Effect of citric acid on the utilization of olive cake diets for laying hens

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Abstract
The study aims at improving the utilization of olive cake (OC) containing-diets for laying hens by citric acid supplementation at 0.1 and 0.2%. Olive cake was collected and dried by sunny warm air at an average temperature of 45°C with continuous stirring until completely dried. Then, the OC was included in isonutritive diets at 0, 10 and 20%. Additionally, citric acid was added at 0, 0.1 and 0.2%. This resulted in 3 (OC levels)×3(citric acid concentrations), producing 9 different treatments. Each treatment was represented by eight replicates of three laying hens each. The diets were fed to laying hens from 40 to 56 weeks of age. Olive cake inclusion up to 20% without citric acid supplementation in the diets of laying hens did not affect laying performance, egg quality or liver function indices, but 10% and 20% OC diets increased feed intake and impaired the feed conversion ratio (FCR) compared with the control group. Citric acid added at 0.1% to the diets of laying hens containing 20% OC yielded similar FCR to the unsupplemented control. In addition, citric acid supplementation at 0.1% significantly decreased relative weight of liver compared with the other levels of citric acid, which is obvious in OC-free diet, and it significantly increased relative weight of ovary compared with the control diet, which is obvious in different diets. In conclusion, OC could be used in the laying hens’ diets at 20% when supplemented with 0.1% citric acid without negative effects on laying performance, egg quality and blood metabolites.

Introduction
Improving the utilization of low nutritive value feed resources is essential for overcoming the limits of available feedstuffs in developing countries (Al-Saffar et al., 2013). Olive cultivation has increased recently for oil production due to its many health benefits (Amici et al., 1991). Extraction of olive oil has resulted in a huge amount of olive by-products, including olive cake (OC), which is rich of residual oil (6.8%) and unsaturated fatty acids (Amici et al., 1991; Abd El-Samee and Hashish, 2011; Heuzé et al., 2011; Al-Harthi, 2015). The literature shows that up to 10% OC has been fed to laying hens with no negative effects on productive performance, egg quality or liver and renal function (Mohebbifar et al., 2011; Zangeneh and Torki, 2011; Zarei et al., 2011; Al-Harthi, 2015). However, OC also contains non-starch polysaccharides (NSP) as xyloglucan and xylan-xyloglucan complexes (Gil-Serrano and Tejero-Mateo, 1988; Coimbra et al., 1995), and glucononayslans with a xylose/glucose at a ratio of 7:1 (Rosa Rio and Domingues, 2002). The negative effect of NSP on gut ecology and function has been highlighted (Chocht, 2006), but experiments on improving OC utilization are limited (Al-Harthi, 2015). On this topic, Islam (2012) reported that citric acid increased villus height/ crypt depth ratio and increased beneficial bacteria while decreasing pathogenic organisms in the feed and gut (Falkowski and Abeine 1984; Eidelburscher and Kirchgessner, 1994; Deepa et al., 2011). Organic acids may be a useful alternative for improving production performance and animal health (Attia et al., 2012, 2013). Organic acids exert their antimicrobial action both in the feed and in the gastrointestinal tract of the animal. (Radcliffe, 2000; Islam, 2012). Organic acids in their undissociated forms are able to pass through the cell membrane of bacteria. Once inside the cell, the acid dissociates to produce H+ ions, which lower the pH of the cell, causing the organism to use its energy trying to restore the normal balance. The RCOO- anions produced from the acid can disrupt DNA and protein synthesis, putting the organism under stress and making it unable to replicate (Islam, 2012). Citric acid can be classified as a growth promoter, acidifier, bacterial inhibitor, antioxidant and antitoxin (Salgado-Tránsito et al., 2011; Rostamzad et al., 2011; Islam, 2012). Yesilbag and Çolpan (2006) reported that a mixture of formic and propionic acids and their ammonium salts increased egg production by accelerating laying capacity and prolonging the laying period, and to a lesser extent, feed efficiency, but did not affect body weight, feed intake or the egg quality traits of laying hens that were fed a diet of corn-wheat-soybean and meat and bone meal. In the literature, Jozefiak and Rutkowski, (2005) and Chowdhury et al. (2009) reported that citric acid lowers the pH in the gut, thus reducing harmful microbiota and modifying the distribution of bacterial species in the gut. These effects boost the health status of chickens, which may be reflected in the improvement of the external and internal egg quality. In addition, organic acid at 0.2% and 0.3% available phosphorus diet showed the best results in hen-housed egg production, soft-shell plus broken egg production, FCR, and egg yolk gamma immune globulin (Park et al., 2009). On the other hand, Boling et al. (2000) indicate that citric acid does not improve the utilisation of dietary P in laying hens fed a corn-SBM diet containing 3.8% Ca. However, no study has been conducted to investigate the effect of citric acid on the nutritive value of OC. This study aims to investigate the effect of different concentrations of citric acid for improving the utilisation of olive cake on productive performance, egg quality, liver function and on the inner organs of laying hens.

Materials and methods
Diet, design and birds
This research was carried out in the Hada Al-Sham Research Station, College of Meteorology, Environment and Arid Land
Citric acid and olive cake in hens

Five hens were chosen randomly to represent five replicates per treatment. Blood samples were collected (5 ml) from the brachial vein into heparinized tubes at week 56 of age and plasma was collected after blood centrifugation at 1500 x g for 20 min. Biochemical parameters of blood plasma were determined using commercial diagnostic kits (Diamond Diagnostics Company, Cairo, Egypt). Plasma total protein and albumin concentrations were done according to Henry et al. (1974) and Doumas et al. (1977), respectively. Globulin concentration was calculated by the difference between total protein and albumin. The activities in (UL) of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzymes were measured according to Reitman and Frankel (1957). At week 56 of age, 8 hens per treatment were slaughtered after being fasted overnight to calculate relative weight of inner organs.

Statistical analysis

The data was analysed using the GLM procedure of Statistical Analyses software (SAS, 2001) using two-way factorial analyses according to the two-factor randomized complete block design of the experiment (3 × 3). In the Model statement, the effects included olive cake, citric acids, and the interaction between them and the replicate. The replicate was the experimental unit. Before analysis, all percentages were subjected to logarithmic transformations (log10 x + 1) to normalize data distribution. Means differences were significantly tested using the Student Newman-Keuls test at (P≤0.05). As significant interactions were found, the differences among the interaction treatments were discussed.

Table 1. Composition and nutrient profiles of the experimental diets containing different concentrations of olive cake.

| Ingredients, g/kg | Olive cake, g/kg |
|-------------------|-----------------|
|                   | 0   | 100 | 200 |
| Maize             | 652.3 | 558.4 | 454.4 |
| Soybean meal, 48% CP | 171.0 | 190.0 | 209.0 |
| Wheat bran        | 78.0  | 33.0  | 0.0  |
| Dried olive cake  | 0.0   | 100   | 200  |
| Di-calcium phosphate | 10.6  | 10.4  | 9.6  |
| Limestone         | 80.0  | 80    | 79.5 |
| Sodium chloride   | 3.0   | 3.0   | 3.0  |
| Vitamin + mineral premix | 3.0 | 3.0 | 3.0 |
| DL-methionine     | 1.1   | 1.3   | 1.4  |
| L-lysine          | 0.4   | 0.3   | 0.0  |
| Antioxidin        | 0.6   | 0.6   | 0.6  |
| Soybean meal oil  | 0.0   | 20.0  | 39.5 |
| Total             | 1000  | 1000  | 1000 |

Determined § and calculated ¶ analysis on DM basis

ME, MJ/kg: 11.33 11.37 11.30
Dry matter, %: 905 906 903
Crude protein, g/kg: 164 162 163
Lysine, g/kg: 7.1 7.0 6.9
Methionine, g/kg: 3.5 3.6 3.6
Methionine + cysteine, g/kg: 6.0 6.0 6.0
Ether extract, g/kg: 27.6 46.9 63.5
Linoleic acid, g/kg: 15.02 22.96 31.5
Crude fibre, g/kg: 31.5 57.5 77.2
Ash, g/kg: 129.3 136.9 154.5
Calcium, g/kg: 35.0 35.0 35.0
Available phosphorus, g/kg: 3.1 3.2 3.1

CP: crude protein; DM: dry matter; ME: metabolizable energy. "Chemical composition of sundried olive cake (AOAC, 1990) showed 5.2% CP; 11.8% ether extract, 14% crude fibre and 20.4% ash, 0.73% Ca, 0.95% total phosphorus, 0.06% methionine, 0.16% methionine and cysteine, 0.03% lysine and 0.67 MJ/kg ME. Provided the following per kg of diet: vitamin A, 12,000 IU; vitamin D3, 7200 ICU; vitamin E, 20 IU; vitamin B1, 2.5 mg; vitamin B2, 5 mg; vitamin K, 1 mg; vitamin B6, 1.5 ppb; pyridoxine, 0.225 ppb; pantothenic acid, 10 mg; niacin, 35 mg; folic acid, 1.5 mg; biotin, 125 mg; Mn, 90 mg; Cu, 7.5 mg; Zn, 65 mg; Fe, 50 mg; Se, 0.4 mg. ¶ Determined values expressed on DM basis (AOAC, 1990). § Calculated analyses values based on National Research Council (1994).
Results and discussion

Body weight and egg production traits

The changes in body weight, egg production traits and survival rates during the period of 40-56 weeks of age are shown in (Table 2). Olive cake and/or the addition of citric acid did not significantly affect the body weight nor the survival rate, showing that OC had no harmful effects on animal liveability.

Laying hens could be fed a diet contained up to 20% OC without effects on laying rate, egg weight and egg mass. Similarly, Al-shanti and Abo Omar (2003), Heuzé et al. (2011), Mohebbifar et al. (2011) Zarei et al. (2011), and Zangeneh and Toki (2011) recommended OC at 10% in laying hens’ diets. This may be due to the maturity of the gastrointestinal tract of laying hens in terms of enzyme secretions, absorptive capacity, and microbial diversity (El-Ghamy et al., 2005).

Feed intake and FCR were significantly affected by OC concentration and/or citric acid. The feed intake of laying hens fed 10% or 20% OC diets was similar, but was significantly higher by 4.4% and 7.0% respectively than that of hens fed an OC-free diet when compared to the other unsupplemented level of OC. It is worth noting that the increase in fibre ratio (3.15%, 5.75% and 7.72%, respectively) as OC increases from 0% to 10% and 20%, and hence nutrient digestibility might be the reason for increasing feed intake as the experimental diets containing similar energy content. It is interesting to note that feed intake may change when diet composition changes, even though the diets is isocaloric and isonitrogenous. The lack of a positive influence of citric acid on laying rate, egg weight and egg mass may be due to the high content of organic acids in olive e.g. succinic, malic, citric and oxalic acids. The sum of these acids among the three strains of olives ranged from 2156.2 to 4671.1 mg / 100 g (Cunha et al., 2001; Ergönül and Nergiz, 2010). The large amounts of organic acids in olives and OC may be adequate for inducing the desired effect on laying performance without the need for further supplementation. In addition, high levels of calcium in the laying hens’ diets may interfere with citric acid affecting pH in the gut towards alkalinity, thus reducing its effectiveness as acidic agent (Nezhad et al., 2007).

Feed intake of hens fed diets containing 10% OC supplemented with citric acid at 0.1 and 0.2% was significantly greater by 5.0 and 13.4%, respectively, than that of hens fed the same feed without citric acid supplementation. In addition, supplementation of 0.1% or 0.2% citric acid to a diet containing 10% OC significantly increased feed intake compared to the same levels of citric acid when supplemented in an OC-free diet as well as in a 20% OC-containing diet. In addition, 0.2% citric supplementation of the 20% OC-containing diet significantly increased feed intake by 7.9% compared to supplementation of the same citric acid concentration in the OC-free diet. Within the 10% and 20% OC diets, increasing the citric acid concentration from 0.1% to 0.2% significantly increased feed intake by 8% and 6%, respectively. The positive effect of citric acid on feed intake may be due to the effect of maintaining feed freshness and thus the palatability of the feed. In the literature, citric acid supplementation has been reported to preserve freshness and to decrease microbial growth and harmful bacteria (E. Coli), thereby maintaining feed quality during storage and in the gut and leading to better animal growth (Falkowski and Aheme 1984; Eidelsburger and Kirchgesssner, 1994; Deepa et al., 2011).

When comparisons were made across the citric acid unsupplemented diets, it was found that FCR using 10% and 20% OC in the feed led to a significant deterioration in FCR, by 6.5% and 7.5% respectively, in comparison to the OC-free feed. The deterioration in FCR for

Table 2. The effect of olive cake concentration with or without citric acid supplementation on performance of laying hens.

| Effect of olive cake concentration, % | BWG40-56 wk, g | Survival rate, % | Laying rate, % | Egg weight, g | Egg mass, g/h/d | Feed intake, g/h/d | FCR, g feed: g gain |
|-------------------------------------|----------------|------------------|----------------|--------------|-----------------|-----------------|------------------|
| Control                             | 163            | 100              | 89.5           | 64.1         | 57.3            | 114^c           | 1.99^c           |
| 10                                  | 154            | 100              | 87.8           | 64.7         | 56.8            | 125^d           | 2.23^d           |
| 20                                  | 160            | 100              | 89.3           | 64.5         | 57.5            | 120^o           | 2.09^o           |
| Effect of citric acid supplementation, % | 160            | 100              | 88.4           | 64.1         | 56.6            | 118^e           | 2.10^e           |
| 0.1                                 | 164            | 100              | 90.1           | 64.0         | 57.6            | 118^e           | 2.05^e           |
| 0.2                                 | 153            | 100              | 88.0           | 65.2         | 57.4            | 124^d           | 2.16^d           |
| Interaction among treatments        | 141            | 100              | 88.6           | 64.5         | 57.1            | 114^c           | 2.00             |
| 0 0.1% citric                       | 187            | 100              | 91.1           | 63.6         | 57.9            | 113^c           | 1.95             |
| 0 0.2% citric acid                  | 151            | 100              | 88.8           | 64.1         | 56.8            | 114^d           | 2.00 ^d           |
| 10 0                                | 159            | 100              | 86.7           | 64.5         | 55.9            | 119^e           | 2.13             |
| 10 0.1% citric acid                 | 165            | 100              | 90.2           | 63.7         | 57.4            | 125^d           | 2.18             |
| 10 0.2% citric acid                 | 139            | 100              | 86.5           | 66.0         | 57.1            | 135^e           | 2.36             |
| 20 0.1% citric acid                 | 180            | 100              | 89.9           | 63.2         | 56.7            | 122^c           | 2.15             |
| 20 0.2% citric acid                 | 105            | 100              | 89.0           | 64.7         | 57.6            | 116^e           | 2.01             |
| 20 0.3% citric acid                 | 169            | 100              | 88.9           | 66.5         | 58.3            | 123^c           | 2.11             |
| SEM                                 | 32.8           | -                | 1.54           | 0.79         | 1.08            | 1.74            | 0.045            |
| Olive cake                          | 0.625          | -                | 0.343          | 0.571        | 0.729           | 0.001           | 0.001            |
| Citric acid                         | 0.727          | -                | 0.237          | 0.104        | 0.512           | 0.001           | 0.019            |
| Interaction                          | 0.085          | -                | 0.651          | 0.291        | 0.921           | 0.001           | 0.059            |

BWG, body weight gain; FCR, feed conversion ratio. ^aDifferences among means within a column within each factor not sharing similar superscripts are significantly (P < 0.05) different among treatments.
the negative effect of OC. However, citric acid intake.
chickens fed low ME tended to increase feed intake.
high percentage of fibre (5.75% and 7.72% vs 3.15%) and percentage of ash (13.69 and 15.45 vs 12.93%) in 10% and 20% OC diets vs OC-free diet on energy utilization (Attia et al., 1998). In the literature, there were negative correlations between feed intake and ME (National Research Council, 1994; Attia et al., 1995); chickens fed low ME tended to increase feed intake.

Obviously, supplementing a 10% OC-containing diet with 0.2% citric acid aggregates the negative effect of OC. However, citric acid added at 0.1% to the diets of laying hens containing 20% OC yielded similar PRC to the unsupplemented control. This may be due to the effect of citric acid on ecology and morphology of the gut and thus on gut health.

Egg shape index and quality of eggshell

Table 3 indicates the effect of OC and/or different levels of citric acid on the shape index and the quality of the eggshell (absolute and relative weight of the shell, shell thickness and shell weight per unit of surface area (SWUSA)).

The egg shape index significantly decreased due to 20% OC compared to the other OC concentrations as unsupplemented groups were compared. These findings are similar to those reported by Al-shanti and Abo Omar (2003).

Concentrations of OC had a significant influence on absolute and relative weight of the shell and SWUSA. It was found that, 10% OC groups produced the best eggshell quality compared to the OC-free diet. Similarly, Mohebbifar et al. (2011), Zangeneh and Torki (2011) and Zarei et al. (2011) revealed that OC in laying hen diets of up to 10% did not affect eggshell quality.

There was a significant effect of citric acid on absolute shell weight and SWUSA. The results indicated that 0.2% citric acid significantly improved eggshell quality compared to 0.1% citric acid and this effect was clear within each OC level, but the magnitude of the increase depends on OC concentration. In the literature, citric acid supplementation at 0.15-0.2% improved eggshell quality. This is probably due to the positive impact of citric acid on phytate phosphorus utilization and thus on the release of minerals bound to the phytate molecule (Nezhad et al., 2008; Islam, 2012; Kaya et al., 2014). In this context, Youssef-Amami et al. (2013) found that sodium formate additions of 0, 0.1, 0.2 and 0.3% significantly increased eggshell quality, probably due to the greater stability of organic acids salts under the gastrointestinal tract rather than to the organic acids themselves.

In the literature, organic acids was found to promote the growth of the epithelial cells of the intestine and thereby improve the absorption and metabolism of Ca (Langhout and Sus, 2005) and improve the digestion and absorption of nutrients (Vogt, 2001; Kaya et al., 2014). However, Abdel-Fattah et al. (2008) showed that the addition of 1.5 or 3% citric acid increased the Ca and P concentrations in the blood serum. However, citric acid did not affect the osteocalcin, or the blood serum content of 1,25 (OH) 2, vitamin (D) or Ca of chickens fed a diet containing 0, 0.25, 0.75 and 1.25% citric acid (Islam et al., 2010). In addition, citric acid affected mineral utilization, but did not affect the plasma mineral contents (Nourmohammadi et al., 2010). These differences in the response to citric acid may be due to strain differences, dietary composition, hygienic conditions and the hormonal balance responsible for maintaining Ca and P homeostasis (Fraser, 1988).

Table 3. The effect of olive cake concentration with or without citric acid supplementation on egg shape index and egg shell quality.

| Effect of olive cake concentration, % | Shape index, % | Shell weight, g | Shell weight, % | Shell thickness, µm | SWUSA, mg/cm² |
|--------------------------------------|---------------|-----------------|-----------------|---------------------|---------------|
| Control                              | 74.5*         | 6.02*           | 9.37*           | 401                 | 81.8*         |
| 10                                   | 74.7          | 6.28*           | 9.78*           | 399                 | 85.4*         |
| 20                                   | 73.2*         | 6.10*           | 9.24*           | 383                 | 81.3*         |
| Effect of citric acid supplementation, % | 73.9          | 6.07*           | 9.52            | 393                 | 82.9*         |
| 0.1                                  | 74.4          | 5.98*           | 9.22            | 389                 | 80.8*         |
| 0.2                                  | 74.2          | 6.35*           | 9.65            | 401                 | 84.9*         |
| Interaction among treatments          | 75.0          | 5.99            | 9.68            | 403                 | 83.5          |
| 0                                    | 74.2          | 5.80            | 8.86            | 393                 | 77.8          |
| 0.1% citric acid                     | 74.2          | 6.28            | 9.56            | 407                 | 84.1          |
| 0.2% citric acid                     | 74.5          | 6.26            | 9.67            | 409                 | 84.7          |
| 10                                   | 74.4          | 6.15            | 9.80            | 383                 | 85.0          |
| 0.1% citric acid                     | 75.2          | 6.43            | 9.88            | 407                 | 86.6          |
| 0.2% citric acid                     | 72.1          | 5.96            | 9.21            | 368                 | 80.6          |
| 20                                   | 74.5          | 6.00            | 9.00            | 391                 | 79.5          |
| 0.1% citric acid                     | 73.1          | 6.33            | 9.50            | 390                 | 83.9          |
| 0.2% citric acid                     | 1.32          | 0.210           | 0.375           | 18.8                | 3.32          |
| SEM                                  | 0.044         | 0.024           | 0.006           | 0.341               | 0.005         |
| P                                    | 0.763         | 0.001           | 0.052           | 0.661               | 0.012         |
| Interaction                          | 0.269         | 0.803           | 0.240           | 0.897               | 0.414         |

SWUSA, shell weight per unit surface area; *Differences among means within a column within each factor not sharing similar superscripts are significantly (P≤0.05) different among treatments
with the OC-free diet. These are consistent with the results of Zarei et al. (2011), who reported that yolk colour increased with the use of OC at 9% in the laying hens’ diets. However, Mohebbifar et al. (2011), Zangeneh and Torki (2011) and Zarei et al. (2011) reported that inclusion of 9% olive pulp did not significantly affect yolk colour, but significantly lowered the Haugh unit more than other diets. Nezhad et al. (2008) found that citric acid at 2% did not significantly affect the Haugh unit, which is similar to the present findings, but increasing citric acid to 4% significantly decreased the Haugh unit.

Citric acid at 0.2% citric acid significantly increased egg yolk colour compared to the 0% and 0.1% concentrations. This was obvious within each level of OC. Similarly, Kaya et al. (2014) found that citric acid significantly increased egg yolk colour. The increase in egg yolk colour of laying hens supplemented with 0.2% citric acid may be due to an increase in the absorption of pigments because of the effect of citric acid on improving the gut ecology and/or the antioxidant effect (Salgado-Tránsito et al., 2011; Islam, 2012).

Blood plasma metabolites

Table 5 describes the effect of OC and/or citric acid on some blood biochemical components and liver function indices. Feeding a 10% OC diet led to a significant reduction in plasma total protein and globulin in comparison to other unsupplemented levels of OC. This is probably due to the low protein digestibility of OC (Rabaya et al., 2001; Abo Omar, 2005; Hassanen et al., 2005) because of its high fibre and ash (Francisco et al., 1989; Al-Harthi, 2015). On the other hand, Rupic et al. (1999), Zarei et al. (2011) and Zangeneh and Torki (2011) demonstrated that OC did not affect blood biochemical profiles or liveability.

The increase in plasma total protein and globulin due to 0.2% citric acid supplementation to OC-free diet compared to some level of citric supplemented to 10 and 20% OC diets is consistent with the findings that adding 0.5% citric acid to chicken feed improved humeral and cellular immunity against the Newcastle disease virus (NDV) (Chowdhury et al., 2009; Das et al., 2011; Islam, 2012). This can be considered a good indicator of the health status (Khatoon et al., 2010; Haque et al., 2010). However, another study Özek et al. (2011) found that organic acids at a rate of 0.2% did not affect the immune response to NDV, infectious laryngotracheitis or infectious bursa disease despite increasing globulin. In the literature, chicks fed acidifiers have a better immune response, as evidenced by increased serum globulin and an allied increase in lymphoid organ weights when compared to a control group (Abdel-Fattah et al., 2008).

Comparing the effect of citric acid across different levels of OC shows that 0.1% citric acid led to a substantial decrease in plasma globulin in the OC-free feed groups compared to the influence of the same level of citric acid in the other OC diets. The decreased plasma protein and globulin of 20% OC diet and globulin of OC-free diet found herein due to citric acid supplementation at 0.1% are in agreement with those cited by El-Afifi et al. (2001) who found a decrease in blood plasma protein with supplementation of different citric acid concentrations (0.0%, 0.2%, 0.4%, 0.6%, and 0.8%) to corn-soybean meal diet for broilers. In a subsequent study, El-Afifi (2003) cited that total plasma protein, albumin and globulin were not affected by citric acid. This contradiction in response to citric acid supplementation could be attributed to the citric acid concentration, dietary composition and hygienic conditions (Islam, 2012).

Citric acid at 0.1% within the OC-free diet led to a significant increase in plasma albumin compared to 0.2% citric acid, a non-specific immune index, and to the albumin to globulin ratio compared to the other concentrations of citric acid. These effects were obvious only with OC-free diet. Similarly, Capcarova et al. (2014), who cited that citric acid did not negatively affect serum total protein but significant-

Table 4. The effect of olive cake concentration with or without citric acid supplementation on interior egg quality of laying hens.

| Effect of olive cake concentration, % | Yolk weight, % | Yolk index | Yolk: albumen ratio | Yolk colour score | Albumen weight, % | Haugh Unit score |
|--------------------------------------|---------------|------------|--------------------|------------------|------------------|-----------------|
| Control                              | 29.5          | 49.6       | 0.454              | 5.08             | 64.5             | 60.9            |
| 10                                   | 29.4          | 56.4       | 0.458              | 5.24             | 64.3             | 64.7            |
| 20                                   | 29.3          | 54.2       | 0.453              | 5.40             | 64.6             | 62.5            |

| Effect of citric acid supplementation, % | Yolk weight, % | Yolk index | Yolk: albumen ratio | Yolk colour score | Albumen weight, % | Haugh Unit score |
|-----------------------------------------|---------------|------------|--------------------|------------------|------------------|-----------------|
| Control                                 | 29.7          | 53.2       | 0.460              | 5.24             | 64.2             | 65.1            |
| 0.1                                     | 29.3          | 54.4       | 0.453              | 5.00             | 64.7             | 56.4            |
| 0.2                                     | 29.2          | 52.8       | 0.453              | 5.49             | 64.5             | 66.6            |

Interaction among treatments

| Effect of citric acid supplementation, % | Yolk weight, % | Yolk index | Yolk: albumen ratio | Yolk colour score | Albumen weight, % | Haugh Unit score |
|-----------------------------------------|---------------|------------|--------------------|------------------|------------------|-----------------|
| 0 0                                     | 29.6          | 47.0       | 0.460              | 5.17             | 64.4             | 60.4            |
| 0 0.1% citric acid                      | 28.9          | 52.0       | 0.443              | 4.75             | 65.3             | 57.4            |
| 0 0.2% citric acid                      | 29.2          | 49.7       | 0.452              | 5.33             | 64.6             | 64.3            |
| 10 0                                    | 29.4          | 59.1       | 0.457              | 5.17             | 64.4             | 68.6            |
| 10 0.1% citric acid                     | 29.9          | 55.4       | 0.467              | 5.08             | 64.0             | 57.3            |
| 10 0.2% citric acid                     | 29.1          | 54.8       | 0.451              | 5.46             | 64.5             | 68.3            |
| 20 0                                    | 29.5          | 53.8       | 0.457              | 5.38             | 64.6             | 65.8            |
| 20 0.1% citric acid                     | 29.1          | 55.1       | 0.448              | 5.17             | 65.0             | 54.5            |
| 20 0.2% citric acid                     | 29.3          | 53.2       | 0.455              | 5.67             | 64.4             | 67.1            |

SEM 1.20                                | 4.07          | 0.020      | 0.277              | 1.19             | 5.95             |

P

Olive cake                          0.956 | 0.222 | 0.752 | 0.054 | 0.889 | 0.730
Citric acid                        0.756 | 0.905 | 0.635 | 0.001 | 0.793 | 0.068
Interaction                       0.874 | 0.920 | 0.615 | 0.886 | 0.966 | 0.966

* Differences among means within a column within each factor not sharing similar superscripts are significantly (P < 0.05) different among treatments.
ly increased serum albumin.

However, citric acid at 0.2% to the feed containing 20% OC significantly decreased the plasma albumin concentration compared to their respective control without citric acid supplementation as well as the same level of citric acid added to the other OC diets. The same level of citric acid (0.2%) also decreased the albumin to globulin ratio compared to the same level of citric acid added to the 10% OC diet. These results indicated that the effect of citric acid on plasma protein, globulin and albumin depends on OC concentrations.

In general, the 10% OC feed caused an increase in the AST concentrations compared to the other OC levels when unsupplemented levels were compared. Citric acid at 0.1% in OC-free feed significantly decreased plasma AST concentration compared to the effect of the same level of citric acid added to other levels of the OC. On the other hand, supplementation of 0.1 and led to a significant increase in plasma AST compared to the unsupplemented 20% OC diet. In addition, 0.2% citric acid to feed containing 20% of the OC led to similar effect of groups fed OC-free feed and in the 10% OC diet supplemented by the same level of citric acid. The decrease in hepatic enzyme AST demonstrates an improvement in liver function with citric acid supplementation. The effect could be attributed to the antioxidant and antitoxin effects of citric acid (Salgado-Tránsito et al., 2011; Rostamzad et al., 2011; Fazayeli-Rad et al., 2014). That there was a significant effect only for citric acid on plasma ALT showed that citric acid at a rate of 0.1 and 0.2% added to different levels of OC led to a significant increase in ALT. These outcomes are in agreement with those reported by Capcarova et al. (2014), who reported that citric acid did not negatively affect ALT, alkaline phosphatase or gamma-glutamyl transferase, which is the most sensitive enzymatic indicator of hepatobiliary disease.

Feeding OC at 10% and 20% to broilers significantly increased AST to ALT in a stepwise manner compared to OC-free feed. In addition, citric acid added at 0.1% to OC-free feed led to broilers led to a significant decrease in the AST to ALT ratio when compared to the unsupplemented control. These changes in liver enzymes are within the natural physiological range and were not accompanied by concomitant changes in laying performance and/or liver percentage, meaning that OC and citric acid had no harmful effects on liver function and protein metabolism. These effects are similar to those cited by Zangeneh and Torki (2011) and Zarei et al. (2011), who noted that the OC has no detrimental effect on blood biochemical components.

### Table 5. The effect of olive cake concentration with or without citric acid supplementation on blood metabolites of laying hens.

| Effect of olive cake concentration, % | Total protein, g/dL | Globulin, g/dL | Albumin, g/dL | A/G ratio | AST, U/L | ALT, U/L | AST/ALT |
|--------------------------------------|---------------------|----------------|--------------|-----------|----------|----------|---------|
| Control                              | 6.76a               | 3.46           | 3.30a        | 0.980a    | 53.5b    | 64.0     | 0.837a  |
| 10                                   | 6.53a               | 3.34           | 3.19a        | 0.958a    | 56.2a    | 64.4     | 0.874a  |
| 20                                   | 6.53a               | 3.52           | 3.01a        | 0.865a    | 56.6a    | 62.9     | 0.903a  |
| Effect of citric acid supplementation, % |                     |                |              |           |          |          |         |
| Control                              | 6.69b               | 3.56b          | 3.13bc       | 0.883b    | 55.3     | 61.9     | 0.895b  |
| 0.1                                  | 6.55b               | 3.27b          | 3.28b        | 1.02b     | 55.0     | 64.9     | 0.849b  |
| 0.2                                  | 6.58b               | 3.49b          | 3.89b        | 0.893b    | 56.1     | 64.5     | 0.869b  |
| Interaction among treatments          |                     |                |              |           |          |          |         |
| 0 0                                  | 6.72bc              | 3.70c          | 3.02cd       | 0.82c     | 54.6d    | 61.8     | 0.853c  |
| 0 0.1% citric                        | 6.70b               | 3.28b          | 3.37a        | 1.25a     | 52.0     | 65.2     | 0.798a  |
| 0 0.2% citric                        | 6.88e               | 3.70e          | 3.16e        | 0.855e    | 54.0d    | 65.0     | 0.831e  |
| 0 10                                  | 6.50f               | 3.30f          | 3.20f        | 0.97f     | 56.6fb   | 62.6     | 0.904f  |
| 0 0.1% citric acid                   | 6.58h               | 3.48h          | 3.10h        | 0.89h     | 56.0hc   | 66.0     | 0.848h  |
| 0 0.2% citric acid                   | 6.50i               | 3.26i          | 3.25i        | 1.00i     | 56.0bc   | 64.6     | 0.867i  |
| 20 0                                  | 6.88e               | 3.70e          | 3.16e        | 0.855e    | 54.6d    | 61.2     | 0.896d  |
| 20 0.1% citric acid                  | 6.36f               | 3.32f          | 3.02f        | 0.91f     | 57.0bf   | 63.6     | 0.896f  |
| 20 0.2% citric acid                  | 6.38f               | 3.52f          | 2.86f        | 0.83f     | 58.2f    | 64.0     | 0.909f  |
| SEM                                  | 0.052               | 0.093          | 0.078        | 0.057     | 0.490    | 0.646    | 0.017   |
| P                                    |                     |                |              |           |          |          |         |

A/G, albumin/globulin; AST, aspartate amino transferase; ALT, alanine amino transferase. * Differences among means within a column within each factor not sharing similar superscripts are significantly (P<0.05) different among treatments.

### Inner body and reproductive organs

Table 6 demonstrates the effect of OC and/or citric acid on the relative weight of abdominal fat, liver, heart, pancreas, gizzard, intestine, ovary and oviduct. The relative weight of abdominal fat was significantly affected by the OC concentration only, showing a similar increase, 90% and 105% respectively, when 10% and 20% OC was fed compared to the OC-free diet. The increase in fat deposition in the abdominal cavity may be due to the increase in the added fat to 2% and 3.95%, even diets were isocaloric. This indicates an increase in ME available beyond the needs for egg formation, even with increasing dietary fibre contents.

The relative weight of liver was significantly affected by citric acid concentration and its interaction with OC levels, indicating that the effect of citric acid on relative weight of liver depends on the OC concentration. The lack of adverse effects of OC in plasma liver enzymes indicated the safety of OC for chicken health. These results could be attributed to the unsaturated fatty acids and polyphenols contents of OC. These can increase lipoprotein synthesis, the precursor for yolk formation, in the liver and boost the antioxidant defence system, respectively. Further evidence of a lack of adverse effects for OC is provided by the lack of...
a negative effect on the survival rate and on the heart percentage. These findings are in agreement with those reported by Al-Shanti and Abo Omar (2003), MohbibiFar et al. (2011), Zarei et al. (2011), Zangeneh and Torki (2011) and Al-Harthi (2015). They reported that inner organs were not negatively affected by 10% OC in laying hens.

Relative weight of pancreas and gizzard was significantly affected only by OC concentrations. Pancreas of the group fed 10% OC significantly increased, amounting to 13.2% and 25.3%, respectively, compared to those fed the control OC-free feed and 20% OC diet. In addition, OC at the 20% level in the laying hens’ diets significantly increased relative weight of gizzard by 22.1% and 12.5%, respectively, compared to OC-free feed and to the 10% OC diet. The increase in relative weight of gizzard could be attributed to the increase in mechanical digestion and the growth of muscles required to digest the olive cake fibre and nucleus (Al-Shanti and Abo Omar, 2003; Al-Harthi, 2015).

Relative weight of intestinal percentage was significantly affected by the OC and by its interaction with the citric acid concentration. The diet containing 10% or 20% OC significantly decreased the relative weight of intestinal by similar extend (36.2%) compared to the OC-free diet. This decrease in relative weight of intestinal is contrary to the 15.6% increase in relative weight of gizzard of the same group and indicates that dietary composition may regulate the growth of different gut segments (Attia et al., 2003).

Relative weight of ovary was significantly affected by OC and citric acid, while relative weight of oviduct was significantly affected only by the OC concentration. Diets contained 10% and 20% OC significantly increased relative weight of ovary by 25.9% and 38.7%, respectively, compared to the OC-free diet, but the OC-free diet significantly increased the relative weight of oviduct percentage by 14% compared to the 10% OC-containing diet. Citric acid at 0.1% significantly increased the relative weight of ovary by 18.3% compared to the unsupplemented control group; nonetheless, the group fed the 20% OC diet was intermediate. These demonstrated an increase in the number of follicles with no signs of fatty liver, as liver enzymes and the relative weight of liver were similar among the different OC-level diets. Thus these adaptive changes did not negatively affect laying rate, egg weight or egg mass output, and are similar to the reports by Al-Shanti and Abo Omar (2003), Abd El-Samie and Hashish (2011) and Al-Harthi (2015). The adaptive changes in abdominal fat and in the ovaries as OC level increased could be attributed, as previously explained, to the changes in diet composition, such as increases in the fibre and oil contents as the OC level increases (Attia et al., 1998; Al-Harthi, 2015). This could affect ME utilization and nutrient re-partitioning in body organs.

Citric acid added at 0.1% to OC-free from OC have led to a significant decrease in the liver weight percentage compared to the unsupplemented controls and to the 0.2% supplemented diets, but significantly increased the liver weight percentage of the 10% OC diet compared to the other OC diets supplemented with the same level of citric acid. In addition, citric acid added at 0.2% to the OC-free diet significantly increased liver weight percentage when compared to the other OC diets supplemented with the same level of citric acid. This increase in relative weight of liver due to citric acid supplementation may indicate an increase in ME availability (Islam, 2012) that did not negatively affect liver enzymes, showing the safety of citric acid as a feed additive for laying hens (Rostamzad et al., 2011; Islam, 2012).

Citric acid at 0.2% to laying hens’ diets containing 10% OC significantly decreased the relative weight of intestinal compared to their counterpart unsupplemented controls and to a 0.1% citric acid supplemented diet. The same

### Table 6. The effect of olive cake concentration with or without citric acid supplementation on internal organs relative weight of laying hens.

| Effect of olive cake concentration, % | Abdominal fat weight, % | Liver weight, % | Heart weight, % | Pancreas weight, % | Gizzard weight, % | Intestinal weight, % | Ovary weight, % | Oviduct weight, % |
|-------------------------------------|-------------------------|----------------|----------------|---------------------|-------------------|---------------------|----------------|------------------|
| Control                            | 2.29<sup>a</sup>        | 2.10           | 0.480         | 0.219<sup>a</sup> | 1.31<sup>a</sup>  | 4.00<sup>a</sup>  | 2.82<sup>a</sup> | 3.64<sup>a</sup> |
| 10                                 | 4.35<sup>a</sup>        | 2.15           | 0.460         | 0.248<sup>a</sup> | 1.40<sup>a</sup>  | 2.53<sup>a</sup>  | 3.55<sup>a</sup> | 3.13<sup>a</sup> |
| 20                                 | 4.65<sup>a</sup>        | 2.08           | 0.475         | 0.198<sup>a</sup> | 1.60<sup>a</sup>  | 2.31<sup>a</sup>  | 3.91<sup>a</sup> | 3.27<sup>a</sup> |
| Effect of citric acid supplementation, % | Abdominal fat weight, % | Liver weight, % | Heart weight, % | Pancreas weight, % | Gizzard weight, % | Intestinal weight, % | Ovary weight, % | Oviduct weight, % |
| Control                            | 3.63                    | 2.17<sup>a</sup> | 0.469         | 0.227               | 1.48              | 3.19               | 3.17<sup>a</sup> | 3.35              |
| 0.1                                | 3.78                    | 1.95<sup>b</sup> | 0.479         | 0.212               | 1.41              | 3.08               | 3.75<sup>b</sup> | 3.25              |
| 0.2                                | 3.89                    | 2.21<sup>c</sup> | 0.467         | 0.225               | 1.42              | 2.76               | 3.36<sup>c</sup> | 3.44              |
| Interaction among treatments       |                         |                |               |                     |                   |                   |                |
| 0 0                                | 2.02                    | 2.25<sup>ab</sup> | 0.487         | 0.234               | 1.41              | 3.90<sup>ab</sup> | 2.80             | 3.36              |
| 0 0.1% citric                      | 2.71                    | 1.56<sup>c</sup> | 0.504         | 0.207               | 1.29              | 3.56<sup>c</sup>  | 3.02             | 3.95              |
| 0 0.2% citric acid                | 2.14                    | 2.48<sup>a</sup> | 0.450         | 0.215               | 1.22              | 4.54<sup>a</sup>  | 2.64             | 3.61              |
| 10 0                               | 4.16                    | 2.04<sup>a</sup> | 0.434         | 0.231               | 1.40              | 3.19<sup>cd</sup> | 3.19             | 3.13              |
| 10 0.1% citric acid               | 3.96                    | 2.35<sup>a</sup> | 0.454         | 0.235               | 1.32              | 2.72<sup>ab</sup> | 3.94             | 2.78              |
| 10 0.2% citric acid               | 4.93                    | 2.65<sup>a</sup> | 0.491         | 0.282               | 1.50              | 1.67<sup>c</sup>  | 3.53             | 3.48              |
| 20 0                               | 4.70                    | 2.22<sup>ab</sup> | 0.486         | 0.216               | 1.63              | 2.48<sup>ab</sup> | 3.52             | 3.56              |
| 20 0.1% citric acid               | 4.66                    | 1.92<sup>c</sup> | 0.478         | 0.199               | 1.63              | 2.98<sup>c</sup>  | 4.29             | 3.03              |
| 20 0.2% citric acid               | 4.60                    | 2.10<sup>ab</sup> | 0.460         | 0.178               | 1.54              | 2.08<sup>c</sup>  | 3.93             | 3.32              |
| SEM                                | 0.260                   | 0.101          | 0.030         | 0.014               | 0.062             | 0.250             | 0.283            | 0.187             |

Probability level

| Olive cake                          | 0.001                  | 0.811          | 0.778         | 0.004               | 0.001             | 0.001             | 0.001            | 0.029             |
| Citric acid                        | 0.626                  | 0.940          | 0.917         | 0.525               | 0.529             | 0.299             | 0.023            | 0.615             |
| Interaction                        | 0.194                  | 0.001          | 0.653         | 0.101               | 0.266             | 0.013             | 0.717            | 0.069             |

<sup>a</sup>Differences among means within a column within each factor not sharing similar superscripts are significantly (P<0.05) different among treatments.
group had also a significantly lower relative weight of intestinal compared to its respective control group on OC-free diet. The decrease in intestinal (%) due to citric acid supplementation at 0.2% for the groups fed diets containing 10% and 20% OC may be a beneficial effect that indicates lower energy consumption by the intestine, which is the largest energy-consuming organs (Al-Harthi, 2015). This could be attributed to the antimicrobial effect of citric acid (Chowdhury et al., 2009; Islam, 2012).

Conclusions

The addition of 10% and 20% OC without citric acid supplementation to the diets of laying hens had no effects on laying performance, egg quality and blood metabolites. However, using these levels induced an increase in feed intake and alternations in the FCR compared with the control group (0% OC). Even so, supplementing the 20% OC diet with 0.1% citric acid overcame the shortage in FCR. An increase in relative weight of abdominal fat and ovary weights was noted. Citric acid supplementation of 0.1% significantly decreased relative weight of liver compared to the other citric acids levels and this is obvious in OC-free diet, but it increased ovary ratios compared with control group.

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