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The effect of a diet containing grape seed meal on inflammatory and antioxidant markers in spleen of weaned piglets

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ABSTRACT

During the weaning period, piglets are exposed to different stress factors (environmental, physiological and nutritional stressors) that leads to dysfunctions of both gastrointestinal and immune systems. The wastes resulted from grape processing are rich in bioactive compounds, like polyphenols, fiber, PUFAs and vitamins. In present study we aimed to investigate the effects of diet containing 8% grape seed meal (GSM diet) to counteract the weaning-induced immune system perturbations in piglets. Briefly, the effects of GSM diet on the markers involved in inflammatory processes and oxidative stress were investigated Also, the GSM diet effects on the in-depth signalling markers involved in both inflammatory and anti-oxidant responses modulation (NF-kB, MAPKs and Nrf2) were evaluated. The results showed that the diet with 8% GSM reduced *IL-6, IL-12, IL-18, IFN-γ* and *MCP1* gene expressions in spleen. Also, GSM diet increased the total antioxidant capacity in spleen and the antioxidant enzymes (CAT, SOD and GPx) activities. These results demonstrated that GSM diet has potent anti-inflammatory and antioxidant properties, attenuating these processes in spleen of weaned piglets. These effects are probably exerted by MAPKs/NF-kB and Nrf2 pathways Further studies are needed for a complete image on GSM diet effects on the immune system in weaned piglets.

Keywords: grape by-product, inflammation, oxidative stress, weaning piglets

INTRODUCTION

During the weaning period, piglets are exposed to different stressors (environmental, physiological and nutritional stressors) that leads to dysfunctions of the gastrointestinal and immune systems, affecting piglet`s growth, health and feed intake (Campbell J. M. 2013). The immune system activation occurring during weaning stress is associated with an increased secretion of TNF-α (tumour necrosis factor-α), interleukins IL-1β, IL-6 and IL-8 pro-inflammatory cytokines; these processes are driven by NF-*k*B (Nuclear factor kappa-light-chain-enhancer of activated B cells) and MAPKs (Mitogen-Activated Protein Kinases) signalling pathways (Katayama M. 2011; Yang T. 2024). Also, a disruption of the antioxidant response and of oxidative balance was noticed in weaned piglets, causing oxidative damage (Yin J. 2014). These immune and oxidative damages were reported to be more frequent and acute in weaning piglets after European Commission banned the antibiotics used as growth promoters in animal feeding (Gallois M. 2009). Also, the accumulation of the residues of antibiotics in animal products and the increase of the resistance of bacteria to antibiotics leaded to an increased interest in finding other alternatives to the in-feed antibiotics (Gallois M. 2009). Of these, a particular interest was given to products rich in bioactive compounds, with positive impact on the health, having anti-inflammatory, antioxidant and anti-microbial properties (Sorrenti V. 2023). A particular interest was attributed to the agro-industry wastes, which are accumulating, causing environmental issues; their recycling and valorisation is feasible due to their high content in bioactive compounds, arguing the use of these wastes in animal nutrition as health enhancers and immunomodulators (Sorrenti V. 2023).

The wastes resulted from grape processing are rich in bioactive compounds, like polyphenols, fiber, PUFAs and vitamins (Baroi A. M. 2023; Chedea V. S. 2018). Our previous studies showed the beneficial effects of some grape wastes (e.g., grape pomace and grape seed meal) in reducing the amplitude of inflammatory and oxidant responses in both *in vitro* models using epithelial intestinal cells (Pistol G. C. 2023; Pistol G. C. 2019) and *in vivo* in colon and mesenteric lymph nodes collected from weaned piglets (Pistol G. C. 2021; Pistol G. C. 2023). In weaning piglets, not only the gastro-intestinal tract is affected by weaning stress, but also spleen is affected by weaning transition in piglets (Chen J. 2022). Starting from these data, in present study we aimed to investigate the effects of diet containing 8% grape seed meal (GSM diet) to counteract the weaning-induced immune system perturbations in piglets. Taking into account the role of spleen in systemic immune response regulation, the effects of GSM diet on the markers involved in different aspects related to the immune activity and regulation: inflammation (by measuring the pro-inflammatory mediators gene and protein expressions: TNF-α, IL-1β, IL-6, IL-8, IL-12, IL-17, IL-18, MCP-1 - monocyte chemoattractant protein-1

and IFN-γ - interferon-gamma) and oxidative stress (lipid peroxidation, TAC - total antioxidant capacity, antioxidant enzymes – catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx)). Also, the GSM diet effects on the in-depth signalling markers involved in both inflammatory and pro-oxidant/antioxidant responses (NF-*k*B, MAPKs and Nrf2) were evaluated.

MATERIALS AND METHODS

Animals and experimental diets:

In this study, ten cross-bred TOPIGS-40 weaned piglets, with an average initial body weight of 9.04 ± 0.13 kg were used. The piglets were assigned to two experimental groups (5 piglets/group/pen) and the experiment was conducted within the experimental base of the INCDBNA - IBNA Balotesti, Romania. Piglets were fed for 30 days with a Control diet (basal diet) and with 8% grape seed meal diet (GSM). During the experimental period, the piglets had access *ad libitum* to water and feed. The composition of GSM and experimental diets was already described in (Pistol G. C. 2021).

Ethics Statements: This experimental protocol was approved by the Ethical Committee of INCDBNA Balotesti (no. 118/2019); the animals were treated in accordance with EU Council Directive 98/58/EC and with Romanian Law 43/2014.

Determination of pro-inflammatory cytokines concentration

ELISA (Enzyme-linked immunosorbent assay) was used to measure the concentration of the pro- inflammatory markers (TNF-α, IL-1 β, IL-6, IL-8, and IFN- γ) as described by (Pistol G. C. 2020). The concentrations of the selected cytokines in the collected supernatants were determined by using ELISA commercial kits, according to manufacturers' instructions. Absorbance was measured at 450 nm using a TECAN reader. Data were analysed using the standard curve, the results being expressed as pg/ml (picograms/ml of sample).

Determination of antioxidant parameters

The thiobarbituric acid reactive substances (TBARS) and the total antioxidant capacity (TAC) in spleen samples were performed as described by (Pistol G. C. 2023), and the results were expressed as nmol/g sample (for TBARS) and as μmol TEAC (Trolox equivalent antioxidant capacity)/g of tissue (for TAC). The activities of antioxidant enzymes (CAT, SOD and GPx) were determined as described in (Pistol G. C. 2023), the results being expressed as μmol/min/g tissue (CAT and GPx activity) and U/g tissue for SOD activity.

Evaluation of genes encoding for antioxidant markers and for signalling pathways

The expression of genes coding for *CAT*, *SOD* and *GPx* antioxidant enzymes, for NF-kB, RELA and Nrf2 nuclear receptors and for *p38/ERKs/JNKs/TAK1* MAPKs was determined using quantitative PCR (qPCR) analysis. Briefly, the total RNA was isolated from spleen samples using specific kits, with respect to manufacturer`s recommendations. The concentration and quality of the extracted RNAs were evaluated, and the revers-transcription into complementary DNA was performed using M-MuLV reverse transcriptase kit. For qPCR analysis, the method and primers described in (Pistol G. C. 2023) were used. The qPCR data analysis was made using the $2^{(-\Delta\Delta CT)}$ method, the results being expressed as Fold-changes (Fc) in relation to the Control group.

Statistical analysis

The obtained results are presented as means ± SEM (Standard Error of the Mean). One-way ANOVA tests were used for the comparison between experimental groups (StatView software 6.0), the statistical significance being considered at $p < 0.05$.

RESULTS

The effects of GSM diet on markers of pro-inflammatory responses in piglets' spleen

Knowing that pro-inflammatory cytokines are defined as master mediators of inflammatory processes, we investigated the effects of experimental diets on the TNF-α, IL-1β, IL-6, IL-8 and IFN-γ concentrations in spleen collected from weaned piglets. The obtained results showed that GSM diet slightly reduced the protein concentration of both IL-1β and IL-6 cytokines (IL-1β: -34%, IL-6: -15%, Figure 1) compared to Control group fed basal diet.

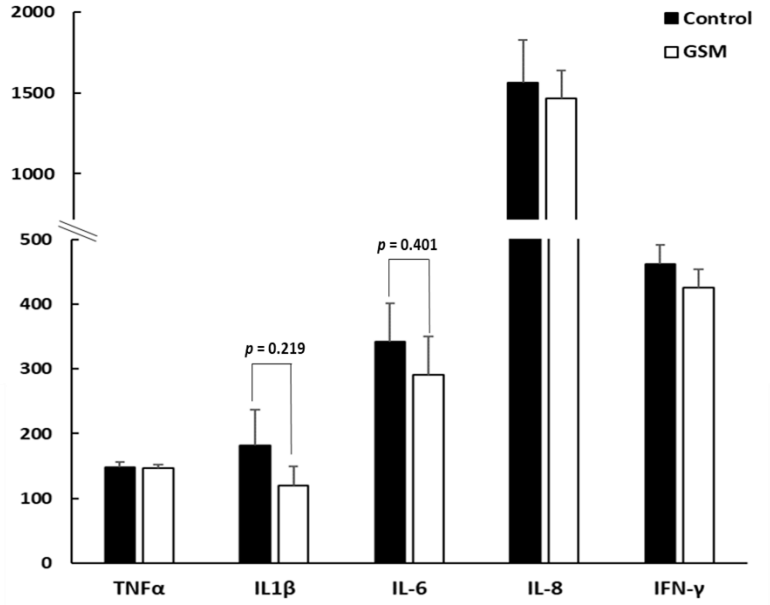

Figure 1. GSM diet effects on pro-inflammatory cytokines concentration in piglets' spleen. Weaned pigs were allocated to two experimental groups and fed for 30 days the control diet (Control group) or 8% GSM diet (GSM group). Spleen samples from all animals (n = 5 piglets/group) were collected at the end of the feeding experiment, and the analysis of the TNF-α, IL-1β, IL-6, IL-8, and IFN-γ cytokines concentrations was performed. The obtained results are presented as means ± SEM.

Next, we analysed the effects of the diets on the gene expression of *TNF-α, IL-1β, IL-8, IL-12, IL-6, IL-17, IL-18, MCP1, IFN-γ* pro-inflammatory cytokines. The qPCR analysis showed that diet with 8% GSM significantly reduced the *IL-6* (-48%, $p = 0.008$), *IL-12* (-33%, $p = 0.003$), *IL-18* (-44%, $p = 0.040$), *MCP1* (-32%, $p = 0.050$) and *IFN-γ* (-49%, $p = 0.002$) mRNAs under the levels of Control group (Figure 2).

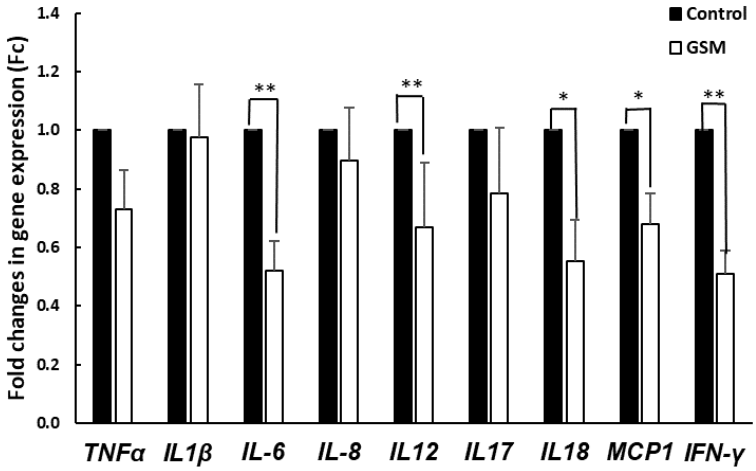

Figure 2 GSM diet effects on the pro-inflammatory mediators coding genes in piglets' spleen. Weaned pigs were allocated to two experimental groups and fed for 30 days the control diet (Control group) or 8% GSM diet (GSM group). Spleen samples from all animals (n = 5 piglets /group) were collected at the end of the feeding experiment, and the analysis of the cytokines mRNAs was performed. The obtained results are presented as means ± SEM. * indicates differences between groups ($p<0.05$)

GSM diet effects on the markers of pro-oxidant/anti-oxidant responses in piglets' spleen

The analysis of lipid peroxidation marker (TBARS) showed no effect of GSM diet compared to Control diet, while total antioxidant capacity (TAC) levels increased significantly in spleen of piglets fed 8% GSM diet (17.71 ± 0.28 µmol/g tissue in GSM group *vs* 14.14 ± 0.29 µmol/g tissue in Control group, $p < 0.001$, Figure 3). The CAT, SOD, GPx enzyme activities were slightly increased by GSM diet in spleen (CAT: +17%, $p > 0.050$ vs Control; SOD: +20%, $p < 0.001$ *vs* Control; GPx: +10%, $p > 0.050$ *vs* Control, Table 1).

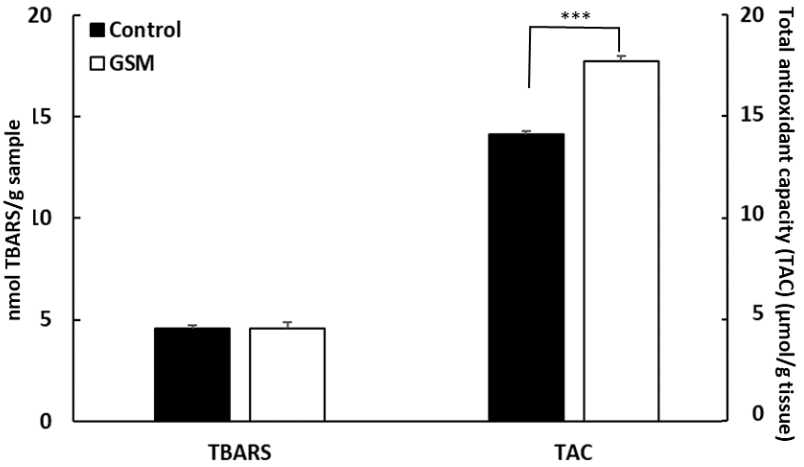

Figure 3. GSM diet effects on lipid peroxidation and total antioxidant capacity in piglets' spleen. Weaned pigs were allocated to two experimental groups and fed for 30 days the control diet (Control group) or 8% GSM diet (GSM group). Spleen samples from all animals (n = 5 piglets /group) were collected at the end of the feeding experiment, and the analysis of the TAC and TBARS levels was performed. The obtained results are presented as means ± SEM.

Table 1. The effects of experimental diets on the antioxidant enzyme activities

Experimental group*	Enzyme activity		
	CAT (µmol/min/g tissue)	GPx (µmol/min/g tissue)	SOD (U/g tissue)
Control	8.75 ± 0.86[a]	22.87 ± 0.59[a]	1185.10 ± 31.57[b]
GSM	10.26 ± 0.68 [a]	27.24 ± 0.31[a]	1243.73 ± 45.61[a]

* Weaned pigs were allocated to two experimental groups and fed for 30 days the control diet (Control group) or 8% GSM diet (GSM group). Spleen samples from all animals (n = 5 piglets /group) were collected at the end of the feeding experiment, and the CAT, SOD and GPx enzymatic activities were analysed. The obtained results are presented as means ± SEM.

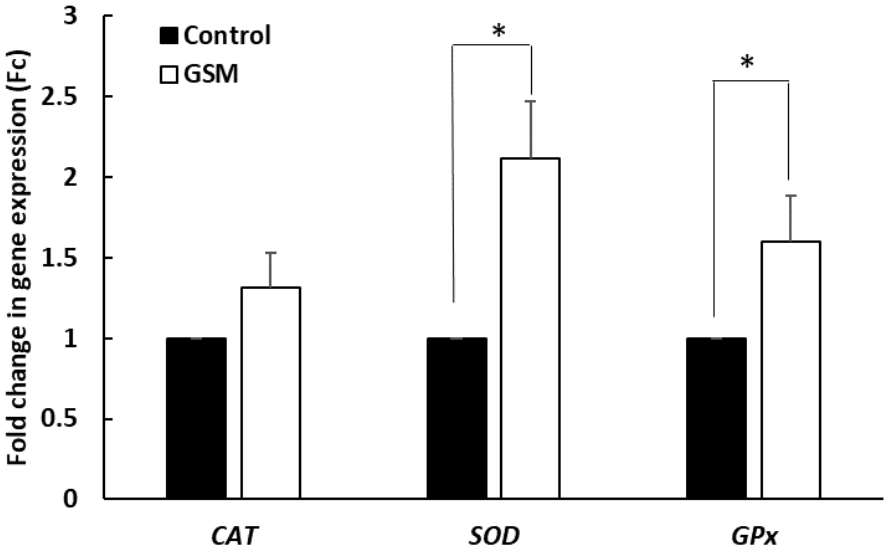

Figure 4. GSM diet effects on the antioxidant enzymes – coding genes in piglets' spleen. Weaned pigs were allocated to two experimental groups and fed for 30 days the control diet (Control group) or 8% GSM diet (GSM group). Spleen samples from all animals (n = 5 piglets /group) were collected at the end of the feeding experiment, and the analysis of the *CAT*, *SOD, GPx* gene expressions was performed. The obtained results are presented as means ± SEM.

The GSM diet effect on the antioxidant enzymes mRNA levels was investigated by qPCR. The results showed that 8% GSM diet significantly over-expressed the expression of *SOD* (+2-fold increase, p = 0.013 *vs* Control) and *GPx* genes (+ 1.6-fold increase, p =0.040 *vs* Control; Figure 4).

GSM diet effects on the in-depth signalling pathways involved in inflammatory and antioxidant responses in piglets' spleen

To investigate the in-depth mechanism of regulation involved in both anti-inflammatory and antioxidant roles of GSM diet, we analysed by qPCR the expressions of genes coding for nuclear receptors involved in the regulation of inflammation (*NF-kB1* and *RELA,* coding for NF-kB transcription factors (R. 2006)) and of oxidative stress (*Nrf2*). The obtained results showed that, in spleen of GSM-fed piglets, a significant decrease of *RELA* gene was found (0.45 ± 0.15 Fc *vs* 1.00 ± 0.00 in Control group, p = 0.049, Figure 5). Also, a slight decrease of *NF-kB1* mRNA was found in GSM group (0.79 ± 0.12 Fc *vs* 1.00 ± 0.00 in Control group, p > 0.050, Figure 5). The *Nrf2* gene was up-regulated in spleen of GSM-fed piglets (1.61 ± 0.59 Fc *vs* 1.00 ± 0.00 in Control group, p > 0.050, Figure 5).

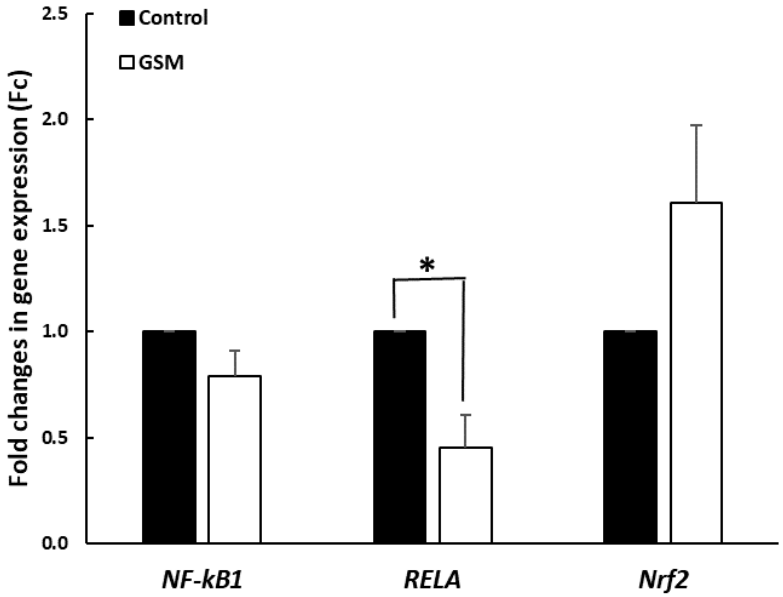

Figure 5. GSM diet effects on the nuclear receptor genes in piglets' spleen. Weaned pigs were allocated to two experimental groups and fed for 30 days the control diet (Control group) or 8% GSM diet (GSM group). Spleen samples from all animals (n = 5 piglets /group) were collected at the end of the feeding experiment, and the analysis of the *NF-kB*, *RELA* and *Nrf2* nuclear receptors was performed. The obtained results are presented as means ± SEM.

Given that the main effect of GSM diet was modulation of inflammation and of its associated nuclear receptors, in the next step we analysed the members of MAPKs (mitogen-activated protein kinases) intracellular signalling pathway involved in controlling the in-depth cellular responses to diverse stimuli, like proinflammatory cytokines (Pearson G. and Xu B. E. 2001). In our study, the genes coding for the members of the three MAPK subfamilies of kinases, p38 mitogen-activated protein kinases (p38α), extracellular signal-regulated kinases (*ERK1/2*), c-Jun N-terminal kinases (*JNK1/2*) and the upstream *TAK1* kinase were analysed. The results presented in Figure 6 showed that GSM inclusion in piglets' diet leaded to a significant decrease in *p38α*, *JNK1* and *TAK1* gene expressions (*p38α:* 0.65 ± 0.10 Fc *vs* 1.00 ± 0.00 in Control group, $p = 0.032$; *JNK1:* 0.47 ± 0.12 Fc *vs* 1.00 ± 0.00 in Control group, $p = 0.013$; *TAK1:* 0.59 ± 0.12 Fc *vs* 1.00 ± 0.00 in Control group, $p = 0.030$, Figure 6).

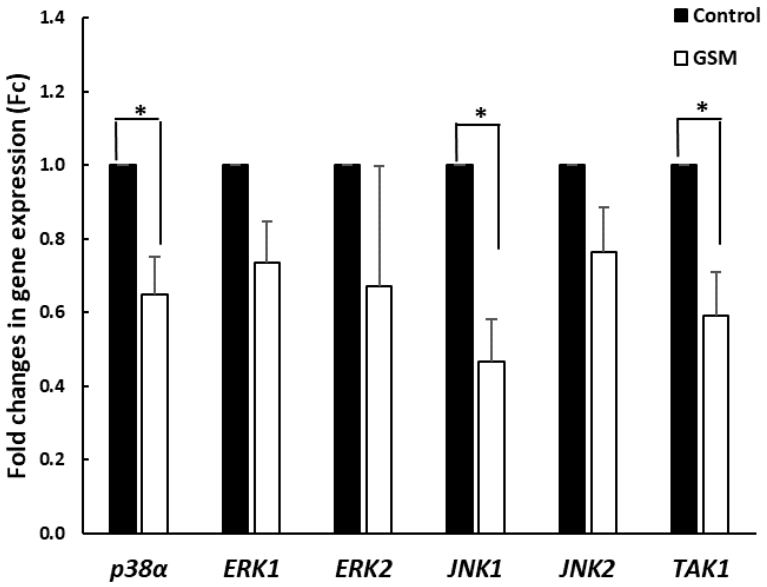

Figure 6. GSM diet effects on the MAPKs genes in piglets' spleen. Weaned pigs were allocated to two experimental groups and fed for 30 days the control diet (Control group) or 8% GSM diet (GSM group). Spleen samples from all animals (n = 5 piglets /group) were collected at the end of the feeding experiment, and the analysis of the *p38α, ERK1/ERK2, JNK1/JNK2* and *TAK1* MAPKs genes was performed. The obtained results are presented as means ± SEM.

DISCUSSIONS

Many studies demonstrated that immature immune function associated with weaning stress induced disruption of immune system in piglets. This immune dysfunction was attributed to an impairment in spleen development and functionality, leading to immune alterations, inflammation and a reduction of antioxidant defense mechanisms (Katayama M. 2011; Yang T. 2024; Yin J. 2014). That is why in piglets research an increased attention was paid to the strategies that can be used to attenuate or even prevent these weaning negative effects. Of these strategies, nutritional approaches based on natural sources of beneficial bioactive compounds are intensely studied. Literature data showed that grape by-products rich in bioactive compounds exerted their benefic effects by modulation of both inflammatory processes and oxidative stress (Baroi A. M. 2022; Nemzer B. 2022). Also, grape wastes could balance the immune response having immunomodulatory properties in different immune organs (thymus, spleen) (Tong H. 2011; Xie Y. 2023). Our previous results showed that 8% GSM diet attenuated dextran sodium sulphate (DSS) – induced experimental colitis in weaned piglets, by (i) attenuation of intestinal inflammation at colon level (reduction of DSS-induced expression of pro-inflammatory cytokines expression and

concentrations, effects modulated via NF-kB signaling pathway) (Pistol G. C. 2020); (ii) improving the antioxidant response in both colon and mesenteric lymph nodes and by Nrf2 signaling pathway regulation (Pistol G. C. 2023). In the present study we supposed that GSM diet could exert immunomodulatory effects at the spleen level, as organ involved in both innate and adaptive responses to inflammatory and oxidant stressors. Although the anti-inflammatory properties of the grape wastes at local level (intestine) are intensively studied in both *in vitro* and *in vivo* experiments, the modulation of immune response in spleen by these wastes was less studied. For example, Bedhiafi et al (Bedhiafi T. 2018) showed that extracts of grape seed and grape skin reduced the concentration of plasma and spleen pro-inflammatory cytokines, for example IL-6, IL-17A, TNF-α, in obese mice; similar modulation by grape wastes was observed in CCL-4 treated rats (Habashy N. H. 2021). Grape seed extract inhibited the TNF-α, IL-12, IL-17, IL-1 β, IL-6, and IFN-γ concentration in spleen cells isolated from mice with autoimmune encephalomyelitis (Wang Q. 2023). In spleen collected from fattening pigs fed grape seed cake diet, a decrease in IL-1β and IFN-γ pro-inflammatory cytokines was registered (Taranu I. 2020). Similarly, in our study, piglets fed 8% GSM diet showed a significant reduction of *IL-6, IL-18, IL-12*, *IFN-γ* and *MCP1* pro-inflammatory genes in spleen (Figure 2).

The antioxidant properties of grape and of bioactive compounds from grape wastes are well-established by a variety range of studies, using different animal models. For example, in CCl-4 treated rats fed with 1.5 g/kg b.w. grape polyphenols the cellular parameters of redox state in spleen were normalized: the ROS, NO, TBARS levels were reduced, while the levels of GSH, TAC and the activities of SOD, GPx were increased by grape polyphenols (Habashy N. H. 2021). In spleen of piglets fed 5% gape pomace diet, strong antioxidant effects of grape polyphenols were detected at the spleen level (for example, increase of total antioxidant capacity and of the activities of SOD and CAT enzymes) (Chedea V. S. 2019). Also, in spleen of broilers fed for 15 days a diet with 9% GP, an increasement of GSH levels in spleen was reported; after 35 days of 9% GP diet, an increase of TAC levels and the decrease of TBARS levels were registered in spleen (Makri S. 2017). In piglets fed 8% GP diet for 15 days, a decrease of TBARS and an increase of GSH levels in different organs including spleen were found (Kafantaris I. 2018). Feeding of weaned piglets with 5% grape seed meal diet restored the pro-oxidant effects induced by DSS challenge in colon and mesenteric lymph nodes (Pistol G. C. 2023). Also, grape seed cake diet increased the CAT and SOD gene expression in spleen of fattening pigs (Taranu I. 2020). Similarly, in our study, 8% GSM diet significantly increases the antioxidant capacity in spleen (Figure 3) and *SOD* and *GPx* gene expressions (Figure 4), supporting the antioxidant effects of GSM.

The in-depth mechanisms regulating both inflammatory and antioxidant responses involved the NF-kB (together with its isoform RELA) and Nrf2 nuclear receptors (Kundu J. K. 2006). This hypothesis is validated by the decrease of *RELA* and the increase of *Nrf2* mRNAs found in our experiment (Figure 5). Our data suggested that the anti-inflammatory properties of GSM are controlled via NF-kB and p38α/JNKs/TAK1 MAPKs. These observations were supported by studies on resveratrol (antioxidant compound found in grape and grape wastes) showing that the anti-inflammatory effects of this polyphenol were mediated by upstream inhibition of the NF-κB (Kundu J. K. 2006); also, the anti-inflammatory effects of resveratrol were related to MAPK pathway, as reviewed by Meng et al. (Meng T. 2021).

Our study highlights another important role of GSM, its antioxidant capacity, supposed to be regulated via Nrf2 pathway. Indeed, this nuclear receptor has an important role in the modulation of antioxidant response genes (Kode A. 2008). Grape polyphenols, including resveratrol, modulated the oxidative stress in pig intestinal epithelial cells (Yang J. 2019), and in rat endothelial cells (Csiszár A. 2015), and this regulation involved the Nrf2 signaling pathway. Additionally, grape polyphenols regulated via Nfr2 pathway the *in vivo* oxidative stress induced in rats (Palsamy P. 2011) and mice (Ungvari Z. 2010), preventing the oxidative damage.

CONCLUSION

In summary, our results demonstrated that 8% GSM diet have potent anti-inflammatory and anti-oxidant properties, attenuating these processes in spleen of weaned piglets. These effects could be mediated via NF-kB/p38/JNK/TAK1 MAPKs for anti-inflammatory effects and via Nrf2 signaling for antioxidant response. Further studies are needed in order to obtain one comprehensive image of the GSM diet effects on the immune system in weaned piglets.

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