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EFFECT OF QUINOA SEEDS AS FOOD SUPPLEMENT ON METABOLIC PROFILE AND GENE EXPRESSION OF ANTIOXIDANT AND IMMUNE MARKERS IN GROWING BARKI LAMBS UNDER DESERT CONDITIONS

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ABSTRACT
This search intended to evaluate the effect of dietary supplementation of quinoa seeds to growing Barki lambs. The study involved twenty apparently healthy lambs which were divided into two equal groups (ten lambs each). The first group considered as control group while the second group supplemented with quinoa seeds for subsequent 60 days. Supplementation of lambs with quinoa has significantly up-regulated the gene expression of immune (*IL1*, *IL6*, *TLR4*, and *Tollip*), antioxidant (*SOD1*, and *CAT*), and lipogenic (*ACACA*, *FASN*, and *SCD*) at day 60 compared with the other group. In lambs fed, the basal diet, CAT was the most up regulated gene at 0 day (1.41±0.14) and day 60 (1.44±0.13); while TLR4 was the most down regulated at 0 (0.35±0.1) and 60 days (0.37±0.1) in control group. In the same respect for lambs supplemented with quinoa, the gene expression of CAT was the most up regulated profile among the investigated markers at 0 (1.45±0.15), and 60 (2.36±0.1) days; while TLR4 was most down regulated genes with values (0.39±0.13) and (0.98±0.15) at 0, and 60 days, respectively. Therefore, our study is the first to explore the alterations in gene expression profile of immune, antioxidant, and lipogenic markers as a result of supplementation of growing Barki lambs with quinoa. In conclusion, supplementation of quinoa seeds enhance the antioxidant defense and immune response that reflect on the health status of lambs.

KEYWORDS: Lambs, Quinoa, Gene expression, Antioxidant, Immune response.

1. INTRODUCTION
Quinoa is a flowering plant in the amaranth family. It is a herbaceous annual plant grown as a crop primarily for its edible seeds. These seeds are rich in dietary fibers, protein, vitamins C, B and E, and dietary minerals in higher amounts.[1-3] It is originated in the Andean region of northwestern south America.[4] After that it is cultivated in many countries such as European countries, United States, India, Kenya.[5] Also quinoa has been cultivated in Egypt since 2005. It can be grown in the winter season as it needs a short day and low temperature. It grows in various lands, even saline and sandy soil, where the crop reveals tolerance to drought and salinity.[4,6,7] Salinity limits the absorption of water by plants resulting in reduction of plant growth.[8]

Natural antioxidant enzymes produced in the body provide an essential protection against oxygen reactive species. SOD, GPX and CAT are the most important enzymes of the antioxidant system. Quinoa seeds contain high percent of protein.[4,5,9] albumins and globulins are the main proteins in quinoa (about 77% of the total proteins). The rest is composed mainly of prolamines.[8] It contains also significant amounts of antioxidant phytochemicals including: phenolic acids, flavonoids, fatty acids, fat soluble vitamins, squalene, and trace elements.[3,4,10] Quinoa is a rich source of minerals such as potassium, calcium, sodium and iron.[7] Many studies proved that it reveals protection against a lot of diseases, particularly cancer, inflammatory diseases, allergy, and may reduce the risk of cardiovascular diseases.[2]

Its nutritional value is also characterized by the presence of vitamins as well as polyunsaturated fatty acids of the omega-3 family.[5,11]

Quinoa has strong antioxidant activities and ability to capture free radicals and reactive oxygen species which can enhance the health of the animal.[10,12,13] These non-enzymatic antioxidants, can prevent oxidative stress. Phenolic antioxidants in quinoa seeds can be found as free or in bound forms attached to cell wall structures.[14]

Quinoa can be considered as good anti-inflammatory and anti-microbialc plant regarding to its composition.[15] Until now, none of reports has dealt with the influence of quinoa seeds upon the antioxidative status in animals.

Changes in the activity of key regulatory enzymes involved in the intermediary metabolism can offer valuable resources for enhancing genetic selection to better adapt livestock to extreme conditions.[16] Regulation of metabolism depends, in part, on the control of gene networks through transcription, involving the assembly of DNA fragments that interact with a transcription factor or nuclear receptor. This interaction serves as a mechanism to regulate the levels of important enzymes within cells. These "worldwide" exchanges can control how quickly genes within the network are converted into mRNA. Investigating the whole genome, smaller networks, or specific genes at the mRNA stage covers the extensive area of genomics.[17]

So, the goal of this research is to evaluate the potential effects of dietary quinoa supplementation on metabolic profile and gene expression of antioxidant and immune markers in growing Barki lambs under desert conditions.

2. MATERIALS AND METHODS

2.1. Animals

The study includes twenty apparently seemingly healthy growing Barki lambs ranged in age from 8 to 9 monthes and had body weight from 15 to 27 kg (mean ± SD: 19.6 ± 6.5). The Mariut Research Station, Desert Research Centre, El Amria, Egypt. served as the experiment's locations. 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. The Ethical Committees gave their approval to all procedures, which were carried out in compliance with the regulations of the Desert Research Centre (Egypt).

2.2. Biochemical analysis of quinoa seeds

Seeds of quinoa (Chenopodium quinoa) were obtained from New Valley where they were cultivated and the biochemical analysis revealed that quinoa seeds composed of 67.5% carbohydrates, 16.0% proteins, 7.5% fat, 3.0% fibers, and 3.5% ash.

2.3. Experimental design

The lambs were allocated into two equal-sized 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 Quinoa seeds which were incorporated daily in the concentrate of each lambs at a rate of 7 % /kg diet for subsequent 60 days. The investigated lambs were fed on 500 g concentrate feed mixture (CFM) plus 500 g alfalfa hay /head/day as shown in table 1. Diet was offered twice a day in the morning and evening with free access to water. Lambs were weighed on days 0 and 60 of experiment, after fasting for twelve hours before the morning feedings.

Table 1: 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

2.4. 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.

2.5. Blood sampling

Ten milliliters of blood was collected from each animal via jugular venipuncture before starting the experiment as control group, and at 60th day 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. The EDTA blood used for RNA extraction. All samples were cooled on crushed ice and were transported immediately to the laboratory for further processing.

2.6. 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 (IL1, IL6,TLR4, and Tollip), antioxidant (SOD1, and CAT), and lipogenic (ACACA, FASN, and SCD) 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 1. The housekeeping gene GAPDH 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 1 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 GAPDH gene was carried out and calculated according to the $2^{-\Delta\Delta Ct}$ method.[18]

2.7. Serum profile of immune and antioxidant markers

Serum biochemical analysiswas 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 Biotech, 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).

2.8. Statistical analysis

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

H_A: Dietary supplementation of Quinoa 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 by using SPSS software (SPSS analytical program for windows version 21). The effect of treatment on each variable in each group was evaluated using analysis of variance with repeated measurements of the general linear model using the Mauchly's sphericity test to detect the significant variations. When there was a significant result, one-way ANOVA with post hoc Duncan multiple comparison tests was used to detect the specific variations. A univariate general linear model (GLM) was used to test interaction effect of gene type and study period (0 and 60) 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 enzymes. Correlation coefficient (r) and p value were considered. A difference was considered significant at $p < 0.05$.

3. RESULTS

3.1. Clinical examination

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

3.2. Changes in body weight in lambs after supplementations with Quinoa seeds.

The weight of all lambs were recorded before and after supplementations with Quinoa seeds, Table 2. There was a significant ($p < 0.05$) increase in the body weight of the lambs after supplementation with Quinoa seeds compared to the control group.

Table 2: Body weight of lambs.

	Control	Before treatment (0 day)	After treatment (60 days)
Body weight	18 ± 1.5	17±2.5	28*±4.3

***Statically significant when p< 0.05.**

3.3. Gene expression pattern of immune and antioxidant markers

Supplementation of lambs with Quinoa has significantly up-regulated the gene expression of immune (*IL1*, *IL6*, *TLR4*, and *Tollip*), antioxidant (*SOD1*, and *CAT*), and lipogenic (*ACACA*, *FASN*, and *SCD*) at day 60 compared with other groups (Figures 1, 2, and 3). In lambs fed the basal diet, CAT was the most up regulated gene at 0 day (1.41±0.14) and day 60 (1.44±0.13); while TLR4 was the most down regulated at 0 (0.35±0.1) and 60 days (0.37±0.1) in control group. In the same respect for lambs supplemented with Quinoa, the gene expression of CAT was the most up regulated profile among the investigated markers at 0 (1.45±0.15), and 60 (2.36±0.1)days; while TLR4 was most down regulated genes with values (0.39±0.13) and (0.98±0.15) at 0, and 60 days, respectively, Table 2.

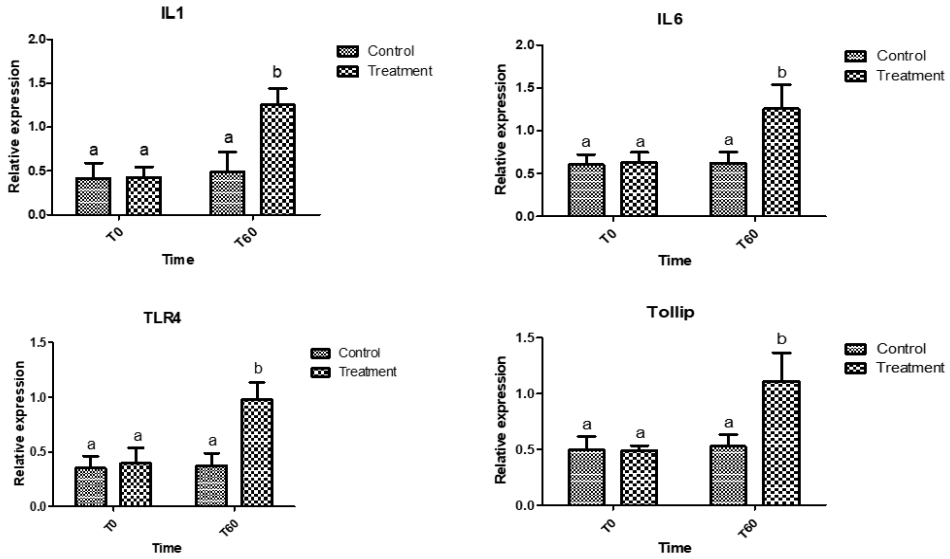

Figure 1: Relative expression patterns of immune markers genes in the control and Quinoa supplemented Barki lambs groups at 0 and 60 days. Small alphabetical letters show significance when (P<0.05).

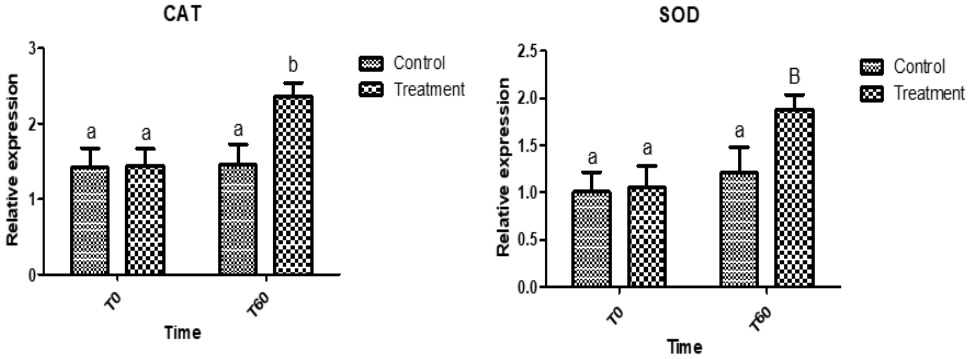

Figure 2: Relative expression patterns of antioxidant markers genes in the control and Quinoa supplemented Barki lambs groups at 0 and 60 days. Small alphabetical letters show significance when (P<0.05).

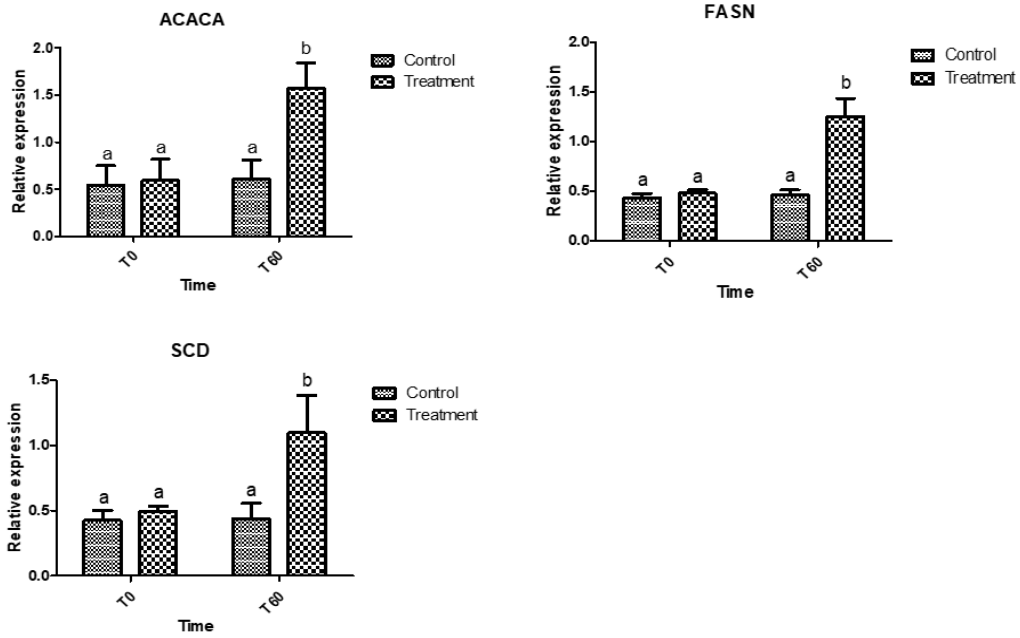

Figure 3: Relative expression patterns of lipogenic markers genes in the control and Quinoa supplemented Barki lambs groups at 0 and 60 days. Small alphabetical letters show significance when (P<0.05).

Table 2: Oligonucleotide primers sequence, annealing temperature and PCR product size of the studied genes.

Gene	Oligonucleotide sequence	Accession number	Annealing temperature (C^0)	Size (bp)
IL1	f5-CCATACATGACGGCTGCTACA-3′ r5-TGTCTCAGGCATCTCCTTATGC-3′	NM_001009808.1	60	180
IL6	f5- TGCAGTCCTCAAACGAGTGG-3′ r5′- CCGCAGCTACTTCATCCGAA - 3′	NM_001009392.1	58	110
TLR4	f5- GGTTCCCAGAACTGCAAGTG -3 , r5-GGATAGGGTTTCCCGTCAGT -3	AY957615	58	117
Tollip	f5-CTGGTGCTGTCCTACACGTC-3 , r5-ACAGTGGGCATTCCTGTGAT-3	NM_001039961	60	122
SOD1	f5-TGATCATGGGTTCCACGTCC-3 r5-CACATTGCCCAGGTCTCCAA-3	NM_001145185.2	60	139
CAT	f5′-CAGTAGGAGACAAACTCAATG-3′ r5′- ACGACTCTCTCAGGAATTCTC - 3′	GQ204786.1	58	121
ACACA	f5,- ATGTGGCCTGGGTAGATCCT-3, r5,-ACGTAACACAAGGCTGATGGTG-3,	NM_001009256.1	60	261
FASN	f5,- GGAAGGCGGGACTATATGGC-3, r5,- CATGCTGTAGCCTACGAGGG-3,	XM_004013447.1	60	278
SCD	f5,- GGCGTTCCAGAATGACGTTT-3, r5,- TGAAGCACAACAGCAGGACA-3,	NM_001009254.1	58	251
GAPDH	f5,- TGACCCCTTCATTGACCTTC-3, r5,- GATCTCGCTCCTGGAAGAG-3,	NM-001034034	60	143

3.4. Serum profile of immune and antioxidant markers

Serum concentrations of cytokines and oxidative stress markers in control and supplemented groups are summarized in Table 3. There was a significant ($p < 0.05$) increase in the serum concentrations of SOD, CAT, TAC, GPx, IL1, and IL-6 in supplemented group in relation to control one.

Table 3: Effect of Quinoa seeds on antioxidant and immune markers of growing Barki lambs.

Parameters	Control	Before Treatment (0 day)	After treatment (60 days)
GPx (U/L)	19.5 ±0.5	17.9 ±1.1	27.2* ± 3.5
Catalase (U/L)	205 ±3.1	234 ±5.5	284* ± 2.1
SOD (U/ml)	862 ± 2	932 ± 7	1088* ± 3
TAC (mM /L)	31.6 ± 2.1	32.1 ± 1.5	52.3* ± 3.6
IL 1 (pg/ml)	5.6 ±0.7	5.3 ±0.2	6.4* ± 0.2
IL 6 (pg/ml)	5.9 ±0.3	5.4 ±0.5	6.5* ± 0.3

Values with an asterisk within the same raw are statistically significant ($p < 0.05$). GPx: Glutathione peroxidase; SOD: Super oxide dismutase; TAC: total antioxidant capacity; IL1: Interlukin 1; IL 6: Interlukin 6.

4. DISCUSSION

In the present study, real time PCR was carried out to quantify mRNA level of immune (*IL1*, *IL6*, *TLR4*, and *Tollip*), antioxidant (*SOD1*, and *CAT*), and lipogenic (*ACACA*, *FASN*, and *SCD*)genes in growing Barki lambs supplemented with Quinoa. Our findings revealed that supplementation of lambs with Quinoafor successive 60 days significantly up-regulated the expression pattern of immune, antioxidant, and lipogenic markers. To the best of our knowledge, there were no previous studies reported the effect of Quinoa supplementation on the gene expression profile of immune, antioxidant, and lipogenic markers in growing lambs. Therefore, our study is the first to explore the alterations in gene expression profile of immune, antioxidant, and lipogenic markers as a result of supplementation of growing Barki lambs with Quinoa.

The phytochemical makeup of quinoa and the antioxidant properties of hydrophilic (such as phenolics, betacyanins) and lipophilic (such as fatty acids, tocopherols, and carotenoids) nutrients may be to blame for the significant alteration in the expression pattern of immune, antioxidant, and lipogenic markers.[19] In addition, it was demonstrated that eating quinoa seeds may protect against oxidative stress.[20] Also**,** quinoa seeds exhibit antioxidant activity because they contain significant active components such polysaccharides.[21] Quinoa is a great source of vitamin E, and because quinoa oil contains more γ-tocopherol than maize oil does, it has a longer shelf life. Quinoa is also a powerful antioxidant. Vitamin E is crucial because it functions as a natural antioxidant to shield fatty acids from destruction by free radicals.[22,23] Six glycosylated flavonols were isolated from quinoa seeds by Zhu et al. (2001), who demonstrated that quinoa may be crucial in the suppression of free radicals.[24] Other reasons for interpretation of alteration in the expression profile of investigated markers is that Pompeu et al. reported that the lectin found in quinoa seed has antimicrobial

potential, which may explain a decrease in the total number of bacteria with an increase in the quinoa seeds additive concentration.[25] Amiri et al. and Eassaway et al. reported that feeding broilers a diet supplemented with quinoa seeds increased the synthesis of digestive enzymes, improving nutrient digestibility and growth efficiency.[26,27]

The enzymatic antioxidants like CAT and SOD are in charge of scavenging the intracellular and extracellular ROS and keep away from lipid peroxidation of cellular plasma membrane.[28] The SOD catalytically converts the $O_2^{\bullet-}$ to hydrogen peroxide and oxygen in the presence of zinc, manganese, or copper as metal ion cofactors. However, the CAT converts hydrogen peroxide to oxygen and water.[29] The antioxidant enzymes inhibit peroxidation of the lipid and preserve the cell membrane structure.[30]

Quinoa seeds have high content of flavonoids and phenolic acids which are powerful antioxidants.[31] The presence of hydroxyl groups in polyphenols provides antioxidant properties through chelating ability to suppress ROS output and scavenging the free radicals, by either up regulation of antioxidant defenses or inhibition of enzymes involved in their creation.[32] The antioxidant capacity of quinoa is duplicated by the gastrointestinal digestion of quinoa flavonoids.[33] It is stated that some quinoa peptides are antioxidants which act free radical scavenger, electron donors to neutralize free radicals, and inhibition of lipid peroxidation.[34] Quinoa seeds revealed gastro protection by prevent lipid peroxidation and increase of gastric mucus secretion, gastric pH, and endogenous antioxidant enzymes.[35]

The supplementations of quinoa seeds in diet of growing Barki lamb's resulted in a significant increase in serum level of CAT, SOD, GPx, and TAC. GPx and SOD are responsible for the defense of the cellular antioxidant system.[36] CAT is responsible for removing the peroxides and converting them into O_2.[37] Also polysaccharide of quinoa was considered a natural antioxidant, and immune-regulating in drug application.[38, 39]

5. CONCLUSION

The dietary supplementation of quinoa seeds alter the metabolic profile and gene expression of immunological and antioxidant indicators in growing Barki lambs. Furthermore, enhance the antioxidant defense and immune response that reflect on the health status of lambs.

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