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EFFECTS OF ADDING NANO-CHITOSAN ON PRODUCTIVE PERFORMANCE OF LAYING HENS

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SUMMARY

The present study evaluated the effects different forms of chitosan (ordinary chitosan "OCh" and chitosan nanoparticles "ChNP") as a dietary supplementation on egg production, egg quality, yolk egg composition, blood serum traits, and intestinal bacteria. A total of 96 hens of Bábolna TETRA-SL at 23 weeks of age were randomly assigned into 4 experimental groups with three replicates each. The control (Co) group, T1 group, and T2 group were fed 0.0 mg/kg diet, 150 mg/kg diet and 300 mg/kg diet, respectively. The control group (Co) were fed the basal diet without the supplement, while birds in the treatment groups T_1, T_2, and T_3 were fed the basal diet supplemented with 200 mg OCh/kg diet, 50 mg ChNP/kg diet, and 200 mg ChNP /kg diet, respectively. The experimental period was 12 weeks (23-34 weeks old). The egg laying rate, average egg weight, and feed conversion ratio of hens were not affected significantly by the experimental groups. Egg mass and feed intake were significantly decreased ($P<0.05$) for all treatments compared to the control group. Albumen height and Haugh unit were significantly increased in the T1 and T3 groups compared to the control group ($P<0.05$). Unsaturated fatty acids (ΣUSFA) were significantly increased in the T1 and T3 compared to the other groups ($P<0.05$). However, saturated fatty acids (ΣSFA) were significantly decreased in the T1 and T3 groups compared to the other groups ($P<0.05$). Malondialdehyde concentration was significantly decreased in the T1 and T3 groups ($P<0.05$) compared to the Co group. Immunoglobulin Y was significantly increased ($P<0.05$) in the T1 group followed by decreasing order in the T3 group. Total bacterial count, including *Lactobacillus* count in the intestine was significantly increased ($P < 0.05$) in the T1 and T3 groups compared to the other groups. These results concluded that both of OCh and ChNP have a positive effect on the productive performance of laying hens.

Keywords *Chitosan, nanoparticles, egg production, egg quality, intestinal bacteria and laying hens.*

INTRODUCTION

As the global population grows, the need for egg production increases as the cheapest source of high-quality animal protein. In poultry, bacterial infections are responsible for economic loss and low poultry production because of the decreased feed intake, egg rate, digestive integrity, mortality, and medication costs. Therefore, international attention has turned to the search for feed additives from natural and safe sources as an alternative to antibiotics, which caused potential adverse effects, such as antibiotic residues, environmental pollution, and emergence of antibiotic-resistant bacteria. Therefore, since March 2006, the European Union (EU) has banned the use of antibiotics as catalysts for growth in animal production (European Union, 2003). In past years, a wide range of functional materials has been used as a substitute for antibiotics to prevent disease and promote growth in poultry production. These substrates include prebiotics, probiotics, plant extracts and other bioactive compounds (Zhou *et al.*, 2012 and Attia *et al.*, 2018). Chitosan is a deacetylated form of chitin, which is extracted from the exoskeletons of invertebrates, such as crabs, shrimps, insects, and squid (Nwe *et al.*, 2009, Singla and Chawla, 2001 and Tømmeraas *et al.*, 2011). After the chitosan was shown to be reliable and safe, it was approved by the U.S. Food and Drug Administration (FDA) in 2001 (Kong *et al.*, 2010 and Wang *et al.*, 2020) Chitosan (Ch) is one of the promising natural polymers that can be developed to polymeric nanoparticles (NPs), and chitosan has attracted great attention due to its attractive properties (Naskar *et al.*, 2019). Chitosan can be used in a wide range of applications, including agriculture, food science, pharmaceuticals, and biomedical fields, due to its unique biological characteristics, including biodegradability, and non-toxicity (Naskar *et al.*, 2019 and Yin *et al.*, 2009). Some studies showed that feeding a diet containing 1.4 grams of chitosan / kg of body weight daily to laying hens, led to hypolipidemic and reduced cholesterol, triglycerol and free fatty acids in serum (Hirano *et al.*, 1990).

Abdominal fat was significantly reduced after chitosan supplementation at a level of 0.025% and 0.05% in broilers (Sirsat Shraddha *et al.*, 2017). However, Egg weight and egg component weights were not affected by chitosan supplementation at a level of 20 or 30g chitosan/kg diet during 29-37 weeks of age (Nogueira *et al.*, 2003). Palmitic and stearic acids contents in eggs were lower in the groups supplemented with 20 or 30g than in the control group, while oleic acid in eggs in the group supplemented with 30 g was higher than in the control group at 37 weeks of age. Egg yolk cholesterol was reduced by a diet containing 30 g/kg chitosan at 35 - 37 weeks of age compared to the control group. Yan *et al.*, (2010) indicated that egg weight, yolk color and Haugh units were linearly improved in a diet containing 0.01% or 0.02% of chitosan, while the egg production was not affected. In another study by Swiatkiewicz *et al.*, (2013) birds that fed a diet containing a high level of distillers dried grains with soluble (DDGS) (20%) or chitosan (0.01%) had a large number of eggs with an increased egg mass. The incorporation of chitosan a level of 0.02% or 0.04% in the diet of laying hens showed a positive effect on laying egg rate, egg quality, Haugh Units, and apparent digestibility of dry matter and nitrogen (Meng *et al.*, 2010). In recent years, a lot of attention has been paid to the use of biodegradable polymeric nanoparticles in poultry nutrition to promote production. There are very limited published studies worldwide on the use of a diet supplemented with chitosan nanoparticles (ChNP) for laying hens. Despite the unique biological properties of Ch, so far little is known about whether ChNP supplement provides measurable health benefits. Therefore, the objective of this study was to evaluate the effects of adding ChNP with a size up to 17 nm to the diet of laying hens during 23-34 weeks of age on egg production, egg quality, yolk egg composition, blood serum traits, and intestinal bacteria.

MATERIALS AND METHODS

Preparation of chitosan nanoparticles:

Chitosan nanoparticles (ChNP) were prepared by an ionic gelation method according to Calvo *et al*. (1997) with some modifications. The method utilizes the electrostatic interaction between the amine group of chitosan (Sigma-Aldrich, USA, molecular weight 50,000-190,000 Da, degree of deacetylation 75-85% and viscosity: 20-300 cP) and a negatively charged group such as sodium tripolyphosphate (TPP) (Sigma-Aldrich, USA). DNA free deionized water (Millipore, USA) was used for preparation and dilutions. Ch aqueous solution (0.2% w/v) was prepared by dissolving Ch in acetic acid solution (1% v/v) at room temperature. Subsequently, TPP solution (0.06% w/v) was added dropwise to Ch solution under vigorous stirring for 30 min. The resulting chitosan particle suspension was centrifuged at 12000 g for 30 min. The pellet was resuspended in deionized water. The chitosan nanoparticles suspension was then freeze-dried before further use or analysis.

Characterization of chitosan nanoparticles:

Actual morphology of the prepared Ch nanoparticles was imaged by High Resolution Transmission Electron Microscope (HR-TEM) operating at an accelerating voltage of 200 kV (Tecnai G2, FEI, Netherlands). Diluted Ch nanoparticle solution was ultra-sonicated for 5 min to reduce the particles aggregation. Using micropipette, three drops from the sonicated solution were deposited on carbon coated-copper grid and left to dry at room temperature.HR-TEM images of the Ch nanoparticle that deposited on the grid were captures for morphological evaluation. Dynamic Light Scattering (DLS) technique was utilized to estimate the average particle size distribution that was measured by zeta sizer (Malvern, ZS Nano, UK). The chemical structure of the prepared Ch nanoparticles was assessed using X- ray Diffraction (XRD) technique. The corresponding XRD pattern was recorded in the scanning mode (X'pert PRO, PAN analytical, Netherlands) operated by Cu K radiation tube =(1.54 A˚) at 40 kV and 30 mA. The obtained diffraction pattern was interpreted by the standard ICCD library installed in PDF4 software. All the preparation and characterization processes were conducted at Nanotechnology and Advanced Materials Central Lab (NAMCL), Agricultural Research Center, Egypt.

Diet and management:

A total number of 96 hens of Bábolna TETRA-SL at 23 weeks of age were randomly divided into 4 experimental groups with three replicates. The experimental period was 12 weeks (23-34 weeks old). The control group Co, T_1, T_2 and T_3 were fed without addition, 200 mg ordinary chitosan/kg diet, 50 mg ChNP /kg diet, and 200 mg ChNP /kg diet, respectively. Laying hens were housed in pyramid shaped batteries, which were equipped with water and feeders (dimensions:45 cm in length, 45 cm in width, and 45 cm in height). Daily temperature and relative humidity were approximately 22 ± 2.0 C^o and 40 ± 3.0% during the experimental period. The batteries were cleaned, vaporized with formaldehyde solution, and washed before the start of the experiment. The lighting program used was 16 L: 8 D (L = light, D = darkness), with an automatic ventilation during the whole experimental period. The conditions of housing and management of birds were similar during the experimental period. The mean weight at the start of the experiment for hens was 1745 ± 41 g. All diets were formulated to provide the nutrient requirements according to Tetra-SL LL guide (2018). The chemical diet composition of the experimental diets (Table, 1) according to (NRC, 1994). Water and feeds were offered *ad libitum* for hens during the experimental period. Determination of crude protein and ether extract for treatments were carried out in the diet according to AOAC (2012).

Laying performance:

During the experimental period (23-34 weeks of age), eggs were collected twice daily at 9:00 am and 2:00 pm. The total number of eggs, egg weight, egg mass, feed intake and feed conversion ratio (feed intake per egg weight) were recorded on a daily basis for a period of 12 weeks.

Table (1): Formulation and diet composition of laying hens.

Ingredient	Treatments			
	Co	**T_1**	**T_2**	**T_3**
Ground yellow corn (8.5% CP)	64.50			
Soybean meal (44% CP)	13.75			
Corn gluten meal (60% CP)	10.00			
L-Lysine HCl (%)	0.21			
DL-Methionine (%)	0.10			
*Premix (%)	0.30			
Sodium chloride (%)	0.30			
Di-calcium phosphate (%)	2.14			
Calcium carbonate (%)	8.70			
Total (kg)	100			
Calculated				
C P (%)	18.04			
ME (kcal /kg diet)	2838			
Lysine (%)	0.84			
Methionine + cystein (%)	0.77			
Methionine (%)	0.44			
Available phosphorus (%)	0.50			
Calcium (%)	3.83			
C F (%)	2.46			
Analysis				
CP (%)	18.00			
EE (%)	4.01			
Ash (%)	4.63			
CF (%)	2.50			

**Each one kg of laying premix contained Vit A, 10.000 I.U or 300 mcg retinol; Vit D3, 3.000 I.U/ 75 mcg cholecalciferol; Vit E, 10 mg /25 mg; Vit K3, 1.0 mg/2.0 mg; Vit B1, 2.0 mg; B2, 6.0 mg; B6, 3.0 mg; B12, 0.02 mg; B5 Pantothenic acid, 10 mg; B3 Nicotinic acid, 30 mg; Biotin, 0.10 mg; B9 Folic acid, 0.1 mg, Choline Chloride 50% 250 mg; Fe, 50 mg; Manganese, 100 mg; Cu 8.0 mg; Zn 80 mg; Iodine 1.3 mg; Selenium 0.3 mg. Co (without supplementation), T_1 200 Ch mg/kg diet, T_2 50 ChNP /kg diet and T_3 200 ChNP /kg diet. (CP) crude protein, (EE) ether extract and (CF) crude fiber.*

Fatty acids, cholesterol content and lipid oxidation of egg yolk:

Six eggs from each group were randomly collected at the end of experimental period (34 weeks of age) to determine their fatty acid (FA) content of yolk in eggs produced by laying hens. The yolks were, separated from albumen and quickly frozen at -20 °C. Total lipids from eggs yolk were extracted by the method of Folch *et al.* (1957). Methyl esters of fatty acid (FA) were prepared to determine the contents of FA as described by Pearson's Chemical Analysis of Food Eighth Edition (1981). Gas chromatography, the HP (Hewlett Packard) 6890 GC model with mass spectrometer (GC/MS) was used to analyze fatty acids in samples. Yolk lipid oxidation was estimated using 6 eggs, which were randomly collected from each group to determine malondialdehyde (MDA) content described by Racanicci *et al.* (2008). Six eggs were randomly collected from each replicate at 34 weeks old. All yolks were cleaned from the albumen, mixed well, and freeze-dried. After freeze-drying, they were weighed, homogenized, vacuum packed in plastic bags, and stored at - 20 °C prior to analysis. Yolk cholesterol concentrations were measured by an enzymatic colorimetric test described by Mannheim, (1989).

Egg quality traits:

At the end of the experimental period, eight fresh eggs from each replicate were randomly selected at 10 am to measure the egg quality traits, including egg weight, yolk weight, shell weight, albumen weight , yolk color, shell thickness (mm), albumen height (mm) and Haugh Unit. The individual weight of eggs was recorded, and then each egg was broken Egg yolk and albumen were carefully separated. Further separation of the chalaza from the yolk was made by carefully rolling the yolk several times on a moistened paper towel. Yolk weight and shell weight of each egg were then recorded. Shell weight included shell membrane was air-dried for 24 h and then was recorded. Albumen weight was calculated by subtracting yolk and shell weight from egg weight. For measurement of albumen high, the egg was broken on a smooth level surface, and the albumen height was determined away from the chalaza. Albumen height was measured using a tripod micrometer. Shell thickness with shell membrane was measured by using an Ames shell thickness gauge reading to the nearest 0.01mm. The DSM Roche Yolk Color Fan was used to measure the color of the egg yolk. Haugh unit was measured according to the method of Haugh, (1937) by the following formula: Haugh Unit = 100 log (H - 1.7 $W^{0.37}$ + 7.6). Where: H= Albumen height and W= Egg weight.

Blood parameters:

Blood samples were collected from 6 hens, which were randomly selected from each group at end of the experiment. Blood was taken from the jugular vein. Then the serum was isolated from samples by centrifugation at 3000 rpm for 15 min and saved at -20 $^{\circ}$C for future uses. The serum total protein was determined according to the methods described by Henry *et al.* (1974). The total cholesterol, high-density lipoprotein, and low-density lipoprotein were determined by calorimetric methods according to (Richmond, 1973), high-density lipoprotein (Lopez *et al.* 1977), and low- density lipoprotein (Wieland and Seidel 1983), respectively. Serum immunoglobulins, including IgA, IgY, and IgM were determined as follows: One ml of 4% sheep red blood cells (SRBCs) was injected into 6 hens from each group through the left-wing vein at 33 wks of age. Seven days after the injection (at 34 wks), blood samples were taken from the hens through the jugular vein. Serum immunoglobulin (IgA, IgY and IgM) concentrations were determined by ELISA (Microplate Reader® - DAS) using a commercial kit as explained by Hogenesch *et al.* (2002).

Microbial populations:

Six intestinal samples were collected from each treatment. The contents of the intestine were removed and placed in a sterile sample bag and put in a cooler box with ice packs (-4 to -10 °C) and immediately transported to the laboratory to be tested bacteriologically within a time limit. *Lactobacillus*, coliform, *Enterococcus*, *Salmonella*, and *Clostridia* counts were determined. Content of samples were then diluted serially from 10^{-1} to 10^{-7}. One-tenth milliliter of each diluted sample was immersed on the appropriate agar media. Bacterial counts were performed using the appropriate dilution and plate culture techniques under aerobic or anaerobic conditions according to Quinn *et al.* (1994) and Rada *et al.* (1999).

Statistical analysis:

Data analysis was performed using SPSS software program package (SPSS, 2001). All data were analyzed based on a completely randomized design using one-way ANOVA and Duncan's multiple range test. Data were presented as means (Means ± SEM) (Duncan, 1955). All statements of statistical significance are based on a probability of ($P < 0.05$).

RESUILTS AND DISCUSSION

Characterization of chitosan nanoparticles:

Physicochemical characterization of the synthesized ChNPs to evaluate its properties using different techniques is shown in Figure **(**1**)**. HR-TEM electrograph showed nearly spherical shape, smooth surface, and size range of about 17 nm Fig. (1A). The particle size distribution curve obtained from DLS measurements are presented in Fig. (1B) and (1C). The nanoparticle surface charge (or zeta potential) was +52.5 mV. X-Ray powder diffraction pattern (XRD) of ChNPs is shown in Fig. (1D). No peak was found in the diffractograms. ChNPs were comprised of a dense network structure of interpenetrating polymer chains cross-linked to each other by TPP counter ions (Tang *et al.*, 2003). The XRD implicated greater disarray in chain alignment in the nanoparticles after crosslinks.

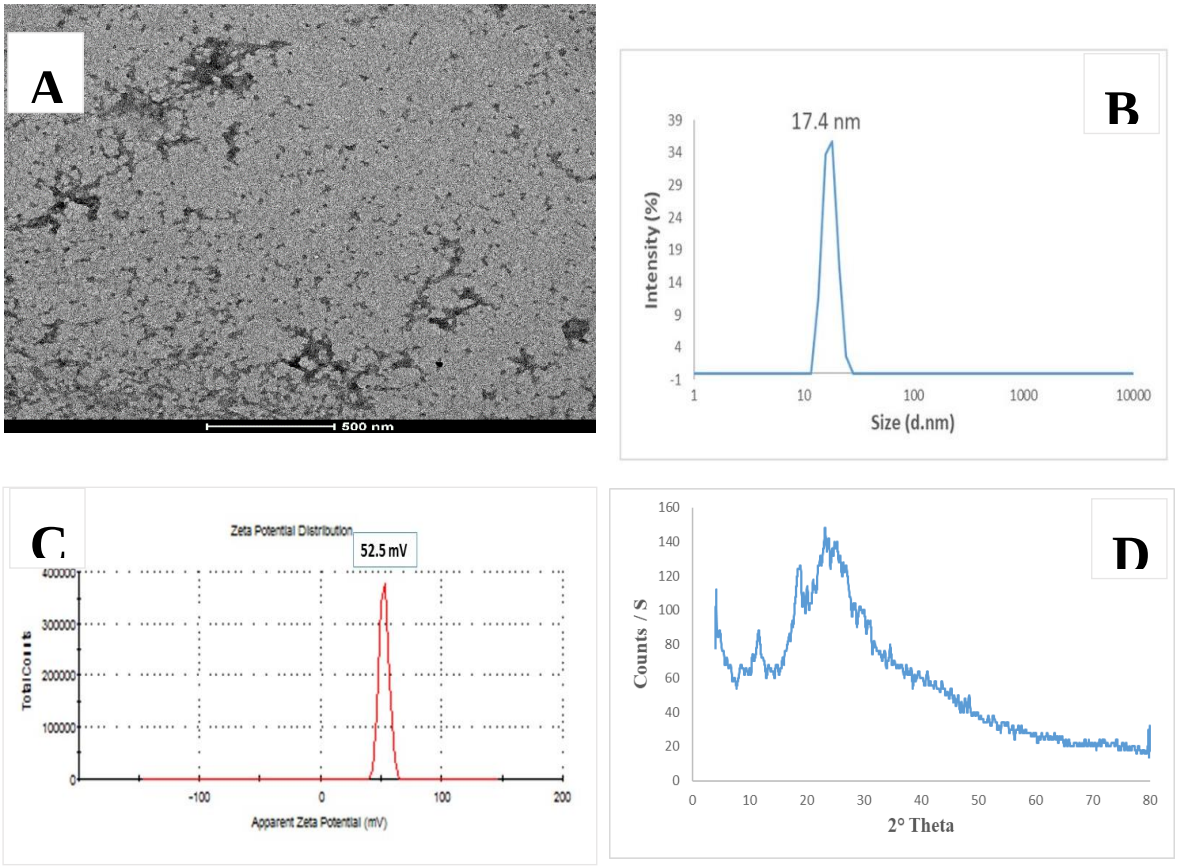

Fig (1). Characterization of chitosan nanoparticles (ChNPs).

(A): *HRTEM image showing nearly spherical shape of prepared chitosan nanoparticles with average size 17 nm.*

(B): *Particle size distribution of prepared chitosan nanoparticles showing the average size of 17 nm.*

(C): *Zeta potential of prepared chitosan nanoparticles showing surface charge, zeta potential, +52.5 mV.*

(D): *XRD pattern analysis indicating the formation of chitosan nanoparticles.*

Laying performance:

The effects of chitosan supplementation on the egg production of laying hens are shown in Table (2). The laying egg rate, average egg weight and FCR of laying hens were not affected significantly by the experimental groups. These results are consistent with the results reported by Nogueira *et al.* (2003) who found that egg weight was not affected by supplementation of 20 or 30g chitosan/kg diet during 29-37 weeks of age. Egg production was not affected by supplementation of chito-oligosaccharides (COS) (0.01%, 0.02%) in the diet Yan *et al.* (2010). The egg mass and feed intake were significantly decreased ($P<0.05$) in all treatments (T1, T2 and T3) compared to the control group, These results may be due to the high viscosity and the slow motility of chitosan in the gastrointestinal tract (Osho and Adeola, 2019). However, knowledge of the effects of ChNP on laying hens is still relatively limited. These results are agreement with the results reported by Xu *et al.* (2019) found that the addition of 200 or 400 mg/kg diet resulted in insignificant reduced feed intake compared to the control group of weaned pigs at 28 days; however, they reported that feed and weight gain were decreased ($P<0.05$) when the

supplementation of ChNP increased to 200 or 400 mg/kg diet for 28 days in weaned pigs. The mortality ratio was not recorded for all treatments during the experimental period.

Table (2): Effect of adding OCh and ChNP on productive performance of laying hens from 23 to 34 weeks of age.

Item	Treatment				SEM	*p*-value
	Co	T1	T2	T3		
Laying egg rate (%)/bird	84.91	80.83	82.75	81.91	0.734	0.249
Average egg weight (g)	60.69	58.26	60.12	59.13	0.386	0.119
Egg mass (Kg)/bird	4.325 ^{a}	3.955 ^{c}	4.125 ^{b}	4.066 ^{bc}	0.032	0.000
Feed intake (Kg)/bird	9.701 ^{a}	8.634 ^{c}	9.187 ^{b}	8.972 ^{bc}	0.089	0.000
Feed conversion ratio	2.24	2.19	2.23	2.21	0.023	0.867
Mortality ratio	ND	ND	ND	ND	-	-

OCh (chitosan ordinary) and ChNP (chitosan nanoparticles). Co (without supplementation), T_1 200 OCh mg/kg diet, T_2 50 ChNP /kg diet and T_3 200 ChNP /kg diet. SEM: standard error of the means. a,b and c mean the same raw having the different superscripts are significantly different (p < 0.05). ND (Not detect).

Egg quality:

Analysis of the data presented in Table (3) showed the effects of chitosan supplementation on egg quality. The addition of chitosan in the diet did not significantly (P<0.05) affect egg weight, yolk weight, shell weight, albumen weight, shell thickness and yolk color. While, the albumen height and Haugh Unit were significantly increased in the T1 and T3 groups compared to the control group. However, knowledge of the effects of ChNP on

Table (3): Effect of adding OCh and ChNP on egg quality of laying hens from 23 to 34 weeks of age.

Item	Treatment				SEM	*p*-value
	Co	T1	T2	T3		
Egg weight (g).	59.13	57.91	58.35	57.78	0.217	0.113
Yolk weight (g).	14.45	14.40	14.68	14.19	0.106	0.451
Shell weight (g).	6.22	6.13	5.90	6.04	0.065	0.273
Albumen weight (g).	38.44	37.37	37.76	37.53	0.157	0.077
Shell thickness (mm).	0.394	0.389	0.392	0.393	0.002	0.800
Yolk color.	7.87	8.04	7.95	8.00	0.090	0.929
Albumen height (mm).	7.24 ^{c}	7.41 ^{a}	7.26 ^{bc}	7.32 ^{b}	0.014	0.000
Haugh Unit.	85.43 ^{c}	86.78 ^{a}	85.77 ^{bc}	86.30 ^{ab}	0.113	0.000

OCh (chitosan ordinary) and ChNP (chitosan nanoparticles). Co (without supplementation), T_1 200 OCh mg/kg diet, T_2 50 ChNP /kg diet and T_3 200 ChNP /kg diet. SEM: standard error of the means. a,b and c mean the same raw having the different superscripts are significantly different (p < 0.05).

the dietary of laying hen appears limited and we have been unable to find any other study to confirm this result. Further study is needed to demonstrate the effect of ChNP addition in the laying hen. A similar finding was noted by Yoo *et al.* (2006) who found that the Haugh unit was improved with chitosan supplementation in the diet of

laying hens. Furthermore, Nogueira *et al.* (2003) found that egg weight and egg component weights were not affected by supplementation of 20 or 30g chitosan/kg diet during 29-37 weeks of age. In other studies, supplementation of chito-oligosaccharides in the diet of laying hens had a positive effect on egg quality Yan *et al.* (2010) and Meng *et al.* (2010).

Fatty acids, lipid oxidation and cholesterol content in egg yolk:

Table (4) shows the effects of chitosan supplementation on fatty acids, cholesterol content and lipid oxidation in the yolk of laying hens. The data revealed that palmitic acid was not affected by treatments. The lowest content of stearic acid was in the egg yolk in the T1 group, which fed a diet containing 200 mg OCh ($P < 0.05$). In contrast, the highest content of hexadecenoic acid, oleic acid, octadecadienoic acid, arachidonic acid and eicosenoic acid was in the yolk of the T1 and T3 groups. ΣUSFAs were significantly increased in the T1 and T3 group compared to the other groups. Conversely, ΣSFA values were significantly decreased in the T1 and T3 groups compared to the other groups. Lipid oxidation was determined by malondialdehyde, which significantly decreased in the T1 and T3 groups. The cholesterol content of yolk was not affected significantly by treatments, although the T1 and T3 groups were decreased numerically compared to the other groups. These results may be due to that the chitosan have the ability to bind to dietary lipids and eliminate them in feces. This is consistent with

Table (4): Effect of adding OCh and ChNP on yolk fatty acid concentrations and cholesterol content of laying hens at 34 weeks of age.

Item	Fatty acid(%)	Treatment				SEM	*p*-value
		Co	T1	T2	T3		
Palmitic acid	C16:0	27.29	24.99	26.86	25.09	0.417	0.100
Stearic acid	C18:0	16.11 ^{a}	12.64 ^{b}	15.15 ^{a}	14.49 ^{a}	0.382	0.004
Hexadecenoic acid	C16:1	2.22 ^{b}	2.81 ^{a}	2.06 ^{b}	2.72 ^{a}	0.087	0.000
Oleic acid	C18:1	39.12 ^{b}	42.60 ^{a}	38.41 ^{b}	41.02 ^{ab}	0.531	0.012
Octadecadienoic acid	C18:2	8.31 ^{b}	10.42 ^{a}	10.11 ^{a}	10.43 ^{a}	0.310	0.033
Arachidonic acid	C20:4	0.76 ^{c}	1.07 $^{a\,b}$	0.96 ^{bc}	1.22 ^{a}	0.049	0.002
Eicosenoic acid	C20:1 n9	0.56 ^{c}	1.43 ^{a}	0.84 ^{b}	1.26 ^{a}	0.077	0.000
ΣUSFA		51.44 ^{b}	58.24 ^{a}	52.50 ^{b}	56.20 ^{a}	0.698	0.000
ΣSFA		43.40 ^{a}	37.63 ^{c}	42.02 ^{ab}	39.58 ^{bc}	0.680	0.005
Malondialdehyde nmol/mL		5.03 ^{a}	4.38 ^{b}	4.97 ^{a}	4.61 ^{b}	0.07	0.003
Cholesterol content (mg/100 g yolk)		1023	968	1016	1001	15.14	0.611

ΣUSFA Unsaturated fatty acids. ΣSFA Saturated fatty acids. OCh (chitosan ordinary) and ChNP (chitosan nanoparticles). Co (without supplementation), T_1 200 OCh mg/kg diet, T_2 50 ChNP /kg diet and T_3 200 ChNP /kg diet. SEM: standard error of the means. a,b and c mean the same raw having the different superscripts are significantly different ($P < 0.05$).

previous studies on hypolipidemic effects of chitosan (Zhang *et al.* 2012 and 2013). These results are consistent with the results reported by Nogueira *et al.* (2003) who found that palmitic and stearic acid contents in eggs were lower in the groups given 20g or 30g than the group given a basal diet, while oleic acid in eggs in the group

given 30 g was higher than the group fed on a basal diet at 37 weeks of age. The egg yolk cholesterol levels were reduced by a diet containing 30 g/kg chitosan at 35 - 37 weeks of age compared to the control group, which fed a basal diet. Also, Abdel-Wahhab *et al.* (2017) reported that serum malondialdehyde was decreased significantly by the high dose of ChNP (280 mg /kg body weight) in rats. Contrary to our results, Li *et al*, (2015) found that the medium and high molecular weight of chitosan increased the serum malondialdehyde.

Blood serum measurements:

Results of blood serum tests are shown in Table (5). There were no differences (P<0.05) in the total serum protein, total cholesterol, high density lipoproteins, low density lipoproteins, immunoglobulin A and immunoglobulin M among all treatments. immunoglobulin Y was significantly increased (P<0.05) for group fed T1 followed by decreasing order by T3. These results may be due to the presence of the amine group in the synthesis of chitosan, which stimulates the immune system to produce antibodies and thus improves the immune response (Li *et al.,* 2016 and Tokura *et al.,* 1999). These results are in agreement with the results reported by Miao *et al.* (2020). Conversely, Xu *et al.* (2019) reported that supplementation of 200 or 400 mg ChNP/kg diet leads to an increased plasma immunoglobulin concentration (IgG) in weaned pigs at 28 days, whereas IgM concentration was not affected by ChNP. Pigs fed with a basic diet containing 50 or 100 mg/kg of low molecular weight chitosan showed an increased levels of serum IgG and IgM compared to the control group, whereas IgA concentration was not affected Zhanga *et al.* (2020). No differences were found among experimental groups (P>0.05) in serum immunoglobulins (IgA and IgM). These results may be due to non-repeated injection of sheep red blood cells (SRBC) before blood samples were-drawn.

Table (5): Effect of adding OCh and ChNP on blood serum parameters of of laying hens at 34 weeks of age.

Item	Treatment				SEM	*p*-value
	Co	T1	T2	T3		
Total protein (mg/dL)	6.27	6.10	6.15	5.97	0.136	0.911
Total cholesterol (mg/dL)	151.25	141.00	144.66	139.50	2.55	0.387
High density lipoproteins (mg/dL)	63.35	69.25	66.02	71.02	1.95	0.098
Low density lipoproteins (mg/dL)	56.50	50.31	53.89	49.91	1.50	0.373
Immunoglobulin A (µg/mL)	78.73	83.06	79.66	82.00	0.921	0.327
Immunoglobulin Y (µg/mL)	378.83^{b}	428.00^{a}	383.33^{b}	404.66^{ab}	6.71	0.025
Immunoglobulin M (µg/mL)	156.83	166.33	158.50	162.83	3.44	0.785

OCh (chitosan ordinary) and ChNP (chitosan nanoparticles). Co (without supplementation), T_1 200 OCh mg/kg diet, T_2 50 ChNP /kg diet and T_3 200 ChNP /kg diet. SEM: standard error of the means. a,b, c and d mean the same raw having the different superscripts are significantly different (P< 0.05).

Microbial population:

Table (6) reveals that total bacterial count, and *Lactobacillus* count of intestine were significantly increased (P<0.05) in the Och and the high dose of ChNP fed groups (T1 and T3) compared to the control group. Conversely, the *Escherichia coli* count was significantly decreased in the T1 and T3 groups. Both of *Salmonella* and *Clostridia* were not found for all groups.In the current study, the intestinal bacterial composition showed

differences among groups. Whereas, laying hens that fed 200 mg OCh or 200 mg/kg diet ChNP enhanced total bacterial count and *Lactobacillus* count in the intestine. Conversely, the *Escherichia coli* was significantly decreased by the same groups. Both 2 ordinary chitosan (200 mg) and ChNPs (200 mg) have a positive effect on the intestinal bacterial community of laying hens. To our knowledge, this is the first study to evaluate the effects of ChNP supplementation with size up to 17 nm in laying hens. These results are consistent with the results reported by Xu *et al.* (2019) who showed that ChNPs might increase the presence of the useful bacterial species and reduce the pathogenic bacterial species. Conversely, Abd El-Naby *et al.* (2019) found that ChNP supplementation in the diet of *Oreochromis niloticus* leads to the inhibition of the growth of intestinal bacterial assemblies. However, to evaluate the diversity changes at a larger scale (i.e. minor bacterial populations), next-generation sequencing combined with specific PCR assays are essential.

Table (6): Effect of adding OCh and ChNP on bacterial count in the intestinal contents of laying hens at 34 weeks of age.

Item	Treatment				SEM	*p*-value
	Co	T1	T2	T3		
Total bacteria count ($x10^7$) [2]	6.42 [b]	8.21 [a]	7.27 [ab]	8.68 [a]	0.29	0.024
Lactobacillus count ($x10^4$) [2]	4.43 [c]	5.52 [ab]	4.81 [bc]	5.83 [a]	0.16	0.003
Escherichia coli ($x10^4$)[2]	8.07 [a]	6.12 [b]	6.95 [ab]	5.62 [b]	0.33	0.045
Salmonella	ND	ND	ND	ND	ND	ND
Clostridia	ND	ND	ND	ND	ND	ND

Co (without supplementation), T_1 200 OCh mg/kg diet, T_2 50 ChNP /kg diet and T_3 200 ChNP /kg diet. SEM: standard error of the means. a,b and c mean the same raw having the different superscripts are significantly different (P < 0.05). ND. Not detected.

CONCLUSION

It could be concluded that addition of chitosan at 200 mg/kg diet of laying hens in both forms (chitosan nanoparticles "with a size of up to 17 nm" or ordinary chitosan) improved some production traits, such as egg quality, egg yolk composition, immunity and intestinal bacteria. Therefore, chitosan nanoparticles (ChNP) could be used as a potential supplement in the diets of laying hens. Future studies for investigation of the host pathogen interaction in the context of the feed additive containing chitosan and chitosan nanoparticles would be useful in corroborating our findings.

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تأثيرات إضافة النانو شيتوزان على الأداء الانتاجى للدجاج البياض

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أجريت هذه الدراسة في محطةِ بحوث الدواجنَ، كليّة الزراعة ، جامعة الأزهر ، القاهرة ، جمهورية مصر العربية. وكان الهدف من هذة الدراسة هو معرفة تأثير إضافة النانو شيتوزان والشيتوزان العادى على الأداءِ الانتاجى وبعض المقاييس الفسيولوجية وصفات جودة البيض وتركيب الاحماض الدهنية لصفار البيض والميكروبات المعوية للدجاج البياض لمدة ١٢ أسبوع (٢٣-٣٤ أسبوع) من العمر أثناء فصل الصيف. وتم استخدام ٩٦ دجاجة بياضة من سلالة (TETRA-SL) عمر يوم ، وتم توزيعهم عشوائيًا إلى ٤ معاملات كل معاملة تحتوى على ٣ مكررات وكل مكررة تحتوى على ٤ عيون وكل عين ٢ دجاجة. وكانت المعاملات كالتالى:-

المجموعة الأولى: مجموعة المقارنة في الظروف المثلى (تغذية حرة حتى الشبع).
المجموعة الثانية: مجموعة تتغذى على نفس العليقة الكنترول مع إضافة ٢٠٠ملجم/كجم علف شيتوزان عادى .
المجموعة الثالثة: مجموعة تتغذى على نفس العليقة الكنترول مع إضافة ٥٠ملجم/كجم علف نانو شيتوزان.
المجموعة الرابعة: مجموعة تتغذى على نفس العليقة الكنترول مع إضافة ٢٠٠ملجم/كجم علف نانو شيتوزان.
و كانت النَتائج المكتسبة لُخّصت كالاتى:

١- الصفات الانتاجية:-

لم يكن هناك فروق معنوية لكلا من معدل انتاج البيض ومتوسط وزن البيض ومعدل التحويل الغذائي بين المعاملات. بينما سجلت المعاملة المقارنة اعلى قيم معنوية لكلا من كتلة البيض والغذاء المأكول. كما انة لم تسجل وفيات خلال التجربة لجميع المعاملات.

٢- صفات جودة البيض:

سجلت المعاملة الثانية والرابعة اعلى قيم معنوية لكلا من ارتفاع الالبيومين و وحدة هيو مقارنة بباقى المعاملات. ولم يلاحظ فروق معنوية بين المعاملات في باقى الصفات (وزن البيض- وزن الصفار – وزن الالبيومين – وزن قشرة البيضة – سمك القشرة – لون الصفار)

٣- تركيب الصفار:

سجلت المعاملة الثانية والرابعة اعلى قيم معنوية لكلا من الاحماض الدهنية غير المشبعة لصفار البيض واقل قيم معنوية للاحماض الدهنية المشبعة مقارنة بباقى المعاملات. كما سجلت نفس المعاملتين اقل قيم معنوية لكلا من محتوى الكوليستيرول وmalondialdehyde مقارنة بباقى المعاملات.

٤- مكونات سيرم الدم:

جميع مقاييس الدم كانت داخل المعدل الطبيعى ، كما انة لم يلاحظ اى فروق معنوية بين المعاملات في الصفات المقاسة (البروتين الكلى -الكوليستيرول الكلى والكوليستيرول منخفض الكثافة والعالى الكثافة- الاجسام المناعية Igg- Igm بينما سجلت المعاملات الثانية والربعة اعلى قيمة معنوية للاجسام المناعية IgY .

الميكروبات المعوية :أشارت النتائج الى أن العد الكلى للبكتريا فى الأمعاء واللاكتوباسلس كان أعلى معنويا فى المعاملات التى تم إضافة الشيتوزان بمعدل ٢٠٠ ملجم في صورة نانو او في الصورة العادية. وبالنسبة لبكتريا E. Coil انخفضت معنويا بشكل ملحوظ فى الطيور التى عوملت بإضافة إضافة الشيتوزان بمعدل ٢٠٠ ملجم في صورة نانو او في الصورة العادية مقارنة بالكنترول. بينما لم يسجل وجود كلا من الكوليستريديا والسلامونيلا لجميع المعاملات.

من النتائج السابقة والتى يمكن من خلالها نستنتج أن إضافة الشيتوزان بمعدل ٢٠٠ ملجم في صورة نانو او في الصورة العادية خلال ٢٣-٣٤ أسبوع من عمر الدجاج البياض يعمل على تحسين مكونات صفار البيض ويرفع المناعة ويحسن بيئة الأمعاء الميكروبية بدون تأثير سلبى على الانتاج ووظائف الجسم.