments (duodenum and colon) and liver. A significant $p < 0.05$ decreased in the concentration of cytokines was observed in duodenum of pigs fed AFB1 contaminated diet in comparison with pig fed control diet: IFN-γ (−15.30%), IL-1β (−42.90%), TNF-α (−34.21%) and IL-6 (−60.80%) as well as a decreasing tendency in the concentration of IL-8. However, GS diet alone decreased also the level of duodenal IL-1β (−25.3%) and TNF-α (−28.3%, $p < 0.05$) cytokines under that of the control proving the intestinal anti-inflammatory properties of bioactive compounds from GS at intestinal level (Table 7). Addition of 8% GS into the diet containing 320 ppb AFB1was also able to enhanced the concentration of pro-inflammatory cytokines.

By contrast, at hepatic and colon level AFB1 diet induced an increase in the concentration of all pro-inflammatory cytokines as compared with control (Table 8, Table 9). Conversely, the level of cytokines decreased near that of the control in GS + AFB1-fed pigs. A slight decrease in IFN-γ, TNF-α and IL-8 was observed in colon of GS treatment alone.

4. Discussion

In the last decade there has been an increasing interest in the use of bio-adsorbing and bio-transforming agents such probiotics (ex. bacteria and yeast) and prebiotics (ex. carbohydrates) as mycotoxin mitigating agents. Recent studies showed that grape waste (ex. grape pomace) is able to sequester rapidly AFB1 from liquid media due to their high content of fibers (Avantaggiato et al., 2014). These agents are also rich sources of bioactive compounds such as polyphenols, polyunsaturated fatty acids, vitamins and minerals with strong antioxidant activity which could increase the defense capacity of animal against the negative action of mycotoxins. The aim of the present study was to investigate the potential of grape seed meal to mitigate the *in vivo* adverse effects produced by an AFB1 contaminated diet on oxidative and inflammatory status of piglets after weaning.

Exposure to aflatoxins in either higher or lower levels affected growth performance in different animal species (pig, chicken, mice, rabbit) (Ali Rajput et al., 2017; Long et al., 2016; Marin et al., 2002; Meissonnier et al., 2007). In the present study the ingestion of 320 ppb of dietary AFB1 reduced significantly the body weight (−25.1%) and the body weight gain (−41.6%) of piglets fed contaminated diet when compared to pigs receiving the control diet. The adverse effect on growth could be attributed to the changes induced by AFB1 in digestive processes from intestinal level. It was shown that like other mycotoxins, AFB1 alter the intestinal absorbing barrier (Ali Rajput et al., 2017) reduce the activity of digestive enzymes (pancreatolipase, amylase, and trypsin) and disturb the energy metabolism of the intestinal cells as well as the gluconeogenesis, fatty acids synthesis etc (Ling et al., 2016; Long et al., 2016; Pinton and Oswald, 2014). The inclusion of 8% GS into the diet contaminated with AFB1 improved body weight (+22.9%) and body weight gain (+28%) of pigs from GS group compared with AFB1 group. Studies of (Gessner et al., 2013) and (Fiesel et al., 2014) showed that the efficacy of grape byproducts in performance improvement is due mainly to the influence of their active biomolecules on microbial composition and to the downregulation of several pro-inflammatory genes in various parts of the intestine and less to the effect on nutrient digestibility. Other products derived from grape (ex. grape seed proanthocyanindin) were also able to restore the weight gain in mice and broilers (Ali Rajput et al., 2017; Long et al., 2016).

Exposure to AFB1 increased plasma AST, ALT, ALP, LDH suggesting certain liver damage (Ali Rajput et al., 2017; Nurul Adilah et al., 2018; Yilmaz et al., 2018) and decreased the total plasma protein considered as the indicator of protein synthesis (Salem et al., 2018). There are

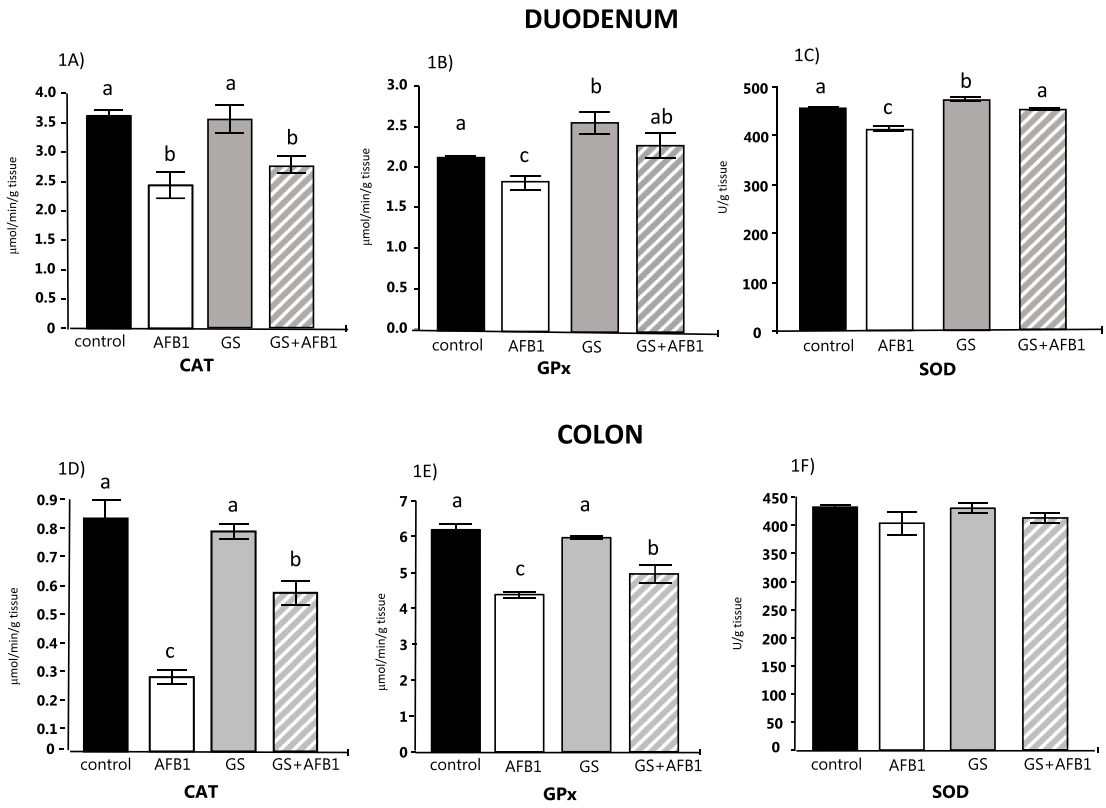

Fig. 1. A, B, C, D, E, F. Effect of GS diet on antioxidant enzymes activity in intestin. Duodenum and colon samples were taken at the end of the trial on day 30 and were analysed for SOD, CAT and GPx activity by using Cayman kits according to the manufacturers' instructions. Results are expressed as µmol/min/g tissue for CAT and GPx activity or U/g tissue for SOD. The means value ± SEM (n = 6) were calculated and presented as histogram (duodenum-1A, 1B, 1C and colon-1D, 1E, 1F). Statistical analysis was performed using one-way ANOVA followed by Fisher test (*$P < 0.05$, control diet (■), AFB1 contaminated diet (□), GS diet (▣) and GS + AFB1 diet (▨)).

Table 5
Effect of AFB1 and GS diet on total antioxidant capacity.

	Control	AFB1	GS	AFB1 + GS	SEM	p-value
Duodenum (µmol/g tissue)	5.02^{a}	2.59^{b}	4.22^{c}	3.29^{d}	0.217	0.0001
Colon (µmol/g tissue)	7.04^{a}	5.83^{b}	7.18^{a}	6.59^{ab}	0.179	0.022
Plasma (mmol/l)	0.338^{a}	0.277^{b}	0.429^{c}	0.337^{ad}	0.012	0.0001
Liver (µmol/g tissue)	58.22^{ac}	45.50^{b}	63.59^{a}	53.85^{c}	1.883	0.001

studies which assign the decrease of growth performance produced by AFB1 to its effect on protein synthesis (Marin et al., 2002; Salem et al., 2018). Other mechanisms by which AFB1 results in reduced performance included the decrease of nutrients absorption and of insulin-like growth factor-1 (IGF-1), a peptide hormone produced in liver that stimulates growth (Ohlsson et al., 2009; Watson et al., 2018). However (Lindemann et al., 1993), reported that an effective and significant effect of AFB1 on protein synthesis depend on the toxin concentration as well as the time of exposure. The results of his study evaluating in weanling pig the effect of both the dose of AFB1 and the time of exposure showed that liver damage was occurred at the highest level of AFB1, 840 ppb. Our data showed no effect of AFB1 on the level of liver endogenous enzymes and only a slight non-significant decrease of total protein and albumin. It is possible that the difference could be due to the concentration of AFB1 in the diet. In the studied mentioned above the AFB1 concentration was higher (1–5 mgAFB1/kg feed) than in the present experiment (0.32mgAFB1/kg feed).

It was found that aflatoxins have a high prooxidant potential resulting from the increase of the ROS (reactive oxygen species) production and the decrease of important components of the antioxidant defense system such as SOD, CAT and GSH-Px enzymes which maintain the intracellular redox balance by scavenging the free radicals (Yener et al., 2009). Moreover, in a recent study (Muhammad et al., 2018), showed that AFB1 enhanced the cytochrome p450 (CYP) enzymes involved in the biotransformation of AFB1 in toxic metabolites (Muhammad et al., 2018). In our experiment, AFB1-treated group had a reduced antioxidant enzymes activity compared to control group. However SOD activity was not affected in plasma and colon. The changes in antioxidant enzymes activity were also accompanied by a decrease of the total antioxidant capacity, a useful and important marker for nutritional interventions involving antioxidant rich food. A significant reduction in the activity of antioxidant enzymes was also found by (Yilmaz et al., 2018) in kidney and heart tissues of rats intoxicated with 0.5 and 1.5 mg AFB1/kg bw/day for 3 and 7 days respectively. These authors hypothesised that the decrease of enzymes activity might either be due to their consumption during the defense process against ROS formation which occurred inside the cells during AFB1 metabolism or due to the inhibitory effect of AFB1 on enzymes activity (Yilmaz et al., 2018). Lipid peroxidation is one of the principal results of ROS alteration provoked by AFB1 exposure which affect the integrity of cell membrane and its functionality leading to cell death (Long et al., 2016; Shen et al., 1995; Yilmaz et al., 2018) have demonstrated that administration of AFB1 increased the level of malonaldialdehyde (MDA), marker of lipid peroxidation as one of its end products which is proportionally with this process. Our results showed also that the concentration of thiobarbituric acid reactive substances (TBARS) as an indicator of lipid peroxidation was increased in plasma and tissues samples derived from pigs fed AFB1 contaminated diet. Similarly, the study of (Wang et al., 2012) demonstrated that zearalenone, other mycotoxins known to induce oxidative stress, increased the level of MDA and decreased the activity of antioxidant enzymes SOD and GPx in serum of pigs. Conversely, in the present work, grape

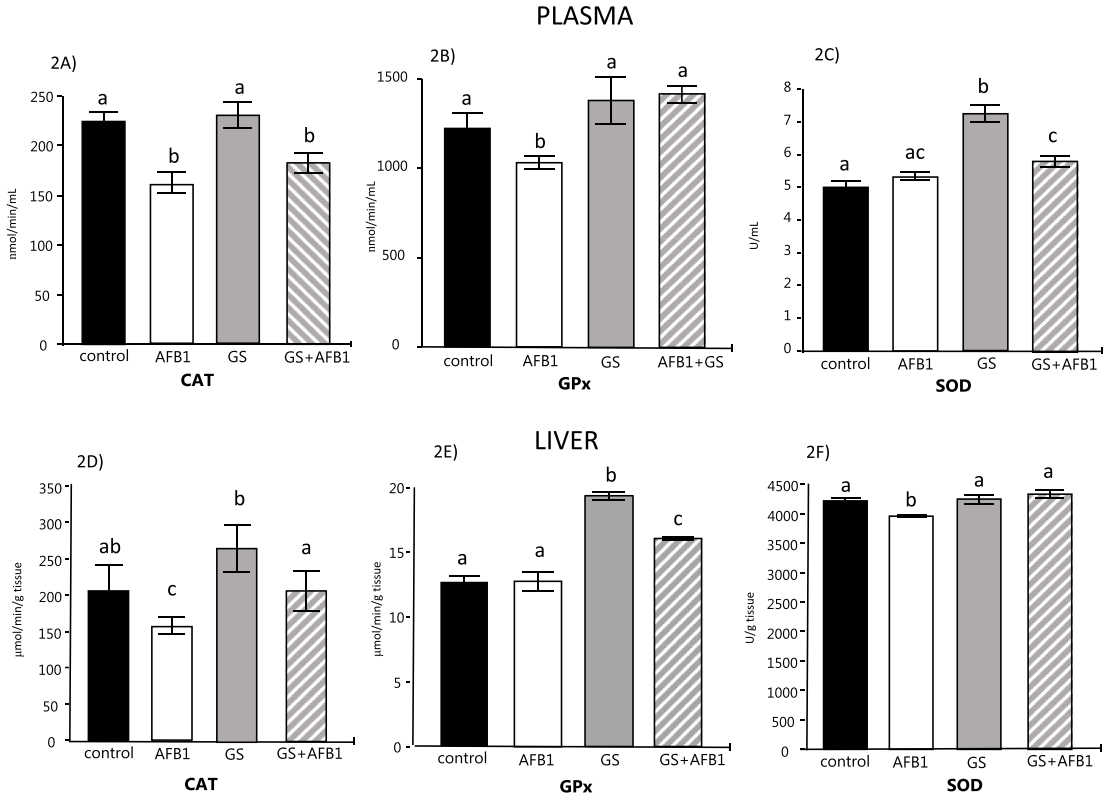

Fig. 2. A, B, C, D, E, F. Effect of GS diet on antioxidant enzymes activity in plasma and liver. Plasma and liver samples were taken at the end of the trial on day 30 and were analysed for SOD, CAT and GPx activity by using Cayman kits according to the manufacturers' instructions. Results are expressed as nmol/min/mL for CAT and GPx activity or U/mL for SOD in plasma (2A, 2B, 2C) and µmol/min/g tissue for CAT and GPx activity or U/g tissue for SOD in liver (2D, 2E, 2F). The means value ± SEM (n = 6) were calculated and presented as histogram. Statistical analysis was performed using one-way ANOVA followed by Fisher test (*$P < 0.05$, control diet (■), AFB1 contaminated diet (□), GS diet (▣) and GS + AFB1 diet (▨)).

Table 6
Effect of AFB1 and GS diet on Thiobarbituric acid reactive substances (TBARS).

	Control	AFB1	GS	AFB1 + GS	SEM	p-value
Duodenum (µmol/g tissue)	1.668^{a}	2.890^{b}	0.815^{c}	2.305^{d}	0.166	0.0001
Colon (µmol/g tissue)	2.603^{a}	3.738^{b}	2.640^{a}	2.673^{a}	0.097	0.0001
Plasma (mmol/l)	0.595^{a}	0.657^{b}	0.440^{c}	0.452^{c}	0.020	0.0001
Liver (µmol/g tissue)	23.18^{a}	33.15^{b}	19.64^{d}	26.99^{c}	1.110	0.0001

Table 8
Effect of AFB1 and GS diet on inflammatory status in colon.

	Control	AFB1	GS	AFB1 + GS	SEM	p-value
IFN-γ (pg/mL)	91.67^{ab}	101.03^{a}	63.08^{bc}	59.58^{c}	29.27	0.018
IL-1-β (pg/mL)	643.83^{a}	2022.51^{b}	883.73^{a}	1199.91^{a}	162.2	0.007
TNF-α (pg/mL)	50.59^{ab}	61.34^{a}	39.42^{bc}	33.79^{c}	3.21	0.005
IL-6 (pg/mL)	799.52^{a}	1068.78^{b}	906.42^{ab}	803.83^{a}	52.6	0.235
IL-8 (pg/mL)	3047.55	3468.12	2919.39	3587.57	184.6	0.543

seed meal alleviated the AFB1-induced antioxidant components reduction. Grape and grape by products are rich sources of bioactive compounds (phenolic compounds, fatty acids, fibre, minerals and vitamins etc), known for their high antioxidant and anti-inflammatory capacity (Doshi et al., 2015; Iora et al., 2015; Melo et al., 2015; Perez-Jimenez and Saura-Calixto, 2008; Sehm et al., 2007) with beneficial effect in preventing oxidative stress and inflammation (Chuang et al., 2011; Fiesel et al., 2014; Hogan et al., 2010). Indeed, in the present study GS diet enhanced the above mention antioxidant defense parameters and was efficient in counteracting the AFB1-induced antioxidant damage at a level closed to that of the control. In the same line other studies observed that dietary bioactive polyphenols improve the antioxidant status in piglets subjected or not to mycotoxins treatment. For example (Chedea and Palade, 2018), showed that 5% grape pomace in the diet of growing pigs reduced lipid peroxidation products in duodenum and colon and in the study of (Wang et al., 2012) the addition of soya isoflavones in the diet contaminated with 2 mg/kg of zearalenone lowered the MDA concentration and increased the activity of SOD and GPx. Amelioration of antioxidative capacity in plasma and decreased MDA level in liver was found in LPS (lipopolysaccharide) challenged pigs fed diet supplemented with ampeopsin a common flavonoid from dry tender stems and leaves (Hou et al., 2014) (Belguendouz et al.,

Table 7
Effect of AFB1 and GS diet on inflammatory status in duodenum.

	Control	AFB1	GS	AFB1 + GS	SEM	p-value
IFN-γ (pg/mL)	46.61^{ac}	39.46^{b}	51.51^{a}	42.36^{bc}	1.46	0.016
IL-1-β (pg/mL)	1077.51^{a}	730.12^{b}	805.66^{ab}	913.39^{ab}	58.07	0.166
TNF-α (pg/mL)	84.10^{a}	54.25^{b}	60.52^{b}	68.21^{ab}	3.51	0.006
IL-6 (pg/mL)	487.99^{ab}	305.39^{c}	580.32^{a}	415.52^{b}	31.96	0.009
IL-8 (pg/mL)	13,616.40^{abc}	11,612.27^{a}	14,600.38^{b}	14,327.07^{c}	446.39	0.034

Table 9
Effect of AFB1 and GS diet on inflammatory status in liver.

	Control	AFB1	GS	AFB1 + GS	SEM	p-value
IFN-γ (pg/mL)	2436.62[a]	4154.06[b]	2267.39[a]	3316.13[ab]	257.8	0.030
IL-1-β (pg/mL)	17,040.87[a]	23,557.07[b]	17,456.00[a]	16,891.60[a]	827.39	0.009
TNF-α (pg/mL)	905.59[a]	1298.59[b]	1287.98[ab]	1277.78[ab]	45.56	0.018
IL-6 (pg/mL)	7599.00[a]	10,850.13[b]	7752.20[a]	7489.07[a]	534.21	0.062
IL-8 (pg/mL)	6358.17[a]	8881.10[b]	6997.40[a]	6027.10[a]	329.30	0.019

1997) reported that resveratrol, a powerful polyphenol, protects porcine LDL against peroxidative degradation by both chelating and free radical scavenging mechanisms.

It has been suggested that the in depth cellular mechanism by which bioactive molecules from grape especially polyphenols stimulate the antioxidant systems is related to the activation of nuclear factor-erythroid 2-related factor-2 (Nrf2), a redox sensitive transcription factor which modulates the anti-oxidative defense system by binding to antioxidant response elements (ARE) and activates several genes encoding for phase II detoxification enzymes as well as for antioxidant enzymes (Gessner et al., 2013; Liu et al., 2018; Lu et al., 2014). For example (Liu et al., 2018), found that dietary procianidin extract (GSPE) attenuated liver injury induced by lead (Pb) in rat through an integrated mechanism associated with the miRNA153 and AKT/glycogen synthase kinase 3 beta/Fyn-mediated Nrf2 activation and (Muhammad and Wang, 2018) reported the chemoprotective effects of turmeric curcumin polyphenols againt AFB1-induced damages by the activation of Nrf2 pathway along with the inhibition of cytochrome P450 enzymes which mediate the biotransformation of AFB1 into harmful metabolites.

The results of the present study showed that pro-oxidant effect of AFB1 was also associated with the biphasic modulation of pro-inflammatory response. Indeed, diet contaminated with AFB1 mycotoxin increased significantly the concentration of IFN-γ, IL-1β, TNF-α, IL-6 and IL-8 in liver (+70.5%, +38.2%, +43.4%, +42.8% and +39.8% respectively) and colon (+10.2%, +103.9%, +21.3%, +33.7%, +13.8%), but decreased duodenum concentration of these cytokines (−15.3%, −42.9%, −34.2%, −60.8% and −5.7%) when compared to control. AFB1 diet included grape seed meal (GS + AFB1) proved to be effective in restoring the level of inflammatory markers toward that of the control. These results suggest that bioactive compounds (especially polyphinols and PUFA) from GS are responsible for anti-inflammatory effect. Indeed (Muhammad and Wang, 2018), demonstrated that AFB1-induced inflammation via NF-kB activation in broiler was counteracted by curcumin, a polyphenolic compound found in turmeric and its inhibitory role on signalling pathway involved in inflammation and oxidation. In their study (Muhammad and Wang, 2018) found that curcumin ameliorated in a concentration dependent manner pro-inflammatory mediators and cytokines by suppressing NF-kB and activation Nrf2/HO-1 pathways in broiler hepatocytes.

Moreover, GS diet alone also reduced several cytokine concentrations in duodenum and colon. Similar trend was found by earlier study of (Gessner et al., 2013) which showed that grape seed and grape marc extract (GSGME) suppressed the inflammatory process via cytokine reduction in the small intestine of pigs after weaning by inhibiting NF-kB responsible for signalling pathway of inflammation. They reported a reduced transactivation of Nrf2 and NF-kB as well as a reduced level of several of their target genes in duodenum mucosa of pigs fed (GSGME) compared to control. Evidence that polyphenols are directly involved in inflammatory and oxidative process come from the study of (Magrone and Jirillo, 2010) which showed that the removal of polyphenols from red wine a rich source in these compounds abrogated the release of nitric oxide and cytokines in human blood mononuclear cells (Magrone and Jirillo, 2010).

5. Conclusion

The diet contaminated with 320 ppb of AFB1 altered pig growth, induced inflammation in liver and colon by increasing pro-inflammatory cytokines level, but decreased duodenum concentration of these cytokines when compared to control. AFB1 altered also oxidative status by reducing antioxidant enzymes activity and total antioxidant capacity and increasing tiobarbituric substances in plasma and organs. The inclusion of grape seed in the diet of AFB1 intoxicated pigs had the potential to mitigate the AFB1-induced toxicity. It enhanced the antioxidant enzymes activity and the other indices of oxidant status. Dietary grape seed meal restored the inflammatory markers and performance of AFB1-treated animals. These findings suggest that grape waste is a promising feeding source in counteracting the harmful effect of aflatoxin B1.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2019.02.020.

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