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ANTIOXIDANT ROLE OF THE GRAPE SEEDS MEAL IN PREVENTING THE DEGRADATION OF FATTY ACIDS-HIGH DIET FORMULATIONS FOR BROILERS

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Abstract

The production of foods rich in polyunsaturated fatty acids is influenced by the structure and nature of the feeds given to animals. The use of feed ingredients rich in polyunsaturated fatty acids causes the feeds to get rancid due to fat oxidation. Hence, the need to use antioxidants blocks oxidation by their reaction with the free radicals. The aim of the work was to investigate ways to prevent the degradation of high-polyunsaturated fatty acids diets used for Hubbard broilers feeding. Two compound feeds formulations, grower and finisher, were used. The control compound feed used corn, wheat, soybean meal and gluten as basic ingredients, plus 2% flax meal which is rich in polyunsaturated fatty acids. The experimental compound feeds differed from the control diet by the use 2% (E1) and 3% (E2) grape seeds meal, which has antioxidant properties. The flax meal had 30.01% protein, 15.59% fat, 75.76 g PUFA/100 g total fatty acids, of which 57.87 g omega -3 fatty acids, and 17.89 g omega-6 fatty acids/100 g total fatty acids, and omega-6/omega-3 ratio of 0.31; the energy level was 20.94 Mj/kg. The grape seeds meal had 12.76% protein, 7.13% fat, 3.68 mg EAG/g polyphenols and 35.30 mM Trolox equivalents/g sample, as antioxidant capacity. The compound feeds for the grower stage had between 20.19% (C) and 20.44% (E2) protein, between 5.44% (C) and 6.06% (E2) fat, between 65.33 g% (C) and 70.10 g% polyunsaturated fatty acids, of which omega-3 polyunsaturated fatty acids between 4.29 g% (E1) and 5.01 g% (E2). The compound feeds for the finisher stage had between 19.84% (C) and 20.09% (E2) protein, between 8.13% (E1) and 8.74% (E2) fat, an average value of 60.80% of polyunsaturated fatty acids, of which omega-3 polyunsaturated fatty acids between 7.31 g% (C) and 7.92 g% (E2). We analysed the evolution of the compound feeds fat degradation indices at 0 and 14 days after manufacture and noticed that the peroxide index at 14 days, compared to 0 days, had the lowest increase in group E2, 4.00%, for the grower compound feeds, compared to 14.63% for group C, and 10.00% for the finisher compound feeds, compared to 26.82% in group C. fat acidity displayed the same evolution, with the lowest increase in group E2: 13.86%, compared to 20.10% in group C for the grower stage, and 1.83% (E2), compared to 4.27% in group C for the finisher stage. The Kreiss reaction was negative for all compound feeds. The use 3% of grape seeds meal as natural antioxidant in the compound feed for group E2, inhibited the reactions of lipid degradation.

Key words*: antioxidants, winery by-products, fatty acids, lipid degradation.*

INTRODUCTION

The consumers are increasingly interested to use in their daily diet foods (vegetal or animal) rich in polyunsaturated fatty acids, omega 3 particularly, because of their beneficial effects on human health (Huang, 2010; Pilkington et al., 2011; Flachs et al., 2014). Animal products can be enriched in polyunsaturated fatty acids through animal nutrition, using diets which include feed ingredients high in polyunsaturated fatty acids. However, the higher proportion of these fatty acids in animal diets also enhances the effect of their lipid oxidation (Ren et al., 2013), which makes it necessary to use feed additives with antioxidant properties. Hence, the increasing interest of the nutritionists and animal producers towards the use of agro-industrial by-products rich in bioactive compounds, as natural antioxidants in animal feeding.

The winery by-products, grape marc, grape seeds and peels, as well as the grape seeds cakes and meal, are a natural source high in

polyphenols, which bestows them with antioxidant properties. The abundance of bioactive polyphenols in these by-products (Radovanovic et al., 2009; Granato et al., 2010), is of real interest for many researchers and companies from the food industry and from the feed additives industry.

MATERIALS AND METHODS

Our feeding trial studied ways to prevent the degradation of the dietary polyunsaturated fatty acids added to the compound feeds for slow-growing Hubbard broilers. We used grape seeds meal as natural antioxidant and manufactured two types of compound feeds, grower and finisher. The diets were formulated with corn, wheat, soybean meal and gluten as basic ingredients, plus 2% flax meal, which is high in polyunsaturated fatty acids. The experimental diet formulations differed from the control compound feed by the addition of 2% (E1) and 3% (E2) grape seeds meal. Compound feeds samples were collected and assayed for the main nutrients and for the evolution of the fat degradation indices at 0 and 14 days from manufacture. We also performed mycological, bacteriological and mycotoxicological analyses to check compound feeds salubriousness.

Standardized methods were used to determine the concentration of the basic nutrients, as follows:

- the dry matter (DM) was determined using the gravimetric method, whose working principle involves the determination of sample mass by drying at 103^{o}C, according to Regulation (CE) 152/2009 and standard SR ISO 6496:2001. We used a Sartorius (Gottingen, Germany) scale and BMT drying closet, ECOCELL Blueline Comfort (Neuremberg, Germany);
- the crude protein (CP) was determined using the Kjeldahl method, whose working principle involves sample digestion by heating with sulphuric acid in the presence of a catalyst, for the conversion of the protein nitrogen into ammonium sulphate. The reaction products are alkalinized with sodium hydroxide, for the release of the trapped ammonia, by distillation in a solution of boric acid in excess, followed by titration in a solution of hydrochloric acid. The method complies with Regulation (CE) 152/2009 and standard SR ISO 5983-2:2009. We used a semiautomatic KJELTEC auto 2300 system – Tecator (Sweden);
- the ether extractives (EE) were determined by extraction is organic solvents, which involves fat extraction by petrol ether, removal of the solvent by distillation, drying and weighing the residue. The method complies with Regulation (CE) 152/2009 and standard SR ISO 6492:2001. We used a SOXTEC-2055 FOSS system – Tecator (Sweden);
- the fatty acids were determined using the chromatographic method, which involved the transformation of the fatty acids from the sample in methyl esters, followed by the separation of the compounds in a chromatographic column and their identification by comparison with standard chromatograms. The method complies with standard SR CEN ISO/TS 17764 -2: 2008. We used a Perkin Elmer-Clarus 500 chromatograph, fitted with a system for injection into the capillary column, with high polarity stationary phase (BPX70: 60m x 0.25mm inner diameters and 0.25µm thick film); or high polarity cyanopril phases, which have similar resolution for different geometric isomers (THERMO TR-Fame: 120m x 0.25mm ID x 0.25 µm film);
- the crude fibre (CF) was determined with the method with intermediary filtration, whose working principle involves weighing the sample boiled successively with solutions of sulphuric acid and sodium hydroxide. The resulting residue was filtered, dried, burnt and weighed. The method complies with Regulation (CE) 152/2009 and standard SR EN ISO 6865:2002. We used a FIBERTEC 2010 system – Tecator (Sweden);
- the ash (Ash) was determined by the gravimetric method, which involves sample decomposition by burning and weighing of the resulting ash. The method complies with Regulation (CE) 152/2009 and standard SR EN ISO 2171:2010. We used a Caloris CL 1206 furnace.
- the gross energy was determined by calculation using the gross chemical analysis (dry matter, protein, fibre, fat, nitrogen-free extractives and ash), using the equations of Burlacu et al. (2002).

The fat degradation indices, peroxide value, fat acidity and Kreiss reaction were determined by the volumetric method, according to STAS 12266-84. The principle of the peroxide value determination presumes the treatment of the fat solution in acid medium with potassium iodide, where the peroxides release the iodine, which is titrated with sodium thiosulphate. The principle of fat acidity determination presumes the neutralization of the free fatty acids from the fat by titration with potassium hydroxide solution. The Kreiss reaction is a qualitative method whose principle involves the condensation reaction between the epihydrinic aldehyde and fluoroglucine, with the formation of a coloured compound whose intensity varies from pink to violet-red, depending on the concentration of epihydrinic aldehyde.

To determine the concentration of polyphenols and the antioxidant capacity of the samples, we first extracted the phenol compounds in acidified methanol (methanol:HCl = 80:20).

The polyphenol content of the methanol extracts has been determined according to the method described by Mihailovic et al. (2013), modified. The reaction mixture consisted of: the methanol extract in proper dilution according to the analysed sample, Folin-Ciocâlteu reagent and a solution of 7.5% de Na_2CO_3. The reaction mixture was maintained for 30 min. at room temperature, thereafter absorbance was read at 765 nm. The total concentration of phenols, was expressed in equivalents gallic acid/g fresh matter (mg EAG/g sample). We used a UV-VIS Thermo Scientific spectrophotometer.

The determination of the antioxidant capacity of the methanol extracts has been done using the DPPH method proposed by Marxen et al. (2007). The antioxidant capacity has been estimated by calculating the difference between the control and the sample, compared to a standard curve which used Trolox (synthetic antioxidant analogue to α-tocopherol), as standard antioxidant. The antioxidant capacity has been expressed in Trolox equivalents/g fresh matter (mMTrolox/g sample). We used a UV-VIS Analytik Jena Specord 250 Plus spectrophotometer with thermostatic carousel.

RESULTS AND DISCUSSIONS

The addition of raw materials rich in polyunsaturated fatty acids to the compound feeds for animals, with the purpose of producing functional foods, favours the lipid oxidation phenomenon, which can be slowed down using antioxidants, whose high content of polyphenols react with the free radicals.

Flax meal, feed ingredient high in polyunsaturated fatty acids, is used the compound feeds for slow-growing Hubbard broilers. The flax meal we used had 30.01% protein, 15.59% ether extractives and 20.94 MJ/kg gross energy, similar to the results reported by Mironeasa et al. (2010) and by Elagamey et al. (2013), i.e. 86.74 – 89.17% dry matter, 6.26 - 9.01% protein and 2.14 – 8.28% ash, similar to the data obtained by researchers Criste et al. (2009) and Panaite et al. (2016).

The grape seeds meal had 12.76% protein, 7.13% fat (Table 1), similar to those reported by Elagamey et al. (2013), Panaite et al. (2016) or by Vlaicu et al. (2017). The polyphenols content of the grape seeds meal was 26.65 mg EAG/g sample and the antioxidant capacity was 148.35 mM Trolox equivalents/g sample. These results are close to those reported by Poudel et al. (2008), cited by En-Qin Xia et al. (2010) or by Ky et al. (2014), who reported polyphenols concentrations in the range of 23.8 – 44.5 mg EAG/g sample in the grape pulp, to 31.6 - 374.9 mg EAG/g in the grape peel samples; they also reported values of the antioxidant capacity in the range of 16.8 - 92 mM Trolox equivalents/g sample in the grape seeds, to 15.7 - 113.3 mM Trolox equivalents/g sample, in the grape peel samples.

Table 1. Feed ingredients content of main nutrients

Sample	DMr (%)	OM (%)	CP (%)	EE (%)	CF (%)	Ash (%)	GE (Mj/kg)
Flax meal	90.75	88.28	30.01	15.59	11.47	4.96	20.94
Grape seeds meal	90.76	88.35	12.76	7.13	35.30	2.92	18.49

Chemical composition expressed on dry matter (DM); DMr-real dry matter; OM-organic matter; CP-crude protein; EE-ether extractives; CF-cellulose; Ash-ash; Ca-calcium; P-phosphorus; GE-gross energy

The values of the lipid degradation indices for the flax meal and for the grape seeds meal (Table 2), which are below the admitted level, show that that could be added to the compound feeds for broilers.

Table 2. Evolution of the fat degradation indices in the feed ingredients

Items	Flax meal	Grape seeds meal	Admitted limits
Peroxide value (ml thiosulfate 0.1 N/g fat);	0.350	0.445	1.2
Fat acidity (mg KOH/g fat);	9.10	11.24	50
The Kreiss reaction	negative	negative	negative

We also determined the fatty acids content in the fat of the feed ingredients in terms of the level of fatty acids saturation (Table 3).

Table 3. Fatty acids content in the fat of the feed ingredients in terms of the level of fatty acids saturation (g fatty acid/100 g total fatty acids)

Items	Flax meal	Grape seeds meal
SFA, %	7.43	14.82
MUFA, %	16.81	17.91
UFA, %	92.57	84.93
PUFA, % of which:	75.76	67.02
Ω3, %	57.87	0.78
Ω6, %	17.89	66.24
Ω6/Ω3	0.31	85.28
SFA/UFA	0.080	0.174
PUFA/MUFA	4.506	3.742

SFA= saturated fatty acids; MUFA= mono-unsaturated fatty acids; PUFA= poly-unsaturated fatty acids; UFA= total unsaturated fatty acids; Ω3-omega 3 poly-unsaturated fatty acids; Ω6- omega 6 poly-unsaturated fatty acids

There was obtained a value of 0.31 for the omega-6/omega-3 (Ω6/Ω3) ratio in the flax meal, which is below 1, value considered to be ideal. These results are close to those reported by Panaite et al. (2016) and by Vlaicu et al. (2017).The evaluation of the basic chemical composition of the compound feeds for broilers, tested for the growing stage, showed a protein content ranging between 20.19% (C) and 20.44% (E2) and a fat content ranging between 5.44% (C) and 6.06% (E2). The compound feeds for the finishing stage had protein content ranging between 19.84% (C) and 20.09% (E2), and a fat content ranging between 8.13% (E1) and 8.74% (E2).

The total content of polyunsaturated fatty acids, PUFA (Table 4) of the studied compound feeds ranged between 60.75 g/100 g fat, in group E1 –finishing stage, and 70.10 g/100 g fat, in group E2 –growth stage. The content of omega-3 fatty acids ranged between 4.29 g/100 g fat, in group E1 –growth stage, and 7.92 g/100 g fat, in group E2 –finishing stage. The omega-6/omega-3 ratio was between 6.68 in group E2 –finishing stage, and 14.59 in group E1 – growth stage. These results are higher than those reported by Olteanu et al. (2017) and Turcu et al. (2017).

As it is known, peroxide concentration is a major indicator of fat degradation by autooxidation, so that the peroxide value must be determined in the samples with more than 4% fat.

Table 4. Fatty acids content of the compound feeds fat, depending on their level of saturation (g acid/100 g total fatty acids)

Items	Growth stage			Finishing stage		
	C	E1	E2	C	E1	E2
SFA, %	11.04	11.14	9.72	14.74	14.75	14.87
MUFA, %	23.62	21.94	20.18	24.11	24.23	24.14
UFA, %	88.96	88.86	90.28	84.96	84.98	84.95
PUFA, % of which:	65.33	66.92	70.10	60.86	60.75	60.82
Ω3, %	4.46	4.29	5.01	7.31	7.36	7.92
Ω6, %	60.87	62.62	65.09	53.55	53.39	52.90
Ω6/Ω3	13.65	14.59	13.00	7.33	7.25	6.68
SFA/UFA	0.124	0.125	0.108	0.17	0.17	0.18
PUFA/MUFA	2.765	3.050	3.474	2.52	2.51	2.52

SFA= saturated fatty acids; MUFA= mono-unsaturated fatty acids; PUFA= poly-unsaturated fatty acids; UFA= total unsaturated fatty acids; Ω3-omega 3 poly-unsaturated fatty acids; Ω6- omega 6 poly-unsaturated fatty acids

The acidity index shows the amount of free fatty acids in the fat.

The Kreiss reaction too is an indicator of the fat getting rancid, as it shows the presence of epihydrinic aldehyde and of other carbonyl compounds resulting from fat degradation. Thus, we evaluated the effect of the grape seeds meal, as antioxidant, added to the compound feeds for the two experimental groups (E1 and E2), compared to the control group (Table 5), by determining the indices of fat degradation in the compound feeds samples collected at 0 and 14 days from manufacture.

Table 5. Evolution of fat degradation indices in the compound feeds

Items		Growth stage			Finishing stage			Admitted limits
		C	E1	E2	C	E1	E2	
Peroxide value (ml thiosulfate 0.1 N/g fat);	0 days	0.41	0.54	0.50	0.41	0.40	0.50	1.2
	14 days	0.47	0.59	0.52	0.52	0.50	0.55	
	Difference (%)	14.63	9.26	4.00	26.82	25.00	10.00	
Fat acidity (mg KOH/g fat);	0 days	15.71	14.42	14.35	17.34	17.42	18.60	50
	14 days	20.10	17.27	16.34	18.08	18.00	18.94	
	Difference (%)	27.94	19.76	13.86	4.27	3.33	1.83	
Kreiss reaction	0 days	negative	negative	negative	negative	negative	negative	negative
	14 days	negative	negative	negative	negative	negative	negative	

After 14 days of storage at room temperature, the lowest difference in the peroxide values, 4.00%, was noticed in the compound feed for group E2, followed by 9.26%, in the compound feed for group E1, compared to 14.63% for group C, for the growth stage. In the compound feed for the finishing stage, the lowest difference in the peroxide values, 10.00%, was noticed also in the compound feed for group E2, followed by 25.00% in the compound feed for group E1, compared to 26.82% for group C. The lowest difference in fat acidity, 13.86%, was noticed in the compound feed for group E2, followed by 19.76% in the compound feed for group E1, compared to 27.94% for group C, for the growth stage. In the compound feed for the finishing stage, the lowest difference in fat acidity, 1.83%, was noticed also in the compound feed for group E2, followed by s 3.33%, in the compound feed for group E1, compared to la 4.27% for group C. As Table 5 shows, the absolute values for the fat degradation indices are below the admitted limit in all compound feeds, for both stages.

Table 6. Mycological, bacteriological and mycotoxicological evaluation of the compound feeds

Items	Growth stage			Finishing stage			Admitted limits *(MO 362bis/2003; 32 EC/2002)*
	C	E1	E2	C	E1	E2	
Mycological assessment							
TFC (col/g);	7000	2500	4000	7000	10500	5250	max. $5x10^4$ col/g;
Bacteriological assessment							
TGC (col/g);	$35x10^3$	$35.5x10^3$	$37x10^3$	$40x10^3$	$38.5x10^3$	$37.5x10^3$	max. $15x10^6$ col/g;
Total coliform(col/g)	0.9	9.5	9.5	0.7	150	110	max. $3x10^3$ col/g
E. coli (col/g)	0.4	4.5	0.9	0.7	0.0	3	max.100 col/g;
Salmonella (col/g);	Absent	Absent	Absent	Absent	Absent	Absent	0 col/g;
Mycotoxicological assessment							
AFLA-total (ppm)	$0.5x10^{-3}$	$0.7x10^{-3}$	$0.8x10^{-3}$	0	$0.1x10^{-3}$	$0.1x10^{-3}$	0.02 ppm

TFC–total fungal count; TGC –total germs count; *E.coli – Escherichia coli*

We also evaluated the sanitary quality of the compound feeds (Table 6), as they may bring risk for the animals, therefore for the foods (Frazzoli and Mantovani, 2010). Hence, the permanent feed safety concern of the food industry and of the compound feeds producers.

The results of the mycological, bacteriological and mycotoxicological determinations on the compound feeds show that they were all below the admitted levels. ANSVSA norm regarding the quality and salubriousness parameters for the production, import, quality inspection, selling and using simple concentrate feeds, compound feeds, feed additives, premixes, energy substances, minerals and special feeds, published in MO 362 bis/2003 and in EC Regulation 32 bis/2002.

CONCLUSIONS

The 2% grape seeds meal added to the compound feeds enriched them in omega-3 polyunsaturated fatty acids, in both stages, with higher values in the finishing stage.

The use of the natural antioxidant, 2% grape seeds meal in the compound feeds for group E1, and 3% grape seeds meal in the compound feeds for group E2, improved the feeding qualities of the compound feeds in both stages, by inhibiting the reactions of lipid degradation. Thus, the highest values of total polyunsaturated fatty acids, of omega-3 fatty acids, were determined in the compound feeds for group E2, higher than in the compound feeds for group E1, and for the control group (C).

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