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# Ameliorative effect of dietary nucleotides supplementation on antioxidative status and molecular expression of growth and immune related genes in broiler chickens

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**ABSTRACT**

The present study aimed to evaluate the effect of dietary nucleoforce® supplementation on the antioxidative status and the molecular expression of IGF-1, IL-1ß and IL-6 genes in broiler chickens. 240, one-day old Ross 308 broiler chicks were randomly allocated into four equal groups, each of which contained 60 birds and was divided into three replicates with 20 birds for each replicate. Birds in the first group fed basal diet and considered as a control group (C), whereas birds of the second (200N), third (350N), and fourth (500N) groups fed diet supplemented with 200, 350 and 500 g/ton nucleoforce®, respectively from zero day till the end of the experiment. At days 21 and 49 of age, two birds from each replicate were randomly chosen, slaughtered, and dissected to collect blood and tissue samples. The concentrations of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in serum were calculated as an indicator for the antioxidant status. Gene expression of insulin-like growth factors 1 (IGF-1) in liver, interleukin-1β (IL-1β) and interleukin-6 (IL-6) in spleen was performed using quantitative real-time PCR (qRT-PCR). The obtained results revealed that the highest nucleoforce® concentration (500g/ton) resulted in time dependent significant increases in both SOD and GPx. This study also indicated that nucleotides supplementation resulted in significant up regulations of growth and immune related gene expression with the best results were obtained with the highest nucleotides concentration. In conclusion, dietary nucleoforce® inclusion can improve the performance of broiler chickens and enhance their antioxidative and immune status.

## Introduction

Food which provides substances that can improve animal health beside its nutritional value is known as functional food (Meyer, 2009). Among these functional substances are nucleotides, which were evidenced to have immense roles in many of the biological reactions that are crucial for life sustenance of both human and animals (Superchi *et al*., 2012). Nucleotides are involved in determining the characteristics of organisms and the structure and functions of body proteins. They also participate in the regulation of various substances of metabolism. They serve as the primary high energy chemicals in the pathway for energy metabolism and crucial messengers in the transmission of cell signals (Zhu *et al*., 2023). In some conditions, endogenous synthesis of nucleotides is insufficient for performing physiological functions (Maldonado *et al*., 2001) so; dietary nucleotides are considered as essential nutrients help in the growth of rapidly dividing cells without the expense of more energy and thereby increase the productivity in birds. Exogenous nucleotides supplementation is greatly substantial in various processes including antioxidant activity, immunomodulatory activity, the maintenance of liver and gastrointestinal functions and optimizing intestinal microbiota (Ding *et al*., 2021), DNA protective activity, the restoration of mitochondrial function, the promotion of cell proliferation (Pérez *et al*., 2004; Gil, 2002; Maribo, 2003; Domeneghini *et al*., 2004; Sauer *et al*., 2011; Che *et al*., 2016; Daneshmand *et al*., 2017). In commercial conditions, birds are exposed to high stocking density and poor hygiene conditions resulting in bacterial contamination, inflammatory (Takahashi *et al*., 2008) and immune system activation, and impaired growth performance (Xie *et al*., 2000). Nucleotides act to increase humoral immunity and cell-mediated immunity, resulting in improved host resistance to bacterial infections (Maldonado *et al*., 2001; Frankič, *et al*., 2006; Hess and Greenberg, 2012).

It was also reported that nucleotides deficiency in diet may impair intestine, immune, liver and heart functions as endogenous source of nucleotide from these tissues are inadequate (Grimble and Westwood, 2000). Therefore, nucleotides are used as functional feed ingredients and often supplemented to diets of livestock in the form of yeast extracts or pure substance (Sauer *et al*., 2012; Alizadeh *et al*., 2016). Depending on these findings, the objective of this study was to estimate the effects of dietary nucleotide supplementation on the antioxidative status and the molecular expression of growth (IGF-1) and immune (IL-1β and IL-6) related genes in broiler chickens.

## Materials and methods

*Nucleotides (Nucleoforce®)*

Nucleoforce® is a balanced concentrate of free nucleotides and active precursors obtained from dried yeast with a minimum concentration of 80%. It was obtained from Bioibérica, S.A., Spain in a powder form composed of 20.34% crude protein, 3.25% protein nitrogen, 12.09% non-protein nitrogen (from nucleotides), 0.1% crude fiber (CF) and 3.38% Ash.

*Birds, housing, and management*

A total number of 240 one-day old broiler chicks (Ross 308) of both sexes with average weight of 43g obtained from a commercial hatchery (El-Desoky Company) were randomly distributed into four equal groups with 60 birds for each group. Then the groups were subdivided into replicates with 3 replicates for each group and 20 birds for each replicate. All birds were reared under the same environmental and management


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conditions. The broiler chicks were vaccinated against most infectious diseases affecting broilers. The birds were housed in clean disinfected well-ventilated rooms bedded with fresh clean wood shaving forming a layer of 4 cm depth. The floor was divided into 12 separate pens of equal size by using wire net and bamboo materials. Intermittent lightening program (23 hours lighting: 1 hour darkness) was used. The environmental temperature was adjusted according to the age of chicks. Feed and fresh water were supplied ad libitum. All the procedures of the experiment were ethically approved by the Ethics Review Committee of the Faculty of Veterinary Medicine, Benha University, Egypt.

*Experimental design*

Birds were randomly allocated into four equal groups and were fed on four experimental diets throughout the experimental period as follow:
Group1: basal diet without any supplementation and considered as control group (C).
Group2: basal diet supplemented with 200g nucleoforce®/ton (200N).

Table 1. The chemical composition of the basal diet during different phases of growth

| Feed Ingredients | Composition (%) mixed feed Broiler ration | | |
|---|---|---|---|
| | Starter (0 to 17d) | Grower (17 to 36d) | Finisher (36d till end of experiment) |
| Yellow corn (crushed) | 53.03 | 55.51 | 60.68 |
| Soya bean meal (CP 46 %) | 35 | 33.7 | 27.5 |
| Corn gluten meal | 4.7 | 3 | 3.5 |
| Soya bean oil | 2.4 | 3.4 | 4.3 |
| Di calcium phosphate | 1.6 | 1.33 | 1.23 |
| Limestone | 1.5 | 1.4 | 1.25 |
| L_Lysine | 0.39 | 0.31 | 0.29 |
| Sodium chloride | 0.33 | 0.31 | 0.31 |
| Vitamin and mineral premix | 0.3 | 0.3 | 0.3 |
| DL_methionine | 0.33 | 0.3 | 0.26 |
| Sodium bicarbonate | 0.13 | 0.13 | 0.13 |
| Anticoccedia | 0.05 | 0.05 | 0.05 |
| Antimycotoxin | 0.05 | 0.05 | 0.05 |
| L_Threonine | 0.1 | 0.1 | 0.1 |
| Anticolesteridia | 0.03 | 0.03 | 0.03 |
| Energy enzyme | 0.03 | 0.05 | 0.05 |
| Lysomax | 0.1 | 0.1 | 0.1 |
| Phytase enzyme | 0.01 | 0.01 | 0.01 |
| Protease B | 0.01 | 0.01 | 0.01 |
| Emulsifier | 0.01 | 0.01 | 0.01 |
| Total | 100 | 100 | 100 |
| Calculated composition | | | |
| Metabolizable energy ME (kcal/kg) | 3001.88 | 3101.78 | 3226.25 |
| CP | 23.02 | 21.54 | 19.51 |
| CF | 3.56 | 3.5 | 3.17 |
| Crude fat | 5.03 | 6.02 | 7.02 |
| Lysine | 1.35 | 1.25 | 1.09 |
| Lysine dig | 1.26 | 1.16 | 1.01 |
| Methionine | 0.67 | 0.62 | 0.55 |
| Methionine dig | 0.63 | 0.58 | 0.52 |
| Methionine+ cysteine | 1.02 | 0.95 | 0.86 |
| Methionine+ cysteine dig | 0.92 | 0.85 | 0.77 |
| Threonine | 0.92 | 0.87 | 0.73 |
| Threonine dig | 0.79 | 0. 75 | 0.62 |
| Calcium | 1.05 | 0.95 | 0.85 |
| Available phosphorus | 0.5 | 0.45 | 0.42 |
| Sodium | 0.18 | 0.17 | 0.17 |
| Chloride | 0.23 | 0.22 | 0.22 |
| Potassium | 0.88 | 0.85 | 0.75 |
| Pellet quality factor | 3.28 | 2.88 | 2.55 |
| Acid base balance (me/kg) | 223.67 | 217.11 | 188.5 |

Vitamin and mineral premix was composed of (Content per: 3.0 kg): vitamin A 12000000 IU; vitamin D 200000 IU; vitamin E 10000 mg; vitamin K3 2000 mg; vitamin B11000 mg; vitamin B2 5000 mg; vitamin B6 1500 mg; Biotin 50 mg; Niacin 30000 mg; Folic acid 1000 mg; D-Calpan 10000 mg; vitamin B12 10 mg; Iron carbonate 3000 mg; Cobalt Carbonate 100 mg; Manganese oxide 60000 mg; Calcium Iodate 1000 mg; Copper sulphate 4000 mg; Se1enium Sodium 100 mg; Zinc (global) 50000 mg and carrier (CaCo3) Up to 3.0 kg. Vitamin and mineral premix produced by MULTI-VITA 6 of October city, Egypt.

Group3: basal diet supplemented with 350g nucleoforce®/ton (350N)
Group4: basal diet supplemented with 500g nucleoforce®/ton (500N).

The basal diet was formulated to provide the nutritional requirements of birds during different phases of age according to National Research Council (1994). Nutrient requirements of the birds for starter ration (from 0 to 17 days) were 23.02% crude protein and 3001.88 Kcal/Kg metabolizable energy, for grower ration (from 17 to 36 days) were 21.54% crude protein and 3101.78 Kcal/Kg metabolizable energy, and for finisher ration (from 36 to 49 days) were 19.51% crude protein and 3226.25 Kcal/Kg metabolizable energy. The composition of the ration used was shown in Table 1.

On days 21 and 49 of age, two birds from each replicate (six birds from each group) were randomly chosen, slaughtered, and eviscerated to collect blood and tissue samples.

*Antioxidant activity*

Blood samples were collected from the jugular vein during slaughtering and loaded into sterile screw capped tubes. Serum was separated by centrifugation of blood samples at 3000 r.p.m for 15 minutes, aspired by automatic pipette then kept in dry sterile tubes in deep freeze at -20ºC till used for subsequent biochemical analysis.

*Determination of superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity*

Superoxide dismutase and glutathione peroxidase activities were measured in serum sample according to the methods of Nishikimi *et al*., (1972) and Paglia and Valentine *et al*., (1967), respectively. All these measurements were performed on the diagnostic kits in a manner that is compliant with the instructions provided by the manufacturer (Biodiagnostic Company, Dokki, Giza, Egypt).

*Molecular studies*

After slaughtering, bleeding and dissection, liver and spleen samples were collected. Insulin-like growth factor 1 (IGF-1), interleukin-1β (IL-1β) and interleukin-6 (IL-6) gene expressions were applied as a molecular marker to evaluate the effect of different nucleoforce® concentrations via quantitative real-time PCR (qRT-PCR).

TRIzols Reagent (15596026, Life Technologies, USA) was applied for total RNA purification from samples. Yield and quality of total RNA were determined spectrophotometrically at 260 and 260/280 nm ratio, respectively. Insulin-like growth factor 1 (IGF-1), interleukin- IL-1β, and interleukin-6 mRNA were determined using Maximas SYBR Green/Fluorescein qPCR Master Mix by Rotor-Gene Q (Qiagen, USA). 1 µg of total RNA was reverse-transcribed into single-stranded complementary DNA by using QuantiTects Reverse Transcription Kit (Qiagen, USA) using a random primer hexamer in a two-step RT-PCR reaction in which any genomic DNA (gDNA) contamination was eliminated using gDNA Wipeout buffer. Total cDNA (30 ng) was used as a template for amplification with the specific primers pair (Table 2) used at a 300 nM final concentration. Each sample was subjected to real-time PCR in duplicate and the mean values of the duplicates were used for subsequent analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as house-keeping gene. Rotor-Gene Q collected data automatically and analyzed the value of threshold Cycle (Ct) which was normalized to an average Ct value of the house-keeping genes (ΔCt) and the relative expression of each representative was calculated as $2^{-\Delta Ct}$.

*Statistical analysis*

It was carried out by Graph Pad Prism software, 2007 version 5.04 (Graph Pad Prism, San Diego, CA, USA) for determining the significant difference between the treatment groups by one way analysis of variance (ANOVA). Duncan's Multiple Range test (LSD) using Costat Computer Program (1986) was used to detect the significance of difference between each two groups. Data were represented as mean±S.E. Significant difference between mean values was determined at $P \leq 0.05$.

Table 2. Gene primer sequences used for quantitative RT-PCR

| RNA target | Probe/primer sequences | References |
|---|---|---|
| IGF-1 | ATGCCCATCACATCCTCC<br>TACATCTCCAGCCTCCTCA | Yang *et al*. (2019) |
| IL-1β | GAAGTGCTTCGTGCTGGAGT<br>ACTGGCATCTGCCCAGTTC | Crhanova *et al*. (2011) |
| IL-6 | GCTACAGCACAAAGCACCTG<br>GACTTCAGATTGGCGAGGAG | Kolesarova *et al*. (2011) |

## Results

The concentrations of superoxide dismutase (SOD) in serum of broiler chickens after dietary nucleoforce® supplementation were illustrated in Table 3. Nucleoforce® supplementation at a concentration of 500 g/ton resulted in significant increases in the concentration of SOD as compared to control, 200N and 350 N groups on 21 days of age and to control and 200 N groups on 49 days of age.

Table 3. The effect of dietary Nucleoforce® supplementation on superoxide dismutase (SOD) (U/ml) in broiler chickens

| Groups | Superoxide dismutase (SOD) | |
|---|---|---|
| | 21 days of age | 49 days of age |
| C | 9.03±0.82$^{b}$ | 22.71± 1.7$^{b}$ |
| 200 N | 11.96±1.6$^{b}$ | 23.23±1.9$^{b}$ |
| 350 N | 13.16±2.2$^{b}$ | 34.17±5.7$^{ab}$ |
| 500 N | 18.71±1.4$^{a}$ | 44.75±3.2$^{a}$ |

Each value is expressed as mean±standard error. Means with different letters in the same column are significantly different (p≤0.05). C= control, 200 N= Nucleoforce® at a concentration of 200g/ton, 350 N= Nucleoforce® at a concentration of 350g/t, 500N= Nucleoforce® at a concentration of 500g/t.

Table 4 showed the effect of dietary nucleoforce® supplementation on glutathione peroxidase (GPx). On day 21 of age the highest concentration of nucleoforce® resulted in a significant increase in GPx as compared to control and 200N supplemented group but the concentration of 350 g/ton significantly increased GPx as compared to the control group only. While on 49 days of age, the highest nucleoforce® concentration significantly increased GPx as compared to control and other treated groups.

Table 4. The effect of dietary nucleoforce® supplementation on glutathione peroxidase (GPx) (mU/ml) in broiler chickens.

| Groups | Glutathione peroxidase (GPx) | |
|---|---|---|
| | 21 days of age | 49 days of age |
| C | 48.41±6.6$^{c}$ | 92.43±5.1$^{b}$ |
| 200 N | 63.27±4.7$^{bc}$ | 96.81±5.7$^{b}$ |
| 350 N | 78.40±8.9$^{ab}$ | 104.28±6.7$^{b}$ |
| 500 N | 89.66±6.6$^{a}$ | 129.22±5.7$^{a}$ |

Each value is expressed as mean±standard error. Means with different letters in the same column are significantly different (p≤0.05). C= control, 200 N= Nucleoforce® at a concentration of 200g/ton, 350 N= Nucleoforce® at a concentration of 350g/t, 500N= Nucleoforce® at a concentration of 500g/t.

Table 5 showed that dietary nucleotides supplementation resulted in a significant (P≤0.05) upregulation of IGF-1 gene expression in broilers liver for two different time periods as compared to control group. This upregulation was dose dependent as the highest nucleotides concentration resulted in the highest IGF-1 upregulation.

Table 5. The effect of dietary Nucleoforce® supplementation on the relative expression of IGF-1 gene in broilers liver.

| Group | Insulin like growth factor 1 (IGF-1) | |
|---|---|---|
| | 21 days of age | 49 days of age |
| C | 1.0±0.10$^{c}$ | 1.0±0.067$^{c}$ |
| 200N | 1.44±0.058$^{b}$ | 1.19±0.033$^{bc}$ |
| 350N | 1.62±0.033$^{b}$ | 1.24±0.033$^{b}$ |
| 500N | 1.96±0.12$^{a}$ | 1.67±0.12$^{a}$ |

Each value is expressed as mean±standard error. Means with different letters in the same column are significantly different (p≤0.05). C= control, 200 N= Nucleoforce® at a concentration of 200g/ton, 350 N= Nucleoforce® at a concentration of 350g/t, 500N= Nucleoforce® at a concentration of 500g/t.

Table 6 showed a significant (P≤0.05) upregulation of IL-1β gene expression level in broilers spleen following dietary nucleotides supplementation for two different time periods as compared to control group. This upregulation was dose dependent with the highest significant result achieved with the highest nucleotides concentration.

Table 6. The effect of dietary Nucleoforce® supplementation on interleukin-1β (IL-1β) gene expression in broiler chickens.

| Group | Interleukin-1β (IL-1β) | |
|---|---|---|
| | 21 days of age | 49 days of age |
| C | 1.0±0.033$^{c}$ | 1.0±0.033$^{d}$ |
| 200N | 1.94±0.033$^{c}$ | 1.74±0.058$^{c}$ |
| 350N | 3.35±0.033$^{b}$ | 3.17±0.033$^{b}$ |
| 500N | 5.47±0.088$^{a}$ | 5.35±0.067$^{a}$ |

Each value is expressed as mean±standard error. Means with different letters in the same column are significantly different (p≤0.05). C= control, 200 N= Nucleoforce® at a concentration of 200g/ton, 350 N= Nucleoforce® at a concentration of 350g/t, 500N= Nucleoforce® at a concentration of 500g/t.

Table 7 revealed that dietary nucleotides supplementation resulted in a significant (P≤0.05) upregulation of IL-6 gene expression level in broilers spleen for two different time periods as compared to control group. This upregulation was directly proportional with the concentration of nucleotides supplemented.

Table 7. The effect of dietary Nucleoforce® supplementation on interleukin-6 (IL-6) gene expression in broiler chickens.

| Group | Interleukin-6 (IL-6) | |
|---|---|---|
| | 21 days of age | 49 days of age |
| C | 1.0±0.00$^{c}$ | 1.0±0.033$^{c}$ |
| 200N | 1.3±0.033$^{c}$ | 1.59±0.033$^{c}$ |
| 350N | 2.3±0.033$^{b}$ | 2.35±0.058$^{b}$ |
| 500N | 3.7±0.033$^{a}$ | 3.53±0.033$^{a}$ |

Each value is expressed as mean±standard error. Means with different letters in the same column are significantly different (p≤0.05). C= control, 200 N= Nucleoforce® at a concentration of 200g/ton, 350 N= Nucleoforce® at a concentration of 350g/t, 500N= Nucleoforce® at a concentration of 500g/t.

## Discussion

Nucleotides are vital metabolites involved in approximately all cellular processes (Liu, 2016). They play critical roles in structural, metabolic, functions, and energetic regulatory processes (Ridwanudin *et al*., 2019). Nucleotides have numerous physiological functions, including cellular agonists, cell signaling, and co-enzyme components (Carver and Walker, 1995; Liu, 2016). It has been theorized that tissues, immune cells, and gastrointestinal cells require nucleotides during the early stages of development with a high metabolism or fast growth (Ringo *et al*., 2012; Liu, 2016).

The balance between oxidative damage and antioxidant capacity is important for maintaining cell homeostasis and physiological activities (Domingues *et al*., 2016). The production of reactive oxygen species (ROS) affects the antioxidant system leading to damage, which in turn results in oxidative stress (Sies, 1991). Broilers are susceptible to different types of stressors, such as heat stress which prompts the generation of ROS. Antioxidant enzymes are found in all organisms and help prevention of cell membrane damage and alteration of nucleic acids. The major enzymes that make up the primary defenses are catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD). SOD catalyzes the dismutation of superoxide radicals to H2O2 and oxygen, while CAT catalyzes the breakdown of H2O2 to H2O and molecular oxygen. GPx is a selenium-based enzyme, which deactivates peroxides using the peptide glutathione (GSH) as its cosubstrate (Halliwell, 2006). Catalase and peroxidases are enzymatic ROS scavengers that decrease the concentration of H2O2, which acts as a source of active radical species. ROS are deemed as critical oxygen mediators and crucial messengers that promote cell division (Buetler *et al*., 2004). The results of this study showed that nucleotides supplementation to broiler diet enhanced their antioxidative status which was represented by significant increases in both SOD and GPx activities. These results are in harmony with those of Rady *et al*., 2023 who found that addition of nucleotides significantly increased GPx and SOD enzymes level in broilers. It was mostly suggested that dietary nucleotides ameliorate the oxidative status by reinforcing the antioxidant capacity and upregulating genes encoding for antioxidant enzymes (Tie *et al*., 2019; de Lima *et al*., 2020). Furthermore, it was evidenced that there is a correlation between exogenous nucleotides inclusion and high mRNA levels associated with antioxidant enzymes (Salobir *et al*., 2005; Frankič *et al*., 2006; Tie *et al*., 2019).

The results of the current study showed that dietary nucleotides supplementation resulted in a significant dose dependent upregulation of IGF-1 gene expression in broilers liver for two different time periods. These resulted were the same as those obtained by Rady *et al*., 2023 who stated that the fold change of insulin-like growth factor (IGF) showed that the addition of nucleotides significantly up regulated (IGF) expression in liver tissue. The regulation of insulin-like growth factor-1 concentrations with nutrient intake links diet and growth, which in turn brings an interface between nutrients and hormones acting together to stimulate growth, while illustrating the cardinal role that nutrients play in the control of gene expression (Thissen *et al*., 1994). Circulating IGF-I probably represents the most meaningful serum index for adequate nutrient intake because of its regulatory mode, its growth-promoting effect, and its close relationship to nitrogen balance. Serum IGF-I level is positively related to nutritional status and affected by other hormones like insulin (Jahreis *et al*., 1992).

Exogenous nucleotides stimulate lymphoid cell maturation and the lymphoproliferative response to alloantigens and mitogens. They also contribute to the response of T lymphocytes, increase delayed cutaneous hypersensitivity, increase resistance to certain infections, increase rejection of grafts, counteract malnutrition-induced immunosuppression, regulate the quantity of natural killer (NK) cells and macrophages and promote the synthesis of immunoglobulins Maldonado *et al*., (2001).

Interleukins are polypeptides produced by cells involved in immune and inflammatory responses to activate and modulate other cells and tissues Kaiser and Staheli (2008). IL-6 is a protein involved in recruiting and controlling cells in both natural and acquired immunities. Such cytokines are needed for successful host immune responses to pathogens. IL-6 aids short-term protection against infection or damage by alerting the immune system to the source of inflammation. However, illness arises from improper control of this molecule. Pro-inflammatory cytokines, such as IL-6, control the immune response by stimulating the proliferation and differentiation of leukocytes that kill pathogens Rodes *et al.* (2013).

Interleukin-1β, a member of the interleukin-1 family, (Awomoyi *et al*., 2005) plays an important role in inflammatory responses and autoinflammatory diseases (He *et al*., 2011). This interleukin is associated with and induces the expression of proinflammatory cytokine genes such as IL-1β, IL-6, and IL-8 by associating and activating the JAK-STAT, NF-κB, PI3K, and JNK signaling pathways (Tsukada *et al*., 1996; Oh *et al*., 2016). Interleukin-1β has been shown, both in vitro and in vivo, to be a growth factor for B cell proliferation due to induction of IL-6, which is often under the control of IL-1β (Dinarello, 2009). Both IL-1β and IL-6 are pleiotrophic cytokines that regulate immune responses, and are commonly referred to as the proinfiammatory cytokines produced as part of the induced innate response (Kaiser *et al*., 2004). The results of our study revealed that dietary nucleotides supplementation resulted in significant upregulations of both IL-1β and IL-6 gene expression in broiler chickens in a dose dependent manner.these results were in agreement with those of El-Nokrashy *et al*. (2021) and Reda *et al*. (2018).

## Conclusion

It could be concluded that dietary nucleoforce® inclusion, as an exogenous source of nucleotides, can improve the performance of broiler chickens and enhance their antioxidative and immune status.

## Conflict of interest

The authors declare that they have no conflict of interest.

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