METABOLISM AND NUTRITION

Comparative evaluation of dietary supplementation with mannan oligosaccharide and oregano essential oil in forced molted and fully fed laying hens between 82 and 106 weeks of age

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ABSTRACT The aim of this study was to investigate the efficacy of feed-grade preparations of mannan oligosaccharides (**MOS**) and oregano essential oil (**OEO**) in forced molted or fully fed 82-week-old, laying hens. A 2 × 3 factorial experiment investigated the influence of molting vs. full feeding and dietary supplements [i.e., unsupplemented control, MOS (1 g/kg) diet, and OEO (24 mg/kg) diet] on production parameters, egg quality, serum stress indicators, blood constituents, tibial characteristics, liver antioxidant status, and cecal microflora composition. A total of 864 Single Comb White Leghorn hens were randomly assigned to 6 treatments, each with 6 replicates of 24 hens each, and studied for 25 wk. Hens were fed a molt diet containing of 50% alfalfa and 50% wheat bran (**aa+wb**) for 12 d, then returned to the laying ration. Results indicate that molt vs. full feed impacted more on most variables measured than supplementation or supplement type. Significant ($P < 0.01$) interactions between molting and diet were observed for the egg production, egg weight, egg mass, and feed conversion ratio (**FCR**). In fully fed hens, MOS supplementation improved ($P < 0.01$) the egg production, egg weight, and FCR, and an OEO addition significantly improved the egg production and FCR in forced molted hens. Molting improved egg quality despite the significant regression in ovary and oviduct weight ($P < 0.01$), though supplements showed no influence. The bone ash ($P < 0.01$) and mineral content ($P < 0.05$) of molted hens were significantly lower than those of fully fed counterparts; however, poor mineralization was not reflected in the bones' mechanical properties. No significant differences were observed among treatments for hematological characteristics. Both the MOS and particularly the OEO supplementation improved ($P < 0.01$) liver antioxidant status and mitigated the significant increase in cecal pathogenic bacteria after molt. Our results indicate that full feeding with an aa+wb diet is an effective non-feed-removal method for molted hens, the benefit of which can be improved with MOS and OEO supplementation.

Key words: Molt diet, mannan oligosaccharide, oregano oil, bone strength, cecal microflora

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INTRODUCTION

Induced molting is an effective means of economically managing laying flocks (Zimmerman et al., 1987; Bell, 2003). Feed withdrawal is the primary procedure used by the poultry industry to induce molt and thereby stimulate multiple egg-laying cycles in hens (Holt, 1995; Koelkebeck et al., 2006). However, concerns regarding the effects of fasting on the welfare of hens and reports suggesting that molting hens can be more susceptible to *Salmonella* infection have prompted calls for feed withdrawal to be banned (Brake, 1993; Bell, 2003). Increased scrutiny of animal welfare and food safety in recent years (Gast and Ricke, 2003; Park et al., 2003) in association with feed-withdrawal methods has supported these calls. Opposition to this traditional method, which has been used by the poultry industry for more than half a century, has encouraged the development of alternative methods that refrain from feed withdrawal (Holt, 2003; Donaldson et al., 2008).

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Alternative molting approaches have applied dietary-modification strategies that involve alternative feedstuffs such as wheat middlings (Seo et al., 2001; Biggs et al., 2003) and alfalfa (Donaldson et al., 2005, 2008; Landers et al., 2005). These studies have shown that feeding strategies involving low-energy, low-calcium feeds can effectively induce molt, improve post-molt production numbers, and inhibit *Salmonella enteritidis* infection.

Such promising evidence of using feedstuffs with high fiber content and offering access ad libitum instead of feed deprivation during molting appear to allow opportunities for the supplementation of in-feed agents such as prebiotics and plant extracts that have proven efficacy against intestinal pathogens (Baurhoo et al., 2009). The results of a recent study carried out on older laying hens have proved the beneficial effects of prebiotic oligofructose on tibia bone-breaking strength and yielding load (Swiatkiewicz et al., 2010). Non-digestible ingredients such fructo-oligosaccharides and mannan oligosaccharides (**MOS**) are widely used as prebiotics in the poultry industry and have proven efficacy as in-feed antimicrobial agents against enteric pathogens in broiler chickens and laying hens (Hernandez et al., 2004; Baurhoo et al., 2009), which in turn enhances their productivity. They also act as quantitatively available substrates for gastrointestinal tract microflora (Roberfroid et al., 1998), enhance the growth of beneficial bacteria (*Bifidobacterium* and *Lactobacillus*), and inhibit pathogenic bacteria such as *Escherichia coli* and *Salmonella* spp. (Xu et al., 2003).

In association with strong *in vitro* and *in vivo* antibacterial properties against food-borne pathogens (Cowan, 1999; Jang et al., 2007), as well as antioxidant properties (Sivropoulou et al., 1996), oregano essential oil (**OEO**) has garnered keen interest in poultry nutrition. OEO's benefits for the digestive tract include stable intestinal microbial ecology, which improves nutrient use and absorption (Diaz-Sanchez et al., 2015) by increasing the activity of digestive enzymes such as trypsin, lipase, and amylase (Basmacıoğlu et al., 2010). Improved laying rate, eggshell quality, bone strength, and immune response were also reported when either MOS or OEO was added to laying hens' diets (Shashidhara and Devegowda, 2003; Kim et al., 2006; Gürbüz et al., 2011; Bozkurt et al., 2012a,b).

With regards to the possible role of MOS and OEO in improving gut function, immunity, antioxidative defenses and the promotion of productive performance, the existing evidence remains rather limited with respect to the conditions of forced molting. In most cases, such evidence is either preliminary or nonexistent, particularly concerning certain essential oils (**EOs**). Hence, understanding their positive roles during molting requires additional experimental studies, particularly studies that address the negative effects of drastic shifts in feeding and lighting regimens upon the intestinal microbial ecology and welfare of hens.

The aim of this study is to compare 2 traditional laying-hen-replacement programs used by the poultry industry. The concept of using wheat bran in molt diet is quite new and supposed to ensure that supplements are more homogenously distributed. Moreover, in an attempt to further increase the efficacy of the molt diet and the regular-laying-hen diet, MOS and OEO were supplemented at proven inclusion rates. The efficacy of the hen-replacement program (i.e., fully fed vs. molted) and the diet (i.e., unsupplemented vs. supplementation with MOS or OEO) was investigated using a 2 × 3 factorial arrangement, in which egg production, egg quality, characteristics of hematology, liver antioxidant status, immune response, bone chemical and mechanical properties, and the cecal microbial composition of laying hens were evaluated from 82 to 106 wk of age.

MATERIALS AND METHODS

Birds and Housing

The Adnan Menderes University Animal Care and Use Committee approved the techniques and procedures involved in the animal care and handling. An experiment was conducted using 864 Single Comb White Leghorn hens of the Lohmann White strain (82 wk of age). The experiment lasted between May 21 and November 11, 2014, for a 25-week period. The hens were housed in a caged layer house of commercial design with water and feed provided ad libitum. Hens were kept under standard management procedures without performance-enhancer feed additive prior to the start of the experiment (i.e., from 17 to 80 wk of age). The experimental house comprised 2 identical blocks of a 3-tier cage facility which were separated by an aisle of 1.40 m width and 15 m length. The experiment used a 2 × 3 factorial design consisting of 2 types of hen-replacement strategy, namely fully fed and molted, and 3 diets (unsupplemented and supplemented with either MOS or OEO). Six replicate groups of 24 hens each (4 adjacent raised wire cages, 60 × 50 × 46 cm, containing 6 hens per cage) were allotted to each dietary treatment in a randomized block design. Thus, fully fed hens were distributed to one of the 2 blocks while assigned to one of the following 3 dietary treatments, and also the molted hens. Birds were weighed individually at 80 wk of age for the aim of eliminating potential culls prior to molt and to ensure similar body weight (**BW**) means among the different treatments prior to the initiation of the experiment. The experiment consisted of a 12-d molt period followed by a 163-d post-molt production period (82 to 106 wk of age). Egg production, egg weight, and egg quality were monitored before the start of the treatments (80 to 82 wk of age) to ensure that all hens were healthy and actively producing according to the recommendations of the breeder (Lohmann LSL, Commercial Management Guide, 2007)[1].

[1]Lohmann LSL–Classic. Layer Management Guide, 2007. Lohmann Tierzucht, Cuxhaven, Germany.

During the molt period (82 and 83 wk of age), a dark-blue nylon cover of 3 m height and 20 m length that allows no light transfer was hung between the 2 blocks in order to isolate the management procedure of the molted hens from that of the unmolted hens. Much effort was paid to be as silent as possible during the application of routine farm practices (e.g., feed distribution, egg collecting, and removing of manure) wherein the section of fully fed hens maintained their egg production. The separate cover was removed at the end of the molt program (i.e., 12 d), and then management practices were the same for the 2 blocks of hens until the end of the experiment.

The experimental house was mechanically ventilated with adjustable windows at side walls and a tunnel ventilation fan with a flow rate of 2.5 m/s. The average daily mean temperature during the experiment in this region was 24°C (mean of highest temperatures 32°C and of the minimum 16°C) and the mean relative humidity value was 58%. The lighting regimen for molted hens changed from 16L:8D to 12L:12D on the first day of feed transition (from regular layer diet to molt diet) and remained there for 12 d, then returned to 16L:8D for the remainder of the trial. Illumination was supplied by 36-W florescent lamps providing 3.8 lux of illumination. Fully fed hens were exposed to a photoperiod of 16 h throughout the experiment.

Experimental Diets

The molting replacement program included the ad libitum consumption of a diet containing 50% alfalfa meal and 50% fine wheat bran for 12 d (Table 1). The nutritional composition of the molt diet is presented in Table 1. The molting regimen provided water for ad libitum consumption. At 13 d post molt, hens from molting treatment were returned to a regular egg-laying diet (Table 1) and kept on ad libitum consumption of the layer diet until the end of the experiment. The fully fed control hens consumed the regular egg-laying diet and water ad libitum throughout the experiment.

Hens in the control group (**CNT**) were given a corn-soybean-based basal diet supplemented with no performance-enhancer additive. Table 1 shows the ingredients and the nutritional composition of the basal laying-hen diet. The remaining 2 groups were given the same basal diet supplemented with an additional 1 g/kg MOS (Bio-Mos®, Alltech Inc., Nicholasville, KY) or 24 mg/kg OEO (WILDMIX®, İnan Tarım-ECODAB® Ltd. Co., Antalya, Turkey). Both were added at the expense of sawdust as inert filler. In order to ensure homogenous distribution, the MOS (1000 g) and OEO (300 g) preparations were added into 1000 g and 1700 g saw dust, 2 kg of each, mixed in a laboratory-type mixer for 30 s, and then supplemented into 1 ton of the basal diet and molt diet.

The OEO is derived from the herb *Origanum minutiflorum*, growing wild in Turkey, by steam distillation.

Table 1. Ingredients and nutrient composition (% as fed basis unless otherwise indicated) of the basal laying hen diet and molt diet.

Ingredients (g/kg)	Basal laying hen diet	Molt diet
Alfalfa meal		49.90
Fine wheat bran		49.90
Corn	55.49	
Soybean meal (48% CP)	21.46	
Sunflower meal (36% CP)	3.05	
Corn gluten meal (60% CP)	5.00	
Soybean oil	3.41	
Di-calcium phosphate	1.89	
Ground limestone	8.60	
NaCl	0.30	
DL-Metionine (99%)	0.15	
L-Threonine	0.04	
L-Lysine	0.01	
Vitamin premix[1]	0.25	
Mineral premix[2]	0.10	
Choline chloride	0.05	
Saw dust[3]	0.20	0.20
Analyzed nutrient content (%)		
Dry matter	88.68	90.93
Crude protein (N × 6.25)	17.47	14.83
Ether extract	5.13	2.87
Crude ash	11.06	7.80
ADF	3.19	22.13
NDF	8.07	38.53
Starch	38.05	3.62
Sucrose	2.98	2.03
Calcium (Ca)	3.94	0.78
Phosphorus (P) (total)	0.71	0.82
Sodium (Na)	0.18	0.06
Chloride (Cl)	0.27	0.32
Specific gravity (g/cm^3)	0.75	0.24
Calculated nutrient content (%)		
P (available)	0.44	0.30
Lysine	0.80	0.60
Methionine	0.43	0.20
Threonine	0.69	0.54
Valine	0.83	0.65
AME (kcal/kg)	2,864	1,093

[1]Vitamin premix contained the following per kilogram of diet: vitamin A (retinyl acetate), 4.12 mg; vitamin D_3 (cholecalciferol), 60 μg; vitamin E (DL- α-tocopheryl acetate), 32.96 mg; vitamin K_3, 3 mg; vitamin B_1, 3 mg; vitamin B_2, 7 mg; vitamin B_6, 4 mg; vitamin B_{12}, 0.02 mg; nicotine amid 40, mg; Ca-D- pantothenate, 8 mg; folic acid, 1 mg; biotin, 0.045 mg; vitamin C, 50 mg; choline chloride, 125 mg.

[2]Mineral premix contained the following per kilogram of diet: Mn, 80 mg; Fe, 40 mg; Zn 60 mg; Cu, 5 mg; I 0.4 mg; Co, 0.1 mg; Se 0.15 mg.

[3]Sawdust was substituted by MOS or OEO preparations.

It contained carvacrol (81.69%), δ-3-caren (4.15%), thymol (2.06%), and p-cymen (2.02%) as the main active components. The essential oil preparation used 920 g of zeolite as a feed-grade inert carrier for each 80 g of OEO. Thus, in the present study, the OEO preparation of 300 g contained 24 g pure oregano oil which provided 19.60 mg carvacrol, 1.00 mg δ-3-caren, 0.49 mg thymol, and 0.48 mg p-cymen per kg of diet. Compounds in the OEO are shown in Table 2. The composition of the OEO was determined using the GC/MS (HP 6890GC/5973 MSD) system. Subsequent analyses were carried out according to the procedure as mentioned by Bozkurt et al. (2014).

The hens were given 2 wk (from 80 to 81 wk of age) to acclimatize to the experimental diets before the initiation of the experiment. All the diets were isonitrogenous and isocaloric, and were fed in mash form. The

Table 2. Bioactive components of the oregano essential oil (steam distillation of *Origanum minutiflorum*).

Compound	Value (%)	Compound	Value (%)
Carvacrol	81.69	β-Myrcene	0.35
δ-3-caren	4.15	Sabinen	0.16
Thymol	2.06	Carvacryl acetate	0.16
p-cymen	2.02	α-amorphene	0.14
β-bisabolene	1.80	(+) Aromadendren	0.13
γ-terpinen	1.54	Camphene	0.13
Trans caryophyllene	1.25	(+) Carvon	0.11
(+) Borneol	1.05	α-terpinolen	0.07
α-Pinen	0.47	Limonen	0.07
α-Phellandrene	0.43	Undefined	2.22

experimental diets were formulated to meet the nutritional requirements for layer hens according to the recommendations of the breeder (Lohmann LSL, Commercial Management Guide, 2007)[2].

Sample Collection and Analyses

All hens were weighed individually at 82, 94 and 106 weeks of age. Hen/day egg production was recorded daily from 82 to 106 wk of age. During this period, a random sample of 36 eggs/treatment/day was collected on two consecutive days every week (6 eggs per replicate per day). Therefore, a total of 1,800 eggs were weighed in each treatment to determine the average egg weight throughout the trial. The feed intake and feed conversion ratio (**FCR**) were determined at 7-d intervals. The FCR was expressed as kilograms of feed consumed per kg of egg produced (kg feed/kg egg). Egg mass was calculated by multiplying egg weight by egg production rate. All production variables were determined on replicate basis. The magnitude of production variables such as feed intake and egg production were adjusted for hen mortalities. Hen mortality was recorded as it occurred.

An additional sample of 24 eggs was randomly collected from each experimental group (4 eggs per replicate) every 28 days to assess eggshell quality parameters. Therefore, 864 eggs in total were analyzed for egg quality. The first examination for egg quality characteristics was performed at the end of the 86 wk of age, then concomitant analyses were executed within stated intervals. Egg shell quality characteristics including egg shell weight, strength, and thickness were measured as described by Bozkurt et al. (2012a). Egg shell weight is defined as a percentage of the egg weight. Yolk height and yolk diameter were measured using a micrometer (model IT-014UT-Mitutoyo, Kawasaki, Japon). The Haugh unit (HU) was calculated as Haugh units (%) = $100 \times \log (H + 7.57 - 1.7\ W^{0.37})$, where H is the height of the albumen and W is the weight of the egg, according the formula proposed by Haugh (1937). The egg yolk with the albumin was placed on a tray, which was enclosed in a completely dark module of the egg quality measuring equipment (SANOVO), and then the intensity of the yolk color was compared with matching color numbers in the Roche yolk color fan (Vuilleumier, 1969) within 4 seconds.

The nutrient content of the diets was determined by proximate analysis (Naumann and Bassler, 1993). The experimental diets were analyzed for dry matter, crude protein, ether extract, crude ash, crude fiber, starch, sugar, total calcium (**Ca**) and phosphorus (**P**) content using methods outlined by the Association of German Agricultural Analysis and Research Institutes (**VDLUFA**) for the chemical analysis of feedstuff (Naumann and Bassler, 1993). Neutral detergent fiber (**NDF**) and acid detergent fiber (**ADF**) were analyzed sequentially (Van Soest et al., 1991). Metabolizable energy (**ME**) concentrations of the laying hen diet and molt diet were estimated using the equation by Carpenter and Clegg (1956): ME (Kcal/kg) = 53 + 38 × [CP (%) + 2.25 × ether extract (%) + 1.1 × starch (%) + 1.05 sugar (%)]. Analyses of experimental diets were also duplicated to guarantee that they were identical regarding chemical composition with the exception of the supplements.

Blood Sampling and Laboratory Analysis

Six d after the beginning of the experiment, 12 birds at 83 wk of age from each treatment were randomly selected (2 birds per replicate), and wing tagged. Blood was drawn from wing vein using sterilized needles and syringes in vacutainer tubes for serum collection. Feed was not withdrawn from the feeder before blood was collected. Blood samples were allowed to stand for 2 h at room temperature to allow proper clotting. The samples were then centrifuged at 1,700 × *g* for 10 minutes. Serum total cholesterol (**CHOL**) (Archem, A2091, Istanbul, Turkey), glucose (**GLU**) (Archem, A2191, Istanbul, Turkey) total protein (Archem, A2301, Istanbul, Turkey), calcium (Archem, ASX2062, Istanbul, Turkey) and phosphorus (Archem, A22291, Istanbul, Turkey) concentration was measured using commercial available test kits at an autoanalyzer (Sinnowa D280, China). Sodium and chloride levels were analyzed at photometer (Sinnowa BS-3000P, China) using ready commercial test kits (Teco Diagnostic, CA). Corticosterone (**CS**) (Enzo Life Sci, ADI-900-097, Farmingdale, NY) was measured using commercial colorimetric competitive enzyme immunoassay kit according to the manufacturer's instructions at ELISA reader (Thermo Multiskan FC, Thermo Scientific, Waltham, MA). Individual serum samples were analyzed for antibody responses against Newcastle disease virus (NDV) by the ELISA technique using commercial kits (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The plates were read at 405 nm on an ELISA reader (Labsystems Multiscan MS, Labsystems, Helsinki, Finland). The same blood samples were used for further analysis of hematological parameters.

[2]Lohmann LSL–Classic. Layer Management Guide, 2007. Lohmann Tierzucht, Cuxhaven, Germany.

Organ Weights and Intestinal Measurements

The birds that had been used for blood sampling before were euthanized by cervical dislocation, eviscerated, and their proventriculus, gizzard, liver, and pancreas, spleen, complete intestines, abdominal fat, ovary, and oviduct were removed. The total length of the small intestine (duodenum, jejunum, and ileum) provided the intestinal length and intestinal weight was determined after the system being absolutely emptied. The weight of these visceral organs and abdominal fat were expressed as a percentage of live body weight. The number of follicles on the ovary, having a diameter greater than 10 mm, was measured using a micrometer (model IT-014UT-Mitutoyo, Kawasaki, Japon). The birds euthanized for organ measurements were also used for further analysis of liver antioxidant status, tibial characteristics, and cecal microbiological analysis.

Measurement of Bone Mechanical Properties

Following the organ sampling, the left and right tibias with some attached flesh were collected. Bones were excised from the fresh carcasses, and all flesh and proximal cartilages were removed. While the left tibias were used for the determination of bone ash and mineral content, the right ones used for measuring the bone mechanical properties and bone size (i.e., bone diameter and wall thickness). The tibias were individually sealed in plastic bags to minimize moisture loss. The sample bags were placed in a plastic container and stored at –20°C until analysis. The bones were thawed at room temperature for 6 h in an air-conditioned room before the measurements began. The bone mechanical properties were determined using the procedure as outlined by Wilson and Ruszler (1996) and Armstrong et al. (2002).

Tibia Ash and Mineral Analysis

Each tibia (i.e., the left ones) was broken into small pieces, weighed, oven-dried at 105°C for 24 h, cooled in a desiccator, weighed, dry-ashed at 600°C for 12 h, cooled in a desiccator, and weighed (AOAC, 1990). Bone weight was determined on dry defatted weight basis. The ash content was expressed as a percentage of the dry bone weight.

The mineral contents of and tibias of 12 samples per treatment were analyzed. The Ca and P concentrations were determined using the following method. Ultrapure HNO_3 (5 mL, Merck) was added to each ash sample until it was completely dissolved; afterwards, 20 mL of de-ionized water was added to each sample. The samples were filtered using WH 42 filter paper. The obtained solutions were diluted with de-ionized water to a final volume of 100 mL. The concentrations of minerals were measured at specific wavelengths for each element with an ICP-OES (Perkin Elmer Optima 2100 DV). Subsequent tests were conducted according the procedure as outlined by Küçükyılmaz et al. (2014).

Determination of Heterophils and Lymphocytes Ratio

Following the procedure of blood sampling, two drops of blood were also collected from the wing vein of each bird and smeared on each of 2 glass slides. The smears were stained with Wright stain for 15 min. One hundred leucocytes, including heterophils (**H**), lymphocytes (**L**), monocytes, basophils and eosinophils, were counted on each slide and the H/L ratio was calculated by dividing the number of heterophils by that of lymphocytes. The means of the 2 slides were calculated for each bird (Gross and Siegel, 1983).

Liver Antioxidant and Oxidant Status

Lipid peroxidation was determined using the procedure described by Yoshioka et al. (1979), in which malondialdehyde (**MDA**), an end product of fatty acid peroxidation, reacts with tribarbituric acid (**TBA**) to form a colored complex with a maximum absorbance at 532 nm. Total antioxidant status (**TAS**) of the supernatant was determined using an automated measurement method with a commercial available kit (Total Antioxidant Status Assay kit, Rel Assay Diagnostics, RL0017, Turkey). Using this method, the antioxidative effect of the sample is measured against the potent free radical reactions, initiated by the reduced hydroxyl radical. The results are expressed as mmoL trolox equiv./mg protein. To measure superoxide dismutase (**SOD**) activity in supernatant incubated with xanthine oxidase solution for 1 h at 37°C. Absorbance was read at 490 nm to generate superoxide anions. SOD activity is determined as the inhibition of chromogen reduction. In the presence of SOD, superoxide anion concentration is reduced, yielding less colorimetric signal (OxiSelect^{TM} Superoxide Dismutase Activity Assay, Cell Biolabs, STA-340, USA). SOD activity was shown in U/mg protein.

Enumeration of Cecal Microflora

During evisceration, the distal intestines were removed aseptically. The intestines were then divided into sections (i.e., ileum, ceca, and colon), ceca were ligated with silk catgut before separating the ceca from the small intestine. The ceca samples were immediately frozen at −80°C, sealed in sterile bags filled with 50 mL ice-cold cryoprotective broth as mentioned by Mountzouris et al. (2007) and immediately stored at −80°C until subsequent analyses.

Cecal digesta contents were then aseptically emptied in a new sterile bag and were immediately diluted tenfold (i.e., 10% w/v) with sterile, ice-cold, anoxic

PBS (0.1 M, pH 7.0) and subsequently homogenized for 3 min in a stomacher (Bagmixer® 100 Minimix, Interscience, Arpents, France). Each cecal digesta homogenate was serially diluted from 10^{-1} to 10^{-7}. Dilutions were subsequently plated on selective agar media, in duplicate, for the enumeration of target bacterial groups.

In particular, total aerobes, coliforms, total anaerobes, *Clostridium* spp., *Lactobacillus* spp., *Bifidobacterium* spp. and gram-positive cocci were enumerated using nutrient agar, MacConkey agar, Wilkens–Chalgren agar, Reinforced Clostridial agar, Rogosa agar, Beerens agar and Azide agar (Tuohy et al., 2002; Mountzouris et al., 2011). Plates were then incubated at 39°C, for 24 to 72 h aerobically (nutrient and MacConkey agars) or 48 to 120 h anaerobically (Wilkens–Chalgren, Clostridial, Beerens, Rogosa and Azide agars) and colonies were counted. Anaerobic incubation was achieved using appropriate catalysts (AnaeroGen®, Oxoid, Hampshire, England) in sealed anaerobic jars (Oxoid, Basingstoke, UK). Results were expressed as $\log_{10}$ CFU/g cecal digesta.

Statistical Analyses

The experiment used a completely randomized design, and each experimental unit was a replicate consisting of 6 groups of adjacently caged layer hens fed as one group. Data regarding the period of 82 to 83 wk were subjected to ANOVA using the SAS Institute's GLM procedure (2001). Data collected between 84 and 106 wk were analyzed on a two-factorial ANOVA using the SAS Institute's GLM procedure (2001). The main effects of replacement program, diet, and the replacement-by-diet interaction were tested. Arc-sin transformation was applied to the percentage values before testing for differences. Duncan's multiple range test was carried out to detect differences among treatments. All differences were considered significant at $P < 0.05$.

RESULTS

Performance During Molt

The effects of the dietary supplements MOS and OEO on the hens' BW, percentage of BW loss, feed intake, and mortality rate during the 12-d molt period are presented in Table 3. At the end of the period, the hens on the CNT, MOS, and OEO diets lost 22.4, 23.4, and 23.3% of their initial BW, respectively; none of these rates differed significantly from each other ($P > 0.05$). Data gathered from the experiment suggested that the non-feed-removal molt program with aa+wb effectively induced BW loss and the total cessation of egg production, while neither the MOS nor the OEO treatment played a distinctive role ($P > 0.05$). All molt treatments effected rapid reductions in egg production, which ceased after 8.0, 8.4, and 8.3 d for the CNT, MOS, and OEO treatment groups, respectively ($P > 0.05$). Hens returned to egg production 9 d after they commenced feeding with the regular laying diet. The mean periods until the resumption of egg production (i.e., the period from initial molt to resumed egg production) in the CNT, MOS, and OEO treatment groups were 21.0, 20.3, and 20.5 d, none of which differed significantly from the others ($P > 0.05$).

During the 12-d molt period, the average feed consumption of molted hens was approximately 16 g/hen/d and did not differ among the varying treatments

Table 3. Body weight, body weight loss, feed intake, egg production rate, and mortality rate of fully fed and molted hens fed on an aa+wb molt diet with or without MOS and OEO for 12 d (at 82 and 83 wk of age).

	Body weight (g)			Feed consumption (g/hen/d)		Egg production rate (%)		
Item[1,2]	At initiation of the molt	At the end of the molt	Body weight loss (%)	Period 1[X]	Period 2[z]	Period 1	Period 2	Mortality (%)
Fully fed[3]	1,806	1,813		106.4	106.9	89.4	89.2	0.00
Molted[4]								
CNT[5]	1,833	1,422	22.4	12.2	18.7	35.2	0.74	0.7
MOS[5]	1,803	1,380	23.4	12.5	19.3	36.8	0.40	1.4
OEO[5]	1,810	1,388	23.3	12.8	19.1	32.5	0.30	0.7
Pooled SEM[6]	18.80	15.39	0.63	0.26	0.22	12.22	0.33	0.85
P-value	0.81	0.12	0.49	0.26	0.25	0.96	0.89	0.81

[1]The statistical comparisons were made among the 3 molt treatments excluding the fully fed CNT treatment. Data regarding fully fed CNT hens are only informative. No performance indices measured during this period were influenced ($P > 0.05$) by dietary treatments in fully fed hens.

[2]Cumulative egg number and egg mass output of the flock between 20 and 81 wk of age was 364.6 and 23.2 kg with a survival rate of 92.8%.

[3]Hens fed ad libitum on a regular layer-hen diet with no MOS or OEO and not subjected to molting.

[4]Hens molted on an aa+wb diet with ad libitum intake for 12 d.

[5]The laying hens were fed on a control diet (CNT) that contained no performance enhancer and was supplemented with preparations of mannan oligosaccharide (1 g/kg of diet; MOS) and oregano essential oil (24 mg/kg of diet; OEO).

[6]Data are means of 6 replicates of 4 adjacent cages with 24 hens each per treatment.

[x]Period 1 includes d 1 to 6 of the 12-d molt period.

[z]Period 2 includes d 7 to 12 of the 12-d molt period.

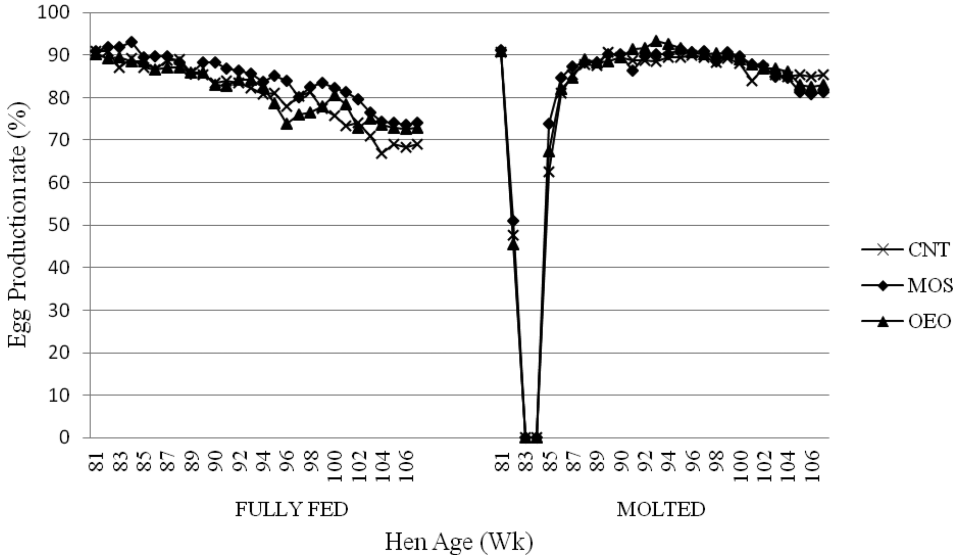

Figure 1. Hen-day egg production of fully fed and molted hens between 82 and 106 wk of age. Molted hens fed on an aa+wb for 12 d (from 82 to 83 wk of age) then returned to regular layer-hen diet. The laying hens were fed on a control diet (CNT) that contained no performance enhancer and was supplemented with preparations of mannan oligosaccharide (1 g/kg of diet; MOS) and oregano essential oil (24 mg/kg of diet; OEO).

($P > 0.05$). The molt diet induced an 85% reduction in average feed consumption compared to that of the full-feeding program. The mortality rate was low and did not differ ($P > 0.05$) among the dietary treatments and replacement programs. No performance indices measured during the period were influenced ($P > 0.05$) by dietary treatments in fully fed hens (Table 3).

Post-Molt Egg Production and Egg Quality

The egg production rates of fully fed and molted hens treated with MOS- and OEO-supplemented diets are shown in Figure 1. The grand mean egg production rate of the flock at the beginning of the experiment (i.e., at 82 wk of age) was 89.4%. At the end of the study, the mean laying rate of the fully fed hens (72%) dropped below the productivity (83.1%) of the molted hens. The molted hens egg production rates reached 25 and 75% of the laying rates of fully fed hens approximately 12 and 16 d after the commencement of the full feeding of the regular-laying diet, respectively, and attained the production level of their fully fed counterparts 30 to 32 d after the implementation of the molt program.

No replacement program and diet interaction was observed for the BW of hens. Molted hens were 51 g lighter ($P < 0.01$) than unmolted hens at 94 wk of age (1716 g vs. 1767 g); however, there was no significant difference ($P > 0.05$) among the treatments at 106 wk of age (mean BW = 1832 g, SEM = 22.6; data not shown). In summary, MOS and OEO did not significantly influence the BW of aged hens during the course of this study ($P > 0.05$).

Table 4 shows the influence of the treatments on egg production rate, egg weight, egg mass output, feed intake, FCR, mortality, and cumulative egg production from the beginning of treatment, when the hens were aged 82 wk, until the end of the experimental period, when the hens were aged 106 wk. Significant interactions between replacement program and diet ($P < 0.01$) characterized egg production rate, egg weight, egg mass, and FCR. In molted hens, supplementation with MOS and OEO increased the egg production rate compared with that of the unsupplemented treatment. Whereas, only MOS increased egg production when hens were fully fed. The supplements did not influence egg weight under the molting regimen. However, the supplementation diet containing MOS and OEO significantly ($P < 0.01$) reduced egg weight as compared to CNT treatment under the full-feeding program, though the effect was far more pronounced in hens fed OEO. Nonetheless, fully fed hens treated with MOS produced eggs of at least 2 g in mass greater than those hens in the CNT and OEO treatments, whereas egg mass output remained unaffected by dietary treatments when the hens were subjected to molting.

The daily feed intake of molted hens was far higher (4.25 g) than those of their fully fed counterparts ($P < 0.01$) and this was evident during the entire post-molt production phase. Dietary supplementation with MOS increased the feed intake of hens by approximately 1 g compared with hens in the CNT treatment ($P < 0.01$), whereas no significant effect was observed in the OEO treatment group compared to CNT treatment. The fully fed hens responded to the MOS supplementation with a significant reduction in FCR ($P < 0.01$); however, this was the case for OEO supplementation when hens were exposed to the molt program.

The overall mean of total hen/housed egg number in hens fed MOS was significantly higher ($P < 0.05$)

Table 4. Effect of the hen-replacement program with and without dietary MOS and OEO supplementation on egg production rate, egg weight, egg mass output, feed intake, feed conversion ratio (FCR), mortality, and total egg yield of hens from 82 to 106 wk of age.[1]

Item	Diet	Egg production rate (%)	Egg weight (g)	Egg mass (g/d)	Feed intake (g/hen/d)	FCR (kg feed/kg egg)	Mortality (%)	Cumulative egg number
Fully fed	CNT	81.26[d]	68.46[b]	55.53[c]	105.7	1.92[a]	2.01	141.9[b]
	MOS	84.86[c]	67.68[c]	57.43[b]	107.2	1.87[b]	4.11	146.1[a]
	OEO	81.63[d]	67.42[d]	55.03[c]	105.9	1.93[a]	2.86	141.4[b]
Molted	CNT	86.46[b]	69.21[a]	59.84[a]	110.8	1.86[b]	2.80	132.9[c]
	MOS	87.58[a]	69.35[a]	60.73[a]	111.5	1.84[b,c]	3.55	134.5[c]
	OEO	87.82[a]	69.30[a]	60.85[a]	109.4	1.81[c]	3.55	135.1[c]
Pooled SEM[2]		0.29	0.09	0.39	0.37	0.01	1.66	1.07
Replacement program								
Fully fed [3]		82.58	67.85	55.98	106.3[b]	1.91	3.01	143.1[a]
Molted[4]		87.29	69.29	60.50	110.6[a]	1.84	3.30	134.2[b]
Diet[5]								
CNT		83.86	68.84	57.70	108.3[b]	1.89	2.25	137.4[b]
MOS		86.22	68.52	59.09	109.4[a]	1.86	3.83	140.3[a]
OEO		84.73	68.36	57.92	107.7[b]	1.87	3.17	138.2[a,b]
Source of variation Probability								
Replacement		0.0001	0.0001	0.0001	0.0001	0.0001	0.83	0.0001
Diet		0.0001	0.0001	0.001	0.0001	0.078	0.71	0.030
Replacement × Diet		0.0001	0.0001	0.008	0.12	0.004	0.90	0.068

[a–d]Means within columns with different superscripts are different at $P < 0.05$.
[1]Data pooled from molted hens between 85 and 106 wk of age are based on calculation and statistical analysis.
[2]Data are means of 6 replicates of 4 adjacent cages with 24 hens each per treatment.
[3]Hens fed ad libitum on a regular layer-hen diet and not subjected to molting.
[4]Hens molted on an aa+wb diet with ad libitum intake for 12 d between 82 and 83 wk of age.
[5]The laying hens were fed on a control diet (CNT) contained no performance enhancer and supplemented with preparations of mannan oligosaccharide (1 g/kg of diet; MOS) and oregano essential oil (24 mg/kg of diet; OEO).

than that of hens in the CNT treatment; however, no significant improvement was obtained by adding OEO to the feed. Fully fed hens cumulatively produced 8.9 more ($P < 0.01$) eggs than their molted counterparts and yielded 143 eggs in total during the 25-wk egg production phase. At the same time, results showed that molted hens are capable of nearly compensating for this deficiency in total egg number by increasing their egg mass output in concert with an improved feed efficiency. The performance observed during 20 to 81 wk of age with a total of 364 eggs and 23.2 kg egg mass output while surviving at a rate of over 92%, modern laying hybrid hens may augment their productive performance to produce approximately 500 eggs while yielding over 32 kg in egg mass until aged 106 wk, with survival rates of roughly 90%. Average hen mortality from 82 to 106 wk of age was approximately 3% and remained unrelated to replacement program and diet ($P > 0.05$).

Egg Quality

The egg quality indices of hens aged from 86 to 106 wk are depicted in Table 5. The replacement program with ad libitum access to the molt diet enhanced eggshell quality and significantly ($P < 0.01$) increased eggshell thickness, eggshell weight, eggshell breaking strength, yolk height, and Haugh unit compared to those variables in the full-feeding program. However, eggshell quality indices of hens remained unaffected by MOS and OEO supplementation compared to the CNT treatment ($P > 0.05$). There was a significant interaction ($P < 0.01$) between replacement program and diet in terms of yolk diameter. In fully fed hens, feeding OEO increased yolk diameter relative to CNT; however, a contradictory pattern was observed for molted hens. The yolk diameters from MOS treatment were intermediate between CNT and OEO treatments. There was no significant difference in yolk color score among the treatments ($P > 0.05$).

Digestive and Reproductive Organs

The relative weight (g/100 g BW) of the hens' digestive and reproductive organs 6 d after the implementation of induced molting is presented in Table 6. There is no significant interaction between the replacement program and diet for any organ measurement ($P > 0.05$). The relative weights of all organs, except the pancreas, were strongly affected by induced molting ($P < 0.001$), but not by any dietary supplementation with OEO or MOS ($P > 0.05$). The only exception is that the liver weight of OEO-fed hens was lighter ($P < 0.01$) than that of hens treated with MOS and CNT, which the latter 2 did not significantly differ from each other. Induced molting evoked significant decreases ($P < 0.01$) in the relative weights of the proventriculus, liver, small intestines, ovaries, and oviduct, as well as in the length of the small intestine ($P < 0.01$). However, marked increases ($P < 0.01$) in relative gizzard and spleen weights as a response to molting were observed. The number of follicles on the ovaries with a radius of greater than 10 mm was

Table 5. Egg quality charecteristics of fully fed and molted hens between 86 and 106 wk of age with and without dietary MOS and OEO.

Item	Shell thickness (μm)	Shell breaking strength (kg/cm^2)	Shell weight (%)	Yolk height (mm)	Yolk diameter (mm)	Haugh unit	Yolk color score
Replacement program							
Fully fed[1]	373[b]	3.73[b]	9.26[b]	16.6[b]	41.2	75.1[b]	5.32
Molted[2]	385[a]	4.92[a]	9.54[a]	16.7[a]	40.8	78.9[a]	5.27
Diet[3]							
CNT	377	4.06	9.48	16.7	41.0	76.9	5.33
MOS	383	4.02	9.39	16.6	41.2	77.1	5.25
OEO	378	3.95	9.34	16.6	40.8	76.9	5.23
Pooled SEM[4]	2.87	0.09	0.06	0.08	0.24	0.69	0.10
Source of variation Probability							
Replacement	0.0001	0.0001	0.0001	0.016	0.10	0.0001	0.26
Diet	0.086	0.50	0.13	0.081	0.45	0.93	0.58
Replacement × Diet	0.60	0.81	0.10	0.51	0.002	0.31	0.15

[a,b]Means within columns with different superscripts are different at $P < 0.05$.
[1]Hens fed ad libitum on a regular layer hen diet and not subjected to molting.
[2]Hens molted on an aa+wb diet with ad libitum intake for 12 d between 82 and 83 wk of age.
[3]The laying hens were fed on a control diet (CNT) contained no performance enhancer and supplemented with preparations of mannan oligosaccharide (1 g/kg of diet; MOS) and oregano essential oil (24 mg/kg of diet; OEO).
[4]Data are means of randomly sampled 24 eggs per treatment (4 eggs per replicate) with 4 w intervals from 86 to 106 wk of age.

Table 6. Relative weight of digestive and reproductive organs of fully fed and molted hens fed on diets with added MOS and OEO 6 d after the molt induction.

Item	Proventriculus (%)	Gizzard (%)	Spleen (%)	Liver (%)	Pancreas (%)	Intestinal length[1]	Small intestine weight (%)	Ovarium (%)	Oviduct (%)	Number of follicles > 10 mm
Replacement program										
Fully fed[2]	0.38[a]	1.35[b]	0.07[b]	2.26[a]	0.22	9.15[a]	2.42[a]	0.62[a]	4.13[a]	5.94[a]
Molted[3]	0.31[b]	1.72[a]	0.10[a]	1.42[b]	0.21	7.46[b]	2.05[b]	0.45[b]	1.51[b]	0.17[b]
Diet[4]										
CNT	0.36	1.48	0.08	1.91[a]	0.22	8.28	2.19	0.55	2.89	3.08
MOS	0.33	1.57	0.09	1.92[a]	0.22	8.51	2.28	0.52	2.78	3.20
OEO	0.34	1.56	0.08	1.69[b]	0.21	8.08	2.24	0.54	2.81	3.03
Pooled SEM[5]	0.02	0.06	0.004	0.07	0.01	0.28	0.11	0.03	0.15	0.24
Source of variation Probability										
Replacement	0.0001	0.0001	0.0001	0.0001	0.48	0.0001	0.0002	0.0001	0.0001	0.0001
Diet	0.25	0.31	0.76	0.007	0.87	0.26	0.72	0.75	0.76	0.50
Replacement × Diet	0.90	0.63	0.93	0.56	0.93	0.31	0.55	0.29	0.26	0.67

[a,b]Means within columns with different superscripts are different at $P < 0.05$.
[1]Relative length of small intestine (cm/100 g body weight).
[2]Hens fed ad libitum on a regular layer-hen diet and not subjected to molting.
[3]Hens molted on an aa+wb diet with ad libitum intake for 12 d between 82 and 83 wk of age.
[4]The laying hens were fed on a control diet (CNT) that contained no performance enhancer and was supplemented with preparations of mannan oligosaccharide (1 g/kg of diet; MOS) and oregano essential oil (24 mg/kg of diet; OEO).
[5]Data are means of 12 birds per treatment (2 birds per each replicate).

nearly zero and substantially fewer ($P < 0.01$) than those of fully fed hens. However, it was observed that the relative mass of the pancreas was not affected by the hen replacement program or supplement type used ($P > 0.05$).

Biomechanical Properties and Ash and Mineral Composition of Hen Tibias

The biomechanical properties of tibias in the laying hens are shown in Table 7. There were no significant differences in bone diameter, cortex thickness, profile area, or shear force among the treatment groups ($P > 0.05$). However, hens fed the diet with MOS showed decreased ($P < 0.05$) shear stress and fracture energy by about 15% compared with those of hens fed OEO and CNT, though the replacement program exerted no significant impact ($P > 0.05$). The data regarding tibia ash and mineral content are also shown in Table 7. Molting induced a decrease of more than 4% ($P < 0.01$) in the percentage of tibia ash, whereas MOS and OEO had no influence ($P > 0.05$). A similar pattern was observed for tibia mineral content (i.e., Ca and P), indicating that bone mineralization was adversely affected by the dietary deprivation of these macro minerals, even if experienced for only 6 d. The absolute weight (grand mean = 8.5 g with SEM = 0.24) and length (grand mean = 6.7 cm with SEM = 0.09) of tibias were not influenced

Table 7. Tibia bone charecteristics of fully fed and molted layer hens fed on dietary regimens with and without MOS and OEO supplementation 6 d after the molt induction.

	Bone mechanical properties						Bone ash and mineral content		
Item	Bone diameter (mm)	Cortex thickness (mm)	Profile area (mm^2)	Shear force (N)	Shear stress (N/mm^2)	Fracture energy (N-mm)	Bone ash (%)	Ca (%)	P (%)
Replacement program									
Fully fed[1]	6.32	0.69	24.4	581	47.8	597	46.4[a]	18.4[a]	8.16[a]
Molted[2]	6.33	0.66	23.4	606	51.3	605	41.1[b]	16.7[b]	7.09[b]
Diet[3]									
CNT	6.29	0.68	23.9	626	52.6[a]	637[a]	44.7	17.9	7.80
MOS	6.37	0.67	24.0	540	45.9[b]	516[b]	43.0	17.7	7.40
OEO	6.31	0.67	23.9	615	51.2[a]	659[a]	43.4	17.0	7.68
Pooled SEM[4]	0.08	0.02	0.92	41.66	2.67	56.94	0.96	0.99	0.44
Source of variation Probability									
Replacement	0.93	0.17	0.21	0.48	0.11	0.86	0.0001	0.032	0.040
Diet	0.57	0.95	0.98	0.088	0.015	0.033	0.19	0.68	0.65
Replacement × Diet	0.38	0.91	0.97	0.64	0.49	0.76	0.91	0.23	0.20

[a,b]Means within columns with different superscripts are different at $P < 0.05$.
[1]Hens fed ad libitum on a regular layer-hen diet and not subjected to molting.
[2]Hens molted on an aa+wb diet with ad libitum intake for 12 d between 82 and 83 wk of age.
[3]The laying hens were fed on a control diet (CNT) that contained no performance enhancer and was supplemented with preparations of mannan oligosaccharide (1 g/kg of diet; MOS) and oregano essential oil (24 mg/kg of diet; OEO).
[4]Data are means of 12 birds per treatment (2 birds per each replicate).

by either the replacement program or diet ($P > 0.05$; data not shown).

Hematological Characteristics and Immune Response

The hematological characteristics and Newcastle disease (**ND**) titers of hens fed MOS and OEO in both rearing procedures are shown in Table 8. Molting did not significantly change the number of lymphocytes, heterophils, or basophils or the heterophil-to-lymphocyte (**H/L**) ratio in hens ($P > 0.05$), yet remarkably increased ($P < 0.01$) the hematocrit compared with those in the fully fed program. There were significant interactions between the replacement program and diet on eosinophil count ($P < 0.01$) and serum ND titers ($P < 0.05$). The number of eozinofils in MOS-fed (9.54) and OEO-fed (8.54) fully fed hens were significantly higher than those of the untreated controls (7.33); however, corresponding values did not differ from each other when hens were subjected to induced molting. The serum ND titer was highest in hens treated with OEO (9,573) in the fully fed program, whereas highest ND titer (9,387) was observed for hens that received the MOS-supplemented diet under molted regimen.

Liver Antioxidant Status and Blood Constituents

The results of liver antioxidant indices and the blood constituents of hens halfway through the 12-d molt period are presented in Table 9. No significant interaction between the replacement program and diet was found for these characteristics ($P > 0.05$). Molting significantly increased the MDA level of the liver ($P < 0.01$); however, molted hens responded to a related increase in this lipid-peroxidation product by increasing ($P < 0.01$) the antioxidant enzyme SOD activity. In contrast to the CNT group, the MDA level of the liver slightly increased in hens given the MOS-supplemented diet, whereas that of hens in the OEO treatment group markedly decreased ($P < 0.01$). Significant ($P < 0.01$) increases were observed in liver SOD concentration following dietary OEO supplementation, whereas there was a numerical yet not significant increase in SOD activity when MOS was added to the feed. The total antioxidant status TAS activity in the liver showed no differences ($P > 0.05$) between the treatment groups. Serum total protein, P, and Ca levels were significantly ($P < 0.01$) lower in hens that molted than those in fully fed hens. Serum concentrations of GLU, CHOL, and CS were significantly higher in molted hens than in those that were fully fed, yet the replacing program did not affect serum chloride (**Cl**) and sodium (**Na**) concentrations ($P > 0.05$), though salt was not added to the molt diet. Supplementing diets with MOS and OEO did not significantly ($P > 0.05$) alter any blood constituents except for the CS level, which both supplements significantly decreased with similar efficacy in relation to the CNT treatment ($P < 0.01$).

Cecal Microbial Composition

The composition of the cecal microflora of laying hens 6 d after the initiation of molting is shown in Table 10. In the present study, the cecal microbial population of hens was strongly affected by the molt program, though

Table 8. Hemotological parameters and ND titers of fully fed and molted hens administered diet with or without MOS and OEO 6 d after the molt induction.

Item	Lymphocyte	Heterophil	Eosinophil	Basophil	H/L[1]	Hemotocrit	ND titers
Replacement program							
Fully fed[2]	63.6	21.2	8.47	2.16	0.33	35.6[b]	8833
Molted[3]	63.6	21.4	7.95	1.95	0.34	40.5[a]	8727
Diet[4]							
CNT	64.6	20.5	7.83	2.27	0.32	37.3	8702
MOS	63.5	21.6	8.59	1.81	0.34	38.4	8757
OEO	63.0	21.9	8.22	2.09	0.35	38.6	8881
Pooled SEM[5]	1.26	1.04	0.35	0.25	0.02	0.84	481
Source of variation Probability							
Replacement	0.86	0.84	0.094	0.29	0.85	0.0001	0.70
Diet	0.40	0.40	0.14	0.16	0.42	0.23	0.89
Replacement × Diet	0.53	0.93	0.001	0.40	0.97	0.11	0.013

[a,b]Means within columns with different superscripts are different at $P < 0.05$.
[1]Heterophil to lympocyt ratio.
[2]Hens fed ad libitum on a regular layer-hen diet and not subjected to molting.
[3]Hens molted on an aa+wb diet with ad libitum intake for 12 d between 82 and 83 wk of age.
[4]The laying hens were fed on a control diet (CNT) that contained no performance enhancer and was supplemented with preparations of mannan oligosaccharide (1 g/kg of diet; MOS) and oregano essential oil (24 mg/kg of diet; OEO).
[5]Data are means of 12 birds per treatment (2 birds per each replicate).

Table 9. Liver antioxidant and oxidant status and blood constituents of fully fed and molted laying hens treated with or without MOS and OEO 6 d after the molt induction.

	Liver antioxidant and oxidant status			Blood constituents							
Item	MDA (μmol/mg protein)	SOD (U/mg protein)	TAS (mmol trolox Equiv./mg protein)	Ca (mg/dL)	P (mg/dL)	Cl (mEq/L)	Na (mEq/L)	Total protein (g/dL)	GLU (mmol/L)	CHOL (mg/dL)	CS (ng/mL)
Replacement program											
Fully fed[1]	9.4[b]	23.8[b]	3.62	17.2[a]	3.15[a]	116	58	3.71[a]	10.7[b]	23[b]	2.96[b]
Molted[2]	11.4[a]	34.2[a]	3.48	3.1[b]	2.15[b]	120	46	1.57[b]	11.7[a]	100[a]	4.99[a]
Diet[3]											
CNT	10.6[b]	26.4[b]	3.59	10.6	2.72	115	53	2.96	11.1	69	6.25[a]
MOS	11.2[a,b]	28.6[a,b]	3.49	9.3	2.54	128	56	2.39	11.2	55	2.82[b]
OEO	9.4[c]	32.0[a]	3.58	10.6	2.70	110	46	2.58	11.2	61	2.85[b]
Pooled SEM[4]	0.23	1.79	0.14	2.13	0.22	9.47	10.15	0.43	0.2	15.64	0.72
Source of variation Probability											
Replacement	0.0001	0.0001	0.24	0.0001	0.0001	0.56	0.16	0.0001	0.0001	0.0001	0.0011
Diet	0.0001	0.009	0.76	0.79	0.67	0.15	0.60	0.41	0.87	0.66	0.0001
Replacement × Diet	0.098	0.73	0.42	0.74	0.78	0.97	0.15	0.63	0.80	0.81	0.50

[a-c]Means within columns with different superscripts are different at $P < 0.05$.
[1]Hens fed ad libitum on a regular layer-hen diet and not subjected to molting.
[2]Hens molted on an aa+wb diet with ad libitum intake for 12 d between 82 and 83 wk of age.
[3]The laying hens were fed on a control diet (CNT) that contained no performance enhancer and was supplemented with preparations of mannan oligosaccharide (1 g/kg of diet; MOS) and oregano essential oil (24 mg/kg of diet; OEO).
[4]Data are means of 12 birds per treatment (2 birds per each replicate).

the effect of MOS and OEO was far less pronounced. The enumeration of cecal bacteria showed that total aerobes, total anaerobes, coliforms, and *Clostridium* spp. significantly ($P < 0.01$) increased in response to molting, whereas numbers of *Lactobacillus* spp. and *Bifidobacterium* spp. lessened. The concentration (i.e., $\log_{10}$ cfu/g in wet cecal digesta) of coliforms in MOS and OEO treatments were numerically but not significantly lower ($P = 0.07$) than in the CNT treatment by 0.39 and 0.54 logs, respectively. A near-significant trend for increased number of *Lactobacillus* spp. was observed in both MOS and OEO treatment groups vs. the unsupplemented CNT group ($P = 0.08$). There were no statistically significant differences for the other examined microbial populations regarding diets administered with MOS and OEO.

DISCUSSION

At present, the interest for feeding molt diets to egg-laying hens remains strong because feed removal is no longer the preferred method owing to food safety and animal welfare concerns (Berry, 2003). Such hen-replacement strategies that do not involve fasting are reported to be effective tools for halting egg production, inducing molting, and enhancing the productivity and sustainability of laying hens throughout the subsequent production period. Researchers have

Table 10 . Effect of hen replacement program and dietary supplementation with or without MOS and OEO on cecal microbial composition (log_{10} CFU/g cecal digesta) of laying hens 6 d after the molt induction.

Item	Total aerobes	Coliform	*Lactobacillus* spp.	Total anaerobes	*Clostridium perfringens*	*Bifidobacterium* spp.
Replacement program						
Fully fed[1]	7.30[b]	7.65[b]	8.09[a]	8.34[b]	3.19[b]	6.70[a]
Molted[2]	8.16[a]	8.42[a]	6.46[b]	9.43[a]	4.32[a]	5.42[b]
Diet[3]						
CNT	7.96	7.90	6.97	9.11	4.04	5.84
MOS	7.62	7.51	7.51	8.80	3.57	6.15
OEO	7.60	7.36	7.35	8.75	3.66	6.18
Pooled SEM[4]	0.25	0.23	0.24	0.31	0.26	0.36
Source of variation Probabilities						
Replacement	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Diets	0.29	0.07	0.080	0.46	0.17	0.58
Replacement × Diet	0.73	0.91	0.91	0.81	0.96	0.79

[a,b]Means within columns with different superscripts are different at $P < 0.05$.
[1]Hens fed ad libitum on a regular layer-hen diet and not subjected to molting.
[2]Hens molted on an aa+wb diet with ad libitum intake for 12 d between 82 and 83 wk of age.
[3]The laying hens were fed on a control diet (CNT) that contained no performance enhancer and was supplemented with preparations of mannan oligosaccharide (1 g/kg of diet; MOS) and oregano essential oil (24 mg/kg of diet; OEO).
[4]Data are means of 12 birds per treatment (2 birds per each replicate).

studied the use of alfalfa (Landers et al., 2005) and wheat middlings (Seo et al., 2001) in molt diets, the results of which indicated their effectiveness as alternatives to traditional feed removal and in producing satisfactory post-molt performance for the commercial poultry industry.

The molt diet used for 12 d in this study produced an 85% reduction in 12-d average feed consumption of the molted hens with respect to the unmolted fully fed hens. In fact, the aa+wb molt diet was bulky due to its high fiber content and low specific gravity, which may have reduced the hen's feed intake by decreasing the feed's palatability. The low Ca and Na content of the molt diet (Table 1) may have also diminished the palatability, thus causing the hens to refrain from eating. Likewise, consuming diets low in Ca and Na (Ross and Herrick, 1981) has also been reported to halt egg production in laying hens. The low energy consumption of molted hens (17 kcal/hen per d) could additionally be an important factor in rapidly reducing egg production (Biggs et al., 2004).

The results show that the efficacy of each preparation differed in response to the hen-replacement program. Feed supplemented with OEO maximized the benefits of the post-molt diet, thereby decreasing the FCR ($P < 0.01$) beyond that of the CNT treatment group, which might be associated with its antibacterial and antioxidant activities (Sivropoulou et al., 1996; Betancourt et al., 2014), ability to stimulate digestion (Jamroz et al., 2003; Basmacıoğlu et al., 2010), and inflammatory potential (Bozkurt et al., 2013). However, MOS conferred positive effects ($P < 0.01$) on both egg mass output and FCR in fully fed hens during the 25-wk experimental period (Table 4). The performance-enhancing potential observed among laying hens fed MOS might be connected with the supplement's positive effect on pro-inflammatory response and intestinal villi morphology (Gürbüz et al., 2011). In addition, MOS-supplemented diets have been reported to promote growth by enhancing birds' resistance to enteric pathogens (Fernandez et al., 2002). Data regarding performance features indicated that laying hens aged more than 80 wk previously screened for their benefits to egg-laying performance, eggshell quality, and immune response at earlier ages benefited from a dietary supplementation of MOS or OEO (Çabuk et al., 2006; Gürbüz et al., 2011; Bozkurt et al., 2012a,b).

Ovarian regression is essential to obtaining long-term egg production and eggshell quality during the second production cycle (Biggs et al., 2004). Data regarding production performance (Table 4) imply that maintaining a feed consumption of approximately 16 g/hen/d during the 12-d period (Table 3) may elicit a regression of the reproductive system with ovarian follicles (Table 6) and enable hens to exhibit acceptable post-molt egg production. This implication agrees with the observation of Donaldson et al. (2005), who also reported that feeding alfalfa meal to hens during molt induced significant weight loss in the ovaries and oviduct, yet maintained an average egg production rate of more than 70% throughout the 39-wk post-molt period.

Typically, most measures of egg quality deteriorate as flocks age and such deterioration affects both interior and external traits (Gast and Ricket, 2003). However, improvements in egg quality become evident after induced molt (Swanson and Bell, 1975). Improvements in eggshell quality could also be associated with the total cessation of egg production during the molting period (Noles, 1966).Verifying these statements, in the present study, a non-feed-removal molting procedure proved effective at initiating a dramatic recovery in both egg quality and rate of egg production.

However, neither MOS nor OEO imparted further benefits to egg quality following molt. Nevertheless, significant improvements in eggshell weight in layer hens fed MOS or OEO have also been reported by Berry and Lui (2000) and Bozkurt et al. (2012a,b). Discrepancies in results among studies suggest that the same product used in a distinct management procedure (i.e., younger hens vs. molted aged hens) can greatly differ in response. Indeed, there is a lack of scientific evidence of the mechanism by which dietary OEO and MOS affect egg quality in hens aged more than 80 wk. We postulate that hens in our study were unable to maintain eggshell quality when treated with either OEO or MOS while trying to replenish lost body stores following feed deprivation during the 12-d molting period.

In the present study, as expected, induced molting elicited substantial decreases in the relative weight of digestive organs, including the proventriculus, liver, and small intestines, in connection with limited access to feed. This finding supports earlier studies (Brake and Thaxton, 1979). A liver-weight reduction of 38% in molted hens in comparision with fully fed hens is expected (Table 6) and indicates a loss of liver energy sources such as glycogen and lipids which are being metabolized in the liver (Berry and Brake, 1985). In contrast, the relative gizzard weight of molted hens was found to be 27% greater than that of fully fed hens. This result is not surprising because it is expected that the higher fiber content of the molt diet might stimulate a contraction of the gizzard for grinding the bulky structure of the aa+wb mixture. The unchanged pancreas weight indicates that pancreatic enzyme activities in molted hens continued despite the marked restriction in nutrient intake.

Bone measurements such as bone-breaking force (Ruff and Hughes, 1985), bone ash content, and mineral content (Akpe et al., 1987) have been used as indicators of bone status in the mineral nutrition of poultry. However, induced molting via feed withdrawal is a potential factor for increased structural bone loss and osteoporosis in laying hens (Park et al., 2003). Previous research has also shown that molt diets (Mazzuco and Hester, 2005; Kim et al., 2006) adversely affect bone mineralization and biochemical properties during molt, which consequently reduces bone mineral densities and bone-breaking force.

The results of the current study revealed reduced bone mineralization as a response to non-feed-removal molt regimen (Table 7). However, there was no observed deterioration in the geometrical characteristics or bone strength of the tibia. These findings indicate that bone mechanical properties were not correlated with bone characteristics measured by conventional assays (i.e., ashing, mineral assay, and histomorphometry). The hypothesis by Fleming et al. (1996) may aid to interpretation of this inconsistency that skeletal integrity during molt becomes compromised in the traditional method because the medullary component of bone contributes to bone strength.

In the current experiment, bone mechanical properties and mineral content did not show any change from MOS. Except for the research findings using fructo-oligosaccharides (Kim et al., 2006) and inulin (Chen and Chen, 2004), the research literature offers little support that supplementing laying hens' diets with MOS increases the availability of Ca or that other minerals could benefit bone mineralization and bone-breaking strength. Differences in the bone quality of laying hens fed diets supplemented with different prebiotic preparations may relate to the situation that their efficiency depends on many factors, including hen age, the composition of the molt diet, the dietary concentration of prebiotics used, and the production of hens at the initiation of molting. Similar to that observed for MOS, the provision of a diet with OEO posed no implications for bone mineralization or bone strength. Overall, the findings of the current study suggest that neither MOS nor OEO can reduce bone mineral losses during molting or aging.

Molting affects differential white blood cell counts primarily by increasing heterophils and decreasing lymphocyte cells in peripheral blood (Holt and Porter, 1992). In the present study, the H/L ratios, which are used to measure the level of stressful conditions (Gross and Siegel, 1983), were not affected by either molting or diet ($P > 0.05$). A possible explanation for unchanged H/L is the adaptation of the hens to physiological stress caused by induced molting, even 6 d after molt commenced. This indicates that hens fed a molt diet progressively decreased H/L until d 6 of the molt period (i.e., 12 d) and thus overcame any acute stress within several days. Similarly, a lack of significant differences in the H/L ratios between non-feed-removal molted and fully fed hens has also been reported (Mazzuco et al., 2011). Earlier, Kogut et al. (1999) observed that the H/L ratio returned to normal 10 d after the start of the feed-restricted program.

Certain alterations in poultry management procedures such as feeding programs can result in increases in circulating CS concentrations (Kogut et al., 1999). In addition to CS, actual increases found in plasma concentrations of GLU (Brake and Thaxton, 1979) and of GLU and CHOL (Gildersleeve et al., 1983) in hens during periods of feed withdrawal support the findings of this study.

Furthermore, the degree of increase in CS concentration depends upon the method used to induce molt (Berry, 2003). Methods such as fasting are associated with larger increases in CS levels, whereas methods that provide limited amounts of feed to induce molt result in a relatively lower increase in CS (Etches et al., 1984). However, in the current experiment, serum concentrations of CS and also GLU and CHOL in hens molting on an aa+wb diet showed significant increases compared to those of fully fed hens. We conclude that the drastic change in the feeding program used to elicit molt

triggered an increase in stress indicators. It is interesting to note that, despite molt-driven stress, hens quickly returned to their normal physiological limits and production performance after the reintroduction of the regular diet for egg-laying hens. This finding supports the proposal by Berry (2003) that CS increase is transitory and decreases toward pre-molt levels as molting progresses. Harvey et al. (1983) found that the mere sight of food was sufficient to cause plasma CS levels to quickly decline in chickens.

Herbal EOs, including oregano (Bölükbaşı et al., 2009; Bozkurt et al., 2012b), have been shown to decrease the serum triglyceride level of laying hens in their peak and post-peak production periods. However, there are no known reports on the implementation of molt diets that monitor the effect of MOS and OEO supplementation on serum stress indicators including CS, GLU, and CHOL. The significant decreases in serum CS concentration in response to MOS and OEO supplementation in hens indicate that such dietary regimens may alleviate hens' physiological stress derived from molting or aging.

It has been demonstrated that birds under acute (Lan et al., 2004) and chronic (Zhang et al., 2013) stress have also elevated oxidative stress. However, little is known regarding the extent that molting triggers oxidative stress in extremely old hens. In the present study, the significantly increased MDA concentration in the liver during molting clearly indicates that the experimental molt program used (12 d) elicited oxidative stress response in hens. However, the potential for dietary supplements OEO and MOS to influence the oxidative status of tissues or plasma in hens exposed to a molt program has not been previously investigated.

OEO is well known for its antioxidant and antimicrobial properties (Sivropoulou et al., 1996) and is therefore of interest to the poultry industry for its potential to improve the oxidative stability of chicken meat (Basmacıoğlu et al., 2004). Nearly all studies reported consistent, positive implications regarding the antioxidant properties of OEO (Brenes and Roura, 2010). In the present study, OEO and, to a lesser extent, MOS significantly improved SOD activity in the liver in concert with a reduction in MDA formation, which is the end product of lipid peroxidation (Table 9). The reduction of MDA in accordance with the increase in hepatic SOD activity indicates that the major components are carvacrol, p-cymene, and thymol, which constitute approximately 86% of the oil (Table 2), exhibiting considerable antioxidant activity. As demonstrated in our earlier study (Bozkurt et al., 2012b), MOS and OEO can be used to alleviate oxidative stress in the livers of younger hens, though antioxidant activity displayed by OEO was more outstanding. We are unable to further discuss the antioxidant potential of in-feed MOS as there is very little available literature supporting that observation.

Management practices to induce hens to molt elicit major chronic stress during the molt period. The stress associated with feed withdrawal allows an increased susceptibility to bacterial infections, particularly those of *S. enteritidis* and *E. coli*, marked by increased intestinal shedding and the invasion of organs (Holt and Porter, 1992; Holt, 1995; Donaldson et al., 2008). The concept tested in this work—namely a short-term, fully fed diet of aa+wb—could not maintain the stability of the cecal microflora composition by impeding colonization by pathogenic organisms, which resulted from the drastic change in the dietary regimen. Our observations contradict Seo et al. (2001), who reported that alfalfa and wheat middlings in molt-induced diets are desirable given the fermentation properties of cecal microflora, causing an overall reduction of *S. enteritidis* colonization in laying hens. In the present study, a lower daily intake of molt diet by hens might fail to confirm such benefits of earlier work.

Interest in using herbal EOs as potential antimicrobial agents in poultry diets has emerged as a result of *in vitro* (Cowan, 1999) and *in vivo* experiments (Jang et al., 2007; Mountzouris et al., 2011) showing that EOs actually have antimicrobial activity against microflora commonly present in chicken guts. Similarly, it is well documented that MOS competitively absorbs the mannose-specific type 1 fimbriae of *E. coli*, *Campylobacter*, and *Salmonella*, thereby limiting their colonization of the intestinal epithelium (Fernandez et al., 2002; Baurhoo et al., 2009). In agreement with these observations, the results of the present study showed that OEO and MOS fairly modulated cecal microflora composition by reducing coliforms and fostering the fortification of gut microflora with purportedly beneficial members such as lactobacilli (Table 10). Indeed, the supplementation of poultry diets with MOS and OEO to improve intestinal balance is quite a new concept, hence further studies are necessary in support of this observation in molted laying hens.

Our results indicate that a non-feed-removal molting procedure is an effective way of bringing about a recovery in both the quality and rate of egg production without compromising hens' survival. In supplemented diets, MOS and OEO can remain effective as prebiotic or phytogenic substances for molted hens on an aa+wb diet. However, in fully fed hens, the consistently positive effects on all performance traits mediated by dietary MOS supplementation are conclusive. Results clearly show that a non-feed-removal molting program that is easy for the industry to implement and uses readily available, inexpensive feed ingredients may extend the economically useful life of laying flocks up to 106 wk of age and achieve production performance of an efficiency comparable to that of a full-feeding hen-replacement program. However, an aa+wb molt diet did not minimize the risk factors of infection of laying hens with food-borne pathogens due to the temporary but drastic change in the feeding schedule.

This study is also proposes that MOS- and OEO-supplemented diets for modern laying hybrid hens may contribute to the breed's ultimate goal in the eyes of the poultry industry: to reap 500 eggs from hens from 20 to 106 wk of age based on the performance of 364.6 cumulative egg number and 23.2 kg egg mass output between 20 to 81 wk of age while surviving at a rate of over 92.8%. However, the deterioration in egg quality with age is an important drawback of any full-feed program.

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