ATATÜRK ÜNİVERSİTESİ YAYINLARI
ATATURK UNIVERSITY PUBLICATIONS

Research Article *Araştırma Makalesi* DOI: 10.17097/agricultureatauni.1424918

Assessment of Phytochemical Characteristics of Walnut (*Juglans regia*) Leaves: Determination of Nutritional Value and Quantitative Content of Phenolic Compounds

Ceviz (*Juglans regia*) yapraklarının Fitokimyasal Özelliklerinin Değerlendirilmesi: Besin Değeri ve Fenolik Bileşiklerin Niceliksel İçeriğinin Belirlenmesi

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ABSTRACT

This study aimed to assess the nutritional value of walnut leaves (*Juglans regia*), investigate the content of major polyphenolic compounds in walnut leaves, and assess the potential of walnut leaves as a phytogenic supplement to poultry diets. The nutritional composition of dried ground walnut leaves (crude protein, crude fat, crude fiber, calcium, phosphorus, vitamin E, b-carotene) was assessed according to generally accepted methods. The quantitative content of individual groups of polyphenols, such as total phenols, hydroxycinnamic acids, flavonoids, and juglon, was determined spectrophotometrically. The content of the sum of tannins was determined by the titrimetric method. Walnut leaves contain a high content of b-carotene (295.0 µg/g) and natural antioxidant vitamin E (128.8 µg/g). The protein content was noted at the level of 12.5%, the fiber content - 12.86%. In a significant amount, calcium and phosphorus accumulated in walnut leaves - 2.04% and 0.23%, respectively. The content of the total hydroxycoric acids in terms of chlorogenic acid in walnut leaves was 24.3 mg/g, the total content of phenolic compounds in gallic acid equivalent was 14.4 mg/g, flavonoids in rutin equivalent was 20.2 mg/g, and juglone was 2.72 mg/g. The content of total tannins in walnut leaves was 124.5 mg/g. The high nutritional value of walnut leaves compared to alfalfa meal and the presence of a number of biologically active polyphenolic compounds in a significant amount give grounds to consider this phyto-raw material not only as a valuable feed component, but also as a source of natural antioxidants.

Keywords: Walnut, Leaves, Nutritional value, Phenolic compounds, Hydroxycinnamic acids, Flavonoids, Tannins, Juglone

ÖZ

Bu çalışma, ceviz yapraklarının (*Juglans regia*) besin değerini değerlendirmeyi, ceviz yapraklarındaki başlıca polifenolik bileşiklerin içeriğini araştırmayı ve ceviz yapraklarının kanatlı hayvan diyetlerine fitogenik bir katkı maddesi olarak potansiyelini değerlendirmeyi amaçlamıştır. Kurutulmuş ve öğütülmüş ceviz yapraklarının besin bileşimi (ham protein, ham yağ, ham lif, kalsiyum, fosfor, vitamin E, β-karoten) genel olarak kabul edilen yöntemlere göre değerlendirilmiştir. Toplam fenoller, hidroksinamik asitler, flavonoidler ve juglon gibi bireysel polifenol gruplarının niceliksel içeriği spektrofotometrik olarak belirlenmiştir. Toplam tanenlerin içeriği titrimetrik yöntemle tespit edilmiştir. Ceviz yaprakları yüksek β-karoten (295,0 µg/g) ve doğal antioksidan vitamin E (128,8 µg/g) içeriğine sahiptir. Protein içeriği %12,5, lif içeriği ise %12,86 olarak kaydedilmiştir. Ceviz yapraklarında önemli miktarda kalsiyum ve fosfor birikmiştir, sırasıyla %2,04 ve %0,23. Ceviz yapraklarında klorojenik asit cinsinden toplam hidroksikorik asit içeriği 24,3 mg/g, gallik asit eşdeğeri olarak toplam fenolik bileşik içeriği 14,4 mg/g, rutin eşdeğeri olarak flavonoidler 20,2 mg/g ve juglon da 2,72 mg/g olarak belirlenmiştir. Ceviz yapraklarında toplam tanenlerin içeriği 124,5 mg/g olmuştur. Ceviz yapraklarının yüksek besin değeri ve önemli miktarda biyolojik olarak aktif polifenolik bileşiklerin varlığı, bu fito hammaddeyi sadece değerli bir yem bileşeni olarak değil, aynı zamanda doğal antioksidan kaynağı olarak da değerlendirmeyi mümkün kılmaktadır.

Anahtar Kelimeler: Ceviz, Yapraklar, Besin değeri, Fenolik bileşikler, Hidroksinamik asitler, Flavonoidler, Tanenler, Juglon

Received / Geliş Tarihi 24.01.2024
Accepted / Kabul Tarihi 11.07.2024
Publication Date / Yayın Tarihi 29.09.2024

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Cite this article: Gaviley, O., Pankova, S., Poliakova, L., & Chorna, G. (2024). Assessment of phytochemical characteristics of walnut (*Juglans regia*) leaves: determination of nutritional value and quantitative content of phenolic compounds. *Research in Agricultural Sciences*, *52*(3), 151-157.

Introduction

The introduction of a ban on the use of feed antibiotics in animal husbandry and strict control over their use in the European Union since 2006 prompted a large-scale search for alternatives. The introduction of so-called phytobiotics into diets instead of antibacterial drugs, plant complexes, the use of which makes it possible to obtain environmentally friendly food, has gained immense popularity. Herbal supplements, unlike drugs, can be used for clinically healthy animals almost throughout their lives.

In this context, much attention is paid to the search for local plant materials that have both nutritional value and the presence of biologically active ingredients with antioxidant and antibacterial properties. These criteria are met by the walnut (*Juglans regia*), whose products, such as husks and leaves, are rich in phenolic compounds and are a valuable source of antioxidants (Shah et al. 2018). Walnut is cultivated worldwide not only for its nutritious nuts but also for its valuable by-products, such as husks and leaves, which have numerous applications due to their rich phenolic composition. Walnut cultivation is particularly important in regions like Ukraine, where it contributes significantly to the agricultural economy. According to FAOSTAT, Ukraine occupies the 7th position in the world in the cultivation of walnuts. In the period from 2018 to 2021, 14,218 hectares of new orchards were planted in the country, 41% of their total area was walnut plantations (Mazur & Gontaruk, 2021). These statistics indicate that Ukraine has a sufficient amount of walnut plant material.

Some studies have demonstrated the antioxidant and antimicrobial activity of various walnut parts, including the leaves (Jahanban-Esfahlan et al., 2019). The phenolic composition of walnut leaves, their antibacterial activity and antioxidant potential have been studied, and the dependence of these parameters on the crop variety has been shown (Pereira et al., 2007). It has been reported that walnut leaves have a significant content of phenolic compounds, in particular flavonoids of the flavone and flavonol groups, hydroxycinnamic and hydroxybenzoic acids, and tannins (Nour et al., 2016). Most of the properties of this raw material are determined by the presence of juglone in its composition. It is a natural antibiotic of a number of naphthoquinones, toxic to fungi and plants, inhibits Helicobacter pillory enzymes, exhibits pronounced antioxidant properties, is active against the bacteria *Pseudomonas aeruginosa*, *Candida albicans*, *Helminthosporium sp.*, *S. aureus*, *Bacillus subtilis* and can have a positive effect on the human body, as well as on the body of animals and birds (Chaudhary et al., 2021).

At the same time, the nutritional potential of walnut leaves is insufficiently studied, the details of its chemical components have not yet been fully elucidated. Such information is almost absent in the Ukrainian scientific community. Leaves are readily available in large quantities, and their collection does not endanger the life of plants.

Thus, the literature contains information about the antioxidant and antimicrobial potential of walnut leaves from trees growing in different regions of the world, but there is no characterization of the nutritional value and polyphenolic profile of walnut leaves growing in the forest-steppe zone of Ukraine. The insufficient level of research on the nutrition and polyphenolic complex of walnut (*Juglans regia*) leaves of this region does not allow to fully use it in poultry feed as a phytogenic antioxidant additive.

The aim of the research is to study the nutritional potential and polyphenolic composition of walnut (*Juglans regia*) leaves.

Methods

Experimental studies on the determination of the nutritional composition and content of polyphenolic compounds in phytogenic raw materials were conducted in the conditions of the testing laboratory of the Department of Quality and Safety Assessment of Poultry Feeds and Products of the State Poultry Research Station of the National Academy of Agrarian Sciences of Ukraine (SPRS NAAS) using available laboratory and analytical equipment.

Plant material preparation

The material for research was walnut leaves without petioles, which were collected in early June in dry weather from adult plants growing on the territory of the SPRS NAAS without the use of phytosanitary treatments. The walnut trees were of the 'Bukovynskiy' variety, a commonly cultivated type in Ukraine known for its high yield and resistance to disease. Plant raw materials were dried under natural conditions at ambient temperature (20–24°C) in a dark, well-ventilated room without access to direct sunlight. Samples were ground in an electric mill to a fraction that passed through a 1-mm mesh sieve and then stored at room temperature in paper bags until analysis.

The chemical composition and nutritional value of raw materials from walnut leaves (content of crude protein, crude fat, fiber, calcium, phosphorus, concentration of vitamin E and β-carotene) were studied using generally accepted methods (Ionov & Shapovalov, 2015).

The amount of tannins in terms of tannin was determined by the classical titrometric method, based on the titration of the infusion of raw materials with potassium permanganate in the presence of indigosulfonic acid as an indicator

(Cobzaru et al., 2019).

About 3 g (exact weight) of the tested walnut leaf powder was extracted with distilled deionized water (dd H_2O) in a 250 ml volumetric flask for 4 hours at room temperature. Then the infusion was filtered through cotton wool into a measuring flask with a volume of 250 ml so that particles of raw materials did not enter the flask, the volume of the solution was brought up to the mark with water and mixed.

25 ml of the obtained infusion was placed in a conical flask with a volume of 1 l, 25 ml of indigosulfonic acid solution and 750 ml of distilled deionized water (dd H_2O) were added. For titration, a 0.1 N aqueous solution of potassium permanganate ($KMnO_4$) was used until the blue color of the solution changed to green. Then a few drops were added until the solution turned golden yellow.

In parallel, a control experiment (blank sample) was conducted by titrating a mixture of 25 ml of indigosulfonic acid solution and 750 ml of distilled deionized water (dd H_2O). All samples were analyzed in duplicate. The content of the total amount of tannins in the sample was expressed in milligrams of tannin per gram of dry leaves.

Quantitative assessment of the content of other polyphenolic compounds (hydroxycinnamic acids, phenols, flavonoids, glycosides) in phytogenic raw materials was carried out by the spectrophotometric method at the appropriate wavelength, using the Beer–Lambert law. A SF-26 spectrophotometer and 10-mm quartz cuvettes were used for all optical density measurements.

Determination of the total amount of hydroxycinnamic acids was carried out in terms of chlorogenic acid (Proskurina et al., 2021).

Basic solution. 2.0 g (exact weight) of the test sample was placed in a flask with a capacity of 200 ml, 50 ml of 70% ethanol was added and heated in a reflux water bath for 30 min. After cooling, the extract was filtered through a Buchner filter paper. The filtrate was transferred to a volumetric flask with a capacity of 200 ml and the volume was brought up to the mark with 70% ethanol.

Test solution. 2 ml of the basic solution was added to a 50 ml volumetric flask and the volume of the solution was adjusted to the mark with 70% ethanol. The optical density of the obtained solution was measured at a wavelength of 325±2 nm. As a comparison solution, 70% ethyl alcohol was used.

The total amount of hydroxycinnamic acids was expressed in milligrams of chlorogenic acid per gram of dry leaves, the specific absorption index of chlorogenic acid at 325±2 nm was 531.

The total content of phenolic compounds present in walnut leaf extracts was determined quantitatively in terms of gallic acid (Fedosov et al., 2018).

Basic solution. An exact weight of the raw material (about 1.0 g) was placed in a conical flask with a capacity of 100 ml with a ground stopper, poured 30 ml of 40% ethanol, closed with a stopper and weighed (error ±0.01 g). The flask was connected to a reflux condenser, the contents were heated in a water bath until boiling, and a gentle boiling was maintained for 30 minutes. After that, the flask was cooled, closed with a cork, weighed, adjusted to the initial mass with 40% ethanol, and the contents were filtered through a dry paper filter into a dry flask with a volume of 50 ml.

Test solution. 1.0 ml of the filtrate was collected with a pipette, transferred to a volumetric flask with a capacity of 50 ml and the volume of the solution was brought up to the mark with 40% ethanol. The optical density was measured on a spectrophotometer at a wavelength of 270±2 nm. The comparison solution was 40 % ethanol.

The total content of phenolic compounds was expressed in milligrams of gallic acid per gram of dry leaves, the specific absorption index of gallic acid was 540.

The total flavonoid content was determined spectrophotometrically, in terms of rutin (Vronska et al., 2015).

Basic solution; An exact weight of the sample (approximately 1.0 g) was placed in a 100 ml flask with a ground stopper, and 30 ml of 70% ethanol was added. The flask was connected to a reflux condenser and heated in a boiling water bath for two hours, periodically shaking to wash off particles of raw materials from the walls. After cooling, the contents of the flask were filtered through a paper filter into a 100 ml flask and made up to the mark with 70% ethanol (solution A).

Test solution; 2 ml of basic solution A was poured into a 50 ml volumetric flask, 2 ml of a 3% solution of aluminum chloride in 96% ethanol and 0.1 ml of diluted acetic acid were added, and the volume of the solution was brought up to the mark with 96% ethanol (test solution). After 40 minutes, the optical density of the solution was measured on a spectrophotometer at a wavelength of 415±2 nm. As a comparison solution, a solution containing 2 ml of solution A, 0.1 ml of diluted acetic acid, and made up to the mark with 96% ethanol in a 50 ml volumetric flask was used. In parallel, the optical density of a standard sample of rutin, prepared similarly to the tested solution, was measured. The total flavonoid content was expressed in milligrams of rutin per gram of dry leaves.

Juglone content in walnut leaves was measured by the spectrophotometric method (Kocaçalışkan et al., 2020).

An exact weight of crushed walnut leaves (about 2.0 g) was placed in a 100 ml flask, 50 ml of petroleum ether was added and stirred for 30 minutes on a magnetic stirrer. After filtration, the filtrate was centrifuged at 18,000 rpm in a cooled centrifuge for 15 min. The supernatant was diluted ten times with petroleum ether and its absorbance was recorded by spectrophotometric measurement at a wavelength of 410±2 nm.

The blank sample was petroleum ether. 10 mg of commercially purified juglone was placed in a 50 mL volumetric flask and sufficient petroleum ether was added to make up the volume. A standard curve obtained from a series of pure juglone solutions in the juglone content range of 0.01, 0.02, 0.03, 0.04, and 0.05 mg was used to determine the juglone content in leaves. Juglone content was expressed in milligrams of juglone per gram of dry leaves.

Microsoft Excel software was used for statistical analysis. All determinations were performed in five replicates for each raw material sample and the results were expressed as mean ± error.

Results and Discussion

The growing interest in the powerful biological activity of plant phenolic substances and the potential of using phytobiotics in animal feed has outlined the need to determine the nutritional properties of walnut leaves and evaluate its potential as an alternative to commonly used plant components of poultry feed. Table 1 lists some of the chemical constituents of ground walnut leaves and alfalfa meal that reflect their nutritional value.

Table 1.
Chemical composition of walnut leaves (Bratyshko et al., 2013)

Parameters	Walnut leaves	Alfalfa meal *
β-Carotene, μg/g	295.0	150-250
Vitamin E, μg/g	128.8	170-215
Crude protein, %	12.5	14.2-17.3
Crude fat, %	1.19	2.4-2.7
Crude fiber, %	12.86	22.0-27.1
Ash, %	11.54	9.0
Moisture, %	8.25	10.5
Calcium, %	2.04	0.92-1.22
Phosphorus, %	0.23	0.21-0.26

A high content of β-carotene (295 μg/g) was found in the studied samples of walnut leaves. This is significantly higher than in alfalfa meal, where the concentration of β-carotene can be from 150 to 250 μg/g, depending on the grade. A similar result regarding the content of β-carotene in walnut leaf powder was obtained in the study by Kravchenko & Pop (2014), as well as Panaite et al. (2019).

The studied raw material has a fairly high content of natural antioxidant - vitamin E (128.8 μg/g). A somewhat higher concentration of vitamin E in walnut leaves was noted by Untea et al. (2020) – at the level of 157.54 μg/g. However, according to this indicator, the studied raw material is inferior to alfalfa meal, in which, depending on the class, the content of vitamin E is noted in the range from 170 to 215 μg/g. At the same time, according to other data, the content of this vitamin in alfalfa meal ranges from 27.55 to 83.77 μg/g (Cort et al., 1983).

The content of crude protein in walnut leaves is 12.5%, which is slightly lower than alfalfa meal (14.2-17.3%). Our results are confirmed by the data of Panaite et al. (2019), in whose study this indicator was at the level of 12.83%. In contrast, in another experiment, the mass fraction of protein in walnut leaf powder was 7.7% (Kravchenko & Pop, 2014). This indicates the existence of differences in the composition of nutrients in plant organs of different varieties and grown in different places.

As for fiber, its content in walnut raw materials was significantly lower than in alfalfa flour. 12.86% fiber is noted in the walnut leaf, in alfalfa meal, most of which consists of plant stems, the crude fiber content is almost twice as high and reaches 22-27.1%. At the same time, the study by Panaite et al (2019) noted the content of crude fiber in walnut leaves at the level of 17.41%.

The analysis of phytoraw material showed the content of crude fat at the level of 1.19%. In alfalfa meal, its content is twice as high - 2.4-2.7%. A significantly higher result was also reported by Untea et al (2020), in whose study walnut leaves contained 2.21% crude fat.

Regarding the content of macroelements in the investigated phytogen, calcium and phosphorus accumulated in significant quantities in the walnut raw material. Thus, the calcium content in walnut leaves was more than 2 times higher than in alfalfa meal, and was 2.04%. The phosphorus content in the studied samples of walnut leaves was at a fairly high level (0.23%), which is at the level of the average indicator in alfalfa meal (0.21-0.26%). Our data do not contradict the results obtained by Turfan N. et al. (2020), in whose research, depending on the age of the plants, the content of calcium in walnut leaves was in the range of 1.713-2.206%, phosphorus - 0.236-0.277%. In another experiment, the variation of the content of these macroelements in the leaves of different varieties of walnuts was determined - calcium at the level of 1.23-1.85%,

phosphorus at the level of 0.17-0.37% (Miłek et al., 2022). At the same time, according to Solmaz & Adiloğlu (2017), the content of calcium and, especially, phosphorus in samples of walnut leaves fluctuated within wider limits - calcium from 0.31 to 2.86%, phosphorus from 0.11 to 12.32%.

Walnut (*Juglans regia*) is rich in phenolic compounds, including phenolic acids, tannins, naphthoquinones, and flavonoids. Phenolic compounds, which are the most common secondary metabolites of plants and contain a wide range of molecules with a phenolic structure, exhibit various types of pharmacological activity: antioxidant, antiradical, antibacterial, antiviral, antimicrobial, anti-inflammatory, antitumor, immunostimulating (Nardini, 2022).

The content of the main groups of phenols and juglone in dry ground walnut leaves was determined, the results are shown in Table 2.

Table 2.

Quantitative content of the main groups of phenols in walnut leaves

Group of phenol compounds	Measurement units	Content in walnut leaves
THCAC	mg CGAEs/g DW	24.3±0.34
TPC	mg GAEs/g DW	14.4±0.72
TFC	mg REs/g DW	20.2±0.19
TTC	mg TAEs/g DW	124.5±13.12
TJC	mg J/g DW	2.72±0.571

THCAC: total concentration hydroxycinnamic acid content; TPC: total phenolic content; TFC: total flavonoid content; TTC: total tannin content; TJC: Total juglone content; CGAEs: chlorogenic acid equivalents; GAEs: gallic acid equivalents; REs: rutin equivalents; TAEs: tannic acid equivalents; J: juglone; DM: dry matter; DW: dry weights.

According to the results of research, it was established that the content of hydroxycinnamic acids in walnut leaves in the equivalent of chlorogenic acid is 24.3 mg/g. At the same time, Gutiérrez Ortiz et al (2018) reported that the content of hydroxycinnamic acids, depending on the period of leaf collection, ranged from 23.49 to 68.59 mg/g. Variations in the content of hydroxycinnamic acids from 8.9 to 26.8 mg/g were noted in the leaves of different varieties of walnut (Medic et al., 2022).

The content of total phenols in terms of gallic acid in walnut leaves was observed at the level of 14.4 mg/g in dry weight. According to a general review, dried leaves have a significant amount of total phenolics ranging from 34 to 194 mg/g in gallic acid equivalent (Jahanban-Esfahlan et al., 2019). According to other data, the total content of phenolic compounds in walnut leaves is 25.3 mg/g in dry matter (Santos et al., 2013). In another study, depending on the plant variety and leaf collection period, the total phenolic content of walnut raw materials ranged from 15 mg/g to 88.1 mg/g in gallic acid equivalent (Kocaçalışkan et al., 2020). Miłek M. et al. (2022) noted the variation of the total content of phenols in the leaves of different varieties from 25.6 to 64.78 mg/g. Such a wide range indicates a high dependence of the content of phenols in the studied plant material on a large number of factors, including also geographical location.

The analysis of the total amount of flavonoids showed their content in walnut raw materials in terms of rutin at the level of 20.2 mg/g. A slightly lower result was obtained by Vieira et al. (2019) - 13 mg/g in green leaf and 17.4 mg/g in yellow. However, other authors report a significantly higher content of flavonoids in walnut leaves - 89.62-93.51 mg/g (Turfan et al., 2020), and even 330 mg/g (Chaleshtori et al., 2011).

The content of the amount of tannins in terms of tannin in walnut leaves in our study is 124.5 mg/g in dry matter. According to Cobzaru et al. (2019), the content of this phenolic compound in the walnut leaf ranged from 91.4 mg/g to 124.7 mg/g, depending on the extraction method. At the same time, a significantly lower content of tannins is noted in fresh walnut leaves, which shows seasonal and varietal variations from 1.98 to 9.04 mg/g (Giura et al., 2019).

As can be seen from the data presented in Table 2, the content of juglone in the studied raw material is 2.72 mg/g. Meanwhile, Cosmulescu et al (2011) reported significant differences in juglone content in leaves between cultivars, ranging from 0.054 to 0.228 mg/g. According to other data, the average value of juglone, depending on the variety, was 2.26-3.51 mg/g, and its seasonal variations from 0.21 to 4.46 mg/g of dry leaves were also noted (Kocaçalışkan et al., 2020). In another study, the content of juglone in walnut leaves was 3.57 mg/g, although its individual variations depending on the place of cultivation of the plants ranged from 0.13 to 15.56 mg/g dry weight (Cahalan et al., 2011).

According to the above results of the analysis of the content of nutrients and phenolic compounds in walnut leaves, it can be assumed that the above-mentioned raw material can be considered as a phyto-additive with a positive effect on animal health and productivity. The assessment of the feed properties of the plant showed that walnut leaves can serve as an alternative to alfalfa meal in poultry feeding, and according to a number of researchers, it has high potential as a feed additive to improve the quality of eggs, in particular egg yolk, in quails (Eratalar et al., 2017) and laying hens (Untea et al., 2020; Abbasi Rad et al., 2014).

At the same time, the high content of biologically active polyphenolic compounds found in walnut leaves allows the use of this phytoraw material in feeding animals and poultry in order to improve metabolic processes in their bodies to achieve higher productivity. Some studies have also shown

the high antioxidant capacity of walnut leaves (Jahanban-Esfahlan et al., 2019), as well as its antimicrobial activity against infections caused by *P. aeruginosa* and *E. coli.* (Badiefar et al., 2022), confirming its powerful health benefits the potential of this phytoraw material.

Conclusion

Walnut leaves, in particular the "Bukovynskyi" variety, grown in the forest-steppe zone of Ukraine, are a by-product of the plant and are obtained in large quantities during its growing season. The high content of natural antioxidants, such as β-carotene and vitamin E, as well as significant amounts of essential minerals, such as calcium and phosphorus, highlight the possibility of using walnut leaves as an alternative to conventional feed components. In addition, the rich polyphenolic profile and the presence of bioactive molecules with antioxidant and antimicrobial properties indicate the potential of walnut leaves as an alternative to antibiotics in animal husbandry. Overall, the results of this study provide grounds for considering walnut leaves as a potential phytobiotic feed additive for organic poultry production. Future studies should focus on the effects of walnut leaf supplements on poultry health and performance to validate their use in feed.

Peer-review: Externally peer-reviewed.
Author Contributions: Concept – OG, SP; Design - OG, SP; Supervision - OG; Resources - OG, SP; Data Collection and/or Processing – LP, GC; Analysis and/or Interpretation - OG; Literature Search - OG, SP; Writing Manuscript - SP; Critical Review - OG
Ethical Statement: State Poultry Research Station NAAS, No. 125, August 1, 2023
Conflict of Interest: The authors have no conflicts of interest to declare.
Financial Disclosure: The work was performed at the State Poultry Research Station NAAS within the State Research Program "Adaptation processes in highly productive agricultural animals under the influence of environmental and climatic factors", approved and funded by the National Academy of Agrarian Sciences of Ukraine.

Hakem Değerlendirmesi: Dış bağımsız.
Yazar Katkıları: Fikir - OG, SP; Tasarım - OG, SP; Süpervizyon - OG; Kaynaklar - OG, SP; Veri Toplama ve/veya İşleme - LP, GC; Analiz ve/veya Yorumlama - OG; Literatür Taraması - OG, SP; Makale Yazımı - SP; Eleştirel İnceleme - OG
Etik Bildirim: State Poultry Research Station NAAS, No. 125, 1 Ağustos 2023
Çıkar Çatışması: Yazarlar, çıkar çatışması olmadığını beyan etmiştir.
Finansal Destek: Çalışma, Ukrayna Ulusal Tarım Bilimleri Akademisi tarafından onaylanan ve finanse edilen "Çevresel ve iklimsel faktörlerin etkisi altında yüksek verimli tarım hayvanlarında adaptasyon süreçleri" Devlet Araştırma Programı kapsamında NAAS Devlet Kümes Hayvanları Araştırma İstasyonu'nda gerçekleştirilmiştir.

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