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Phytogenic Administration and Reduction of Dietary Energy and Protein Levels Affects Growth Performance, Nutrient Digestibility and Antioxidant Status of Broilers

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The aim of this study was to investigate the effect of reduced dietary energy (ME) and protein (CP) levels along with administration of a phytogenic feed additive (PFA) based on oregano, anise and citrus essential oils, on broiler growth performance, nutrient digestibility, meat and blood biochemical parameters and total antioxidant capacity (TAC).

Depending on dietary ME and CP level down regulation compared to a corn-soybean meal basal diet A used as positive control, three diet types [A, B(=A-3%) and C(=A-6%)] were implemented. Depending on the inclusion or not of PFA at 125 mg/kg diet, 450 1-d old, male Cobb broilers were randomly allocated in six treatments according to a 3×2 factorial arrangement with 5 replicates of 15 broilers; A: diets formulated optimally to meet broiler nutrient requirements for maximizing protein content of meat for starter, grower and finisher growth periods; APh: A+PFA; B: suboptimal in ME and CP levels by 3%; BPh: B+PFA; C: suboptimal in ME and CP levels by 6%; CPh: C+PFA.

Feed conversion ratio (FCR) was improved in birds fed diet A compared to diet C during the grower period (P_D=0.021) and overall (P_D=0.010). Phytogenic supplementation resulted in higher ($P_{D\times Ph}$=0.020) total tract apparent digestibility of fat in birds fed diet C compared to diet A. Birds fed diet A had higher (P_D=0.001) plasma cholesterol, compared to birds fed diet C. In addition, birds fed diets A and B had higher (P_D=0.002) breast protein content compared to C. Overall, PFA inclusion reduced cholesterol (P_{Ph}=0.002) and increased plasma TAC (P_{Ph}<0.001). Moreover, PFA increased breast (P_{Ph}=0.001) and thigh (P_{Ph}=0.01) TAC. In conclusion, a reduction in dietary ME and CP levels, adversely affected the FCR, whereas PFA supplementation tended (P_{Ph}=0.089) to compensate these effects. Moreover, the addition of PFA reduced plasma cholesterol and improved plasma and meat TAC.

Key words: broilers, dietary energy, digestibility, phytogenic, protein level

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Introduction

Reduction of dietary energy and protein levels whilst maintaining poultry performance and health are currently considered among strategies to reduce feed cost in poultry production. So far, various studies have examined the effect of dietary energy and / or protein levels on broiler growth performance (Aletor *et al*., 2000; Bregendahl *et al*., 2002; Kamran *et al*., 2004; Corzo *et al*., 2005; Kamran *et al*., 2008; Steiner *et al*., 2008), nutrient digestibility (Aletor *et al*., 2000; Bregendahl *et al*., 2002), blood biochemical parameters (Tabeidan *et al*., 2005; Steiner *et al*., 2008) as well as body composition and biochemical properties (Neto *et al*., 2000; Laudadio *et al*., 2012).

In addition, the effect of certain bioactive feed additives in poultry performance and health has been attracting contemporary research interest. In this respect, phytogenic feed additives (PFA) are being actively researched for their effects on broiler performance (Hernandez *et al*., 2004; Cross *et al*., 2007), nutrient digestibility (Hernandez *et al*., 2004; Malayoglu *et al*., 2010), blood biochemical parameters (Goni *et al*., 2007; Ciftci *et al*., 2010; Polat *et al*., 2011), meat cholesterol

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levels (Ciftci *et al*., 2010) and total antioxidant capacity (Lopez-Bote *et al*., 1998; Lee *et al*., 2004; Hoffman-Pennesi and Wu, 2010). In particular, several studies have researched the effects of PFA containing essential oils extracted from oregano (Botsoglou *et al*., 2002; Giannenas *et al*., 2003; Lee *et al*., 2003; Luna *et al*., 2010; Akyurek and Yel, 2011; Roofchaee *et al*., 2011; Hashemipour *et al*., 2013), anise (Ciftci *et al*., 2005; Soltan *et al*., 2008; Amad *et al*., 2011) or citrus peels (Ani *et al*., 2015), on many of the aforementioned parameters in broiler diets. Moreover, the dietary effects of PFA based on a blend of essential oils derived from oregano, anise and citrus peels, have been investigated on broiler performance nutrient digestibility (Mountzouris *et al*., 2011; Hong *et al*., 2012) and biochemical parameters (Hong *et al*., 2012).

It may be possible to compensate for potential undesirable effects of reduced dietary energy and protein levels via the application of certain bioactive feed additives such as PFA. For example, when only dietary energy was down regulated by 2% compared to optimal levels it was shown that supplementation of broiler diets with essential oils compensated performance (Bravo *et al*., 2011). However, depending on broiler genetics, overall diet and housing conditions a better understanding of the effects of reduced dietary energy and protein levels on broiler performance and health is warranted.

The aim of this work was to evaluate the effect of reduced dietary metabolisable energy (ME) and crude protein (CP) levels while maintaining a constant ME:CP ratio, with or without inclusion of a PFA comprising a blend of oregano, anise and citrus essential oils with carvacrol, anethol and limonen being the main active ingredients, on broiler growth performance, nutrient digestibility, blood and meat biochemical parameters and total antioxidant capacity (TAC).

Materials and Methods

Animals and Experimental Treatments

For the purpose of the experiment, four hundred and fifty, 1-d old, male Cobb 500 broilers were obtained from a commercial hatchery. Birds were vaccinated at hatch for Marek, Infectious Bronchitis and Newcastle Disease.

The nutritional programme was based on a three-phase feeding scheme matching starter (d 1 to 14), grower (d 15 to 28) and finisher (d 29 to 42) growth period requirements. For each growth period three diet types (A, B and C) were formulated. Diet type A served as a positive control and was formulated according to Cobb recommendations for maximizing white meat yield. Diet types B and C were down regulated in terms of ME and CP by 3% and 6%, respectively (Table 1). The ME:CP ratio was maintained constant at all three diet types per growth period including the total sulfur amino acids (TSSA), threonine (Thr) and tryptophane (Try) ratios to lysine (Lys). Depending on the inclusion of a PFA at 125 mg/kg diet or not, the experiment had the following six treatments (i.e. A, APh, B, BPh, C and CPh); A: diet optimal for ME and CP; APh: A+PFA; B: diet with reduced ME and CP by 3% compared to A; BPh: B+PFA; C: diet with reduced ME and CP by 6% compared to A; CPh: C+PFA.

All diets were based on maize-soybean meal and were supplied in mash form. The PFA used was a blend of oregano, anise and citrus essential oils with carvacrol, anethol and limonen being the main active ingredients and fructo-oligosaccharides acting as a carrier (Digestarom P.E.P., Biomin Holding GmbH, Getzersdorf, Austria). The PFA had a concentration of active ingredients of 115 g/kg PFA, of which carvacrol was the main active compound at 102 g/kg PFA. On a weekly basis, PFA was thoroughly blended in the basal diet at the expense of maize. Three complete feed samples per diet type and growth phase were analyzed for their carvacrol content and found to be according to the expected product specifications.

Chicks were randomly allocated in the six experimental treatments, explained above, each treatment having five (n=5) replicates with 15 broilers per replicate. Each replicate was assigned to a clean floor pen (1 m^2) and birds were raised on rice hull shavings litter. Heat was provided with a heating lamp per pen. Except for day 1, a 23-h light to 1-h dark lighting program was applied during the experiment. The experiment lasted for 6 weeks and feed and water were available *ad libitum*.

The overall housing and care of the animals conformed to the Faculty of Animal Science and Aquaculture of the Agricultural University of Athens research ethics guidelines. The experimental protocol was in accordance with the current European Union Directive on the protection of animals used for scientific purposes (EC 43/2007; EU 63/2010) and was approved by the relevant national authority.

Broiler Performance Responses

Broiler growth performance responses such as body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR), were determined on a weekly basis during the 6 experimental weeks. Mortality was recorded on a daily basis. For practical reasons performance data (i.e. BWG, FI and FCR) were presented per growth period (i.e. starter, grower and finisher) basis. In addition, overall BWG, FI and FCR were calculated and presented for the entire duration of the experiment.

Total Tract Apparent Digestibility (TTAD) of Nutrients

The digestibility experiment took place at the final week of the trial. In particular, on day 35, four broilers per floor pen were randomly selected and removed so that a total of 20 birds per treatment were obtained and subsequently randomly divided in groups of five and placed in four (n=4) battery cages (i.e. each treatment having four replicate-cages of 5 birds each) with wire mesh bottom and excreta collection trays. Each cage was equipped with one feeding and two water troughs placed outside on front, left and right sides of the cage, respectively. The digestibility experiment had a 4-d pre-experimental adaptation period and a 3-d collection period. During the 3-d collection period, excreta from each cage were collected 4 times daily (i.e. with 6 h intervals) and stored in sealed bags at −20℃. Remaining feed in the excreta trays was carefully removed and weighed. Feathers were also removed from the excreta. For the total tract apparent digestibility (TTAD) determination, excreta collected

Table 1. **Ingredient (g/kg) and calculated chemical composition (g/kg as fed) of the experimental diets**

	Starter (1–14 d)			Grower (15–28 d)			Finisher (29–42 d)		
Ingredients	A	B	C	A	B	C	A	B	C
Maize	542.5	567.7	591.7	575.7	596.9	617.9	606.8	622.2	637.0
Soybean meal (427 gr CP / kg)	310.7	320.0	330.0	268.1	282.8	297.9	233.6	256.2	280.0
Protein concentrate (580 gr CP / kg)[1]	73.2	51.1	28.7	74.8	50.0	25.0	69.2	40.0	10.0
Vegetable fat[2]	40.3	26.5	13.5	50.2	37.4	24.9	60.0	49.2	38.7
Limestone	8.9	9.6	10.3	9.1	9.9	10.7	8.7	9.6	10.5
Monocalcium phosphate	13.6	13.8	14.1	12.5	12.8	13.0	11.9	12.2	12.5
NaCl	3.9	4.1	4.3	3.1	3.4	3.6	2.9	3.2	3.5
L-lysine HCl[3]	0.5	0.8	1.0	0.2	0.5	0.7	0.5	0.8	1.1
DL-methonine[4]	2.2	2.2	2.2	2.1	2.1	2.1	2.3	2.4	2.4
L-threonine[5]	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2	0.3
Vitamin premix[6]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Mineral premix[7]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Coccidiostat[8]	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.0	0.0
Phytogenic (PFA)[9]	—	—	—	—	—	—	—	—	—
Calculated chemical composition / (determined chemical composition)									
AME_n, (MJ/kg diet)	12.6	12.2	11.8	13.0	12.6	12.2	13.4	13.0	12.6
Dry matter	877.5	875.6	873.7	877.9	876.0	874.1	878.3	876.4	874.5
	(*908.8*)	(*901.2*)	(*907.4*)	(*901.0*)	(*905.1*)	(*906.7*)	(*893.9*)	(*891.1*)	(*893.1*)
Crude protein	220.0	213.4	206.8	205.0	198.9	192.7	190.0	184.3	178.6
	(*215.2*)	(*209.6*)	(*202.3*)	(*201.2*)	(*196.1*)	(*189.0*)	(*187.9*)	(*182.0*)	(*175.8*)
Ether extract	70.0	56.3	43.3	80.0	67.2	54.5	89.7	78.5	67.7
	(*69.9*)	(*56.4*)	(*42.6*)	(*81.7*)	(*69.8*)	(*56.4*)	(*88.9*)	(*77.4*)	(*65.1*)
Crude fiber	36.1	36.6	37.0	33.9	34.6	35.3	32.1	33.1	34.1
Lysine	13.0	12.6	12.2	11.7	11.4	11.0	10.9	10.6	10.3
TSAA (methionine+cysteine)	9.6	9.3	9.0	9.1	8.9	8.6	8.9	8.6	8.4
Threonine	8.8	8.5	8.1	8.2	7.9	7.5	7.6	7.4	7.1
Calcium	9.0	9.0	9.0	8.8	8.8	8.8	8.4	8.4	8.4
Available phosphorus	4.5	4.5	4.5	4.2	4.2	4.2	4.0	4.0	4.0
Sodium	2.0	2.0	2.0	1.7	1.7	1.7	1.6	1.6	1.6

[1] Vegetable protein concentrate Nuevopro (Nuevo SA, Nea Artaki, Greece).
[2] Vegetable hydrogenate palm oil stearine triglicerides (Norel SA, Spain).
[3] L-Lysine HCl 99% feed grade (Ajinomoto Eurolysine S.A.S, Paris, France).
[4] DL-Methionine 99% feed grade (DSM Nutritional Products Ltd, Basel Switzerland).
[5] L-Threonine 98.5% feed grade (Ajinomoto Eurolysine S.A.S, Paris, France).
[6] The vitamin premix for starter and grower periods (Rovimix 11 Bro Basic, DSM, Netherlands) provided per kg of diet: 3.6 mg retinol (Vit.A), 100 μg cholecalciferol (Vit.D_3), 80 mg Vit.E, 9 mg Menadione (Vit.K_3), 3 mg Thiamine, 7 mg Riboflavin, 6 mg Pyridoxine, 25 μg Cyanocobalamin, 50 mg Nicotinic acid, 15 mg Pantothenic acid, 1.5 mg Folic acid, 150 μg Biotin. The vitamin premix for the finisher period (Rovimix 12 Bro Basic, DSM, Netherlands) provided per kg of diet: 3.6 mg retinol (Vit.A), 75 μg cholecalciferol (Vit.D_3), 50 mg Vit.E, 7 mg Menadione (Vit.K_3), 3 mg Thiamine, 6 mg Riboflavin, 6 mg Pyridoxine, 25 μg Cyanocobalamin, 40 mg Nicotinic acid, 12 mg Pantothenic acid, 1.2 mg Folic acid, 150 μg Biotin.
[7] The mineral (Rovimix Bro M, Roche, DSM, Netherlands) provided per kg of diet: 400 mg choline chloride, 250 μg Co, 1.5 mg I, 300 μg Se, 50 mg Fe, 130 mg Mn, 20 mg Cu and 100 mg Zn.
[8] Coccidiostat (Clinacox 0.5%) was added in the feed separately from the phytogenic feed additive (PFA).
[9] The phytogenic feed additive (PFA) was added at 125 mg/kg in treatment APh, BPh and CPh, at the expense of maize.

per cage were pooled and represented one replicate. Feed and excreta samples were analyzed for nutrients, uric acid and energy according to Mountzouris *et al*. (2011).

Biochemical Parameters and Total Antioxidant Capacity (TAC) in Blood Plasma

At 42-d of broiler age, 10 broilers per treatment (i.e. 2 birds per treatment replicate) were randomly selected and blood samples were collected from the birds' wing vein in heparinized tubes. Blood samples were subsequently stored in ice, centrifuged at 2,500×*g* for 10 min at 4℃ and the plasma stored at −80℃ until pending analyses.

Blood plasma cholesterol was determined via a coupled enzyme assay kit resulting in colorimetric product (510 nm) proportional to the cholesterol present in the sample (Biosis LTD, Athens, Greece). Blood plasma protein, was determined with an assay kit (Biosis LTD) based on the reduction of copper in the presence of a chromogenic reagent resulting in colorimetric product (540 nm) proportional to the concentration of protein present in the sample. Glucose and triglycerides were determined by appropriate enzymatic kits

(Biosis LTD). Blood plasma total antioxidant capacity (TAC) was determined using the oxygen radical absorbance (ORAC) assay (Cao and Prior, 1999). In particular, appropriately diluted plasma samples in phosphate buffered saline (PBS) were used and their ability to delay the decay of phycoerythrin fluorescence under the presence of [2,2-azobis(2-methylpropionamidine) dihydrochloride] (APPH) used as oxidant was compared to that of Trolox (6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid) used as an antioxidant standard and was expressed as concentration of Trolox equivalents (mmol TE/mL of serum).

Breast and Thigh Meat Total Antioxidant Capacity (TAC) and Crude Protein (CP) Content

At the end of the experiment, 10 broilers per treatment (i.e. 2 birds per treatment replicate) were randomly selected and euthanized. Subsequently, from each bird, the breast and thigh parts were removed and stored at −80℃ until pending analyses

The determination of TAC in breast (*pectoralis major*) and thigh (*biceps femoris*) samples was evaluated with the ORAC assay (Cao and Prior, 1999) and in accordance to the above was expressed as concentration of Trolox equivalents (mmol TE/g of meat). Prior to the ORAC assay meat samples were minced. A representative minced meat portion (1 g) was thoroughly homogenized in phosphate buffer (pH＝7) using a tissue grind tube. Subsequently, the homogenate was centrifuged for 10 min at 12,000×g at 4℃, the supernatant collected and centrifuged again for 30 min at 50,000×g at 4℃ and finally the supernatant was collected and stored at −80℃, until analysis within a month following appropriate dilution with PBS.

Breast and thigh meat homogenate supernatant samples prepared for the ORAC assay were additionally analysed for their protein content using the Bradford Assay (Bradford *et al*., 1976).

Breast and thigh meat CP, was analyzed as 6.25×Kjeldahl nitrogen using a Kjeltec 2300 analyser unit (Foss Tecator AB, Hoganas, Sweden; AOAC, 1991). Thigh cholesterol concentration was determined using an appropriate enzymatic kit (Biosis LTD).

Statistical Analysis

Experimental data were tested for normality using the Kolmogorov – Smirnov test and found to be normally distributed. Data were analyzed with the general linear model (GLM) – general factorial ANOVA procedure using diet type (A, B, C) and PFA addition (0 and 125 mg/kg diet) as fixed factors. Statistically significant effects were further analyzed and means were compared using Tukey's honestly significant difference (HSD) multiple comparison procedure. Statistical significance was determined at $P \leq 0.05$. All statistical analyses were done using the SPSS for Windows Statistical Package Program, Version 8.0.0 (SPSS Inc., Chicago, IL).

Results

Broiler Growth Performance

Diet type and PFA addition had no effect ($P > 0.05$) on broiler BWG and FI during the starter, grower and finisher growth phases as well as for the whole experiment (Table 2). On the contrary, broilers fed diet type A had significantly better FCR compared to diet C during the grower phase ($P_D = 0.021$) and significantly better FCR compared to diet C for the whole experiment ($P_D = 0.010$). In addition, PFA addition tended to improve broiler FCR during the starter ($P_{Ph} = 0.090$) and grower ($P_{Ph} = 0.057$) phases and for the whole experiment ($P_{Ph} = 0.089$). Overall mortality did not differ between treatments and averaged 3.56% (data not shown).

Total Tract Apparent Digestibility (TTAD) of Nutrients

The TTAD of DM and CP and the AME of diets were not affected ($P > 0.05$) by diet type and PFA inclusion (Table 3). Broilers fed diet C showed higher ($P_D = 0.046$) TTAD of EE compared to diet B. In addition, a significant interaction ($P_{D\times Ph} = 0.020$) between diet type and PFA addition was noted for TTAD of EE.

Biochemical Parameters and TAC in Blood Plasma

The effect of diet type and PFA on plasma TAC, protein, cholesterol and triglycerides concentration of 42-d old broilers is shown in Table 4. Plasma TAC was affected ($P_D < 0.001$) by diet type, with broilers fed diet A showing higher plasma TAC compared to diets B and C. Moreover, a significant interaction ($P_{D\times Ph} = 0.035$) between diet type and PFA supplementation was found for blood plasma TAC, with the lower specified diets B and C resulting in similar plasma TAC levels, with diet A after PFA administration. In addition, a significant interaction ($P_{D\times Ph} = 0.002$) regarding blood plasma protein concentration was shown with treatment BPh having higher values compared to B treatment. Triglyceride levels were not affected either by diet type or PFA inclusion (Table 4). Moreover, plasma TAC was increased by PFA addition ($P_{Ph} < 0.001$), the effect being significant in diets B and C ($P_{D\times Ph} = 0.035$). Plasma total cholesterol level in broilers fed diet type A was higher ($P_D < 0.001$) compared to the respective one in diet C. Moreover, PFA reduced ($P_{Ph} = 0.002$) plasma cholesterol levels.

Breast and Thigh Meat TAC and CP

Diet type had no effect on breast and thigh meat TAC (Table 5). The inclusion of PFA increased TAC of breast ($P_{Ph} < 0.001$) and thigh meat ($P_{Ph} = 0.010$). In addition none of the two factors studied had any effect ($P > 0.05$) on the soluble protein content of the samples analyzed for TAC (data not shown). Thigh meat cholesterol concentration was not affected by PFA inclusion in the diets. Diet type affected ($P_D = 0.002$) the CP of breast meat, with diets A and B resulting in broilers having higher CP content compared to diet C. Thigh meat cholesterol concentration tended to be higher ($P_D = 0.054$) in diet A compared to B, with diet C not differing significantly from the other two diets (Table 5).

Discussion

In this work, the effect of reduced dietary ME and CP levels in conjunction with PFA administration on broiler performance, nutrient digestibility and selected blood and meat biochemical parameters and total antioxidant capacity was evaluated.

Table 2. **Broiler growth performance during the starter (1 to14d), grower (15 to 28d) and finisher (29–42d) growth periods and for the entire experiment**

Factors	BWG Gain (g/bird)[1]				Feed Intake (g/bird)[1]				Feed Conversion Ratio (g FI/g BWG)[1]			
	Starter	Grower	Finisher	Overall	Starter	Grower	Finisher	Overall	Starter	Grower	Finisher	Overall
Diet type (D)[2]												
A	384.0	895.3	1167.9	2447.2	561.6	1384.0	2134.6	4080.2	1.47	1.55^{B}	1.83	1.68^{B}
B	386.9	892.6	1152.4	2431.9	563.7	1439.3	2172.1	4175.1	1.46	1.61^{AB}	1.89	1.72^{AB}
C	384.1	874.5	1125.0	2383.6	578.2	1420.5	2151.6	4150.3	1.51	1.62^{A}	1.91	1.74^{A}
Phytogenic (Ph)[3]												
No	381.4	876.9	1152.2	2410.4	582.5	1417.4	2156.4	4156.4	1.53	1.62	1.88	1.73
Yes	388.7	898.1	1144.7	2431.4	553.1	1411.8	2149.2	4114.1	1.43	1.57	1.88	1.69
Interactions (treatments)												
A	374.8	868.9	1167.1	2410.8	576.3	1367.6	2109.6	4053.5	1.54	1.57	1.81	1.68
APh	393.2	921.7	1168.7	2483.6	546.8	1400.3	2159.7	4106.8	1.40	1.52	1.85	1.65
B	387.6	885.2	1147.6	2420.4	578.5	1438.3	2176.7	4193.6	1.49	1.63	1.90	1.74
BPh	386.2	900.1	1157.1	2443.4	548.9	1440.3	2167.5	4156.6	1.43	1.60	1.88	1.70
C	381.6	876.6	1141.8	2400.0	592.8	1446.3	2182.9	4222.0	1.56	1.65	1.91	1.76
CPh	386.6	872.4	1108.2	2367.2	563.6	1394.8	2120.3	4078.7	1.46	1.60	1.92	1.72
SEM[4]	10.53	23.44	32.42	45.16	18.70	34.33	39.70	61.25	0.071	0.028	0.040	0.023
P-value												
D	0.952	0.633	0.421	0.355	0.630	0.280	0.645	0.293	0.742	0.021	0.129	0.010
Ph	0.403	0.280	0.780	0.575	0.066	0.843	0.825	0.406	0.090	0.057	0.841	0.089
D×Ph	0.634	0.476	0.781	0.514	1.000	0.474	0.380	0.294	0.861	0.886	0.719	0.955

[1] Data shown per diet type represent treatment means from $n=10$ replicate floor pens (e.g. treatments A+APh). Data shown for PFA represent means from $n=15$ replicate pens (e.g. A+B+C). Within the same column means with different superscript (A, B) per diet type, differ significantly ($P<0.05$).
[2] Diet type (A, B and C): for each growth phase diet type A optimal for energy and protein; B and C suboptimal for protein and energy compared to A by 3 and 6% respectively.
[3] Phytogenic supplementation (No=0 mg/kg diet and Yes=125 mg/kg diet).
[4] Standard error of the means.

Diet type did not affect FI in this study. Reducing ME level by 2% (i.e. from 3,000 to 2,950 kcal/kg) whilst keeping CP level constant did not affect FI in broilers following PFA (i.e. 100 mg/kg) supplementation (Bravo *et al*., 2011). In addition, FI was not affected in two other studies whereby diets were kept isoenergetic but CP level was lowered by 3% (Kamran *et al*., 2004) and 4% (Corzo *et al*., 2005). However, lowering CP level by 32% (i.e. 225 to 153 g CP/kg) at constant dietary ME, in 3 to 6 weeks old broilers significantly increased FI (Aletor *et al*., 2000). Moreover, lowering dietary CP level by 6% (i.e. 23 to 17%) and ME level by 16% (i.e. from 3,146 to 2,645 kcal/kg), whilst maintaining a constant ME: CP ratio, linearly increased FI during grower, finisher, and overall periods (Kamran *et al*., 2008). It is obvious that among other factors the degree of reduction in dietary CP and ME levels could be considered as the main factor for the discrepancies in the results shown above (Aletor *et al*., 2000).

In this study, it was shown that diet C having the lowest ME and CP specifications compared to diet A had poorer FCR at the grower period and for the whole experiment. Previous studies have shown that reduction of CP level resulted in poorer FCR (Aletor *et al*., 2000; Bregendahl *et al*., 2002), even when diets were kept isoenergetic (Waldroup *et al*., 2005). It is possible that certain non-essential amino acids (AA) become limiting below a certain level of dietary CP in broilers. This fact could explain the reduced performance of chicks fed low CP diets, even when essential AA were provided at requirement levels (Berres *et al*., 2010).

In addition, Kamran *et al*. (2008) indicated that decreasing CP levels with constant ME:CP ratio worsened FCR during all growth periods except the starter period. Similarly, in the present study, diet C had poorer FCR by 4.3% at the grower period and overall by 3.5% compared to diet type A. Further to the lower levels of essential AA in diet C compared to A, other potentially limiting non-essential AA due to the lower CP levels could also justify the an increase in FCR (Waldroup *et al*., 2005; Kamran *et al*., 2008) as in diet C compared to optimal diet A.

Concerning the supplementation of broiler diets with PFA, the majority of studies indicate that the addition of essential oils mixtures containing carvacrol reduced FI (Lee *et al*., 2003; Hashemipour *et al*., 2013) but did not affect BWG (Botsoglou *et al*., 2002; Barreto *et al*., 2008; Akyurek and Yel, 2011) and consequently resulted in improved FCR (Lee *et al*., 2003; Hashemipour *et al*., 2013). In the present study, PFA supplementation did not affect BWG, and tended to decrease FI by 5.1% in the starter phase. In addition, trends for improved FCR by 6.5% and 3.1% at the starter and grower phase, respectively, and overall by 2.3% compared to the un-supplemented diets were evidenced. Moreover, the

Table 3. **Total tract apparent apparent digestibility of nutrients and apparent metabolisable energy (AME) content at 40–42d old broilers**

Factors	Dry matter[1]	Ether extract[1]	Crude protein[1]	AME (Mj/kg)[1]
Diet type (D)[2]				
A	72.6	64.4^{AB}	66.4	12.89
B	72.9	60.2^{B}	68.7	12.67
C	72.1	69.0^{A}	70.6	12.78
Phytogenic (Ph)[3]				
No	73.3	66.0	69.2	12.89
Yes	71.8	63.0	68.0	12.67
Interactions (treatments)				
A	74.0	71.4^{ab}	68.0	13.34
APh	71.2	57.4^{b}	64.8	12.44
B	72.9	60.7^{ab}	67.4	12.50
BPh	72.8	59.8^{ab}	70.1	12.84
C	72.8	66.0^{ab}	72.0	12.82
CPh	71.4	71.9^{a}	69.1	12.74
SEM[4]	1.47	3.23	4.59	0.273
P-value				
D	0.874	0.046	0.669	0.720
Ph	0.241	0.270	0.760	0.350
D×Ph	0.668	0.020	0.772	0.097

[1] Data shown per diet type represent treatment means from $n=8$ replicate cage pens (e.g. treatments A+APh). Data shown for PFA represent means from $n=12$ replicate cage pens (e.g. A+B+C). Within the same column means with different superscript (A, B) per diet type and (a, b, c) per interaction means (treatments), differ significantly ($P<0.05$).
[2] Diet type (A, B and C): for each growth phase diet type A optimal for energy and protein; B and C suboptimal for protein and energy compared to A by 3 and 6% respectively.
[3] Phytogenic supplementation (No=0 mg/kg diet and Yes=125 mg/kg diet).
[4] Standard error of the means.

dietary supplementation of broilers with a similar essential oils blend with the one used in this study resulted in improved FCR (Mountzouris *et al*., 2011; Hong *et al*., 2012) by significantly (Mountzouris *et al*., 2011) or numerically (Hong *et al*., 2012) decreasing overall FI and / or increasing BWG. Generally, apart from differences in the actual essential oil components, the different growth performance responses may be also due to other factors such as PFA dietary inclusion levels, the type of basal diet and feeding conditions (Ertas *et al*., 2005).

The TTAD of nutrients may be related with the degree of reduction of dietary ME and CP levels, the balance of amino acids, the ME:CP ratio (i.e. constant or not), and the age and breed of broilers (Aletor *et al*., 2000). According to amino acids and their correlation with digestibility estimates, Ravindran *et al*. (2005) stated that the apparent digestibility coefficients could be affected by the level of dietary protein and/or amino acid concentrations. For example at low protein / amino acid intakes, TTAD of nutrients could be lower due to greater proportion of endogenous protein present at the terminal ileum. In this study, diet type did not affect TTAD of DM, CP and AME content. However, it affected the TTAD of EE. Specifically birds fed on diet C had higher TTAD of EE compared to the ones on diets B. In addition, from the interaction between diet type and PFA administration regarding the TTAD of EE, it appears that the addition of PFA was more beneficial in improving EE digestibility in suboptimal diet C (i.e. treatment CPh) compared to the optimal diet A (i.e. treatment APh). This result indicates that PFA addition could improve EE digestibility in diets with lower than the optimal specification of ME and CP. This finding could be considered in line with Aletor *et al*. (2000) who showed dietary energy to be more efficient in low versus high dietary protein diets, and as such it may provide a potential nutritional benefit for broilers.

The supplementation of PFA significantly increased blood plasma TAC by 19.4%. This is agreement with other studies where phytogenics increased antioxidative capacity of blood plasma (Goni *et al*., 2007; Zhang *et al*., 2009; Ciftci *et al*., 2010; Polat *et al*., 2011). Moreover, Hoffman-Pennesi and Wu (2010) evaluated antioxidative capacity with the ORAC assay and showed that thymol which is an isomer of carvacrol significantly increased the antioxidative status of broiler blood plasma. Similarly with our study, the supplementation of essential oils derived for oregano, exerted higher serum antioxidant activity compared to the control

Table 4. **Total antioxidant capacity (TAC) and concentration of protein, cholesterol and triglycerides in blood plasma of 42d old broilers**

Factors	TAC (mmol TE/ l plasma)[1]	Protein (g/ dL plasma)[1]	Cholesterol (mg/ dL plasma)[1]	Triglycerides (mg/ dL plasma)[1]
Diet type (D)[2]				
A	13.67^{A}	5.93	182.65^{A}	65.64
B	11.50^{B}	6.16	166.32^{AB}	62.86
C	9.87^{B}	6.16	153.14^{B}	57.04
Phytogenic (Ph)[3]				
No	10.42^{x}	5.95	177.44^{y}	64.63
Yes	12.93^{y}	6.22	157.30^{x}	59.06
Interactions (treatments)				
A	13.53^{a}	5.92^{ab}	186.00	65.66
APh	13.80^{a}	5.95^{ab}	179.30	65.61
B	9.26^{b}	5.51^{b}	180.75	65.64
BPh	13.74^{a}	6.80^{a}	151.88	60.08
C	8.47^{b}	6.41^{ab}	165.56	62.59
CPh	11.26^{ab}	5.91^{ab}	140.71	51.49
SEM[4]	0.793	0.242	7.408	5.526
P-value				
D	$<$0.001	0.568	0.001	0.292
Ph	$<$0.001	0.169	0.002	0.223
D$\times$Ph	0.035	0.002	0.289	0.609

[1] Data shown per diet type represent treatment means from $n=20$ broilers (e.g. 10 from treatment A+10 from treatment A+Ph). Data shown for PFA represent means from $n=30$ replicate pens (e.g. A+B+C). Within the same column means with different superscript (A, B) per diet type, (x, y) per phytogenic and (a, b, c) per interaction means (treatments), differ significantly ($P<0.05$).
[2] Diet type (A, B and C): for each growth phase diet type A optimal for energy and protein; B and C suboptimal for protein and energy compared to A by 3 and 6% respectively.
[3] Phytogenic supplementation (No=0 mg/kg diet and Yes=125 mg/kg diet).
[4] Standard error of the means.

treatments in a dose dependent manner (Roofchaee *et al*., 2011). The improvement of plasma antioxidant capacity by dietary phytogenics could be due to the active components and their phenolic group constituents which exhibit a strong antioxidant effect (Polat *et al*., 2011). In addition, the improvement in the antioxidant status may have resulted from the induction of antioxidant enzyme activities (Ciftci *et al*., 2010; Roofchaee *et al*., 2011). The reduction of ME and CP levels, in the present study, significantly reduced blood plasma TAC. Given the significant interaction noted between diet type and PFA administration it could be speculated that within the diet type range examined (i.e. levels of energy and protein reduction) the higher energy and protein levels have stimulated host resistance to oxidation, the effect being stronger when PFA was administered. Moreover, while in lower specified diets blood plasma TAC was reduced, the addition of PFA increased plasma TAC levels to the levels of diet A.

In this study, protein concentration was measured among other blood parameters in order to examine the effect of ME and CP levels reduction and PFA administration on protein blood plasma balance in *ad libitum* fed broilers. It was shown that blood plasma protein concentration was not affected either by diet type or PFA inclusion. Regarding diet type, the above is in agreement with other studies in which low CP diets (Corzo *et al*., 2005) including diets with constant ME:CP ratio (Steiner *et al*., 2008) did not affect broiler plasma protein concentration. However, from the interaction of diet type with PFA it was shown that when dietary ME and protein level was reduced by 3%, PFA supplementation resulted in higher blood plasma protein concentration by 19%. Given that there we no differences in FI, it is possible that the properties of essential oils to protect cells and inhibit non enzymatic oxidation (Ghazalah and Ali., 2008) could explain the increased broiler blood plasma protein levels observed by other studies (Ghazalah and Ali, 2008; Al-Jaff., 2011; El-Ghousein *et al*., 2009).

In the present study, ME and CP reductions resulted in a reduction of blood plasma cholesterol, with diet A having higher cholesterol compared to diet C by 16.2%. Blood plasma cholesterol was measured in this study in order to assess the potential hypocholesterolemic properties of the PFA used. The inclusion of PFA resulted in blood cholesterol reduction by 11.4%. Administration of PFA such as herbs, essential oils and their mixtures reduced blood plasma cholesterol (Soltan *et al*., 2008; Ciftci *et al*., 2010; Al-Jaff., 2011; Hajati *et al*., 2011; Polat *et al*., 2011). In particular, the supplementation in broiler diets of PFA containing essential oils derived from oregano, anise and citrus peels similar to our study, reduced serum levels of cholesterol

Table 5. **Meat total antioxidant capacity (TAC), cholesterol and protein content of 42d old broilers**

Factors	TAC (mmol TE/g)[1]		Cholesterol (mg/100 g)[1]	Protein content - Kjedahl (%)[1]	
	breast	thigh	thigh	breast	Thigh
Diet type (D)[2]					
A	59.42	51.54	221.70	23.92^{A}	21.45
B	59.18	48.11	190.79	23.57^{A}	21.33
C	57.73	45.64	186.33	22.78^{B}	20.86
Phytogenic (Ph)[3]					
NO	53.62^{x}	44.46^{x}	206.43	23.53	21.16
YES	63.94^{y}	52.40^{y}	192.78	23.32	21.27
Interactions (treatments)					
A	53.57	45.29	221.64	24.01	21.44
APh	65.27	57.79	221.76	23.83	21.46
B	56.45	45.87	197.87	23.59	21.37
BPh	61.91	50.35	174.79	23.56	21.30
C	50.83	42.22	199.79	23.00	20.68
CPh	64.64	49.05	181.79	22.56	21.04
SEM[4]	3.473	3.633	15.534	0.315	0.338
P-value					
D	0.871	0.273	0.054	0.002	0.190
Ph	0.001	0.010	0.287	0.411	0.698
D×Ph	0.463	0.529	0.736	0.803	0.792

[1] Data shown per diet type represent treatment means from $n=20$ broilers (e.g. 10 from treatment A+ 10 from treatment APh). Data shown for PFA represent means from $n=30$ replicate pens (e.g. A+B+ C). Within the same column means with different superscript (A, B) per diet type and (x, y) per phytogenic, differ significantly ($P<0.05$).
[2] Diet type (A, B and C): for each growth phase diet type A optimal for energy and protein; B and C suboptimal for protein and energy compared to A by 3 and 6% respectively.
[3] Phytogenic supplementation (No=0 mg/kg diet and Yes=125 mg/kg diet).
[4] Standard error of the means.

(Hong *et al*., 2012). The reduction of plasma cholesterol could be due to the down regulation of HMG-Co A reductase activity by PFA components such as carvacrol and thymol (El-Ghousein *et al*., 2009; Hayati *et al*., 2011). This enzyme is the rate controlling enzyme of the mevalonate pathway which produces cholesterol and other isoprenoids. Ciftci *et al*. (2010) noticed that inhibition of HMG-CoA reductase reduced cholesterol synthesis and its inhibitors were very effective in lowering plasma cholesterol in most animal species, including humans. The concentration of plasma triglycerides was not affected by diet type and/or PFA inclusion. This result is in agreement with other studies (Soltan *et al*., 2008; Najafi and Torki, 2010) in which supplementation of phytogenics had no effect on blood plasma triglycerides.

In the present study, PFA supplementation increased TAC of breast and thigh meat by 16.1 and 15.2%, respectively. It is known that herbs and their essential oils have antioxidative effects on meat lipid oxidation, the effect being dose dependent (Lee *et al*., 2004). Moreover, broiler breast and thigh meat antioxidant capacity was found to be significantly increased in other studies following dietary phytogenic supplementation containing essential oils derived from oregano (Botsoglou *et al*., 2002; Young *et al*., 2003; Luna *et al*., 2010; Hashemipour *et al*., 2013). The increase in meat TAC could be explained by the antioxidant properties of several polyphenolic compounds such as carvacrol in this study, which some herbs (e.g. oregano) and their essential oils contain that extend the shelf life and improve the meat quality by delaying lipid oxidation (Botsoglou *et al*., 2002; Brenes and Roura, 2010). Moreover, dietary supplementation of plant antioxidants enhances membrane stability and reduces lipid peroxidation in broilers meat due to their preservation of thiol-containing enzymes such as glutathione reductase and gluthatione peroxidase (Lopez-Bote *et al*., 1998).

As it was expected based on the diet formulation, reduced levels of dietary ME and CP levels in the present study resulted in a significant reduction of breast meat CP, with broilers fed diet type C having lower CP values by 4.8 and 3.4% compared to diets A and B, respectively. This result is in agreement with the results of studies in which lowering ME:CP levels in broiler diets, resulted in decreasing meat CP concentration (Neto *et al*., 2000; Kamran *et al*., 2004; Laudadio *et al*., 2012). In addition, the reduction of cholesterol in thigh meat is in line with the decrease of dietary ME: CP levels, with diet A resulting in higher cholesterol by 16% compared to diet type C.

It is clear that diets with lower than optimal ME and CP levels, although more economical in principle, yet they may

result in higher FCR. In this study, PFA administration tended to improve FCR, while in other studies using a PFA similar to the one used in this work, FCR was improved significantly (Mountzouris *et al*., 2011; Hong *et al*., 2012). Therefore, the overall economic benefit of formulating diets using lower ME and CP specifications and the potential of using PFAs compared to other bioactive feed additives such as enzymes to compensate for losses in growth performance will depend on the actual feed additive costs.

In conclusion, the results of the present study showed that a reduction in dietary ME and CP levels at a constant ME:CP ratio adversely affected broiler feed efficiency, whereas supplementation with PFA tended to compensate these effects at the starter, grower phase and overall. Moreover, PFA administration significantly improved plasma and meat TAC and reduced plasma cholesterol compared to the non-supplemented controls. Given the significant lower cost of down regulated diet types B and C by approximately 8.7 and 17.4% for the grower and 8.9 and 17.8% for the finisher phase compared to the respective diet type A, the inclusion of PFA to ameliorate the negative impact of lower energy and protein levels could be considered as an option worth for further consideration.

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