



Original Research Article

Growth performance, nutrient digestibility, antioxidant capacity, blood biochemical biomarkers and cytokines expression in broiler chickens fed different phytogetic levels



Vasileios Paraskeuas^a, Konstantinos Fegeros^a, Irida Palamidi^a, Christine Hunger^b,
Konstantinos C. Mountzouris^{a,*}

^a Department of Nutritional Physiology and Feeding, Agricultural University of Athens, 118 55 Athens, Greece

^b BIOMIN Holding GmbH, Erber Campus 1, 3131 Getzersdorf, Austria

ARTICLE INFO

Article history:

Received 18 August 2016

Accepted 24 January 2017

Available online 30 January 2017

Keywords:

Chicken
Phytogetic
Antioxidant
Menthol
Anethol
Eugenol

ABSTRACT

The effects of inclusion levels of a phytogetic feed additive (PFA), characterized by menthol anethol and eugenol, on broiler growth performance, nutrient digestibility, biochemical biomarkers and total antioxidant capacity (TAC) of plasma and meat, as well as on the relative expression of selected cytokines, were studied in a 42-d experiment. A total of 225 one-day-old male Cobb broiler chickens were assigned into 3 treatments, with 5 replicates of 15 chickens each. Chickens were fed maize-soybean meal basal diets following a 3 phase (i.e., starter, grower and finisher) feeding program. Depending on PFA inclusion level, treatments were: no PFA (PFA-0), PFA at 100 mg/kg (PFA-100) and PFA at 150 mg/kg (PFA-150). Feed and water were available *ad libitum*. Feed conversion ratio (FCR) during finisher phase was improved quadratically ($P < 0.05$) with increasing PFA level. Overall, increasing PFA level increased body weight gain (BWG) in a linear ($P < 0.05$) and quadratic ($P < 0.05$) manner with treatments PFA-100 and PFA-150 being greater ($P < 0.05$) compared with PFA-0. Total tract apparent digestibility of dry matter increased linearly ($P < 0.05$) and quadratically ($P < 0.05$) with increasing PFA level. The apparent metabolizable energy corrected for nitrogen (AMEn) also increased linearly ($P < 0.05$). Increasing PFA level resulted in a linear ($P < 0.05$) increase in blood plasma TAC. Expression of pro-inflammatory cytokine interleukin -18 (*IL-18*) was reduced linearly ($P < 0.05$) in spleen with increasing PFA level. In conclusion, PFA inclusion at 100 mg/kg diet positively influenced performance, whereas PFA inclusion at 150 mg/kg resulted in a stronger improvement in AMEn and plasma TAC. Finally, PFA inclusion resulted in a pattern of reduced pro-inflammatory biomarker *IL-18* at spleen. Overall, this study provides evidence for the beneficial role of PFA as a natural growth and health promoter in broiler chickens that needs to be further confirmed in field studies.

© 2017, Chinese Association of Animal Science and Veterinary Medicine. Production and hosting by Elsevier B.V. on behalf of KeAi Communications Co., Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

The ban of antimicrobial growth promoter (AGP) use in animal nutrition in the European Union has urged the research for effective

alternatives. The use of phytogetic compounds (i.e., herbs, spices, their essential oils and mixtures or their single active components) in broiler chicken diets as alternatives to AGP has started to attract interest due to their growth promoting potential (Ghazalah and Ali, 2008; Mountzouris et al., 2011), their antioxidant (Ciftci et al., 2010; Polat et al., 2011), and hypocholesterolaemic properties (Ciftci et al., 2010; Safamehr et al., 2012).

Moreover, nutrient digestibility improvements via digestive enzyme stimulation by phytogetics have been reported (Malayoğlu Basmacioğlu et al., 2010; Safamehr et al., 2012). Furthermore, phytogetic feed additives (PFA) are thought to exert effects on the immune system by reducing inflammation in cecal tonsils (Lu et al., 2014), or reducing pro-inflammatory intestinal cytokines (Kim et al., 2013).

* Corresponding author.

E-mail address: kmountzouris@aua.gr (K.C. Mountzouris).

Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.



The efficacy of a PFA is possibly related, among other factors, to the phytogetic inclusion levels and its bioactive compound constituents (Brenes and Roura, 2010). The former has been investigated in several studies in broiler chickens (Malayoğlu Basmacıoğlu et al., 2010; Mountzouris et al., 2011). For example an inclusion level-dependent effect on performance (Mountzouris et al., 2011) and on meat lipid oxidation have been shown (Botsoglou et al., 2002).

Among the various PFA bioactive constituents, menthol, anethol and eugenol are currently being researched for their effects in broiler chickens. All of these components have been shown to promote growth performance (Erhan et al., 2012; Fallah et al., 2013; Hong et al., 2012) and nutrient digestibility (Amad et al., 2011; Khodhambashi Emami et al., 2012). However, despite the high antioxidant properties of menthol, anethol and eugenol (Petrovic et al., 2012), contradicting results have been shown for their effects on blood cholesterol, triglycerides, protein and glucose concentration (Hong et al., 2012; Hosseinzadeh and Farhoomand, 2014) as well as for their immune effects (Hong et al., 2012; Kim et al., 2013; Toghyani et al., 2010).

Therefore, the aim of this work was to assess the effect of dietary inclusion levels of a PFA comprising a mixture of menthol, anethol and eugenol on growth performance, nutrient digestibility, selected biochemical responses, total antioxidant capacity (TAC) of blood plasma and meat as well as cytokine gene expression in spleen and caecal tonsils in broiler chickens.

2. Materials and methods

2.1. Animals and experimental treatments

In total, 225 one-day-old male Cobb broiler chickens were obtained from a commercial hatchery. The broilers were vaccinated at hatch for Marek, Infectious Bronchitis and Newcastle Disease and randomly allocated to 3 experimental treatments, for 6 weeks. Each treatment had chickens arranged in 5 replicates of 15 broiler chickens. Each replicate was assigned to a clean floor pen (1 m²) and birds were raised on rice hulls 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. Depending on the level of PFA addition to basal diet (BD), the experimental treatments were: PFA-0 (no addition of PFA in BD), PFA-100 (PFA added at 100 mg/kg BD) and PFA-150 (PFA added at 150 mg/kg BD). All diets were provided in mash form and were formulated for starter (d 1 to 14), grower (d 15 to 28) and finisher (d 29 to 42) broiler chicken growth periods (Table 1). The PFA used was comprised of a selected combination of different plant materials including herbs, spices, essential oils and extracts. The PFA had an active ingredient concentration of 350 g/kg PFA, mainly essential oils from mint (*Mentha arvensis*), star anise (*Illicium verum*) and cloves (*Syzygium aromaticum*), mixed with carrier (silicium dioxide and sodium chloride). The content of essential oil was determined by a method from the European Pharmacopoeia 5.0 (EDQM, 2005). The PFA was mixed weekly into the BD. Throughout the experiment, experimental diets 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.

2.2. Broiler performance responses

Broiler chicken growth performance responses, such as body weight gain (BWG), feed intake (FI), mortality corrected feed

conversion ratio (FCR) and livability were determined on a weekly basis during the 6 experimental weeks. Performance data were presented on a growth period (i.e., starter, grower and finisher) basis. In addition, overall BWG, FI, FCR and livability were calculated and presented for the entire duration of the experiment.

2.3. Total tract apparent digestibility of nutrients and apparent metabolizable energy corrected for nitrogen (AMEn)

The total tract apparent digestibility (TTAD) of nutrients was determined via the addition of chromic oxide (Cr₂O₃) as an analytical marker. In particular, chromic oxide was carefully added and mixed in each of the 3 experimental diets for the digestibility experiment at a concentration of 0.5 g Cr₂O₃/kg diet at the expense of maize. For each treatment, on 35 d of age, 4 broiler chickens per floor pen were removed and pooled per treatment (i.e., 20 broilers per treatment). Broiler chickens were subsequently placed in battery cages so that each treatment had 4 replicate-cages of 5 birds each. Each cage had wire mesh bottom and excreta collection trays. The experimental set up, the sampling and analytical protocol followed was according to Mountzouris et al. (2011). The digestibility experiment had a 4-d adaptation period and a 3-d collection period. During the collection period, excreta from each cage were collected 4 times daily (in 6-h intervals) and stored in sealed bags at –20 °C. Remaining feed in the excreta trays was carefully removed and weighed. Feathers were also removed from the excreta. Excreta collected per cage were pooled and represented one replicate.

Feed and excreta samples were subsequently analyzed in duplicate for dry matter (DM), ether extracts (EE), crude protein (CP) and gross dietary energy. Excreta samples were additionally analyzed for uric acid nitrogen. Feed and excreta samples were analyzed for chromic oxide concentration and all the analyses above were according to Mountzouris et al. (2011).

2.4. Biochemical biomarkers and total antioxidant capacity of blood plasma

At 42 d of age, 10 broilers per treatment (2 birds per replicate) were randomly selected and blood samples were collected from the bird's wing vein in heparinized tubes. Blood samples were stored on ice, centrifuged at 2,500 × g for 10 min at 4 °C and the plasma stored at –80 °C until pending analyses.

Blood plasma cholesterol, protein, glucose and triglycerides were determined by commercial enzymatic kits (Biosis LTD, Athens Greece). Blood plasma total antioxidant capacity (TAC) was determined, using the oxygen radical absorbance (ORAC) assay (Cao and Prior, 1999) to evaluate the hydrophilic antioxidants (Prior et al., 2003). Appropriately diluted plasma samples in phosphate-buffered saline (PBS) were used and the ability to delay the decay of phycoerythrin fluorescence under the presence of 2,2'-azobis (2-methylpropionamide) dihydrochloride (APPH) used as oxidant was compared with that of trolox (6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid) used as an anti-oxidant standard. Data were expressed as concentration of trolox equivalents (TE) (mmol/L of serum).

2.5. Breast and thigh meat total antioxidant capacity

At the end of the experiment, 10 broilers per treatment (2 birds per replicate) were randomly selected and euthanized. The breast (pectoralis major) and thigh (biceps femoris) parts were removed from each bird and stored at –80 °C until pending analysis.

Breast and thigh TAC was determined by the ORAC assay (Cao and Prior, 1999) to evaluate the hydrophilic antioxidants (Prior et al., 2003) and the results were expressed as concentration of

Table 1
Ingredient and chemical composition of the basal experimental diets (as fed basis).

Item	Maize based diets		
	Starter (d 1 to 14)	Grower (d 15 to 28)	Finisher (d 29 to 42)
Ingredients, g/kg			
Maize	540.6	570.0	607.8
Soybean meal (460 g crude protein/kg)	371.4	332.8	294.6
Soy-oil	25.0	34.3	0.0
Vegetable fat ¹	20.3	21.5	59.5
Limestone	13.5	13.0	12.2
Mono calcium phosphate	16.2	15.6	14.4
Salt (NaCl)	4.7	3.9	3.7
L-lysine	1.1	1.3	1.2
DL-methionine	2.6	2.7	2.4
L-threonine	0.0	0.3	0.3
Vitamin premix ²	2.0	2.0	2.0
Mineral premix ³	2.0	2.0	2.0
Cocciostat (Monteban 100)	0.6	0.6	0.0
Phytogenic feed additive ⁴	–	–	–
Calculated (determined) chemical composition, g/kg			
AME _n , MJ/kg diet	12.5	12.9	13.15
Dry matter	883.6 (903.2)	884.0 (913.3)	883.2 (898.4)
Crude protein	215.0 (217.3)	200.0 (201.8)	185.0 (187.9)
Ether extract	70.5 (73.0)	81.8 (82.1)	85.7 (84.5)
Crude fiber	32.1	31.0	29.9
Lysine	12.4	11.6	10.5
TSAA (methionine + cysteine)	9.2	8.9	8.2
Threonine	8.2	7.8	7.2
Calcium	10.0	9.6	9.0
Available phosphorus	5.0	4.8	4.5
Sodium	2.0	1.7	1.6

TSAA = total sulphur amino acids.

¹ Lecithinised fat powder with 6% lecithin (BERGAFAT HTL-306, Berg & Schmidt, Hamburg, Germany).

² The vitamin premix for starter and grower periods (Rovimix 11 Bro Basic, DSM, Netherlands) provided per kg of diet: 3.6 mg retinol (vitamin A), 100 µg cholecalciferol (vitamin D₃), 80 mg vitamin E, 9 mg menadione (vitamin K₃), 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 (vitamin A), 75 µg cholecalciferol (vitamin D₃), 50 mg vitamin E, 7 mg menadione (vitamin K₃), 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.

³ The mineral (Rovimix Bro M, 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.

⁴ The phytogenic feed additive (Digestarom Poultry, Biomin Phytogenics GmbH, Germany) was added at 100 and 150 mg/kg diet at the expense of maize in the respective diets.

TE (mmol/g of meat). Prior to the ORAC assay meat samples were minced and prepared as follows. A 1-g minced meat portion was then 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 °C, the supernatant collected and centrifuged again for 30 min at 50,000 × g at 4 °C and finally the supernatant was collected and stored at –80 °C until analysis, within a month following appropriate dilution with PBS.

2.6. RNA isolation and reverse-transcription PCR

At 42 d of age, 5 broilers per treatment were randomly selected and cecal tonsils and spleen samples were excised aseptically and stored at –80 °C until pending analyses. Subsequently, spleen and cecal tonsils per broiler were homogenized with pestle and mortar under nitrogen and extraction of total RNA was performed using Trifast Reagent (PEQLAB Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's protocol. RNA quantity was determined by spectrophotometry (NanoDrop-1000, Thermo

Fisher Scientific, Waltham, United Kingdom). Prior to complementary DNA (cDNA) synthesis, DNase treatment was applied. Ten microgram of RNA were treated with 1 U of DNase I (M0303, New England Biolabs Inc, Ipswich, UK) and 10 µL of 10× DNase buffer for 1 h at 37 °C. The DNase was inactivated by the addition of 1 µL of 0.5 mol/L ethylene diamine tetraacetic acid at 75 °C for 10 min. RNA integrity was assessed by agarose gel electrophoresis.

For cDNA preparation, 500 ng of total RNA from each sample were reverse transcribed to cDNA by PrimeScript RT Reagent Kit (Perfect Real Time, Takara Bio Inc., Shiga-Ken, Japan) according to the manufacturer's recommendations. All cDNAs were then stored at –20 °C.

2.7. Quantitative real-time PCR

Chicken transforming growth factor beta 4 (*TGF-β4*), interferon gamma (*IFN-γ*), interleukin -18 (*IL-18*), *IL-2*, *IL-10*, inducible nitric oxide synthase 2 (*iNOS2*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) primers were designed with the PerlPrimer program v.1.1.19 (Marshall, 2004) using the GenBank sequences deposited on the National Center for Biotechnology Information and US National Library of Medicine (NCBI) shown in Table 2. The primers were selected so as to hybridize within the coding region of the transcripts. Furthermore 1 of the 2 primers was selected to hybridize at an intron/exon junction so as to exclude hybridization to even tiny residuals of genomic DNA. Afterwards primers were checked using the PRIMER BLAST algorithm against *Gallus gallus* genomic and mRNA databases to ensure that there was no genomic amplification and that there was a unique amplicon. Primers were checked for amplification efficiency which was found to range between 1.9 and 2.0 for all primers and the respective R squared (RSQ) values were presented in the Results section.

Real-time PCR was performed in 96 well microplates with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) and KAPA SYBR FAST qPCR Kit (KAPA Biosystems, Wilmington, MA, USA). Each reaction contained 12.5 ng RNA equivalents as well as 200 to 250 nmol/L of forward and reverse primers for each gene. The reactions were incubated at 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s 60 or 62 °C (depends on the target gene) for 20 s, 72 °C for 33 s. This was followed by a melt curve analysis to determine the reaction specificity. Each sample was measured in duplicates. Relative expression ratios of target genes were calculated according to Pfaffl (2001) using *GAPDH* as a reference gene.

2.8. Statistical analysis

The experimental data per diet type were analyzed with the general linear model (GLM) – ANOVA procedure using the SPSS for Windows statistical package program, version 8.0.0 (SPSS Inc., Chicago, IL). 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* < 0.05. Linear and quadratic effects of dietary PFA inclusion level were studied using polynomial contrasts.

3. Results

3.1. Broiler growth performance

There were no significant differences between the experimental treatments (PFA-0, PFA-100 and PFA-150) regarding body weight gain (BWG) and FI at any of the 3 growth phases as shown in Table 3. In the finisher period increasing PFA level improved feed

Table 2
Oligonucleotide primers used for quantitative real time PCR.

Target	Primer sequence (5' to 3') ¹	Annealing temperature, °C	PCR product size, bp	GenBank accession No.
<i>GAPDH</i>	F: GCTGAATGGGAAGCTTACTG R: AAGGTGGAGGAATGGCTG	60	216	NM_204305.1
<i>IFN-γ</i>	F: AGTCCCGATGAACGAC R: CAGGAGTCTATAAGATGCCA	62	151	NM_205149.1
<i>TGF-β4</i>	F: GGACGGATGAGAAGAAGCTG R: ACGACCACCATATTGGA	62	296	M31160.1
<i>IL-18</i>	F: GTTGTTCGATTTAGGGAAGGAG R: TCAAAGGCCAAGAACATTCC	60	146	NM_204608.1
<i>IL-10</i>	F: GACCAGCACCAGTCATCAG R: CCGTTCTCATCCATCTTCTCG	62	159	EF554720.1
<i>IL-2</i>	F: AGTCTTACGGGTCTAAATCACAC R: GGACAGCAGATTAGTTAGCCA	62	219	AF000631.1
<i>iNOS</i>	F: AAAGAAAGGGATCAAAGGTGGT R: CAAGCATCTCTTCAAAGTCTG	60	296	NM_204961.1

GAPDH = glyceraldehyde 3-phosphate dehydrogenase; *IFN-γ* = interferon-γ; *TGF-β4* = transforming growth factor beta 4; *IL* = Interleukin; *iNOS* = inducible nitric oxide synthase.

¹ F: Forward, R: Reverse.

conversion ratio (FCR) quadratically ($P < 0.05$), with treatment PFA-100 having lower ($P < 0.05$) FCR compared with treatments PFA-0 and PFA-150. Overall, increasing PFA level enhanced BWG in a linear ($P < 0.05$) and quadratic ($P < 0.05$) manner with treatment PFA-100 and PFA-150 having higher ($P < 0.05$) BWG compared with treatment PFA-0. Finally, no significant differences were observed regarding livability during the starter, grower, finisher period, and overall of each treatment (Table 3).

3.2. Total tract apparent digestibility of nutrients and AMEn

Increasing PFA level increased TTAD of DM linearly ($P < 0.05$) and quadratically ($P < 0.05$) with treatment PFA-150 being greater ($P < 0.05$) compared with treatments PFA-0 and PFA-100. Moreover, increasing PFA level linearly ($P < 0.05$) increased AMEn with treatment PFA-150 being greater ($P < 0.05$) compared with treatments PFA-0 and PFA-100 (Table 4).

3.3. Biochemical biomarkers and blood plasma TAC

Increasing PFA level linearly increased ($P < 0.05$) blood plasma TAC, with treatment PFA-150 having greater ($P < 0.05$) TAC, compared with treatment PFA-0. The level of PFA inclusion did not affect protein, glucose, triglycerides, and cholesterol concentration in the plasma of 42-d-old broiler chickens (Table 5). Breast and thigh meat TAC were not affected by PFA inclusion level (Table 6).

3.4. Cytokines

Gene expression of *TGF-β4*, *IFN-γ*, *IL-10*, *IL-18*, *IL-2* and *iNOS* was evaluated in spleen and the cecal tonsils of 42-d-old broiler chickens (Table 7). The gene expression of *IL-10* in spleen was low with cycle threshold values greater than 30 and was therefore excluded from further analysis. Increasing PFA level, linearly ($P < 0.05$) decreased *IL-18* in spleen. There were no significant differences, in the expression of *TGF-β4*, *IL-2*, *IFN-γ* and *iNOS* in spleen between the experimental treatments (Table 7). Increasing PFA level did not affect the expression of *TGF-β4*, *IFN-γ*, *IL-10*, *IL-18*, *IL-2* and *iNOS* in the cecal tonsils (Table 7).

4. Discussion

In this study, beneficial effects of PFA administration were shown for FCR in the finisher phase and for the overall BWG. Improvements in BWG have been reported following supplementation of anise oil, cloves oil or mentha extracts (Ciftci et al., 2005;

Table 3
Broiler growth performance responses during starter, grower, finisher period and overall.

Item	Treatments ¹			SEM ²	Statistical analysis ³		
	PFA-0	PFA-100	PFA-150		ANOVA	Polynomial contrasts	
					<i>P</i> -value	<i>P</i> linear	<i>P</i> quadratic
Starter period (d 1 to 14)							
BWG, g	306	333	336	15.7	0.157	0.086	0.379
FI, g	431	457	465	21.2	0.285	0.136	0.636
FCR	1.41	1.38	1.39	0.087	0.947	0.823	0.816
Livability, %	98.7	100.0	96.0	1.72	0.100		
Grower period (d 15 to 28)							
BWG, g	836	856	867	39.8	0.739	0.451	0.906
FI, g	1,371	1,380	1,404	90.4	0.931	0.721	0.923
FCR	1.64	1.61	1.63	0.098	0.954	0.872	0.799
Livability, %	100.0	100.0	98.4	1.36	0.397		
Finisher period (d 29 to 42)							
BWG, g	1,006	1,122	1,064	47.4	0.089	0.251	0.055
FI, g	1,938	1,962	2,120	120.7	0.299	0.158	0.536
FCR	1.93 ^{ab}	1.75 ^a	1.99 ^b	0.078	0.007	0.400	0.009
Livability, %	100.0	100.0	100.0	0.00	–		
Overall (d 1 to 42)							
BWG, g	2,149 ^a	2,312 ^b	2,267 ^b	42.7	0.007	0.018	0.016
FI, g	3,740	3,799	3,989	179.5	0.381	0.191	0.681
FCR	1.74	1.65	1.76	0.069	0.254	0.778	0.109
Livability, %	98.67	100.00	94.66	2.309	0.099	–	–

PFA = phytogenic feed additive; BWG = body weight gain; FI = feed intake; FCR = feed conversion ratio.

^{a,b} Means with different superscripts within the same row differ significantly ($P \leq 0.05$). Data represent treatment means from $n = 5$ replicate floor pens per treatment.

¹ PFA-0 (basal diet with no PFA addition), PFA-100 (basal diet containing 100 mg PFA/kg diet), PFA-150 (basal diet containing 150 mg PFA/kg diet).

² Pooled standard error of means.

³ The statistical analysis tests the differences between treatments (ANOVA) and the linear and quadratic effect of PFA inclusion levels (polynomial contrasts).

Ertas et al., 2005; Hong et al., 2012). However, menthol and anethol may not increase BWG (Amad et al., 2011; Erhan et al., 2012; Ocak et al., 2008). The latter does not preclude improvements in FCR by phytogenic administration which may come as the result of a reduced FI at a largely unchanged BWG (Brenes and Roura, 2010). In the present study FI was not affected by PFA inclusion levels. Despite the fact that menthol, anethol and eugenol have been proposed as appetite stimulants (Frankic et al., 2009; Kumar et al., 2014), yet their effect on broiler chickens FI is controversial. In particular, FI was decreased (Erhan et al., 2012), not affected (Amad et al., 2011; Ciftci et al., 2005; Ertas et al., 2005) or increased (Khodhambashi Emami et al., 2012) with supplementation of menthol and/or anethol. Factors such as the phytogenic inclusion

Table 4
Total tract apparent digestibility (%) of nutrients and apparent metabolizable energy (AMEn) of 42-d-old broiler chickens.

Item	Treatments ¹			SEM ²	Statistical analysis ³		
	PFA-0	PFA-100	PFA-150		ANOVA		
					P-value	P _{linear}	P _{quadratic}
Dry matter	73.9 ^a	73.9 ^a	77.1 ^b	0.65	0.001	0.001	0.023
Crude protein	73.2	77.1	76.7	4.98	0.823	0.503	0.621
Ether extract	80.7	77.7	82.8	3.13	0.320	0.521	0.177
AMEn, MJ/kg	13.2 ^a	13.4 ^{ab}	13.9 ^b	0.21	0.013	0.005	0.455

PFA = phytogenic feed additive.

^{a,b} Means with different superscripts within the same row differ significantly ($P \leq 0.05$). Data represent treatment means from $n = 5$ replicate floor pens per treatment.¹ PFA-0 (basal diet with no PFA addition), PFA-100 (basal diet containing 100 mg PFA/kg diet), PFA-150 (basal diet containing 150 mg PFA/kg diet).² Pooled standard error of means.³ The statistical analysis tests the differences between treatments (ANOVA) and the linear and quadratic effect of PFA inclusion levels (Polynomial contrasts).**Table 5**
Blood plasma total antioxidant capacity (TAC) and concentration of protein, triglycerides, cholesterol and glucose of 42-d-old broiler chickens.

Item	Treatments ¹			SEM ²	Statistical Analysis ³		
	PFA-0	PFA-100	PFA-150		ANOVA		
					P-value	P _{linear}	P _{quadratic}
TAC, mmol TE/L ⁴	10.4 ^a	10.9 ^{ab}	13.0 ^b	1.14	0.048	0.031	0.461
Protein, g/dL	6.8	7.3	7.6	0.64	0.445	0.214	0.835
Triglycerides, mg/dL	33.2	33.6	28.6	4.62	0.549	0.445	0.437
Glucose, mg/dL	267.1	320.2	298.7	13.81	0.074	0.167	0.064
Cholesterol, mg/dL	118.0	105.4	138.3	22.30	0.073	0.153	0.068

PFA = phytogenic feed additive; TAC = total antioxidant capacity; TE = trolox equivalents.

^{a,b} Means with different superscripts within the same row differ significantly ($P \leq 0.05$). Data represent treatment means from $n = 10$ replicate floor pens per treatment.¹ PFA-0 (basal diet with no PFA addition), PFA-100 (basal diet containing 100 mg PFA/kg diet), PFA-150 (basal diet containing 150 mg PFA/kg diet).² Pooled standard error of means.³ The statistical analysis tests the differences between treatments (ANOVA) and the linear and quadratic effect of PFA inclusion levels (Polynomial contrasts).⁴ Total antioxidant capacity data were expressed as concentration of TE (mmol/L of plasma).**Table 6**
Meat total antioxidant capacity (TAC) of 42-d-old broiler chickens.¹

Item	Treatments ²			SEM ³	Statistical analysis ⁴		
	PFA-0	PFA-100	PFA-150		ANOVA		
					P-value	P _{linear}	P _{quadratic}
TAC, mmol TE/g ⁵	37.4	43.1	38.1	3.73	0.259	0.836	0.106
Breast	31.6	34.1	35.3	3.73	0.600	0.327	0.832
Thigh							

PFA = phytogenic feed additive; TAC = total antioxidant capacity; TE = trolox equivalents.

¹ Data represent treatment means from $n = 10$ replicate floor pens per treatment.² PFA-0 (basal diet with no PFA addition), PFA-100 (maize basal diet containing 100 mg PFA/kg diet), PFA-150 (maize basal diet containing 150 mg PFA/kg diet).³ Pooled standard error of means.⁴ The statistical analysis tests the differences between treatments (ANOVA) and the linear and quadratic effect of PFA inclusion levels (Polynomial contrasts).⁵ Total antioxidant capacity data were expressed as concentration of TE (mmol/g of meat).

levels, active components and composition of basal diet may explain the variability in broiler chickens responses seen (Hong et al., 2012; Ocak et al., 2008).

In this study PFA inclusion level at 100 mg/kg improved finisher phase FCR by 9.3% compared with treatment PFA-0. Moreover, due to the quadratic pattern of FCR response with increasing PFA level, finisher phase FCR in treatment PFA-150 was significantly worse than PFA-100 but did not differ from PFA-0. The necessity of optimizing phytogenic inclusion level with respect to growth performance responses has been documented by a number of recent studies (Mountzouris et al., 2011; Traesel et al., 2011). Generally, it has been shown that inclusion of phytogenic compounds in broiler chickens diets improved FCR (Fallah et al., 2013; Mountzouris et al., 2011; Traesel et al., 2011). More specifically, menthol sources (i.e., herbs or extracts) improved FCR (Erhan et al., 2012; Fallah et al.,

Table 7Relative gene expression of *TGF-β4*, *IL-18*, *IL-10*, *iNOS*, *IL-2* and *IFN-γ* in spleen and cecal tonsils of 42-d-old broiler chickens.¹

Item	Treatments ²			SEM ³	Statistical analysis ⁴		
	PFA-0	PFA-100	PFA-150		ANOVA		
					P-value	P _{linear}	P _{quadratic}
Spleen							
<i>TGF-β4</i> ⁵	0.96	0.77	0.88	0.135	0.391	0.575	0.217
<i>IL-18</i>	1.09	0.76	0.59	0.201	0.074	0.027	0.666
<i>iNOS</i>	1.61	0.87	0.51	0.579	0.193	0.081	0.714
<i>IL-2</i>	1.00	0.73	1.08	0.228	0.326	0.732	0.152
<i>IFN-γ</i>	1.93	0.95	1.04	0.584	0.307	0.152	0.311
Cecal tonsils							
<i>TGF-β4</i>	1.02	1.27	0.90	0.326	0.537	0.729	0.298
<i>IL-10</i>	1.43	0.97	0.66	0.378	0.170	0.066	0.830
<i>IL-18</i>	0.88	1.13	1.51	0.738	0.701	0.411	0.927
<i>iNOS</i>	1.47	0.90	1.60	0.632	0.523	0.848	0.271
<i>IL-2</i>	1.15	1.17	1.20	0.369	0.989	0.886	0.993
<i>IFN-γ</i>	0.96	1.27	1.22	0.332	0.481	0.456	0.539

TGF-β4 = transforming growth factor beta 4; *IL* = Interleukin; *iNOS* = inducible nitric oxide synthase; *IFN-γ* = interferon-γ.¹ Data represent treatment means from $n = 5$ broilers per treatment (i.e., one broiler per replicate floor pen).² PFA-0 (basal diet with no PFA addition), PFA-100 (maize basal diet containing 100 mg PFA/kg diet), PFA-150 (maize basal diet containing 150 mg PFA/kg diet).³ Pooled standard error of means.⁴ The statistical analysis tests the differences between treatments (ANOVA) and the linear and quadratic effect of PFA inclusion levels (polynomial contrasts).⁵ Relative expression ratios of target genes were calculated according to Pfaffl (2001) using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as a reference gene.

2013). In addition supplementation of anise oil (Ciftci et al., 2005; Soltan et al., 2008) and cloves oil (Ertas et al., 2005) improved FCR. Improvements in FCR could be linked with nutrient

digestibility improvements. Example cases include anise oil (Amad et al., 2011), mentha extracts (Hernandez et al., 2004; Khodhambashi Emami et al., 2012) as well as thyme and star anise essential oils (Cho et al., 2014). In this study, PFA administration improved TTAD of DM and AMEn, both increasing according to a strong linear motive with increasing PFA inclusion level. As a result, treatment PFA-150 displayed greater TTAD of DM and AMEn by 4.2% and 5.0% compared with treatment PFA-0, respectively. These could be considered in line with the linear pattern of increase in overall BWG noted. Similar PFA inclusion level-dependent effects on DM and AMEn were shown in an earlier experiment using a different PFA (Mountzouris et al., 2011). Generally, the phyto-genic-mediated improvements in nutrient and energy digestibility in broiler chickens may stem from phyto-genic properties to enhance digestion via the enhancement of digestive secretions (Brenes and Roura, 2010; Lee et al., 2004). The role of menthol anethol and eugenol as digestion stimulant factors has been highlighted by other studies (Frankic et al., 2009; Kumar et al., 2014). The supplementation of a PFA containing star anise as one of its lead active components improved nutrient digestibility (Amad et al., 2011). Mentha extracts in particular have been reported to improve digestibility (Hernandez et al., 2004; Khodhambashi Emami et al., 2012), via the control of microbial growth in the intestine (Erhan et al., 2012). Therefore, the improvement of the aforementioned growth performance parameters in the present study could partly be explained in the light of improvements in dietary AMEn and digestibility of dry matter.

Blood plasma TAC was significantly increased by PFA in a linear pattern. In particular, treatment PFA-150 showed greater plasma TAC by 20% compared with treatment PFA-0. These results are in good agreement with other studies in which the supplementation PFA resulted in improvements on the antioxidant status of broilers (Ciftci et al., 2010; Hoffman-Pennesi and Wu, 2010; Polat et al., 2011). In particular, the antioxidant properties of menthol, anethol and eugenol have been reported in many studies (Kamkar et al., 2010; Kim et al., 2013). Furthermore, the study of Petrovic et al. (2012) reported that cloves essential oil showed the strongest antioxidant activity among tested herb extracts. The improvement of plasma antioxidant capacity by dietary phyto-genics could be due to the active components and their phenolic group constituents which exhibit a strong antioxidant effect (Polat et al., 2011). Antioxidant status improvements may also result from the induction of antioxidant enzyme activities (Ciftci et al., 2010), however this needs to be specifically studied. In addition, homeostasis is maintained by prooxidant/antioxidant balance, and the imbalance in favor of the pro-oxidant system results in oxidative stress (Lin et al., 2004). For the reason above, the role of PFA may be critical for broiler chickens health due to its effects on the homeostatic mechanisms by affecting blood plasma TAC.

Regarding plasma protein, triglycerides, glucose and total cholesterol, there were no effects of PFA inclusion levels in agreement with previous studies using menthol anethol and eugenol sources (Hosseinzadeh and Farhoomand, 2014; Petrovic et al., 2012; Toghyani et al., 2010). However, addition of anethol oil reduced plasma cholesterol (Hong et al., 2012) and glucose concentration (Soltan et al., 2008). The aforementioned discrepancies could perhaps be explained due to PFA inclusion levels, its bioactive compounds, and overall feed composition (Toghyani et al., 2010).

The supplementation of PFA had no effect on the antioxidant status of broiler chicken breast and thigh meat. However, in other studies, dietary phyto-genic supplementation increased the antioxidant capacity of broiler chicken meat (Botsoglou et al., 2002; Goni et al., 2007). It is known that some essential oil components may get quickly absorbed after oral administration and metabolized and/or eliminated by the kidneys without significant

deposition in the body (Lee et al., 2004). The latter could thus explain the absence of changes in the antioxidant capacity of meat despite the increased blood plasma TAC in this study.

A palette of important factors known for their pro-inflammatory (*IL-18*, *iNOS*, *IL-2*, *IFN- γ*) and anti-inflammatory (*TGF- β 4*, *IL-10*) roles, were evaluated for their expression in spleen and cecal tonsils of broiler chickens. Increasing PFA level significantly decreased in a linear manner the pro-inflammatory cytokine *IL-18* in spleen. *Interleukin-18* is primarily produced by macrophages, targeting T helper type-1 (Th1) cells that subsequently secrete *IFN- γ* (Cox et al., 2010). Furthermore, *IL-18* belongs to the *IL-1* family of pro-inflammatory cytokines and is actively involved in an inflammatory response and the secretion of antibody under hyperthermia in animal models (Jang et al., 2014). In the present study, the fact that there was no effect of PFA inclusion level in any of the factors studied in broiler cecal tonsils could be justified by the absence of pathogenic challenge and the good zootechnical and veterinary practices followed during the experiment. Overall, the reduction of pro-inflammatory *IL-18* at systemic level could be considered concomitant with the improved performance in PFA supplemented broiler chickens. Essential oil supplementation may have both pro-inflammatory and anti-inflammatory roles (Miguel, 2010; Tsai et al., 2011). However, in a similar line with this study, anethol supplementation in non-parasite-infected (*Eimeria acervulina*) broiler chickens also led to a decrease in the expression of intestinal pro-inflammatory cytokines, compared with the non-supplemented control (Kim et al., 2013).

5. Conclusion

In conclusion, it was shown that inclusion of a PFA characterized by menthol, anethol and eugenol at 100 and 150 mg/kg diet improved growth performance, enhanced digestibility, increased plasma total antioxidant capacity and resulted in a pattern of reduced expression of pro-inflammatory cytokine *IL-18* at spleen. Overall, this study provides evidence for the beneficial role of PFA as a natural growth promoter with a potential to favor broiler chickens health. Further research is required to assess PFA effects on immune responses and gut microbiota indices under a variety of diet formulations combined or not with pathogenic challenge.

Acknowledgements

The authors would like to thank Mr. Kokkosis for his assistance during the experiment. The authors would like to thank Biomin Holding GmbH for provision of the PFA and acknowledge for funding (grant no 73.00.00.06.0012) this research work. Finally, we would like to dedicate this paper to the memory of our esteemed colleague Prof. George Theodoropoulos.

References

- Amad AA, Männer K, Wendler KR, Neumann K, Zentek J. Effects of a phyto-genic feed additive on growth performance and ileal nutrient digestibility in broiler chickens. *Poult Sci* 2011;90(12):2811–6.
- Botsoglou NA, Florou Paneri P, Christaki E, Fletouris DJ, Spais AB. Effect of dietary oregano essential oil on lipid oxidation in raw and cooked chicken during refrigerated storage. *Meat Sci* 2002;62(2):259–65.
- Brenes A, Roura E. Essential oils in poultry nutrition: main effects and modes of action. *Anim Feed Sci Tech* 2010;158(1–2):1–14.
- Cao G, Prior R. Measurement of oxygen radical absorbance capacity in biological samples. *Method Enzymol* 1999;299:50–62.
- Cho JH, Kim HJ, Kim IH. Effects of phyto-genic feed additive on growth performance, digestibility, blood metabolites, intestinal microbiota, meat color and relative organ weight after oral challenge with *Clostridium perfringens* in broilers. *Livest Sci* 2014;160:82–8.

- Ciftci M, Güler T, Dalkılıç B, Ertas NO. The effect of anise oil (*Pimpinella anisum L.*) on broiler performance. *Int J Poult Sci* 2005;4(11):851–5.
- Ciftci M, Simsek UG, Yuce A, Yilmaz O, Dalkılıç B. Effects of dietary antibiotic and cinnamon oil supplementation on antioxidant enzyme activities, cholesterol levels and fatty acid compositions of serum and meat in broiler chickens. *Acta Vet Brno* 2010;79(1):33–40.
- Cox CM, Summers LH, Kim S, McElroy AP, Bedford MR, Dalloul RA. Immune responses to dietary β -glucan in broiler chicks during an *Eimeria* challenge. *Poult Sci* 2010;89(12):2597–607.
- Erhan MK, Bolukbaşı SC, Urusan H. Biological activities of pennyroyal (*Mentha pulegium L.*) in broilers. *Livest Sci* 2012;146(2–3):189–92.
- Ertas ON, Talat Güler T, Ciftçi M, Dalkılıç B, Simsek UG. The Effect of an essential oil mix derived from oregano, clove and anise on broiler performance. *Int J Poult Sci* 2005;4(11):879–84.
- EC 43. Council Directive of 28 June 2007 laying down minimum rules for the protection of chickens kept for meat production. *OJEU L* 2007;182:19–28.
- EDQM. European Directorate for the Quality of Medicines and HealthCare 2.8. Methods in pharmacognosy, 2.8.12. Determination of essential oils in vegetable drugs. *European Pharmacopoeia* 5.0. European Directorate for the Quality of Medicines and HealthCare, Strasbourg, France 2005: 217–218.
- EU 63. Directive of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. *OJEU L* 2010;276: 33–79.
- Fallah R, Kiani A, Azarfaz A. Effect of artichoke leaves meal and mentha extract (*Mentha piperita*) on immune cells and blood biochemical parameters of broilers. *GV J* 2013;10(1):99–102.
- Frankic T, Voljc M, Salobir J, Rezar V. Use of herbs and spices and their extracts in animal nutrition. *Acta Agric Slov* 2009;94(2):95–102.
- Ghazalah AA, Ali AM. Rosemary leaves as a dietary supplement for growth in broiler chickens. *Int J Poult Sci* 2008;7(3):234–9.
- Goni I, Brenes A, Centeno C, Viveros A, Sauro Calixto F, Rebda A, et al. Effect of dietary grape pomace and vitamin E on growth performance, nutrient digestibility and susceptibility to meat lipid oxidation in chickens. *Poult Sci* 2007;86(3):508–16.
- Hernandez F, Madrid J, Garcia V, Orengo J, Megias MD. Influence of two plant extracts on broiler performance digestibilities and digestive organ size. *Poult Sci* 2004;83(2):169–74.
- Hoffman-Pennesi D, Wu C. The effect of thymol and thyme oil feed supplementation on growth performance, serum antioxidant levels, and cecal Salmonella population in broilers. *J Appl Poult Res* 2010;19(4):432–43.
- Hong JC, Steiner T, Aufi A, Lien TF. Effects of supplemental essential oil on growth performance, lipid metabolites and immunity, intestinal characteristics, microbiota and carcass traits in broilers. *Livest Sci* 2012;144(3):253–62.
- Hosseinzadeh Z, Farhoomand P. The effects of *Artemisia dracunculoides* powders different levels on blood parameters and internal organs weight broiler chickens. *Int J Adv Biol Biom Res* 2014;2(3):661–8.
- Jang IS, Ko YH, Moon YS, Sohn SH. Effects of vitamin C or E on the pro-inflammatory cytokines, heat shock protein 70 and antioxidant status in broiler chicks under summer conditions. *Asian-Australas J Anim Sci* 2014;27(5):749–56.
- Kamkar A, Javan AJ, Asadi F, Kamalinejad M. The antioxidative effect of Iranian *Mentha pulegium* extracts and essential oil in sunflower oil. *Food Chem Toxicol* 2010;48(7):1796–800.
- Kim DK, Lillehoj HS, Lee SH, Jang SI, Park MS. Immune effects of dietary anethole on *Eimeria acervulina* infection. *Poult Sci* 2013;92(10):2625–34.
- Khodhambashi Emami N, Samie A, Rahmani HR, Ruiz-Feria CA. The effect of peppermint essential oil and fructooligosaccharides as alternatives to virginiamycin, on growth performance, digestibility, gut morphology and immune response of male broilers. *Anim Feed Sci Tech* 2012;175(1–2):57–64.
- Kumar M, Kumar V, Roy D, Kushwaha R, Vaiswani S. Application of herbal feed additives in animal nutrition – a review. *Int J Livest Res* 2014;4(9):1–8.
- Lee KW, Everts H, Beynen AC. Essential oils in broiler nutrition. *Int J Poult Sci* 2004;3(12):738–52.
- Lin H, Decuyper E, Buyse J. Oxidative stress induced by corticosterone administration in broiler chickens (*Gallus gallus domesticus*). *Comp Biochem Physiol* 2004;139(4):737–44.
- Lu H, Adedokun SA, Adeola L, Ajuwon KM. Anti-inflammatory effects of non-antibiotic alternatives in *Coccidia* challenged broiler chickens. *J Poult Sci* 2014;51(1):14–21.
- Malayoglu Basmacıoğlu H, Baysal S, Misirlioglu Z, Polat M, Yilmaz H, Turan N. Effects of oregano essential oil with or without feed enzymes on growth performance, digestive enzyme, nutrient digestibility, lipid metabolism and immune response of broilers fed on wheat–soybean meal diets. *Br Poult Sci* 2010;51(1):67–80.
- Marshall OJ. PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. *BMC J* 2004;20(15):2471–2.
- Miguel MC. Antioxidant and anti-inflammatory activities of essential oils: a short review. *Molecules* 2010;15(12):9252–87.
- Mountzouris KC, Paraskeuas V, Tsirtsikos P, Palamidi I, Steiner T, Schatzmayr G, et al. Assessment of a phyto-genic feed additive effect on broiler growth performance, nutrient digestibility and caecal microflora composition. *Anim Feed Sci Tech* 2011;168(3–4):223–31.
- Ocak N, Erener G, Sungu M, Altop A, Ozmen A. Performance of broilers fed diets supplemented with dry peppermint (*Mentha piperita L.*) or thyme (*Thymus vulgaris L.*) leaves as growth promoter source. *Czech J Anim Sci* 2008;53(4): 169–75.
- Petrovic V, Marcincak S, Popelka P, Simkova J, Martonova M, Buleca J, et al. The effect of supplementation of clove and agrimony or clove and lemon balm on growth performance, antioxidant status and selected indices of lipid profile of broiler chickens. *J Anim Physiol Anim Nutr* 2012;96(6):970–7.
- Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001;29(9):900.
- Polat U, Yesilbag D, Eren M. Serum biochemical profile of broiler chickens fed diets containing rosemary and rosemary volatile oil. *J Biol Environ Sci* 2011;5(13): 23–30.
- Prior RL, Hoang H, Gu L, Wu X, Bacchiocca M, Howard L, et al. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORAC)) of plasma and other biological and food samples. *J Agric Food Chem* 2003;51(11):3273–9.
- Safamehr A, Mirahmadi M, Nobakht A. Effect of nettle (*Urtica dioica*) medicinal plant on growth performance, immune responses, and serum biochemical parameters of broiler chickens. *Intl Res J Appl Basic Sci* 2012;3(4):721–8.
- Soltan MA, Shewitta RS, El-Katcha MI. Effects of diary anise seeds supplementation on growth performance, immune response, carcass traits and some blood parameters of broiler chickens. *Int J Poult Sci* 2008;7(11):1078–88.
- Toghyani M, Gheisari A, Ghalamkari G, Mohammadrezaei M. Growth performance, serum biochemistry and blood hematology of broiler chicks fed different levels of black seed (*Nigella sativa*) and peppermint (*Mentha piperita*). *Livest Sci* 2010;129(1–3):173–8.
- Traesel CK, Wolkmer P, Schmidt C, Silva CB, Paim FC, Rosa AP, et al. Serum biochemical profile and performance of broiler chickens fed diets containing essential oils and pepper. *Comp Clin Pathol* 2011;20(5):453–60.
- Tsai ML, Lin CC, Lin WC, Yang CH. Antimicrobial, antioxidant and anti-inflammatory activities of essential oils, from five selected herbs. *Biosci Biotechnol Biochem* 2011;75(10):1977–83.