# Gene expression of heat shock protein 70 and antioxidant enzymes, oxidative status, and meat oxidative stability of cyclically heat-challenged finishing broilers fed *Origanum compactum* and *Curcuma xanthorrhiza* essential oils

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**ABSTRACT** Heat stress in poultry is a serious problem in many countries and has been associated with oxidative stress. Hence, nutritional interventions with antioxidants might be beneficial. Therefore, the effects of dietary *Curcuma xanthorrhiza* (CX) and *Origanum compactum* (OC) essential oils on mRNA levels of heat shock protein 70 and antioxidant enzymes, oxidative status, and meat oxidative stability of heat-challenged broilers were studied. Starting on d 25 of age, a control diet and 4 diets containing 200 or 400 mg/kg feed of CX or OC (CX200, CX400, OC200, OC400 diets) were fed to 3 pen replicates of 20 Ross 308 chickens each. From d 28 of age on, the temperature was increased from 22 to 34°C with 50% RH for 5 h daily during 2 wk. Dietary CX or OC did not affect zootechnical performance. Feeding CX400 and both levels of OC increased the a* value in stored breast meat ($P < 0.05$), and OC diets tended to decrease the thiobarbituric acid reactive substances values in fresh breast meat ($P = 0.061$). Compared with control, at d 31, feeding CX400 and OC400 reduced mRNA levels of heat shock protein 70 and increased mRNA levels of catalase in kidney and liver ($P < 0.05$). The mRNA levels of superoxide dismutase were increased at d 31 on the OC400 diet in kidney and on the CX400 diet in heart ($P < 0.05$). In heart, at d 31, both dietary levels of CX and OC200 resulted in higher glutathione peroxidase activity ($P < 0.05$). Feeding CX400 increased superoxide dismutase activity in liver, kidney, and heart at d 31 ($P < 0.05$). Catalase activity was increased in the CX200 and OC400 groups at d 42 ($P < 0.05$). Feeding CX at both levels and OC200 decreased plasma malondialdehyde concentrations at d 42 ($P < 0.05$). In conclusion, dietary essential oils rich in simple phenolic compounds offer potential for improving the antioxidant defense against heat stress–induced changes.

**Key words:** broiler, heat shock protein, high temperature, meat quality, oxidative status

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## INTRODUCTION

Broilers can properly perform if they are not subjected to suboptimal environmental conditions. It has been well documented that exposing broilers to continuously high temperatures, especially during the finisher phase, leads to chronic heat stress and may reduce performances (Sahin et al., 2003; Ahmad et al., 2006). Ambient temperatures above the thermoneutral zone of birds have been associated with oxidative stress (Lin et al., 2006), as reflected in elevated lipid peroxidation products in blood and tissues, causing protein and DNA oxidation (Floyd and Carney, 1992). Besides impairing the oxidative status in vivo, it is also documented that elevated temperatures may result in a higher incidence of pale, soft, exudative meat, which is less color stable and prone to a shorter shelf life (Tankson et al., 2001).

Exposing broilers to a heat shock increases the activity of antioxidant enzymes such as superoxide dismutase (**SOD**) and glutathione peroxidase (**GSH-Px**) in the short term to protect cells from the negative consequences of excessive generation of free radicals (Lin et al., 2006). In contrast, after a long-term heat challenge, there might be a decrease in their activity in some tissues because of the development of lesions in cells and increased excretion of minerals such as Zn, Cu, and Se that act as cofactors for antioxidant enzymes, and vitamins such as vitamin C and E (Sahin and Kucuk, 2003). Hence, the activities of the antioxidant enzymes


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might be insufficient to counteract the initiation and propagation of oxidative damage, and using antioxidants in the diet can be considered as a mechanism for cells to increase their protection against damage induced by free radicals in the case of long-term heat challenge.

Typically an upregulation of heat shock proteins is observed upon heat stress (Craig and Gross, 1991). Heat shock proteins are a group of proteins produced in all cells and tissues to protect against harmful effects of environmental stressors, especially elevated temperatures. The most important member of these proteins with respect to heat stress is heat shock protein 70 kDa (**HSP70**; Yahav et al., 1997). The transcription factors AP-1 and NFκB involved in the stimulation of antioxidant enzymes and heat shock protein (**HSP**) transcription factor (HSF-1) are sensitive to the redox status of the cell (Khassaf et al., 2003). Hence, antioxidants supplied by the diet may interact with the activity of antioxidant enzymes by maintaining the reducing state of cells and thereby interfering with the activation of these transcription factors.

Numerous studies have been performed in poultry on the effects of various dietary antioxidants on the bird oxidative status and the oxidative stability of their meat postmortem, e.g., reviewed by Brenes and Roura (2010) for essential oils, Bou et al. (2009), and Surai (2014). Synthetic antioxidants and some other antioxidant additives are under significant public scrutiny and have limitations in their use, e.g., their inclusion levels in all feeding stuffs of all species of meat animals are regulated in EU countries. Therefore, there is currently a great interest in exploring the effects of natural compounds.

*Origanum compactum* (**OC**), a member of the genus *Origanum* belonging to the mint family (*Lamiaceae*), is native to warm-temperate and southwestern Eurasia and the Mediterranean region. *Curcuma xanthorrhiza* (**CX**, commonly known as temulawak or Javanese turmeric in Indonesia) is native to tropical South-Asia and is found both in the wild and cultivated in Indonesia. The essentials oils of OC and CX contain high amounts of simple phenols, and their antioxidant activity is well documented (Sinurat et al., 2009; Luna et al., 2010). The antioxidant activity of OC is mainly attributed to its main components carvacrol and thymol, and for CX mainly refers to ar-curcumene, β-curcumene, and xanthorrizhol. Carvacrol, thymol, and xanthorrizol are simple phenolic compounds bearing different aliphatic side chains on the aromatic ring. In previous works, we showed that using plant extracts rich in phenolic compounds could positively affect broiler gut health and metabolic parameters when reared under high ambient temperatures (Akbarian et al., 2013a,b). The positive effects of oregano, curcuma, or their essential oils on the antioxidant status of poultry have already been reported (Sinurat et al., 2009; Avila-Ramos et al., 2012), but to the best of our knowledge, no information is available concerning the potential effects of these products in heat-challenged broilers. Thus, it was hypothesized that these 2 essential oils may maintain the reducing state of the cells through improving the antioxidant capacity of broilers when subjected to daily increased temperatures. Therefore, this study was set up to assess the effects of OC and CX on performance, oxidative status, and meat quality of broilers reared under hot conditions.

## MATERIALS AND METHODS

The experiment was approved by the Ethics Committee of Ghent University (Belgium) for the humane care and use of animals in research (number EC2011–194).

### Essential Oils

The CX essential oil was obtained from PT.Phytochemindo Reksa (Bogor, Indonesia). According to the compositional data provided by the supplier, the main bioactive compounds were ar-curcumene (11.4%), β-curcumene (8.5%), and xanthorrhizol (hydroxy-ar-curcumene; 28.0%). The OC essential oil was provided by Pranarôm International SA (Ghislenghien, Belgium). According to the compositional data provided by the supplier, the main bioactive compounds were carvacrol (44.9%) and thymol (16.4%).

### Chicks and Housing

Three hundred 1-d-old broiler chicks (Ross 308; mixed sexes) were purchased from a commercial hatchery (Vervaeke-Belavi, Tielt, Belgium). At d 0, chicks were allocated randomly to 15 pens (5 treatments × 3 replicate pens per treatment) with 20 chicks each representing an initial density of 14.8 birds per square meter. The floor was covered with fresh wood shavings, and no additional filling or cleaning of the floor was executed during the trial. Heat was provided with heating lamps and automatically stirred heating elements maintaining a stable temperature of 32 to 28°C, 28 to 25°C, and 25 to 22°C during the first 10 d, from d 11 until 17, and from d 18 until 28, respectively. The lighting program was 23L:1D during the entire period. The broilers were vaccinated against Newcastle disease (spray) and infectious bronchitis (spray) at the hatchery. At d 16, the vaccination against Newcastle disease was repeated (Nobilis ND Clone 30, Intervet, Belgium NV, Mechelen, Belgium), and the chicks were vaccinated against Gumboro (Nobilis Gumboro D78, Intervet). Twice daily birds and housing facilities were inspected for the general health status, constant feed and water supply, temperature and ventilation, dead birds, and unexpected events.

### Diets and Heat Challenge

The chicks were raised up to 25 d of age on a basal diet. The basal diet was formulated to correspond to

nutrient requirements that were equal to or slightly lower than those outlined by the Ross 308 broiler management guide (Aviagen, 2011) but adapted to the Iranian situation. Ingredients and chemical and analyzed compositions of the basal diet are shown in Table 1. From d 25 until the end of the trial (d 42), 5 experimental diets were fed to 3 replicate pens in a completely randomized design. These experimental diets were prepared through including different levels of the essential oils into the basal diet. The 5 dietary treatments were as follows: control diet (**CON**; basal diet, without essential oils), CON + 200 mg/kg CX (**CX200**), CON + 400 mg/kg CX (**CX400**), CON + 200 mg/kg OC (**OC200**), and CON + 400 mg/kg OC (**OC400**). The essential oils were first mixed intensively with the associated corn oil and then gradually added to the basal diet. Feed and water were offered ad libitum. To accustom the chickens to the experimental diets, a 3-d adaptation period was included before imposing the heat challenge. Starting on d 28, a cyclic chronic heat challenge model (34°C–22°C–34°C) was applied. The basal temperature was 22°C. Between 0800 and 1000 h, the temperature was gradually increased to 34°C, and this high temperature was then maintained for 5 h. Afterward, the temperature was decreased to the basal level of 22°C within 2 h (by 1700 h) and then maintained at this level for the rest of the day. Air humidity was kept at 50 to 60% during the experimental period.

**Table 1.** Ingredients and calculated and analyzed compositions of the basal diet

| Item | Amount in basal diet |
|---|---|
| Ingredient (%) | |
| Corn | 56.19 |
| Soybean meal 48HP | 36.07 |
| Corn oil | 4.00 |
| Limestone | 1.12 |
| Dicalcium phosphate | 1.47 |
| Common salt | 0.43 |
| L-Lysine HCl | 0.10 |
| DL-Methionine | 0.12 |
| Vitamin and mineral premix[1] | 0.50 |
| Calculated composition (% unless otherwise stated) | |
| ME (kcal/kg) | 3,000 |
| CP | 20.10 |
| Available phosphorus | 0.43 |
| Lysine | 1.05 |
| Methionine | 0.40 |
| Met + Cys | 0.80 |
| Analyzed composition (%) | |
| DM | 88.8 |
| Crude ash | 5.61 |
| Crude fat | 6.84 |
| CP | 20.4 |
| Crude fiber | 5.18 |
| Phosphorus | 0.69 |

[1]Vitamin and mineral premix supplied the following per kilogram of diet: vitamin A (from vitamin A acetate), 10,000 IU; vitamin $D_3$, 9,790 IU; vitamin E (DL-α-tocopheryl acetate), 30 IU; vitamin $B_{12}$, 20 μg; riboflavin, 4.4 mg; calcium pantothenate, 40 mg; niacin, 22 mg; choline, 840 mg; biotin, 30 μg; thiamin, 4 mg; zinc sulfate, 60 mg; copper sulfate, 100 μg; selenium (sodium selenate), 0.2 mg; iodine, 1 mg; manganese oxide, 60 mg.

## Bird Performance

Average pen weight was recorded at d 25, 31, and 42. Feed intake was recorded for each period (d 25 to 31 and d 31 to 42) and for the entire experimental period (d 25 to 42). Broiler performance at pen level (daily weight gain, g/d; daily feed intake, g/d; and feed:gain, g/g) was calculated per period. Mortality was recorded for each pen and per period. Corrections for average daily feed intake, when calculating bird performances, were done using the number of "broilerdays" (number of broilers × days alive). Broilerdays for the birds that died were calculated counting the number of days alive minus one, due to the unknown moment of the death and the expected reduction in feed intake of a sick bird.

## Sampling

On d 31 (3 d after starting the heat challenge), 4 male birds per pen (12 per treatment) were sampled at random between 1100 and 1400 h. Blood samples (5 mL) were taken with a 23-gauge needle from the vena brachialis and collected in 2 tubes. The first tube containing EDTA was centrifuged (3,000 × *g*, 15 min, 4°C) to obtain plasma for determination of malondialdehyde (**MDA**), and GSH-Px and was stored at −20°C pending analysis. The second tube containing heparin together with Dulbecco's PBS was used to obtain red blood cells (**RBC**) for glutathione redox quantification, i.e., for the determination of reduced (**GSH**) and oxidized (**GSSG**) glutathione. Terminal anesthesia was induced by sodium pentobarbital (250 mg/kg of BW) in the vena brachialis before exsanguination. Then heart, liver, and kidneys were carefully excised. Two subsamples of each tissue were snap frozen in liquid $N_2$ and stored at −80°C for determination of *HSP70* and antioxidant enzyme gene expression and for measuring the activity of these enzymes [SOD, GSH-Px, and catalase (**CAT**)]. Six samples (out of 12 birds that had already been sampled) of each tissue were taken at random for pursuing the analyses of gene expression and activity of antioxidant enzymes in heart, liver, and kidney.

On d 42 (2 wk after starting the heat challenge, end of trial), another 4 male birds per pen were sampled at random (12 per treatment). The blood and tissue sampling procedure was identical as described above. In addition, breast and thigh muscle were sampled for determination of the antioxidant enzymes and for assessing lipid and color oxidative stability after simulated retail display, on fresh meat and after long-term frozen storage. Therefore, pH of the right breast muscle was immediately determined after slaughter. The eviscerated carcasses were subsequently chilled at 4°C. The pH and color of the right breast muscle were determined 24 h postmortem. Subsamples of the right breast and thigh were snap frozen in liquid $N_2$ and stored at

−80°C for determination of GSH-Px and SOD activity. Another set of subsamples of the right breast and thigh were subjected to simulated retail display, whereas subsamples of the left breast and thigh were vacuum packed and stored at −20°C for 7 mo before being subjected, after thawing, to simulated retail display. For the simulation of retail display, samples were wrapped in oxygen-permeable foil and continuously displayed at 4°C under fluorescent light (approximately 1,200 lx) for 10 d.

### Meat Quality and Oxidative Stability

At 0 and 24 h postmortem, pH was measured using a portable pH meter (Knick Portamess 654, Knick Elektronische Messgeräte GmbH, Berlin, Germany) with Schott N5800A electrode (Schott Instruments, Mainz, Germany). The pH meter was calibrated by measuring buffer solutions (pH = 4 and pH = 7). Color L*, a* and b* values were determined with a HunterLab Miniscan color meter (D65 light source, 10° standard observer, 45°/0° geometry, 1-inch light surface, white standard, Hunterlab Associates Laboratory Inc., Reston, VA). Lipid oxidation was assessed spectrophotometrically at the wavelength of 532 nm by the thiobarbituric acid reactive substances method as described by Tarladgis et al. (1960) and expressed as nanograms of MDA per gram of meat.

### Plasma Oxidative Status and Glutathione in Erythrocytes

The GSH-Px activity and MDA concentration in plasma were determined according to Michiels et al. (2012).

Glutathione redox status in erythrocytes (RBC; ratio between GSH and GSSG) was quantified as described by Degroote et al. (2012). Briefly, hemolyzed RBC were homogenized, and an acid extract was made. This was followed by a derivatization procedure including the reaction of iodoacetic acid with thiols to form S-carboxymethyl derivatives and the formation of chromophores of the primary amines with Sanger's reagent, 2,4-dinitrofluorobenzene. Finally, derivatized thiols were separated on an EC250/4.6 Nucleosil 120–7 $NH_2$ column (aminopropyl column; Machery-Nagel, Düren, Germany) protected by the same $NH_2$ guard column (CC8/4). The concentrations of GSH and GSSG were determined by HPLC (Agilent Technologies, 1200 series, Degasser, Germany).

### Tissues Antioxidant Enzymes Activities

Activities of SOD, GSH-Px, and CAT were determined in liver, heart, and kidney samples taken at d 31 and 42 and on stored breast and thigh (not CAT), which were taken only at d 42. After thawing, all samples were kept on ice during the procedure. A 2-g sample of liver, heart, and kidney and 5-g sample of fresh breast and thigh muscles were homogenized in 10 mL of 0.05 *M* phosphate buffer (pH = 7.0) and centrifuged at 4°C for 20 min at 7,000 × *g*. The supernatant fraction was filtered through glass wool before determining enzyme activities. The CAT activity was determined according to the method of Aebi (1983). One unit of CAT activity was defined as the amount of extract needed to decompose 1 μmol of $H_2O_2$ per min at room temperature. The activity of GSH-Px was determined by measuring the oxidation of NADPH according to Hernandez et al. (2004). One unit of GSH-Px activity was defined as the amount of extract needed to oxidize 1 μmol of NADPH per min at 25°C. The SOD activity assay was performed as described by Marklund and Marklund (1974) by measuring the inhibition of pyrogallol autoxidation. One unit of enzyme activity was defined as the amount of extract needed to inhibit the rate of oxidation by the control (no SOD) by 50%. In the case of kidney, no activity of CAT was detected.

### HSP70 and Antioxidant Enzymes mRNA Levels in Heart, Liver, and Kidney

Quantitative real-time PCR was performed to determine the levels of inducible *HSP70* and antioxidant enzymes mRNA in different tissues. Tissues of heart, liver, and kidney were disrupted and homogenized. Tissues were dissolved in TRIZOL reagent for total RNA extraction according to standard instructions. The total RNA concentration was quantified by Nanodrop photometer (ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE). Ratios of absorption (260/280 nm) of all preparations were in the range of 1.9 and 2.1. To verify the integrity of ribosomal RNA bands, 5 μL of each obtained RNA was separated by electrophoresis on agarose gels under denaturing conditions.

After extraction of total RNA, AMV reverse transcriptase (Promega, Madison, WI) was used for reverse transcription. Reverse transcription was performed according to the manufacturer's instruction.

The pooled sample, made by mixing equal quantities of total RNA from all samples, was used for optimizing PCR conditions and tailoring standard curves for the target gene. Two microliters of 10-fold dilution reverse-transcription products was used for PCR in a final volume of 25 μL containing 0.4 to 0.8 μ*M* primers and 12.5 μL of QuantiTect SYBR Green master mix (Cat. no. 204143+204163, Qiagen, Benelux B.V, KJ Venlo, the Netherlands). Real-time PCR was performed in StepOnePlus Real-Time PCR System (AB Applied Biosystems, Foster City, CA). The PCR cycling program was as follows: 1 min at 95°C then followed by 42 cycles of 1 min at 95°C, 20 s at 60°C. Melting curves were executed to ensure a single specific PCR product for

**Table 2.** Nucleotide sequences of specific primers

| Target gene[1] | Product length (bp) | Primer sequence (F: forward, R: reverse) | Gene bank accession |
|---|---|---|---|
| *HSP70* | 145 | F: ATGCTAATGGTATCCTGAACG<br>R: TCCTCTGCTTTGTATTTCTCTG | NM_001006685.1 |
| *B2M* | 1,038 | F: ACCAAGAACGTCCTCAACTGC<br>R: CGGGATCCCACTTGTAGACC | Z48921 |
| *CAT* | 245 | F: ACCAAGTACTGCAAGGCGAA<br>R: TGAGGGTTCCTCTTCTGGCT | NM_001031215.1 |
| *GSH-Px* | 141 | F: TTGTAAACATCAGGGGCAAA<br>R: ATGGGCCAAGATCTTTCTGTAA | NM_001163245.1 |
| *SOD* | 122 | F: AGGGGGTCATCCACTTCC<br>R: CCCATTTGTGTTGTCTCCAA | NM_205064.1 |
| *TBP* | 196 | F: TTTAGCCCGATGATGCCGTATG<br>R: CTGTGGTAAGAGTCTGTGAGTGG | NM_205103 |

[1]*HSP70* = heat shock protein 70; *B2M* = β-2-microglobulin; *CAT* = catalase; *GSH-Px* = glutathione peroxidase; *SOD* = superoxide dismutase; *TBP* = TATA box binding protein.

each gene. Numbers of controls were set to monitor the possible genomic and environmental contamination of DNA both at the stages of reverse transcription and PCR. The special forward and reverse primers were designed using Primer Express Software (Primer Express Software version 3.0, Leusden, the Netherlands) for *HSP70* and antioxidant enzymes *CAT*, *SOD*, and *GSH-Px* amplification. The TATA box binding protein and β-2-microglobulin were used as reference genes for normalization purposes. Primer sequences and information are listed in Table 2. The $2^{-\Delta\Delta Ct}$ method was used to analyze the real-time PCR data relative to the average value of control.

### Statistical Analysis

Statistical analysis was done using the general linear models procedure, followed by Tukey's multiple comparison test (version 9.1, SAS Institute Inc., Cary, NC). For performance and meat quality traits, a model with the fixed effect of diet was used, and orthogonal contrasts were applied to test the effect of the essential oil across doses. The activity of antioxidant enzymes in tissues and oxidative status of blood were analyzed using a model with the fixed effects of diet, day of sampling, and diet × day of sampling. All statements of significance are based on probability $P < 0.05$.

## RESULTS

### Bird Performance

Mortality data were not subjected to statistical analysis because just one case of mortality in the control treatment was observed throughout the trial. Dietary treatments did not affect daily weight gain, daily feed intake, and feed:gain of chickens (data not given) during d 25 to 31, d 31 to 42, and the total period of feeding the experimental diets (d 25 to 42; $P > 0.05$). However, there was a tendency for improved feed:gain in those chicks fed with OC, irrespective of dose compared with the control group during d 31 to 42 of age and the total experimental period (d 25 to 42; 1.68 vs. 2.10 for OC and CON, respectively, in period d 31 to 42, $P = 0.081$; 1.56 vs. 1.80 for OC and CON, respectively, in period d 25 to 42, $P = 0.065$).

### Meat Oxidative Stability and Antioxidant Enzymes

With regard to breast muscle characteristics, there were no differences for pH and color variables ($P > 0.05$), except for the a* value, for which the birds fed the control diet had lower values than those fed OC400 after storing the breast meat for 7 mo and then exposing for 10 d to simulated retail display ($P < 0.05$; data for L* and b* color values are not shown; Table 3). The a* values for fresh breast and thigh were numerically higher on the supplemented diets compared with the control diet, as was also the case for stored thigh ($P > 0.05$). Regarding thiobarbituric acid reactive substances values in fresh breast, the orthogonal contrast of OC versus CON was significant ($P = 0.005$), indicating a reduction in lipid peroxidation. Thiobarbituric acid reactive substances values increased substantially upon storage at −20°C for 7 mo, in particular for thigh.

Activity of SOD in breast and thigh muscle was not affected by treatment ($P > 0.05$; Table 3). In contrast, supplementation of the diets with essential oils increased GSP-Px activity compared with the CON diet, both in breast and thigh, with the difference being significant for several means ($P < 0.05$).

### Plasma and Erythrocyte Oxidative Status

In general, dietary effects were most pronounced on d 42 (Table 4). Only feeding CX400 decreased MDA at d 31 of age compared with the CON, whereas supplementing CX at both levels and OC200 decreased MDA at d 42 of age ($P < 0.05$). None of the treatments exerted an effect on plasma GSH-Px activity at both ages. Interestingly, whereas GSH-Px activity in plasma increased with age duration of and heat challenge, MDA concentrations declined (both $P = 0.001$).

**Table 3.** Effect of *Curcuma xanthorrhiza* and *Origanum compactum* essential oils at 200 and 400 mg/kg of diet on fresh and stored meat oxidative stability and activities of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) in broilers subjected to heat challenge (n = 12)

| | | *C. xanthorrhiza* | | *O. compactum* | | | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|
| Item | Control | 200 | 400 | 200 | 400 | Pooled SEM | Model | CX[1] | OC[2] |
| Fresh meat | | | | | | | | | |
| Breast pH 0 h postmortem | 6.71 | 6.63 | 6.71 | 6.64 | 6.56 | 0.052 | 0.124 | 0.461 | 0.053 |
| Breast pH 24 h postmortem | 5.68 | 5.69 | 5.78 | 5.70 | 5.70 | 0.031 | 0.095 | 0.101 | 0.464 |
| Breast a*[3] | 5.42 | 5.92 | 5.33 | 4.75 | 5.17 | 0.328 | 0.093 | 0.499 | 0.229 |
| Thigh a*[3] | 7.00 | 7.33 | 7.83 | 7.83 | 7.67 | 0.239 | 0.190 | 0.107 | 0.035 |
| Breast TBARS[4] (ng of MDA/g)[5] | 258 | 224 | 231 | 180 | 186 | 29.9 | 0.061 | 0.234 | 0.005 |
| Thigh TBARS[4] (ng of MDA/g) | 221 | 223 | 238 | 186 | 206 | 26.7 | 0.712 | 0.779 | 0.451 |
| Meat after 7 mo of storage at −20°C | | | | | | | | | |
| Breast a*[3] | 5.41[c] | 5.60[bc] | 6.56[ab] | 6.45[ab] | 6.85[a] | 0.351 | 0.017 | 0.120 | 0.005 |
| Thigh a*[3] | 8.10 | 8.33 | 8.42 | 8.66 | 8.27 | 0.272 | 0.678 | 0.394 | 0.263 |
| Breast TBARS[4] (ng of MDA/g) | 356 | 361 | 371 | 330 | 311 | 46.1 | 0.885 | 0.855 | 0.535 |
| Thigh TBARS[4] (ng of MDA/g) | 1,432 | 1,176 | 1,292 | 1,473 | 1,272 | 169.2 | 0.722 | 0.345 | 0.776 |
| Antioxidant enzymes activities | | | | | | | | | |
| Breast GSH-Px (U/g) | 178[b] | 222[ab] | 294[a] | 264[ab] | 283[a] | 53.3 | 0.005 | 0.001 | 0.006 |
| Breast SOD (U/g) | 33.9 | 38.2 | 38.5 | 35.9 | 37.1 | 8.32 | 0.873 | 0.526 | 0.295 |
| Thigh GSH-Px (U/g) | 402[b] | 457[ab] | 497[a] | 489[ab] | 446[ab] | 29.9 | 0.029 | 0.018 | 0.008 |
| Thigh SOD (U/g) | 39.2 | 39.3 | 39.1 | 39.4 | 39.6 | 0.22 | 0.289 | 0.157 | 0.868 |

[a–c]Means within a row lacking a common superscript differ ($P < 0.05$).

[1]Orthogonal contrast of *C. xanthorrhiza* versus control.

[2]Orthogonal contrast of *O. compactum* versus control.

[3]Redness, determined after 10 d of simulated retail display.

[4]Thiobarbituric acid reactive substances, determined after 10 d of simulated retail display.

[5]MDA = malondialdehyde.

**Table 4.** Effect of *Curcuma xanthorrhiza* and *Origanum compactum* essential oils at 200 and 400 mg/kg of diet on plasma oxidative status and glutathione in erythrocytes of broilers at 31 and 42 d of age subjected to heat challenge (n = 12)

| | 31 d of age (3 d after heat challenge) | | | | | 42 d of age (2 wk after heat challenge) | | | | | *P*-value | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *C. xanthorrhiza* | | *O. compactum* | | | *C. xanthorrhiza* | | *O. compactum* | | | | | |
| Item | Control | 200 | 400 | 200 | 400 | Control | 200 | 400 | 200 | 400 | Pooled SEM | Diet | Day | Diet × day |
| Plasma | | | | | | | | | | | | | | |
| MDA[1] (nmol/mL) | 14.7[a] | 14.1[a] | 12.5[b] | 13.4[ab] | 13.5[ab] | 12.9[a] | 11.9[b] | 11.7[b] | 11.7[b] | 12.3[ab] | 0.28 | 0.019 | 0.001 | 0.639 |
| GSH-Px[2] (U/L) | 995 | 921 | 934 | 946 | 842 | 1,318 | 1,123 | 1,240 | 1,311 | 1,196 | 50.3 | 0.303 | 0.001 | 0.859 |
| Red blood cells | | | | | | | | | | | | | | |
| GSH[3] (nmol/mL) | 0.45 | 0.34 | 0.43 | 0.54 | 0.53 | 0.57[b] | 0.78[a] | 0.84[a] | 0.78[a] | 0.75[a] | 0.052 | 0.001 | 0.003 | 0.002 |
| GSH/GSSG[4] | 10.4 | 9.7 | 12.4 | 11.6 | 11.3 | 7.54 | 9.59 | 7.05 | 6.54 | 9.63 | 0.066 | 0.647 | 0.906 | 0.576 |

[a,b]Means within a row and within day of sampling lacking a common superscript are significantly different ($P < 0.05$).
[1]Malondialdehyde.
[2]Glutathione peroxidase.
[3]Reduced glutathione.
[4]The ratio of reduced glutathione to oxidized glutathione.

Feeding both levels of CX and OC noticeably elevated the concentration of GSH in RBC compared with the CON at d 42 ($P = 0.001$). There was no difference for the GSH/GSSG ratio among treatments ($P > 0.05$).

### Antioxidant Enzyme Activities in Liver, Kidney, and Heart

Supplemented diets appeared to enhance antioxidant enzyme activities (Table 5). However, this effect was largely dependent on the tissue and sampling day, i.e., time after starting the heat challenge. More effects were found on d 31 than on d 42. Day had in all cases an effect on the antioxidant enzyme activities (all $P < 0.05$). A significant interaction between diet and day was found in a few occasions.

In heart, at d 31 of age, both levels of CX and OC400 resulted in higher GSH-Px activity compared with CON ($P < 0.05$). The CAT activity in heart at 31 d of age was increased by feeding CX400 and OC400 ($P < 0.05$). Feeding CX400 increased SOD activity of liver, kidney, and heart at d 31 ($P < 0.05$). Also, dietary supplementation with OC400 resulted in higher SOD activity in kidney at d 31 ($P < 0.05$). None of the treatments affected the liver CAT and both the liver and kidney GSH-Px activities at d 31 ($P > 0.05$).

Fewer effects were observed at d 42. There was no effect of treatment on the kidney and heart enzyme activities at d 42 of age. Feeding CX200 increased the activity of GSH-Px in liver at d 42 ($P < 0.01$). A higher activity of CAT in liver was observed for the treatments CX200 and OC400 compared with the control ($P < 0.01$) at d 42. None of the treatments affected the activity of SOD in liver at d 42 ($P > 0.05$).

### HSP70 and Antioxidant Enzyme mRNA Levels in Heart, Liver, and Kidney

Effects of dietary supplemental essential oils on mRNA levels of inducible *HSP70* and antioxidant enzymes of heart, liver, and kidney are presented in Figure 1. Compared with CON, mRNA levels of *HSP70* were reduced at d 31 of age by feeding CX400 and OC400 in kidney and liver tissues, respectively ($P < 0.05$). The mRNA levels of *SOD* were increased at d 31 in the OC400 group in kidney and CX400 diet in heart ($P < 0.05$). Feeding CX400 and OC400 increased the mRNA levels of *CAT* at d 31 of age ($P < 0.05$). At d 42, only CX400 decreased *HSP70* mRNA levels in the heart ($P < 0.05$). None of the treatments affected the *GSH-Px* mRNA levels in any of the tissues.

## DISCUSSION

In the current study, the effects of 2 essential oils rich in simple phenolic compounds (xanthorrhizol in CX oil, and the monoterpenes thymol and carvacrol in OC oil) on the gene expression of heat shock pro-

**Table 5.** Effect of *Curcuma xanthorrhiza* and *Origanum compactum* essential oils at 200 and 400 mg/kg of diet on activities (U/g) of catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) in liver, kidney, and heart of broilers at 31 and 42 d of age subjected to heat challenge (n = 6)

| | 31 d of age (3 d after heat challenge) | | | | | 42 d of age (2 wk after heat challenge) | | | | | *P*-value | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *C. xanthorrhiza* | | *O. compactum* | | | *C. xanthorrhiza* | | *O. compactum* | | | | | |
| Item | Control | 200 | 400 | 200 | 400 | Control | 200 | 400 | 200 | 400 | Pooled SEM | Diet | Day | Diet × day |
| Liver | | | | | | | | | | | | | | |
| CAT | 198 | 218 | 189 | 180 | 193 | 50.1$^{b}$ | 86.1$^{a}$ | 72.9$^{ab}$ | 63.1$^{ab}$ | 85.2$^{a}$ | 8.61 | 0.008 | 0.001 | 0.608 |
| GSH-Px | 142 | 146 | 139 | 130 | 138 | 77$^{b}$ | 141$^{a}$ | 120$^{ab}$ | 95$^{ab}$ | 111$^{ab}$ | 5.8 | 0.004 | 0.001 | 0.030 |
| SOD | 70.4$^{b}$ | 70.3$^{b}$ | 74.1$^{a}$ | 71.5$^{b}$ | 71.1$^{b}$ | 78.7 | 79.0 | 78.9 | 80.3 | 81.0 | 0.34 | 0.002 | 0.001 | 0.001 |
| Kidney | | | | | | | | | | | | | | |
| GSH-Px | 554 | 552 | 527 | 546 | 533 | 598 | 633 | 617 | 663 | 584 | 14.7 | 0.317 | 0.001 | 0.520 |
| SOD | 81.1$^{b}$ | 106$^{ab}$ | 114$^{a}$ | 112$^{ab}$ | 122$^{a}$ | 76.5 | 83.8 | 78.3 | 79.6 | 78.7 | 3.6 | 0.004 | 0.001 | 0.012 |
| Heart | | | | | | | | | | | | | | |
| CAT | 269$^{b}$ | 290$^{ab}$ | 313$^{a}$ | 292$^{ab}$ | 320$^{a}$ | 282 | 281 | 269 | 264 | 265 | 8.3 | 0.003 | 0.004 | 0.089 |
| GSH-Px | 637$^{b}$ | 702$^{a}$ | 714$^{a}$ | 672$^{ab}$ | 698$^{a}$ | 593 | 626 | 638 | 568 | 609 | 19.6 | 0.003 | 0.001 | 0.907 |
| SOD | 64.6$^{b}$ | 66.4$^{a}$ | 66.6$^{a}$ | 64.9$^{b}$ | 65.7$^{ab}$ | 78.1 | 78.3 | 77.7 | 78.6 | 79.7 | 0.41 | 0.001 | 0.001 | 0.127 |

$^{a,b}$Means within a row and within day of sampling lacking a common superscript are significantly different ($P < 0.05$).

tein 70 and antioxidant enzymes, oxidative status, meat oxidative stability, and bird performance were tested in heat-challenged finishing broilers. Two main outcomes can be deduced from the results. First, these essential oils could alleviate some of the negative effects on the bird oxidative status associated with the pathophysiology of heat stress. Second, these essential oils may improve the oxidative stability of meat, and there were indications that feed efficiency is improved in OC-fed broilers. These effects might be a consequence of the beneficial effects on the antioxidant system.

The antioxidant properties of phenolic compounds are well established and involve radical scavenging, hydrogen or electron donating, and metal chelating activity (Bravo, 1998; Balasundram et al., 2006). Their efficiency of antioxidant activity in vivo or in food systems depends among other factors mainly on the molecular structure, the bioavailability, and the tissue or matrix considered. In addition, polyphenols have a large array of biological activities including modulation of cell signaling pathways and gene expression effects (Rodrigo et al., 2011). Hence, their overall effects are very complex and not consistent among studies. Their use in chicken diets should be critically evaluated (Surai, 2014).

## Plasma and RBC Oxidative Status

In the present study, feeding CX400 and both levels of OC caused a decrease in plasma MDA. Consistent with our results, Seven et al. (2009) reported that using plant extracts rich in phenolics leads to a decrease of the MDA concentration in heat-stressed broilers, confirming the direct antioxidant activity of phenolic compounds.

Glutathione is a tripeptide present at high concentrations intracellularly and known as a protector against toxic effects of lipid peroxidation (Nordberg and Arner, 2001). Interestingly, an enhancement in GSH concentrations had arisen after 14 d of dietary supplementation with essential oil. In other words, the antioxidant system had been successful in adapting itself via upregulation of glutathione synthesis through the phenolic compounds.

## Effects of the Essential Oils on the Antioxidant System in Tissues of Heat-Challenged Broilers

The applied heat-stress model can be ascribed as a cyclic chronic heat-challenge protocol. Birds were sampled between 1 and 4 h after the daily increased temperature (34°C) was achieved. In these conditions, physiological responses of sampled birds can be considered acute heat-stress responses.

It has been reported that elevated temperatures lead to mitochondrial damage and consequently excessive production of free radicals, hence oxidative stress (Lin et al., 2006). Exposing broilers to acute heat shock leads

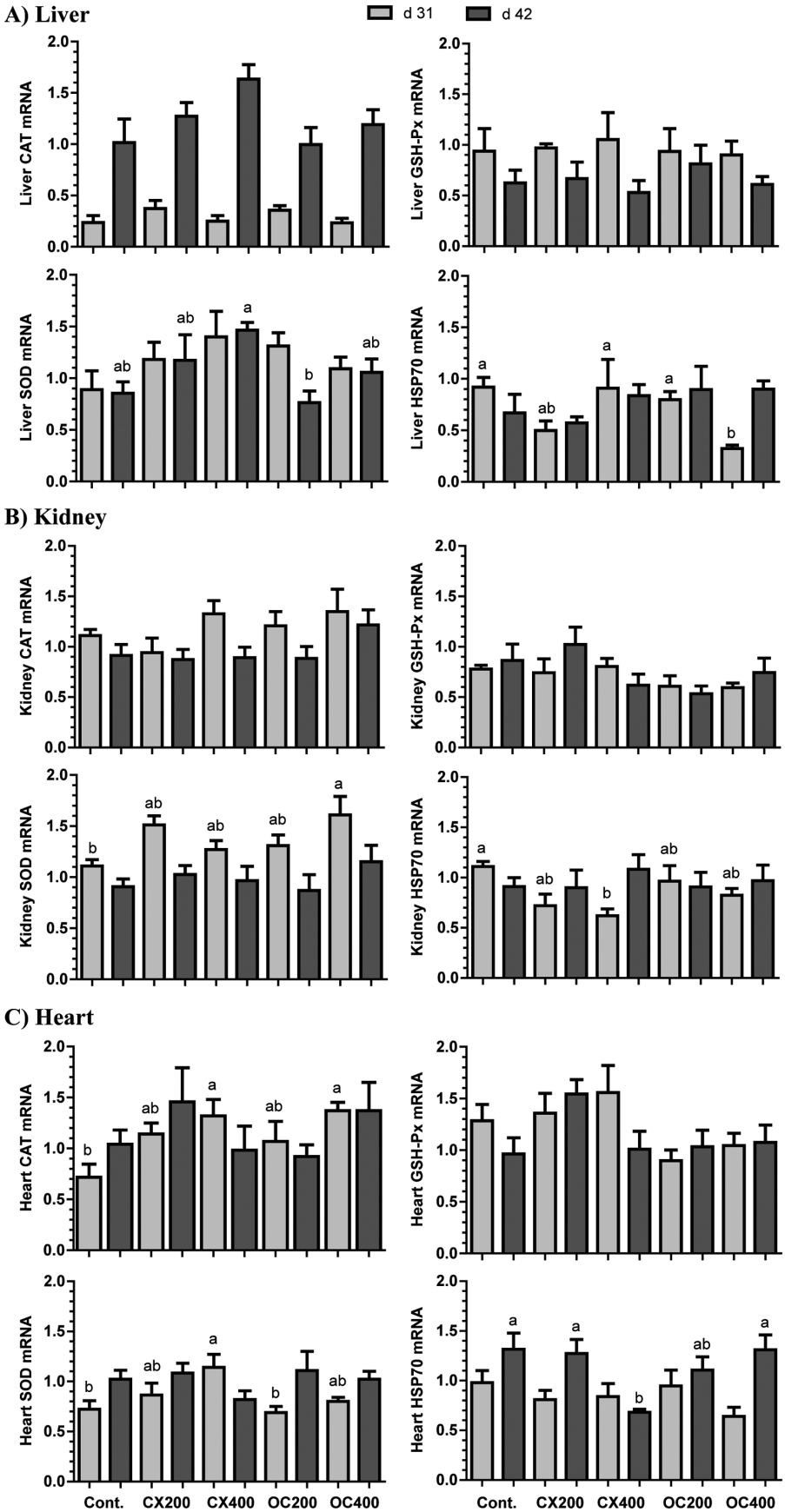


**Figure 1.** Effect of *Curcuma xanthorrhiza* and *Origanum compactum* essential oils on gene expression of heat shock protein 70 (*HSP70*) and antioxidant enzymes in heart, liver, and kidney (n = 6) of broilers at 31 and 42 d of age subjected to heat challenge. (A) Liver; (B) kidney; (C) heart. Each bar on the graph represents a treatment mean ± SEM. Means within day of sampling lacking a common letter (a,b) are significantly different ($P < 0.05$). Cont. = control; CX200 and CX400 = *C. xanthorrhiza* essential oil at 200 and 400 mg/kg, respectively; OC200 and OC400 = *O. compactum* essential oil at 200 and 400 mg/kg, respectively. *CAT* = catalase; *SOD* = superoxide dismutase; *GSH-Px* = glutathione peroxidase.

to an increase in the activity of antioxidant enzymes as a protective mechanism (Lin et al., 2006). In contrast, after a longer term of heat challenge (i.e., cyclic or constant chronic heat exposure), there might be a decrease in their activity, rendering it insufficient to counteract the induced oxidative damage (Sahin and Kucuk, 2003; Sahin et al., 2013). This was confirmed by the findings of the present study, i.e., a drastic reduction in the activities of CAT (~4-fold) and GSH-Px (~2-fold) in the liver at d 42 compared with d 31 in control groups was observed.

In the course of the trial, the effect of age of the birds and the adaptation to the daily heat stress might superimpose to the acute heat-stress effects. This may explain the clear differences in oxidative status between d 31 and 42 in the current study, shown by alteration of SOD activity in liver, kidney, and heart after 3 d of heat shock (as an acute heat-stress model) compared with 2 wk after heat challenge (as a cyclic chronic heat-stress model). These findings suggest that effects of the essential oils on the antioxidant enzymes were modified by the duration of the heat challenge, or from another view, it sounds that those birds in the control group that did not receive plant oils had become acclimated to high ambient temperature. A stimulating effect of dietary supplementation with phenolic compounds (gallic acid, ferulic acid, and p-coumaric acid) on the activity of GSH-Px, SOD, and CAT in heart of male Sprague-Dawley rats was reported by Yeh et al. (2009) and was accompanied by similar changes in mRNA levels.

## Effects of the Essential Oils on Meat Oxidative Stability

Some positive effects of essential oils on meat characteristics such as reducing the MDA concentration and stabilizing the redness of the meat (a* value) during storage were found in the present study. Avila-Ramos et al. (2012) reported a reduction in lipid oxidation when diets were supplemented with oregano essential oil. As described by Yanishlieva et al. (1999), the preventive effects of oregano on lipid peroxidation could be due to blocking the radical chain process by intervening with peroxide radicals and by enhancing the activity of antioxidant enzymes. Also, these authors suggested that during the peroxidation of lipids at high ambient temperature, thymol is a more effective antioxidant than carvacrol, because of the fact that the former has greater steric hindrance of the phenolic group than the latter.

Dai et al. (2009) showed that high ambient temperature caused a decrease in the a* value of meats harvested from broilers. The higher a* value in CX400 and both OC groups after simulated retail display in our study is in line with this, hence showing a role for essential oils antioxidants in retarding oxidation of myoglobin to metmyoglobin and hence maintaining meat redness longer during fresh or frozen storage. In line with our results, Sayago-Ayerdi et al. (2009) reported that chickens fed grape antioxidant fiber had redder meat with lower lipid oxidation.

## HSP70 and Antioxidant Enzyme mRNA Levels

The synthesis of HSP70 is temperature dependent and is induced to conserve cells that are exposed to stress, so that the role of HSP70 is considered as a cellular thermometer. It is well documented that high temperatures can induce *HSP70* mRNA synthesis via increasing either the amount or the activity of the heat-shock transcription factor and consequently the HSP70 concentration (Craig and Gross, 1991). On the other hand, most of the factors that induce the HSP response are also involved in the production of reactive oxygen species (Mahmoud et al., 2004). Thus, oxidative stress has been proposed as an indirect mechanism to induce HSP synthesis. Thereupon, the heat-shock response may be used as an analytical tool to better understand the response of the bird to elevated temperatures on a molecular basis.

Higher levels of HSP70 and oxidative stress in different tissues of poultry after environmental stressors such as increased temperature have been reported (Craig and Gross, 1991). A strong relationship between lipid oxidation and HSP70 synthesis in stressed cells has also been noticed (Mahmoud et al., 2004). Based on these findings it can be concluded that the supplementation with essential oils in the current study was protective against the onset of oxidative damage. Supplementation of plant extracts caused a decrease in both plasma MDA concentration and kidney and heart *HSP70* mRNA levels in the CX400 group. The abundance and inducibility of heat shock proteins has been shown to vary according to organ and developmental stage in different organisms (Givisiez et al., 2003). The abundance of *HSP70* mRNA at d 42 in different tissues was as follows in the present study: heart > kidney > liver. Chickens given the diet without essential oils had higher inducible *HSP70* mRNA levels in liver and kidney than the other groups at d 31 of age. In other words, by the end of 3 d of heat challenge, the resistance to high temperature was improved in the birds fed plant extracts as evidenced by lower *HSP70* gene expression in OC400 and CX400. At d 42, although adding CX400 decreased the inducible *HSP70* gene expression in heart, there were no significant differences for *HSP70* mRNA concentration among the treatment groups in liver and kidney. The increase in *HSP70* levels in heat-challenged birds at higher age demonstrates that even not a very severe but permanently increased environmental temperature leads to a cellular stress response. An adaptive response to *HSP70* mRNA had occurred in liver and kidney after prolonged high-temperature exposure, but heart was still suffering from long-term exposure to high temperature. The varying changes of *HSP70*

mRNA levels in heart tissue compared with liver and kidney may be attributed to differences in heart tissue antioxidant capacity (Cao et al., 1996). In this regard, Lin et al. (2006) noted that liver has more antioxidant power than heart, possibly due to the role of liver in nutrient storage and metabolism.

## Conclusion

To summarize, even though dietary essential oils did not exert a pronounced effect on chicken performance, evidence was present that feeding CX and OC at 200 and 400 mg/kg in the diet is beneficial for the broiler antioxidant system when subjected to long-term cyclic heat challenge. In particular, CX and OC at 400 mg/kg showed desirable effects on meat quality and activity of GSH-Px in both breast and thigh. Effects of the dietary supplementation on the activity and gene expression of antioxidant enzymes and *HSP70* in different tissues displayed a complex pattern, illustrating that the effects depend on age of the birds, tissue, and duration of high ambient temperature.

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