



Chlorella vulgaris supplementation effects on performances, oxidative stress and antioxidant genes expression in liver and ovaries of New Zealand White rabbits



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ABSTRACT

Oxidative stress is an exclusive biochemical complication affecting reproduction; hence, dietary antioxidant supplementation for its attenuation is a required nutrition – reproduction improvement strategy. On this background, *Chlorella vulgaris* (a natural antioxidant) was supplemented to grower female rabbits to maturity. The rabbits were thirty-five in number randomly distributed into five experimental groups in a completely randomized design. Control group was fed only basal feed while treatment groups were fed diets containing 40 %, 60 %, 80 % and 100 % *Chlorella vulgaris* biomass as T1, T2, T3 and T4 respectively at 500 mg per animal body weight (kg) along with the basal feed daily. Performance records were obtained, blood was collected, and at the end uterus, ovaries and liver were removed from sacrificed animals for analysis. Serum, uterus and liver oxidative stress status were determined while RNA isolated from liver and ovaries samples were used for antioxidant genes expression analysis. Oxidative stress status and antioxidant enzymes activities were determined using chemical assays while antioxidant gene expression levels were determined using real-time quantitative PCR system. There was significant difference in feed intake ($p < 0.014$), final body weights ($p < 0.008$), empty carcass weights ($p < 0.001$) and commercial carcass weights ($p < 0.001$) of the rabbits as results of the microalgae supplementation. There was also significant difference in malondialdehyde (MDA) concentrations ($p < 0.050$), total antioxidant capacity (TAC) ($p < 0.050$) and protein carbonyl (PCO) concentrations ($p < 0.050$) due to the supplementation of the microalgae; in addition, supplementation of the microalgae significantly improved activities of superoxide dismutase (SOD) ($p < 0.050$), catalase (CAT) ($p < 0.050$) and reduced glutathione (GSH) concentration ($p < 0.050$). Furthermore, there was significant difference in relative expression of primary antioxidant genes *sod1* ($p < 0.050$) and *gpx1* ($p < 0.050$); however, there was no significant difference in relative expression of *bre* ($p > 0.050$) and *ucp1* ($p > 0.050$). The study concluded from the outcomes stated above that supplementation of microalgae *Chlorella vulgaris* improved performances of rabbits through attenuation of oxidative stress, enhancement of antioxidant enzymes activities as well as up-regulation of primary antioxidant genes. Hence, it was recommended as dietary supplement for protection against oxidative stress and improved productivity in rabbits and other food producing mammalian species. In addition, further studies into assessment of its effects on expression of transcripts and immune modulation genes in rabbits and other animals is warranted as future studies in order to established its potential as beneficial nutraceutical for animals and human.

1. Introduction

Oxidative stress negatively affects reproductive system and reproductive outputs starting from oocytes production, conception, and embryo development to parturition. Oxidative stress complexity could be

trace to mitochondria leakages of electrons during stimulation of NADPH via oxidative phosphorylation causing cascades of cellular and tissue damage as results of compromised internal antioxidant defence against prooxidants [1, 2, 3]. Dietary supplementation of antioxidants is one of the most valid strategies for its management because oxidative stress is

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almost inevitable especially in animals due to increased energy requirement associated with growth and economic activities such as reproduction, milk and meat yield [4]; additional antioxidants supplied exogenously complement innate antioxidant apparatus of the body together for protection against activities of free radical generations and halting cascades of oxidative stress damages [5].

Oxidative stress as an imbalance between prooxidants and antioxidants compromise animal performance and production, reduce animal productivity and negatively impact animals' health; therefore, because antioxidants are capable of attenuating the menace, antioxidant supplementation is not only a nutritional strategy for improving productivity but also a palliative health promotion strategy for increasing animal production outputs [6]. However, commonly used synthetic antioxidants including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butyl hydroquinone (TBHQ) for management of oxidative stress in food producing animals are becoming source of growing concerns because of their carcinogenic tendencies which remains a major threat to health; therefore, exploration of natural antioxidants for oxidative stress attenuation is a required animal production and health research [7].

Microalgae are examples of natural sources of antioxidants for potential exploration because they contained modified metabolites of functional benefits suitable for improving quality of human and animal lives [8]. Some of these secondary metabolites in microalgae are biological resources with industrial applications such as thickeners and emulsifiers in food processing, complementary drugs in nutraceutical and pharmaceuticals industries; and biomedical materials in alternative medicine practices as well as feed additives in animals and fisheries production [9]. Their mechanisms of actions are diverse from direct scavenging on free radicals or prooxidant to inhibiting cascades of biochemical processes leading to prooxidants generation of oxidative stress products.

Chlorella vulgaris is a popular microalga commercially cultivated across the world and rich in bioactive compounds including carotenes, astaxanthin, lutein and fucoxanthin; in addition to these compounds, it was also reported to contain *eicosapentaenoic acid* (EPA) and *docosahexaenoic acid* (DHA) which are all bioactive compounds of functional values [10]. The microalga is also identified as direct scavenger of free radicals causing oxidative stress; and according to Rodriguez-Garcia et al. [11] the microalga has higher antioxidants power compared with synthetic antioxidants commonly used in foods and animal production; it is a natural growth promoter, immune booster, and tissues rebuilder [12, 13]. Therefore, this study because of antioxidant potential of *Chlorella vulgaris* investigated effects of its supplementation on performance and oxidative stress in rabbits through assessment of oxidative stress products generation, antioxidant enzymes activities and expression of primary antioxidant genes.

2. Materials and methods

2.1. Location and duration of the study

The experiment duration was 120 days; animal experimentation took place at the Laboratory Animal Section of Experimental Livestock Unit (ELU) of ICAR – National Institute of Animal Nutrition and Physiology (NIANP), Bangalore, India. The institute is located at Adugodi a suburb of Bangalore city; Karnataka, India. It is a Tropical Savannah area located on coordinates longitude 12.97° N and latitude 77.56° E; average monthly temperature of the area is 23.9 °C while warmest temperature is 27.6 °C, annual rainfall is 970 mm usually spread clearly in a season different from dry season. Approval of the animal experimentation protocol was given from a joint consideration of Institutional Animal Ethics Committee (IAEC) of ICAR – NIANP, Bangalore; India and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.2. Animals and their management

Thirty-five female growing New Zealand White rabbits were used for the study. The animals were divided into 5 experimental groups of 7 animals per group in a complete randomized design experiment. The animals were sourced from Biogen Biotechnology (P) Ltd., Laboratory Animal Facility, Attibele; Anekal - Taluk, Bangalore - 562107, India. Biogen is a laboratory animal breeding company in India with registration number 971/PO/RcBiBt/S/2006/CPCSEA. Upon delivery of the rabbits to the animal house, they were randomly distributed into the experimental groups and acclimatized for a period of 14 days before commencement of the trial.

The rabbits were housed individually in aluminium coated wire mesh cages; animals in control group were fed only basal feed without *Chlorella vulgaris* supplements while animals in treatment groups were supplemented with 200, 300, 400, and 500 mg *Chlorella vulgaris* biomass as T1, T2, T3 and T4 respectively along with the basal feed daily throughout the experimental period while water and basal feed (Krishna Valley Agrotech, India) were provided *ad-libitum* via automatic nipple drinkers and feeding trough. Nutri-functional properties of the supplement diets were determined through subjected to proximate and *in-vitro* free radical attenuation analysis (Table 1).

Chlorella vulgaris used in this study was an organically produced commercial microalga rich in antioxidant and other bioactive beneficial compounds procured from Seagrass Tech (Pvt.) Ltd, 600087 – India. The microalgae was manufactured organically without use of synthetic chemical in closed batch production system, it was delivered as a fine green powder biomass screened for its safety of use in animal experimental consumption by evaluation of its heavy metal and microbial compositions. These assessments indicated that the microalga in its biomass form used in this study is safe for animals' consumption (Table 2).

2.3. Observation and recording of animal performances

Performance parameters were recorded daily, weekly and at the end of the study. Feed intake was recorded daily from evaluation of differences in feed served and remnants by each animal in gram (g). On weekly basis, body weight change was determined from difference in body weight of the current week and body weight of the previous week for each animal using small digital scale with accuracy of 0.2 g (Atom Scale, India).

2.4. Determination of serum and tissues oxidative stress status

2.4.1. Sample collection and storage

Blood was collected from each animal into vacutainers kept at room

Table 1

Chemical and nutri-functional composition of the *Chlorella vulgaris* supplement diets.

Parameters	T1	T2	T3	T4	Units
Dry matter	93.95	94.38	96.54	92.33	% dry matter
Crude protein	30.46	44.36	53.86	61.15	% of dry matter
Total ash	2.23	3.59	4.30	5.28	% of dry matter
Metabolizable energy	3433	3025	3183	3648	Kcal/Kg
Crude fibre	0.92	1.75	1.71	2.90	% of dry matter
Ether extract	1.22	1.13	1.18	0.80	% of dry matter
<i>Chlorella vulgaris</i>	200	300	400	500	mg/500 mg
Total phenolic content	39.38	36.28	41.16	43.93	mg/g ellagic acid equivalent
Total antioxidant concentration	670.30	601.21	760.00	902.54	µmol/g ascorbic equivalent
H ₂ O ₂ Scavenging Power	47.44	23.26	59.58	41.14	%
FRAP	1.34	1.20	1.52	2.48	-n/a-

Source: Laboratory analysis. Ferric Reducing Antioxidant Power (FRAP).

Table 2Sample information of *Chlorella vulgaris* biomass used in this study.

Parameters	Methods	Specification	Analysis
Physical appearance	Visual examination	Fine green powder	Fine green powder
Particle sizes	AOAC 973.03	100 % pass through 100 mesh	Complies
Arsenic as As	AOAC 993.14	<0.5 ppm	Below detection limit
Lead as Pb	AOAC 993.14	<1 ppm	Below detection limit
Aerobic plate count	AOAC 966.23	<200,000 cfu/g	300 cfu/g
<i>E. Coli</i>	AOAC 991.14	Absent	Absent
<i>Salmonella sp</i>	AOAC 998.09	Absent	Absent
<i>Staphylococcus aureus</i>	AOAC 975.55	Absent	Absent
Coliforms	AOAC 966.24	<10 cfu/g	<10 cfu/g
Yeast/mold	AOAC 997.02	<500 cfu/g	<10 cfu/g
Chlorophyll	AOAC 942.04	0.25 %	2.67 %
Beta carotene	AOAC 955.10	Nil	0.1 mg/g
Packaging	Bagging	Dry, cool away from light	1 kg/bag

Source: Seagrass Tech Lab (2018).

temperature for approximately 15 min allowing clotting then centrifuged at 2000 rpm for 10 min using bench top centrifuge (Remi Centrifuge, India). Supernatant layers were collected into test bottles and kept at -80°C for downstream analysis. For tissue oxidative stress markers, at the end of the experiment the animals were randomly selected from each group and sacrificed by sharp cutting through jugular vein after being fasted overnight. Liver and uterus of the sacrificed animals were removed into phosphate buffer (pH 7.4), snap frozen in liquid nitrogen then stored at -80°C for downstream enzymes analysis.

2.4.2. Preparation of tissue homogenates

Tissues homogenates were prepared as 10 % in Tris HCl buffer (1 mM ethylene diamine tetra acetic acid, 1 mM dithiothreitol and 50 mM Tris HCl) used for antioxidant enzymes analysis. The tissues homogenates were prepared with 100 mg of each tissue sampled in triplicates then lysed under cold liquid nitrogen and added with 1 mL Tris HCl buffer as homogenizer followed by centrifuge at 3500 rpm for 15 min at 4°C . Collected supernatants were stored at -80°C for downstream antioxidant enzymes activities.

2.4.3. Protein estimation

Amount of protein in tissue homogenates was determined using Bicinchoninic Acid (BCA) Protein Assay Kit (G-Biosciences, USA). According to the manufacturers' protocol; briefly, it involves preparation of protein standards of known concentrations using bovine serum albumin (BSA) through serial dilution of 300 μL of BSA from stock into 150 μL Tris HCl buffer; then to 25 μL of each homogenate sample and standards, 200 μL BCA working solution was added and incubated at 37°C (Labnet Inc., USA). After the incubation, absorbance of the samples and the standards were determined at 562 nm using Thermo Multiskan GO micro plate reader (Thermo Fisher Scientific, Finland).

2.4.4. Serum and tissues superoxide dismutase enzymes activities

Enzymatic activities of superoxide dismutase were determined using chemical assay developed on the background of pyrogallol autooxidation in the presence of diethylenetriaminepentaacetic acid (DEPTA) at pH 8.5 by 50 %; this principle was established and verified by Marklund and Marklund [14] and its modification according to Guvala et al [15] was adopted for use in this study. The chemical assay comprised of 50mM Tris cacodylate buffer, 1mM DEPTA and 20mM Pyrogallol solution prepared in 10 mM HCl. The assay was used to produce reaction mixtures of each sample comprising Tris buffer, Pyrogallol and respective serum and tissues samples. The reaction mixtures were individually set for absorbance

reading at wavelength 420 nm in UV/Vis spectrophotometer (Biochrom Libra S32, UK). The reaction time was 180 s and at the end of spectrometer absorbance readings, inhibition of pyrogallol autooxidation and activity of the superoxide dismutase enzymes in the samples were calculated.

2.4.5. Serum and tissues catalase enzymes activity

Serum and tissues samples prepared as described above were used for determination of catalase activities based on their ability to decompose hydrogen peroxide (H_2O_2) into H_2O and O_2 (EC 1.11.1.6). The catalase enzyme assay composition includes 50 mM of potassium phosphate buffer (pH-7.0) prepared from di-potassium hydrogen phosphate, potassium dihydrogen phosphate; and 100 μM hydrogen peroxide. Blank (reaction mixture without sample) and test reaction mixture comprising the assay and samples were subjected to spectrophotometer reading at 240 nm; absorbance changes were noted at every 30 s throughout 180 s reaction time for each sample. Catalase enzyme activities were calculated from changes in absorbance at 240 nm per minutes using milli molar extinction coefficient of H_2O_2 at 240nm.

2.4.6. Serum and tissues reduced glutathione

Reduced glutathione was determined following protocol of Moron et al [16] on the principles that the enzyme is capable of reacting with Ellman's reagent 5,5'- dithiobis (2-nitrobenzoic acid) to form 5'-thio-2--nitrobenzoic acid (TNB) which absorb UV – light at 412 nm. The assay involved precipitation of the samples in 20 % trichloro acetic acid then centrifuge the mixture at 2000 rpm for 2 min at 4°C (Eppendorf Centrifuge 5424 R, Germany). Supernatant of 50 μL were obtained after the centrifuge; for each reaction mixture, the supernatant was added with 100 μL of phosphate buffer (200 mM, pH 8.0) and 50 μL of 5mM 5,5'-dithiobis (2-nitrobenzoic acid) then incubated for 10 min at room temperature. Reduced glutathione standards were prepared and alongside with the test samples were put up for absorbance reading at 412 nm in micro plate reader (Thermo Fisher Scientific, Finland); activities of reduced glutathione in each reaction was calculated μmol of GSH per g of the sample for tissue and μmol per mL for serum samples.

2.4.7. Serum and tissue lipid peroxidation

The levels of lipid peroxidation in the serum and tissues were determined according to procedures of Ohkawa et al [17]. Serum samples were used undiluted while 10 % tissue homogenates prepared using potassium chloride was used for tissues lipid peroxidation analysis. The assay reaction mixture comprised 0.5 mL samples, 0.2 mL 8 % sodium *n*-dodecyl sulphate, 1 mL 20 % acetic acid and 1 mL 0.8 % 2-thiobarbituric acid (TBA). These reaction mixtures were boiled for 1 h at 95°C in water bath (Brookefield-Ametek, India); the reactions were terminated by cooling on ice for 10 min each. After cooling a mixture of *n*-butanol and pyridine (15:1) was added to each reaction vortex briefly then centrifuge at 4000 rpm for 10 min. Organic supernatant layers were taken from the samples and set for absorbance readings in micro plate reader at 532 nm; molar extinction coefficient of malondialdehyde (MDA) was used for estimation of the lipid peroxidation products quantity expressed in nanomole (nmol) per mg for tissue and per mL for serum samples.

2.4.8. Serum and tissues protein carbonylation

Protein carbonyl was determined based on protocol of Colombo et al [18] a procedure involving labelling of protein carbonyl with 2,4-dinitrophenylhydrazine (DNPH) to form its conjugate dinitrophenylhydrazones (DNP). For serum 100 μL of serum was used while for tissue 1 mg protein sample per tissue was used; both serum and tissue sample were individually incubated with 100 μL of DNPH (20 mM prepared in 2N HCl) for 60 min in dark room. After the incubation, 1 mL 20 % acetic acid was added and incubated on ice for 15 min then centrifuge at 10000 rpm for 5 min at 4°C ; the supernatant was discarded while the protein pellets were washed in ethyl acetate and ethanol mixture (1:1) then vortex mixed

followed by centrifuge at 10000 rpm for 5 min at 4 °C.

The procedure of washing and pellet collection was repeated twice for each sample after which the pellets were resuspended in 1 mL guanidine hydrochloride (6 M prepared in 50 mM phosphate buffer pH 2.3). The process of re-suspension involved incubation of the pellets in the guanidine solution at 37 °C for period of 30 min. After complete dissolution of the pellets in the guanidine solution, they were put up for absorbance reading at 366 nm in microplate reader Thermo Multiskan GO micro plate reader (Thermo Fisher Scientific, Finland). Protein carbonyl concentration ($\mu\text{M/mL}$) was calculated using molar absorption coefficient of $22,000 \text{ M}^{-1}\text{cm}^{-1}$.

2.4.9. Serum and tissues total antioxidant capacity

Overall antioxidant capacities of the serum and tissues samples were determined using protocol described by Benzie and Strain [19]; the principle of the assay is based on redox reduction of iron (III) to iron (II) at low pH. In this study, a chemical assay containing 25 mL sodium acetate buffer (300 mM, pH 3.6), 2.5 mL of 10 mM 2,4,6-Tri-(2 pyridyl)-s-triazine prepared in 40 mM HCl and 2.5 mL Ferric chloride (20 mM prepared in distilled water) was prepared and used working assay. Reaction mixture comprising 50 μL of samples and 200 μL of the FRAP working solution were incubated for 30 min at 37 °C (Labnet Inc., USA) after which absorbance was measured at 493 nm using microplate reader. Ascorbic acid was used as standard for calculating antioxidant power associated with colour changes and reduction of Fe^{3+} to Fe^{2+} ; total antioxidant concentration was expressed as μmol per mL for serum and as μmol per gram for tissues.

2.5. Quantification of antioxidant genes expression in liver and ovaries of the rabbits

2.5.1. Sample collection and RNA isolation

200 mg liver and ovary samples were collected in triplicate from each sacrificed animal into 200 μL of RNeasy lysis buffer (Qiagen Bioservices, USA) and stored at -80 °C ahead of downstream analysis. For RNA isolation, 50 mg of collected samples each in triplicates were crushed under liquid nitrogen after being washed in phosphate buffer (pH 7.4); after proper lysis they were homogenized TRIzol reagent (Life Technologies, USA).

After homogenization of the samples, they were subjected to mechanical cell disruption for separation of membranes from the cells using a mechanical disruptor (Scientific Industries, USA). The disruption was followed by 5 min incubation at room temperature and phases separations by addition of 1 mL chloroform followed by rigorous mixing for 15 s each. Then each of the samples was centrifuge at 12000 rcf for 15 min at 4 °C. After the centrifuge 500 μL RNA clear aliquot were obtained from each sample into 500 μL isopropanol. The mixture of RNA and isopropanol were then gently mix for 15 s each and incubated for 10 min for RNA precipitation; then centrifuge again for 10 min at 12000 x g at 4 °C. After this centrifuge, the RNA pellets were collected while the isopropanol was discarded then the pellets were washed with 1 mL ethanol and centrifuge for 5 min at 12000 x g at 4 °C and allowed to evaporate. After evaporation the RNA pellets were dissolved by adding 50 μL nuclease free water, then subjected to purity and concentration evaluation using Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., USA).

2.5.2. cDNA synthesis

Revertaid cDNA synthesis kit was used for synthesizing of cDNA from isolated RNA samples according to the manufacturers' protocol (Thermo Fisher Scientific, USA); after synthesizing, from respective cDNA templates, 50 ng/ μL of each sample in triplicates were used for real time quantification using StepOne Real-Time PCR system (Applied Biosystem, USA).

2.5.3. Real-time PCR quantification of relative expression of the target genes

Takara SYBR® premix (Takara Bio Inc., Japan) was used for the

comparative quantification of relative expression of the target genes in the animals. The quantification reaction followed Takara SYBR® premix protocol for Applied Biosystems' 7300/7500/7500 Fast Real-Time PCR System and StepOne Real-Time PCR System. The first step of the reaction ran for 30 s at 95 °C; the second step (denaturation) ran first at 95 °C which for 5 s while annealing of the reaction mixture at 60 °C which ran for a period of 60 s in a continuous cycle of 40 runs followed by melt curve stage.

2.5.4. Primers and primer designing

Primers used for the real-time quantifications were designed using NCBI database given below (Table 2); while glyceraldehyde-3-phosphate dehydrogenase (gapdh) was used as internal reference control gene. The gene product yield was confirmed in 1.8 % agarose gel electrophoresis (Tarsons Product Pvt. Ltd., India).

2.6. Statistical analysis of data

Performance records, oxidative stress biomarkers, antioxidant enzymes activities and fold changes of relative expression of the genes were subjected to one - way analysis of variance (ANOVA); significant means were determined at $p < 0.05$ while means separation was done with Duncan test procedure of SPSS version 20.0. In addition, there was comparative analysis of relative expression of primary antioxidant genes in liver and ovaries using Mann-Whitney U test for comparative ranking of the genes expression patterns in both tissues.

3. Results

3.1. Animals' performances characteristics

There was no significant difference in initial weights of the rabbits; mean initial weight was $1060 \pm 29.42 \text{ g}$ ($p < 0.17$). However, there was significant difference in final weights of the rabbits, the finishing weight was $2383 \pm 40.99 \text{ g}$ ($p < 0.008$). There was also significant difference in feed intakes ($p < 0.014$); mean feed intake was $100.56 \pm 1.22 \text{ g}$ while minimum and maximum feed intakes were 90.60 g and 110.26 g respectively. Furthermore, there was significant difference in commercial carcass weight ($p < 0.001$); similarly, significant difference was observed in the empty carcass weight ($p < 0.001$) as well as the rabbits' skin weight ($p < 0.001$). Weight after slaughtering was $2325 \pm 39.07 \text{ g}$ ($p < 0.004$), blood weight was $58.94 \pm 5.54 \text{ g}$ ($p < 0.006$), empty carcass weight $1801.74 \pm 52.78 \text{ g}$ ($p < 0.001$), commercial carcass weight was $1419.72 \pm 34.36 \text{ g}$ ($p < 0.001$), skin weight was $236.96 \pm 7.2 \text{ g}$ and feed conversion ratio was 10.32 ± 0.45 ($p > 0.05$) respectively (Table 3).

3.2. Oxidative stress status and antioxidant enzymes activities

There was significant difference in serum oxidative stress status of the rabbits; mean malondialdehyde (MDA) concentration was $18.40 \pm 0.91 \text{ nmol/mL}$ ($p < 0.010$) while minimum and maximum concentrations were 11.51 and 27.60 nmol/mL respectively. Total antioxidant concentration (TAC) was $65.00 \pm 8.60 \mu\text{mol/mL}$ ($p < 0.005$) while minimum and maximum total antioxidant concentrations were 14.10 and 170.57 $\mu\text{mol/mL}$ respectively. Protein carbonyl (PCO) concentration was $7.28 \pm 0.34 \mu\text{mol/mL}$ ($p < 0.001$) while minimum and maximum concentrations were 5.45 and 10.23 $\mu\text{mol/mL}$ respectively. Superoxide dismutase (SOD) activities was $172.40 \pm 7.8 \text{ U/mL}$ ($p < 0.042$) while minimum and maximum activities were 123.10 and 261.50 U/mL respectively. Catalase (CAT) enzyme activities was $4.06 \pm 0.26 \text{ U/mL}$ ($p > 0.05$) while minimum and maximum activities were 1.72 and 6.19 U/mL respectively. Reduced glutathione (GSH) concentration was $10.11 \pm 0.62 \mu\text{mol/mL}$ ($p < 0.017$) while minimum and maximum concentrations were 6.11 and 17.39 $\mu\text{mol/mL}$ respectively (Table 4).

Similarly, there was significant difference in malondialdehyde (MDA) concentration of the rabbits' liver $6.61 \pm 1.54 \text{ nmol/mg}$ ($p < 0.001$)

Table 3

List of target genes and their primers evaluated in the rabbits.

S/ N	Gene Name	Primers type	Primer Sequence (5' to 3')	Primers Length (bp)	Product size (bp)	Accession No
1	Oviductal glycoprotein 1 (OVGP1)	F	TTCTTGCCTGAGACAGTT	20	121	NM_001105687.1
		R	CTCTCTCTCGGGTGGTCATC	21		
2	Uncoupling protein 1 (UCP1)	F	CCTAACAACTGGAGGCGTGG	20	177	NM_001171077.1
		R	GGAGTTGTCCCTTTCCACAGA	21		
3	Oxidative stress responsive 1 (OXSRI)	F	GTTTGCCTAAAGTGGGTGG	20	120	XM_008252717.2
		R	AGCAGGCACACATCACTG	20		
4	Brain and reproductive organ-expressed (TNFRSF1A modulator) (BRE)	F	TCAGTCACITTTGGCACAGGT	20	120	XM_002709945.3
		R	GGAAAAACAGGGGACAGTCA	20		
5	Oryctolagus cuniculus superoxide dismutase 1 (SOD1)	F	GGTGGTCAAGGGACGCATAA	20	205	NM_001082627.2
		R	CACATCAGCCACACCATTCG	20		
6	Oryctolagus cuniculus glutathione peroxidase 1 (GPX1)	F	TTTGGGCATCAGGAGAACGC	20	211	NM_001085444.1
		R	TGATGAACITGGGGTCGGTC	20		
7	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	F	TCGGAGTGAACGGATTGGGC	20	181	NM_001082253.1
		R	TTCCCGTCTCAGCCTTGAC	20		

while minimum and maximum concentrations were 0.15 and 19.65 nmol/mg respectively. Superoxide dismutase (SOD) enzyme activities was 169.70 ± 18.3 U/mg ($p < 0.002$) while minimum and maximum activities were 73.40 and 376.10 U/mg respectively. Catalase (CAT) enzyme activities in the liver was 8.70 ± 0.30 U/mg ($p < 0.010$) while minimum and maximum activities were 7.14 and 13.37 U/mg respectively. Reduced glutathione (GSH) concentration was 9.71 ± 0.49 μ mol/g ($p < 0.001$) while minimum and maximum glutathione reduced were 6.09 and 15.37 μ mol/g respectively (Table 5).

There was also significant difference in malondialdehyde (MDA) concentration of the rabbits' uterus, mean concentration was 1.70 ± 0.46 nmol/mg ($p < 0.001$) while minimum and maximum concentrations were 0.03 and 5.48 nmol/mg respectively. Total antioxidant concentration (TAC) was 244.00 ± 18.00 μ mol/mg ($p < 0.012$) while minimum and maximum antioxidant capacity were 138.00 and 510.00 μ mol/mg respectively. Superoxide dismutase (SOD) enzyme activities in the uterus was 144.40 ± 18.4 U/mg ($p < 0.002$) while minimum and maximum activities were 48.10 and 350.90 U/mg respectively. Catalase (CAT) enzyme activities in the uterus was 3.87 ± 0.02 U/mg ($p < 0.001$) while minimum and maximum activities were 3.73 and 3.97 U/mg respectively. Reduced glutathione (GSH) concentration was 9.52 ± 0.48 μ mol/g ($p < 0.001$) while minimum and maximum glutathione reduced were 6.88 and 14.36 μ mol/g respectively (Tables 6 and 7).

3.3. Patterns of primary antioxidant genes expression levels

In the liver, there was significant difference in relative expression of the primary antioxidant genes *sod1* ($p < 0.009$) and *gpx1* ($p < 0.004$) but there were no significant differences in relative expression of *bre* ($p < 0.65$) and *ucp1* ($p < 0.58$). Mean relative expression of *sod1* was 2.02 ± 0.35 fold changes while minimum and maximum expressions were 0.34

Table 5Effect of *Chlorella vulgaris* supplements on serum oxidative stress biomarkers in the rabbits.

Parameters	Control	T1	T2	T3	T4	p - values
MDA (nmol/mL)	23.90 $\pm$ 1.66 ^a	15.37 $\pm$ 2.24 ^b	16.88 $\pm$ 0.94 ^b	19.09 $\pm$ 0.92 ^b	16.78 $\pm$ 1.32 ^b	0.01
PCO (μ mol/mL)	7.74 $\pm$ 0.55 ^b	5.88 $\pm$ 0.22 ^a	6.99 $\pm$ 0.49 ^{ab}	9.61 $\pm$ 0.23 ^c	6.23 $\pm$ 0.30 ^a	0.001
TAC (μ mol/mL)	37.9 $\pm$ 4.50 ^a	112.5 $\pm$ 21.3 ^c	62.80 $\pm$ 20.10 ^{ab}	63.4 $\pm$ 20.05 ^{ab}	80.3 $\pm$ 5.75 ^{bc}	0.005
SOD (U/mL)	158.5 $\pm$ 9.50 ^{ab}	203.8 $\pm$ 22.97 ^b	146.2 $\pm$ 14.70 ^a	157.7 $\pm$ 13.10 ^{ab}	196.2 $\pm$ 3.80 ^b	0.04
CAT (U/mL)	3.80 $\pm$ 0.35	4.64 $\pm$ 0.57	5.13 $\pm$ 0.53	3.33 $\pm$ 0.58	3.41 $\pm$ 0.50	0.10
GSH (μ mol/mL)	7.18 $\pm$ 0.54 ^a	9.05 $\pm$ 0.40 ^{ab}	10.21 $\pm$ 0.22 ^{abc}	10.92 $\pm$ 0.47 ^{bc}	13.19 $\pm$ 2.27 ^c	0.02

a,b,c Means with different superscript in the same row are different ($p < 0.05$) for each of the parameters.

Control - group without supplement diet.

T1 - group supplemented with 200 mg/kg BW *Chlorella vulgaris* biomass.

T2 - group supplemented with 300 mg/kg BW *Chlorella vulgaris* biomass.

T3 - group supplemented with 400 mg/kg BW *Chlorella vulgaris* biomass.

T4 - group supplemented with 500 mg/kg BW *Chlorella vulgaris* biomass.

and 5.18 fold changes respectively. Antioxidant gene *gpx1* was up-regulated in all the treatment groups with mean fold change value of 5.89 ± 0.76 while minimum and maximum fold changes were 0.22 and 9.88 respectively. All treatment groups had significantly higher relative expression of *gpx1* compared with control group (Fig. 1). Relative expression of *bre* was not significantly different among the groups; mean

Table 4Effect of *Chlorella vulgaris* supplementation on performances of the rabbits at finishing stage.

Parameters	Control	T1	T2	T3	T4	SEM	p-value
Feed intake (g)	100.32 ^b	105.92 ^b	101.81 ^b	101.11 ^b	93.67 ^a	1.22	0.014
Initial body weight (g)	1031.50	987.55	1199.25	1065.25	1019.25	29.42	0.170
Final body weight (g)	2152.30 ^a	2322.70 ^{ab}	2545.30 ^{bc}	2428.20 ^{bc}	2471.30 ^{bc}	40.99	0.008
Weight after slaughter (g)	2103.10 ^a	2268.30 ^{ab}	2503.50 ^{bc}	2374.00 ^{bc}	2376.20 ^{bc}	39.07	0.004
Blood weight (g)	49.20 ^a	54.40 ^a	41.80 ^a	54.20 ^a	95.10 ^b	5.54	0.006
Commercial carcass weight (g)	1269.10 ^a	1332.70 ^{ab}	1666.10 ^b	1428.50 ^b	1402.20 ^b	34.36	0.001
Skin weight (g)	203.20 ^a	240.40 ^b	285.50 ^{bc}	234.20 ^b	221.50 ^{ab}	7.20	0.001
Feed conversion ratio	10.12	10.95	10.84	10.42	8.21	0.45	0.324

a,b,c Means with different superscript in the same row are different ($p < 0.05$) for the parameters measured.

Control - group without supplement diet.

T1 - group supplemented with 200 mg/kg BW *Chlorella vulgaris* biomass.

T2 - group supplemented with 300 mg/kg BW *Chlorella vulgaris* biomass.

T3 - group supplemented with 400 mg/kg BW *Chlorella vulgaris* biomass.

T4 - group supplemented with 500 mg/kg BW *Chlorella vulgaris* biomass.

Table 6Effect of *Chlorella vulgaris* supplements on liver oxidative stress biomarkers of the rabbits.

Parameters	Control	T1	T2	T3	T4	p - values
MDA (nmol/mg)	18.91 ± 0.38 ^a	0.54 ± 0.15 ^b	2.58 ± 1.00 ^{bc}	5.05 ± 1.31 ^d	5.99 ± 1.89 ^d	0.001
PCO (nmol/100mg)	7.00 ± 0.60	5.14 ± 0.32	8.08 ± 0.52	6.11 ± 1.53	7.00 ± 1.00	0.23
TAC (μmol/mg)	50.47 ± 1.08	59.10 ± 3.70	65.90 ± 4.92	60.3 ± 4.38	60.4 ± 1.06	0.28
SOD (U/mg)	110.10 ± 22.4 ^a	190.40 ± 18.10 ^b	135.3 ± 4.40 ^{bc}	126.213.18 ^{bc}	286.70 ± 52.4 ^c	0.002
CAT (U/mg)	9.30 ± 0.11 ^{bc}	8.33 ± 0.15 ^{ab}	8.05 ± 0.37 ^{ab}	8.00 ± 0.14 ^{ab}	10.35 ± 1.03 ^c	0.01
GSH (μmol/g)	6.30 ± 0.10 ^b	6.58 ± 0.38 ^b	5.30 ± 0.35 ^{ab}	5.00 ± 0.13 ^{ab}	8.85 ± 1.12 ^c	0.001

^{a,b,c} Means with different superscript in the same row are different (p < 0.05) for the parameters measured.

Control - group without supplement diet.

T1 - group supplemented with 200 mg/kg BW *Chlorella vulgaris* biomass.T2 - group supplemented with 300 mg/kg BW *Chlorella vulgaris* biomass.T3 - group supplemented with 400 mg/kg BW *Chlorella vulgaris* biomass.T4 - group supplemented with 500 mg/kg BW *Chlorella vulgaris* biomass.**Table 7**Effect of *Chlorella vulgaris* supplements on uterus oxidative stress biomarkers in the rabbits.

Parameters	Control	T1	T2	T3	T4	p-value
MDA (nmol/mg)	5.25 ± 0.10 ^c	2.57 ± 0.24 ^b	0.22 ± 0.10 ^a	0.38 ± 0.20 ^a	0.08 ± 0.01 ^a	0.001
PCO (nmol/100mg)	25.49 ± 3.66	22.96 ± 4.15	14.53 ± 1.08	24.57 ± 6.90	23.00 ± 2.88	0.40
TAC (μmol/mg)	149.50 ± 6.00 ^a	332.20 ± 64.15 ^b	255.50 ± 13.14 ^b	248.10 ± 9.80 ^b	235.00 ^{ab}	0.01
SOD (U/mg)	84.80 ± 13.2 ^a	165.10 ± 10.5 ^a	110.10 ± 9.6 ^a	100.90 ± 5.8 ^a	261.50 ± 5.8 ^b	0.002
CAT (U/mg)	3.85 ± 0.01 ^b	3.89 ± 0.02 ^c	3.93 ± 0.14 ^{bc}	3.94 ± 0.12 ^d	3.75 ± 0.01 ^a	0.001
GSH (μmol/mg)	8.36 ± 0.01 ^{ab}	7.59 ± 0.32 ^a	10.94 ± 0.39 ^{bc}	11.58 ± 1.02 ^c	9.17 ± 1.14 ^{abc}	0.02

^{a,b,c} Means with different superscript in the same row are different (p < 0.05) for the parameters measured.

Control - group without supplement diet.

T1 - group supplemented with 200 mg/kg BW *Chlorella vulgaris* biomass.T2 - group supplemented with 300 mg/kg BW *Chlorella vulgaris* biomass.T3 - group supplemented with 400 mg/kg BW *Chlorella vulgaris* biomass.T4 - group supplemented with 500 mg/kg BW *Chlorella vulgaris* biomass.

fold change for the gene was 1.28 ± 0.39 (p < 0.65); there were also no significant differences expression of *ucp1* 1.32 ± 0.46 (p < 0.58) minimum and maximum fold changes of the gene were 0.06 and 7.06

respectively.

Pattern of relative expression of primary antioxidant genes in the ovaries is also comparable with relative expression levels in the liver because antioxidant gene *gpx1* was significantly up-regulated although there were no significant differences in relative expression of *sod1* and *bre*; mean fold changes of *gpx1* was 2.07 ± 0.39 (p < 0.030) while minimum and maximum fold changes were 0.05 and 5.47 respectively. Mean relative expression of *sod1* gene was 1.76 ± 0.61 fold changes (p < 0.42) while minimum and maximum fold changes were 0.11 and 8.60 respectively. Relative expression of *bre* was 1.03 ± 0.24 fold changes (p < 0.23) while minimum and maximum fold changes were 0.12 and 2.86 respectively. The relative level of antioxidant genes expression in the ovaries indicated *sod1* and *gpx1* were higher in the treatment groups compared with control group (Fig. 2).

Comparative analysis of variance between relative expression of *sod1*, *gpx1* and *bre* using Mann-Whitney U statistics indicated that there was significant difference in level of *gpx1* expression between liver and ovaries (p < 0.001) but there were no significant differences in expression of *sod1* (p < 0.25) and *bre* (p < 0.48); however relative expression of the two genes ranked higher in liver than ovaries (Figs. 3 and 4).

4. Discussion

4.1. Effects of *Chlorella vulgaris* supplementations on performances of the rabbits

Chlorella vulgaris is a microalgae commonly cultivated on commercial scale in different parts of the world; its applications cut-across food industries, pharmaceuticals and animal production [20]. In animal

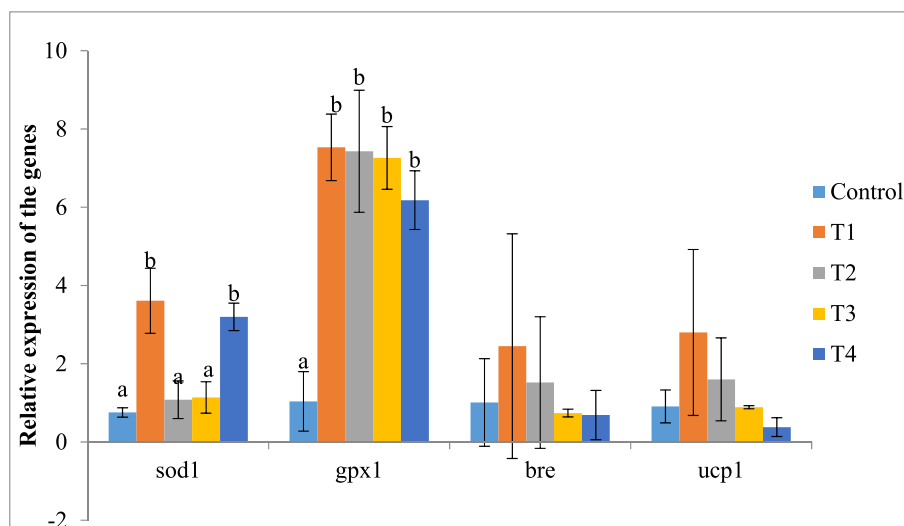


Fig. 1. Relative expression of primary antioxidant genes in liver of the rabbits. Bars with different labels are significantly different in expression of the genes determined (p < 0.05). Control - group without supplement diet. T1 - group supplemented with 200 mg/kg BW *Chlorella vulgaris* biomass. T2 - group supplemented with 300 mg/kg BW *Chlorella vulgaris* biomass. T3 - group supplemented with 400 mg/kg BW *Chlorella vulgaris* biomass. T4 - group supplemented with 500 mg/kg BW *Chlorella vulgaris* biomass.

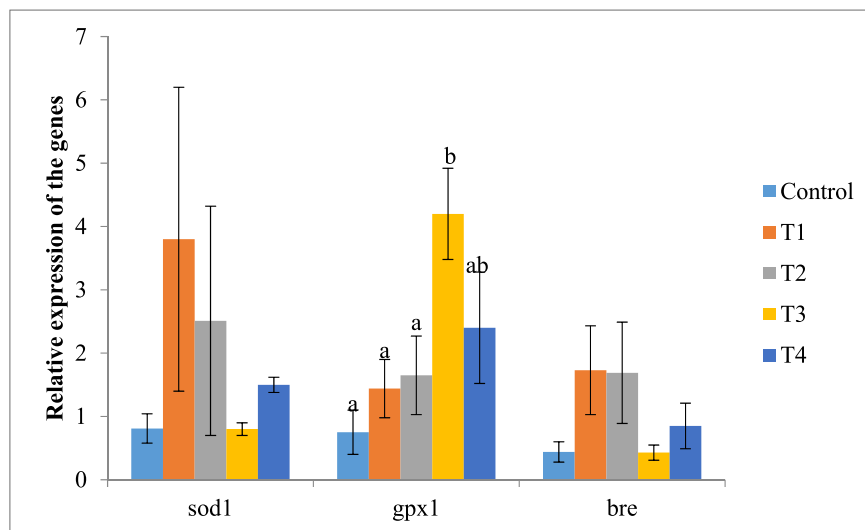


Fig. 2. Relative expression of primary antioxidant genes in ovaries of the rabbits. Bars with different labels are significantly different in expression of the genes determined ($p < 0.05$). Control - group without supplement diet. T1 - group supplemented with 200 mg/kg BW *Chlorella vulgaris* biomass. T2 - group supplemented with 300 mg/kg BW *Chlorella vulgaris* biomass. T3 - group supplemented with 400 mg/kg BW *Chlorella vulgaris* biomass. T4 - group supplemented with 500 mg/kg BW *Chlorella vulgaris* biomass.

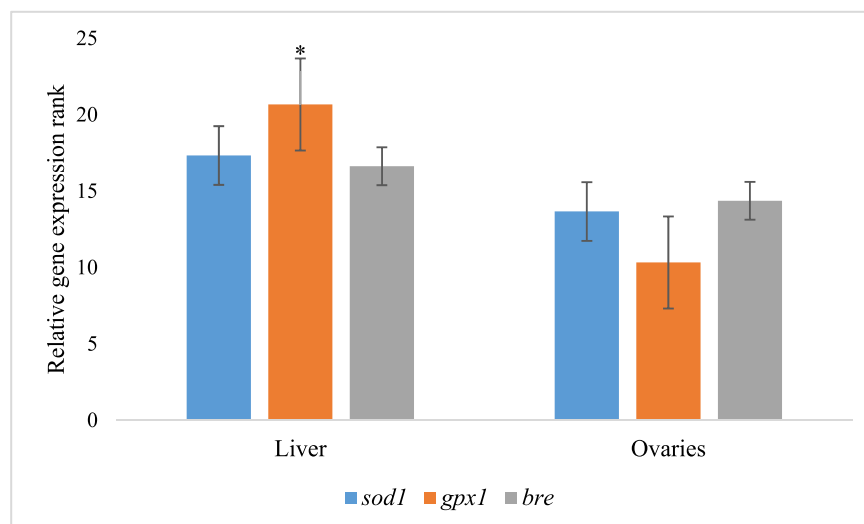


Fig. 3. Comparative expression of primary antioxidant genes in the rabbits' liver and ovaries (* $p < 0.05$). Control - group without supplement diet. T1 - group supplemented with 200 mg/kg BW *Chlorella vulgaris* biomass. T2 - group supplemented with 300 mg/kg BW *Chlorella vulgaris* biomass. T3 - group supplemented with 400 mg/kg BW *Chlorella vulgaris* biomass. T4 - group supplemented with 500 mg/kg BW *Chlorella vulgaris* biomass.

production, it is being used as animal and fish feeds additives because of its nutritional profile [21]. In this present study, supplementation of the microalgae to rabbits showed that it is a potential for improving production performances and efficiencies of feeds utilization because despite reduction in feed intake as results of the supplementation final body weight of the rabbits in the treatment groups were higher compared with control group (Table 4). The reduction in feed intake was not because of non-palatability of the microalgae or as results of toxicity but due to improved efficiency of feed utilization of the animals which was evident with their higher final body weights compared with control; furthermore, feed conversion ratio of the control was higher compared with the treatment groups. All these are indications that supplementation of the microalgae has significant economic benefits since it reduced feed intake, improved feed conversion ratio and increased productivity.

Chlorella vulgaris supplementation in this study due to reduction in feed intakes however cannot be interpreted to be that the rabbits rejected feed due to the supplement because productivity was not compromised in fact it increased. This justification agreed with submission of Halle et al [22] who observed that supplementation of *Chlorella vulgaris* in laying birds led to reduction in feed intake without reduction in productivity of

the birds. In fact, all the birds' productivity indicators increased in the supplemented groups; according to the report production performances were significantly higher compared with control group because the supplemented groups had higher egg weight, higher laying percentages, higher hen day production and lower feed conversion ratio.

Similarly, supplementation of *Chlorella vulgaris* in fish was also reported to improved productivity, dietary lipid utilization and muscle pigmentation which are all identified as products quality improvement in fish [23]. *Chlorella vulgaris* supplementation and reduced feed intake in this study could be linked with improved biodiversity of intestinal microbes of the rabbit thereby contributing to better gut health. Supplementation of the microalga since it is a green alga with enormous amount of starch and cellulose led to increased population of lactic acid bacteria and ability of the gut microflora to degrade algal polysaccharides and other complex plant polymers in the basal feed increased and in overall led to better digestive efficiency of the animals which was the reason why despite the reduced feed intake, there was higher product yields [23].

This is possible in rabbits because they are hindgut fermenters capable of degrading complex biomolecules in their caecum as result of the increased microflora population and diversity. Again, as a way of

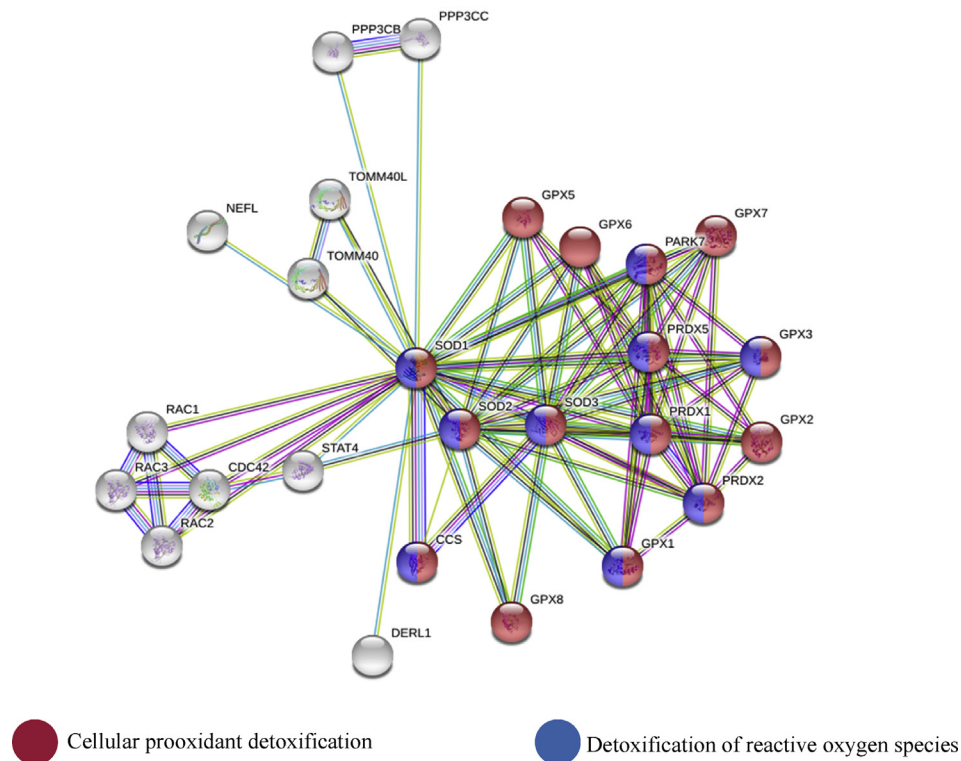


Fig. 4. Functional enrichment analysis of superoxide dismutase gene expression biological impacts in the rabbits.

improving gut health, the diversity is capable of reducing population of non-beneficial microbes in the guts of the rabbit and hence contributed to improvement of the animals' health and productivity [24]. It can be deduced from this study thereof that supplementation of *Chlorella vulgaris* is a strategy for reducing rabbits feed intake, improving gut health and productivity. Higher finishing weights of the supplemented groups, higher commercial carcass weight as well as higher skin weight are indications attestation to improved nutritional efficiencies as result of the supplementation. These observations are in agreements with previous studies on *Chlorella vulgaris* feeding in other animal species where it was regarded as microalga with potential nutritive values capable of enhancing some biochemical and physiological functions for improved immunity and growth [25].

4.2. Effects of *Chlorella vulgaris* supplements on oxidative stress status of the rabbits

Oxidative stress products generation is a major patho-physiological mechanism of oxidative stress damages; malondialdehyde and protein carbonyls are leading among these products and their increasing concentration corresponds with poor performance [26]. In this study, supplementation of *Chlorella vulgaris* significantly reduced generation of these products on one hand and on the other hand it led to increased total antioxidant concentration in the serum, uterus and liver of the rabbits. Significant reduction of these products in all these tissues indicated that the supplement diets contributed to overall antioxidant defense system of the rabbits across different tissues of the animals' body. Specifically, the supplementation led to inhibition of malondialdehyde production which is a cellular mechanism protecting cell membrane from lipid peroxidation damage; and a cellular mechanism for promoting animal production and performance. Therefore, it could be derived that protection against oxidative stress due to the supplementation is a strategy for promoting animal production performances [27].

Chlorella vulgaris is a rich source of polyphenolic compounds [28];

hence, reduction in oxidative stress products concentration in this study can be linked with antioxidant capabilities of the polyphenolic contents of the microalgae. Polyphenols are bioactive compounds capable of reducing oxidative stress products if they are biologically available in animals' gut for absorption. Since rabbits are hind-gut fermenters, there is possibility of maximum bioavailability of the polyphenols because both enzymatic and microbial degradation of bounded polyphenols takes place within the gastro-intestinal tracts [29]. The higher bioavailability of polyphenols as results of these multiple absorption was responsible for the increased total antioxidant concentration in the supplemented animals in this study. The absorbed polyphenols from the microalgae at cellular level hence inhibited oxidative stress and its associated compromise while several repeats of this process could lead to non-disturbance of cellular activities and therefore reduced oxidative stress products generation [30]. In this present study, significantly decreased generation of oxidative stress products across different body tissues and systems implied that at the minimal and maximal levels of supplementation used in this study, oxidative stress can be attenuated using microalgae *Chlorella vulgaris* because of bioactive compounds it contained. Some of these bioactive compounds including carotenes, zeaxanthin, astaxanthin, fucoxanthin, vitamins and minerals were reported in studies where similar microalgae fed to rabbits led to decrease in production of oxidative stress products including malondialdehyde [31, 32].

Lipid peroxidation is the chief oxidative stress effect and most prevalent form of oxidative stress compromise at cellular level; it is a toxic process to the cells causing cell loss of membrane integrity, cell death and activation of genetic programme cell death [33]. Compromised reproductive inefficiencies in male such as spermatozoa dysfunctions, low epididymis sperm concentrations, poor sperm motility and abnormal sperm morphology are also linked with increasing lipid peroxidation [34]. In female, infertility triggered by endometriosis is also reportedly linked with increased production of oxidative stress products specifically increasing levels of malondialdehyde; furthermore, lipid peroxidation is

also mechanism of oxidative stress associated with decline oocytes quality [35, 36]. Therefore, for improving reproduction and promotion of reproductive health according to this present study, *Chlorella vulgaris* supplementation is an option due to its capacity to inhibit lipid peroxidation. In agreement with this, earlier studies also reported inhibition of lipid peroxidation and improved performances using varied derivatives of *Chlorella vulgaris* in different models [37, 38, 39, 40].

4.3. Effects of *Chlorella vulgaris* supplementations on antioxidant enzymes activities and reduced glutathione concentrations in the rabbits

In this study, supplementation of microalgae *Chlorella vulgaris* led to enhanced antioxidant enzymes activities as well as increased concentration of reduced glutathione; these are indicators that the alga has strong antioxidant capabilities. In animal production, this is beneficial because it demonstrated that at minimal supplementation levels the microalgae is an additive whose supplementation is capable of improving productivity. In a similar submission Lee et al [41] stated that short-term *Chlorella vulgaris* supplementation improved activities of antioxidant enzymes. The enhancement of antioxidant status by supplementation of *Chlorella vulgaris* can be biologically explained to be as results of complementary roles of antioxidants in the microalga which contributed to free radical scavenging capacities of the internal antioxidant system serving as second line of defence and thereby increase activities of primary antioxidants enzymes activities. This underlying biochemical mechanism led to conservation of primary antioxidant enzymes depletion hence higher activities [42].

Modulation of antioxidant enzymes by *Chlorella vulgaris* in this study is in agreement with previous reports that supplementation of *Chlorella vulgaris* in human or animal models [43]. *Chlorella vulgaris* increases total antioxidant capability of body systems and tissues, and kept degradation of cellular membrane at bay due to inhibition of lipid peroxidation. These are the reasons for increased activities of the antioxidant enzymes [44, 45, 46]. Higher concentrations of reduced glutathione in animals of the treatment groups compared with control also signified that supplementation of *Chlorella vulgaris* led to adequate elimination of hydrogen peroxide because glutathione as a non-enzyme antioxidant has complex metabolic role and capable of decomposing hydrogen peroxides.

Biochemical activities of glutathione apart from decomposition of hydrogen peroxides also involve enhancement of the primary antioxidant enzymes activities, and participation in third line of defence such as cellular protection against toxic agents resulting from lipid peroxidation and restoration of cellular balance of reduced and oxidized glutathione [47]. These mechanisms of reduced glutathione in oxidative stress protection is complex antioxidant defence process that can be associated with the supplementation of *Chlorella vulgaris* in this study because apart from the increase concentration of glutathione, there was also significant higher activities of catalase antioxidant enzymes in the treatment groups compared with control which indicated that the treatment groups had better hydrogen peroxide decomposition potential compared with the control group [48].

4.4. Effects of *Chlorella vulgaris* supplementations on relative expression of primary antioxidant genes in liver and ovaries of the rabbits

This study outcomes of gene expression showed that supplementation of *Chlorella vulgaris* to the rabbit exhibited protection against oxidative stress across all lines of oxidative stress defence systems because significant up-regulation of the primary antioxidant genes *sod1* and *gpx1* indicated that at immediate cellular defence stage; there is adequate protective actions of antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase in suppression, detoxification and activation of free radicals dismutation as well as capabilities for decomposition of hydrogen peroxides and overall prevention of free radicals formation by inhibition of mechanisms leading to their production [49].

It is also evident that the up-regulations of these genes in the

supplemented rabbits confirmed molecular relevance of *Chlorella vulgaris* as a source of antioxidants capable of preventing metabolic and reproductive oxidative stress damages. This could be attributed to complementary actions of the microalga antioxidant components including carotenoids, astaxanthin, fucoxanthin, vitamins and minerals which are bioactive compounds capable of chain breaking, scavenging and neutralizing free radicals. These are also factors leading to up-regulation of these genes [50].

Sod1 a protein-coding gene with capacity of binding copper and zinc ions is one of the enzymes primarily responsible for protection against superoxide radical destruction. It is also active against superoxide radical and hydrogen peroxide in both the cytoplasm and mitochondria therefore its up-regulation as result of *Chlorella vulgaris* supplementation indicated complete cellular protection. *Sod1* is also involved in pharmacokinetic and reactive oxygen species detoxification pathways hence the microalga is a drug candidate in development of therapeutic solution for oxidative stress attenuation [51, 52].

In this study, it is also evident that as the rabbit matures there are increasing metabolic activities due to growth and development of reproductive organs leading to cellular generation of reactive oxygen species and superoxide radicals [53]. However, in the supplemented group the superoxide radicals were converted to hydrogen peroxide (H_2O_2) by superoxide dismutase activities. This is because in the supplemented group there was higher adequate cyclical reaction of neutralizing H_2O_2 into water and molecular oxygen; this was why the supplemented rabbit had lowered levels of oxidative stress status via reduced malondialdehyde concentration, reduced protein carbonyl concentration and higher total antioxidant capacity [54]. Molecular basis of these protective mechanisms was the up-regulation of the antioxidant genes.

Furthermore, up-regulation of *sod1* due to supplementation of *Chlorella vulgaris* in this study indicated that the microalga is an excellent source of antioxidant for oxidative stress attenuation because *sod1* gene is associated with several other genes working together for cellular prooxidants detoxification, response to toxic compounds in the cells, and detoxification of reactive oxygen species. This is in agreement with Ismail et al [55] who reported that supplementation of *Spirulina* which is also a microalga contributed to oxidative stress protection in a network of gene relationships linked with up-regulation of *sod1*. These genes along with *sod1* jointly contribute to cellular prooxidant detoxification and specifically detoxification of reactive oxygen species (Fig. 3).

Glutathione peroxidase (*gpx1*) is another antioxidant gene up-regulated as result of the supplementation of *Chlorella vulgaris* in this study; the gene is also a protein coding gene and it belongs to peroxidase family whose responsibilities include protection against oxidative stress. The gene when expressed is responsible for reduction of organic hydroperoxides and detoxification of hydrogen peroxide. Up-regulation of these genes is associated with hydrogen peroxide detoxification and catalytic activation of drugs against oxidative stress and selenious acid related therapeutic agents [56, 57, 58]. Molecular advantage of this is that the microalgae *Chlorella vulgaris* can be exploited as feed additive and or drug agent with bioactive compounds that can contribute to favourable cellular transcriptions.

The gene is expressed in both liver and ovaries indicating that metabolically and reproductively there was adequate oxidative stress protections in the rabbits. This is ultimate benefits from *Chlorella vulgaris* supplementation because up-regulation of *gpx1* is a promoter of oxidoreductase catalytic activities at cellular levels. This is a molecular protective catalysis of an oxidation-reduction (redox) reaction; it is a reversible chemical reaction in which oxidation state of an atom or atoms within a molecule is altered; two substrates are involved and one of the substrate acts as electron donor then becomes oxidized, while the other acts as electron acceptor and becomes reduced [59]; this cellular mechanism is responsible for interactive relationship between *gpx1* and *sod1* for protection against oxidative stress. Molecular function of *gpx1* expression is also related to enhancement of glutathione-transferase

activities; biological cellular mechanism promoting total antioxidant protection. The implication of this is that up-regulation of *gpx1* could enhance activities of glutathione-S-transferase which again signified that the microalga supplementation contributed to total antioxidant protection [60].

Specific benefits of this gene in enhancement of glutathione-S-transferase activities is that the enzyme has capacity to protect against neuron oxidative stress damages and by extension it could be deduced that supplementation of microalga *Chlorella vulgaris* can be exploited as supplement nutraceutical for protection of oxidative stress associated with nervous systems [61]. Deficiency of glutathione peroxidase expression is related to occurrence of many diseases including haemolytic anaemia, diabetes mellitus, ischemia and retina vascular diseases among others; what is common to all these diseases however is oxidative stress complexities. Therefore, it up-regulation in the treatment groups could explain the reason for reduced lipid peroxidation products, higher antioxidant potential and enhanced antioxidant enzymes activities since its deficiency is related to higher oxidative stress damages [62].

Co-expression of glutathione peroxidase and *sod1* can be regarded as another important biological benefit of *Chlorella vulgaris* supplementation effects in oxidative stress protection in this study because this interaction inhibits cellular modification of amino acids metabolism and can be used to adjudge *Chlorella vulgaris* as source of antioxidants with wide range of biological protective activities against oxidative stress. The interactive co-expression identified between *sod1* and *gpx1* in this present study agreed with submissions efficacy of *Chlorella vulgaris* as source of antioxidant; the co-expression was responsible for effective protection against oxidative stress [63, 64].

5. Conclusions

Oxidative stress is an inevitable biochemical process compromising animal performances and the implications transcend beyond physiological dysfunction to economic loss since it manifested as relevant mediator of reduced animal productivity in form of lower finishing weights as discovered in this study. Dietary supplementation of *Chlorella vulgaris* could serve as nutritional strategy for attenuation of oxidative stress for improving animal performance and reproduction because its supplementation led to higher finishing weights, higher commercial carcass yields as well as uteri-ovarian oxidative stress protector which could contribute to better reproduction. Supplementation of *Chlorella vulgaris* also manifested its antioxidant capabilities in multiple folds; from inhibition of oxidative stress products generation, to enhancement of antioxidant enzymes activities and up-regulation of primary antioxidant genes in liver and ovaries as important organs responsible in coordination of metabolic and reproductive activities. Therefore, the microalgae *Chlorella vulgaris* was recommended as supplement for protection against oxidative stress and improved productivity in rabbits and other food producing mammalian species. In addition, further studies into assessment of its effects on expression of transcripts and immune modulation genes in animal models is warranted in order to established its potential as nutraceutical.

Declarations

Author contribution statement

Sikiru A.B.: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Arangasamy A.: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Alemede I.C., Egena S.S.A.: Conceived and designed the experiments;

Wrote the paper.

Guvvala P.R.: Analyzed and interpreted the data. Ippala J.R., Bhatta R.: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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