

Active ingredients from oil by-products modulate spleen inflammatory and antioxidant response in pigs

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ABSTRACT

Grape seed cakes (GSC) resulted from grape seed oil extraction represent a by-product rich in bioactive compounds such as polyphenols, polyunsaturated fatty acids, fibres, minerals, vitamins etc known for their beneficial anti-inflammatory, anti-microbial, anti-oxidative and immune-modulatory effects. In the present study, we have investigated the effects of dietary grape seed cakes bioactive compounds on several anti-inflammatory and antioxidative biomarkers in spleen of pigs during fattening phase. Twelve crossbred TOPIG hybrid fattening pigs were allocated to two experimental treatments: 1) commercial diet (control group) and 2) a diet including 5% grape seed cakes (GSC group) for 24 days. At the end of experimental period (day 24) pigs were sacrificed and spleen samples were collected and stored at -80°C until analysis. The results showed that GSC diet lowered the gene expression as well as the protein concentration of pro-inflammatory markers: interleukin 1 beta (IL-1 β , -52.66%, $p < 0.05$) and interferon gamma (IFN- γ , -42.13%, $p < 0.05$) and had a tendency to decrease that of interleukin 6 (IL-6, -13.25%), tumour necrosis factor alpha (TNF- α , -9.06%) and interleukin 8 (IL-8, -11.08%) when compared to control diet confirming the anti-inflammatory properties of GSC's active ingredients. The total splenic antioxidant capacity (TEAC) and gene expression of antioxidant enzymes (catalase-CAT and glutathione peroxidase-GPx) were higher in spleen of pigs fed GSC diet than in control group suggesting also the antioxidative potential of GSC. The results related to the molecular mechanism showed a higher expression of gene encoding for PPAR- γ and for Nrf2 in spleen of pigs receiving the GSC diet (Fc 1.81 and 1.58) suggesting that the anti-inflammatory and antioxidant effect of this diet is probably exerted by PPAR- γ and Nrf2 pathway. Further, other percent of dietary GSC inclusion need to be investigated in order to obtain a more complex response related to biomarkers which characterise the fattening/obesity process.

Keywords: pig, spleen, oil by-products, inflammation, antioxidant response.

INTRODUCTION

Grape seed cakes resulted from grape seed oil extraction is a waste rich in bioactive polyphenolic compounds such as proanthocyanidins, flavanols (catechin, epicatechins, gallic catechin, epigallocatechin, procyanidins), stilbenes as well as polyunsaturated fatty acids especially linoleic- ω -6 and oleic- ω -9 fatty acid (Grases et al., 2015) which are known for their beneficial anti-inflammatory, anti-cancer, anti-microbial, anti-oxidative and immunomodulatory effects on animal and human health (Cho et al., 2013; Torres et al., 2002). These active compounds generated lower level of cholesterol, hepatic inflammatory and oxidative biomarkers in animals and decreased the incidence of cardiovascular diseases and hypercholesterolemia in human (Farkhondeh et al., 2020; Ionelia Taranu, 2017) Grape seed and skin extract (GSSE), for example, was used as an anti-obesity and anti-lipotoxic agent alone or in combination with orlistat in obese rats (Bedhiafi et al., 2018). These authors reported recently that GSSE in combination with orlistat neutralised spleen lipotoxicity, oxidative stress and inflammation induced *in vivo* by a high fat diet. It counteracted body weight gain, adiponectin deficiency in plasma and spleen as well as the accumulation of cholesterol and triglyceride along with lipase activity in spleen.

Recently investigations showed that spleen, very known as one of the central immune organs where the immune response is built is also implicated in lipid metabolism (Bedhiafi et al., 2018; Jhun et al., 2013). Obesity represent a lipid metabolic disease characterized by a low grade of chronic inflammation linked also to an impaired immune response on vaccination, infection and tumours (Boi et al., 2016). The strong correlation between pro-inflammatory biomarkers such as pro-inflammatory cytokines, interleukin-6 (IL-6), tumor necrosis factor (TNF)- α and obesity suggested a possible common pathophysiologic link between immunity and obesity (Jhun et al., 2013). Spleen as an important part of the immune system are sensitive to factors linked to inflammation and obesity.

The most and common model utilised to study obesity has been on mice. Thus, ICR male mice fed with a normal or high fat diets supplemented or not with 3 and 5% FCC (a powder mixture of 98: Cheonggukjang and 2: Chaga extracts fermented with *Lactobacillus acidophilus* KCTC3925) showed that FCC attenuated the body weight and inflammatory responses increased in liver and spleen by the high fat diet (Na et al., 2019).

Taken fattening pig as animal model we investigated in the present study the anti-inflammatory and antioxidative potential of bioactive compounds (especially polyphenols, unsaturated fatty acids) from grape seed cakes included 5% in the diet on pigs during fattening phase, a situation that may have similarities with human obesity which involved a low degree of chronic inflammation. We choose pig as animal model for two reasons: i) there are few studies investigating dietary compounds and their interrelation with inflammation and obesity in spleen; ii) pig is a more suitable animal model for human due to their similarities in term of general physiology, intestine development, proportional organs and fat cells size, body fat absorption and distribution as well as the diet quality dependence (Chedea et al., 2019; Guilloteau et al., 2010; Houpt et al., 1979; Rauw et al., 2007; Spurlock and Gabler, 2008).

MATERIALS AND METHODS

Ethics statement

The study protocol was approved by the Ethical Committee of the National Research-Development Institute for Animal Nutrition and Biology, Balotesti, Romania. All animals were healthy during nutritional trial period. The animals were cared for in accordance with the Romanian Law 206/2004 for handling and protection of animals used for experimental purposes and the EU Council Directive 98/58/EC concerning the protection of farmed animals.

Animals, treatments, samples collection

The experiment was carried out on twelve pigs (crossbred TOPIG hybrid pigs) with an average body weight of 75.53 ± 1.0 kg. They were housed in boxes (6 pigs/group), individually identified by ear tags and allocated to two experimental treatments: 1) control diet; 2) control diet containing 5% seed cakes by replacing corn (Table 1). Grape seed cakes (GSC) resulted from grape pomace oil extraction and were provided by S.C. DIONISOS S.R.L., Bucuresti, Romania, a local commercial. Brute composition of the feed diets as well as of grape seed cakes was determined according to the ISO methods (ASRO-SR EN ISO, 2010, Table 1).

Pigs received feed and water ad libitum during the experimental period. Pigs received feed and water ad libitum during the experimental period. At the end of experimental period (day 24) pigs were sacrificed and spleen samples were collected and stored at -80°C until analysis.

Table 1. Composition and calculated nutrient content of experimental diets

Ingredients (%)	Fattening-Finishing phase ¹	
	Control diet	Grape seed cakes diet
Corn meal	55.84	52.83
Rice meal	15.00	15.00
Sunflower meal (31.94% CP)	13.00	10.00
Soybean meal (44% CP)	9.00	10.00
Sunflower oil	3.00	3.00
Grape seed cakes	-	5.00
Monocalcium phosphate	0.35	0.46
Limestone	2.03	1.90
NaCl	0.40	0.40
Metionină	-	0.04
Lisine	0.28	0.27
Choline premix	0.10	0.10
Mineral vitamin-premix ²	1.00	1.00
Mineral vitamin-premix ²	1.00	1.00
TOTAL	100.00	100.00
Calculated Nutrient content		
CP (%)	15.29	15.05
DP (%)	12.07	11.94
Fat (%)	4.29	4.43
Crude fiber (%)	5.68	6.36
ME (Kcal/kg)	3073	3065
Lysine (%)	0.28	0.88
Digestible Lysine (%)	0.73	0.73
Met + Cys (%)	0.59	0.59
Calcium (%)	0.90	0.87
Phosphorus (%)	0.60	0.59

¹BW range 75.53 to 99.59 kg

²mineral-vitamin premix (1%) supplied per kg diet as follows: vit. A 6000 IU, vit. D3 800 IU, vit. E 20 IU, vit. K1 1.0 mg, vit. B₁ 1.0 mg, vit. B₂ 3.0 mg, d-pantothenic acid 6.3 mg, niacin 10 mg, biotin 30 µg, vit. B₁₂ 20 µg, folic acid 0.3 mg, vit. B₆ 1.5 mg, Fe 80 mg, Zn 25 mg, Mn 30 mg, I 0.22 mg, Se 0.22 mg, Co 0.3 mg, antioxidants 60 mg and maize starch as carrier.

Polyphenols determination

The total polyphenols concentration from grape seed cakes was determined by using Folin-Ciocalteu method as described by Taranu et al., 2017. The absorbance was read at 750 nm on a UV-VIS diode array spectrophotometer (Specord 250, Analytic Jena), and the total polyphenol concentration was expressed as Gallic acid equivalents (mgGAE)/100g sample.

Polyphenols identification was done by HPLC-DAD-MS according with the method described by (Dulf et al., 2015) using an Eclipse, XDB C18 (4.6 × 150 mm, 5 µm) column and based on polyphenols individual compounds retention times, UV-vis and the mass spectrum as well as their standards. The detection and calculation of catechins, its derivatives and anthocyanins were done as described by (Taranu et al., 2018).

Measurement of fatty acids

Fatty acids from seed cakes and spleen tissue were determined by gas chromatography (Perkin Elmer gas chromatograph, Clarus 500, USA) after lipid extraction and their transformation into methyl esters by transmethylation. They were separated on a BPX70 capillary chromatographic column for fatty acid methyl esters (60 m × 0.25 mm i.d. × 0.20 µm, Agilent, column flux being 50 mL/min, and the split ratio 1:100), identified by comparison with standard chromatograms and were calculated as percentage.

Measurement of antioxidant defence

The antioxidant capacity of grape seed cakes was measured by the DPPH method (515nm) using the stable radical DPPH as previously described by (Garcia et al., 2012) while the total antioxidant capacity of spleen tissue by using TAC kit (QuantiChrom – BioAssay Systems, USA, TAC, 570 nm) according to the manufacturer's instructions. The DPPH results were expressed as µM TRE (trolox equivalents)/g sample or µmol/g spleen tissue (TAC) using a trolox standard curve and a microplate reader (TECAN, Infinite M200 PRO, Austria).

Antioxidant activity of enzyme superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in spleen tissue was measured by using specific Cayman kit according to the manufacturer's instructions as described by (Giriwono et al., 2010).

Measurement of lipid peroxidation in spleen (TBARS assay)

Lipid peroxidation was assessed by measuring the thiobarbituric acid reactive substances (TBARS). Spleen tissue (0.2g) was homogenized with 8mL phosphate buffer, incubated at 95°C for 15 min and then cooled. TBARS fluorescence was measured at 515 nm excitation and 548 emission with a Tecan Sunrise, Austria and expressed as nmol/g tissues.

Analysis of inflammatory and antioxidant gene expression (qPCR)

Gene expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, IFN-γ), antioxidant enzymes (SOD, CAT, GPx) and related signalling nuclear factors molecules (NF-κB Nrf2 and PPAR-γ) were determined by using quantitative PCR technique (qPCR) as described by (Taranu et al., 2018). Briefly, spleen tissue was disrupted in liquid nitrogen and 100 mg of tissue

powder was homogenized in RTL buffer (QIAGEN GmbH, Germany) with an Ultra-Turrax (IKA®-Werke GmbH & Co. KG, Germany). Total RNA was extracted using Qiagen RNeasy midi kit (QIAGEN GmbH, Germany), according to the manufacturer's recommendations. The qPCR was performed using the Rotor-Gene-Q (QIAGEN GmbH, Germany) machine, primers and the cycling conditions as described by (Pistol et al., 2018). Best housekeeping genes were selected from a panel of five reference genes (*ACTB*; *GAPDH*; *CYP*; *B2m*; *RPL32*) for data normalization by using NormFinder software. Results were expressed as relative fold change (Fc) compared with control group.

Detection of cytokine protein concentration (ELISA)

Pro-inflammatory cytokine (IL-8, IL1- β , IL-6, TNF- α and IFN- γ) protein concentration was measured in spleen lysates obtained by homogenisation of 2g of spleen tissue in phosphate buffer containing 0.5% sodium deoxycholate, 1% IGEPAL, 0.1% SDS and complete protease inhibitor cocktail tablets (EDTA-free). After keeping on ice for 30 minutes and two centrifugations at 10,000 g and 4°C for 10 minutes cytokines concentration was measured by ELISA using ELISA commercial kits (R&D Systems, Minneapolis, MN 55413, USA and Biosource International, Inc., Camarillo, USA) according to the manufacturer's recommendations. Capture antibody consisting in anti-swine TNF- α (MAB6902), IL-1 β (MAB6811), IL-6 (MAB686), IFN- γ (ASC4934) and IL-8 (MAB5351) were used in conjunction with biotinylated anti-swine TNF- α (BAF690), IL-1 β (BAF681), IL-6 (BAF686) IFN- γ (ASC4839) and IL-8 (BAF535). Streptavidin horseradish peroxidase (HRP) (Biosource, Camarillo, USA) and tetramethylbenzidine (TMB) (Sigma) was used for detection along with an ELISA microplate reader (Tecan, SunRise, Austria) and 450 nm optical density. The results were expressed as microgram (μ g) of cytokine/g of spleen tissue quantified by using bovine serum albumin as standard (Pierce® BCA Protein Assay Kit, Thermo Fischer Scientific, USA).

Statistical analyses

For all parameters analysed one-way ANOVA analysis was performed to measure the statistical differences between groups. All the results are expressed as mean \pm standard error of the mean (SEM). Significant differences between means were determined by the least square difference Fisher procedure (StatView software 6.0, SAS Institute, Inc., Cary, NC). Values of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Chemical composition of grape seed cakes

Chemical analysis of feed showed that the inclusion of grape seed cakes in the experimental diet increased the percentage of bioactive compounds such as total polyphenol content by 30.40% and the antioxidant activity by 71.8% when compared to control diet. Grape seed cakes are not rich in protein (10.64%), but is an important source of fibres (37.91%) and active ingredients (Table 2). It is a notably source of polyphenols especially proanthocyanidins (Tong et al., 2011). In the present study the total content in polyphenols of GSC was 5.355 gGAE/100g (Table 2) and their profile (Table 3) measured by LC-MS was diverse highlighting a high level of proanthocyanidins among which flavanols (catechin, epicatechins, galocatechin, epigallocatechin, procyanidins) was the main group. Isorhamnetin 3-O-glucoside was in the highest concentration (56.60g/100g) followed by catechin and epicatechin (48.93g/100g and 48.23g/100g respectively) and procyanidin dimers (26.79g/100g and 18.34g/100g). Grape seed cakes used in the present study was also rich in unsaturated fatty acids (84.66g%g FAME-fatty acids methyl esters). A high level of unsaturated omega-6 fatty acid (e. g. linoleic acid, 62.26 g%g FAME) and omega-9 (oleic acid, 17.05%g% g FAME) was found in GSC (Table 4) which was similarly with results reported by (Garavaglia et al., 2016) for grape seed oil and higher than other oils or by-products, e.g. sea-buckthorn oil (12.12 mg/100g of linoleic acid and 8.77 mg/100g oleic acid), rapeseed meal (20% linoleic acid) and fish oil (1.67 mg/100g linoleic acid, 9.67 mg/100g oleic acid) (Czaplicki et al., 2017; Demers et al., 2020; Tran G., 2020; Zielińska and Nowak, 2017).

Table 2. Chemical composition and antioxidant activity of grape seed cakes (g/100g sample)

	Grape seed cakes
Dry matter (DM)	90.74
Crude protein (CP)	10.64
Ether extract (EE)	5.97
Crude fiber (CF)	37.91
Carbohydrates	71.16
Neutral detergent fiber (NDF)	66.22
Acid detergent fiber (ADF)	59.95
Ash	2.97
Metabolisable energy (ME, kcal/kg)	2268
Total polyphenols (GAE)	5.355
DPPH (μ M TRE/g sample)	32.70
Unsaturated fatty acids (g%g FAME)	84.66

Table 3. Polyphenols composition of grape seed cakes

Items	R _t (min)	[M-H] ⁺	Grape seed cakes (g/100gsample)
Procyanidin trimer	11.67	867, 290	10.94
Procyanidin trimer	13.01	867, 290	9.84
Catechin	13.29	291	48.93
Procyanidin dimer	14.29	579, 290	18.34
Epicatechin	15.08	291	48.23
Gallocatechin	15.74	307	7.22
Epigallocatechin	16.58	307	12.41
Procyanidin dimer	17.02	579, 290	26.79
Petunidin 3-O-glucoside	17.68	479, 317	5.25
Procyanidin dimer	17.91	579, 290	-
Malvidin 3-O-glucoside	18.35	493, 331	4.65
Malvidin 3-O-(6''- coumaroyl -glucoside)	20.07	639, 331	3.69
Isorhamnetin 3-O-glucoside	23.30	479, 317	56.60

Table 4. Fatty acid composition of grape seed cakes (g/100g FAME)

Saturated fatty acids		Unsaturated fatty acids	
Caprylic acid (10:0)	0.02	Pentadecenoic (C15:1)	0.29
Lauric acid (12:0)	0.11	Palmitoleic (C16:1n-7)	0.28
Miristic acid (C14:0)	0.16	Oleic cis acid (C18:1n-9)	17.05
Pentadecanoic acid (15:0)	0.43	Eicosanoic acid (C20:1n-9)	1.78
Palmitic acid (C16:0)	10.05	Erucic acid (C22:1n-9)	0.26
Heptadecanoic acid (17:0)	0.07	Linoleic acid (C18:2n-6)	62.26
Stearic acid (C18:0)	3.67	Eicosadienoic acid (C20:2n-6)	0.10
Arahdic acid (C20:0)	0.26	Eicosatrienoic acid (C20:3n-6)	0.13
		Docosatetraenoic acid (22:4n-6)	0.04
		Arachidonic acid (C20:4n-6)	0.08
		α -Linolenic (C18:3n-3)	2.32
		Eicosatrienoic acid (C20:3 n-3)	0.07
Other fatty acids	0.49		
Total			
Σ Saturated fatty acids	14.85		
Σ Unsaturated fatty acids	84.66		

Effect of GSC diets on splenic inflammatory markers

Active ingredients from grape by-products demonstrated in many nutritional trials healthy beneficial anti-inflammatory and anti-oxidants

properties being able to counteract inflammation and oxidative stress in human and animals with more effectiveness in free radical scavengers than vitamin C and E (Rodríguez-Pérez et al., 2019; Tong et al., 2011). They possess also a large range of immunomodulatory properties by stimulating for example the humoral and cellular immune response and promoting the weight and functions of important immune organs such as thymus and spleen (Tong et al., 2011). Oral administration of either grape seed proanthocyanidin extract (GSPE) or grape seed and skin extract (GSSE) to obese mice and respectively obese rats resulted in significant decrease of Th17 cells and of pro-inflammatory interleukin 17 (IL-17) in spleen as well as of IL-6, TNF- α and IL-17A in plasma (Bedhafi et al., 2018; Jhun et al., 2013). Accordingly, in the present study the consumption of dietary grape seed cakes decreased in spleen of fattening pigs the protein concentration for pro-inflammatory cytokines, IL-1 β (-52.7%, $p < 0.05$) and IFN- γ (-42.1%, $p < 0.05$) and had a tendency to decrease that of IL-6 (-13.3%, TNF- α (-9.1%) and IL-8 (-11.1%, Figure 1). Real time PCR results showed also a decreased in the gene expression encoding for these inflammatory markers (IL-1 β , -38.0%, IL-8, -37.0%, IFN- γ , -17.0, Table 5). Similarly, grape seed proanthocyanidin extract (GSPE) reduced significantly the spontaneous production of TNF- α and IL-17 in splenocytes of mice in a study investigating the therapeutic effect of GSPE on collagen-induced arthritis (Cho et al., 2013).

Table 5. Effect of dietary GSC on pro-inflammatory cytokine gene expression in spleen*

	Diets			
	Control		Grape Seed cakes	
	Mean	SEM	Mean	SEM
<u>gene expression</u>				
IL-1 β (Fc)	1.00 ^a	0.0	0.62 ^b	0.3
IL-8 (Fc)	1.00 ^a	0.0	0.63 ^b	0.3
TNF- α (Fc)	1.00	0.0	0.97	0.2
IFN- γ (Fc)	1.00 ^a	0.0	0.83 ^b	0.2
IL-6 (Fc)	1.00	0.0	1.06	0.2

*Pigs received two different diets: control diet and 5% GSC (grape seed cakes) diet for 24d. At the end of the experiment, spleen samples were collected and analyzed for the mRNA expression of cytokines using quantitative real-time PCR. Results are expressed as fold change after normalization of target gene expression to the selected internal reference genes (ACTB RPL32 and Bm2) expression. ANOVA (one-way) followed by Fisher's tests were performed to analyze the effect of the diets on cytokine expression. Mean values with their standard errors, $n = 6$. Fc= fold change

^{a,b} = Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

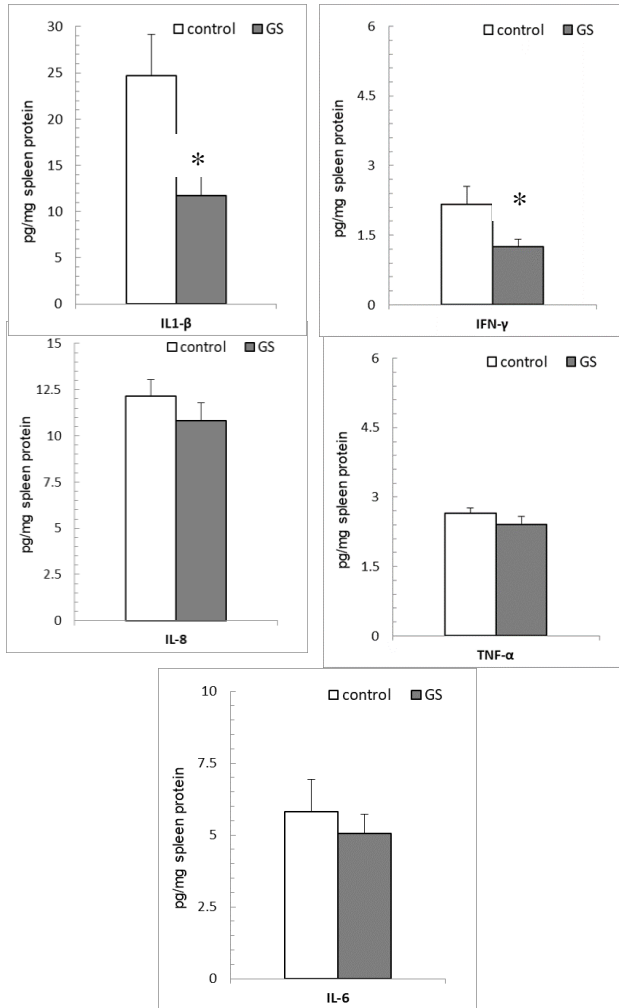


Figure 1. Pigs received two different diets: control diet and 5% GSC (grape seed cakes) diet for 24d. At the end of the experiment, spleen samples were collected, and the total concentration of pro-inflammatory cytokines from splenic tissue was measured by ELISA. Results are expressed as pg of cytokines/g of spleen protein (mean values with their standard errors, n= 6). ANOVA (one-way) followed by Fisher's tests were performed to analyze the effect of the diets on cytokine levels. * = (P<0.05).

Several studies conducted by Gessner and collaborators (Gessner et al., 2013; Gessner et al., 2017; Gessner et al., 2012) in pigs during weaning phase in which diarrhoea caused by pathogenic bacteria (*E. coli*, *Salmonella*) increase inflammatory process reported that pigs fed diet supplemented with a grape seed and grape marc extract had low concentration of relative abundance of

genes involved in inflammation like intercellular adhesion molecule 1 (ICAM1), chemokine (C-C motif) ligand 2 (CCL2), tumor necrosis factor α (TNF- α), interleukin 8 (IL-8) and acute phase response serum amyloid A (SAA) in duodenal mucosa than control pigs.

Effect of GP and GS diets on antioxidative status (TAC, enzymes activity and gene expression) in spleen

We further evaluated the antioxidant properties of grape seed cakes included in the diet of fattening pigs by their effect on gene expression and activity of antioxidant enzymes SOD, CAT and GPx as well as on total antioxidant capacity in spleen. Our results showed that GSC diet significantly increased in spleen the gene expression of GPx and CAT and had no effect on that encoding for gene expression of SOD (Table 6). No effect on spleen enzymes activity was recorded. The inconsistency between enzyme gene expression and enzyme activity is sometimes due to the much more complex structure of the protein than of the gene fragment (Bustin et al., 2009).

An important number of studies demonstrated the antioxidative properties of actives ingredients from grape by-products. Thus, the supplementation with grape seed proanthocyanidins extract (GSPE) in dosage between 100 and 400 mg/kg/b.w. significantly reduced the oxidative stress markers i.e., malondialdehyde (MDA), thiobarbituric acid reactive substance (TBARS), protein carbonyls (PC) and increased the total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities of the tested tissue (Li et al., 2015, Wang et al., 2019, Rodriguez Perez et al., 2019). Also, oral administration of grape seed proanthocyanidins extract (GSPE) reduced the oxidative stress assessed by nitrotyrosine expression in liver and spleen (Jhun et al., 2013) and the grape seed and skin extract (GSSE) was efficient in counteracting the oxidative stress parameters (decrease SOD, CAT and GPx) induced by a high-fat diet (HFD) in spleen of obese rats (Bedhafi et al., 2018). In the same line in the present study the grape seed cake diet decreased the level of TBARS by 20.7% and increased the total antioxidant capacity (TEAC) by 49% when administered of fattening pigs for 24 days (Table 6).

The molecular mechanism related to the anti-inflammatory and antioxidative properties of grape by-products compounds involved the modulation of several cellular pathways. The inhibition of the entire inflammatory cascades including MAPKs (p-38, ERK, JNK, c-jun) in the cytoplasm and NF- κ B in the nucleus was reported by (Chuang et al., 2011) and (Fraga and Oteiza, 2011).

Table 6. Effect of GSC diet on antioxidant enzyme and antioxidant capacity in spleen*

	Diets			
	Control		Grape Seed cakes	
	Mean	SEM	Mean	SEM
<u>Enzyme gene expression</u>				
SOD (Fc)	1.00	0.0	1.13	0.1
CAT (Fc)	1.00 ^a	0.0	3.09 ^b	0.1
GPx (Fc)	1.00 ^a	0.0	8.03 ^b	0.1
<u>Enzyme activity</u>				
SOD (U/mL lysate)	241.73	14.2	247.57	5.97
CAT ($\mu\text{mol}/\text{min}/\text{mL}$ lysate)	2.94	0.2	2.69	0.2
GPx ($\mu\text{mol}/\text{min}/\text{mL}$ lysate)	30.92	2.8	31.00	3.6
<u>Antioxydant capacity</u>				
TEAC ($\mu\text{mol}/\text{mL}$ lysate)	1.922	0.2	2.856	0.56
<u>Lipid peroxidaion</u>				
TBARS (nmol/mL lysate)	4.58	0.6	3.63	0.3

* Pigs received two different diets: control and 5% GSC (grape seed cakes) diet for 24d. At the end of the experiment, spleen samples were collected and analyzed for the mRNA expression, activity of antioxidant enzymes, TEAC and TBARS using quantitative real-time PCR and Cayman kits.

For gene expression, results are expressed as fold change after normalization of target gene expression to the arithmetic mean of selected reference genes (ACTB and GAPDH) and enzymes activity was reported per gram of mL lysate.

Total antioxidant capacity is expressed as trolox equivalent. All values are represented as mean with their standard errors; n= 6. ANOVA (one-way) followed by Fisher's tests were performed to analyze the effect of diets on enzyme expression.

a,b = Mean values within a row with unlike superscript letters were significantly different (P<0.05).

Reseveratrol, a powerful grape polyphenol exerts its anti-inflammatory potential in HUVECs cells by up-regulated the gene expression of SIRT1 (histone deacetylase sirtuin 1), a NAD-dependent histone deacytilase, contributing to the inhibition of p-38 MAPK/NF-kB pathway and of KSRP (KH-type splicing regulatory protein), a central post-transcriptional regulator of many pro-inflammatory mediators (Bollmann et al., 2014; Pan et al., 2016). In the study herein the analyses of mRNA expression of NF-kB and Nrf2 resulted in an increase of Nrf2 expression (Fc 1.58, +58%) in spleen of pigs fed dietary grape seed cakes compared to the control diet and no effect on NF-kB was recorded (Table 7). By contrast the gene expression of PPAR- γ was higher in spleen of pigs receiving the GSC diet (Fc 1.81, +81%) suggesting that the anti-inflammatory effect of this diet is exerted by PPAR- γ pathway. Negative correlation of PPAR- γ and pro-inflammatory cytokines in muscle and spleen was reported by (Zhan et al., 2009) in finishing pigs fed a flaxseed diet, flaxseed

being also known as a rich source of bioactive compounds like PUFA and polyphenols which might inhibit pro-inflammatory cytokines by activating PPAR- γ .

Table 7. Effect of diet GSC on regulatory gene expression in the spleen*

	Diets			
	Control		Grape Seed cakes	
	Mean	SEM	Mean	SEM
NF-kB (Fc)	1.00	0.0	1.05	0.3
Nrf2 (Fc)	1.00 ^a	0.0	1.58 ^b	0.3
PPAR γ (Fc)	1.00 ^a	0.0	1.81 ^b	0.3

* Pigs received two different diets: control diet and 5% GSC (grape seed cakes) diet for 24d. At the end of the experiment, spleen samples were collected and analyzed for the mRNA expression of NF-kB, Nrf2 and PPAR- γ signaling molecules using quantitative real-time PCR.

Results are expressed as fold change (Fc) after normalization of target gene expression to the average level of selected internal reference genes (ACTB and RPL32) expression. ANOVA (one-way) followed by Fisher's tests were performed to analyze the effect of treatment on cytokine expression. All values are expressed as mean with their standard errors; n= 6.

^{a,b} = Mean values within a row with unlike superscript letters were significantly different (P<0.05).

CONCLUSION

In conclusion the present study highlighted the modulatory effect of diet included 5% grape seed cakes on inflammation and oxidative stress in spleen, the largest lymphoid organ with a key role in immune response and lipid metabolism. This diet decreased the gene expression and protein concentration of pro-inflammatory biomarkers, IL-1 β , IFN- γ and had a tendency to decrease that of IL-6, TNF- α and IL-8. It also decreased the level of TBARS, a marker of lipid peroxidation and increased the total splenic antioxidant capacity (TAC) as well as the gene expression of antioxidant enzymes GPx and CAT.

The results obtained herein could be important for human nutrition swine being considered a very good model for human immune system due to the high homologies with human at the tissue level. These data confirm and complete previous finding related to the anti-inflammatory properties of polyphenols and other bioactive compounds exerted in human plasma and blood cells and provide supplementary information about inflammatory metabolic processes which happened during fattening/obesity in less accessible tissue like spleen involved not only in immune response but also in lipid metabolism.

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