Effect of xenobiotic compounds from grape waste on liver function and oxidative status in pigs

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SUMMARY

The objective of this study was to characterize several bioactive xenobiotic phytochemicals from a dried grape pomace (GP) derived from a Romanian winery and further to evaluate their effect on inflammation and oxidative markers in liver of pig. 20 crossbred TOPIG hybrid fattening pigs were randomly assigned (n = 10) to two experimental treatments: a normal diet (control group) and a diet included 5% grape pomace (GP group) for 24 days. The total polyphenols concentration of pomace was 36.2g gallic acid equiv. /100g. The pomace was rich in polyphenols from the flavonoids group, the main class being flavanols (epicatechins, catechin, epigallocatechin, procyanidins) and antocyanins (Malvidin 3-O-glucoside). The highest concentration was recorded for epicatechin (51.96g/100g) and procyanidin dimer (22.79g/100g). A high concentration of total polyunsaturated fatty acids (PUFA) especially ω-6 fatty acids (59.82 g/100g fat) were found also in grape pomace. The GP diet lowered the gene expression and protein concentration of IL-1β, IL-8, TNF-α and IFN-γ cytokines suggesting an anti-inflammatory effect of GP diet. Concentration of hepatic TBARS also decreased significantly. The total antioxidant capacity (liver TEAC) and activity of antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) did not differ between the GP and control diet while their gene expression tended to decrease. The results showed that GP diet exerted an anti-inflammatory effect, but 5% dietary inclusion modulated only partially the oxidative response and suggest other rate of inclusion need to be investigated.

Keywords: xenobiotics, diet liver, pigs, inflammation, oxidative status

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INTRODUCTION

Romanian viticulture and wine industry produced a large amount of waste (grape pomace) that needs to be managed in the context of EU waste recycling concerns (The Roadmap to a Resource Efficient Europe (COM (2011) 571), H2020). Grape pomace (GP) consists basically of grape seed, skin and stems accounting for about 20% of the original grape processed into wine (Taranu et al., 2017). This residue contains a lot of bioactive compounds such as polyphenols (anthocyanins, flavonols, phenolic acids and quercetin), polyunsaturated fatty acids especially linoleic-ω-6 fatty acid, minerals (iron, copper, zinc) fibres (Evans et al., 2014) which have recognized beneficial effect on human and animals. There is literature evidence that the mechanism of their action involved anti-inflammatory, anti-cancer, anti-microbial, anti-oxidative and immune-modulatory effects (Rahman et al., 2006; Cho et al., 2013; Gessner et al., 2013; Gessner et al., 2017a) in human and animals. In rats, for example, diets containing grape compounds stimulated the antioxidative activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GpX) decreasing the free radicals (Rho and Kim, 2006). This improvement of antioxidative status may decrease the health risk such as cardiovascular diseases and plasma cholesterol (Afman et al., 2014). Indeed, *in vivo* studies showed that the consumption of lyophilized grape preparation reduced cholesterol accumulation in guinea pigs (Zern et al., 2003). Also, (Han et al., 2016) stated that the administration of grape pomace and omija fruit extract in low and high concentration to overweight or obese subjects decreased the pro-inflammatory IL-1beta and TNF-alpha level, increased the antioxidant capacity of erythrocytes and decreased plasma total- cholesterol, HDL and LDL –cholesterol compared with the control group or the baseline levels.

The concern to ensure the sustainable recycling of agricultural waste, co-products and by-products intensified in the last decade investigations on their valorisation in animal nutrition. Thus, studies of (Gessner et al., 2013; Fiesel et al., 2014; Gessner et al., 2017b) reported the inhibition or prevention of inflammatory processes in the intestine and improve performance on piglets after the consumption of diet rich in polyphenols derived from grape marc meal extract. In another study in pig, the use of grape seed, grape marc and hop extracts decreased some pathogenic bacteria (*Streptococcus spp*., and *Clostridium spp*.), the expression of several pro-inflammatory intestinal genes and improve the gain-to-feed ratio suggesting the antimicrobial and anti-inflammatory effect of bioactive compounds from grape waste (Fiesel et al., 2014). In dairy cows, the administration of feed containing isoflavones (genistein, daidzein) such as soybean meal or red glover silage led to the increase in their concentration in blood and milk (Cools et al., 2014). Also in cow, dietary inclusion of grape waste increased the concentration of polyunsaturated fatty acids in

milk, prevented the oxidation of fatty in milk and improve the general health of cows (Santos et al., 2014), (Moate et al., 2014). Despite these examples, the number of studies regarding the effects of xenobiotic phytochemicals from grape waste on different aspects related to inflammation and oxidative stress in pig remains rather low, so far. That is why, the aim of the present study was to assess the effect of xenobiotic compounds derived from grape pomace included in the diet for pig on several hepatic specific markers related to inflammation and antioxidant system. Liver is one of the key organs involved in xenobiotic biotransformation and synthesis of a new nutrient and also in the immune homeostasis and immune response (Park et al., 2013). Effect on antioxidant enzyme and inflammatory cytokine gene expression, protein concentration and activity, liver total antioxidant capacity and liver lipid peroxidation was investigated. Pig was used as a target and also as a model species for humans in order to understand the effect of grape phytochemicals specific organs less accessible in human studies.

MATERIAL AND METHODS

Animals and diets

A total number of 20 crossbred TOPIG hybrid [(Landrace × Large White) × (Duroc × Pietrain)] pigs individually identified by ear tag were housed in pens and fed with two experimental diets for 24 days using basal diet (control group) and basal diet with 5% grape pomace (GP group). The grape pomace consisting of stems, skins and seeds derived from Valea Calugareasca Romanian winery, was provided by a local producer. GP raw material dried in a heated air flow was milled to a particle size of less than 6 mm in a Cyclone Mill -MC5 (Tecator, Höganäs, Sweden) and incorporated in the conventional feed compound in a proportion of 5%. The experimental diets were formulated to meet all nutritional requirements for fattening pigs as indicated by NRC (2012). The ingredients and nutrient composition of these diets are presented in Table 1. Brute composition of the diets (dry matter-DM, crude protein-CP, fat-EE, crude fibers-CF and ash) as well as of grape pomace was determined according to the ISO methods (ASRO-SR EN ISO, 2010), (Tables 1 and 2).

Pigs had *ad libitum* access to feed and water during the experimental period and feed intake was recorded daily per pen. Body weights of all pigs were recorded at the beginning and the end of the trial. The average daily gain (ADG), average daily feed intake (ADFI), and gain-feed ratio (G:F) were calculated at the end of the trial.

The experimental procedures used in this trial were 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. The study protocol was approved by the Ethical Committee of the National Research-Development Institute for Animal Nutrition and Biology, Balotesti, Romania. All animals remained healthy during the experimental period and no veterinary drugs were used. All efforts were made to minimize suffering at the slaughter.

Table 1 Composition and calculated nutrient content of experimental diets

Ingredients (%)	Fattening-finishing phase[1]	
	Control diet	Grape pomace diet
Corn	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 pomace	-	5.00
Monocalcium phosphate	0.35	0.46
Limestone	2.03	1.90
NaCl	0.40	0.40
Methionine	-	0.04
Lysine	0.28	0.27
Choline premix	0.10	0.10
Mineral vitamin-premix[2]	1.00	1.00
Calculated nutrient content		
CP (%)	15.29	15.05
DP (%)	12.07	11.94
Fat (%)	4.29	4.43
Crude fibre (%)	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	0.90
Phosphorus (%)	0.60	0.60
Total	100.00	100.00

[1]BW range 75.53 to 99.59 kg

[2]mineral-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. B_1 1.0 mg, vit. B_2 3.0 mg, d-pantothenic acid 6.3 mg, niacin 10 mg, biotin 30 µg, vit. B_{12} 20 µg, folic acid 0.3 mg, vit. B_6 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.

Sample collection and measurements

At the end of the experimental period (day 24), all pigs were sacrificed and their internal organs were removed; tissue samples of liver were collected and stored at –80°C until analysed for inflammatory parameters

including cytokines (gene expression and protein concentration), antioxidant status assessed by TBARS-MDA concentration, total antioxidant capacity (TCA) and antioxidant enzymes, SOD, CAT and Gpx (gene expression and activity).

Table 2. Chemical composition of grape pomace

Items	Grape pomace
Dry matter (%)	87.63
Crude protein (%)	10.32
Ether extract (%)	5.14
Crude fibre (%)	25.01
Neutral detergent fibre (%)	58.01
Acid detergent fibre (%)	52.26
Ash (%)	5.75
Metabolisable energy (ME, kcal/kg)	1912
Total polyphenols (mg GAE/100g)	3619.80
DPPH (μM TRE/g sample)	27.05

Measurement of total phenolic content and polyphenols composition of grape pomace

The polyphenols from grape pomace were extracted in 80% acetone (ratio sample; solvent 1:7 w/v) at 37°C with continuous shaking for 20 hours and the total content was determined using Folin–Ciocalteu method adapted to a microscale (Arnous et al., 2001) as described by (Taranu et al., 2017) (Table 2).

The composition in polyphenols of the GP acetone extract was determined by HPLC-DAD-MS based on their retention times, UV-Vis spectra (200 to 600 nm) and the mass spectrum of individual compounds according to the method of (Garcia et al., 2012; Dulf et al., 2015) described by (Taranu et al., 2017). Briefly, the mobile phases gradient consisted of 0.1% acetic acid in distilled water (v/v) (solvent A) and 0.1% acetic acid in acetonitrile (v/v) (solvent B) at a flow rate of 0.5 mL/min for 30 min using the following gradient elution program: 0 to 2 m (5% B) 2 to 18 m (5 to 40% B) 18 to 20 m (40 to 90% B) 20 to 24 m (90% B) 24 to 25 m (90 to 5% B) 25 to 30 m (5% B). The column used was an Eclipse, XDB C18 (4.6 × 150 mm, 5 μm) (Ag Technologies, U.S.A.).

The catechins and derivatives were detected at 280nm and the anthocyanins at 520nm. Data analysis was performed using Agilent ChemStation Software (Rev B.04.02 SP1, Palo Alto, California, U.S.A.). The catechins and derivatives were calculated as equivalents of catechin (mg catechin/100g DW of substrate) (r^2 = 0.9985). Anthocyanin levels were determined using cyanidin chloride as external standard and expressed as equivalents of cyanidin (mg cyanidin/100g DW of substrate) (r^2 = 0.9951).

Antioxidant activity of grape pomace

The antioxidant activity was measured using the stable radical, DPPH as described by (Garcia et al., 2012). An aliquot of 25µl of diluted sample was added to 975µl of DPPH solution (60 µM in MeOH) and vortexed, the absorbance was read at 515 nm at t = 0 and t = 30 min. The results were expressed as µM trolox equivalents (TRE).

Fatty acid composition of grape pomace

Fatty acid composition from GP was determined by gas chromatography using a Perkin Elmer gas chromatograph (Clarus 500, USA) and 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) as described by (Taranu et al., 2014).

Liver antioxidant capacity

Antioxidant capacity in liver of pigs fed with control or GP diets was measured using total antioxidant capacity kit (TAC QuantiChrom – BioAssay Systems, USA) as described by (Taranu et al., 2014). 20µL of liver tissue lysate or Trolox standard solution plus 100µl Working Reagent were added to a 96-well microplate, mixed by tapping and incubated at room temperature for 10 min according to the manufacturer's instructions. The end point absorbance was read at 570 nm using a microplate reader (TECAN, Infinite M200 PRO, Austria).

Liver lipid peroxidation (MDA-TBARS assay)

0.2g of liver was homogenized with 8mL phosphate buffer and the liver lipid peroxidation was evaluated by measuring thiobarbituric acid reactive substances (TBARS). The mix was incubated at 95^0C for 15 min, then cooled and the TBARS adducts were collected and the TBARS fluorescence was measured at 515 nm excitation and 548 emission with a Tecan Sunrise, Austria. MDA-TBARS are expressed as nmol/g tissues.

Analysis of inflammatory and antioxidant gene expression (qPCR)

Gene expression of pro-inflammatory markers (TNF-α, IL-1β, IL-6, IL-8, IFN-γ), antioxidant enzymes (SOD, CAT, GPx1) and related signalling molecules (nuclear factors NF-κB and Nrf2) were determined using quantitative PCR technique (qPCR). Frozen samples of liver were disrupted in liquid nitrogen and then 100 mg of tissue powder was homogenized in RTL buffer (QIAGEN GmbH, Germany) with an Ultra-Turrax homogenizer (IKA®-Werke GmbH & Co. KG, Germany). Total RNA was extracted using Qiagen RNeasy midi kit (QIAGEN GmbH, Germany), according to the manufacturer's recommendations.

After extraction, RNA was treated with a ribonuclease inhibitor (RNasin® Plus RNase Inhibitor Promega Corp., USA) and the integrity of RNA was verified by agarose gel electrophoresis. The quantity and quality of the total extracted RNA was measured on a Nanodrop ND-1000 spectrophotometer (Thermo Fischer Scientific, USA). Total liver RNA and M-MuLV Reverse Trascriptase kit (Fermentas, Thermo Fischer Scientific, USA) was used to generate cDNA according to the manufacturer's protocol (Pistol et al., 2015). 5µl of cDNA (diluted 1:10), 12.5µl Maxima SYBR Green/ Fluorescein qPCR Master Mix 2X (Fermentas, Thermo Fischer Scientific, USA) and 0.3µM each of gene-specific primer (Table 3) in a total reaction volume of 20µl were used for qPCR reactions in order to evaluate above mentioned gene expression.

Table 3. Nucleotide sequences of primers for Real-Time PCR

Gene	Accession no.	Primer source	Primer sequence (5`→3`)	Orientation	Tm (ºC)	Amplicon length (bp)	References
TNF-α	NM_214022	Pig	ACTGCACTTCGAGGTTATCGG GGCGACGGGCTTATCTGA	forward reverse	60 60	118	(Grenier et al., 2012)
IL-8	NM_213867.1	Pig	GCTCTCTGTGAGGCTGCAGTTC AAGGTGTGGAATGCGTATTTATGC	forward reverse	58 54	79	(Grenier et al., 2012)
IL-6	NM_214399	Pig	GGCAAAAGGGAAAGAATCCAG CGTTCTGTGACTGCAGCTTATCC	forward reverse	57 61	87	(Grenier et al., 2012)
IL-1β	NM_214055	Pig	ATGCTGAAGGCTCTCCACCTC TTGTTGCTATCATCTCCTTGCAC	forward reverse	62 59	89	(Royaee et al., 2004)
IFN-γ	NM_213948.1	Pig	TGGTAGCTCTGGGAAACTGAATG GGCTTTGCGCTGGATCTG	forward reverse	54 55	79	(Royaee et al., 2004)
iNOS	NM_00114369 0.1	Pig	GGAGCCATCATGAACCCCAA GTAGAAGCTCGTCTGGTGGG	forward reverse	60 62	73	Primer 3
eNOS	NM_214295.1	Pig	CCCTACAACGGCTCCCCTC GCTGTCTGTGTTACTGGATTCCTT	forward reverse	64 50	129	(Chai et al., 2005)
COX2	NM_214321.1	Pig	CCCAATTTGTTGAATCATTT TCTCATCTCTCTGCTCTGGT	forward reverse	55 55	119	(Jung et al., 2007)
CAT	NM_214301.2	Pig	CTTGGAACATTGTACCCGCT GTCCAGAAGAGCCTGAATGC	forward reverse	55 55	241	(Blomberg et al., 2005)
GPx	NM_214201.1	Pig	GGAGATCCTGAATTGCCTCAAG GCATGAAGTTGGGCTCGAA	forward reverse	56 57	62	(Hostetler et al., 2006)
SOD	NM_00119042 2.1	Pig	GAGACCTGGGCAATGTGACT CTGCCCAAGTCATCTGGTTT	forward reverse	62 58	139	(Marin et al., 2013)
NF-kB	NM_00111428 1.1	Pig	CGAGAGGAGCACGGATACCA GCCCCGTGTAGCCATTGA	forward reverse	55 54	62	(Chatelais et al., 2011)
GAPDH	NM_00120635 9.1	Pig	ACTCACTCTTCTACCTTTGATGCT TGTTGCTGTAGCCAAATTCA	forward reverse	57 55	100	(Avramovic et al., 2012)
ACTB	NM_213978.1	Pig	GGACTTCGAGCAGGAGATGG GCACCGTGTTTGCGTAGAGG	forward reverse	60 62	230	(Meadus et al., 2002)
RPL32	NM_00100163 6	Pig	TGCTCTCAGACCCCTTGTGAAG TTTCCGCCAGTTCCGCTTA	forward reverse	62 60	106	(Grenier et al., 2012)
Cyclophilin A	NM_214353.1	Pig	CCCACCGTCTTCTTCGACAT TCTGCTGTCTTTGGAACTTTGTCT	forward reverse	54 55	92	(Devriendt et al., 2009)
Beta-2 microglobulin	NM_213978.1	Pig	TTCTACCTTCTGGTCCACACTGA TCATCCAACCCAGATGCA	forward reverse	55 50	162	(Hyland et al., 2006)

The cycling conditions were: UDG pre-treatment at 50°C for 2 min, initial denaturation step at 95°C for 15 s, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 15 s with a single fluorescence measurement; a final elongation step was carried out at 72°C for 10 min.

The relative product levels were quantified using the $2^{(-\Delta\Delta C_T)}$ method (Meurens et al., 2009) as described by (Taranu et al., 2017).

Measurement of cytokine protein concentration (ELISA)

Protein concentration of pro-inflammatory cytokine (IL-8, IL1-β, IL-6, TNF-α and IFN-γ) was measured in the liver lysates by ELISA from undiluted supernatants using commercially available kits (R&D Systems, Minneapolis, MN 55413, USA and Biosource International, Inc., Camarillo, USA) according to the manufacturer's instructions. Capture antibodies including purified fractions of anti-swine cytokines IL-8 (MAB5351), TNF-α (MAB6902), IL-1β (MAB6811), IL-6 (MAB686) and IFN-γ (ASC4934) were used in conjunction with biotinylated anti-swine cytokines IL-8 (BAF535), TNF-α (BAF690), IL-1β (BAF681), IL-6 (BAF686) and IFN-γ (ASC4839). Recombinant swine IL-8, TNF-α, IL-1β and IFN-γ diluted protein were used to generate standard curve and streptavidin horseradish peroxidase (HRP) (Biosource, Camarillo, USA) along with tetramethylbenzidine (TMB) (Sigma) was used for detection. Optical density was measured on an ELISA microplate reader (Tecan, SunRise, Austria) at 450 nm and results were expressed as picrogram (pg) of cytokine/mg liver protein quantified by using bovine serum albumin as standard (Pierce® BCA Protein Assay Kit, Thermo Fischer Scientific, USA).

Antioxidant enzyme (SOD, CAT, GPx) activity

Antioxidant enzyme activity was measured by using Cayman kit (Ann Arbir, MI) as described by (Giriwono et al., 2010). Frozen liver (1g/sample) was homogenised in chilled phosphate buffer and centrifuged at 1500 or 15000 × g for 15 min at 4^0C according to the manufacturer's recommendations for each specific enzymes. The supernatants then were used as already described in the Cayman kit for the activity measurement of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). The absorbance was measured using a Tecan microplate reader (Tecan, SunRise, Austria).

Statistical analyses

All the results are expressed as mean ± standard error of the mean (SEM). One way ANOVA analysis was performed to investigate the statistical differences between groups for all parameters analysed. 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

Polyphenols composition and antioxidant activity of GP

Folin-Ciocalteu reaction showed that GP is a good source of polyphenols, the total content rising 3.62g/100g with a DPPH activity of 27.05 μM TRE/g sample) (Table 2). The polyphenol profile highlighted that the flavonoids group is the most important the main class being flavanols (catechin, epicatechins, gallocatechin, epigallocatechin, procyanidins) and antocyanins (Petunidin 3-O-glucoside, Malvidin 3-O-glucoside) (Table 4). The highest concentration was recorded for epicatechin (51.96g/100g) followed by procyanidin (ex. 22.79g/100g for procyanidin dimer), catechin (11.63 g/100g) and so one (Table 4).

Table 4. Polyphenols composition (g/100g of sample) of grape pomace

Items	R_t (min)	$[M-H]^+$	Grape pomace
Procyanidin trimer	11.67	867, *290*	10.62
Procyanidin trimer	13.01	867, *290*	10.16
Catechin	13.29	291	11.63
Procyanidin dimer	14.29	579, *290*	-
Epicatechin	15.08	291	51.96
Gallocatechin	15.74	307	6.90
Epigallocatechin	16.58	307	9.20
Procyanidin dimer	17.02	579, *290*	22.79
Petunidin 3-O-glucoside	17.68	479, *317*	-
Procyanidin dimer	17.91	579, *290*	-
Malvidin 3-O-glucoside	18.35	493, *331*	6.75
Malvidin 3-O-(6''- coumaroyl – glucoside)	20.07	639, *331*	7.44
Isorhamnetin 3-O-glucoside	23.30	479, 317	-

Polyunsaturated fatty acid composition

The content of polyunsaturated fatty acids (PUFA) was higher than that of saturated fatty acids (79.92 g/100g fat vs 19.71g/100g fat). GP is rich in omega-6 and omega-9 fatty acids, the highest concentration being registered for linoleic acid (58.99g/100g fat), followed by oleic acid (16.86g/100g fat) Table 5.

Animal performance

The average daily weight gain and also the daily feed intake did not differ between control and GP group, the results for these parameters been very close: 0.972 kg ± 0.12 /pig/day GP diet *vs* 1.017 kg ± 0.10 kg/pig/day control diet for ADG, and 3.42 kg ± 0.32/day GP diet *vs* 3.41 kg ± 0.30 /day control diet for ADFI, respectively.

Table 5. Fatty acid composition (g/100g of ether extract) of the grape pomace

Saturated fatty acids		Unsaturated fatty acids	
Caprylic acid (10:0)	0.26	Pentadecenoic (C15:1)	0.12
Lauric acid (12:0)	0.07	Palmitoleic (C16:1n-7)	0.53
Myristic acid (C14:0)	0.39	Oleic cis acid (C18:1n-9)	16.86
Pentadecanoic acid (15:0)	0.18	Eicosanoic acid (C20:1n-9)	0.30
Palmitic acid (C16:0)	14.36	Erucic acid (C22:1n-9)	0.13
Heptadecanoic acid (17:0)	0.11	Linoleic acid (C18:2n-6)	58.99
Stearic acid (C18:0)	3.89	Eicosadienoic acid (C20:2n-6)	0.03
Arachidic acid (C20:0)	0.39	Eicosatrienoic acid (C20:3n-6)	0.037
		Docosatetraenoic acid (22:4n-6)	0.26
		Arachidonic acid (C20:4n-6)	0.14
		α -Linolenic (C18:3n-3)	2.19
		Eicosatrienoic acid (C20:3 n-3)	-
Other fatty acids	0.38		
Σ Saturated fatty acids	19.71		
Σ Unsaturated fatty acids	79.92		
Σ n-6	59.82		
Σ n-3	2.19		
Σ n-6/Σ n-3	27.32		
Linoleic / α-Linolenic	26.94		

Effect of GP diet on hepatic inflammatory markers

The examination of hepatic pro-inflammatory cytokines gene expression showed that the consumption of GP diet significantly decreased the expression of IL-6 (-64.4%), IL-8 (-57.7%) and IFN-γ (-52.0%) cytokines (Table 6) in comparison with control diet. The qPCR analysis showed also that GP diet down regulated the expression of eNOS and COX2 another inflammatory mediators (Table 6). A similar effect on the pro-inflammatory cytokines was noticed at the protein level (Table 7).

Table 6. Effect of GP diet on inflammatory cytokine gene expression in liver*

Cytokines	Diets			
	control		Grape pomace	
	Mean	SEM	Mean	SEM
IL-1β (Fc)	1.00	0.0	1.18	0.1
IL-8 (Fc)	1.00[a]	0.0	0.42[b]	0.1
IL-6 (Fc)	1.00[a]	0.0	0.36[b]	0.1
TNF-α (Fc)	1.00	0.0	0.67	0.1
IFN-γ (Fc)	1.00[a]	0.0	0.48[b]	0.1
Other inflammatory molecules				
iNOS (Fc)	1.00	0.0	0.81	0.1
eNOS (Fc)	1.00[a]	0.0	0.33[b]	0.1
COX2	1.00[a]	0.0	0.37[b]	0.2

* Pigs received two different diets: control diet and 5% grape pomace (GP) for 24d. At the end of the experiment, liver samples were collected and analyzed for cytokines mRNA expression using q PCR. Results are expressed as fold change (Fc) after normalization of target gene expression to the selected internal reference genes (CYPA and RPL32). All values are represented as mean 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).

Table 7. Effect of GP diet on protein concentration of inflammatory cytokine in liver*

Cytokines	Diets			
	control		Grape pomace	
	Mean	SEM	Mean	SEM
IL-1β (µg/g tissue)	13.42	1.23	11.27	1.79
IL-8 (µg/g tissue)	6.40[a]	0.91	3.60[b]	0.47
IL-6 (µg/g tissue)	7.88	0.84	6.19	1.09
TNF-α (µg/g tissue)	23.35	2.41	19.76[b]	2.87
IFN-γ (µg/g tissue)	5.39[a]	0.41	2.92[b]	0.63

* Pigs received two different diets: control diet and 5% grape pomace (GP) for 24d. At the end of the experiment, liver samples were collected and the protein concentration of cytokines from hepatic tissue was measured by ELISA. Results are expressed as pg of cytokines/mg of liver protein. All values are represented 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).

Effect of GP diet on liver antioxidative status (TAC, lipid peroxidation, gene expression and activity of antioxidant enzymes)

GP diet reduced significantly the TBARS concentration (-20.44%, p < 0.0188) and had no effect on total antioxidant capacity (Table 8). Gene expression of antioxidant enzyme SOD, CAT and GPx tended to be lower (p<0.1) in liver o pigs fed GP diet (Table 8). Enzymes activity decreased slightly for catalase and no effect on that of SOD and GPx was found when compared with control diet (Table 8).

Table 8. Effect of GP diet on liver antioxidant enzyme and antioxidant status*

Items	Diets			
	control		Grape pomace	
	Mean	SEM	Mean	SEM
SOD1 (Fc)	1.00	0.0	0.52	0.0
CAT (Fc)	1.00	0.0	0.47	0.0
GPx (Fc)	1.00	0.0	0.52	0.2
Enzyme activity				
SOD (U/mg tissue)	5.392	0.06	5.332	0.06
CAT (mmol/min/g tissue)	0.810	0.09	0.750	0.06
GPx (µmol/min/g tissue)	0.704	0.06	0.715	0.02
TAC (µmol/g tissue)	179.68	11.1	181.19	8.3
TBARS (nmol/g tissue)	257.67[a]	13.9	205.00[b]	14.9

* Pigs received two different diets: control diet and 5% grape pomace (GP) for 24d. At the end of the experiment, liver samples were collected and analysed for the mRNA expression and activity of antioxidant enzymes.

For gene expression, results are expressed as fold change after normalization of target gene expression to the mean of selected reference genes (CYPA and RPL32) and enzymes activity was reported per g of tissue. Total antioxidant capacity is expressed as trolox equivalent. All values are represented 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).

Effect of GP diet on regulatory molecules NF-kB and Nrf2

Expression of NF-kB and Nrf2 important genes for the regulation of inflammatory cytokines and antioxidant enzymes was not influenced by the GP treatment (Table 9).

Table 9. Effect of GP diet on regulatory gene expression in the liver*

	Diets			
	control		Grape pomace	
	Mean	SEM	Mean	SEM
NF-kB (Fc)	1.00	0.0	0.97	0.1
Nrf2 (Fc)	1.00	0.0	0.80	0.1

* Pigs received Pigs received two different diets: control diet and 5% grape pomace (GP) for 24d. At the end of the experiment, liver samples were collected and analysed for the mRNA expression of Nf-kB and Nrf2 molecules using qPCR. Results are expressed as fold change (Fc) after normalization of target gene expression to the average level of selected internal reference genes (CYP and RPL32). 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).

DISCUSSION

Grape by-products resulting from wine processing are rich sources of xenobiotics (polyphenols, unsaturated fatty acids etc.) whose possible beneficial effects have been scarcely investigated in farm animals (Gessner et al., 2013). This study evaluated the effects of xenobiotic bioactive compounds from grape pomace (5% inclusion in the diet) on performance and several parameters related to inflammation, immune response and antioxidative defence in liver of fattening pigs.

The results showed that consumption of diets including grape pomace had no effect on the growth performance of fattening pigs. It was suggested that the effect of grape by-products on animal performance is correlated with the concentration of polyphenols in the diet which influence or not the nutrient digestibility (Taranu et al., 2017).

The effects of grape by-products on inflammation was investigated in the liver, a key organ for the metabolization and detoxification of xenobiotic substances (Cui et al., 2014) important also for innate immunity and immune homeostasis (Mbimba et al., 2012; Hong et al., 2013; Pistol et al., 2014). Hepatic inflammation is mediated mainly by cytokines such as IL-6, IL-1β, TNF-α etc. (Park et al., 2013). The results obtained at the end of this trial showed that IL-6, TNF-α, IL-8 and IFN-γ gene expression was lowered in liver of pigs fed GP diet as well as their protein secretion.

(Cho et al., 2013) also observed that a combination of grape pomace and omija fruit extracts down-regulated the gene encoding for IL-6, TNF-α and of the NF-kB (Perez-Cano et al., 2013), in obese mice. The decrease of NF-kB, the signalling transcription factor involved in the regulation of pro-inflammatory cytokines genes (Park et al., 2012) could be one of the way by

which grape compounds exert their anti-inflammatory action (Cho et al., 2013).

It was shown that this action include also other members of inflammatory cascade like (p-38, ERK, JNK, c-jun) (Chuang et al., 2011; Park et al., 2012) and related inflammatory molecules. In this study the GP down-regulated the gene expression of the pro-inflammatory COX2, iNOS and eNOS. Furthermore, investigations with resveratrol an active polyphenol present in grapes showed that this compound inhibited the inflammation in HUVECs cells by increasing the expression of two important regulator genes: SIRT1 (histone deacetylase sirtuin 1), a NAD-dependent histone deacytilase, which contributes to the inhibition of p-38 MAPK/NF-kB pathway and KSRP (KH-type splicing regulatory protein), a central post-transcriptional regulator of many pro-inflammatory mediators which bind to the AU-rich elements (AREs) and recruits enzymes involved in mRNA decay (Bollmann et al., 2014; Pan et al., 2016). Reseveratrol did not reduce iNOS, IL-8 and TNF-α mRNA expression in the cells with diminished KSRP expression (Bollmann et al., 2014). Other bioactive compounds present in grape by-products possessing anti-inflammatory properties such as PUFA might be responsible for the inflammatory reduction of GP diet. PUFA and especially omega-3 PUFAs, affect for example the phosphorylation of nuclear receptors (Umezawa et al., 2000; Xu et al., 2006) and inhibits the upstream pathway of MAPKs through the activation of GPR120 receptor (Eder et al., 2002) leading to anti-inflammatory effects (Zhan et al., 2009; Farmer et al., 2010; Hur et al., 2012; Taranu et al., 2014).

By contrast an up-regulation of NF-kB, IL-10, IGF-1 and Caspase 3 was observed in the liver of young pigs (Sehm et al., 2011) fed with dietary apple pomace.

Previous studies showed that the bioactive compounds from grape extract or grape by-products reduced lipid peroxidation measured by TBARS-MDA (Rho and Kim, 2006; Goutzourelas et al., 2014). The intake of grape pomace and whole grape caused a decrease in plasma and liver lipid peroxide content in Sprague-Dawley male rats and the grape pomace-ingesting group had the lowest level (Rho and Kim, 2006). In the present study, hepatic TBARS-MDA value was significantly decreased in the liver homogenates derived from pigs fed with dietary GP in comparison with control diet. It was suggested that the antioxidants content of grape by-products (flavonoids, phenols, PUFA) are effective in diminishing the TBARS level by their ability to scavenge free radicals (Giribabu et al., 2015). No effect on total antioxidant capacity was observed similar with (Zhang et al., 2014) in weaned pigs. Beside the decrease in TBARS level many findings reported an increase in the three antioxidant enzymes (SOD, CAT and GPx) activity under grape compounds action. Thus, (Bobek et al., 1998)

reported that in Wistar rats fed with 5% dietary grape pomace for 8 weeks the level of GSH-Px was increased by 38 %. An increased in hepatic antioxidant enzymes activity was observed also by (Rho and Kim, 2006) in rats. Gessner et al., (2013) reported that the modulation of antioxidant enzymes by grape compounds involved the activation of nuclear transcription factor (Nrf2) followed by a rapid induction of antioxidant enzymes. By contrast, in the present work GP diet modulated differently the expression as well as the activity of SOD, GPx and CAT in liver of pigs; gene expression of hepatic antioxidant enzymes and Nrf2 was down-regulated by GP diet in comparison with control. The activity of CAT was also decreased and no changes in that of SOD and GPx was produced by GP diet A decreasing in antioxidant enzymes by bioactive substances from different pomace was also shown by (Bobek et al., 1998) and (Gessner et al., 2013) who observed a decreasing of Nrf2 gene activation and its target genes in piglets after consumption of grape extracts (seed and marc).

In conclusion, the results obtained in this study showed that 5% dietary grape pomace did not influence the pig performance. Other rates of grape by-product inclusion in pig diet need to be tested in order to see if an effect on performance could be obtained. However, there are finding showing that grape pomace improved the quality of pig meat (Garrido et al., 2011). The utilization of grape waste (grape pomace in this case) produced a significant effect on liver function. GP diet generated lower level of inflammatory cytokines (gene expression and protein secretion) and decreased the gene level of antioxidant enzymes and their activity partially. The results obtained herein could be important for human nutrition, taking into account the high similarity between the pig and human liver metabolism (Soucek et al., 2001).

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