

# Integrated In Silico Modeling and In Vivo Trials to Investigate the Functional Role of *Nigella sativa* Seeds as a Feed Additive for Enhancing Performance, Physiology, and Stress Resilience in Heat-Stressed Farâfra Lambs

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## Research Article

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# Abstract

**Background and Objective:** Heat stress (HS) is one of the stressors that impairs various physiological and immunological conditions in an animal's body. Small ruminants have a principal role in the economy. Lambs are young sheep under one year and have a huge nutritional and economic value. *Nigella sativa* (NS) is a perennial growing plant from the *Ranunculaceae* family. Both NS seeds (NSS) and NS oil have a wide range of therapeutic properties as immunomodulators, antimicrobials, and anti-inflammatories. This research aims to assess the functional potential of NSS as a natural nutritional supplement to enhance growth, antioxidant defense, immune function, and physiological resilience in heat-stressed Farâfra lambs to preserve the livestock.

**Materials and Methods:** NS compounds were explored through computational molecular docking, where seventeen bioactive ligands were screened against 473 *Ovis aries* protein structures from the AlphaFold database to identify key immune-related molecular interactions. After that, an *in vivo* study was performed using eighteen Farâfra lambs (7-8 months old,  $38 \pm 2.56$  kg), which were randomly allocated to three groups: control (0 g NSS/day), NS-S6 (6 g NSS/day), and NS-S12 (12 g NSS/day), with six lambs per group. The feeding trial lasted 60 days following a 7-day adaptation period. Growth performance, blood biochemical indices, and physiological variables, including rectal temperature, skin temperature, breathing rate, and heart rate, were measured throughout the experiment.

**Results:** Molecular docking analysis showed strong binding of alpha-longipinene to the melatonin receptor 1A, suggesting possible immune-enhancing activity, while Frizzled-4 was identified as another key target involved in Wnt-mediated immune regulation. Experimentally, NSS supplementation improved average daily gain, feed efficiency, antioxidant capacity, and immune parameters while stabilizing physiological responses to HS.

**Conclusion:** Supplementation with NSS enhanced growth performance, immunity, and stress tolerance in Farâfra lambs exposed to HS. The combination of *in silico* and *in vivo* findings highlights the immunomodulatory and antioxidant potential of NSS, supporting its use as a natural, functional feed additive to encourage livestock resilience and productivity under thermal stress.

## 1. Introduction

The accelerating progression of global warming and the accompanying increase in extreme climate events continue to impose substantial economic losses on agriculture and livestock production, raising urgent concerns regarding global food security. The worldwide climate change is predicted to increase by 1.5°C between 2030 and 2052 (Barati et al., 2024). Livestock systems are expected to experience more frequent heat waves, prolonged warm seasons, and reduced periods of thermal comfort. Like global patterns, the New Valley region has shown a notable rise in ambient temperature across the governorate Soliman et al. (2024).

Although controlling surrounding temperature and relative humidity (RH) is the majority of the direct methods for mitigating heat stress (HS), the high cost of environmental regulation restricts its practical implementation in livestock operations (Ji et al., 2020). As a result, nutritional strategies, particularly the utilization of functional feed ingredients such as minerals, vitamins, medicinal plants, amino acids, and probiotics, have become economically feasible and biologically effective alternatives for reducing HS-induced physiological and metabolic disturbance (Qin et al., 2023; Souza et al., 2023).

*Nigella sativa* (NS), commonly recognized as black seed, is a well-established medicinal plant of the *Ranunculaceae* family, long utilized across North Africa, Asia, and the Middle East for its therapeutic value. Its seeds contain a diverse profile of bioactive compounds, with thymoquinone (TQ) identified as the principal constituent responsible for potent antioxidant, anti-inflammatory, antimicrobial, and immunomodulatory behaviors (Dabeer et al., 2022; Shad et al., 2021). Other constituents, including essential fatty acids, volatile oils, alkaloids, flavonoids, vitamins, and minerals, further enhance its pharmacological potential (Mazaheri et al., 2019). Numerous animal studies have reported improvements in growth performance, immune competence, oxidative balance, and physiological resilience following dietary NS supplementation (Odhaib et al., 2018; Rahmy et al., 2024). In addition, NS has been demonstrated to enhance growth, milk yield, and reproductive performance in multiple livestock species (Mamdouh Elsayed et al., 2025; Fathi et al., 2024; Mohammed and Al-Suwaiegh, 2023). Despite these advances, evidence regarding its effects on growing lambs, particularly under HS conditions, remains insufficient.

Recent progress in computational biology, especially molecular docking, has accelerated the identification of molecular targets and mechanisms underlying bioactive compound activity. These tools provide rapid, cost-efficient predictions that complement and refine *in vivo* investigations.

Accordingly, the present study integrates *in silico* and *in vivo* approaches to evaluate the functional potential of NS seeds in heat-stressed Farâfra lambs. The *in silico* analysis involved docking 17 NS-derived ligands against 473 *Ovis aries* protein structures to identify putative targets linked to immune regulation and metabolic adaptation. The *in vivo* experiment assessed the impact of nourishment of NS supplements on growth performance, antioxidant capacity, immune responses, and physiological indicators under HS. This combined methodology offers a comprehensive understanding of the molecular and physiological mechanisms through which NS may enhance thermotolerance and overall health in sheep.

## 2. Materials and Methods

### 2.1. Ligand preparation

The ligands were selected in accordance to Abdel-Razek et al. (2024) and then retrieved from the PubChem database. Energy lowering was accomplished utilizing the MMFF94 (Halgren, force field implemented in Avogadro 1.2.0 (Hanwell et al., 2012) program to acquire ideal configurations of the ligands.

2.2. Protein preparation

The set of available *Ovis aries* (sheep) protein structures was obtained from AlphaFold using the UniProt API (500 structures). All protein structures were processed for molecular docking by adding hydrogen atoms using the PrepareReceiver4.py script. The structures were then converted to PDBQT format, which is in harmony with molecular docking software.

2.3. Active site prediction and molecular docking

The binding positions for each protein were anticipated using the CB-Dock2 (Liu et al., 2022) server, which applies a cavity verification algorithm to discover possible binding pockets. Molecular docking simulations were executed by QuickVina-2 (QVina2) (Alhossary et al., 2015) software to estimate the binding exchanges between the prepared proteins and all ligands.

2.4. Experimental site and ethical approval

This trial was conducted at the Department of Animal Production Farm, Faculty of Agriculture, New Valley University, during the summer season. Every operation and experimental methodology is aligned with Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010, legislation on the protection of animals used for scientific purposes. The experimental procedures were approved by the New Valley University Scientific Research Ethics Committee (Ethical code: NVREC 03-3-7-2025-17).

2.5. Source and preparation of NS

The NS were obtained from a local medicinal plant market in New Valley, Egypt. The seeds were finely ground and saved in a black container until they were integrated into the experimental diets in line with the study framework.

2.6. Meteorological data and temperature humidity index (THI) Estimation

Air temperature (AT) (°C) and RH within the farm construction were evaluated every week, on four occasions at 8.00, 10.00, 14.00, and 16.00 hours through a digital thermo-hygrometer. THI was calculated as follows 
$$\text{THI} = (0.8 \times \text{AT}) + [(\text{RH}/100) \times (\text{AT} - 14.4)] + 46.4$$
 (Mader et al., 2006). Where Ta (°C) is the temperature (°C), and RH is the relative humidity.

Table (1). Means of AT (°C), RH (%), and THI during the experimental period.

| Month                                                                       | AT (°C) | RH (%) | THI   |
|-----------------------------------------------------------------------------|---------|--------|-------|
| June                                                                        | 36.03   | 15.75  | 76.46 |
| July                                                                        | 34.65   | 16.25  | 75.30 |
| August                                                                      | 34.08   | 16.30  | 74.82 |
| AT: Air temperature, RH: Relative humidity, THI: Temperature humidity index |         |        |       |

Table (1) presents the average monthly values of AT, RH, and THI recorded during the experimental period. The estimated average monthly air temperature (°C) was 36.03, 34.65, and 34.08, and the RH was 15.75, 16.25, and 16.30% during June, July, and August. The corresponding THI values were 76.45, 75.30, and 74.82, indicating that Farâfra lambs suffer from HS conditions during June, July, and August in the Animal Production Experimental Farm, New Valley University.

2.7. Experimental design and Animal management

A total of eighteen male Farâfra lambs at 12 ± 0.24 months of age and an average weight of 38.01 ± 2.56 kg were used. All lambs were housed in identical environmental, sanitary, and managerial circumstances and preserved under similar conditions of accommodation networks in shaded regions. Lambs were fed matching diets [2.5% of initial body weight (BW)], considering the NRC guidelines twice a day at 08:00 am and 04:00 pm. The elements and chemical structure of the diet are listed in Table (2). Throughout the experimental time, all lambs had constant access to freshwater. Before starting the experiment, lambs were medicated for internal and external parasites, as well as inoculated against the most common infectious diseases.

Table (2)

Ingredients of concentrated feed mixture (CFM) and chemical composition of CFM and wheat straw (% dry matter (DM) basis).

| Ingredients          | %    | Item | CFM   | Wheat straw |
|----------------------|------|------|-------|-------------|
| Corn                 | 55   | DM   | 89.76 | 92.36       |
| Wheat bran           | 26.5 | OM   | 93.72 | 89.42       |
| Soya bean meal%      | 15   | CP   | 14.76 | 1.88        |
| Limestone            | 1.5  | CF   | 14.65 | 37.81       |
| Di-calcium phosphate | 0.5  | EE   | 3.39  | 1.09        |
| Yeast                | 0.2  | Ash  | 6.28  | 10.58       |
| Bicarbonate          | 0.3  | NFE  | 60.92 | 48.64       |
| Sodium chloride      | 1    |      |       |             |

CFM: Concentrate feed mixture, DM: Dry matter, OM: Organic matter, CP: Crude protein, CF: Crude fiber, EE: Ether extract, NFE: Nitrogen-free extract.

The lambs were randomly split into 3 categories (8 lambs each) depending on the therapy kind, as the control and NSS groups. Supplementations such as control, this group didn't receive NSS, NS-S6, this group administrated 6 grams NSS, and NS-S12, this group administrated 12 grams NSS. All administrations were performed directly to every lamb with the morning daily meal. The duration of the experiment was 60 days, including a 7-day adaptation period.

## 2.8. Biometry and thermoregulatory parameters

Throughout the treatment duration, the initial and final body weights of lambs were recorded. Afterward, the BW gain was determined by subtracting the initial BW (IBW) from the final BW (FBW). The average daily gain (ADG) was computed by dividing the disparity between FBW and IBW by the number of days. The feed conversion ratio (FCR) was measured by dividing the mean of total dry matter intake (TDMI) by the ADG.

The length (dorsal–ventral distance) and width (mid-lateral diameter) of the testicular mass (right and left sides) were quantified. An elastic tape was utilized to measure the circumference of the scrotum at the point of maximum circumference of the paired testes. The real testicular volumes were determined by water displacement.

Finally, the rectal temperature (RT), respiration rate (RR), and pulse rate (PR) were noted in all studied groups.

## 2.9. Blood collection and serum analysis

Blood was collected from each lamb post-8-hour fasting via the jugular vein. Hematological parameters were measured using an automatic cell counter (Dirui BCC-3600). Serum, obtained after centrifugation at 3500 rpm for 20 min, was kept at -20°C until examination. Biochemical parameters included the concentration of blood glucose (BG), total cholesterol (TC), total protein (TP), albumin, globulin, urea, and creatinine, and the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed by laboratory kits. Further, the pro-oxidant and antioxidant markers involved thiobarbituric acid reactive substances (TBARS), glutathione peroxidase (GPx), reduced glutathione (GSH), and total antioxidant capacity (TAC) were measured via established methods. Serum testosterone was determined by radioimmunoassay with a sensitivity of 0.2 ng/ml and a coefficient of variation < 10%.

## 2.10. Statistical analysis

Statistical data assessment was undertaken using SAS Version 9.1 software (Marasinghe and Koehler, 2018). Duncan's multiple range test was used to assess the differences among variable treatments (Duncan, 1955). All information is displayed as means  $\pm$  standard error of the mean (SEM). Statistically significant variations were approved as ( $P < 0.05$ ). The model used was  $Y_{ij} = \mu + A_j + \epsilon_{ij}$

Where  $Y_{ij}$  = Observation traits,  $\mu$  = Overall mean,  $A_j$  = Experimental treatment and  $\epsilon_{ij}$  = Random error.

## 3. Results

### 3.1. Molecular docking

Considering the data offered in **Table (3)**, the molecular docking study systematically evaluated binding interactions between a focused set of bioactive ligands and a comprehensive protein library from *Ovis aries* (sheep). A total of 7.875 protein-ligand interactions were simulated, involving 473 unique protein receptors and 17 unique ligands. The binding affinities, expressed in kcal/mol, demonstrated considerable variation across the dataset, with values ranging from -8.8 kcal/mol (strongest predicted binding) to -2.4 kcal/mol (weakest predicted binding). The distribution of binding affinities showed a mean value of -5.0 kcal/mol and a median of -4.9 kcal/mol, indicating a relatively symmetric distribution of moderate to strong binding interactions across the tested combinations.

**Table 3. Summary statistics of molecular docking results**

| Metric                                | Value |
|---------------------------------------|-------|
| Total protein-ligand interactions     | 7.875 |
| Protein receptors                     | 473   |
| Ligands tested                        | 17    |
| Strongest binding affinity (kcal/mol) | -8.8  |
| Weakest binding affinity (kcal/mol)   | -2.4  |
| Mean binding affinity (kcal/mol)      | -5    |
| Median binding affinity (kcal/mol)    | -4.9  |

### 3.2. Top-ranking ligand-protein interactions and binding affinity analysis

According to the information given in **Table (4)**, the top-ranking interactions exhibited notably strong predicted binding affinities, with the 20 highest-scoring complexes ranging from -8.8 to -7.9 kcal/mol, and among the 17 tested ligands, two sesquiterpene compounds, alpha-longipinene ( $\alpha$ -longipinene) and longifolene, emerged as the most promising candidates, appearing repeatedly in the highest-affinity interactions. This consistency suggests superior binding compatibility with multiple sheep protein targets compared to the other tested compounds.

**Table 4. Top 20 protein-ligand interactions ranked by binding affinity**

| Rank | Protein Target                                                     | UniProt ID | Ligand                 | Binding Affinity (kcal/mol) |
|------|--------------------------------------------------------------------|------------|------------------------|-----------------------------|
| 1    | Melatonin receptor type 1A                                         | P48040     | $\alpha$ -longipinene  | -8.8                        |
| 2    | Cyclin-dependent kinase 4                                          | B2MVY4     | Longifolene            | -8.5                        |
| 3    | Carboxylesterase 5A                                                | Q3T930     | Longifolene            | -8.4                        |
| 4    | Cyclin-dependent kinase 4                                          | B2MVY4     | $\alpha$ -longipinene  | -8.4                        |
| 5    | 11 $\beta$ -hydroxysteroid dehydrogenase type 2                    | P50168     | Longifolene            | -8.3                        |
| 6    | Carboxylesterase 5A                                                | Q3T930     | $\alpha$ -longipinene  | -8.3                        |
| 7    | Frizzled-4                                                         | A0A6P7DAJ6 | $\alpha$ -longipinene  | -8.2                        |
| 8    | Estrogen receptor                                                  | A0A6P7ECT5 | Longifolene            | -8.1                        |
| 9    | Uroporphyrinogen decarboxylase                                     | Q8HY31     | $\alpha$ -longipinene  | -8.1                        |
| 10   | Adrenocorticotrophic hormone receptor (ACTH)                       | Q9TU77     | $\alpha$ -longipinene  | -8                          |
| 11   | Progesterone receptor                                              | Q29175     | Longifolene            | -8                          |
| 12   | Melatonin receptor type 1A                                         | P48040     | Longifolene            | -8                          |
| 13   | Estrogen receptor                                                  | A0A6P7ECT5 | $\alpha$ -longipinene  | -7.9                        |
| 14   | 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (11 $\beta$ -HSD2) | P50168     | $\alpha$ -longipinene  | -7.9                        |
| 15   | Progesterone receptor                                              | Q29175     | $\alpha$ -longipinene  | -7.9                        |
| 16   | Frizzled-4                                                         | A0A6P7DAJ6 | Longifolene            | -7.9                        |
| 17   | Adrenocorticotrophic hormone receptor                              | Q9TU77     | Longifolene            | -7.9                        |
| 18   | Uroporphyrinogen decarboxylase                                     | Q8HY31     | Longifolene            | -7.9                        |
| 19   | Carboxylesterase 5A                                                | Q3T930     | $\beta$ -caryophyllene | -7.9                        |
| 20   | Cyclin-dependent kinase 4                                          | B2MVY4     | $\beta$ -caryophyllene | -7.9                        |

### 3.3. Functional classification of the top 20 ligand-protein interactions

**Table 5** lists the top 20 ligand-protein interactions found by molecular docking studies along with their functional distribution. With 50% of all interactions falling into this category, hormone receptors and regulatory proteins were the largest group. Important targets included the progesterone, estrogen, melatonin, ACTH, and 11 $\beta$ -HSD2 receptors. These results demonstrate how strongly the bioactive chemicals of NS bind to immunoregulatory and endocrine pathways. Twenty percent of the top interactions were made up of proteins involved in metabolic and cell cycle control, indicating possible roles in both metabolic regulation and cellular growth. Frizzled-4, a signal transduction protein, made up 10% of the total, suggesting that Wnt signaling pathways connected to inflammatory and immunological responses may have been modulated.

**Table 5. Distribution of top 20 interactions by functional protein class**

| Functional category              | No. of interactions | Percentage (%) | Representative proteins                                                                           |
|----------------------------------|---------------------|----------------|---------------------------------------------------------------------------------------------------|
| Hormone receptors and regulation | 10                  | 50%            | Estrogen receptor, progesterone receptor, melatonin receptor, ACTH receptor, and 11 $\beta$ -HSD2 |
| Cell cycle control               | 4                   | 20%            | Cyclin-dependent kinase 4                                                                         |
| Metabolic enzymes                | 4                   | 20%            | Carboxylesterase 5A, and uroporphyrinogen decarboxylase                                           |
| Signal transduction              | 2                   | 10%            | Frizzled-4                                                                                        |

### 3.4. Hydrophobic interaction profile between the top ligand and the target protein

The hydrophobic interactions between the top protein target and the best-performing ligand are shown in **Table 6**. Important amino acid residues like VAL127, ILE128, VAL207, LEU270, and PHE195 are involved in the interactions, which are mostly of the alkyl and  $\pi$ -alkyl types. The interaction lengths, which range from roughly 3.8 to 5.4 Å, show that the ligand's considerable binding affinity is mostly due to stable hydrophobic interactions.

**Table 6.** Analysis of alkyl and  $\pi$ -alkyl hydrophobic contacts in the UNL1-protein complex

| Interaction         | Distance (Å°) | Category    | Type     |
|---------------------|---------------|-------------|----------|
| A:VAL127 - A:UNL1   | 4.67936       | Hydrophobic | Alkyl    |
| A:ILE128 - A:UNL1   | 5.28525       | Hydrophobic | Alkyl    |
| A:VAL207 - A:UNL1   | 5.40713       | Hydrophobic | Alkyl    |
| A:LEU270 - A:UNL1   | 5.44697       | Hydrophobic | Alkyl    |
| A:UNL1:C - A:VAL207 | 3.82288       | Hydrophobic | Alkyl    |
| A:PHE195 - A:UNL1   | 5.26476       | Hydrophobic | Pi-Alkyl |
| A:PHE195 - A:UNL1:C | 4.03356       | Hydrophobic | Pi-Alkyl |

### 3.5. Feed intake, growth performance, scrotal and testicular biometry, and testosterone concentration (ng/ml)

Data in **table (7)** exhibited that feeding lambs with diets supplemented with NSS significantly ( $P < 0.05$ ) improved total dry matter intake, weight gain, average daily gain, and feed conversion ratio contrasted to the control group. Further, FBW increased from 48.38 kg in the control to 51.90 kg in NS-fed lambs. Scrotal circumference and testicular dimensions (length and width) were also significantly higher in supplemented groups. However, testicular volume showed no significant differences. Moreover, the serum testosterone levels increased ( $P < 0.05$ ) to 4.59 and 4.71 ng/ml in the NS-S6 and NS-S12 groups versus 4.22 ng/ml in the control. Overall, NSS supplementation enhanced growth performance and reproductive traits of lambs.

**Table 7.** Productive performance and biometry of scrotum and testis (right and left) of lambs in the experimental groups

| Parameter                                      | Means of experimental groups |                     |                     | SEM   | P-values      |
|------------------------------------------------|------------------------------|---------------------|---------------------|-------|---------------|
|                                                | Control                      | NS-S6               | NS-S12              |       |               |
| Total number of lambs                          | 8                            | 8                   | 8                   | --    | --            |
| <b><u>Average daily DM intake, g/h/d:</u></b>  |                              |                     |                     |       |               |
| TDMI                                           | 1352.5 <sup>b</sup>          | 1510.2 <sup>a</sup> | 1512.5 <sup>a</sup> | 28.48 | <b>0.0211</b> |
| FCR                                            | 8.93 <sup>a</sup>            | 8.15 <sup>b</sup>   | 7.65 <sup>b</sup>   | 0.198 | <b>0.0144</b> |
| <b><u>Growth performance of lambs</u></b>      |                              |                     |                     |       |               |
| Initial body weight, kg                        | 39.25                        | 40.76               | 40.01               | 0.478 | 0.5093        |
| Final body weight, kg                          | 48.38 <sup>b</sup>           | 51.87 <sup>a</sup>  | 51.90 <sup>a</sup>  | 0.646 | <b>0.0281</b> |
| Total weight gain, kg                          | 9.12 <sup>b</sup>            | 11.12 <sup>a</sup>  | 11.89 <sup>a</sup>  | 0.388 | <b>0.0019</b> |
| Average daily gain, g                          | 152.1 <sup>b</sup>           | 185.2 <sup>a</sup>  | 198.1 <sup>a</sup>  | 6.589 | <b>0.0019</b> |
| <b><u>Scrotal and testicular biometry:</u></b> |                              |                     |                     |       |               |
| Scrotal circumference (cm)                     | 29.55 <sup>b</sup>           | 31.53 <sup>a</sup>  | 31.65 <sup>a</sup>  | 0.318 | <b>0.0016</b> |
| Right testis length (cm)                       | 10.88 <sup>b</sup>           | 11.38 <sup>a</sup>  | 11.98 <sup>a</sup>  | 0.160 | <b>0.0077</b> |
| Left testis length (cm)                        | 10.60 <sup>b</sup>           | 11.10 <sup>ab</sup> | 11.58 <sup>a</sup>  | 0.143 | <b>0.0090</b> |
| Right testis width (cm)                        | 6.38 <sup>b</sup>            | 6.68 <sup>a</sup>   | 6.78 <sup>a</sup>   | 0.065 | <b>0.0226</b> |
| Left testis width (cm)                         | 6.43 <sup>b</sup>            | 6.63 <sup>ab</sup>  | 6.70 <sup>a</sup>   | 0.047 | <b>0.0402</b> |
| Testicular volume (ml)                         | 332.3                        | 339.3               | 340.8               | 1.672 | 0.0940        |
| Testosterone (ng/ml)                           | 4.22 <sup>b</sup>            | 4.59 <sup>a</sup>   | 4.71 <sup>a</sup>   | 0.082 | <b>0.0116</b> |

Values represent mean  $\pm$  SEM. Means with different superscripts in the same row are significantly different at  $P < 0.05$

### 3.6. Thermoregulatory response

NSS supplementation exerted a significant influence on thermoregulatory parameters. As shown in **Fig. 1**, lambs receiving NS-S6 and NS-S12 exhibited markedly lower RT, RR, and PR as opposed to the control group ( $P < 0.05$ ). These findings showed that NSS supplementation effectively enhances thermoregulatory efficiency and supports physiological stability in lambs exposed to HS.

### 3.7. Hematological results

Hematological data were presented in **Table (8)** and showed significant elevation in Hb concentration by 12.57% in NS-S6 and 14.82% in NS-S12 groups in comparison to the control group. However, there wasn't a substantial variation of RBCs, WBCs, and HCT in both NS-S6 and NS-S12 compared to the control group.

**Table 8. Mean values, SEM, and P-values of hematological parameters of lambs in the experimental groups**

| Parameter                   | Means of the experimental groups |                    |                    | SEM   | P-values      |
|-----------------------------|----------------------------------|--------------------|--------------------|-------|---------------|
|                             | Control                          | NS-S6              | NS-S12             |       |               |
| Hb (g/dl)                   | 10.66 <sup>b</sup>               | 12.00 <sup>a</sup> | 12.24 <sup>a</sup> | 0.258 | <b>0.0039</b> |
| RBCs ( $10^6/\mu\text{l}$ ) | 7.73                             | 8.46               | 8.54               | 0.192 | 0.1233        |
| WBCs ( $10^3/\mu\text{l}$ ) | 12.76                            | 12.38              | 12.28              | 0.120 | 0.5708        |
| HCT %                       | 34.08                            | 35.63              | 35.48              | 0.474 | 0.3027        |
|                             |                                  |                    |                    |       |               |

Values represent mean  $\pm$  SEM. Means with different superscripts in the same row are significantly different at  $P < 0.05$

### 3.8. Serum biochemical parameters results

BG, TC, TP, albumin, globulin, and urea levels in serum were assayed in different studied groups and showed no apparent variance between the control and NS-S6 and NS-S12 groups ( $p < 0.05$ ). However, creatinine concentration revealed a reduction of nearly 9.43% and 22.01% in NS-S6 and NS-S12, respectively, compared to the control group. Moreover, ALT and AST activities were decreased by 16.16% and 14.42% in NS-S6, respectively, compared to the control. Also, in NS-S12, the activity of ALT was diminished by nearly 17.17% and AST by 11.58% to the control group (**Table 9**).

**Table 9. Mean values, SEM, and P-values of biochemical parameters of lambs in the experimental groups**

| Parameter          | Means of the experimental groups |                    |                   | SEM   | P-values      |
|--------------------|----------------------------------|--------------------|-------------------|-------|---------------|
|                    | Control                          | NS-S6              | NS-S12            |       |               |
| BG (mg/dl)         | 65.4                             | 67.2               | 70.0              | 1.864 | 0.5835        |
| TC (mg/dl)         | 161.8                            | 161.4              | 155.8             | 4.957 | 0.8564        |
| TP (g/dl)          | 6.97                             | 7.07               | 7.06              | 0.148 | 0.9555        |
| Albumin (g/dl)     | 3.45                             | 3.56               | 3.64              | 0.055 | 0.3594        |
| Globulin (g/dl)    | 3.52                             | 3.51               | 3.42              | 0.153 | 0.9587        |
| Urea (mg/dl)       | 25.28                            | 22.95              | 23.10             | 0.587 | 0.1450        |
| Creatinine (mg/dl) | 1.59 <sup>a</sup>                | 1.44 <sup>ab</sup> | 1.24 <sup>b</sup> | 0.058 | <b>0.0113</b> |
| ALT (U/l)          | 19.8 <sup>a</sup>                | 16.6 <sup>b</sup>  | 16.4 <sup>b</sup> | 0.546 | <b>0.0011</b> |
| AST (U/l)          | 41.6 <sup>a</sup>                | 35.6 <sup>b</sup>  | 36.8 <sup>b</sup> | 1.059 | <b>0.0158</b> |
|                    |                                  |                    |                   |       |               |

Values represent mean ± SEM. Means with different superscripts in the same row are significantly different at  $P < 0.05$

### 3.9. Pro-oxidant and antioxidant markers

Pro-oxidant parameter represented in TBARS level was estimated in all clusters and displayed a reduction of approximately 32.08% in NS-S6 and 49.89% in NS-S12 than the control group (**Fig.2.A**).

Moreover, the specific activity of GPx proved a significant increase in NS-S6 and NS-S12 by (0.58 and 1.12-fold), respectively, in comparison to control (**Fig.2.B**). Further GSH content was discovered to be promoted in NS-S6 by 13.74% and in NS-S12 by 29.43% to control (**Fig.2.C**). The significant elevation of TAC was also in NS-S6 and NS-S12 by (46.52 and 47.44%), correspondingly, compared to the control group (**Fig.2.D**).

## 4. Discussion

The current study offers strong proof that supplementing with NSS has a variety of positive impacts on heat-stressed individuals' growth performance, physiological stability, reproductive development, antioxidant capacity, and general health. Lambs from Farâfra have positive implications for heat-stressed individuals' growth performance, physiological stability, reproductive development, antioxidant capacity, and general health. These findings reinforce the growing body of literature supporting the value of natural feed additives, particularly NSS, in enhancing livestock productivity and resilience under challenging environmental conditions.

Consistent with reports by (Mamdouh Elsayed *et al.*, 2025; Rahmy *et al.*, 2024), dietary inclusion of NSS significantly improved DM intake, ADG, FBW, and feed conversion efficiency. The enhanced feed utilization may be attributed to the high nutritional value and diverse phytochemical composition of NS, including TQ, phenolics, and polyunsaturated fatty acids. These constituents are known to enhance metabolic activity, stimulate appetite, and support efficient nutrient assimilation. The stimulatory effect on thyroid hormone activity, as previously suggested by Farhangi *et al.* (2016), may further explain the increased feed intake and growth rates observed in NS-supplemented lambs.

A notable finding of this study is the marked improvement in testicular biometric traits among NSS-treated lambs. Increases in scrotal circumference and testicular dimensions reflect enhanced reproductive development and potential improvements in spermatogenic capacity. These outcomes align with earlier reports demonstrating improved testicular histoarchitecture and elevated testosterone levels following NS supplementation (Umar *et al.*, 2017; Wani *et al.*, 2022). The androgenic and antioxidant properties of TQ likely contribute to the preservation of testicular tissue integrity and stimulation of Leydig and Sertoli cell activity. The concurrent rise in serum testosterone further supports the hypothesis that NSS enhances endocrine functions related to male fertility.

The thermophysiological responses observed in this investigation highlight the capability of NSS to enhance heat tolerance in lambs. Significant reductions in RT, PR, and RR in the supplemented groups indicate improved autonomic regulation and reduced metabolic heat load under thermal stress. These effects may be ascribed to the strong anti-inflammatory and antioxidant actions of NSS bioactive, which mitigate cellular oxidative damage and support systemic homeostasis during heat exposure. Similar findings have been reported in other herb-supplemented livestock, underscoring the role of plant-derived antioxidants in improving thermal resilience.

Hematological and biochemical assessments further illustrate the health-promoting capacity of NSS. The significant increase in Hb suggests enhanced erythropoiesis and improved oxygen transport, likely driven by the iron-rich and antioxidant nature of NSS (Ekanayake and Mudaliar, 2023). Although RBCs and WBCs values, and HCT percentage did not differ significantly, numerical improvements reflect stabilized immune and hematopoietic functions (Witeska *et al.*, 2023).

The non-significant influence of NS-S6 and NS-S12 on TP, albumin, globulin, and urea levels in the current study is in accordance with a prior study (Hassan *et al.*, 2024). However, the marked reductions in creatinine concentration and ALT, AST activities were noted and provide strong evidence of nephroprotective and hepatoprotective effects, consistent with earlier findings on the organ-protective properties of TQ and related phytochemicals (M. Elsayed *et al.*, 2025).

HS is accompanied by the generation of reactive oxygen species (ROS) and free radicals (FRs) in the animal's body (El-Zeftawy *et al.*, 2020). The current findings of NS-S6 and NS-S12 exposed a reduction of pro-oxidant and elevation of antioxidant issues, and this is related to the antioxidant power of NSS, which relies on its radical scavenging activity to FRs created from HS (Dalli *et al.*, 2021). In addition, NSS is distinguished by a high content of flavonoids and phenolic acids, and is characterized by the existence of hydroxyl groups in its chemical structure, which facilitate trapping of the ROS (Kurnia *et al.*, 2025). Also, TQ is one of the vital ingredients in NSS that can reduce lipid peroxidation and enhance the antioxidant capacity (Kavyani *et al.*, 2023). These findings corroborate preceding studies (El-Gindy *et al.*, 2020) and confirm the robust antioxidant potency of NSS. The enhancement of enzymatic and non-enzymatic antioxidant systems highlights the ability of NSS to fortify endogenous defenses while also providing direct free radical scavenging through its diverse phytochemical constituents.

The molecular docking results of NSS exhibited the presence of multiple components that offer mechanistic insight into the observed physiological improvements. Strong binding affinities of NSS ligands, particularly  $\alpha$ -longipinene and longifolene, to key proteins, melatonin receptor 1A and frizzled-4.

The melatonin receptor 1A protein is a subtype of melatonin (MT) (Okamoto *et al.*, 2024). MT and its subtypes have a vital signaling antioxidant effect via detoxifying the ROS and diminishing their concentration under HS by elimination of hydrogen peroxide (Hassan *et al.*, 2022). Further MT enhances some polyamines, such as spermine and spermidine, that have a defensive role against HS (Jahan *et al.*, 2019). Further MT boosts the expression of heat shock protein 90, which is involved in HS tolerance and regulates cell proliferation and protein folding (Zhao *et al.*, 2021) and promotes the antioxidant enzyme activities, such as GPx, catalase, and superoxide dismutase (Cao *et al.*, 2024). Moreover, frizzled-4 protein is a subclass of frizzled receptors (Pascual-Vargas and Salinas, 2021). It modulates the HS by enhancing the transcriptional activation of the  $\beta$ -catenin pathway (Rickman *et al.*, 2023) and norrin/Wnt cascade (Mitton *et al.*, 2025).

The predominance of hydrophobic interactions and the stability of ligand-protein complexes underscore the potential of NSS constituents to modulate multiple biological pathways simultaneously. These molecular predictions align closely with the *in vivo* findings and highlight the multifunctional nature of NSS in enhancing thermotolerance and metabolic resilience.

## 5. Conclusion

Collectively, the findings of this investigation confirm that *Nigella sativa* seeds (NSS) supplementation provides substantial physiological, metabolic, and reproductive benefits for growing lambs under heat stress. The convergence of *in vivo* outcomes and *in silico* predictions underscores the multifactorial mechanisms through which NSS enhances animal health and performance. This study therefore supports the strategic use of NSS as a natural, cost-effective functional feed additive to improve sheep productivity and resilience in arid and heat-challenged production environments.

## Declarations

### Author contribution

A. Soliman, Shymaa Mourad, Marwa El-Zeftawy, and Amira Mohamed shared in designing the experimental plan, evaluating the results, and writing the manuscript. A. soliman and Marwa El-Zeftawy participated in the collection of the samples. Shymaa Mourad, Marwa El-Zeftawy, and Amira Mohamed shared in the analyses of samples. A. soliman, H. Hamdon, and A. Kassab shared in the statistical analyses. A. Soliman, Shymaa Mourad, Marwa El-Zeftawy, and Amira Mohamed contributed to the review, finalization, and publication of this paper.

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### Availability of data

All data generated or analyzed during this study are included in this published article.

### Conflicts of interest/ Competing interests

The authors declare that they have no competing interests.

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## Figures

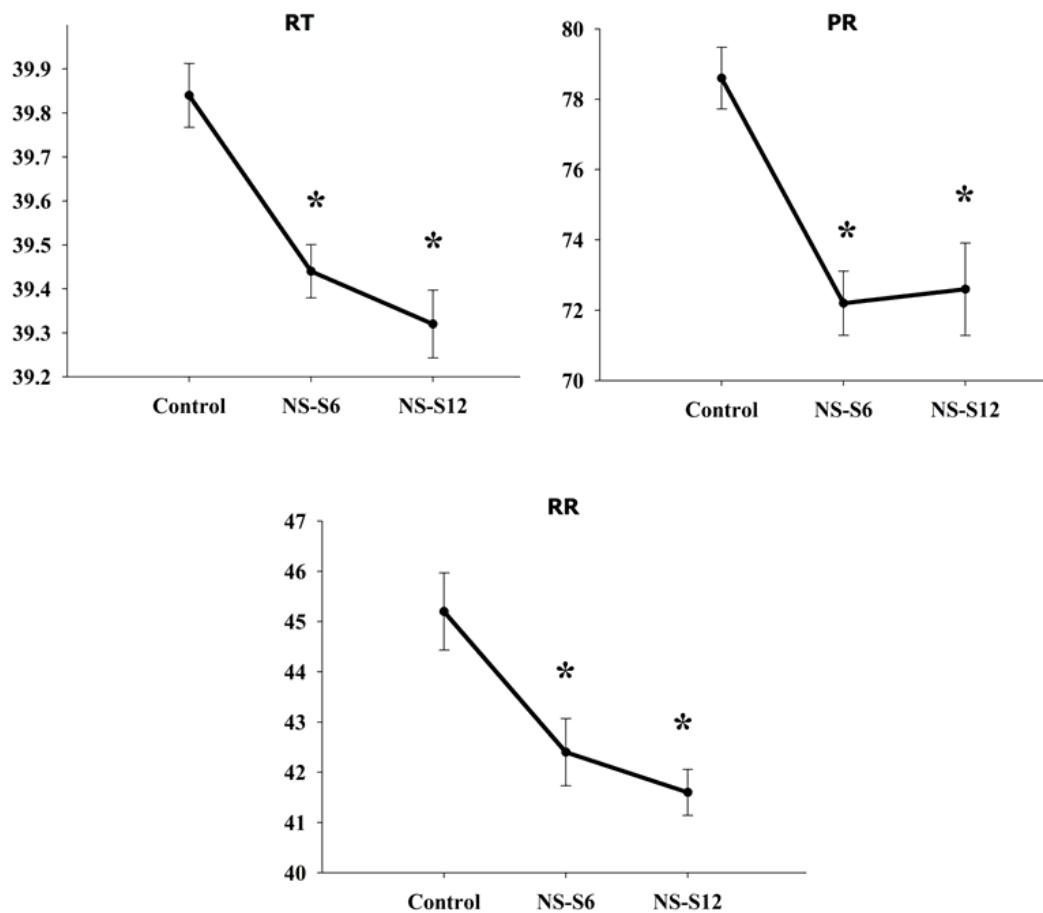


**Figure 1**

Thermoregulatory responses of lambs after being treated with NS-S6 and NS-S12 (n=6). The asterisks show significant differences between NS-S6 and NS-S12 groups compared to the control (\* $p < 0.05$ ).

**Abbreviations:** RT, Rectal temperature; PR, pulse rate; RR, respiration rate.

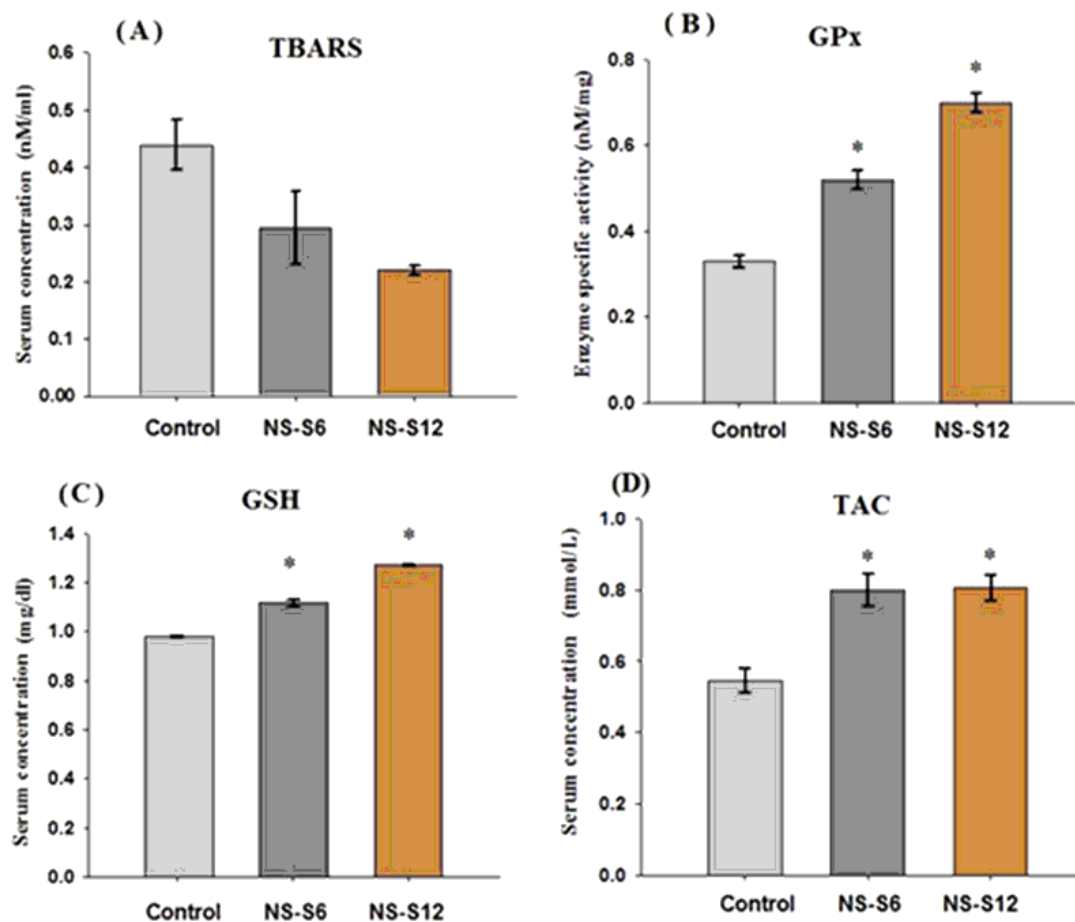


**Figure 2**

Effect of NS-S6 and NS-S12 on serum pro-oxidant and antioxidant issues in lambs. (n= 6). **(A)** Effects on serum TBARS concentration, **(B)** Effects on the serum GPx specific activity, **(C)** Effects on the serum GSH concentration, and **(D)** Effects on serum TAC concentration. The asterisks show significant differences between NS-S6 and NS-S12 groups compared to the control (\*p < 0.05).

**Abbreviations:** TBARS, thiobarbituric acid reactive substances; GPx, glutathione peroxidase; GSH, reduced glutathione; TAC, total antioxidant capacity.