

Effect of dietary inclusion of a commercial polyherbal formulation on some physiological and immune parameters in healthy and stressed hens

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Citation: Cecchini S., Rossetti M., Caputo A.R., Bavoso A. (2019): Effect of dietary inclusion of a commercial polyherbal formulation on some physiological and immune parameters in healthy and stressed hens. Czech J. Anim. Sci., 64, 448–458.

Abstract: The effects of the dietary inclusion of a polyherbal formulation based on three powdered herbs (*W. somnifera*, *T. cordifolia*, *O. sanctum*) on some physiological and immune parameters were studied in healthy and stressed laying hens. The effects of the dietary polyherbal formulation were also compared with those of dietary ascorbic acid (AA) supplement, nowadays considered one of the most potent immunostimulant substances widely used as a food supplement. Experimental data did not show any positive effects, or very low ones, on the assessed parameters in healthy hens as a consequence of the two dietary supplementations. On the contrary, the dietary inclusion of the polyherbal mixture or AA partially counteracted the adverse effects in hens subjected to a moderate and transient dexamethasone-induced stress, when ameliorating effects on natural IgM antibody level, specific antibody response, total immunoglobulin content, respiratory burst activity and total antioxidant capacity were shown. The obtained results justify the ethnomedical use of this polyherbal mixture in stressed laying hens in which faster recovery has been demonstrated, whereas healthy specimens did not seem to substantially benefit from the dietary integration, neither with the polyherbal product nor with AA. Thus, the presence of nutraceutical compounds in several herbal plants exerting no side-effects might be useful for exploring them as an alternative to allopathic substances for preventive or therapeutic purposes in poultry.

Keywords: dietary supplement; immunity; laying hens; physiology; stress

In the European Union the use of antibiotic growth promoters (AGPs) as feed additives was allowed in the poultry industry for a long time, but their use was banned in 2006, mainly due to cross-resistance against pathogens and residues in tissues (Castanon 2007). Even if AGPs are still allowed in most non-EU countries (Van Boeckel et al. 2015), their ban took away a valuable tool from the European farmers that also acted as a control means of subclinical diseases. Nowadays most of the problems in poultry are due to a combination of several environmental factors that act in synergy as stressors (Lutful Kabir 2009). Moreover, where

farm animals are kept in close co-existence, like in intensive farming, the immune system is constantly exposed to numerous opportunistic pathogens. Since the innate immune response provides the first line of defence for the host, the regulation of its activity by the use of natural compounds, as an alternative to AGPs, represents a fundamental strategy to increase the survival during the production cycle, the quality of products intended for human consumption and, more generally, to ensure animal welfare (Hajati et al. 2014).

Among possible alternatives to AGPs, a useful aid may come from medicinal plants that include

<https://doi.org/10.17221/189/2019-CJAS>

herbs, spices, seaweeds, herbal extracted substances and commercial plant-derived products. Although medicinal plants have been used as immunostimulants in human medicine for thousands of years, the application of natural compounds represents an alternative to the use of antibiotics in farm animals. Many studies have been performed to identify nutraceutical substances in herbs or herbal mixtures of traditional medicine. Plants have several bioactive compounds; among them, antioxidant substances (tannic acid, flavonoids, tocopherol, carotenoids, ascorbate, etc.), which exert several biological functions in living beings, are believed to act synergistically (Scartezzini and Speroni 2000). Plant-derived polyphenols, the main secondary plant metabolites, have received considerable attention for the control of chronic diseases in human medicine (Knekt et al. 2002). Their biological functions are principally assumed as results of the polyphenolic radical-scavenging properties. However, even if there is a lack of information on their *in vivo* absorption and metabolism, polyphenols are believed to exert several modes of action in *in vivo* systems, in which their antioxidant properties do not seem to represent the pivotal activity (Surai 2014).

In poultry, several beneficial effects regarding the dietary inclusion of herbs, vegetables or their extracted substances were shown and the topic has recently been reviewed by Surai (2014). Among these beneficial effects, immune activity regulation, increased survival during the production cycle, reduction of oxidative stress, prebiotic effects enhancing the gut health and increased oxidation stability of products intended for human consumption are noteworthy and are the main mechanisms by which these substances exert helpful effects on growth and health of poultry. As regards the effect of plant- or plant extract-enriched diets on production traits of chickens, the results are inconsistent amongst different studies (Erener et al. 2011; Viveros et al. 2011; Shahid et al. 2013; Farahat et al. 2016).

The enhancement of the oxidative stability of meat by the use of polyphenol-enriched diets is obtained by the reduction of oxidative processes leading to meat lipid peroxidation, as shown by the decrease of the malondialdehyde (MDA) content in broiler meat (Farahat et al. 2016). The same authors showed an increased level of reduced glutathione in liver tissue, one of the most important

antioxidants inside living cells, a useful indicator of the antioxidant status in tissue (Farahat et al. 2016). At the same time, polyphenol-enriched diets are responsible for the modulation of the chicken immune system against Newcastle disease and coccidiosis (Farahat et al. 2016). One of the most important biological effects of plant extracts is to guarantee the health status of chicken directly, preserving the gut health by increasing mucus production and epithelial cell proliferation, and modulating the activity of the gut-associated immune system (Awati et al. 2012). According to Viveros et al. (2011), dietary inclusion of polyphenol-rich grape products in broilers influences gut morphology and acts as a prebiotic, promoting the gut microflora biodiversity.

However, despite that the information about their effectiveness and safety is not exhaustive yet and is sometimes inconsistent, some polyphenol-based feed additives for livestock are marketed. Thus, this study was performed to evaluate the effects of dietary inclusion of a polyherbal formulation marketed under the name ImmuPlus by Global Herbs Ltd. (Chichester, UK) on some physiological and immune parameters in laying hens. This product, suggested as a nutritional supplement for horses, is based on three powdered herbs in equal proportions (*Withania somnifera*, *Tinospora cordifolia* and *Ocimum sanctum*). These herbs are known in Ayurveda, the Indian traditional medicine, for their immunostimulant efficacy for human and animal beings (Devasagayam and Sainis 2002). In a previous paper, Cecchini et al. (2014) evaluated the *in vitro* antioxidant potential of this herbal mixture by assessing different methods, showing high polyphenol and flavonoid contents responsible for the observed antioxidant and scavenging activities. The obtained results suggested the effective role of the polyherbal mixture as a good source of antioxidants in animal feeding and stimulated us to test it in an *in vivo* animal model (Cecchini et al. 2014). Moreover, to compare the resulting biological activities obtained with the polyherbal formulation with a substance currently recommended for the immune system modulation, ascorbic acid (AA) was employed to enrich the diet of other animal groups. In fact, AA is nowadays considered one of the most potent antioxidant and immunostimulant substances being widely used as a food supplement both in human and veterinary medicine.

MATERIAL AND METHODS

Experimental design, immunization and stress induction. The experiment was performed at a local farm and all the procedures were conducted in strict accordance with European legislation regarding the protection of animals used for scientific purposes (European Directive 2010/63/EU), as recognized and adopted by the Italian law (DL 2014/26). No animals died during or as a consequence of the conducted experiment.

Sixty ISA Brown (Rhode Island Red × Rhode Island White hybrid) young hens of 16 weeks of age were kept in floor pens in an environmentally controlled room ($20 \pm 1^\circ\text{C}$) and tagged for identification. Animals were provided free access to water and feed, using a commercial corn-soybean diet for layers, whose metabolizable energy and chemical composition are reported in Table 1. After 3 weeks of acclimatization when the light schedule was progressively modified at 15 h light and 9 h darkness, hens were randomly divided into six groups of ten hens per pen and their diet was supplemented as follows: basal diet of 2 groups was supplemented with ImmuPlus (1% w/w), representing the ImmuPlus groups; basal diet of 2 groups was supplemented with L-AA (0.02% w/w), according to Asli et al. (2007), representing the ascorbic acid groups; basal diet of 2 groups was without supplementation, representing the control groups.

On the first day of differentiated feeding (day 0), all animals were subjected to the first blood sampling, which was repeated 2, 4, 5, 6, 7 and 9 weeks from the beginning of the experiment. Blood samples were drawn from the brachial vein and collected into microtubes containing EDTA as the anticoagulant. Plasma was obtained by centrifugation at 2500 g for 15 min at 4°C (Centrifuge 5810 R; Eppendorf, Germany) and stored at -80°C until analysed.

At week 4 and after blood sampling, all animals were immunized by intramuscular injection in the breast with 5 mg of human- γ -globulins (HyG) (Sigma-Aldrich, USA) in 0.5 ml of saline solution as previously described (Cecchini et al. 2016). Further, after the immunization the specimens of one group for each differentiated diet were submitted for 6 consecutive days to intramuscular injection of dexamethasone (DEX) (Sigma-Aldrich) at the dosage of approximately 1.5 mg/kg body weight as previously described (Cecchini et al. 2016), to in-

Table 1. Metabolizable energy (MJ/kg) and chemical composition (%) of the basal diet of laying hens

Composition	
Metabolizable energy (MJ/kg)	11.60
Crude protein (%)	16.80
Fat (%)	3.50
Fibre (%)	3.80
Ash (%)	13.50
Lysine (%)	0.80
Methionine + cysteine (%)	0.70
Calcium (%)	4.00
Phosphorus (%)	0.65
Sodium (%)	0.30
Vitamin and mineral premix ¹	

¹provided per kilogram of diet: vitamin A 6750 IU, cholecalciferol 2500 IU, vitamin E 25 mg, vitamin K 2.5 mg, thiamine 2 mg, riboflavin 5 mg, pyridoxine 2 mg, vitamin B12 0.01 mg, niacin 30 mg, calcium pantothenate 10 mg

duce a short/medium-term stress condition (stressed groups). At the same time, hens of the other group for each diet received by injection 0.5 ml of saline solution for the 6 consecutive days (no stressed groups). The experimental protocol is shown in Figure 1.

Analytical methods. Plasma glucose concentrations (glycaemia) were measured by the glucose oxidase colorimetric method (GAGO-20; Sigma-Aldrich). Total protein and albumin concentrations were measured by the biuret method using a total protein reagent (T1949; Sigma-Aldrich) and by the bromocresol green method (Doumas et al. 1971), respectively, expressing data as bovine serum albumin equivalents (BSA; mg/ml). Total immunoglobulin concentrations (tot Ig) and total non-Ig protein concentrations (not-Ig prot) were analysed by the precipitation method (Sechman et al. 2004) and data were expressed as BSA equivalents (mg/ml).

Respiratory burst was analysed as nitroblue tetrazolium (NBT) test, measuring the formazan production from NBT by intracellular superoxide anion (O_2^-), as indicated by Ruane et al. (1999). Data were expressed as formazan ($\mu\text{g/ml}$) derived from a standard curve obtained by solubilising increasing amounts of reduced NBT formazan.

Total antioxidant capacity was evaluated performing the FRAP assay (Benzie and Strain 1996) as shown by Fazio et al. (2014). Experimental data are presented as iron sulphate heptahydrate ($\text{FeSO}_4 \times 7\text{H}_2\text{O}$) equivalents (μM).

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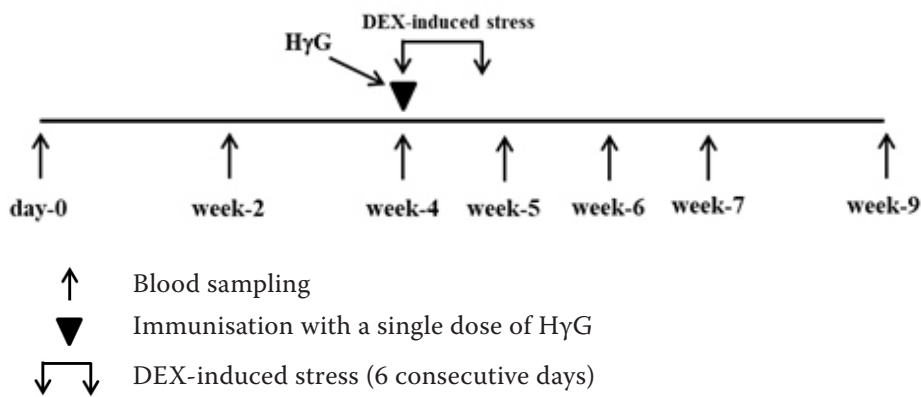


Figure 1. Scheme of the experimental protocol

HyG = human- γ -globulins,
DEX = dexamethasone

Lipid peroxidation damage was analysed as thiobarbituric acid reactive substances (TBARS) as previously described by Cecchini et al. (2018). Data were expressed in terms of MDA equivalents (μM) using an external standard calibration curve obtained from MDA generation by 1,1,3,3-tetraethoxypropane (Sigma-Aldrich) hydrolysis.

All the optical readings of the spectrophotometric methods were acquired with a SmartSpec 3000 UV/Vis Spectrophotometer (Bio-Rad Laboratories Inc., USA).

Levels of natural antibodies (NAb) binding TNP-BSA, both isotype IgY and isotype IgM, and specific antibody (IgY) response against HyG (SpAb) were measured by indirect ELISA, as indicated by Cecchini et al. (2016). Briefly, different microtiter plates were coated (100 μl per well) with antigens (5 and 10 $\mu\text{g}/\text{ml}$ for TNP-BSA (BioResearch Technologies, USA) and HyG, respectively) in coating buffer. Blocking of residual sites was obtained with 0.5% fish gelatine in phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS-T). After blocking, 100 μl of diluted plasma samples (1:400 and 1:2000 for NAb and specific Ab response, respectively) were tested in duplicate. After incubation (1 h at 37°C), 100 μl of polyclonal Ab rabbit anti-chicken IgY-HRP or polyclonal Ab goat anti-chicken IgM-HRP (Sigma-Aldrich) were used to detect bound antibodies. Between the different steps the plates were extensively washed with PBS-T and PBS. The enzymatic reaction was obtained with 100 μl o-phenylenediamine (OPD) plus hydrogen peroxide (H_2O_2) in citrate-phosphate buffer and the reaction was stopped with 50 μl 2 M H_2SO_4 . Subsequently, optical densities (OD) were read at 492 nm on a microplate reader (Model 550, Bio-Rad Laboratories, Inc.). The upper negative limit with a 99.9% confidence was obtained by positive

value (cut-off) set at the mean OD value plus three times standard deviation of the mean of wells in which blocking buffer replaced the antigen for NAb evaluation and at the mean OD of ten negative (pre-immune) controls plus three times standard deviation of the mean for SpAb response assay, as previously reported by Cecchini et al. (2016).

Statistical analysis. Sixty hens were included in this study and randomly divided into six groups of ten animals each subjected to three different diets (i.e. control diet, control diet supplemented with ImmuPlus or AA; two groups for each diet). At week 4 from the beginning of the experiment, one group for each differentiated diet was exposed to DEX-induced stress. Blood samples were collected on the first day of differentiated feeding (day 0) and 2, 4, 5, 6, 7 and 9 weeks later (time points $n = 7$). Normal distribution of analytical data was confirmed using the Kolmogorov-Smirnov test ($P > 0.05$). Two-way analysis of variance (ANOVA) for repeated measures was used to analyse the effect of the independent variables (diet and DEX-induced stress) on the obtained analytical data along with the interaction between the independent variables.

When a significant overall difference was detected, differences among means were determined by Tukey's pairwise comparisons. P -values less than 0.05 were considered statistically significant. All statistical analyses were performed using SigmaPlot Version 11.0 for Windows™ statistical software.

RESULTS

Two-way repeated measures ANOVA showed that neither diets nor DEX-induced stress affected total protein, TBARS and NAb-IgY levels ($P > 0.05$, data not shown), although a slight increase

($P = 0.10$) in the protein level was observed in the stressed groups only at the first sampling after the end of DEX treatment (week 5). As regards the other assessed parameters, some significant differences appeared starting 4 weeks (week 4) from the beginning of the experiment (Figures 2–5).

At week 4 and before the DEX-induced stress only respiratory burst (Figure 2B) and total non-Ig protein concentrations (Figure 4A) were affected by the independent variable diet; respiratory burst was significantly enhanced only in hen groups fed AA-enriched diet ($P < 0.05$), while total non-Ig protein contents were increased in groups fed both AA- and polyherbal formulation-enriched diet ($P < 0.05$). Starting from the end of the DEX-induced stress (week 5 and subsequent weeks), some assessed parameters (glycaemia, respiratory burst, total antioxidant capacity, total Ig content,

not-Ig protein content, albumin content, NAb-IgM level, SpAb) were shown to be statistically affected mainly by DEX-treatment and some of them also by the interaction between the two independent variables (diet \times stress) (Figures 2–5).

Although the differences are almost always limited over time, especially in the first (week 5) or in the second week (week 6) after the induced stress, it is interesting to note that the interaction between the two independent variables (diet \times stress) statistically influenced the respiratory burst (Figure 2B), total Ig content (Figure 3B), albumin content (Figure 4B), NAb-IgM level and SpAb response against human- γ -globulins (Figure 5).

As for the comparison between the herbal mixture and AA, obtained results have demonstrated similar biological responses, but AA-enriched diet seems to have better counteracted, although

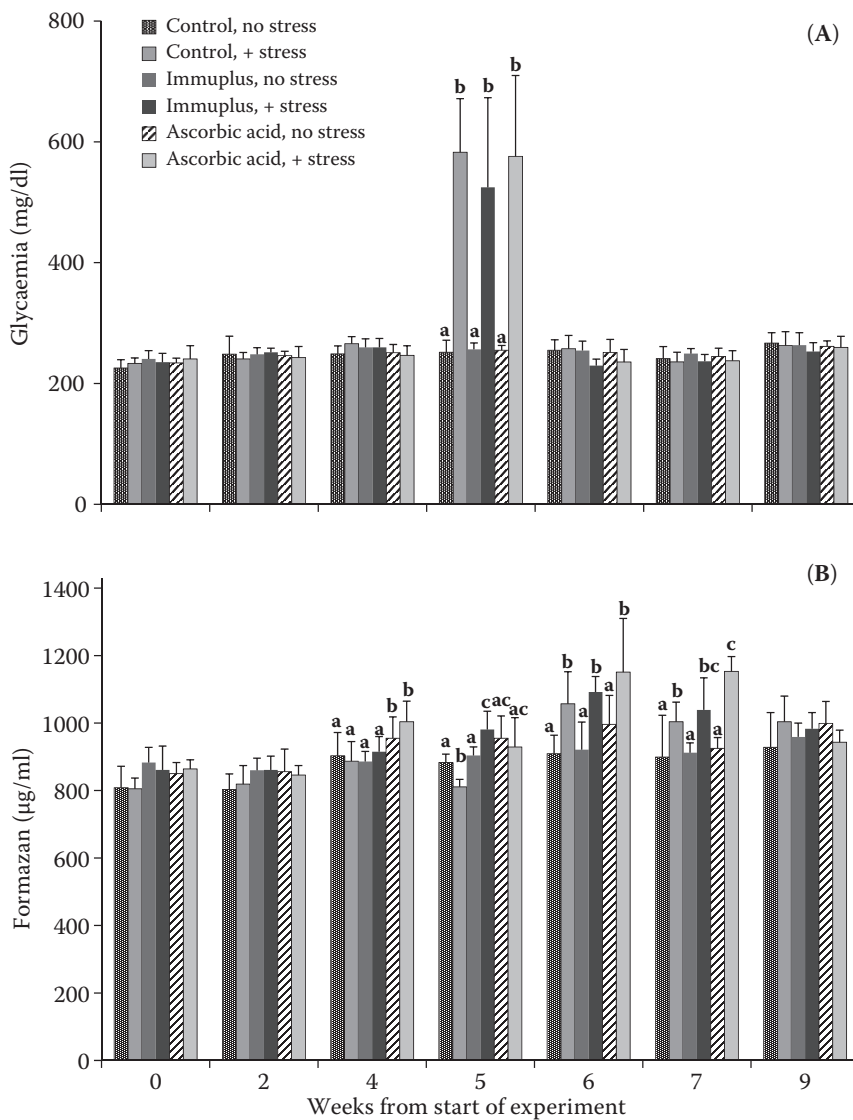


Figure 2. Influence of dietary supplement ImmuPlus and ascorbic acid on glycaemia (A) and respiratory burst (B) in laying hens

Values are expressed as means \pm standard deviation. Means with different letters at the same sampling time are statistically different ($P < 0.05$). For the time window of dexamethasone-induced stress see Figure 1

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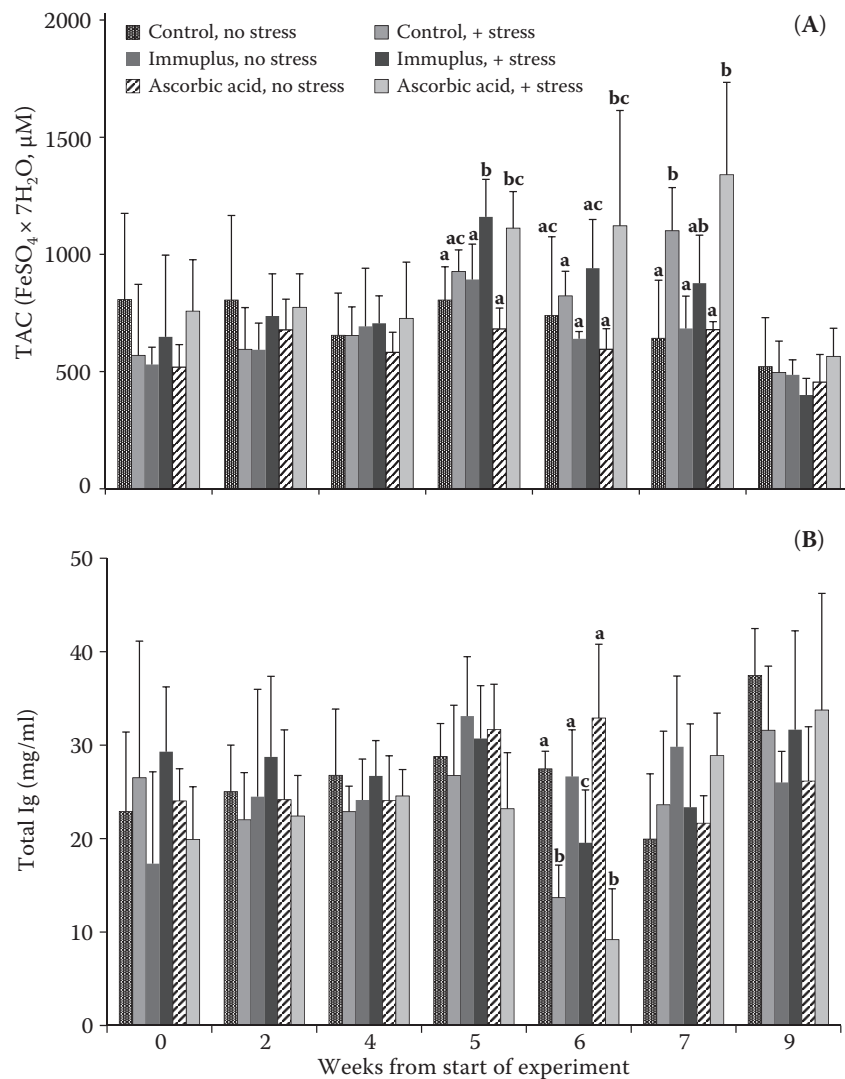


Figure 3. Influence of dietary supplement ImmuPlus and ascorbic acid on total antioxidant capacity (A) and total immunoglobulin content (B) in laying hens

Values are expressed as means \pm standard deviation. Means with different letters at the same sampling time are statistically different ($P < 0.05$)

not significantly in comparison with the herbal mixture-enriched diet, the immune depression of the SpAb response already starting from the first sampling (week 5) after DEX-treatment (Figure 5B). At the following sampling time (week 6), the SpAb responses of DEX-stressed hens fed polyherbal formulation- or AA-enriched diet were not statistically different from each other but they were significantly higher in comparison with the SpAb response of stressed hens fed the control diet.

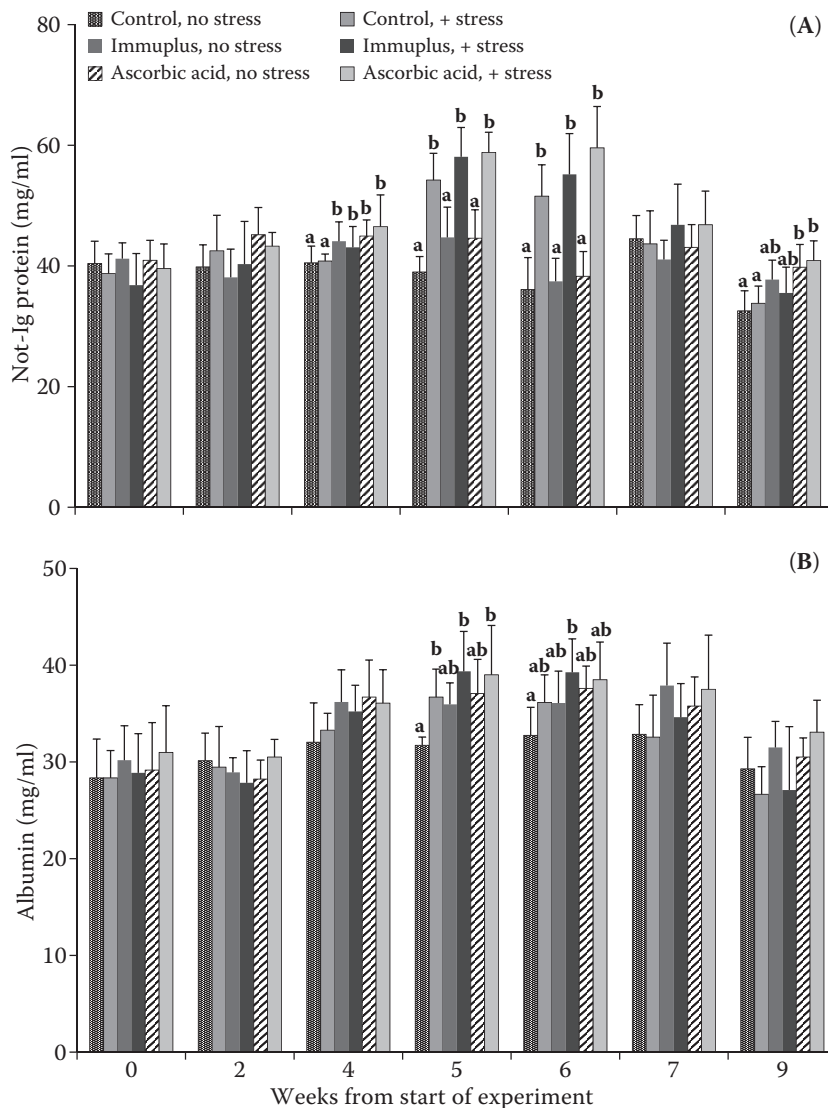
DISCUSSION

In the present study, the effects of a dietary polyherbal formulation on some physiological and immune parameters in healthy and stressed young laying hens were investigated. To compare

the resulting biological activities not only versus a control diet but also versus a well-known immunostimulant and antioxidant substance, the experimental plan also included the administration of AA in the diet, nowadays one of the substances employed to enrich diets mainly when animals are exposed to environmental stressors. In fact, if the effect of dietary AA supplementation in poultry is well known (Naseem et al. 2005; Asli et al. 2007; Mirfendereski and Jahanian 2015), less information is available on the potential use of medicinal plants or phytobiotics in which active compounds are secondary plant constituents (Hajati et al. 2014).

The marketed polyherbal formulation applied in the present experiment is based on three powdered herbs in equal proportions (*W. somnifera*, *T. cordifolia* and *O. sanctum*) that are well-known for their use in human medicine, being scientifically proven to be adaptogenic and to have immunomodulatory,

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(A)

Figure 4. Influence of dietary supplement ImmuPlus and ascorbic acid on not-immunoglobulin protein content (A) and albumin content (B) in laying hens

Values are expressed as means \pm standard deviation. Means with different letters at the same sampling time are statistically different ($P < 0.05$)

(B)

free radical scavenging and antioxidant rejuvenating properties (Devasagayam and Sainis 2002). Thus, the extracts of this formulation contain phytochemicals that exhibit high antioxidant activities, even when compared to pure AA, due to a high content of polyphenols and, among them, of flavonoids (Cecchini et al. 2014).

These plants, of which the marketed product is composed, possess antioxidant and immunostimulant properties (Devasagayam and Sainis 2002). Among them, *T. cordifolia* is known to have a strong lipid peroxidation inhibitory activity (Premanath and Lakshmidevi 2010), mainly due to several compounds, like polyphenols, flavonoids and glucosides, acting in a synergistic way (Scartezzini and Speroni 2000). Furthermore, *W. somnifera* and *O. sanctum* are also known as excellent growth promoters in the poultry industry (Dhama et al. 2015).

According to literature, when extracts of these herbs are added to poultry diet, some positive effects on the function of the immune system have been shown. Administration of *W. somnifera* extracts to broiler chick diet leads to an increase in some haematological parameters and specific antibody response against viral diseases, suggesting a pivotal role in the modulation of the immune system (Mushtaq et al. 2012; Pant et al. 2012). Kolte et al. (2007), studying the effect of feeding *T. cordifolia* in cyclophosphamide-immunosuppressed broilers, showed a significant rise in antibody titre against the Newcastle disease (ND) virus along with increased inflammatory reaction to a skin contact sensitivity test. At the same time, the mortality and the severity of the disease symptoms in *Escherichia coli*-infected chicks receiving *T. cordifolia* stem extract supplementation are reduced and last for a

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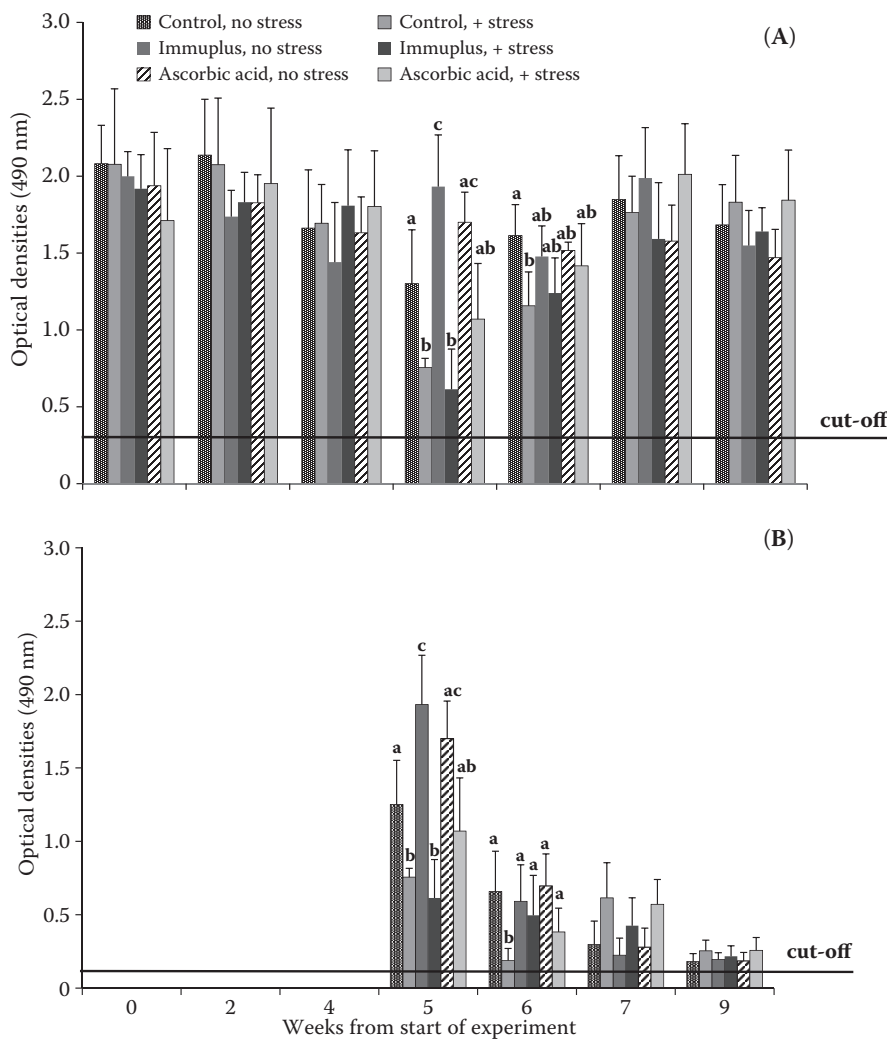


Figure 5. Influence of dietary supplement ImmuPlus and ascorbic acid on natural antibodies isotype IgM (A) and specific antibody response against human- γ -globulins (B) in laying hens

Values are expressed as means \pm standard deviation. Means with different letters at the same sampling time are statistically different ($P < 0.05$)

shorter time if compared to chicks fed the control diet (Mamta and Jakhar 2016). According to Ariuchelvan et al. (2012), the use of crude extracts of *O. sanctum* in the diet of broiler chickens helps to counteract the immunodepressive effect during antibiotic treatment. Moreover, oral administration of *W. somnifera* root and *O. sanctum* leaf powder is shown to prevent cadmium-induced peroxidation of liver and kidney tissues in broiler chicks (Bharavi et al. 2010).

As reported in the cited literature, the dietary supplementation of extracts of these herbs seems to produce, when studied, beneficial effects also in healthy animals. On the contrary, in the present experiment no positive effects, or very low ones, of the two dietary supplementations were observed in healthy hens not subjected to DEX-induced stress, thus making dietary integration unnecessary in healthy specimens. Furthermore, it should be added that the DEX treatment for

6 consecutive days is a valid tool to induce a moderate and transient immune depression (Cecchini et al. 2016). Thus, this study showed that, when hens are subjected to a short/medium-term stress condition, the dietary supplementation based on this commercial mixture can partially counteract the negative effects of the induced stress and, even if it is not able to undo its effects, it can ensure a faster animal recovery. In fact, although some studied parameters were affected neither by stress nor by dietary supplementation (total protein, TBARS, NAb-Ig γ levels), some of them were effective to assess the stress status and the subsequent recovery (glycaemia, respiratory burst, total antioxidant capacity, total Ig content, not-Ig protein content, albumin content, NAb-IgM level, SpAb response) and to highlight the positive effects on immunity in stressed specimens receiving the dietary supplementations (respiratory burst, total Ig content, albumin content, NAb-IgM level

and SpAb response). In particular, if the hyperglycaemia was a result of the transient DEX-induced diabetes, also evidenced by notable polydipsia and by aqueous stools (data not shown), the assessed immune parameters were modulated by dietary supplements in stressed animals. Among them, the observed modulation of NAb-IgM, representing a direct linkage between the innate and acquired immune system, should be highlighted for their influence on specific antibody response and the maturation of the immune system of poultry (Lammers et al. 2004; Cecchini et al. 2016).

Surprisingly, we observed no effect of the induced stress on lipid peroxidation, analysed as TBARS. Although we cannot exclude that the transient DEX-induced stress did not cause any peroxidative damage, it must be emphasized that TBARS assay is widely criticized for low specificity and artefact formation and for its inaccuracy to return expected results (Halliwell and Chirico 1993; Celi 2011). Moreover, the limited number of specimens included in the present study and the great individual variability may also be further responsible for the inaccuracy of the obtained results.

As for the comparison between the herbal mixture and AA, obtained results have demonstrated similar biological responses, without substantial differences, in the different DEX-treated groups. This means that the two different dietary supplementations are able to counteract the effect of induced stress and to ensure faster animal recovery in comparison with the control diet.

Therefore, since the immune system is an indicator of the health status, its protection can also be afforded by dietary supplementation of this marketed mixture based on three herbs known for their ethnomedical use. Thus, this herbal formulation might be useful as an alternative to the allopathic substances in poultry, to prevent the harmful effects of a multitude of long-term and short-term stressors to which animals are subjected during their lifespan.

CONCLUSION

The presence of nutraceutical compounds in several herbal plants giving no side-effects might be useful for exploring them as an alternative to allopathic substances for preventive or therapeutic purposes in poultry. The present results revealed

that dietary supplementation of the tested polyherbal formulation has some beneficial effects on immune parameters in stressed hens comparable with those achieved with AA integration, mainly ensuring faster recovery. On the contrary, no substantial effects on healthy hens were observed, making its use unnecessary in healthy animals. However, further studies are warranted to ascertain the effect of the studied marketed product and to conclusively optimize the level of supplementation in practical diets as an immune enhancer in stressed specimens.

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Received: 2019–09–02

Accepted: 2019–11–04