Received: 31 October 2023, Accepted: 13 November 2023

Original Research

Journal of Advanced Veterinary Research
(2023) Volume 13, Issue 9, 1871-1880

Effect of Dietary Supplementation of *Nannochloropsis* on Gene Expression and Serum Profile of Metabolic and Lipogenic Markers in Growing Barki Lambs

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Abstract

The objective of this study was to investigate the effects of dietary supplementation of *Nannochloropsis* on gene expression and serum metabolic profile in growing Barki lambs. For this reason, twenty apparently healthy growing Barki lambs were allocated into two equal groups (10 lambs each). Control group received the basal diet whereas the supplemented group received the basal diet with *Nannochloropsis* powder which given in the concentrate at a rate of 10 g /kg/day for subsequent 30 days. A blood sample was collected from each lamb via jugular venipuncture before starting the experiment (T0), (T15) and (T30) after supplementation for hematobiochemical examination and real time PCR. Gene's expression of ACACA, FASN, SCD and FABP4 were significantly up-regulated in supplemented lambs at (T15) and (T30) while DGAT1, insulin, glucokinase and glucose transporter showed a significant up-regulation at day 30 compared to control ones. Supplemented lambs had a significant ($P < 0.05$) increase of final body weights, daily weight gain and feed intake with significant ($P<0.05$) decrease of feed conversion ratio. From the third week onwards, the body weight, body length and body condition score were significantly ($P > 0.05$) higher in the supplemented group. There was a significant ($P<0.05$) increase in Hb, RBC, WBC, serum level of glucose, insulin, triglyceride, total protein, globulin and BUN with significant ($p<0.05$) decrease of cholesterol, ALT and AST in supplemented lambs at T30. The results suggest that *Nannochloropsis* could be used as a useful supplement to improve health status and body growth of growing Barki lambs.

KEYWORDS
Nannochloropsis, Gene expression, Growth performance, Metabolic variables, Barki lambs

INTRODUCTION

There is an increasing interest in the use of microalgae as a feed or feed supplement for ruminants (Gomaa *et al*., 2018; Lamminen *et al*., 2019). Dietary supplementation of small amounts of microalgae had positive effects on animal physiology, productivity and feed conversion by improving gut and immune functions (Camacho, 2019).

Microalgae are a diverse group of autotrophic an photosynthetic microorganisms with various unique biological characteristics, including high photosynthetic energy transfer efficiency, have the ability to synthesize biologically complex components such as lipids, proteins, carbohydrates, pigments, and polymers (De Morais, 2015), have a high content of bioactive compounds, including protein, polysaccharides (Mohamed, 2008), and vitamins such as vitamins A, C, E, K, thiamine (B1), pyridoxine (B6), riboflavin (B2), nicotinic acid, biotin, and tocopherol (Chew *et al*., 2017; Khan MI, 2018), excellent adaptability to various environments and capability of producing a broad variety of bioenergy (Levering *et al*., 2015). Thus, the inclusion of microalgae in ruminant's diets has been shown as an effective nutritional strategy to enrich cow's (Glover *et al*., 2012) and goat's (Kouřimská *et al*., 2014) milk with PUFA. The products of microalgae have various applications, particularly in pharmaceuticals, cosmetics, biofuel production, and aquaculture (Borowitzka, 2013). For example, the members of *Nannochloropsis* genus are marine Eustigmatophyceae microalgae with advantages such as fast growth and easy cultivation, capable of storing large triacylglycerols (TAGs) under particular culture conditions and are therefore an environmentally friendly biodiesel feedstock with great developmental potential (Kilian *et al*., 2011).

Nannochloropsis is a unicellular microalga species with a polysaccharide cell wall structure that contains one chloroplast, this genus contains 6 different species namely as *N. gaditana*, *N. salina, N. limnetica, N. granulata, N. oceanica,* and *N. oculata* (Hibberd, 1981). Numerous studies (*in-vitro* and *in-vivo*) have verified the positive role of *N. oculata* on palatability, lack of toxicity (Gbadamosi and Lupatsch, 2018), easy digestion (Kholif *et al*., 2020), antioxidant actions (Elsheikh *et al*., 2018), immunity (Bule *et al*., 2018), anti-inflammatory and anti-cancer (Md *et al*., 2018) on several animal, additionally to possibility to use as a substitute source of the conventional protein on animals diet, they also constitute a good alternative source of Eicosapentaenoic acid (EPA, C 20:5 n3) (Becker, 2007).

The studies of hematobiochemical profile provide ample clue about nutritional and health status and well-being of an animal so these can be used for evaluating the health condition of animal generally (Faraz A, 2019). Observation of a deviation of certain blood parameters from their normal limits could be a guide for differential diagnosis of diseases and may give an assessment

ISSN: 2090-6277/2090-6269/

of the degree of damage of host tissues as well as severity of infection (Van Saun, 2000).

Variations in gene expression of several regulatory enzymes of the intermediary metabolism can provide useful tools to improve genetic selection towards adaptation of livestock to harsh environments (Van Harten *et al*., 2013). Metabolic regulation relies partly on transcriptional control of gene networks, collection of DNA segments, which interacts with a transcription factor or nuclear receptor as a mechanism controlling the concentration of key enzymes in cells. These "global" interactions can govern the rates at which genes in the network are transcribed into mRNA. The study of the entire genome, sub-networks, or candidate genes at the mRNA level encompasses the broad field of genomics (Loor, 2010).

To the best of the author's knowledge, the relationships between dietary supplementation of *Nannochloropsis* and body growth, growth performance, metabolic profile and genetic polymorphism of lipid genes are lacking in growing Barki lambs. Therefore, the aim of the present study was to evaluate the effects of dietary supplementation of *Nannochloropsis* on gene expression of lipogenic (FABP4 DGAT1, ACACA, FASN and SCD) and metabolic (insulin, Glucokinase and Glucose transporter) in growing Barki lambs. Another aim was to link the gene expression with body growth, growth performance, and metabolic profile.

MATERIALS AND METHODS

Ethical approval

All procedures were performed in accordance with the guidelines of animal and poultry health department, Desert Research Center, ministry of agriculture and land reclamation, Egypt and approved by its Ethical Committees.

Animals and experimental design

The study included twenty 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 (10 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 that 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 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 RR, 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 SY, 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 NRC (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.

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	1862.7
Calcium	229
Potassium	798
Magnesium	173
Quantitative constituents of Amino acids profile (mg/g) in microalgae *Nannochloropsis oculata*	
Methionine	69.52
Cystine	17.3
Phenylalanine	16.24
Lysine	15.2
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

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. A particular concern was given to the following vital signs: rectal temperature, heart rate, respiratory rate, and visible mucous membrane color. Thorax and abdomen were examined thoroughly. Body weight was also checked using the standard scale.

Table 2. Composition of the concentrate feed mixture (CFM).

Ingredients	Quantity
Corn	560 kg
Wheat bran	210 kg
Soya bean	120 kg
Cotton seed	80 kg
Sodium chloride	10 kg
Calcium carbonate	15 kg
Pure mineral plus	5 kg
Pure yeast	2 kg

Body Conformation Measurements

At weekly intervals, each lamb was individually assessed for chest girth (CG), withers height (WH), body length (BL), live weight (BWT) measurements and BCS. CG is the body circumference measured at just behind the forelegs (Afolayan *et al*., 2006). WH was the distance between the highest peak over the scapulae and the ground (Sowande and Sobola, 2008). BL refers to the span between the base of the neck, the vertebrae between the scapulae, to the far point of the pubic bone (Sowande and Sobola, 2008). BCS was subjectively measured (Phythian *et al*., 2012), always by the same researcher, gauging fat depth on a 0-5 point scale as described by (McLeod *et al*., 2010). BWT was monitored using an electronic balance (Shanghai Yaohua Weighing System Co., Ltd – Model: XK 3190-A19E). Body conformation measurements in centimeters were taken using the same measuring tape. During assessment it was ensured that lambs were gently restrained in a relaxed state on all four legs with their heads comfortably erect.

Blood sampling

Ten milliliters of blood were collected from each animal via jugular venipuncture before starting the experiment (T0), and at the 15th day (T15) and the 30th day (T30) after supplementation. The collected blood was added to plain tubes (i.e., without anticoagulants) and to others containing EDTA to yield serum or whole blood, respectively. All samples were cooled on crushed ice and were transported immediately to the laboratory for further processing. Serum biochemical analyses using commercial test kits according to the standard protocols of the suppliers were carried out. The following kits were used to quantify serum concentration of total protein, albumin, glucose, cholesterol and blood urea nitrogen (BUN) (Gamma Trade Company, Egypt); triglyceride levels (Spinreact Company, Spain); and AST (aspartate aminotransferase), and ALT (alanine aminotransferase) (Spectrum Company, Egypt) on a selective chemistry analyzer (Apple 302, USA); insulin levels (NeoBiolab, Cat. no. SI0011, Cambridge, USA), Globulin was calculated by subtracting albumin values from total serum protein. Tubes containing whole blood were used for CBC and real time PCR.

RNA extraction and reverse transcription

Total RNA was extracted from whole blood sample using Direct-zol RNA Kits according to the manufacturer's instructions (Direct-zolTM RNA MiniPrep, Zymo-research, USA, catalog No. R2050). The quantity and purity were measured by using a nano-spectrophotometer (UV-Vis spectrophotometer Q5000, Thermofischer, USA) and the integrity was evaluated by gelelectrophoresis. The cDNA of each sample was synthesized following the manufacture protocol (SensiFastTMcDNA synthesis kit, Bioline, catlog No. Bio-65053). The reaction mixture was carried out in a total volume 20 µL consisted of total RNA up to 1 µg, 4 µL 5x TransAmp buffer, 1 µL reverse transcriptase and DNase free water up to 20 µL. The final reaction mixture was placed in a thermal cycler and the following program was carried out; primer annealing at 25°C for 10 minutes, reverse transcription at 42°C for 15 minutes followed by inactivation at 85°C for 5 minutes. The samples were held at 4°C.

Quantitative Real Time PCR

Relative quantification of mRNA level of lipogenic (FABP4, DGAT1, ACACA, FASN and SCD) and metabolic (insulin, Glucokinase and Glucose transporter) markers in growing Barki lambs blood was performed by real-time PCR using SYBR Green PCR Master Mix (2x SensiFastTM SYBR, Bioline, catlog No. Bio-98002). Primer sequences, annealing temperature and the size of each amplified PCR product are shown in Table 3. The house keeping gene GAPDH was used as an internal control. The reaction mixture was carried out in a total volume 20 µL consisted of 10 µL 2x

Table 3. Oligonucleotide primers sequence, annealing temperature and PCR product size of the studied genes.

Gene	Oligonucleotide sequence	Accession number	Annealing temperature (°C)	Size (bp)
ACACA	f5'- ATGTGGCCTGGGTAGATCCT-3' r5'-ACGTAACACAAGGCTGATGGTG-3'	NM_001009256.1	60	261
FASN	f5'- GGAAGGCGGGACTATATGGC-3' r5'- CATGCTGTAGCCTACGAGGG-3'	XM_004013447.1	62	278
SCD	f5'- GGCGTTCCAGAATGACGTTT-3' r5'- TGAAGCACAACAGCAGGACA-3'	NM_001009254.1	58	251
FABP4	f5,- TCCTTCAAATTGGGCCAGGA-3, r5,- TGGTAGCAGTGACACCGTTC-3	NM_001114667.1	60	190
DGAT1	f5,- TGACCTACCGCGATCTCTAC-3, r5,- TGCGGGAGTAGTCCATGTCC-3	NM_001110164.1	62	216
Insulin	f5,- GAGAGCGCGGCTTCTTCTAC-3, r5,- CGGGGCAGGTCTAGTTACAG-3	XM_027959829.2	62	198
Glucokinase	f5,- TCATCACCTGGCCAGACCTA-3, r5,- GAACCACAGACCACTCAGGG-3	NM_001287471.1	60	175
Glucose transporter	f5,- GGTGCCTACTTCAAGCTGACT-3, r5,- AACCAGTTGGTGAGGACGC-3	AF495799.1	60	248
GAPDH	f5'- TGACCCCTTCATTGACCTTC-3' r5'- GATCTCGCTCCTGGAAGAG-3'	NM-001034034	62	143

SensiFast SYBR, 3 µL cDNA, 5.4 µl H_2O (d.d water), 0.8 µl of each primer. The PCR cycling conditions were as follows: denaturation program 94°C for two minutes; amplification and quantification program repeated 40 cycles of denaturation temperature 94°C for 10 seconds, annealing temperature for 30 seconds (Table 3), and extension temperature 72°C for 20 seconds. 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 the gene in each sample versus a control in comparison to GAPDH gene and calculated according to the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001).

Statistical analysis

Statistical analyses were carried out using a statistical software program (SPSS, ver.20, In c., Chicago, USA). Descriptive statistics were performed for all parameters. Repeated measures ANOVA was used to test the effect of *Nannochloropsis* supplementation on body growth, selected hematobiochemical variables and gene expression pattern of lipogenic and metabolic markers of growing Barki lambs. Results were considered statistically significant at $P < 0.05$.

RESULTS

Clinically, all lambs were healthy, and no evidence of disease has occurred. 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.

Supplementation of growing lambs with *Nannochloropsis* could modulate gene expression profile of lipogenic and metabolic markers (Figure 1). Levels of ACACA, FASN, SCD and FABP4 genes expression were significantly up-regulated in lambs supplemented with *Nannochloropsis* at days 15 and 30 than control ones. In the same respect, DGAT1, insulin, glucokinase and glucose transporter expression profile showed a significant up-regulation at day 30 compared to control and day 15.

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 lipogenic and metabolic markers. In lambs fed the basal diet, FABP4 is the most up regulated gene at 0 day and 30 day (0.66±0.16 and 0.77±0.16, respectively) while DGAT1 is the most up regulated gene at day 15 (0.74±0.2). The mRNA levels of SCD (0.52± 0.09), insulin (0.51±0.15) and glucose transporter (0.52± 0.13) were the most down regulated at 0, 15 and 30 days respectively in control group. In the same respect for lambs supplemented with *Nannochloropsis*, SCD was the most up regulated profile among the investigated markers at 0 and 15 day (0.75±0.09 and 1.53±0.15, respectively) while DGAT1 was the most up regulated gene at day 30 (2.26±0.32). Insulin was the most down regulated at 0 and 30 day (0.54±0.07 and 1.8±0.2, respectively), while glucokinase was the most down regulated at 15 day (0.65±0.11) for the supplemented lambs

There was a significant ($P<0.05$) increase of final body weights, daily weight gain and feed intake with significant ($P<0.05$) decrease of feed conversion ratio in supplemented lambs as compared with the control ones (Table 4). The weekly means and standard errors of the growth parameters are mentioned in Table 5. From the third week onwards, the body weight, body length and body condition score were significantly ($P > 0.05$) higher in the supplemented group as compared with the control one (ta-

Table 4. Effect of *Nannochloropsis* powder on growth performance of growing Barki lambs (n=20).

Item	Control	Treatment
Initial weight (kg)	31.5±0.4	32.3±0.2
Final weight (kg)	33.9 ±4.0	37± 0.6*
Daily weight gain (kg/day)	0.080±0.007 (80 g/day)	0.166± 0.01* (166 g/day)
Daily feed intake (kg/day)	0.85±0.01 (850g/day)	0.96± 0.02* (960g/day)
Feed conversion ratio	10.7±0.9	6.1± 0.5*

Table 5. Mean and Standard Errors for weekly body weight (kg), body length (cm), body height (cm), chest girth (cm) and BCS of 20 Barki growing lambs.

Week		Weight	Length	Height at withers	Circumference of chest	Body condition score
0	Control	30.5±1.4	67.0±0.1	69.6±1.1	82.4±1.7	2.9±0.03
	Treatment	31.9±2.2	68.8±1.75	73.0±1.6	85.0±1.5	3.0±0.04
1	Control	30.8±0.4	69.6±1.5	71.0±1.0	83.2±1.6	2.9±0.02
	Treatment	31.1±0.8	71.2±1.2	72.2±1.8	87.8±2.5	3.0±0.03
2	Control	32.0±0.8	68.2±0.3	71.8±0.8	85.6±1.0	3.0±0.02
	Treatment	11.58±0.75	71.2±0.4	73.6±1.0	89.0±1.1	3.1±0.03
3	Control	32.6±0.18	71.4±1.5	71.8±0.8	86.8±0.8	3.0±0.02
	Treatment	35.0±0.9*	76.0±1.1*	73.4±0.9	90.5±0.9	3.2±0.02*
4	Control	34.3±0.3	71.4±0.5	73.1±0.9	90.8±0.7	3.0±0.02
	Treatment	37.0±0.3*	78.2±1.0*	75.0±1.0	92.4±2.1	3.3±0.02*

ble 5). There was a significant (P<0.05) increase in Hb, erythrocyte count and leukocyte count (WBC) at T30 in supplemented lambs (14.2±0.6, 11.5±0.47 and 13.7±0.1, respectively) when compared with the control group (11.3±0.4, 9.8±0.4 and 11±0.2, respectively) (Table 6). At T30 of experiment, there was a significant (p < 0.05) increase in the serum level of glucose, insulin, triglyceride, total protein, globulin and BUN with significant (p < 0.05) decrease in the serum value of cholesterol, ALT and AST in supplemented lambs in relation to control ones (Table 7).

Correlation between gene expression pattern and serum profile in supplemented Barki lambs

At day 15 in the supplemented group, mRNA levels of ACACA were negatively correlated with the serum levels of AST (r= -0.999 and p=0.03), mRNA levels of FASN were negatively correlated with serum levels of total protein (r= -1 and p=0.01) and mRNA levels of SCD were positively correlated with serum levels of cholesterol (r= 0.998 and p=0.04) and negatively correlated

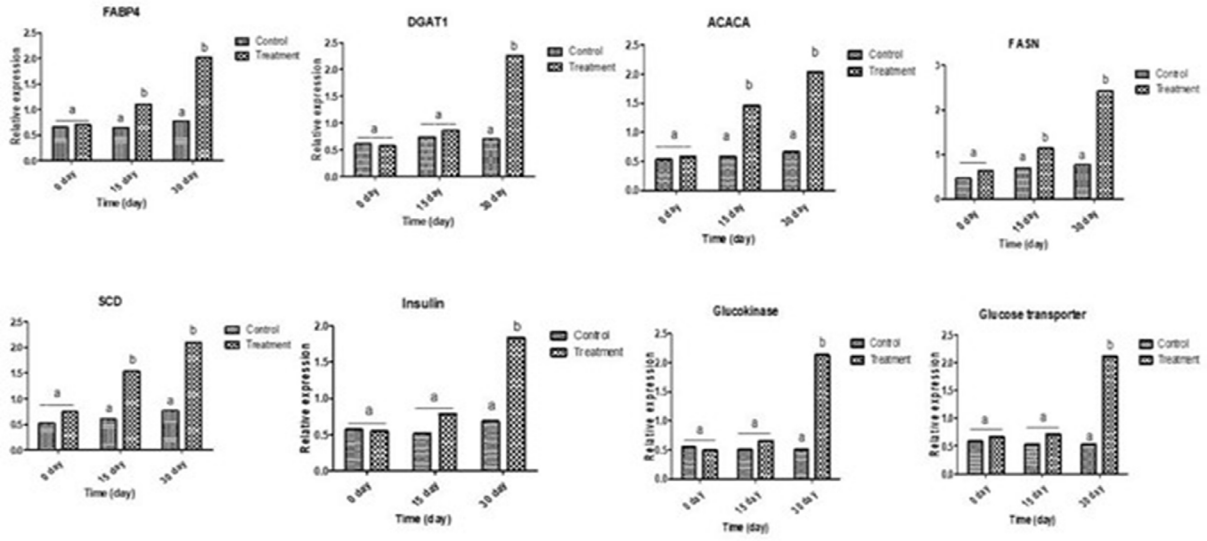

Figure 1. Relative expression patterns of lipogenic and metabolic genes in control and growing Barki lambs supplemented with *Nannochloropsis* at 0, 15 and 30 days. Results are expressed as means±SEM. Small alphabetical letters show significance when (P<0.05)

Table 6. Complete blood counts (mean±SE) in Barki lambs during the growing period (n=20).

Days		WBC (x10^3)	RBC (x10^6)	HB (g/dl)	PCV (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)
1	Control	11.1±0.3	10.8±0.2	10.9±0.3	34.6±1.4	32.1±1.7	10.1±0.11	31.7±2.0
	Treatment	11.3±0.5	11.3±0.6	11.1±0.3	34.0±1.0	32.9±1.3	10.7±0.4	32.7±1.2
15	Control	11.1±0.5	10.8±0.2	10.9±0.3	34.6±1.4	30.12±0.61	10.1±0.1	31.6±2.0
	Treatment	11.3±0.2	10.3±0.6	11.0±0.3	34.0±1.0	32.42±0.55	10.7±0.3	32.6±1.2
30	Control	11.0±0.2	9.8±0.4	11.3±0.4	33.8±0.4	33.3±1.7	10.3±0.1	31.6±0.4
	Treatment	13.7±0.1*	11.5±0.5*	14.2±0.6*	34.6±0.3	33.0±0.05	10.8±0.1	23.3±0.4

WBC: Total leukocytes count; RBC: Erythrocytes count; HB: Hemoglobin; PCV: Packed cell volume; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration.

Table 7. Effect of *Nannochloropsis* powder on biochemical parameters (mean±SE) of growing Barki lambs (n=20).

Parameters	The 1st sampling (0 day)		The 2nd sampling (15 day)		The 3rd sampling (30 day)	
	Control	Treatment	Control	Treatment	Control	Treatment
Glucose (mg/dl)	62.0±3.5	69.0±4.3	62.0±3.7	70.0±3.4	65.0±3.4	87.0±3.7*
Insulin (ng/mL)	1.5±0.2	1.6±0.06	1.5± 0.06	1.7 ±0.05	1.6±0.05	2.1±0.1*
Cholesterol (mg/dl)	59.3±2.3	57.0±7.9	64.6 ±9.8	52.0±5.6	71.0±3.7	39.3±5.3*
Triglyceride (mg/dl)	44.3±6	36.6±1.4	45.0±2.8	48.3±1.7	46.3±3.3	63.6±4.4*
Total protein (g/dl)	4.9±0.2	5.6±0.4	4.9±0.1	5.6± 0.5	4.8±0.2	7.5±0.4*
Albumen (g/dl)	2.5±0.4	3.1±0.1	2.4±0.1	2.6±0.1	2.7±0.1	3.2±0.2
Globulin (g/dl)	2.6±0.6	3.1±0.2	3.0±0.08	3.6±0.3	2.9±0.2	4.8±0.2*
BUN (mg/dl)	44.0±0.5	3.8±3.0	44.0±1.1	42.3±1.4	47.0±2.5	59.6±3.5*
AST (U/L)	76.3±3.0	74.6±3.9	85.6 ±6.1	70.6±2.7	74.6±3.9	50.0±0.5*
ALT (U/L)	41.0±1.5	35.0±2.6	38.3 ±1.2	33.6±2.9	37.3±1.8	29.3±2*

BUN: blood urea nitrogen; AST: aspartate aminotransferase; ALT: alanine transaminase.

serum levels of globulin (r= -0.996 and p=0.03). At day 30 in the supplemented group, mRNA levels of ACACA were positively correlated with serum levels of insulin (r= 1 and p=0.001), mRNA levels of FASN were negatively correlated with serum levels of ALT (r= -0.999 and p=0.04), mRNA levels of SCD were positively correlated with serum levels of triglyceride (r= 0.999 and p=0.02), mRNA levels of DGAT1 were negatively correlated with serum levels of total protein (r= -0.999 and p=0.02) and mRNA levels of insulin were negatively correlated with serum levels of BUN (r= -0.999 and p=0.02).

DISCUSSION

The feeding strategy can modify fatty acid profile through altering the lipid metabolism related gene expression (Dervishi *et al*., 2011). To the best of our knowledge, there were no previous studies reported the effect of microalgae supplementation on the gene expression profile of lipogenic and metabolic markers in growing lambs. Therefore, our study is the first to explore the alterations in gene expression profile of lipogenic and metabolic markers as a result of supplementation of growing Barki lambs with *Nannochloropsis* microalgae.

In the present study, real time PCR was carried out to quantify mRNA level of lipogenic (FABP4 DGAT1, ACACA, FASN and SCD) and metabolic (insulin, Glucokinase and Glucose transporter) 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 ACACA, FASN, SCD and FABP4 at day 15 and day 30 day than control ones. Additionally, DGAT1, insulin, glucokinase and glucose transporter were significantly up-regulated at day 30 compared to control and day 15.

Fatty acid binding protein 4 (FABP4), also known as adipocyte FABP (A-FABP), is a protein found in abundance in the mammary gland, and also in mature adipocytes and adipose tissue (Hunt *et al*., 1986). The gene for FABP4 (FABP4) is expressed during lactation (Bionaz and Loor, 2008) and the main function of this protein is thought to be in lipid metabolism, where it binds both long-chain fatty acids and retinoic acid and delivers them to receptors in the nucleus of adipocytes (Spiegelman and Green, 1980). Triglyceride synthesis is catalyzed by DGAT1. This enzyme plays a major role in intestinal fat absorption, lipoprotein synthesis, the development of adipose tissue and lactation in higher eukaryotes (Cases *et al*., 1998). The bovine DGAT1 gene, which is located on the centromeric end of BTA14, has been reported as a candidate gene for QTLs associated with fat content and milk yield (Spelman *et al*., 2002) .

Acetyl-CoA carboxylase alpha (ACACA), fatty acid synthase (FASN), and stearoyl-CoA desaturase (SCD) are considered key lipogenic enzymes, and therefore, research has been directed toward the identification of factors regulating the activity of these enzymes in ruminant species (Bernard *et al*., 2009). ACACA, FASN and SCD have been studied in Barki ewes (El-Sayed *et al*., 2019), lactating sheep (Ticiani *et al*., 2016) and in different sheep breeds (Izadi *et al*., 2016). The acetyl-CoA carboxylase-α is a key regulated enzyme in de novo fatty acid synthesis, which is decreased by feeding trans-10, cis-12 conjugated linoleic acid. In the ovine, the acetyl-CoA carboxylase-α gene is expressed from three main tissue specific promoters (PI, PII and PIII) (Ticiani *et al*., 2016). FASN is a part of multi-functional enzyme complex that catalyzes the synthesis of saturated fatty acids, including myristate, palmitate, and stearate (Pewan *et al*., 2020). It plays an important role in de novo lipogenesis in mammals, and is a key enzyme in the conversion of acetyl-CoA and malonyl-CoA to triglycerol (Kompan and Komprej, 2012; Pewan *et al*., 2020). The SCD gene encodes for delta-9 desaturase enzyme. It is an iron-containing endoplasmic reticulum enzyme (Gu *et al*., 2019) that catalyzes a rate-limiting step in the conversion of SFA (saturated fatty acid) into MUFA (mono unsaturated fatty acid) in mammalian adipose cells (Pewan *et al*., 2020) and particularly effective in the formation of milk fatty acid composition (Kompan and Komprej, 2012).

In the current study, the marked up-regulation in the lipogenic genes indicating that *Nannochloropsis* microalgae supplementation enhances the growth of growing lambs. This finding might be due to that the microalgae are rich in all essential amino acids, vitamins including vitamin A, minerals, carotenoids and fatty acids, especially gamma-linolenic acid which has several health benefits (Howe *et al*., 2006). Furthermore, microalgae has previously been shown to decrease rumen protein degradation and produce changes in bacterial community composition with a subsequent increase the efficiency of rumen microbial crude protein production (Panjaitan *et al*., 2010). In the same respect, the effects of microalgae on ruminal VFAs (volatile fatty acids), which led to changes in ruminal fermentation, enhanced available energy for growth, and thus improved production (Boeckaert *et al*., 2007). Also, microalgae could improve the growth, of the animal through improving feed intake, feed conversion, nutrient absorption and utilization, body weight gain (El-Desoky *et al*., 2013; Evans *et al*., 2015).

Glucose homeostasis requires hormone and neural mechanisms to provide a continuous glucose supply to the central nervous system (CNS) and to face the metabolic needs of peripheral tissues. Glucose is required as an energy substrate, but also functions as a signaling molecule in primary processes. Indeed, alterations of normoglycaemic levels have deleterious consequences that increase morbidity and mortality rates. Glucose sensors, molecular systems that accurately detect glucose concentrations in the extracellular space, contribute to maintaining glucose homeostasis by controlling several key processes. The first glucose sensor was described in the pancreatic β-cell (Matschinsky, 1990) and is constituted by glucose transporter (GLUT-2) and glucokinase (GK). The marked up-regulation in insulin, Glucokinase and Glucose transporter may be attributed to the fact that microalgae contain higher content of polysaccharides (Balachandran *et al*., 2006).

A way from our results, (Urrutia *et al*., 2016) showed that feeding of lambs with linseed with algae decreased the expression of SCD in subcutaneous (SC) adipose tissue (AT) ($P < 0.01$) and in the intramuscular adipose tissue (AT), the expression of SCD was down regulated in both linseed and linseed with algae group lambs ($P < 0.001$). The authors adding that, expression of ACACA gene was significantly reduced by adding linseed and more intensively by linseed with algae in both subcutaneous (SC) ($P < 0.05$) and intramuscular (IM) adipose tissue (AT) ($P < 0.001$). Furthermore, ACACA expression decreased in linseed with algae group lambs compared to linseed group ($P < 0.01$). The authors suggested that adding PUFA (poly unsaturated fatty acid) may decrease the de novo FA synthesis, at least at the transcriptional level. The later results were in agreement with those published by (Hiller *et al*., 2011), who reported that there was a negative relation between ACACA gene expression and n-3 FA (grass-silage based diet) of steers in both SC AT and IM AT, and by (Dervishi *et al*., 2011), who found alfalfa grazing lambs had lower levels of ACACA and consequently lower levels of fatty acid (FA) synthetized de novo. (Fan *et al*., 2019) found a negative relationship between SCD gene expression and algae supplementation diet in beef cattle and lambs, respectively. The authors attributed these results to that feeding diet supplemented with algae altered rumen biohydrogenation, causing the production of specific intermediates that may affect SCD expression (Angulo *et al*., 2012). In the previous studies in goats (Ollier *et al*., 2009) and in lambs (Fan *et al*., 2019) the FASN mRNA levels showed no modulation by algae feeding across experimental groups and the authors attributed these results to that, algae supplemented diet induced changes in the content of PUFAs might not be mediated by changes in the mRNA abundance of these particular candidate genes. The present results support the hypothesis that the algae supplemented diet not only changes fatty acid profile of Barki lambs but also strongly impairs the transcription of genes related to PUFAs synthesis and metabolism.

The supplemented lambs with *Nannochloropsis* significantly increase (P<0.05) the final body weights, daily weight gain and feed intake with significant (P<0.05) decrease of feed conversion ratio compared with the control group which could be attributed to high nutrient density of *Nannochloropsis* as well as stimulation of the secretion of extracellular enzymes by the gut microflora (Tovar *et al*., 2002). Additionally, *Nannochloropsis* contains several nutrients, especially vitamins, minerals, essential fatty acids, amino acids and other nutrients that may promote faster growth (Chew *et al*., 2017; Khan MI, 2018). Furthermore, *Nannochloropsis* has previously been shown to decrease rumen protein degradation and produce changes in bacterial community composition with a subsequent increase the efficiency of rumen microbial crude protein production in steers (Panjaitan *et al*., 2010). These results are consistent with previous reports in cattle (Kulpys *et al*., 2009), sheep (Holman *et al*., 2012), goat (Abdullah, 2015; Assar *et al*., 2023), lambs (EL-Sabagh *et al*., 2014; Sucu *et al*., 2017), growing Friesian calves (Riad *et al*., 2019), chicken broilers (Abdel-Moneim *et al*., 2020; Elbaz *et al*., 2022) and growing rabbits ((Abd El-Hamid *et al*., 2022; Alazab *et al*., 2020), but away from that reported by (Ghattas *et al*., 2019) and (Seyidoğlu and Galip, 2014) who showed non-significant increase in the body weight of treated calves, and rabbits, respectively compared to control ones.

The weekly means and standard errors of the growth parameters are mentioned in Table 4. At the initial stage, all growth parameters, viz., body weight, body height, chest girth, body length and BCS did not differ significantly (P > 0.05) between the treatment and control groups. At the end of the first and second week, the live body weight, body length and body condition score (BCS) were higher in the treatment group as compared with the control group, though the difference was not statistically significant. However, from the third week onwards, the live body weight, body length and body condition score were significantly higher in the treatment group as compared with the control group. These findings were in agreement with those given by several researchers (Holman *et al*., 2012) in Australian Sheep and (Assar *et al*., 2023) in lambs.

Our observation could be explained to that excessively high dietary protein intake is known to suppress optimal sheep growth. This effect stems from the negative correlation between protein accretion and fat deposition rates, the latter being exacerbated by high feed protein levels (Mitchell, 2007). Excess protein gets deaminated and lost in the urine or gets broken down in the liver and could lead to conditions of fatty liver and ketosis. *Nannochloropsis* contains many essential fatty acids including γ-linolenic acid (Olkiewicz *et al*., 2015) that get deposited subcutaneously (underneath the skin) as triacylglycerols in the adipose tissue, thus explaining the observed proportional increase of BCS with *Nannochloropsis* supplementation. However, the insignificant influence of *Nannochloropsis* on CG and WH in the current study suggests that the physiological mechanisms involved are not fully understood at the moment. Thus, clarification of nutrient partitioning into different tissues including muscle, adipose and wool would allow greater insight into the underlying mechanisms in this species since lamb productivity is a function of genetic and environmental interactions (Oddy and Sainz, 2002). Thus, prime lamb productivity depends on not only environmental factors such as feed ration quality and quantity, but also on genetic factors in terms of lamb sire breed and sex (Malau-Aduli *et al*., 2009).

The effects of *Nannochloropsis* supplementation on the level of Hb, PCV, and RBC count on growing Barki lambs are presented in Table 5. The inclusion of *Nannochloropsis* in growing Barki lamb's diet showed a significant increase in Hb, erythrocyte count and leukocyte count (WBC) at T30 compared with the control group. Leucocytes play an important role in non-specific or innate immunity and their count can be considered as an indicator of relatively lower disease susceptibility (Matanović *et al*., 2007). The benefits of *Nannochloropsis* on hematological parameters may be due to the high content of polysaccharides components, folic acid and vitamin B12 and their better absorption in *Nannochloropsis* (Nedeva *et al*., 2014). The results of present study were consistent with (Assar *et al*., 2023; EL-Sabagh *et al*., 2014) in fattening lambs, (Ghattas *et al*., 2019; Ilona *et al*., 2018) in calves, (Zhang *et al*., 2001) in mice, (Watanuki *et al*., 2006) in fish, but unlike to the finding reported by (Alazab *et al*., 2020) who found non-significant (P ≥ 0.05) improvement of all tested hematological parameters of growing rabbits treated with Spirulina platensis compared to the control group.

Biochemically, hyperglycemia and hyperinsulinemia were evident in the studied lambs with significant decreased of the serum level of CHO in the supplemented group. These results indicate that *Nannochloropsis* may enhance energy and lipid metabolisms. Although the mechanism by which the *Nannochloropsis* reduces CHO has not been fully examined, the hypocholesterolemic actions of *Nannochloropsis* might be attributed to its potential to inhibit cholesterol synthesis and absorption in the gut (Nagaoka *et al*., 2005). Furthermore, its content of polyphenols may inhibit the pancreatic lipase activity and thereby decrease blood lipid concentration (Abdel-Moneim *et al*., 2020). This finding was in line with previous findings in hamisters (Riss *et al*., 2007) in rabbits (Cheong *et al*., 2010), in human (Deng and Chow, 2010), in lambs (Assar *et al*., 2023; EL-Sabagh *et al*., 2014; Sucu *et al*., 2017) and in broiler chickens (Abdel-Moneim *et al*., 2020; Elbaz *et al*., 2022), but unlike to that found in rabbits (Abd El-Hamid *et al*., 2022) and in goats (Kholif *et al*., 2020) who found no significant differences were found in serum glucose and CHO concentrations among groups.

The unexpected increase in the TG in the supplemented fed lambs in this study may imply that the *Nannochloropsis* dose might be not enough to affect serum TG or the supplementation period was not long enough for *Nannochloropsis* to exert its lipid-modulating properties. These results were inconsistent with those shown in fattening lambs (Assar *et al*., 2023), but unlike to the finding reported by (Abdel-Moneim *et al*., 2020; Elbaz *et al*., 2022) in broiler chickens who found a significantly reduced in the serum levels of triglyceride in supplemented group as compared with control. Further trails are required to characterize the efficacy of *Nannochloropsis* in lowering blood lipid in ruminants.

Serum level of total protein and globulin was significantly higher in the *Nannochloropsis* supplemented group. The increased concentrations of globulin may be related to the high contents of protein, essential amino acids, vitamins, minerals, phospholipids and antioxidants in *Nannochloropsis* (Farag *et al*., 2016; Schulze *et al*., 2016; Tibbetts *et al*., 2015). Increased globulin levels are thought to be associated with a stronger innate response in lambs and indicate higher resistance (Matanović *et al*., 2007). This result is supported by increased total leukocytic count in *Nannochloropsis* fed group (table 6). This finding was in line with previous findings in calves (Ghattas *et al*., 2019; Heidarpour *et al*., 2011), in fattening lambs (EL-Sabagh *et al*., 2014) and in rabbits (Abd El-Hamid *et al*., 2022), but unlike to that found in broiler chickens (Abdel-Moneim *et al*., 2020) and in lambs (Assar *et al*., 2023) who reported non-significant differences in serum concentrations of total protein between supplemented and control groups.

In the current study, there was a significant decrease in the serum activity of AST and ALT in the supplemented group when compared with control one indicating that *Nannochloropsis* may play a protective role against liver dysfunctions (EL-Sabagh *et al*., 2014). This finding was in consistency with previous findings in rabbits (Abd El-Hamid *et al*., 2022; El-Ratel, 2017) and in lambs (Assar *et al*., 2023) but away from the finding reported in growing Frisian calves (Riad *et al*., 2019) and in broiler chickens (Abdel-Moneim *et al*., 2020; Elbaz *et al*., 2022) who found non-significant difference of AST and ALT between supplemented and control group.

CONCLUSION

Nannochloropsis supplemented diet shows profound enhanced body growth, growth performance, Hb, erythrocyte

count, leukocyte count (WBC), serum level of globulin, total protein, triglyceride, glucose, insulin. Meanwhile, feed conversion ratio, cholesterol and liver enzymes activities tend to decrease in supplemented Barki lambs. In addition, *Nannochloropsis* supplementation increases the expression of mRNA level of lipogenic (FABP4 DGAT1, ACACA, FASN and SCD) and metabolic (insulin, Glucokinase and Glucose transporter). Further experiments with implementation of different levels of *Nannochloropsis* with higher replications and varying feeding practices are worthwhile to evaluate the nutritional value of *Nannochloropsis* more accurately and precisely. Also, further work needs to elucidate the molecular and biochemical mechanisms controlling the synthesis and deposition of these fatty acids.

ACKNOWLEDGMENTS

The authors acknowledge staff members of Animal Health and Poultry Department, Desert Research Center, Egypt.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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