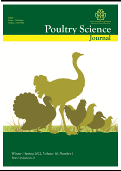

Poultry Science Journal
ISSN: 2345-6604 (Print), 2345-6566 (Online) http://psj.gau.ac.ir
DOI: 10.22069/psj.2022.19534.1733

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Supplementing a Herbal Product (NBS Superfood) in Diets Varying in Energy and Protein Levels: Effects on Productive Performance, Immune Response and Blood Parameters in Commercial Laying Hens

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Poultry Science Journal 2022, 10(1): 45-58

Keywords
Hen
NBS
Performance
Blood parameters
Energy and protein

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Article history
Received: September 20, 2021
Revised: February 15, 2022
Accepted: February 23, 2022

Abstract
This study was conducted to determine the effects of Nutrition Bio-Shield Superfood® (NBS) supplementation in diets varying in energy (ME) and crude protein (CP) levels on the performance of Hy-line W-36 laying hens. The experiment was performed in a 5 × 2 factorial arrangement in a completely randomized design with five dietary levels of NBS (0, 0.5, 1.0, 1.5, 2.0 g/kg of diet) and two dietary nutrient levels (standard and 5% reduced ME and CP). The experiment was conducted during 63-74 weeks of age using 420 laying hens in 10 treatments and 6 replicates of 7 birds each. The egg production was not significantly affected by diet dilution, NBS levels, and their interactions. The diet dilution, NBS powder, and their interaction did not significantly affect egg mass. Except that, it was significantly decreased ($P < 0.05$) by the diluted diet during 66-68 weeks of age. Feed intake (FI) was not significantly affected by diet dilution, NBS levels, and their interactions. Except that, the diluted diet significantly ($P < 0.05$) decreased during 66-68 weeks. Feed conversion ratio (FCR) was not significantly different between standard and 5% diluted diet, among the NBS levels and the interaction means. Diluting the diet and NBS supplementation did not significantly affect blood parameters; however, diluted diet significantly increased ($P = 0.05$) blood LDL-c concentration compared to the standard diet. Anti-SRBC titers were not significantly affected by the diet dilution, dietary levels of NBS powder, or their interactions. The treatments did not affect eggshell strength and the eggshell Ca and P percentages. In conclusion, reducing dietary ME and CP by 5%, adding NBS to the diet and their interaction had no significant effect on the layers' performance, immune response, blood parameters but diluting the diet increased blood LDL-c concentration.

Introduction

Feed additives have been used for decades since the poultry industry became a business. According to the report by Markets and Markets (2019), the feed additives market has globally increased from USD 33.0 billion in 2018 to USD 44.3 billion by 2023. However, some of these additives, such as antibiotics, appeared to be harmful to the animals and people due to bacterial resistance to antibiotics and antibiotics residues (Swann *et al.*, 1969). Antibiotic growth promoters have a threat on the humans through animal products like meats or eggs and animals' health as well as the environment. As a result, the European Union banned the use of antibiotics in 2006 which was followed by other countries worldwide (Yakhkeshi *et al.*, 2011). Because of that, researchers and producers decided to usenon-antibiotic feed additives to improve production and also to prevent the adverse effects on human and animal health (Consleg, 2003). Therefore, alternatives such as probiotics, prebiotics, synbiotics, medicinal plants

Please cite this article as Salah Mahdi Alsherify, Ahmad Hassanabadi, Saeed Zerehdaran & Hassan Nassiri Moghaddam. 2022. Supplementing a Herbal Product (NBS Superfood) in Diets Varying in Energy and Protein Levels: Effects on Productive Performance, Immune Response and Blood Parameters in Commercial Laying Hens. Poult. Sci. J. 10(1): 45-58.

and organic acids attracted more attentions during recent years.
One of the feed additives is "plant-derived products". Their compounds are natural and can improve the health and production of animals. Panda *et al.* (2000) referred that many herbs can be used in animal nutrition because of their anthelmintic, coccidiostatic, antimicrobial, immunomodulatory, and palatability effects. Recently, European Union accepted the usage of many herbs in the nutrition of domestic animals (Frankič *et al.,* 2009). The mechanism by which herbal extracts improve poultry health and performance (growth, carcass quality, nutrient digestibility, feed efficiency), most likely is to affect the gut microbiota (Schleicher *et al.*, 1998; Panda *et al.*, 2000; Gardzielewska *et al.*, 2003; Yakhkeshi *et al.*, 2011). Wheat germ is a by-product obtained from the milling process of wheat. This product contains fibers and important and valuable nutrients such as vitamins, minerals and amino acids (Hassan *et al.*, 2010; Tolouie *et al.*, 2018). It is also considered as a good source of amino acids such as methionine, threonine, and lysine (Jensen and Jensen, 1983) and fermented wheat germs has had a good effect on poultry production and immunity (Mueller and Voigt, 2011). A wheat germ product used in the current study is known by the trading name of NBS (www.nbs-superfood.com; Bayat *et al.*, 2021). It is a natural, healthy and viable herbal supplement prepared from wheat grains by a green route (NBS organic company, Turkey). The NBS powder which is wheat germ extract, contains fat- and water-soluble vitamins (e.g. B1, B2, B3, B5, B6, C, A, D, E, K), minerals (e.g. Ca, P, K, S, Mn, Zn, Cu, Fe, B) and fatty acids including omega-3, omega-6, and omega-9 (Bayat *et al.*, 2021). The company claims that NBS increases efficiency and productivity in breeders and laying hens, and helps to produce enriched eggs with better taste (www.nbs-superfood.com).

Apart from feed additives, energy and protein are two main factors in poultry diets that have a very important effect on poultry production (Kamran et al., 2008). Almeida *et al.* (2012) found that the interaction between ME and CP affectsegg production, egg mass, and feed intake (FI) in Hy-Line W-36 layers during 20-32 weeks of age. Since the diet represent about 70% of the total cost for the poultry producers (Kamran *et al.*, 2010), using a low ME and CP diet supplemented with a herbal product may save a fortune to the producers and keep the production in good shape. Therefore, this study aimed to investigate the effect of different doses of a commercial herbal product in diets with different ME and CP contentson productive performance, immunity, blood metabolites, eggshell strength, and eggshell Ca and P contents in laying hens during 63-74 weeks of age.

Materials and Methods

Experimental design, diets and management

A total of 420 Hy-Line W-36 hens with an initial average BW of 1.6 kg at 63 wk of age were used in this study. The experiment was performed in a 5 × 2 factorial arrangement in a completely randomized design with 5 dietary levels of NBS (0, 0.5, 1.0, 1.5, 2.0 g/kg of diet) and 2 dietary levels of ME and CP (standard and 5% reduced ME and CP) during 63-74 weeks of age for 12 wk. The birds were randomly allocated in 10 treatments with 6 replicate cages (experimental unit) of 7 birds each. Cage dimensions were 60 cm length × 60 cm width × 40 cm height. The cages were provided with two nipple drinkers and one trough feeder. During the experiment, birds received feed and water as ad libitum. The house temperature, lighting program and relative humidity were set to 23-25 °C, 16 hours light: 8 hours dark, and 60% during the experiment, respectively. Wood partitions were used to prevent cross-feeding between the cages (replicates). The chemical composition of NBS supplement powder is presented in Table 1.

Table 1. Chemical composition of NBS supplement powder[1].

Composition	Amount (%)	Minerals	Amount (%)	Vitamins	Amount (mg/kg)
Moisture	8.40	Total phosphorus	0.44	B1	0.66
Total ash	1.80	Potassium	2.31	B2	0.28
Fiber	11.26	Sulfur	0.28	B3	2.70
Digestible nutrients	61.90	Magnesium	0.32	B5	0.89
Carbohydrate (g/100g)	42.53	Calcium	1.67	B6	0.89
Gross energy (kcal/kg)	4300	Boron	0.62	C	52.40
Ether extract	7.20	Iron (mg/kg)	241	E	0.97
Crude protein	20.60	Manganese (mg/kg)	49.80	A (IU)	530.0
Sugar	3.70	Zinc (mg/kg)	26.90	D (IU)	483.0
Cellulose	6.00	Copper (mg/kg)	13.6	K (µg/kg)	63.60
Omega-3 fatty acids (mg/g)	48.42				
Omega-6 fatty acids (mg/g)	60.62				
Omega-9 fatty acids (mg/g)	22.16				

[1]Analyzed in Technology Development Center for Medicinal Plants. Department of Research and Development of Knowledge Based Green Drug Researchers Company.
NBS: Nutrition Bio-Shield Superfood®.

Experimental diets (Table 2) were formulated based on the Hy-Line W-36 management guide for commercial layers (Hy-Line International, 2020). The chemical composition of the diet ingredients and the complete diet were determined based on the AOAC (2019). The samples were ground and analyzed for crude protein (Kjeldahl; N × 6.25; method 990.03), dry matter (DM; method 930.15) and total ash (method 942.05). The contents of calcium (Ca) and total phosphorus (P) in the diets and eggshell samples were measured using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) instrument (Spectro Arcos, Kleve, Germany; method 968.08).

Table 2. Ingredients and nutrient composition of basal diets during 63 - 74 weeks, *as-fed basis*.

Ingredients (%)	Standard	5 % Diluted
Corn	40.86	46.68
Soybean meal (44% CP)	22.44	19.38
Wheat	20.20	20.22
Limestone	10.54	10.54
Vegetable oil	3.28	0.38
Dicalsium phosphate	1.58	1.59
Common salt	0.26	0.26
NaHCO3	0.1	0.1
Mineral premix[1]	0.25	0.25
Vitamin premix[2]	0.25	0.25
DL-Methionine	0.24	0.26
L-Lysine HCL	-	0.09
Calculated analysis, %		
ME (kcal/kg)	2823	2698
CP	15.24	14.44
Calcium	4.32	4.32
Available P	0.41	0.41
Dig Lysine	0.67	0.67
Dig Methionine	0.45	0.45
Dig Met + Cys	0.67	0.67
Sodium	0.17	0.17
Chlorine	0.20	0.21
Potassium	0.67	0.63
Dietary cation-anion balance	216.3	205.3
Determined analyses, %		
DM	93.26	92.63
CP	15.99	15.52
Ash	13.83	14.66
Calcium	3.36	2.91
Total P	0.45	0.55

[1]Provided per kg of diet: vitamin A (retinol), 8,800 IU; vitamin D3 (cholecalciferol), 3,300 IU; vitamin E (DL-α-tocopheryl acetate), 18.5 IU; vitamin K3 (menadione), 2.2 mg; vitamin B1 (thiamin), 2.2 mg; vitamin B2 (riboflavin), 5.5 mg; vitamin B3 (niacin), 28.0 mg; vitamin B5 (pantothenic acid), 6.6 mg; vitamin B6 (pyridoxine), 3.5 mg; vitamin B9 (folic acid), 0.7 mg; vitamin B12 (cyanocobalamin), 0.02 mg; biotin, 0.05 mg; antioxidant 1.0 mg.
[2]Provided (mg/kg of diet): Mn (manganese sulfate) 80.0, Fe (iron sulfate) 75.0, Zn (zinc sulfate) 64.0, Cu (copper sulfate) 6.0, Se (Sodium Selenite) 0.3.

Sample collection

Eggs were collected daily at 8:00 am. Then, they were weighed using a 0.001 g digital scale (0.001 g digital scale, model GF 400, A&D Weighing, San Jose, CA, USA). Egg production and the egg mass were calculated daily based on the following formulas.

Egg production (%) = number of eggs per replicate/number of birds per replicate × 100

Egg mass (g/hen/d) = egg weight per replicate × egg production % per replicate / 100

Daily FI was measured every three weeks by taking the difference of given feed at the beginning and remaining feed in the feeders at the end of the period and then divided by 21. The FCR was calculated by dividing FI (g) over the egg mass (g) (Bakhshalinejad *et al*., 2018). No mortality occurred during the experiment.

Blood parameters measurements

On the last day of the study, one bird from each replicate was randomly selected. About 5 mL of blood was taken from the wing vein and placed into a vacuum tube. Blood samples were kept at room temperature for about 2 hours until clotting and centrifuged at (3000 g; 10 min; 4°C). The collected serum was stored at –20 °C for further analysis. Biochemical analyses were performed according to the standard protocol using commercial laboratory kits (Pars Azmoon Co. Tehran, Iran). Glass tubes containing ethylenediaminetetraacetic acid (EDTA) were used for blood collecting to determine the levels of hematocrit (HCT), hemoglobin (HGB), red blood cells (RBC), white blood cells (WBC), phosphorus (P), calcium (Ca), triglycerides (TG), total cholesterol (Chol), low density lipoprotein cholesterol (LDL-c), and high density lipoprotein cholesterol (HDL-c). Blood samples were analyzed using Hematoanalyzer (model KX21, Sysmex Corporation, Kobe, Japan). All samples were analyzed in duplicate and done immediately to avoid variations.

Humoral immune response

To assay the humoral immune response against sheep red blood cells (SRBC), 10 mL of blood sample was taken from a ram and shed in a glass tube containing ethylenediaminetetraacetic acid (EDTA). The red cells were washed three times with phosphate-buffered saline (PBS) solution and then a 5% solution of red blood cells (RBC) was prepared in saline phosphate buffer. For clarifications, all the steps above were done in sterile conditions. At 71 weeks of age, one hen per replicate was injected with 0.5 mL of SRBC in the wing vein. A color spray was used to to differentiate injected and non injected birds.To measure the primary and secondary antibody responses against SRBC, 2 mL of blood sample was taken from the wing vein seven days after the firstinjection. After blood clotting, serum was removed by centrifuge (3000 g; 10 min; 4 °C). Collected sera were placed for half an hour at 56 °C for measurement of total anti-SRBC titers, IgG and IgM. The titers of antibody were presented as the log2 of the highest dilution level of serum that agglutinated 0.05 mL of 2.5% suspension of SRBC in phosphate-buffered saline (Eftekhari *et al*., 2018).

Eggshell strength

At the end of the study period, one egg was randomly selected from each replicate (n= … per treatment). The eggs were transferred to the laboratory. Eggshells strength was determined using a strength meter (model H5KS, Tinius Olsen Co, Horsham, PA, USA) with a maximum power of 50 Newton and a speed of 10 mm per minute (Bakhshalinejad *et al*., 2018). The egg was placed on its side (horizontally) and the maximum force imposed by the impact of a falling steel rod on eggshells was recorded (Bakhshalinejad *et al*., 2018).

Eggshell calcium and phosphorus

To determine the concentration of calcium and total phosphorus in the eggshells, one egg was randomly selected from each replicate (60 eggs in total, n= 6 per treatment) on the last day of the study. The eggs were kept at 4 °C for further analysis. On the day of analysis, the eggs were individually broken and the shell was separated from the yolk and albumen. Eggshells were then washed with distilled water and left 48 hours to dry at room temperature. After drying, the eggshells were ground and placed in a separate plastic bag specified for each replicate. Finally, Ca and P concentrations in the eggshells were measured using a spectrophotometer (inductively coupled plasma-optical emission spectroscopy, Spectro Arcos Co., Kleve, Germany) with standard methods (AOAC, 2019).

Body weight changes

The hens were weighed by a digital scale at the beginning and at the end of the experiment by randomly taking three replicates from each treatment (Bakhshalinejad *et al*., 2018).

Statistical analysis

Data were analyzed using PROC GLM of SAS software (SAS, 2012) for variance analyzes in a completely randomized design. The experiment was performed as a 2 × 5 factorial arrangement. The first factor was the diet dilution (standard and 5% diluted), and the second factor was the levels of NBS herbal product (0, 0.5, 1, 1.5, 2 g/kg of diet). Duncan's multiple range test was applied to separate treatment means ($P < 0.05$). PROC REG was used to test linear and quadratic responses to increasing dietary levels of NBS.

Results

Productive performance

The egg production percentage was not significantly affected either by the diet dilution, dietary levels of NBS powder, or their interactions in all experimental periods during the study (Table 3). However, diluting the diet caused a non-significant reduction in egg production percentage during all experimental periods of the study (Table 3).

Table 3. Effects of herbal product (NBS) supplementation in diets with different energy and protein levels on egg production (%) in Hy-line W-36 laying hens during 63-74 weeks of age[1].

Treatments		Period 1 63-65wk	Period 2 66-68wk	Period 3 69-71wk	Period 4 72-74wk	Total 63-74wk
Diet dilution	Standard	85.56	85.35	80.82	81.45	83.29
	5% diluted	85.24	83.04	79.75	79.68	81.93
SEM		0.07	0.07	0.08	0.09	0.07
NBS (g/kg diet)	0	85.03	84.18	80.27	80.67	82.54
	0.5	86.00	85.15	82.09	81.46	83.67
	1	86.51	84.75	81.46	82.09	83.70
	1.5	82.99	81.69	77.55	76.59	79.70
	2	86.45	85.20	80.05	82.03	83.43
SEM		0.19	0.19	0.21	0.22	0.19
Dilution × NBS						
Standard	0	84.92	83.56	79.59	80.16	82.06
	0.5	87.53	87.64	82.88	81.97	85.00
	1	86.62	86.85	84.13	85.26	85.71
	1.5	82.65	82.65	77.33	78.23	80.22
	2	86.06	86.05	80.16	81.63	83.47
5 % diluted	0	85.15	84.81	80.95	81.18	83.02
	0.5	84.47	82.65	81.29	80.95	82.34
	1	86.40	82.65	78.80	78.91	81.69
	1.5	83.33	80.72	77.78	74.94	79.19
	2	86.85	84.35	79.93	82.43	83.39
SEM		0.37	0.37	0.42	0.44	0.37
				P- Value		
Dilution		0.807	0.083	0.509	0.333	0.293
NBS		0.402	0.417	0.444	0.286	0.252
Dilution × NBS		0.877	0.595	0.712	0.675	0.748
Linear		0.854	0.629	0.910	0.638	0.764
Quadratic		0.857	0.664	0.772	0.679	0.832

NBS: Nutrition Bio-Shield Superfood®.
[1]Each mean represents six observations.
SEM: Standard error of the mean.

Table 4. Effects of herbal product supplementation (NBS) in diets with different energy and protein levels on egg mass (g of egg/hen/d) in Hy-line W-36 laying hens during 63-74 weeks of age[1].

Treatments		Period 1 63-65wk	Period 2 66-68wk	Period 3 69-71wk	Period 4 72-74wk	Total 63-74wk
Diet dilution	Standard	55.70	56.46[a]	53.07	53.51	54.69
	5% diluted	55.38	53.92[b]	51.83	51.93	53.27
SEM		0.11	0.13	0.13	0.15	0.11
NBS (g/kg diet)	0	55.08	54.63	52.17	52.55	53.61
	0.5	55.64	55.16	53.17	52.94	54.23
	1	56.42	56.83	53.26	53.60	55.03
	1.5	54.24	53.49	51.10	50.48	52.33
	2	56.34	55.83	52.54	54.04	54.67
SEM		0.28	0.34	0.32	0.36	0.27
Dilution × NBS						
Standard	0	55.43	54.55	52.22	52.47	53.67
	0.5	56.62	56.86	53.85	53.59	55.23
	1	56.43	59.84	55.15	55.51	56.73
	1.5	54.11	54.35	51.20	52.13	52.95
	2	55.94	56.71	52.91	53.87	54.86
5 % diluted	0	54.72	54.71	52.12	52.63	53.55
	0.5	54.67	53.46	52.49	52.30	53.23
	1	56.41	53.83	51.37	51.70	53.33
	1.5	54.38	52.63	51.00	48.84	51.71
	2	56.74	54.96	52.16	54.21	54.51
SEM		0.57	0.67	0.64	0.73	0.54
				P- Value		
Dilution		0.717	0.018	0.218	0.168	0.093
NBS		0.494	0.338	0.646	0.325	0.277
Dilution × NBS		0.884	0.424	0.765	0.679	0.721
Linear		0.965	0.744	0.800	0.621	0.982
Quadratic		0.978	0.767	0.738	0.597	0.995

[a–b]Values in the same column with different letters are significantly different ($P \leq 0.05$).
NBS: Nutrition Bio-Shield Superfood®.
[1]Each mean represents six observations.
SEM: Standard error of the mean.

Egg mass was not significantly affected by the diet dilution, dietary levels of NBS powder, or their interactions in all experimental periods during the study. Except that, it was significantly decreased ($P < 0.05$) by the diluted diet during 66-68 weeks of age. Furthermore, diluting the diet at other study periods non-significantly reduced the egg mass percentage (Table 4).

Feed intake of the hens was not significantly affected by the diet dilution, dietary levels of NBS powder, or their interactions in the study. Except that, it was significantly decreased ($P < 0.05$) by the diet dilution (106.58 vs. 102.26 g/hen/d) during 66-68 wk. In addition, diluting the diet non-significantly reduced the FI during the whole experimental period (Table 5).

There was not significant difference between standard and 5% diluted diets, among the herbal plant product levels and also among the interaction means for feed conversion ratio of the birds (Table 6). This means ME and CP levels and the different supplementation levels of NBS had no significant effect on the FCR in this experiment.

Blood characteristics and immune response

Both ME and CP levels and NBS supplementation did not have any significant effect ($P > 0.05$) on blood parameters of RBC, WBC, HGB, and HCT (Table 7). Also, no significant effect was observed on blood biochemical characteristics of TG, Chol, HDL, LDL, Ca, and P during this study (Table 8). However, as shown in Table 8, diluting the diet significantly increased ($P = 0.05$) LDL-C concentration in blood compared to the standard diet (65.53 vs. 59.10 mg/dl, respectively). Total anti-SRBC titer, immunoglobulin G and immunoglobulin M were not significantly affected by the diet dilution, dietary levels of NBS powder, or their interaction in this study (Table 9).

Table 5. Effects of herbal product (NBS) supplementation in diets with different energy and protein levels on feed intake (g/hen/d) in Hy-line W-36 laying hens during 63-74 weeks of age.

Treatments		Period 1 63-65 wk	Period 2 66-68 wk	Period 3 69-71 wk	Period 4 72-74 wk	Total 63-74 wk
Diet Dilution	Standard	106.45	106.58[a]	99.26	104.49	104.20
	5% diluted	106.24	102.26[b]	100.67	102.50	102.92
SEM		0.09	0.09	0.10	0.13	0.10
NBS (g/kg diet)	0	104.68	104.46	100.72	103.90	103.44
	0.5	106.62	104.20	100.09	102.43	103.33
	1	105.14	102.56	100.21	99.13	101.76
	1.5	108.04	103.76	99.68	105.18	104.16
	2	107.25	107.13	99.14	106.84	105.09
SEM		0.24	0.23	0.25	0.32	0.25
Dilution × NBS						
Standard	0	103.04	102.57	97.36	98.92	100.47
	0.5	107.64	105.99	97.69	103.74	103.77
	1	105.16	105.36	100.81	100.96	103.07
	1.5	108.46	108.78	99.96	109.38	106.64
	2	107.94	110.23	100.50	109.47	107.04
5 % diluted	0	106.32	106.35	104.08	108.87	106.41
	0.5	105.59	102.41	102.48	101.13	102.90
	1	105.12	99.76	99.62	97.29	100.45
	1.5	107.62	98.74	99.40	100.98	101.69
	2	106.56	104.03	97.78	104.22	103.15
SEM		0.47	0.47	0.50	0.64	0.50
				P- value		
Dilution		0.922	0.038	0.542	0.597	0.579
NBS		0.824	0.703	0.995	0.743	0.921
Dilution × NBS		0.934	0.299	0.639	0.594	0.589
Linear		0.756	0.318	0.969	0.384	0.593
Quadratic		0.896	0.252	0.960	0.309	0.517

[a–b]Values in the same column with different letters are significantly different ($P \leq 0.05$).
NBS: Nutrition Bio-Shield Superfood®.
[1]Each mean represents six observations.
SEM: Standard error of the mean.

Table 6. Effects of herbal product (NBS) supplementation in diets with different energy and protein levels on FCR (g of feed/g of egg mass) in Hy-line W-36 laying hens during 63-74 weeks of age[1].

Treatments		Period 1 63-65 wk	Period 2 66-68wk	Period 3 69-71 wk	Period 4 72-74wk	Total 63-74 wk
Diet Dilution	Standard	1.92	1.90	1.88	1.96	1.91
	5% diluted	1.92	1.90	1.95	1.99	1.94
SEM		0.01	0.01	0.01	0.02	0.01
NBS (g/kg diet)	0	1.90	1.92	1.94	1.99	1.94
	0.5	1.92	1.89	1.89	1.95	1.91
	1	1.86	1.82	1.89	1.86	1.85
	1.5	2.00	1.95	1.96	2.09	2.00
	2	1.91	1.92	1.89	1.99	1.92
SEM		0.03	0.03	0.04	0.05	0.04
Dilution × NBS						
Standard	0	1.86	1.88	1.87	1.89	1.88
	0.5	1.90	1.87	1.82	1.95	1.88
	1	1.86	1.78	1.83	1.82	1.82
	1.5	2.01	2.01	1.96	2.11	2.02
	2	1.94	1.95	1.90	2.05	1.95
5 % diluted	0	1.95	1.95	2.02	2.09	2.00
	0.5	1.94	1.92	1.96	1.95	1.94
	1	1.87	1.85	1.94	1.90	1.89
	1.5	1.98	1.88	1.96	2.08	1.97
	2	1.88	1.90	1.87	1.94	1.90
SEM		0.07	0.07	0.07	0.10	0.07
				P- value		
Dilution		0.869	0.935	0.145	0.777	0.584
NBS		0.354	0.431	0.803	0.544	0.477
Dilution × NBS		0.806	0.518	0.682	0.829	0.712
Linear		0.822	0.315	0.815	0.640	0.647
Quadratic		0.909	0.273	0.845	0.562	0.592

NBS: Nutrition Bio-Shield Superfood®.
[1]Each mean represents six observations.
SEM: Standard error of the mean.

Table 7. Effects of herbal product (NBS) supplementation in diets with different energy and protein levels on blood parameters in Hy-line W-36 laying hens on 74 weeks of age[1].

Treatments		RBC (10^{12}/L)	WBC (10^{9}/L)	HGB (g/dL)	HCT (%)
Diet Dilution	Standard	2.35	10.60	11.52	31.10
	5% diluted	2.35	10.56	11.81	31.17
SEM		0.01	0.03	0.03	0.10
NBS (g/kg diet)	0	2.41	10.10	12.15	31.75
	0.5	2.46	11.10	11.82	32.75
	1	2.30	10.48	11.44	30.50
	1.5	2.29	10.42	11.37	30.33
	2	2.29	10.27	11.54	30.33
SEM		0.02	0.08	0.07	0.26
Dilution× NBS					
Standard	0	2.44	10.76	11.95	32.33
	0.5	2.45	11.30	11.50	32.50
	1	2.23	10.11	10.97	29.33
	1.5	2.28	10.44	11.23	30.33
	2	2.35	10.41	11.93	31.00
5 % diluted	0				
	0.5	2.39	10.49	12.35	31.17
	1	2.48	10.90	12.13	33.00
	1.5	2.39	10.85	11.92	31.67
	2	2.30	10.41	11.50	30.33
SEM		2.23	10.14	11.15	29.67
		0.04	0.15	0.14	0.52
Dilution			*P*- Value		
NBS		0.904	0.850	0.186	0.934
Dilution × NBS		0.203	0.229	0.159	0.230
Linear		0.618	0.558	0.144	0.606
Quadratic		0.568	0.711	0.078	0.699
Diet Dilution	Standard	0.863	0.481	0.163	0.980

NBS: Nutrition Bio-Shield Superfood®.
[1]Each mean represents six observations.
RBC: red blood cells; WBC: white blood cells; HGB: hemoglobin; HCT: hematocrit.
SEM: Standard error of the mean.

Table 8. Effects of herbal product (NBS) supplementation in diets with different energy and protein levels on blood metabolites in Hy-line W-36 on 74 weeks of age[1].

Treatments		TG (mg/dL)	Chol (mg/dL)	HDL-c (mg/dL)	LDL-c (mg/dL)	Ca (mg/dL)	P (mg/dL)
Diet Dilution	Standard	1455.9	200.83	65.33	59.10[b]	20.44	6.37
	5% diluted	1367.9	185.93	65.83	65.53[a]	20.86	6.27
SEM		13.44	0.23	0.12	0.12	0.04	0.03
NBS (g/kg diet)	0	1328.0	200.42	66.92	66.17	20.73	6.48
	0.5	1376.1	209.67	64.42	63.08	20.70	6.32
	1	1646.8	196.00	66.17	59.92	21.00	6.40
	1.5	1215.6	190.83	67.33	61.75	20.27	6.22
	2	1492.9	170.00	63.08	60.67	20.58	6.12
SEM		33.60	0.57	0.31	0.29	0.11	0.08
Dilution × NBS							
Standard	0	1293.8	199.7	67.50	59.33	20.37	6.25
	0.5	1555.0	225.0	65.67	58.83	20.52	6.43
	1	1853.3	200.7	65.67	55.83	20.73	6.77
	1.5	1229.7	195.5	64.67	65.83	20.53	6.35
	2	1347.5	183.3	63.17	55.67	20.07	6.07
5 % diluted	0	1362.2	201.2	66.33	73.00	21.08	6.70
	0.5	1197.2	194.3	63.17	67.33	20.88	6.20
	1	1440.3	191.3	66.67	64.00	21.27	6.03
	1.5	1201.5	186.2	70.00	57.67	20.00	6.08
	2	1638.3	156.7	63.00	65.67	21.08	6.32
SEM		67.20	1.14	0.62	0.59	0.21	0.15
		P-Value					
Dilution		0.402	0.224	0.889	0.050	0.317	0.621
NBS		0.108	0.324	0.936	0.761	0.858	0.908
Dilution × NBS		0.185	0.911	0.966	0.257	0.810	0.445
Linear		0.661	0.761	0.701	0.369	0.765	0.799
Quadratic		0.728	0.377	0.711	0.547	0.904	0.618

[a–b]Values in the same column with different letters are significantly different ($P \leq 0.05$).
NBS: Nutrition Bio-Shield Superfood®.
[1]Each mean represents six observations.
TG: triglycerides; Chol: total cholesterol; HDL-c: high density lipoproteins -cholesterol; LDL-c: low density lipoproteins - cholesterol; Ca: calcium; P: phosphorus.
SEM: Standard error of the mean.

Table 9. Effects of herbal product (NBS) supplementation in diets with different energy and protein levels on immune response in Hy-line W-36 laying hens on 72 weeks of age[1].

Treatments		Total Ig	Ig G	Ig M
Diet Dilution	Standard	7.83	7.30	0.59
	5% diluted	8.07	7.27	0.80
SEM		0.04	0.04	0.03
NBS (g/kg diet)	0	8.17	7.33	0.83
	0.5	7.58	6.83	0.75
	1	7.92	7.17	0.75
	1.5	8.50	8.17	0.45
	2	7.58	6.92	0.67
SEM		0.10	0.10	0.07
Dilution × NBS				
Standard	0	8.83	8.17	0.67
	0.5	7.83	7.33	0.50
	1	7.67	7.00	0.67
	1.5	7.67	7.67	0.20
	2	7.17	6.33	0.83
5 % diluted	0	7.50	6.50	1.00
	0.5	7.33	6.33	1.00
	1	8.17	7.33	0.83
	1.5	9.33	8.67	0.67
	2	8.00	7.50	0.50
SEM		0.20	0.20	0.13
			P- value	
Dilution		0.538	0.932	0.199
NBS		0.486	0.218	0.643
Dilution × NBS		0.119	0.101	0.495
Linear		0.735	0.580	0.541
Quadratic		0.721	0.621	0.679

NBS: Nutrition Bio-Shield Superfood®.
[1]Each mean represents six observations.
SEM: Standard error of the mean.

Eggshell characteristics and body weight

Eggshell strength and the eggshell Ca, and P percentages were not affected by the study factors (Table 10). However, BW was significantly affected by diet dilution ($P < 0.05$). Hens fed with standard diet had significantly ($P < 0.05$) higher BW compared to the 5% diluted diet (1.62 vs. 1.57 kg; Table 10).

Table 10. Effects of herbal product (NBS) supplementation in diets with different energy and protein levels on eggshell strength, body weight, eggshell Ca, and P in Hy-line W-36 laying hens at 74 weeks of age[1].

Treatments		Egg shell strength (N)	Final BW (kg)	Ca (%)	P (%)
Diet Dilution	Standard	34.66	1.62[a]	37.38	0.13
	5% diluted	35.02	1.57[b]	36.90	0.14
SEM		0.09	0.02	0.05	0.01
NBS (g/kg diet)	0	34.92	1.60	37.31	0.14
	0.5	34.83	1.58	37.24	0.11
	1	35.29	1.59	37.55	0.17
	1.5	33.50	1.62	36.66	0.10
	2	35.64	1.61	36.94	0.15
SEM		0.23	0.04	0.13	0.02
Dilution × NBS					
Standard	0	36.25	1.65	38.06	0.16
	0.5	34.78	1.59	36.37	0.09
	1	35.28	1.60	37.70	0.15
	1.5	33.97	1.64	37.50	0.11
	2	33.00	1.64	37.26	0.16
5 % diluted	0	33.59	1.54	36.56	0.12
	0.5	34.89	1.57	38.11	0.13
	1	35.31	1.57	37.40	0.20
	1.5	33.03	1.61	35.82	0.10
	2	38.28	1.57	36.62	0.14
SEM		0.45	0.08	0.26	0.05
		P- value			
Dilution		0.850	0.014	0.459	0.853
NBS		0.963	0.701	0.919	0.198
Dilution × NBS		0.743	0.491	0.469	0.535
Linear		0.780	0.842	0.940	0.941
Quadratic		0.774	0.721	0.847	0.912

[a–b]Values in the same column with different letters are significantly different ($P \leq 0.05$).
NBS: Nutrition Bio-Shield Superfood®.
[1]Each mean represents six observations.
SEM: Standard error of the mean.

Discussion

Productive performance

Diet dilution, NBS supplementation, and their interactions had no significant effect on the egg production percentage in the current study. This means a 5% diluted diet (5% low ME and CP) used in this study had approximately the same results compared to the standard diet. Therefore, having a good egg production percentage with reduced ME levels and dietary CP benefits egg producers. In several studies, the egg production was not affected by the ME levels in the diet that agree with our findings (Harms *et al.,* 2000; Bean and Leeson, 2003; Wu *et al.,* 2005; Yuan *et al.,* 2009). Also, egg production was not affected by the CP levels in the diet, which agrees with results published by Torki *et al. (*2016). In agreement with our results, Hassan *et al.* (2013), Manangi *et al.* (2015), and Nguyen *et al.* (2021) indicated that using different ME and CP levels did not show a significant effect on the egg production of laying hens.

NBS has recently been developed, and there is no available data on its effect on laying hens performance. However, current study showed that the egg production was not affected by the addition of the supplementation of NBS powder. Yalcın *et al.* (2006) reported that using a herbal product (garlic powder) in the diet of laying hens did not affect egg production. There are no available reports about the effect of NBS in laying hens. However, other herbal extracts used in poultry diets improved growth performance (Panda *et al.,* 2000) and carcass quality (Schleicher *et al.,* 1998). Nevertheless, in agreement with our results, Gumus *et al.* (2018) concluded that adding feed additives (sumac and turmeric) with different ME and CP levels had no significant effect on the egg production of laying hens. This may be due to the source of feed additives or the way of preparing them. In the current study, the ME and CP levels (diet dilution), NBS supplementation, and their interaction had no significant effect on egg mass. Therefore, egg producers can reduce the ME and CP levels of the diets by 5% and still obtain good egg mass. Egg mass in this study was not significantly affected by the ME levels, which is similar to the data obtained by (Wu *et al.*, 2005; Yuan *et al.*, 2009); also, it was not affected by the CP levels in the diet, which is similar to the data reported by (Torki *et al.*, 2016). In addition, Hassan *et al.* (2013), Nguyen *et al.* (2021), and Scappaticcio *et al.* (2021) referred that using different ME and CP levels did not significantly affect the egg mass in laying hens. Egg mass was not affected by the NBS supplementation, which is similar to the results obtained by (Gumus *et al.*, 2018) when they added sumac and turmeric to layers' diet.

Diet dilution, NBS supplementation, and their interaction in the current study did not significantly affect feed intake. Having a good FI (low ME and CP) with no effect on the production will decrease the costs. These results are similar to the results reported by (Bean and Leeson, 2003; Yuan *et al.*, 2009). Also, they agree with the results of Torki *et al. (*2016) and Choi *et al. (*2018) when they reported that using different CP levels had no significant effect on the FI of laying hens of the same age. Moreover, using different ME and CP levels did not show a significant effect on the FI of laying hens, as stated by other researchers (Hassan *et al.*, 2013; Manangi *et al.*, 2015; Gumus *et al.*, 2018; Nguyen *et al.*, 2021; Scappaticcio *et al.*, 2021). A study revealed that hens fed with low ME diets had higher FI, whereas hens fed with high ME diets had lower FI (Harms *et al.*, 2000). Also, Wu *et al.* (2005) reported that as the ME level increased, the FI decreased. Saleh *et al.* (2014) found that dietary supplementation of a herbal product (summer shield) decreased broilers' FI. Feed intake of laying hens in our study was not affected by the dietary herbal product, which is similar to results reported by Yalcın *et al.* (2006).

The FCR was not affected by the diet dilution, NBS supplementation, or their interaction. This means ME and CP levels and the different supplementation levels of NBS had no significant effect on the FCR in this experiment. Then, diluting the diet may help to decrease production costs. Almeida *et al.* (2012) reported that high ME and CP levels resulted in a proper FCR. In the current study, no difference was found between higher and lower ME and CP levels on FCR. FCR and egg mass in this experiment were 1.91, 54.69 and 1.94, 53.27 for standard and 5% diluted diet, respectively. Which most likely a good result compared to some other studies when they reported that FCR and egg mass were 2.21, 50.7 and 2.07, 44.2 with 2700 and 3100 kcal/kg ME, respectively for layers (Almeida *et al.*, 2012). In addition, using different ME and CP levels had no significant effect on the FCR of laying hens (Hassan *et al.*, 2013; Manangi *et al.*, 2015; Gumus *et al.*, 2018; Nguyen *et al.*, 2021; Isabirye *et al.*, 2021). FCR was not significantly affected by the herbal plant product, which is similar to the results obtained by (Yalcın *et al.*, 2006).

Blood parameters

Blood parameters of RBC, WBC, HGB, and HCT were not significantly affected by the diet dilution, herbal product, or their interactions. This means that 5% diluted diet and the standard diet had approximately the same results. Similar results were reported by Lombardi *et al.* (2020) for laying hens. In addition, Lai *et al.* (2018) reported that using different ME and CP levels with varying doses of bile acids did not significantly affect the blood parameters (RBC, WBC, and HGB) of broilers chickens. Moreover, Toghyani *et al.* (2010) reported that using different ME and CP levels with feed additive supplements (antibiotic and thyme) in the broiler diets did not significantly affect blood parameters of RBC, WBC, HGB, and HCT, which is similar to the results of this study. Blood parameters were not significantly affected by the NBS, which is consistent with the results obtained by Gumus *et al.* (2018) when they added sumac and turmeric feed additives to the diets of laying hens.

No significant effects were found for ME and CP levels, herbal product, and their interaction on blood biochemical traits. Therefore, the low ME and CP diet (5% diluted diet) had approximately the same results as the higher ME and CP diet (standard diet). The results of our study agreed with the results of Hassan *et al.* (2013) when they reported that using different ME and CP levels in the laying hens' diet had no significant effect on blood Chol, TG, and HDL-c concentrations. Herbal product in the current study had no significant impact on the blood biochemical traits, which is similar to the results indicated by Yakhkeshi *et al.* (2011) when they used herbal extract for broilers. However, some herbal extracts reduced TG and Chol concentrations in the blood of laying hens and pigs (Yalcın *et al.*, 2006). Egg yolk contains different types of lipids, including cholesterol, triglycerides, fatty acids, and phospholipids. These lipids may be harmful to many people because they may increase their amounts in the blood, leading to coronary heart diseases or cancer. Egg yolk lipid composition can be affected by feed, age, and strain of the laying hens (Świątkiewicz *et al.*, 2020). In this study, feeding a 5% diluted diet significantly increased blood LDL-c concentration compared to the standard diet. With aged laying hens (63 weeks), LDL-c level was increased with a low level of CP and high level of ME (Dong & Tong, 2019), which is consistent with our results.

Decreasing Chol and LDL-c is a good sign for improving animal health and in the end, having healthy eggs for consumers. Moreover, Chinese herbal mixture (CHM), a mixture of pine needle and *Artemisia annua* reduced blood Chol, TG, and LDL-c concentrations (Li *et al.*, 2016).

Immune response

The immune system was not affected by diet dilution, NBS supplementations, or their interactions. This means that using a 5% diluted diet with low ME and CP had almost the same results as the high ME and CP diet (standard one). Similar results were obtained by Manangi *et al.* (2015) who indicated that using different ME and CP levels did not cause a significant effect on the immune response of laying hens. Also, Pourakbari *et al.* (2016) reported that different dietary levels of ME and CP with varying doses of a feed additive (probiotic**)**, had no significant effect on the immune response (IgT, IgM, and IgG) of broiler chickens. NBS is a new herbal product, and as far as the authors know, there is no available report on its effect on poultry. Therefore, the results of this study cannot be compared with any data from other literature. However, only one available published data tested NBS on immune system response in broilers (Bayat *et al.*, 2021). This study concluded that NBS increased immunity by increasing WBC and neutrophil count while decreasing lymphocytes. In our study, hens were injected by the SRBC and the birds' immune response was not affected by either ME and CP or NBS levels, which agrees with Yakhkeshi *et al.* (2011), who observed that total antibody, IgG, and IgM titers against SRBC were not significantly affected by a herbal extract.

In a recent study, supplementation of different NBS levels in broiler diets had no significant effect on the total and IgG antibody titers against SRBC. A herbal extract named *Andrographis paniculata* improved broilers' immune response (Mathivanan and Kalaiarasi, 2007); however, it was not supported with the current study in laying hens. Plant extract called fermented wheat germ extract (FWGE) increased the humoral antibody levels in layer parents after challenging with Newcastle disease (ND), inflammatory bowel disease (IBD), Ehlers-Danlos syndrome (EDS) and AE immunization. In addition, *Andrographis paniculata* herbal extract that has been used in poultry diets revealed beneficial results through improving health and the immune system (Mathivanan and Kalaiarasi, 2007).

Eggshell characteristics and body weight

These parameters were not significantly affected by the diet dilution, NBS supplementation, or their interactions. Using a low ME and CP diet resulted in the same eggshell strength and eggshell Ca and P percentage with the high ME and CP diet except for BW in the current experiment. Eggshell breaking strength was not significantly affected by the ME and CP levels in the present study, which agrees with the results reported by others (Hassan *et al.*, 2013; Manangi *et al.*, 2015; Nguyen *et al.*, 2021; Scappaticcio *et al.*, 2021). In addition, eggshell strength was not affected by CP levels in the laying hens' diet, as mentioned by Choi *et al.* (2018). Body weight was significantly high with the higher level of ME and CP and low for the lower one, which is similar to the results reported by Mikulski *et al.* (2020). Therefore, decreasing ME and CP levels up to 5% has no significant adverse effect on layer hens' performance.

NBS Herbal product had no significant effect on the eggshell strength, which agrees with the results published by Li *et al.* (2016), who used Chinese herbal mixture (CHM), a mixture of pine needle and Artemisia Annua. In addition, other researchers did not observe any differences or beneficial effects of using herbal plant extracts for improving eggshell quality (Bozkurt *et al.*, 2012; Świątkiewicz *et al.*, 2013). Gumus *et al.* (2018) concluded that adding sumac and turmeric feed additives had no significant effect on egg quality characteristics which is similar to the results of our study. Moreover, another study that used a herbal product (garlic powder) in the diet of laying hens concluded that it has no significant effect on BW and egg breaking strength (Yalcın *et al.*, 2006).

Conclusion

Overall laying hens' performance was not significantly affected by the study factors (ME and CP levels) and the different dietary levels of the herbal plant product (NBS). Then, reducing the diet ME and CP by 5% is recommendable for the laying hens during 63 to 75 weeks of age.

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