

USE OF HIGH POLYPHENOLS GRAPE SEEDS CAKES TO MODULATE THE INFLAMMATORY STATUS AND PIGLET HEALTH DURING THE POST-WEANING PERIOD

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Abstract

Weaning is a very difficult period for the pig, during which the nature and quality of the feeds is of great influence on the developing systems of digestion and defence. The weaning time is correlated with an increase of local inflammatory response. The supplementation of the weaning diet with ingredients rich in bioactive compounds with antimicrobial properties was lately investigated. This is the case of the polyphenols, which have been studied in depth for their beneficial effect on health and for their antioxidant action, but to a lesser extent, for their antimicrobial action. Grape by-products (grape seeds, grape pomace) could be alternative and cheaper sources with anti-microbial potential which could be used in the weaning diet. In the present study we analysed the effects of 5% grape seed cakes (GS) inclusion in the diet on the blood biochemical parameters and on pro- and anti-inflammatory markers in spleen and lymph nodes. A total of 12 weaned pigs were fed with a control or 5% grape seed cakes (GS) diets for 30 days. Pigs were sacrificed after 30 days, blood and organs were collected and stored at -80°C until analyses. Our results showed that diet included 5% GS did not influence the health status determined by plasma biochemical parameters. Only a tendency for a slight increase of the biochemical parameters associated with energetic profile (glucose, cholesterol, triglycerides) was observed. Also, GS diet had no effect on pro- and anti-inflammatory cytokines content in spleen and lymph nodes tissue. Further experiments are needed in order to investigate other rate of dietary inclusion which could provide more evidence about the effect of grape bioactive compounds on general health status and inflammation in weaning piglets.

Key words: weaning piglets, inflammation, polyphenols, by-products

Weaning is a very difficult period for the pig, during which the nature and quality of the feeds is of great influence on the development of the digestive and immune systems. Recent immunological researches have shown that the weaning time is correlated with a transitory increase of local inflammatory response (Lalles *et al*, 2007, Pie *et al*, 2004). As the use of antibiotics as growth promoters has been banned in the European Union (2006), researches have been conducted to find alternative feeding solutions to prevent or reduce inflammations and mortality in weaning piglets (Willing *et al*, 2011). The supplementation of the weaning diet with ingredients rich in bioactive compounds with antimicrobial and anti-inflammatory properties was investigated during the past decade. Polyphenols are among these studied phyto-components, and

the studies revealed their beneficial role for the human health and their antioxidant role.

The by-products from wine and oil production (grape pomace and grape seeds cakes) are sources of bioactive compounds, particularly due to their high level of polyphenol compounds such as anthocyanins, flavonoids and phenol acids. It was shown that many of these compounds have beneficial effects on human and animal (rodents) health. Gessner *et al* (2016) reported that diet supplementation with a grape seeds extract and grape pomace, high in polyphenols, reduced the activity of inflammation markers in the duodenal mucosa of the piglets. Recent studies have shown the beneficial effects of proanthocyanidins from the grape seeds on the intestinal microflora (Campos-Salinas *et al*, 2014). Further studies are, however, necessary to determine the effect of the grape seeds cakes before taking them into

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consideration as commercial feed additive for weaning piglets. This study analysed the effects of 5% grape seed cakes (GS) inclusion in the diet on the blood biochemical parameters, as markers of the general health state, and on pro- and anti-inflammatory markers of the local immune response in key organs of the immune system (spleen and lymph nodes) of weaned piglets.

MATERIAL AND METHOD

Animals and experimental diets: The feeding experiment was conducted in the experimental farm of IBNA on 20 weaned hybrid [(Landrace × Large White) × (Duroc × Pietrain)] piglets, with an average initial body weight of 9.83 ± 0.4 kg, for 30 de days. The piglets were allowed one week for accommodation before the on set of the experimental protocol. The animals were assigned to two groups (10 piglets/group): group 1: basal diet formulation (control CF) and group 2: basal formulation with 5% grape seeds cakes (CF-GS). The basal formulation had 45% corn, 15% wheat, 9% rice meal, 15% soybean meal, 4% sunflower meal, 4% corn gluten, 3% powder milk, 0.29% L-lysine, 1.73% calcium carbonate, 0.85% monocalcium phosphate, 0.20% salt, 1% mineral-vitamin premix. CF-GS formulation had a similar composition with the control formulation, with the difference of 42.68% corn and 5% grape seeds cakes. The quality characteristics of the two formulations were as follows: control CF (C): metabolisable energy 3115 Kcal/kg, 18.36% crude protein, 0.90% calcium, 0.65% phosphorus, 4% fibre, 1.05% gross lysine, 0.90% digestible lysine, 0.65% methionine + gross cysteine, 0.53% methionine + digestible cysteine; experimental CF-GS: metabolisable energy 3104 Kcal/kg, 18.25% crude protein, 0.90% calcium, 0.65% phosphorus, 3.80% fibre, 1.05% gross lysine, 0.90% digestible lysine, 0.65% methionine + gross cysteine, 0.53% methionine + digestible cysteine.

The animals were housed in separate pens for each treatment, which allowed the exact measurement of the amount of ingested feeds and of the leftovers for each treatment. The animals had free access to the feeds and water. The animals were slaughtered in the end of the trial, according to the recommendations of the Ethics Committee of IBNA and to the European regulations; blood and organ samples were collected to evaluate the effect of CF-GS formulation on some biochemical and immune parameters.

Polyphenols extraction and determination of the total phenol content in the grape seeds cakes: The grape seeds cakes polyphenols were extracted using a solution of acetone 80% (1:7 w/v sample/solvent ratio) for 20 hours, at 37°C with continuous stirring. The total amount of polyphenols was determined using the Folin–Ciocalteu method adapted for microscale (Arnous *et al.* 2001) and a standard gallic acid curve for calibration. Absorbance at 750 nm was measured with a UV-VIS spectrophotometer (Specord 250, Analytic Jena), and the total concentration of polyphenols was calculated using the correlation between sample absorbance and

the gallic acid concentration. The results were expressed in gallic acid equivalents (mgGAE)/100g sample.

Determination of the composition and concentration of polyphenols in the grape seeds cakes by HPLC couple with mass spectroscopy (HPLC-DAD-MS): The polyphenols composition of the acetone extracts from grape seeds cakes was determined by HPLC-DAD-MS using the retention times, the UV-Vis spectra (from 200 to 600 nm) and the mass spectra of the individual compounds using standard compounds according to the methods of Dulf *et al.*, and Garcia *et al.* (Garcia *et al.* 2013; Dulf *et al.*, 2015) with slight modifications.

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

Biochemical analysis of the plasma biochemical parameters: The plasma samples collected in the end of the feeding trial were used to analyse the effect of the diet formulations with grape seeds cakes on the general health state by determining some blood biochemical parameters, markers of the glucydes-energy (glucose), lipid (total cholesterol and triglycerides), protein (total protein, albumin), and mineral (Ca, P, Mg) metabolism, as well as on the liver metabolism (aspartate aminotransferase-AST, alanin-aminotrasferase-ALT, gamma glutamiltrasferase-GGT, bilirubin) and renal metabolism (urea, creatinine). All these biochemical parameters were measured with an automatic BS-130 Chemistry analyser (Bio- Medical Electronics Co., LTD, China).

Analysis of the tissular parameters of the local inflammatory response : The tissular concentration of the pro- and anti-inflammatory markers (IL-1 β , IL-8, TNF- α , IL-6, IFN- γ , IL-10, IL-4) were measured by ELISA. The collected organ samples (spline, lymph nodes) were lysed in saline phosphate buffer PBS with 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS and a cocktail of protease inhibitors. The samples were lysed for 30 minutes on ice, and centrifuged twice at $10,000 \times g$ at 4°C for 10 minutes. The cytokines concentrations were determined by ELISA, using commercial kits (R&D Systems, Minneapolis, USA, and Biosource International, Camarillo, USA) according to manufacturers' instructions. Absorbance was measured at 450 nm using a microplate reader (Tecan Infinite M200 PRO).

RESULTS AND DISCUSSIONS

Polyphenols content of the grape seeds cakes: The extracts from grape seeds cakes contain a large amount of total polyphenols, 5355.48 mg GAE/100 g sample.

HPLC-MS analysis revealed the following classes of polyphenols: gallic acid, catechins,

procyanidins as dimers) (Table 1)

Table 1. Amounts of phenol compounds expressed in mg/100g (catechin equivalent for catechin and derivatives; cyanidin equivalent for anthocyanins)

Peak	Analyte-polyphenol classes	mg/g
1	Procyanidin trimer	10.94
2	Procyanidin trimer	9.84
3	Catechin	48.93
4	Procyanidin dimer	18.34
5	Epicatechin	48.23
6	Gallocatechin	7.22
7	Epigallocatechin	12.41
8	Procyanidin dimer	26.79
9	Petunidin 3-O-glucoside	5.25
10	Procyanidin dimer	-
11	Malvidin 3-O-glucoside	4.65
12	Malvidin 3-O-(6''- coumaroyl -glucoside)	3.69
13	Isorhamnetin 3-O-glucoside	56.60

CF-GS effect on plasma biochemical parameters: CF-GS effect on the general health state of the animals was evaluated via some blood biochemical parameters which reflect its influence on glucydes, lipid, protein and mineral metabolism as well as on the hepatic and renal metabolism.

CF-GS effect on plasma glucydes and lipid profile: As shown in Figure 1, the plasma glucose level was higher in CF-GS piglets (89.45 ± 11.41

mg/dl) than in CF-control piglets (77.93 ± 1.42 mg/dl). GS group also displayed a slight increase of plasma cholesterol (80.57 ± 4.71 mg/dl) compared to group C (73.47 ± 3.37 mg/dl). The same trend was also noticed for the triglycerides, GS group having a higher level of plasma triglycerides (61.88 ± 7.76 mg/dl) compared to group C (51.08 ± 7.95 mg/dl).

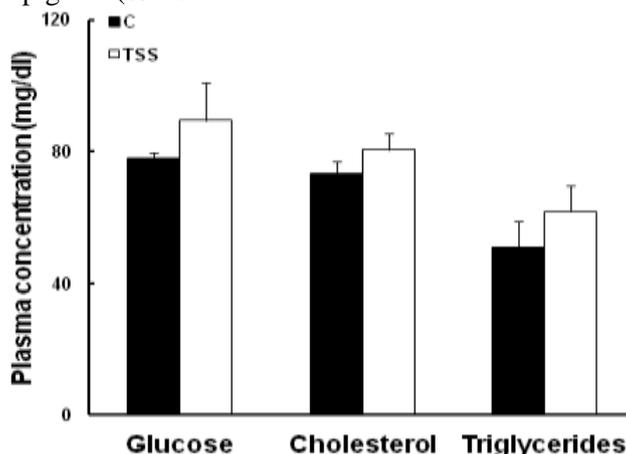


Figure 1 CF-GS effect on the level of plasma glucose, cholesterol and triglycerides. The columns show the mean ± ES for each analysed parameter.

GF-GS effect on plasma protein profile: Concomitantly with the biochemical analysis of the plasma level of glucose, triglycerides and cholesterol, we also evaluated the effect of GS diet

on the plasma concentration of proteins and of some protein metabolism products (urea, creatinine and bilirubin) (Table 2).

Table 2.

CF-GS effect on the profile of plasma proteins

Experimental group	Total proteins (g/dL)	Albumin (g/dl)	Creatinin (mg/dL)	Bilirubin (mg/dl)	Urea (mg/dL)
Control	5.57 ± 0.28	3.17 ± 0.24	0.73 ± 0.14	0.15 ± 0.02	27.28 ± 1.89
TSS	6.28 ± 0.26	3.70 ± 0.13	0.92 ± 0.05	0.10 ± 0.00	23.93 ± 1.15

The level of total proteins and plasma creatinine was slightly higher in GS animals (by 12.74% and 25.55%, respectively) compared to the control group, while the plasma albumin level remained unchanged in both groups. On the other hand, the level of plasma urea was lower in GS animals (23.93 mg/dL) compared to the control group (27.28 mg/dL). The same trend was also noticed for the plasma bilirubin level, which was 33.33% lower in GS piglets than in the control piglets.

CF-GS effect on plasma hepatic enzymes profile: Another important set of biochemical parameters that can be correlated with the health state and liver functionality is the profile of several hepatic enzymes: alanine aminotransferase (ALAT/GPT), aspartate aminotransferase (ASAT/GOT), alkaline phosphatase, gamma-glutamyl-transferase (Gama GT), lactate dehydrogenase (LDH) and creatininkinase (CK). Figures 2 and 3 show the effect of the CF-GS on these enzymes.

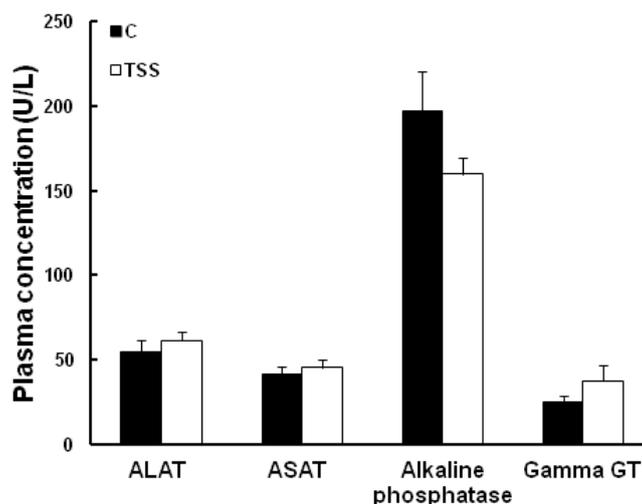


Figure 2 CF-GS effect on the level of plasma alanil-aminotrasferase (ALAT), aspartat-aminotrasferase (ASAT), alkaline phosphatase, gamma-glutamyltransferase (gamma GT). The columns show the mean \pm ES for each analysed parameter.

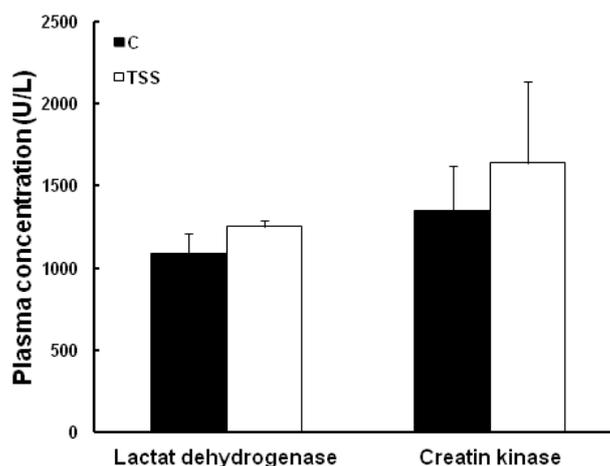


Figure 3 CF-GS effect on the level of plasma lactate dehydrogenase (LDH), creatininkinase in weaned piglets. The columns show the mean \pm ES for each analysed parameter.

CF-GS increased the plasma level of ALAT, ASAT and gamma GT (ALAT: 61.27 ± 5 U/L; ASAT: 45.33 ± 4.80 U/L; gamma GT: 37.53 ± 9.42 U/L) compared to the control group (ALAT: 55.00 ± 6.45 U/L; ASAT: 41.56 ± 4.66 U/L; gamma GT: 24.69 ± 4.08 U/L). On the other hand,

the plasma level of the alkaline phosphatase decreased in GS piglets (159.90 U/L) compared to the control group (196.64 U/L).

Our results have shown that CF-GS didn't have significant effects on the plasma biochemical parameters. Our observations are in line with those

of Han et al. (2014), who proved that various sources of polyphenols, among which the grape seeds, don't have significant effects on the plasma biochemical parameters in weaned piglets. A study conducted on mature pigs showed that there was no effect of the 5% GS treatment on the blood biochemical parameters (Taranu et al, 2017).

CF-GS effect on the concentration of pro- and anti-inflammatory cytokines in the spleen and lymph nodes

In order to evaluate the effect of GS treatment on the immune and inflammatory status, we used ELISA to investigate the main markers of the immune response, the pro-inflammatory cytokines TNF- α , IL-1 β , IFN- γ , IL-6 and IL-8 and the anti-inflammatory ones, IL-4 and IL-10.

Figure 4 shows that CF-GS didn't have any effect on tissue concentrations of TNF- α and IL-1 β in the spleen and lymph nodes. On the other hand, IFN- γ concentration increased by 29% in both analysed tissues.

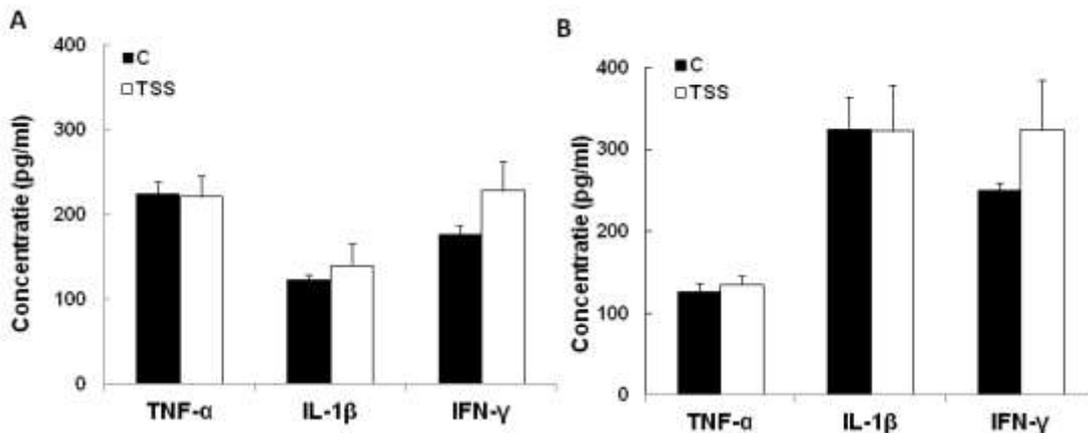


Figure 4 CF-GS effect on the level of cytokines TNF- α , IL-1 β and IFN- γ in the spleen (A) and lymph nodes (B). The columns show the mean \pm ES for each analysed parameter.

The analysis of IL-6 concentration in the spleen and lymph nodes revealed no difference between the piglets from the control and GS groups. IL-8 concentration decreased by 23% in GS piglets (Figure 5).

The anti-inflammatory cytokines IL-4 and IL-10 had a different profile in the analysed organs.

Thus, while the concentration of IL-10 in the spleen and lymph nodes remained unchanged by the GS treatment, the concentration of IL-4 decreased by 58% in the spleen and by 38% in the lymph nodes in the piglets treated with CF-GS (Figure 6).

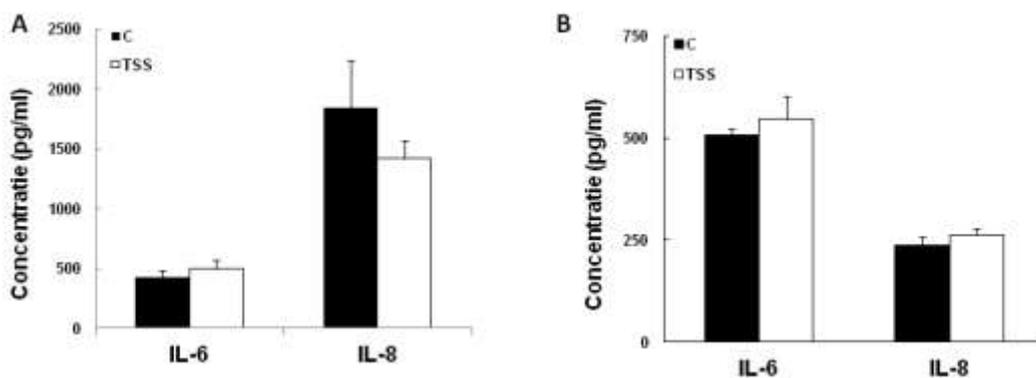


Figure 5 CF-GS effect on the level of IL-6 and IL-8 in the spleen (A) and lymph nodes (B). The columns show the mean \pm ES for each analysed parameter.

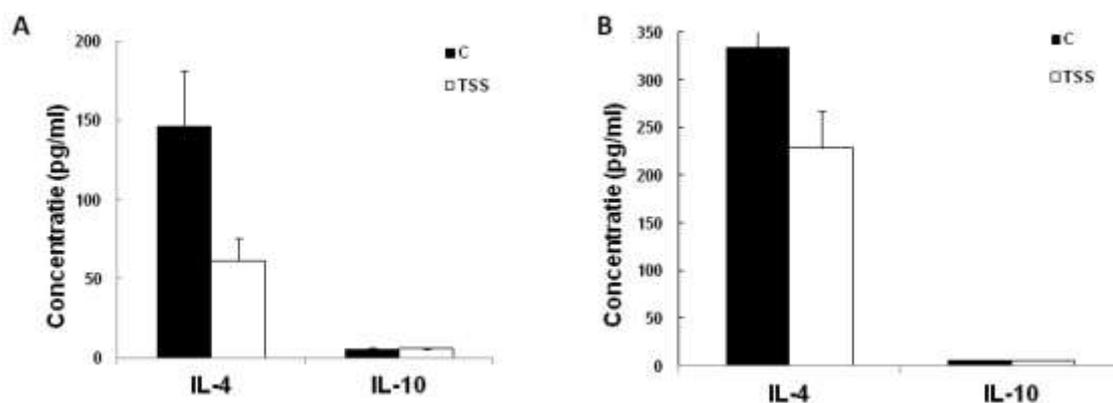


Figure 6. CF-GS effect on the level of IL-4 and IL-10 in the spleen (A) and lymph nodes (B). The columns show the mean \pm ES for each analysed parameter.

There are many studies showing the anti-inflammatory role of the polyphenols from different sources, and within different systems (*in vitro* or *in vivo*). However, a recent study by Gessner et al (2016) has shown that the grape seeds don't have an effect on the genes involved in the development of the inflammatory processes in weaned piglets. These results suggest that further studies are required in order to evaluate the mechanisms of action and the in-depth intracellular effects of the polyphenols from these sources.

CONCLUSIONS

The results of this study show that grape seeds contain a large amount of total polyphenols, the most important ones being the gallic acid, catechins and proanthocyanidins in the form of dimers. However, the diet formulation with grape seeds cakes didn't have significant effects on the plasma biochemical parameters and on the content of pro- and anti-inflammatory markers in the main organs of the immune system, the spleen and lymph nodes. This suggests that further studies are required in order to determine the optimal processes of including other winery by-products in piglet formulations in order to improve compound feeds quality, the immune status, health state and piglet performance after weaning.

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