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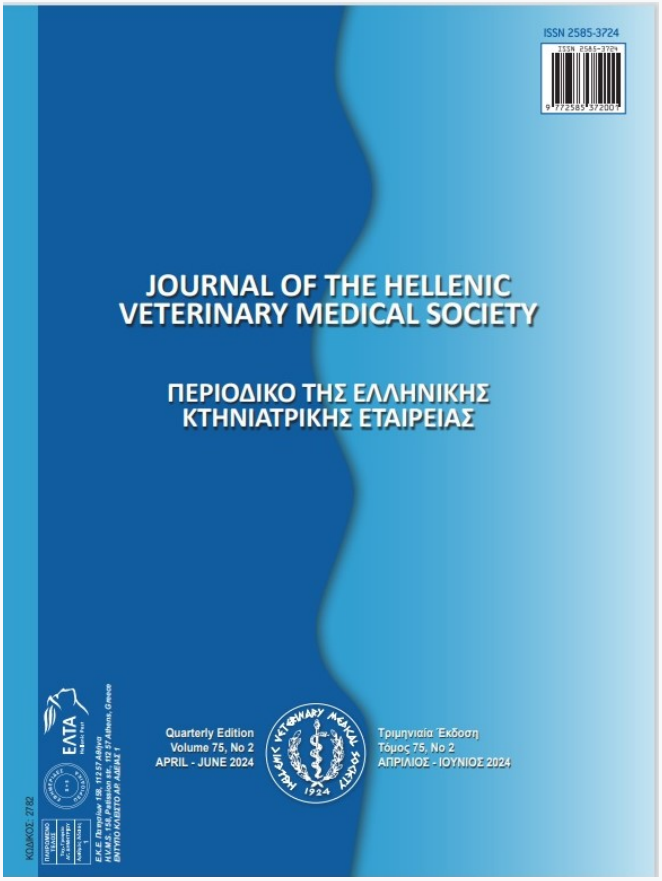

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Effect of dietary supplementation of *Nannochloropsis* on gene expression and metabolic profile of immune and antioxidant markers in growing Barki lambs

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ABSTRACT: The aim of the current study was to elaborate the effect of dietary supplementation of *Nannochloropsis* on gene expression and metabolic profile of immune and antioxidant markers in growing Barki lambs. Sixty apparently healthy growing Barki lambs were enrolled in this study. Lambs were randomly allocated into two equal groups (30 lambs each). The first group considered control group, whereas the second group supplemented with commercially available *Nannochloropsis* powder for subsequent 30 days. Supplementation of growing lambs with *Nannochloropsis* could modulate gene expression profile of immune and antioxidant markers. Levels of immune (*IL1B*, *IL2*, *Il6*, *IL8, RANTES*, *NFKB*, *MASP2* and *TNFα*) and antioxidant (*SOD3*, *GPX4*, *PRDX2*, and *Nrf2*) genes expression were significantly up-regulated in lambs supplemented with *Nannochloropsis* at day 30 than control and day 15. Serum concentrations of SOD, CAT, TAC, GPx, NO, GSH, lysozyme activity, TNF-α, IL1, IL-6, and IgA were significantly ($p < 0.05$) higher in the supplemented group compared to the control group, whereas MDA levels were significantly lower. Among the examined groups, there was no significant difference ($P > 0.05$) in the levels of IgG and IgM. The results herein confirm that there was profound immunological, antioxidant alterations associated with dietary supplementation of *Nannochloropsis* in growing Barki lambs. Serum profile and gene expression could be used as substitute markers to track immune health and the development of an efficient management strategy for enhancing the health of growing Barki lambs.

Keywords: Barki lambs; gene expression; *Nannochloropsis*; antioxidant; immune response

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INTRODUCTION

Ruminant diet and feed supplements containing microalgae are becoming more and more popular (Gomaa et al., 2018, Lamminen et al., 2019). Small doses of microalgae supplementation exhibited positive effects on animal physiology, productivity, and feed conversion by improving gastrointestinal and immune processes (Camacho et al., 2019)**.** According to (de Morais et al., 2015), microalgae are a broad class of photosynthetic, autotrophic microorganisms that possess a number of unique biological characteristics, including high photosynthetic energy transfer efficiency and the ability to synthesize biologically complex substances like proteins, lipids, carbohydrates, pigments, and polymers. Microalgae also include a high concentration of healthy components, including protein, polysaccharides, and vitamins A, C, E, and K, thiamine (B1), pyridoxine (B6), riboflavin (B2), nicotinic acid, biotin, and tocopherol (Chew et al., 2017, Khan et al., 2018)**,** excellent environmental adaptability and the potential to produce a wide range of bioenergy (Levering et al., 2015)**.**

Natural antioxidants found in microalgae include phenols, flavonoids, carotenoids, and chlorophyll, which strengthen the body's defense against free radicals (Ben et al., 2017). In fact, when their meals included microalgae, humans (Panahi et al., 2013), broilers (Elbaz et al., 2022), and fattening lambs (EL-Sabagh et al., 2014) all showed an improvement in their antioxidant status. Oxidative stress in ruminants is caused by an imbalance between the generation of reactive oxygen species (ROS) and the capacity of antioxidant mechanisms to neutralize those ROS. This imbalance can arise from a number of environmental, physiological, and dietary factors (Sies, 1991).

High levels of naturally occurring antioxidant compounds in feedstuffs may protect animals from oxidative stress and ease consumer concerns about safety. The body frequently uses a range of antioxidant processes, both enzymatic and non-enzymatic (metabolites, for instance), to combat oxidative stress (Ye et al., 2015). Blood and milk contain a number of endogenous enzymes that are essential to the intracellular antioxidant defense mechanisms that control the build-up of reactive oxygen species (ROS) in tissues (Sordillo, 2013). These enzymes include superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT), glutathione transferase (GST), and glutathione peroxidase (GSHPx) (Board & Menon, 2013).

In the genus *Nannochloropsis*, there are six different species, including *N. gaditana, N. salina, N. limnetica, N. granulata, N. oceanica, and N. oculata.* They all have polysaccharide cell walls that contain one chloroplast (Hibberd, 1981). Numerous studies (both in-vitro and in-vivo) have demonstrated the beneficial effects of *N. oculata* on palatability, lack of toxicity (Gbadamosi & Lupatsch, 2018) easy digestion (Kholif et al., 2020, Md et al., 2018), antioxidant actions (Elsheikh et al., 2018)**,** immunity (Bule et al., 2018)**,** anti-inflammatory and anti-cancer (Md et al., 2018) on several animal. They can be substituted for conventional protein in animal diets and are also a good alternative source of eicosapentaenoic acid (EPA, C 20:5 n3) (Becker, 2007).

Variations in the microalgal's immunoreactivity were assessed by measuring T and B lymphocyte proliferation and T cell cytokine production. T cells, also referred to as thymocyte cells, are a subset of lymphocytes that are essential for cell-mediated immunity. B cells, a subset of white blood cells, are involved in humeral immunity, which is a component of the adaptive immune system, by secreting antibodies (Nutt et al., 2015). In addition, cytokines, which include chemokines, interferons, and interleukins, are cell signaling molecules that are crucial for the immune system (Borish & Steinke, 2003).

Measuring the concentration of each antioxidant separately does not provide a reliable indicator of the antioxidant capability since antioxidants cooperate to counteract oxidative damage (Abdullah, 2015). Therefore, a deficiency in one antioxidant does not necessarily mean that all antioxidant defense mechanisms are compromised. As a result, numerous techniques have been created to determine the overall antioxidant capacity. In order to anticipate a herd's susceptibility to production disorders, it has recently been shown that examining the gene expression of antioxidant biomarkers offers a trustworthy technique to monitor animal health and productivity (Lager & Jordan, 2012).

According to (Van Harten et al., 2013), variations in the gene expression of a number of regulatory enzymes involved in the intermediate metabolism can serve as effective tools for improving genetic selection for the adaptation of livestock to challenging conditions. One aspect of metabolic regulation is the transcriptional regulation of gene networks, which are collections of DNA segments that interact with transcription factors or nuclear receptors to regulate the concentration of critical enzymes in cells. These

"global" interactions may control how quickly the network's genes are translated into mRNA. The vast area of genomics includes research on the complete genome, sub-networks, or candidate genes at the mRNA level (Loor, 2010).

The serum antioxidant and immunological profile of developing lambs receiving nutritional supplements containing microalgae are not well documented, based on existing knowledge. Additionally, no research have previously explored the impact of microalgae supplementation on the expression profile of immune and antioxidant genes in growing lambs. Therefore, the goal of the current study was to track the pattern of gene expression of immunological (*IL1B, IL2, Il6, IL8, RANTES, NFKB, MASP2,* and *TNFα*) and antioxidant (*SOD3, GPX4, PRDX2*, and *Nrf2*) genes in growing Barki lambs supplemented with *Nannochloropsis*. Exploring the relationship between gene expression pattern and the serum profile of immunological and antioxidant biomarkers was another goal of the investigation.

MATERIALS AND METHODS

Animals and experimental design

The study included sixty apparently healthy growing Barki lambs aged between 8 and 10 months and had average body weight of 28.45 ± 4.2 kg. The study was conducted at Mariut Research Station, Desert Research Center, El-Amria, Alexandria, Egypt. The animals were given a prophylactic dose of broad spectrum anthelmintic (Ivermectin/Clorsulan [AVICO], Amman, Jordan) as recommended. All animals had no history of metabolic or concurrent ailments and were kept under identical conditions of housing throughout the study period. Lambs were randomly allocated into two equal groups (30 lambs each). The first group received the basal diet without feed supplement and considered control group, whereas the second group received the same basal diet but supplemented with commercially available *Nannochloropsis* powder was incorporated daily in the concentrate of each lamb at a rate of 10 g *Nannochloropsis* /kg concentrate (Tsiplako, 2016) for subsequent 30 days, with a pre-trial period of one week for adaptation to diets and facilities. The dried powder *Nannochloropsis* algae used in the present study was produced by National Research Center (NRC), Dokki, Giza, Egypt. The microalga *Nannochloropsis oculata (N. oculata)* used in the present study was prepared and kindly provided by the Biotechnology Microalgae Culture Unit, National Research Center (NRC), Giza, Egypt. Microalgae were maintained in standard F/2 Guillard's media (Guillard & Ryther, 1962)**.** The collected microalgae were stored in the refrigerator at 4 ºC until the culture period was finished and then harvested by centrifugation. The technique for microalgae *N. oculata* extraction was used as described by (Hassan, 2015)**.** The chemical composition of microalgae *N. oculata* extract was determined by gas chromatography-mass at complex laboratories of National Research Centre, Dokki, Giza, Egypt. The identification and quantitative measurements of microalgae *N. oculata* extract constituents are presented in Table 1. The basal diet was formulated to meet the lamb's nutrient requirements in order to meet their energy and nutrient requirements according to Ruminants, (2007) recommendations. The experimental design was a complete randomized design. The investigated lambs were housed individually in soil-surfaced pens (1.5 m2 / lamb) and were fed on 500 g concentrate feed mixture (CFM) plus 500 g alfalfa hay/head/day. Diet was offered twice a day in the morning and evening with free access to water. Feed and refusals were recorded daily. The composition of the basal diet is presented in Table 2. Lambs were weighed on days 0, 15 and 30 of experiment, after fasting for twelve hours before the morning feedings. The protocols were approved by the Ethics Committees and executed in accordance with the guidelines established by the Desert Research Centre, Egypt.

Clinical examination

All lambs were clinically examined prior to the experiment according to the defined methods descried previously and the observed clinical findings were recorded simultaneously. Vital indicators such as rectal temperature, heart rate, breathing rate, and color of the visible mucosal membrane were of special interest. Abdomen and thorax were carefully inspected. Additionally, body weight was measured on a standard scale.

Blood sampling

Each animal had ten milliliters of blood drawn from its jugular vein at the beginning of the trial (T0), at the 15th and 30th days after supplementation. To produce serum or whole blood, respectively, the collected blood was added to simple tubes (i.e., tubes without anticoagulants) and tubes with EDTA. RNA was extracted from the EDTA blood. All samples were sent to the lab for additional processing after be-

Table 1: The quantitative measurements of *Nanochloropsis oculata* constituents by GC mass.

Chemical composition (g/100g) of microalgae *Nannochloropsis oculata*	
Moisture	7.15
Crude protein	55.78
Fat	6.61
Ash	12.29
Quantitative constituents of minerals profile (mg/100g) in microalgae *Nannochloropsis oculata*	
Fe	29.35
Zn	1.02
Sodium	1862.70
Calcium	229
Potassium	798
Magnesium	173
Quantitative constituents of Amino acids profile (mg/g) in microalgae *Nannochloropsis oculata*	
Methionine	69.52
Cystine	17.30
Phenylanlanine	16.24
Lysine	15.20
Isoleucine	55.95
Leucine	65.11
Aspartic acid	30.16
Glutamic acid	15.07
Histidine	13.22
Tyrosine	87.69
Threonine	39.21
Valine	50.36
Serine	11.64
Glycine	9.98
Proline	31.52
Alanine	20.24
Arginine	8.56

Table 2 Composition of the concentrate feed mixture (CFM) fed to growing Barki lambs

Ingredients	Quantity
Corn	400 kg
Wheat bran	300 kg
Soya bean	250 kg
Sodium chloride	10 kg
Calcium carbonate	20 kg
Premix	1 kg
Netro-Nill	0.5 kg
Fylax	0.5 kg

ing chilled on crushed ice.

Total RNA extraction, reverse transcription and quantitative real time PCR

Total RNA was extracted from lamb blood using Trizol reagent following the manufacturer instructions (RNeasy Mini Ki, Catalogue no.74104). The amount of extracted RNA was quantified and qualified using NanoDrop® ND-1000 Spectrophotometer. The cDNA of each sample was synthesized following the manufacture protocol (Thermo Fisher, Catalog no, EP0441). The gene expression pattern of genes encoding immune (*IL1B*, *IL2*, *Il6*, *IL8*, *RANTES*, *NFKB*, *MASP2* and *TNFα*) and antioxidant (*SOD3*, *GPX4*, *PRDX2*, and *Nrf2*) was assessed using quantitative RT-PCR using SYBR Green PCR Master Mix (2x SensiFastTM SYBR, Bioline, CAT No: Bio-98002). Relative quantification of mRNA level was performed by real-time PCR using SYBR Green PCR Master Mix (Quantitect SYBR green PCR kit, Catalog no,

204141). Primer sequences were designed according to the PubMed published sequence of *Ovis aries* as shown in Table 3. The housekeeping gene *ß. actin* was used as a constitutive control for normalization. The reaction mixture was carried out in a total volume of 25 µl consisted of total RNA 3 µl, 4 µl 5x Trans Amp buffer, 0.25 µl reverse transcriptase, 0.5 µl of each primer, 12.5 µl 2x Quantitect SYBR green PCR master mix and 8.25 µl RNase free water. The final reaction mixture was placed in a thermal cycler and the following program was carried out: reverse transcription at 50 °C for 30 mins, primary denaturation at 94 °C for 10 mins followed by 40 cycles of 94 °C for 15 s, annealing temperatures as shown in Table 3, and 72 °C for 30 s. At the end of the amplification phase, a melting curve analysis was performed to confirm the specificity of the PCR product. The relative expression of each gene per sample in comparison with *ß. actin* gene was carried out and calculated according to the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001).

Serum profile of immune and antioxidant markers

Serum biochemical analysis was carried out using commercial test kits according to the standard protocols of the suppliers. The following commercial kits were used according to the standard protocol of the suppliers to quantify each of: malondialdehyde (MDA) (Biodiagnostic Egypt, CAT No: MD2529), glutathione peroxidase (GPx) (Biodiagnostic Egypt, CAT No: GP 2524), catalase (CAT) (Biodiagnostic Egypt, CAT No: CA252417); glutathione reduced (GSH) (Biodiagnostic Egypt, CAT No: GR 2511), total antioxidant capacity (TAC) (Biodiagnostic Egypt, CAT No: TA25 13), nitric oxide (NO) (Biodiagnostic Egypt, CAT .No.NO2533), super oxide dismutase (SOD) (Biodiagnostic Egypt, CAT No: SD 25 20) while serum lysozyme activity was determined using turbidimetric assay. IL 1 alpha ELISA Kit (Ray Bio-

Table 3. Oligonucleotide primers sequence, accession number, annealing temperature and PCR product size of immune and antioxidant genes used in real time PCR.

Gene	Primer	Product length (bp)	Annealing Temperature (°C)	Accession number
IL1B	F5′- TGGGACGTTTTAGAGGTGGC-3 R5′- GTCCTCGGGGTTATTCAGCC -3′	108	60	NM_001009465.2
IL2	F5′- GTTGCAAACGGTGCACCTAC-3′ R5′- GAGAGCTTGAGGTTCTCGGG - 3′	122	60	NM_001009806.1
IL6	F5- TGCAGTCCTCAAACGAGTGG-3′ R5′- CCGCAGCTACTTCATCCGAA - 3′	110	58	NM_001009392.1
IL8	F5′- GACCCCAAGGAAAAGTGGGT-3′ R5′- CCACACAGTACTCAAGGCACT - 3′	183	62	NM_001009401.2
RANTES	F5′- GGACGCCTTGAACCTGAACT -3′ R5′- GTGGAATTGTGCCCTCCCAG - 3′	116	58	EF681968.1
NFKB	F5′-GCCTTTGGGGACTTCTCTCC-3′ R5′- GCAGGAACACGGTTACAGGA - 3′	109	58	AF283892.1
MASP2	F5′- TGCCGTGTGGGCTATATTCTC-3′ R5′- TATTGTTAGGTCCGAGGGGGC - 3′	143	60	XM_027975646.2
TNFα	F5′-CTGCTGCACTTCGGGGTAA-3′ R5′-RTGACGTCAGGGTCTTAACCA-3′	94	58	DQ153000.1
SOD3	F5′-GACCAGGTCCAGCAGCAGATGG-3′ R5′- CATCATCTCCTGCCAGATCTC - 3′	90	62	NM_001285675.1
GPX4	F5′-CTGGCCAGCACCATGTGCGCGT-3′ R5′- AGCTGAGTGTAGTTTACGTCAG - 3′	179	62	GU131344.2
Nrf2	F5′- GCAGTTCACTCAGTGCCATC-3′ R5′- TACCTCTCGACTTACCCCGA-3′	249	58	XM_012132956.4
PRDX2	F5′- TTACCCACGCAGCTTTCAGT-3′ R5′- TCGCAGTTCAGCTTGTGGAA - 3′	233	60	XM_012177564.3
ß. actin	F5′- CGTGCTGCTGACGGAGGCCCC-3′ R5′- GCACAGCCTGGATGGCCACATAC -3′	113	60	AF481159.1

- IL1B= Interleukin 1 beta; IL2= Interleukin 2; IL6= Interleukin 6; IL8= Interleukin8; RANTES= regulated on activation, normal T cell expressed and secreted; NFKB= Nuclear factor kappa B; MASP2= Mannan-binding lectin serine protease 2; TNFα= Tumor necrosis factor alpha; SOD3= Superoxide dismutase 3; GPX4= Glutathione peroxidase 4; Nrf2= Nuclear factor-erythroid factor 2-related factor 2and PRDX2= Peroxiredoxin 2.

tech, Inc, CAT No: ELR-IL1a), IL 6 (BOSTER BIOLOGICAL TECHNOLOGY, CAT No: EK0412) and TNF-α ELISA Kit (AVIVA SYSTEM BIOLOGY). Immunoglobulin G (IgG): (Cell Sciences company, CAT No: CKR004A), Immunoglobulin A (IgA): EAGLE BIOSCIENCE company and Immunoglobulin M (IgM): (Genemed Synthesis, CAT. NO EK-6480).

Statistical analysis

H0: Dietary supplementation of *Nannochloropsis* could not modulate gene expression and metabolic profile of immune and antioxidant markers in growing Barki lambs.

HA: Dietary supplementation of *Nannochloropsis* could modulate gene expression and metabolic profile of immune and antioxidant markers in growing Barki lambs.

All data obtained were expressed as mean ± SEM (standard error) and statistically analyzed using SPSS software (SPSS analytical program for windows version 21). Analysis of variance was used to assess the impact of each treatment on each variable in each group. The general linear model was repeatedly measured, and the Mauchly's sphericity test was used to identify significant variations. One-way ANOVA with post hoc Duncan multiple comparison testing was performed to identify the individual variations where there was a significant result. A univariate general linear model (GLM) was used to test interaction effect of gene type and study period (0, 15, and 30) on gene expression results in control and supplemented groups where data represented as mean±SD. Pearson correlation was performed to assess the correlation between biochemical parameters and gene expression of tested markers. Correlation coefficient (r) and p value were considered. A difference was considered significant at $p < 0.05$.

RESULTS

Clinical examination

Clinically, all vital signs of investigated lambs were within the normal reference range (Jackson et al., 2002), and the animals remained healthy and showed no detectable clinical abnormality throughout the study period. No evidence of gastrointestinal abnormalities was also documented.

Gene expression pattern of immune and antioxidant markers

Supplementation of growing lambs with *Nannochloropsis* could modulate gene expression profile of immune and antioxidant markers (Figures 1 and 2). Levels of immune (*IL1B*, *IL2*, *Il6*, *IL8, RANTES*, *NFKB*, *MASP2* and *TNFα*) and antioxidant (*SOD3*, *GPX4*, *PRDX2*, and *Nrf2*) genes expression were significantly up-regulated in lambs supplemented with *Nannochloropsis* at day 30 than control and day 15.

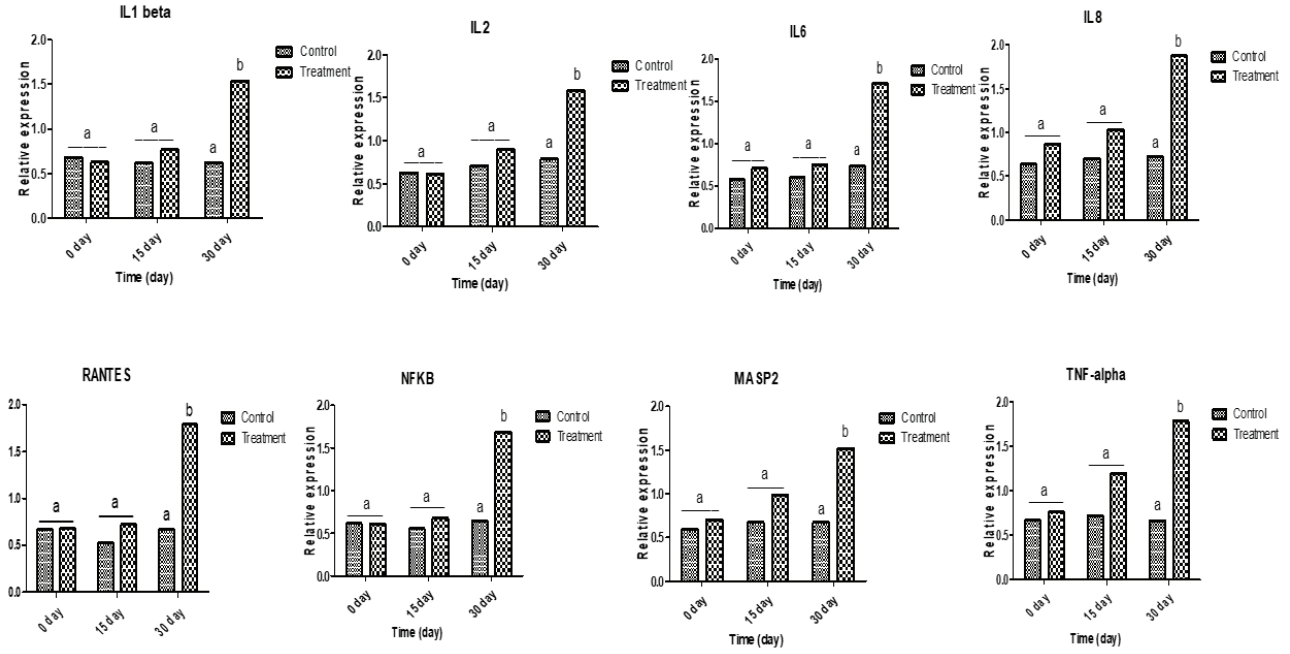

Figure 1. Relative expression patterns of immune genes in control and growing Barki lambs supplemented with *Nannochloropsis* at 0, 15 and 30 days. Results are expressed as means ± SEM. Small alphabetical letters show significance when P<0.05.

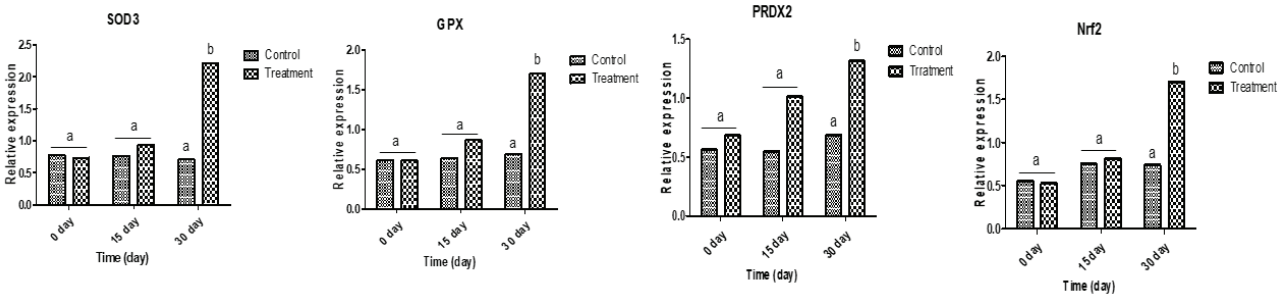

Figure 2. Relative expression patterns of antioxidant genes in control and growing Barki lambs supplemented with *Nannochloropsis* at 0, 15 and 30 days. Results are expressed as means ± SEM. Small alphabetical letters show significance when P<0.05.

There was a significant interaction between type of gene and the period of feeding for control and lambs supplemented with *Nannochloropsis* on mRNA levels of immune and antioxidant markers. In lambs fed the basal diet, *SOD3* is the most up regulated gene at 0 day (0.78 ± 0.10) and day 15 (0.76 ± 0.12); while *IL2* is the most up regulated gene at day 30 (0.78 ± 0.12). The mRNA levels of *Nrf2* (0.55± 0.09), *RANTES* (0.52 ± 0.09) and *ACACA* (0.57± 0.24) were the most down regulated at 0, 15 and 30 days respectively in control group. In the same respect for lambs supplemented with *Nannochloropsis*, the gene expression profiles of *IL8* (0.86 ± 0.08), *TNFα* (1.19 ± 0.24) and *SOD3* (2.21± 0.29) were the most up regulated among the investigated markers at 0, 15 and 30 days respectively; while *Nrf2* (0.52± 0.27), *NFKB* (0.67 ± 0.05) and *PRDX2* (1.31 ± 0.33) were the most down regulated genes.

Serum profile of immune and antioxidant markers

Serum concentrations of cytokines and oxidative stress markers in control and supplemented groups are summarized in Table 4. There was a significant ($p < 0.05$) increase in the serum concentrations of SOD, CAT, TAC, GPx, NO, GSH, lysozyme activity, TNF-α, IL1, IL-6 and IgA with significant decrease in MDA level in supplemented group in relation to control ones. While IgG and IgM levels were not significantly ($P > 0.05$) differed among the tested groups.

Correlation between gene expression pattern and serum profile of immune ad antioxidant markers in supplemented Barki lambs

AT 0 day in the supplemented group, the serum levels of catalase were positively correlated with mRNA levels of *NFKB* (r= 1 and p=0.01), serum levels of GSH were negatively correlated with mRNA levels of *ACACA* (r= -0.999 and p=0.03), serum levels of

Table 4. Effect of ***Nannochloropsis*** powder on antioxidant and immune markers of growing Barki lambs

parameters	1st sampling (0 day)		2nd sampling (15 day)		3rd sampling (30 day)	
	Control	Treatment	Control	Treatment	Control	Treatment
lysozyme (u/ml)	4.1 ± 1	5.4 ± 0.36	4.7 ± 0.4	5.7 ± 0.3	5.5 ± 0.6	8.1* ± 0.5
GSH (mg/dl)	38 ± 0.5	44.3 ± 2.6	38.6 ±0.8	47* ± 1.5	39.6 ± 0.8	54.3* ± 2.6
GPx (U/mL)	45 ± 1	52 ± 3.5	44.5 ±0.5	54.2 ± 3	45 ± 1	60* ± 3.4
MDA (nmol / mL)	10.7 ±0.1	9.6 ± 0.4	10.2 ±0.3	9.2± 0.1	10.2 ± 0.6	7* ± 0.1
Catalase (U/L)	32.3 ±2.9	37 ± 2.6	36.3 ±3.1	40.6 ± 2.1	38.3 ± 1.4	53.3* ± 1.7
NO (µmol / L)	4.9 ± 0.2	4.7 ± 0.1	4.9 ± 0.1	4.9 ± 0.4	4.8 ± 0.2	5.8* ± 0.1
SOD (U/ml)	44 ± 0.5	48 ± 2	46 ± 2	51.3 ± 2	44.6 ± 2.8	73.3* ± 2.9
TAC (mM /L)	40.3 ±2.4	42 ± 1.7	37.6 ±1.4	44.6 ± 2.8	37.6 ± 2.6	59.3* ± 4.8
IL 1 (pg/ml)	6± 0.2	5.4 ± 0.03	5.8 ±0.7	6.1 ± 0.2	5.2 ± 0.6	7.8* ± 0.4
IL 6 (pg/ml)	5.8 ± 0.4	5.2 ± 0.1	6 ±0.3	6.1 ± 0.3	6 ± 0.1	7.4* ± 0.3
TNFα (pg/mL)	20 ± 1.1	17.3 ± 1.4	23 ±1.5	19.5 ± 0.2	21 ± 2	28.6* ±0.8
IgG (ng/ml)	41.3 ±2.4	1.2 ± 0.2	43± 2	41.6 ±1.2	40.3 ± 0.8	39.6. ± 3.1
IgA (ng/ml)	1.2 ± 0.1	1.2 ± 0.2	1.2± 0.1	1.4 ±0.08	1 ± 0.05	2.6* ± 0.1
IgM (ug/ml)	4.9 ±0.05	5 ± 0.1	5.3± 0.1	5.3 ±0.08	5 ± 0.08	5.1 ± 0.2

*Values with an asterisk within the same raw are statistically significant ($p < 0.05$). GSH: Glutathione reduced; GPx: Glutathione peroxidase; MDA: Malondialdhyde; NO: Nitric oxide: SOD: Super oxide dismutase: TAC: Total antioxidant capacity: IL1: Interlukin 1; IL 6: Interlukin 6; TNF-α: Tumer necrosis factor alpha; IgG: Immunoglobulin G; IgA: Immunoglobulin A: IgM: Immunoglobulin M.

MDA were negatively correlated with mRNA levels of *IL6* (r= -1 and p= 0.001), serum levels of NO were negatively correlated with mRNA levels of *SOD3* (r= -0.997 and p=0.04), serum levels of SOD were positively correlated with mRNA levels of *IL6* and *MASP2* (r= 0.999 and p=0.03, r=0.999 and p=0.03, respectively), serum levels of lyzosyme were negatively correlated with mRNA levels of *RANTES* and *ACACA* (r= -0.999 and p=0.02, r= -0.998 and p=0.04, respectively) and positively correlated with mRNA levels of *PRDX2* (r=0.997 and p=0.04), serum levels of IgM were positively correlated with mRNA levels of *IL1β* and *IL2* (r= 0.999 and p=0.03, r=0.998 and p=0.03, respectively) and serum levels of IgG were positively correlated with mRNA levels of *NFKB* and *GPX* (r= 0.999 and p=0.02, r=0.998 and p=0.04, respectively).

AT 15th day in the supplemented group, the serum levels of catalase were positively correlated with mRNA levels of *NFKB* and *SCD* (r= 1 and p=0.04, r=0.997 and p=0.045, respectively), serum levels of GPX were positively correlated with mRNA levels of *SCD* and *FABP4* (r= 1 and p=0.006, r=0.999 and p=0.02, respectively) and serum levels of lyzosyme were positively correlated with mRNA levels of *SCD* (r= 0.999 and p=0.02).

AT 30th day in the supplemented group, the serum levels of catalase were negatively correlated with mRNA levels of *SCD* (r= -1 and p=0.001), serum levels of GPX were negatively correlated with mRNA levels of *NFKB* and *Nrf2* (r= -0.998 and p=0.03, r= -0.998 and p=0.04, respectively), serum levels of NO were positively correlated with mRNA levels of *IL6* (r= 0.999 and p=0.033), serum levels of SOD were positively correlated with mRNA levels of *PRDX2* (r= -0.999 and p=0.023), serum levels of TAC were negatively correlated with mRNA levels of *IL2* (r= -0.999 and p=0.0.21) and serum levels of TNFα were negatively correlated with mRNA levels of IL8, *GPX* and *PRDX2* (r= -0.998 and p=0.035, r= -1 and p=0.011, r= -0.999 and p= 0.03, respectively).

DISCUSSION

In the present study, real time PCR was carried out to quantify mRNA level of immune (*IL1B*, *IL2*, *Il6*, *IL8*, *RANTES*, *NFKB*, *MASP2* and TNFα) and antioxidant (*SOD3*, *GPX4*, *PRDX2*, and *Nrf2*) genes in growing Barki lambs supplemented with *Nannochloropsis*microalgae. Our findings revealed that supplementation of lambs with *Nannochloropsis* for successive 30 days significantly up-regulated the expression pattern of immune and antioxidant markers at 30th day than 0 and 15th days. To the best of our knowledge, no prior studies have looked at the impact of microalgae supplementation on the gene expression profile of immunological and antioxidant markers in growing lambs. As a result, we are the first to investigate how adding *Nannochloropsis* microalgae to growing Barki lambs affects the gene expression profile of immunological and antioxidant indicators. However, the cyanobacteria *Spirulina platensis* and *Chlorella vulgaris*, when given orally to pigs around weaning, increased the expression levels of *IL-8* and *IL-1*, respectively, in the ileum without altering *TNF-*, *IL-1*, *IL-1*, *IL-10*, or *TGF-β*, according to research by (Furbeyre et al., 2018)**.** Additionally, oral administration of *Tetraselmis chuii* increased the expression of a number of immune system-related genes, including IgM, T-cell receptor beta (TCR-beta), and genes of the major histocompatibility complex (Cerezuela et al., 2012).

In inflammatory circumstances, serum cytokines like (IL1B, Il2, IL6, IL8, TNF-α , and NFKB) serve as indirect markers (Salim et al., 2016)**.** TNF-α is a crucial pro-inflammatory cytokine in the immune response. B lymphocytes, T lymphocytes, NK (natural killer) lymphocytes, and LAK (lymphokine-activated killer) cells are just a few of the immune system cells that TNF-α activates, along with other substances (Benedict et al., 2003). TNF-α also triggers the release of a wide variety of other cytokines (Bradley, 2008). On chromosome BTA23q22, the gene that codes for TNF-α has four exons and three introns (Lester et al., 1996). Although it is expressed by a wide variety of mammalian cells, macrophages and monocytes do so most effectively. Lipopolysaccharide (LPS), which is present in the bacterial cell wall, stimulates the production of TNF-α in these phagocytic cells. According to (Bannerman, 2009), In LPS-stimulated macrophages, TNF-α gene expression triples, mRNA levels increase by approximately 100 times, and the protein itself may be released at a rate up to 10,000 times higher.

According to research by Fremond et al., 2004, NFKB activation and cytokine production aid in bacterial identification. Many cells, including blood lymphocytes, express the chemokine regulated on activation normal T-cell expressed and secreted (RANTES), also known as CCL5 chemokine, in response to inflammatory signals (Oliva et al., 1998)**.** According to

Taub et al., 1995, it controls the activation and movement of both inflammatory and non-inflammatory cells. It also has a role in the acute phase response (Tavares &Miñano, 2004). The main protease in the complement system is mannose-binding lectin-associated serine protease 2 (MASP2) (Fu et al., 2016). According to (Ytting et al., 2011), polymorphisms in the *MASP2* gene are strongly associated with autoimmune diseases. MASP2 serum levels and gene polymorphisms have been linked to a number of inflammatory diseases and infections (Wu et al., 2015). Additionally, the *MASP2* gene has been linked to rheumatoid arthritis, TB susceptibility, and an elevated risk of ischemic stroke (Tsakanova et al., 2018). The polymorphisms of the *MASP2* gene and their association to mastitis and milk production in Chinese Holstein cattle were described by Zhang et al., 2019, despite the fact that the function of *MASP2* and its polymorphisms in ruminants is yet unknown.

Free radicals are combated by antioxidants through scavenging, detoxifying, sequestering the transition metals that create them, or inhibiting their production (Masella et al., 2005). These mechanisms include endogenous antioxidant defenses made by the body, such as SOD, CAT, and glutathione peroxidase (GPx), as well as non-enzymatic antioxidant defenses (Glasauer & Chandel, 2014). The peroxiredoxin (PRDX) family of antioxidant enzyme oxido-reductase proteins functions as a catalyst for hydrogen peroxide (H2O2) because of a conserved ionized thiol. Thiol-specific peroxidase functions as a sensor of hydrogen peroxide-mediated signaling events and contributes to cellular defense against oxidative stress by detoxifying peroxides and sulfate-containing radicals (Ateya et al., 2021). The Nrf2 stress response system is the primary inducible defense against oxidative stress and regulates the expression of cytoprotective genes (Yamamoto et al., 2018).

Growing lambs supplemented with *Nannochloropsis* microalgae showed a marked up-regulation in the expression pattern of immune and antioxidant markers. This may be explained by the fact that polysaccharides from microalgae have been shown to activate immune responses from immune cells through the recognition of toll-like receptors (Balachandran et al., 2006). It's important to note that microalgae have strong antioxidant properties. (Belay et al., 1996). Previous research has shown that supplementing small ruminants with microalgae increases their antioxidant activity (EL-Sabagh et al., 2014). Furthermore, it was claimed that microalgae had stronger immunological and antioxidant qualities since they include phenolic substances such beta-carotene zeaxanthin, α-tocopherol, and phycocyanins. Additionally, it was claimed by Yadav and Kumar (2018) that *spirulina* boosts the immune response, particularly the initial response, by promoting the activities of macrophages, phagocytosis, and IL-1 production. Growing Barki lambs supplemented with *Nannochloropsis* allowed us to identify the mechanism underlying the up-regulation of the expression profile of immunological (*IL1B, IL2, Il6, IL8, RANTES, NFKB, MASP2*, and *TNFα*) and antioxidant (*SOD3, GPX4, PRDX2*, and *Nrf2*) genes.

The inclusion of *Nannochloropsis* in growing Barki lamb's diet resulted a significant increase in serum level of SOD, CAT, TAC, GPx, NO, GSH and lysozyme with significant decrease in MDA level. The first line of defense for the cellular antioxidant system is made up of GPx and SOD (Ighodaro & Akinloye, 2018), and MDA levels show how much lipid peroxidation is occurring in a living cell (Mesalam et al., 2021). CAT is involved in the second step, which involves removing the peroxides and converting them into O2 (Yu, 1994).

The ability of mitochondria to produce intracellular reactive oxygen species is well recognized (Vakifahmetoglu-Norberg et al., 2017). Oxidative stress can result from an increase in ROS levels caused by mitochondrial ROS generation that exceeds cellular antioxidant capability (Liemburg-Apers et al., 2015). The ability of *Nannochloropsis* to reduce oxidative stress by reducing oxidant indicators like MDA and carbonyl proteins may be responsible for the improvement in the antioxidant defense mechanism of lamb given the *Nannochloropsis* diet (Bendimerad, 2019). In order to protect its antioxidant components like carotenoids, fucoxanthin, astaxanthin, and vitamins, it also has the capacity to retrieve free radicals and suppress lipid peroxidation (Yaakob et al., 2014). Our results were in agreement with previous reports in human (Panahi et al., 2013)**,** in goats (Yadav &Kumar, 2018), calves (Ghattas et al., 2019), fattening lambs (EL-Sabagh et al., 2014), rats (Nacer, 2020), in rabbits (Abd El-Hamid et al., 2022) and in broilers (Abdel-Moneim et al., 2022, Elbaz et al., 2022). In contrast to the findings of the present investigation, other authors (Sucu et al., 2016) found no statistically significant change in the serum levels of MDA and GPx between lambs supplemented with microalgae and the control group.

Proinflammatory cytokines like TNF-α, IL-1, and

IL-8 as well as anti-inflammatory cytokines like IL-10 all play a significant part in the inflammatory cascade (Al-Batshan et al., 2001). Proinflammatory cytokines initiate an effect against external pathogens, but anti-inflammatory cytokines are essential for reducing the heightened inflammatory response and preserving homeostasis for the proper operation of key organs. Anti-inflammatory properties were previously found for various marine diatoms, such as *Porosira glacialis*, *Attheya longicornis* (Ingebrigtsen et al., 2016), *Cylindrotheca closterium*, *Odontella mobiliensis*, *Pseudonitzschia pseudodelicatissima (Lauritano et al., 2016)* and *Phaeodactylum tricornutum* (Samarakoon et al., 2013), for the dinoflagellate *Amphidinium carterae* (Samarakoon et al., 2013) and the green algae *Dunaliella bardawil* and *Dunaliella tertiolecta* (Lavy et al., 2003). TNFα was examined by Ingebrigtsen et al. (2016) and Lauritano et al., 2016 in lipopolysaccharide (LPS)-stimulated monocytic leukaemia cells (THP-1) as one of the primary inflammatory effectors. Nitric oxide (NO), one of the inflammatory mediators was examined on LPS-induced RAW macrophages as a positive indicator of anti-inflammatory activity (Samarakoon et al., 2013).

The TNF-α, IL1, IL-6 and IgA level showed a significant ($P<0.05$) increase in supplemented group as compared with the control one. IgG and IgM levels were not significantly ($P > 0.05$) differed among the tested groups. These changes could be attributed to the microalgal-derived compounds that have shown immune-stimulatory activity, such as polysaccharides which were rich in (β1→3, β1→4)-glucans, (α1→3)-, (α1→4)-mannans, and anionic sulphated heterorhamnans, sulfated lipids, polyunsaturated fatty acids, and astaxanthin. All these compounds are able to activate macrophage cells (Chen et al., 2019), T-cell (Chuang et al., 2014), or dendritic cells (Manzo et al., 2019). Interleukin-4 (IL-4), interferon- (IFN-γ), interleukin-2 (IL-2) and NK cell production are all increased, which has immunological modifying effects (Barry et al., 2014). They are also the most effective molecules that function as adjuvants (Manzo et al., 2019); they can promote maturation; and they can trigger certain immune responses (Carolina, 2019). Our finding was similar to that obtained by Ghattas et al. (2019); but away from those obtained in fish (Amer, 2016), in rabbits (Abd El-Hamid et al., 2022) and in broiler chickens (Elbaz et al., 2022). The level of IgG and IgM was found to be significantly higher in the *Spirulina platensis* supplemented groups compared to the control group. Calves given supplemental milk replacer containing algae high in docosahexaenoic acid showed non-significant increases in blood IgA levels, according to Flaga et al., 2019.

This study is unique in that it used two methods to assess the health status of developing lambs. The serum biochemical profiles and gene expression of immunological and antioxidant indicators were used in the investigation. It's interesting to notice that there were significant positive and negative associations between the serum profiles and gene expression of the studied indicators. Supplementation of lambs with *Nannochloropsis* for successive 15 days revealed that the serum levels of catalase were positively correlated with mRNA levels of *NFKB* and *SCD*, serum levels of GPX were positively correlated with mRNA levels of *SCD* and *FABP4* and serum levels of lyzosyme were positively correlated with mRNA levels of *SCD*. In the same respect, at 30 day, the serum levels of catalase were negatively correlated with mRNA levels of *SCD*, serum levels of GPX were negatively correlated with mRNA levels of *NFKB* and *Nrf2*, serum levels of NO were positively correlated with mRNA levels of *IL6*, serum levels of SOD were positively correlated with mRNA levels of *PRDX2*, serum levels of TAC were negatively correlated with mRNA levels of *IL2* and serum levels of TNFα were negatively correlated with mRNA levels of *IL8*, *GPX* and *PRDX2*.

CONCLUSION

In growing Barki lambs, dietary supplements of *Nannochloropsis* may alter the serum profile and gene expression of immunological and antioxidant indicators. This polymorphism may be utilized as a biomarker to monitor the immune status of these growing lambs and to develop a successful management strategy that enhances health via prudent breeding practices, dietary decisions, and vaccination schedules. To more reliably and accurately assess the nutritional content of *Nannochloropsis*, new studies with larger replications and alternative feeding strategies are suggested.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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