INFLUENCE OF *CHLORELLA VULGARIS* SUPPLEMENTED DIETS ON OXIDATIVE STRESS AND REPRODUCTION PERFORMANCE OF NEW ZEALAND WHITE RABBITS

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MARCH, 2021

ABSTRACT

This study investigated antioxidant capacity of microalga *Chlorella vulgaris* on oxidative stress attenuation for improved reproduction in rabbits. The study commenced with an *in-vitro* evaluation of the microalga as a source of antioxidants followed by supplementation of 0, 200, 300, 400, and 500 mg *Chlorella vulgaris* biomass per kilogram bodyweight of rabbits in groups designated as Control, T1, T2, T3, and T4 respectively in studies cutting across the different physiological states including pre-pubertal, gestation, lactation, and finishing stages. Forty weaner rabbit does each were studied for the prepubertal, gestation and finishing stages, while 35 and 75 rabbit does and male rabbit kits of the does were studied during the lactation and prepubertal stages respectively. The experimental design implemented at each of the physiological stage was completely randomized design. The animal management practices include the provision of individual housing for the rabbits in cages, *ad-libitum* provision of basal feed, and water, and provision of kindling boxes towards period of parturition. Data on the rabbit's performances, serum, and tissues oxidative stress biomarkers were obtained while in addition, patterns of regulation and expression of genes associated with oxidative stress, reproductive performance, immune, and growth regulations were determined in the rabbit kits and the weaner rabbits reared from weaning to finishing stage. The microalga intakes improved reproductive index of the rabbit does whereby the group with highest intake of the microalga had a reproductive index of 7.00 against the control which had a reproductive index of 5.36 ($p = 0.01$). Serum gestational malondialdehyde concentration was reduced because 13.54, 14.05, 11.82, and 13.54 nmol/mL were determined in the serum of rabbits in the groups T1, T2, T3, and T4 respectively, against 28.85 nmol/mL determined in the serum of rabbits in the control group during the gestation period ($p = 0.003$). The microalga intake reduced feed intakes without reduction in commercial carcass yield and feed to meat production of the rabbits studied till finishing stage ($p = 0.001$). The supplementation of the microalga led to the up-regulation of genes including SOD1, GPX1, GSTP1, and CYP1A1 in rabbits of the treatment groups against the control ($p = 0.05$). Furthermore, gestational maternal intake of the microalga led to kindling of rabbit kits with higher birthweights whereby 40.22, 39.33, 41.73, and 50.23 g, were recorded for the kits kindled by rabbits in T1, T2, T3, and T4 respectively, against 34.89 g, for the Control ($p = 0.04$). Following the analyses of the recorded data, it was concluded that supplementation of *Chlorella vulgaris* biomass in rabbits at different physiological states protected the rabbits against oxidative stress and led to improved reproductive index, higher productivity, and growth. Therefore, intakes of the microalga biomass at 200, 300, 400, and 500 mg per kg bodyweight of rabbits were recommended as suitable dosages for performances improvement in rabbits from pre-pubertal to finishing stages.

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ABBREVIATIONS, GLOSSARIES, AND SYMBOLS

ALT	-	Alanine Amino Transferase
ANOVA	-	Analysis of Variance
AOAC	-	Association of Official Analytical Chemists
AST	-	Aspartate Amino Transferase
ATP	-	Adenosine Tri Phosphate
BHA	-	Butylated Hydroxyl Anisole
BHT	-	Butylated Hydroxyl Toluene
BRE	-	Brain and reproductive organ-expressed (TNFRSF1A modulator)
BW	-	Body Weight
CAT	-	Catalase
CDNB	-	1-chloro, 2,4-dinitrobenzene
CF	-	Crude Fibre
CP	-	Crude Protein
CuZnSOD	-	Copper-Zinc SuperOxideDismutase
CV	-	*Chlorella vulgaris*
DFID	-	Department for International Development
UNGA	-	United Nations General Assembly
DM	-	Dry Matter
DMRT	-	Duncan Multiple Range Test
EDTA	-	Ethylene Diamine Tetra Acetic
ELISA	-	Enzyme-Linked Immunosorbent Assay
ELU	-	Experimental Livestock Unit
FAO	-	Food and Agricultural Organization
FDA	-	Food and Drugs Administration
FRAP	-	Ferric Reducing Antioxidant Power assay

GAPDH	-	Glyceraldehyde-3-phosphate dehydrogenase
GHI	-	Global Hunger Index
GnRH	-	Gonadotropin Releasing Hormones
GNRHR	-	Gonadotropin releasing hormone receptor
GPX	-	Glutathione Peroxidase
GPX1	-	*Oryctolagus cuniculus* glutathione peroxidase 1
Hb	-	Haemoglobin
HPO	-	Hypothalamic-Pituitary-Ovarian
HPS	-	Hydrogen Peroxide Scavenging Potential
HSP - CUPRAC	-	Hydrogen Peroxide Scavenging - Cupric Reducing Antioxidant Capacity
ICAR	-	India Council for Agricultural Research
IFPRI	-	International Food Policy Research Institute
KISS1	-	KiSS-1 metastasis-suppressor
KNDY	-	Kisspeptin-Neurophin-and-Dynorphin
LH	-	Luteinizing Hormones
LPHSI	-	Livestock and Poultry Heat Stress Indices
ME	-	Metabolizable Energy
MnSOD	-	Manganese SuperOxideDismutase
MY	-	Milk Yield
NCBI	-	National Centre for Biotechnology Information
NFE	-	Nitrogen Free Extract
NIANP	-	National Institute of Animal Nutrition and Physiology
NIFA	-	National Institute of Food and Agriculture
NLM	-	National Library of Medicine

NO	-	Nitrogen Oxide
NR3C1	-	Nuclear receptor subfamily 3 group C member 1
OVGP1	-	Oviductal glycoprotein 1
OXSR1	-	Oxidative stress responsive 1
PCO	-	Protein Carbonyl
PCV	-	Packed Cell Volume
PGK1	-	Phosphoglycerate kinase 1
PHA	-	Phytohaemagglutinin
RBC	-	Red Blood Cell
RI	-	Reproductive Index
RNA	-	Ribonucleic acid
RNS	-	Reactive Nitrogen Species
ROS	-	Reactive Oxygen Species
RT-qPCR	-	Real Time Quantitative Polymerase Chain Reaction
SDGs	-	Sustainable Development Goals
SLC2A1	-	Solute carrier family 2 member 1
SLC2A5	-	Solute carrier family 2 member 5
SLC6A16	-	Solute carrier family 6 member 16
SOD	-	Superoxide Dismutases
SOD1	-	*Oryctolagus cuniculus* superoxide dismutase 1
STP	-	Serum Total Protein
THI	-	Temperature-Humidity-Index
TPC	-	Total Phenolic Content
TWH	-	Trivers-Willard Hypothesis
UAR	-	Understanding Animal Research

UCP1	-	Uncoupling protein 1
UN	-	United Nations
UNICEF	-	United Nations International Children's Emergency Fund
USA	-	United States of America
WBC	-	White Blood Cell
WFP	-	World Food Programme
WGL	-	Weight Gain of the Litter
WHO	-	World Health Organization
WRSA	-	World Rabbit Science Association

CHAPTER ONE

1.0. INTRODUCTION

1.1. Background of the Study

Reproduction primarily is a process of duplicating and or replicating a given entity. Biologically, reproduction is a process of producing offspring through sexual or asexual means and it is synonymous to breeding; procreation; propagation; and multiplication of a living organism. In sexual reproduction, there is usually an input by both male and female partners whereby there is a fusion of male and female gametes in a process known as fertilization to form a zygote which later developed into an offspring. While sexual reproduction involves a fusion of gamates from both sexes; asexual reproduction, on the other hand, leads to the production of an offspring from a single organism through a process known as cell division to produce daughter cells (Dictionary.com, 2018).

In livestock production, reproduction has both biological and economic importance because it ensures the existence of animals and also determines returns to producers while directly affecting consumers' spending on procurement of meat and other animal products for consumption. Optimum reproduction in a given animal population promotes the availability and existence of the animal species and in addition is capable of increasing revenue of the animal producers. This makes reproduction an integral part of livestock production and management. Meanwhile, the productivity of foods and raw materials for human consumption and industrial uses from animals depend on reproductive efficiency of the animals. Failure in reproduction is the cause of economic loss currently facing livestock production; therefore, a leading priority of animal

production research is improved reproductive outputs of animals according to recommendations of National Institute of Food and Agriculture (NIFA, 2018).

Reproductive failures limiting the productivity of animals are being caused by several factors including abnormal reproductive cycles, embryonic or foetal loss, neonatal mortality, delayed attainment of puberty, poor sperm production and poor fertility rates. In all the listed reproductive inefficiencies, there is a common biochemical mechanism that contributes significantly to their pathophysiological development; the mechanism is oxidative stress. It is a metabolic process of imbalance between prooxidants and antioxidants favouring abundance and overbearing effects of the prooxidants. When this occurs in the body, there is disruption of redox signals, compromised biochemical pathways and dysfunctional controls leading to damages in the body systems. Oxidative stress based on sources of generation of the stress is categorized as photo-oxidative stress, drug-dependent oxidative stress, metabolic oxidative stress, and environmental oxidative stress (Garcia, 2013).

However, irrespective of sources of generation and causes; what is common to oxidative stresses is that there is always an imbalance between prooxidants and antioxidants favouring prooxidants anytime there is oxidative stress occurrence (Woÿniak *et al.*, 2003). Food shortage, hunger, and poverty are leading challenges of the developing world; available data suggest that there are insufficient supply and intakes of foods across the developing countries especially protein intakes. According to reports of the Food and Agricultural Organization (FAO) of the United Nations (UN), as of the year 2001, estimated daily animal protein intake in developing countries including Nigeria was 4.5 g *per capita* which was very low compared with the minimum global requirement of 35 g *per capita*. Even after more than a decade into the millennium, *per*

capita intake of animal protein was still 10 g/head/day in Nigeria which is less than average for middle-income developing countries (16 g/head/day) not to even mention the global recommended average; these are indications that malnutrition especially shortage of animal protein intake in Nigeria and other developing countries is a problem (FAO, 2015).

Similarly, World Food Programme (WFP, 2016), also reported that one third of children less than five (5) years old in Nigeria are stunted due to poor nutrition and this is twice the rate of such incidence in Thailand and three times that of Tunisia which are also developing countries like Nigeria – hence case of severe malnutrition in the country is worse compared with her peers. This phenomenon also suggests that children in many remote communities across Nigeria are four times more likely to experience malnutrition than children in less remote communities. Furthermore, according to reports of the International Food Policy Research Institute (IFPRI, 2016), about 48.5 percent of women of reproductive age in Nigeria are malnourished and anaemic; a situation ranking Nigeria 172nd best out of 185 countries in the world examined for the menace.

The situations above are problems requiring solutions through increased production of animals in the right quantity and quality capable of addressing poor animal protein intake in Nigeria. Apart from increasing animal protein supply for human consumption, efforts towards increased animal production will also be contributing to eradication of hunger and poverty which are part of United Nations (UN) mission for sustainable development by the year 2030; the mission entails the comprehensive efforts to ensure that every man, woman, and child enjoy their right to adequate food; women are

empowered; priority is given to family farming, and food systems everywhere are sustainable and resilient (UN, 2015).

Nigeria a country characterized by high ambient temperature considering its location within the Tropics is naturally prone to stress arising from environmental factors; these have a great toll on animal performances. For example, according to Mailafia *et al*. (2010), it was stated that the high ambient temperature which is common in Nigeria has negative implications on both male and female rabbits' reproductive performances; in male rabbits, it reduces fertility while in female rabbits, it causes embryo mortality. Apart from these, environmental borne stress contributes to reduced growth and product yield and qualities. The occurrence of diseases and survival of pathogenic organisms are also not left out of the negative effects of environmental stress. Direct implication of environmental stress is the promotion of an underlying pathophysiological condition known as oxidative stress which is a mechanism favouring increasing production of reactive oxygen species and compromising of animals' performance. Therefore, exploring and correcting poor livestock productivity associated with oxidative stress using *Chlorella vulgaris* biomass in rabbits as a model for amelioration of stress for improved food production animals was the focus of this study. This aimed at testing the efficacy of natural antioxidants present in the microalga *Chlorella vulgaris* on the attenuation of oxidative stress and improve performances at physiological reproductive stages of pre-pubertal, gestation and lactation as well as its foetal programming effects on the rabbits' offspring.

1.2. Statement of the Research Problem

Reproductive challenges are leading problems facing the development and growth of the livestock industry worldwide without the exception of Nigeria (Sheldon and Dobson, 2003). These challenges require the attention of animal scientific investigations because the supply of animal products including milk, meat, and egg all depends on successful reproductive processes. There will be no production of any animal products if animals are not reproductively efficient; therefore, enhancements of fertility and prolificacy of animals in the face of current challenges are major problem of animal production science (Gordon, 2018). Advancement in the fields of molecular and genomics studies has also contributed to identification of poor reproduction as factor limiting productivity of animals because its application has led to identification of oxidative stress impacts on regulations of fertility and allied genes as major factors affecting reproductive mechanisms from oocytes production to embryo development, and early post-birth performance of animals (Slimen *et al*., 2016; Baruselli *et al*., 2017).

Significant increase in the exposure to oxidative damages over time is associated with reproductive inefficiencies because of the reduced antioxidant defense due to the accumulation of prooxidants. Hence, it was hypothesized that oxidative stress damage occasioned by overproduction of prooxidants negatively affect reproduction because oxidative stress negatively affects reproductive mechanisms associated with oocytes production, embryo development, litter size, conception rates, lactation performance, and weaning performances (Stier *et al*., 2012). Also, reproductive activities require more investment of energy which makes it naturally susceptible to oxidative stress via leakages of free radicals within the electron transfer chain (ETC) from oxidative stimulation of NADPH via phosphorylation causing cellular and tissue damages; this

according to Bryant, (1997); Speakman, (2008); and Merkling *et al*. (2017) is inevitable endogenous cause of oxidative stress associated with reproduction. However, the specific antioxidant roles of some of the microalgae in attenuation of oxidative stress and promotion of animal performances still remain largely not established.

1.3. Justification of the Study

Livestock production and their development are important for the survival of human society because, despite the continuous increase in food production, millions of people are still experiencing serious hunger and or malnutrition. The bulk of these malnourished people are living in the developing and poor countries where population growth is out-powering food production. To solve this problem, development of affordable livestock production is a way out to increase food (protein) supply for human consumption. This will not only provide food on the table of poor people but can also serve as a source of income generation and employment in addition to its roles as a contributor to nutritional management against malnutrition.

Rabbits used as model animals in this study are justified because rabbits are a practically cost-effective and efficient animal to speed-up research and development in reproductive science. These animals are small, they have a short lifespan, making costs, space, and time required to perform research manageable. Rabbits are the most common model animals used across the world for investigations into animals and human ailments because the genome sequence of rabbits is similar to most mammals and man. Reproductive studies on sperm and oocytes transportation, spermatozoa maturation and fertilization, genetics of gametes, oviduct physiology, embryo *in-vitro* culture and developmental biology of the embryo have all been conducted using rabbit models (Bernd *et al*., 2012).

This present study hopes to establish the potential of *Chlorella vulgaris* as a natural source of antioxidants for the promotion of performances in the rabbit as a model for the establishment of the alga as functional feed resources in animal production. Natural antioxidants in the microalga were identified as promising bioactive compounds capable of eliminating damaging effects of oxidative stress at systems, tissues, cellular and molecular levels. The microalgae contained bioactive compounds with de-radicalizing power using their double bonds to neutralize free radicals and are also capable of modulating gene expression of antioxidant pathways (Di Pietro *et al*., 2016; Zuluga, 2017). These include astaxanthin, beta-carotene, alpha-carotene, glutathione, and lutein present in the microalga whose mechanisms of action is formation of ortho-dihydroxyl-conjugated polyene which is a chain-breaking antioxidant complex (Naguib, 2000; Santocono *et al*., 2007; McNulty *et al*., 2008).

Exploring and correcting poor livestock productivity associated with the negative effects of oxidative stress using *Chlorella vulgaris* biomass for promotion of food security and sufficiency using rabbits as models need to be investigated. This will aid in testing the efficacy of natural antioxidant present in the microalga *Chlorella vulgaris* on the attenuation of oxidative stress and improve performances at different physiological states such as pre-pubertal, gestation, and lactation as well as nutritional foetal programming of rabbit offspring against oxidative stress. Rabbits are suitable animal models for these types of studies because rabbits are animals reported for use in investigating and understanding the impact of the embryo and foetal development on offspring and adult health. These are backed-up with pieces of evidence from functional genomics studies by Learndri *et al*. (2009), Picone *et al*. (2011), and Durathon *et al*. (2012).

Finally, the low *per capita* protein intake and malnutrition in humans in Africa and beyond which required increased livestock output and the need to overcome the growing menace of poor reproduction in food-producing animals, as well as health hazard associated with the use of synthetic antioxidants justified exploration of this microalga as antioxidant source as carried out in this study. Therefore, there is a need for scientific research focusing on cost effective means of using microalgae for oxidative stress management and improved productivity in food-producing animals instead of the hazardous synthetic antioxidants.

1.4. Aim and Objectives of the Study

This study aim is assessment of the *in-vitro* antioxidant capacities of *Chlorella vulgaris* biomass and its *in-vivo* oxidative stress attenuation in rabbit at different reproductive stages.

The aim was achieved through the following objectives:

i. determination of chemical and proximate composition of *Chlorella vulgaris* biomass and its *in-vitro* antioxidant capabilities.

ii. determination of serum and tissues concentrations of malondialdehyde, protein carbonyl and total antioxidant capacities as biomarkers of oxidative stress in rabbits supplemented with *Chlorella vulgaris* at pre-pubertal, gestation, lactation and finishing stages.

iii. determination of antioxidant enzymes activities as mechanisms of oxidative stress attenuation in rabbits supplemented with *Chlorella vulgaris* at pre-pubertal, gestation, lactation and finishing stages.

iv. assessment of improvement in performance characteristics and reproductive indices of rabbits supplemented with *Chlorella vulgaris* at pre-pubertal, gestation, lactation and finishing stages.

v. assessment of estrogen and testosterone levels and serum biochemical profiles in F1 progenies of rabbits does which were supplemented with *Chlorella vulgaris* during gestation period.

vi. determination of the serum and tissues concentrations of malondialdehyde, protein carbonyl content and total antioxidant capacities as biomarkers of oxidative stress in F1 rabbit progenies foetal programmed with *Chlorella vulgaris* biomass.

vii. determination of enzymatic and non-enzymatic antioxidant activities as mechanisms for oxidative stress inhibition in F1 rabbit progenies foetal-programmed using *Chlorella vulgaris* biomass.

viii. elucidation of genes and proteins expression patterns in finisher rabbits supplemented with *Chlorella vulgaris* and F1 rabbit progenies foetal programmed with *Chlorella vulgaris* biomass.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Food Insecurity, Poverty and Livestock Production

Food insecurity occurs in form of malnutrition and hunger resulting from insufficient or lack of nutrient intake from foods causing health problems that may be either physically or mentally inclined. Malnutrition is a risk factor for high profile diseases while improved agricultural practices, poverty reduction, improved sanitation, and women empowerment were suggested ways of preventing the problem. Approximately close to a billion people (793 million) are living under threats of malnutrition globally, and over 400,000 annual deaths were linked to its effects as of the first decade of the 21st century (UNICEF, 2010; Jonathan *et al.*, 2011; Masset *et al.*, 2012; FAO, 2015a).

Malnutrition is either poor or too much intake of nutrients and it is categorized into two broad conditions as under-nutrition and over-nutrition. Under-nutrition is characterized by stunted growth, poor weight gain, and deficient intakes of vitamins, proteins, and minerals from foods; while in over-nutrition, there is over-weight, obesity and non-communicable food-related ailments. Malnutrition in any case (under or over – nutrition) is a global phenomenon affecting millions of people worldwide. Around 1.9 billion adults worldwide were reported to be overweight while 462 million were underweight; an estimated 41 million children under the age of 5 years are overweight or obese, while some 159 million and 50 million are stunted and wasting respectively. Adding to this burden are 528 million or 29 % of women of reproductive age around the world suffering anaemia as a result of poor nutrition (WHO, 2016). These indicators are confirmation that malnutrition is indeed a global challenge threatening human existence.

Internationally, the United Nations General Assembly (UNGA) established years 2016 - 2025 as years of action against malnutrition in support and promotion of efforts towards combating all forms of malnutrition worldwide. This action becomes necessary because one out of every nine persons in the world is experiencing chronic hunger - the inability of an individual to consume sufficient quantity of food required to meet up his or her nutritional requirements. It is paradoxical, however; that despite the increase in agricultural productivity across the world, millions of people still live-in chronic hunger and despite several efforts dedicated to fighting hunger the menace is still growing. The paradox can be linked with uneven technological advancement used for increased agricultural productivity, climate change, wars and other socio-political instabilities affecting different parts of the world (FAO, 2003a; Emile *et al*., 2006; WFP, 2016).

The World Food Programme (WFP) reported the prevalence of hunger across the world in a document called the hunger map. The report showed that many developing countries, especially in Africa, fall under the categories of hunger ridden countries whereby 15 – 34.9 % of their population are experiencing hunger (WFP, 2016). Hunger has both physical and mental depression effects; it causes weakness of the body functions, decreased mental concentration and increase susceptibility to diseases with overall reduced productivity and income-generating capacity of an individual human being and whence promoting poverty (Carol, 2009; David, 2012). The relationships between hunger, malnutrition and poverty, complexes the situation beyond health and performance effects because it also involves economic inefficiencies which in a multi-dimensional way affects human societies. Livestock production is continually on increase in developing countries due to increasing demand for animal products a situation that has led to the development of the agricultural sub-division (livestock production) as the fastest-growing compared with other sub-divisions including crop

production and aquaculture. Livestock production is the source supplying up to one-third of protein for human consumption as well as the promotion of quantitative and qualitative nutrition (Randolph *et al*., 2007).

Therefore, livestock production in developing countries from a tripod perspective can be described to stand in-between poverty alleviation and food insecurity because livestock production is an important part of household income and food supply. Jimmy and Simplice (2015) reported that livestock production contributes especially to rural incomes, nutrition and food security in African rural communities. The authors also reported that livestock production provides a mobile and liquid asset to rural households in addition to its roles as a promoter of food security. Furthermore, increasing demand for livestock products stimulated by high population, increased household income, and urbanization are oppourtunities for livestock production to reduce poverty and contribute to achievement of sustainable development goals –SDGs. Meanwhile, these are unexploited and they are strong potentials that could be used to further promote livestock production as a bridge between poverty and food insecurity according to the submissions of Alexandratos and Bruinsma (2012).

Livestock production has the potential for sustainable development through the efforts of both the commercial and small-scale producers. Investment in livestock production by both groups (commercial and small-scale producers) can lead to increase national income, poverty reduction, food, and nutritional security as well as the promotion of environmental sustainability (Jimmy and Simplice, 2015). Through support from governments in terms of the promulgation of policies supporting livestock production, both small and commercial livestock producers can contribute to poverty reduction, food, and nutritional security. In another opinion, the promotion of smallholders'

livestock production capacity could lead to metamorphosing of the smallholders from being an ordinary traditional livestock keeper into becoming private commercial livestock entrepreneurs taking advantage of government incentives (Herrero *et al*., 2014). This is a potent strategy for social inclusiveness of the poor people living in the rural areas since it could have a direct consequence on the rural development because it will lead to provision of infrastructures for human development in the rural areas.

Apart from developing countries in Africa, developing countries in other parts of the world are also facing hunger problem; an example of such country is India which was reported to have about 217 million people facing challenges of food insecurity ranked 63 out of 69 nations in the Global Hunger Index – GHI by the International Food Policy Research Institute (2013). The majority of the people reported to be facing this problem are children and women of childbearing ages (Deaton and Dreze, 2009). Therefore, livestock production was identified as capable of being explored for overcoming these challenges ravaging the country because it was identified as suitable for the improvement of nutrition and at the same time bringing income to the rural dwellers which could be used for other activities (Rupasi *et al*., 2014).

Livestock production with special emphasis on productivity and product yield were observed as major means of promoting food security by proper breeding of animals, and the introduction of innovative feeding strategies for the promotion of optimum animal performance and health which are necessary for higher productivity (Rupasi *et al*., 2014). The proper breeding programme focused on cross-breeding of indigenous and exotic breeds of animals will contribute to the conservation of top genetic traits while nutritional management focusing on optimum use of crops and crop residues will

promote livestock productivity and contribute to environmental management by conversion of waste to wealth (Singh *et al*., 2003).

2.2. Rabbit Production – an Option for Fighting Malnutrition and Poverty

Rabbit husbandry require less capital compared with other animal production entreprise because resources needed for its successful operation are less expensive hence, it can serve as a cheap source of animal protein for poor families; additionally, it can also serve as an alternative potentially unexploited source of income in Nigeria and other sub-Sahara African countries. Rabbits are highly prolific and excellent converter of fodders not consumable by man to produce meat of high nutrients composition. Carlos and Julian (2010) reported that rabbits are next to broiler chickens in the conversion of protein consumed into meat; although rabbits are comparable with poultry in terms of performance but are better than poultry because they are capable of utilizing cellulose and fibre feed resources hitherto not utilizable by poultry to produce meat. Again, unlike poultry which competes with man for edible cereals and grains of nutritional importance such as maize and soybean; rabbits can subsist on fibre not consumable by humans like agro-industrial by-products to produce meat.

Rabbits among domestic animals are a great contributor to family food security especially in rural communities of sub-Saharan Africa (Mailafia *et al.*, 2010); they are supplies meat for household consumption as well as provide an alternative income for rural farmers. Similarly, according to Oseni *et al*. (2008), the majority of people rearing rabbits in southwest parts of Nigeria were civil servants, unemployed graduates, and artisans who keep rabbits as alternative sources of income to augment income generation from their main vocations. In Africa, Nigeria is a leading rabbit producer with family-owned backyard rearing widely distributed across the country. These family

owners of rabbits are not rearing rabbits as a result of any National policy promoting rabbit production despite its suitability for supply of meat for household consumption unlike in another African country (Ghana) where there was a deliberate government policy promoting rabbit production in order to improve nutrition of her citizens.

In Ghana, the National policy encouraged each family household to have a unit of three to six rabbits for the supply of household meat consumption as a measure for improving animal protein nutrition as reported by the World Rabbit Science Association WRSA, (2017). Rabbit in Nigeria and many other Africa countries are reared for reasons including provision of quality meat compared with beef, it also has wider acceptability as its meat irrespective of culture, religious and or any other social affiliations is eaten compared with meat like pork (Oseni *et al*., 2008; Mailafia *et al*., 2010; Okpanachi *et al*., 2010).

Rabbits also survive well on cheap locally available feed resources and forage which makes rabbit production highly affordable even among rural dwellers who cannot afford the huge investment required for the establishment of larger animal production units (Schiere and Corstiaensen, 2008). Almost half of the farmers producing rabbits in Nigeria use local cheap materials for building rabbits housing facilities. Common material reported for construction of rabbit housing includes waste woods from lumbering works, bamboo, sacks, wire and waste tyres from automobile industries (Oseni *et al*., 2008). These are advantages associated with rabbit production in Nigeria and similar developing countries; they are reasons why rabbit production stands out as a major contributor to human well-being in developing countries.

Conversely, all the stated advantages associated with rabbit production were refuted by Lukefahr (1998) who reported the opposite for all the perceived advantages of rabbit production in Uganda. The author reported that rabbit production in Uganda can better be described as “rabbit craze” because rabbit production in the country is as a result of luring people into a syndrome of getting rich quick which at the end of the day only profits the promoters (usually sellers of breeding stock) rather than the farmers. The author described rabbit production as a non-profitable livestock venture and stated that it is less realistic to make a profit in rabbit production than the way it is been promoted. The author justified this position by quoting huge sum of Uganda Shillings required for the establishment of a rabbit unit that cannot be recovered from sales of rabbits or rabbit meat in the country.

The work also stated that the market for rabbit meat in the country is not as common as that of other meat types, and rabbit production ventures can be regarded as loss for rabbit producers. The work also stated that rabbit meat consumption is not traditional to Ugandan culture and the meat has no wide acceptability in the country; the author corroborated the claims of an earlier study conducted on the subject matter which doubts the availability of markets for rabbit meat in Uganda (Lukefahr, 1998).

However, despite all the challenges highlighted against the prosperity of rabbit production in Uganda, the author finally agreed that with proper strategies in place; rabbit is a suitable livestock production capable of contributing to the reduction of rural poverty in Uganda. The author also stated that rabbit production is capable of improving nutrition conditions by reducing malnutrition and in addition could serve as sources of alternative commercial income generation entreprise especially for women in the rural communities of the country. The author also identified huge feed resources available in

the country which can be harness for rabbit production among other stated socio-economic potentials linked with rabbit production in Uganda.

In a similar report, Schaeffer (2004) stated that rabbit is not a get rich quick business enterprise in the United States of America; the author maintained that rabbit production requires high cost for the provision of adequate housing facilities, maintenance of breeding stocks and provision of other supplies. The author also attributed a high cost to annual feeding, medications, general management of the rabbits and maintained that rabbit production is labour intensive. This submission however could be reflecting the situation in the Americas where the author was reporting from; but it becomes a subject of investigation to actually establish whether rabbit production requires huge capital at start-up and whether it is not a get rich quick enterprise in the developing countries. This is because of the similar report from Uganda (a low-income country in Africa) with potential of rabbit production as a contributor to sustainable development and the United States of America (a high-income country) where rabbit production could definitely be expensive.

Nigeria as the most populous country in Africa currently faces challenges of food production and food supply because of population growth; there is no commensurable supply of foods of both plant and animal origins capable of catering for the population increase and this results into malnutrition as results of shortages in animal protein supply. Therefore, the production of animals capable of providing meat within a short interval becomes highly important and necessary. In addition to short generation interval and economic benefit of producing rabbit, another reason and or benefit of rabbit production in Nigeria is that rabbit meat is cheaper when compared with other commonly available meat in Nigerian markets such as beef and chicken meat; if made

available, rabbit meat will be highly affordable by many people and hence will ameliorate protein malnutrition in Nigerian societies (Ozor and Madukwe, 2005; Okpanachi *et al*., 2010).

Despite the potential of rabbit production for increasing meat supply in Nigeria and associated benefits, it is clear and glaring that rabbit production still remains a green area as far as livestock production is concern. Rabbit production is grossly unorganized, very small scale with poor market, poor productivity and it is the least commercialized livestock entreprise (Mailafia *et al*., 2010). Therefore, Nigeria livestock production stakeholders need to support and promote the production of rabbits to increase meat supply, combat malnutrition, increase income generation and provide gainful employment, especially for women. Development of rabbit production entreprise will not only be of local importance or benefits, it can grow to become a new source of foreign exchange earnings for Nigeria considering high demand for rabbit meat, hide and skin as well as fur in different parts of the world; and its meat superiority as results of low saturated fatty acids composition which is one of the reasons for its high acceptability especially in Europe.

Rabbit production because of its restriction mostly to backyard production system hardly features in most countries National livestock production statistics. Current records of rabbits' production hardly feature in Nigeria and or any other West African countries as major producers of rabbit meat because the production of rabbits still remain largely primitives, backyard in nature and not commercialize either for food production or for research. According to Dalle-Zotte (2014), in the lead among rabbit production in the world is Asia where global rabbit production share of 48.8 % currently exist then followed by Europe with a production share of 28.4 %; the Americas with a

production share of 18.1 % and Africa with a production share of 4.7 %. Meanwhile, previous production distribution according to Lebas and Colin (1992) indicated that rabbit production majorly concentrated in Europe which was the major centre of rabbit production but the current production and supply levels put Asia in the forefront of rabbit meat production and consumption around the world (Table 2.1) according to data of Food and Agricultural Organization of the United Nations as reported by Dalle-Zotte (2014).

The current status of rabbit production which put Asia ahead of Europe began at the beginning of the 21st century when the tune of rabbit production changed and since then has not remained the same again in favour of Europe as the leading rabbit producing countries. China has overtaken Italy, the former USSR and France to become the highest rabbit producing country in the world. According to the report of Wu and Hang (2015), rabbit is now the leading meat produced in China leaving behind beef, poultry and pork in annual growth where meat increase from 6.29 % annually rabbit.

Table 2.1: Rabbit meat production and consumption around the world

World Region	Output per year (tons)
North America	318
Central America	4360
South America	327849
Western and Southern Europe	339334
North Africa	64338
Central and South Africa	20931
Eastern Europe	89012
Central Asia	5573
West Asia	887481

Source: Dalle-Zotte (2014).

2.3. The Challenges and Prospects of Rabbit Production in Nigeria

Rabbit production can be regarded as one of the least developed livestock production practices in Nigeria. Problems responsible for this include poor reproductive performances and challenges of unfavourable climatic factors such as high-temperature range which characterized most parts of the country, diseases and poor markets. Poor reproductive performance as a factor is the leading challenge facing the growth of the rabbit industry in Nigeria. Meanwhile, reproductive inefficiency in the rabbits can be linked with oxidative damage of the reproductive system arising from heat stress due to high temperature; it causes reduced fertility in male and embryo mortality in female rabbits (Mailafia *et al.*, 2010). Another challenge crippling the growth of the Nigerian rabbit production sector is unorganized production; small scale dominated owners rather than large and well-organized rabbit farms while most people involved in rabbit farming do so only for the shake of doing and not committed to a strong increase in productivity and profits (Oseni *et al.*, 2008).

Feeds and feeding are another set of challenges facing rabbit production development in Nigeria. Full advantages of rabbit nutrition potential have not been taken into account in Nigeria and this currently limits interest in rabbit production (Okpanachi *et al.*, 2010). In addition to nutrition, another challenge to rabbit production is poor documentation of rabbit meat consumption records. Records of rabbit meat *per capita* consumption in Nigeria are very hard to come by considering the situation of the rabbit industry which is grossly unorganized and small scale in nature. However, despite all these challenges, rabbit production still remains a livestock unit in Nigeria that can contribute meaningfully to Nigeria's agricultural development because of its low capital outlay

which makes it highly suitable and economical for production by both poor and rich farmers.

Rabbit production is an unexplored source of quality meat supply across all social classes. Wool from rabbit production can also be processed into sewing threads for clothing and the garment industries while it is also suitable in making cotton for textiles. Rabbit production is another potential source of regular income for farmers due to the prestigious position of the rabbit as a “domesticated bush-meat” which has high regard among Nigerian societies (Oguniyi *et al.*, 2015). Increased household protein consumption is another potential from rabbit production in Nigeria since it is cheaper and less capital intensive as well as requires less technical know-how to produce compared with other animals such as cattle, sheep, goat, and poultry (Ebenebe, 2000).

In a related submission by Abu *et al.* (2008), which stated that rabbit production remain one of the most sustainable ways of producing protein of high quality for human consumption from the perspectives of rabbits’ body structural conformation (small size), products yield, production cycle, genetic diversity, environmental adaptation and feeding while another prospect of rabbit production in Nigeria is the large population of young active people who have energy (ies) sufficient enough to push rabbit production capacity beyond doubtful levels. This was agreed to by Oguniyi *et al.* (2015) who reported that most people involved in mini-livestock production such as rabbits in Nigeria are young farmers who are still in active working ages. Nigeria can explore rabbit as foreign exchange if they are traded with countries such as China where rabbit meat consumption is very popular and well-organized markets for rabbits in China and it has large consumers of rabbit meat while another huge quantity of rabbit hides and skin also goes to its shoes and belt manufacturing industries.

2.4. Rabbit: Model Animal and its Applications in Bioscience Research

The rabbit was discovered in Spain about 100 B.C. by the Phoenicians; although some attempts at domestication were made during the Greek and Roman eras, true domestication was not initiated until about the l6th century. Rabbit has been used in almost all areas of biomedical research and has been a significant contributor in many specialized areas of scientific investigations. The first embryo transfer work which was performed by Walter Heape in 1891 used rabbits to answer a basic scientific question concerning the influence of the uterine environment on the phenotype of the developing embryo. Rabbit has also been used in all types of basic science studies, including nutrition, reproduction, embryology and monoclonal gammopathies (Ron, 1989). The rabbit was the first animal model used in the study of cancer in humans. It is also a source for the production of antibodies and investigation into immune system. In the study of cardiovascular-related diseases, rabbits were used for investigating and understanding hypertension and atherosclerosis (UAR, 2015).

In the study of oxidative stress-related diseases, Maria *et al.* (2011) reported the suitability of rabbits as an animal model because rabbits have the facility to generate atherosclerosis with specific diets, especially cholesterol-rich diets. This is a feature similar to natural human condition whereby atherosclerosis develops as a result of high consumption of cholesterol-rich diets. Inclusion of cholesterol and other fat sources at varying levels over a short period of time could lead to development of atherosclerosis in rabbits in a similar manner like in human beings. Atherosclerosis was developed in rabbits by feeding between 0.5 % and 4 % cholesterol in diets for a period of 3 and 12 weeks as reported in studies of Zulli *et al*. (2005), Shakuto *et al*. (2005), Zhang *et al*. (2005), and Pfister (2006).

Rabbits as model animals for understanding human reproductive challenges were used for studies associated with mechanisms of periconceptional programming and its effects on metabolic health in adulthood. This was reported in the work of Bernd *et al*. (2012) which stated that the use of rabbits as model animal periconceptional studies has led to the elucidation of even the smallest changes in metabolism and development in the embryo. Viebahn (2001) also stated that rabbits were model animals used for the earliest investigations in embryology and reproductive biology.

Structural and functional properties of rabbit genital tracts as reported by Bernd *et al*. (2012) facilitated landmark achievements in the studies of seminal functions including endocrine and paracrine regulations in the embryo. These interactions between the maternal uterine environment and the embryo contributed to the understanding of Ashrman's syndrome which is a cause of infertility and recurrent pregnancy loss. The functional physiology of the genital tract of rabbits has also led to the understanding of developmental activities in reproductive science ranging from fertilization to blastocyst formation and entire embryonic development.

Furthermore, according to Manjeet *et al*. (2012); rabbits are a model animal for scientific experimentation because they are docile, non-aggressive and easy to handle as well as good for observation for obtaining biological data. They have short reproductive life-cycle hence, they are excellent for reproductive studies involving data on puberty attainment, gestation, and lactation. Wang *et al.* (1998) also reported that rabbits are commonly used animals for the screening of implant materials prior to testing in a larger animal model in order to determine biocompatibility and mechanical stability as well as safety of newly developed implant materials. Himanshu *et al.* (2011) stated that rabbits are one of the most commonly used animal models in most dental experiments

despite dissimilarities existing between rabbits and human bones because rabbits are easy to handle and have rapid sexual maturity.

Bosze and Houdebine (2006) described rabbits as a translational model of choice because they are valuable animals whose experimental findings have direct applications in biomedical investigations. Rabbits are bioreactors where the production of monoclonal and polyclonal antibodies being carried out and recently, they are being used for the production of recombinant proteins. Rabbits unlike other animal models that are primarily used in the discovery phases of research, are more suitable for pre-clinical and translational investigations because they are phylogenetically closer to primates and offer more diverse genetic background than rodents; hence, better for experimentation as a model for human studies (Kingfisher, 2017).

2.5. Factors Affecting the Reproductive Performance of Rabbits

Reproductive performances and their improvements are leading aspects of food-producing animal production and husbandry research. In recognizing the importance of reproductive studies in rabbit production; rabbit experts established the International Collaboration in Rabbit Reproduction Research Group in 1996 as a unit of the World Rabbit Science focusing on the promotion of reproductive studies in rabbits. The group sets a guideline for conducting reproductive trials and experiments in rabbit reproductive studies which include instructions regarding size of sample, housing conditions, breeding and feeding systems, experimental designs and duration of experiments. The group also specifies reproductive performance parameters for evaluation of rabbit experimentation performances which include sexual receptivity, fertility, prolificacy and neonatal mortality, growth of young and overall productivity of rabbits. According to the group, pure New Zealand White or hybrids rabbits of

commercial values are recommended for reproductive studies research in rabbit (Theau-Clément *et al.,* 2005).

Reproductive performance of rabbit requires improvement in order to increase productivity and or supply of meat from rabbit production for human consumption. Rabbit productivity depends on the reproductivity of rabbit – which is measured in terms of the number of rabbits kits a rabbit doe can successfully kindle within a given period. Although improvement has been so far reported through reproductive activities such as artificial insemination, cycled production and breeding better genetic strains of rabbits; but despite all these approaches, there is excessive rabbit doe replacement, high mortality and low fertility currently being experienced on rabbit farms. The rate of rabbit does replacement for sustainability of rabbit farms has been reported to be between 80 % and 150 % while corresponding life span of rabbit does was reported to be 4.7 litters per doe (Castellini *et al.,* 2010).

Apart from these, there are associated health problems arising from different practices aimed at increasing the productivity of rabbits; some of the problems are infectious and pathogenically important while some of the problems also affect the welfare of the rabbit. In addition to sub-productivity, health and welfare; poor reproductive performance of rabbits also results in economic loss; meanwhile, economic gains and or benefits are the major reason why many people keep a rabbit. This is the reason why improving reproductive performance of rabbit will form basis for promotion of rabbit production research and studies in order to secure increase contribution of rabbit to animal products supply (Maertens *et al.*, 1995; Castellini, 1996; Facchin *et al.*, 1999; Bolet *et al.*, 2004; Castellini, 2007; Xiccato *et al.*, 2007). Improved reproductive performance of rabbit remained the most important factor that can guarantee sustainable

development of commercial rabbit production in the current face of safety and health issues associated with meat consumption (Castellini *et al.*, 2010).

To improve the reproductive performance of rabbits, some positions were put forward; among them is modification of Hypothalamic-Pituitary-Ovarian (HPO) axis which has led to better success of assisted reproductive technologies such as artificial insemination, *in-vitro* fertilization, and oestrus synchronization, fewer cases of abortion and stillbirth. Apart from the modification of the hormonal axis, treatment of feedstuff has also been suggested as a means of achieving improved and better reproductive efficiency in the rabbit. Neuroendocrine system (hormones) control reproductive performance activities in rabbit but feeding, genetics, and management practice are reported as factors having an overall effect on rabbit reproductive performance as they affect and or modify hormones (Castellini *et al*., 2019).

2.5.1. Nutrition as a factor affecting reproductive performances of rabbits

Nutrition and interaction with reproductive performance in the rabbit is an established science playing significant roles for improving rabbit production and reproductive efficiency. Nutrients are reported to modify the endocrine system of reproductive importance (Nagatani *et al.*, 1998; Alemede *et al.,* 2014). The process of modification depends on nutrients types, availability and metabolites arising from the consumption of the nutrients. Alfonzo (2016) reported that levels of nutrition before and after breeding (mating) influence secretion, coordination and activities of the reproductive hormones. An example of such hormone is leptin; leptin can be simply regarded as a signal that alerts the brain about levels of energy reserve in animal whether it is enough to support optimum reproductive performance or not. According to the reports of Zieba *et al.* (2008), leptin is a requirement for puberty because an animal lacking leptin will not be

able to determine whether nutritionally reproduction is feasible or not feasible. Nutrition and animal reproduction interface remain one of the most important basis of animal production in any livestock enterprise because it is the interface that ensures both productivity and multiplicity of animals which guarantee sustainability and profit maximization.

Nutritional investigation for improving rabbit reproductive performance was conducted by Ajayi *et al*. (2005) using maize milling wastes as a way of producing food (meat) from cheap feed resources. The study was carried out on the background of rabbit capacity for digestion and utilization of unconventional feedstuffs to enhance digestibility and test its impact on reproductive performance using some maize milling wastes autoclaved then incorporated into the experimental diets. The study concluded that simple diets comprising maize milling waste and groundnut cake can support normal growth and reproduction of rabbits for meat production. In the study, un-autoclaved maize milling waste was used in the control group while autoclaved maize milling waste inclusions at 25 %, 50 %, 75 %, and 100 % were used as treatment groups.

In rabbit fed autoclaved maize milling waste; there were better conception rates ($p < 0.05$) compared with the control group. Although this is impressive, but the authors did not recognize this to be because of the feed resources or its treatment (autoclaving); they rather reported that poor conception rates in the control group fed un-autoclaved maize milling waste may be due to parity of the does, genetic factors and pathogenic infections in the reproductive tract. So, it can be deduced from this study that autoclaving maize milling waste prior to its inclusion in rabbit diet support normal growth and reproduction but no scientific linkage between the treatment of the maize

milling waste by autoclaving and observed high conception rates in the rabbit fed maize milling waste at 25 %, 50 %, 75 %, and 100 % inclusion was established. The results of this study also reported high abortion rates ($p > 0.05$) in the rabbit fed autoclaved maize milling waste.

This observation requires a probe to know whether autoclaving only improves the digestibility of the feed resources (maize milling waste) or its improved digestibility and also removed anti-nutritional principles as well in order to further explore the unconventional feed resources (maize milling waste) for rabbit production. Akinmuyisitan *et al*. (2015) in a bid to improve reproductive performance and fertility of rabbit through nutrition, investigated influence of a rare earth element compound (cerium oxide) as an additive in rabbit doe diets based on positive performances from supplementation of the compound in feeds of cattle and laying birds. The results from their study indicated that the compound improved litter size ($p < 0.05$), litter weight, embryo survival and led to higher conception rates in rabbits supplemented with the compound. The compound was also reported to lead to the superior performance ($p < 0.05$) of the rabbit kits which is an indication that the compound is capable of influencing milk yield of the rabbit does. The study concluded that supplementing rabbit does with cerium oxide at 100 and 200 ppm had no negative impact on rabbit performance and is capable of improving fertility of the rabbit does.

The improved performance of the rabbit reproductively was also reported to be a result of interactions between the compound and reproductive hormones in the rabbit. This is in line with reports of studies by He *et al.* (2001) and Adu (2005) who all carried out studies involving supplementation of rare earth elements in animals. Although there is still a need to further establish the type of interactions and associated biochemical

mechanism for better exploitation of the use of rare earth elements benefits. Another observation of note in the study was better survival of the rabbit kits which may be due to high milk yield and quality of the milk produced nutritionally.

Nutrient level manipulation in animal diets has also been used for improving reproductive performance of rabbit as well as making rabbits be resilient to Tropical climatic condition in order to maintain good level of reproduction; this was done in a study by Salma *et al*. (2004) where protein levels in diets of female rabbits were varied. Bearing in mind that nutrients and their availability in diets affect animal performance, varying levels of nutrient supply in animals is capable of affecting their productive performance such as reproduction but in this study; it was reported that most post-partum reproductive performance features (gestation length, litter size, litter size, and litter weight) were not significantly affected in the animals but post-partum reproductive performance such as kits weight gain and kits weight at weaning were affected ($p > 0.05$).

In addition, conception rates in rabbits with the highest level of protein were the highest but since that was not corresponding in other traits, it was concluded by the researchers that conception success was a result of management practices of mating rather than nutrition. The protein source was a green grass (*Hymenachne psuedointerrupta*); therefore, there is need to investigate what the reproductive performance of rabbit does will look like if oil seed-based protein feed resources were varied because the grass may contain anti-nutritional principles limiting better derivation of nutrients by the rabbits.

2.5.2. Breeding and genetics in reproductive performances of rabbit

Breeding can be simply described as a system of exchanging genetic materials between parents and offspring; while genetics can simply be described as preservation or manipulation of traits of economic values in a given breed of an organism for transmission to coming generations of the organisms. In Nigeria, breeding and genetics for livestock improvement can be described to be grossly un-explored for livestock improvement and this has been described as the major reason for poor growth, low fertility, poor feeds utilization, small mature size and generally poor economic values of Nigerian breeds of livestock (Oke and Iheanacho, 2011). Hence it can be inferred that livestock breeding and genetics have both biological and economic importance.

Although there is good acceptability of rabbit as an animal for livestock production in Nigeria, there is a poor record of breeding for improvement. This problem is not only associated with rabbit, however, with almost all livestock production enterprises in Nigeria, but that of rabbit can be described to be the worst as this is evident in poor rabbit holdings and meat supply in Nigeria. However, excellent use of breeding and genetics is capable of contributing to improved rabbit performance reproductively because Kumar *et al*. (2001) reported that breeding system irrespective of animal breeds can lead to improved variations in reproductive performances. Rabbit is primarily bred for two major traits which are production and reproduction traits. The production traits have to do with food (meat) production and this includes growth rate, feed efficiency, carcass, and meat yield while reproductive traits include kindling interval, litter size, lactating weight gain, weaning weight and survival of the rabbit kits. Production traits are mostly associated with paternal lines while reproductive traits have to do with maternal lines of rabbit (Alfonso, 2016). Therefore, when selecting rabbits for improved

product performance, the focus should be on paternal lines and when selecting rabbits for improved reproductive performance; the focus is on maternal lines.

Crossbreeding is the most used system of breeding for improving rabbits; in doing this, offspring from two different maternal lines are selected for desirable traits to produce a crossbred female - this type of crossbred female rabbits are most suitable as foundation stock in commercial farms (Alfonso, 2016). This crossbred female serves as a maternal super breed (having desirable reproductive performance) which is then used in second crossing with males from a paternal line selected for productive traits (mainly growth). Crossing both the crossbred female and crossbred male usually, give combined traits of production and reproductive performance. This form of crossing is complementary and usually inclusive for both reproductive and productive performances and it is termed as three-way crossing. However, under limited facilities availability, a single breed of rabbits can be selected for both productive and reproductive performance especially in developing countries where access to facilities and resources for selection and breeding are limited and or too expensive to procure (Bunger *et al*., 2005).

The role of breeding and genetics cannot be overlooked in rabbit production and reproduction performance because both productivity and reproductivity determine profit maximization in rabbit farming and they have been reported to have both positive and negative relationships with each other. Rauw *et al*. (1998) and Sanchez *et al*. (2012) reported that continuous selection of rabbits for growth performance efficiency does result in physiological disorder and or reproductive problems leading to disease condition that physically may not be observed because such rabbits do have good body condition when scored. Researchers differ in their reports and positions on relationships between productive performance (growth) and reproductive performance (mainly

fertility and litter size). Some agreed that there is a positive relationship between ovulation which is a physiological trait that determines acceptability and subsequently conception rates in rabbit; litter size and increasing body weight from 1.2 kg to 6 kg while some posit that continuous selection for bodyweight negatively affects reproductive performance.

Bunger *et al*. (2005) agreed on the former position while some other workers disagreed with these positions and they reported a negative relationship between body weight and reproductive performance. Some reported that continuous selection for increasing body weight has negative effects on fertility (Rochambeau *et al*., 1989; Tusell *et al*., 2011). Lavara *et al*. (2008) and Lavara *et al*. (2011) reported a negative relationship between growth rate and sperm quality; however, they could not establish whether the selection for improved growth rate affected the physiological mechanisms of sperm production in the poor-quality sperm.

The workers who posited positive correlations between body weight selection and reproductive performance were in line with foundation studies carried out in different breeds of rabbits which suggested that both hormonal and genetics activities affect correlation between body weight and reproductive traits while in some breed, it was reported that there were regression and the relationships did not hold for both traits. It was also reported that categorizing rabbits into three breeds based on weight as a light, medium and heavy breeds showed that medium weight rabbits have higher reproductive performance than light and heavy weight rabbits. Therefore, to overcome problems of contradictory reports, it is better to identify a rabbit breed as light, medium or heavy breed before commencing improvement studies with the rabbit using body weight ranges of 1.2 kg to 6 kg because this was the bodyweight ranges used for confirmation

of the variations in the relationships between body weight and reproductive performance in both earliest and present studies in rabbit (Gomez *et al*., 1999; Garcia and Baselga, 2002; Bolet *et al*., 2004; Bunger *et al*., 2005).

Understanding breed performance in a rabbit is not only important for biological reasons, but it is also important for the provision of most suitable environmental conditions for optimum performance of the rabbits and it also affects reproductive performances. According to Lazzaroni *et al*. (2012), breed of animals is among factors influencing the reproductive performance of the animals; this made the study and understanding of the effects of rabbit breeds on their reproductive performance to be important.

In a study carried out by Apori *et al*. (2014) in Ghana, it was discovered that seasonal changes have different impacts on different breeds of rabbit being kept under the same production management. Reproductive performance influenced by seasonal changes in Blue Vienna and Chinchilla breeds of rabbits used in the study includes litter size at birth and weaning, litter weight at birth and weaning, kindling interval, age at first kindling and gestation length. Apori *et al*. (2014) in their study reported that both breeds (Blue Vienna and Chinchilla) of rabbit have very good litter size under both rainy and dry seasons in Ghana. They attributed this trait to their superior mothering ability and hence recommend them to be suitable rabbits with the potential for meat production across different climatic conditions in Ghana similar to earlier reports on litter size in rabbits by Oseni and Ajayi (2010); Lazzaroni *et al*. (2012); Saidj *et al*. (2012).

Oke and Ihenacho (2011) conducted an experimental study based on this hypothesis by investigating the effect of breeds and breeding (mating) systems on the reproductive performance of rabbits does. In the study, there were four groups of rabbits does belong to two breeds (New Zealand White and Chinchilla). These breeds were two groups each in the experiment; one group were artificially mated from each of the breeds while another one group were naturally mated from each of the breeds using the same set of rabbit buck corresponding to the same type of the does (New Zealand buck x New Zealand doe and vice-versa for Chinchilla). The study revealed that differences in breed types had no statistically significant ($p > 0.05$) differences in reproductive performance although Chinchilla breed of rabbit used in the experiment had numerically higher conception rates when compared with the other breed. The similarities in the reproductive performance of the rabbit despite breed differences was attributed to non-purity of rabbit breeds available in many places in Nigeria. However, breeding (mating) systems had an effect on reproductive performance statistically ($p < 0.05$) because naturally mated rabbits in the experiment for both breeds had higher conception percentages.

Therefore, it can be derived from the work of Oke and Iheanocho (2011) that prominent rabbit breeds under commercial production in Nigeria are New Zealand White and Chinchilla rabbits because they were chosen for the study based on their abundance in many rabbit production units in Nigeria. Breed type of a rabbit have no significant effect on reproductive performance while the mating system has a significant effect because naturally mated rabbit does had higher conception rates. Hence, from this work, natural mating should be employed for rabbit breeding and improving practicability of artificial insemination are highly recommended for studies in rabbit's reproductive performance and technologies for husbandry in commercial rabbit farms.

In order to fully explore and derive maximum benefits of applied breeding and genetics in rabbit production, three-way cross-breeding of good performing breeds to produce foundation terminal sire in paternal line for improved growth performance traits is a promising approach. Also, three-way crossbreeding for the production of superior dams can lead to the production of does with super reproductive performances including higher fertility and litter size. After achieving these two lines; cross-breeding the lines may now lead to production of a breed with both good productive and reproductive performances. In order to enhance transmission of the derived traits to new generations, manipulation of exogenous factors such as management practices, nutrition, housing, and many other production management factors can be highly promising.

2.5.3. Ovulation physiology and reproduction in rabbit does

Rabbit does are classified as induced ovulators because they are a group of mammals who display ovulation after mating because they release Gonadotropin Releasing hormone (GnRH) and Luteinizing Hormones (LH) after mating. Animals such as this have no specific oestrus cycle. They are different from spontaneous ovulators that have specific oestrus cycle and secrete gonadotropin-releasing hormones and luteinizing hormones from anterior pituitary before mating takes place. Examples of food-producing animals that fall into the category of spontaneous ovulators are cattle, sheep, and goats.

Rabbit like some other induced ovulators have their ovulations taking place after mating. Harkness *et al.* (2010) stated that due to induced ovulation in rabbits, there is no regulated oestrus cycle and this made rabbit to display irregular reproductive behaviour. Rabbits does are usually more or less permanently on heat whenever they accept male rabbits for mating and otherwise whenever they refuse to accept male rabbits for

mating. According to Gogol (2009), the non-spontaneous ovulation nature of rabbits is neither hormonal or environmentally determined. Rabbit has special reproductive behaviour whereby a female rabbit has its back arched downwards while its hindquarters are raised up when on heat and this is generally accepted as a confirm behaviour for female rabbit willingness to accept male rabbit for mating (Cervantes, 2011).

The process of inducing ovulation in rabbit is not well understood and few reports have been able to clearly substantiate the mechanisms behind induce ovulation but, the mechanical role of male reproductive organ was identified as a factor because Cervantes (2011) reported that in some earlier studies in rabbit, mating of artificially inseminated rabbit with sterile male rabbit after insemination gave a better conception rates compared with non-mated rabbits. Although the process still remains not well understood, there is agreement on the fact that there are ovulation inducing factors that may be responsible for inducing ovulation; in camelid (for instance *Ilama*), it was reported that the ovulation inducing factor is contained in seminal plasma of the male animals.

Based on this background, Cervantes (2011) conducted studies to investigate this in rabbit but concluded that there could be ovulation inducing factor responsible for induced ovulation in rabbits but the process still requires further investigation because the outcome of the study did not support the hypothesis that seminal plasma contained ovulation inducing factor responsible for inducing ovulation in rabbit. It is however very important to investigate what could be the factor for the induced ovulation because Spies *et al*. (1997) reported that after mating in rabbit there is stimulation of pituitary

glands which result in an increase in the concentration of luteinizing hormone circulation that causes ovulation.

2.5.4. Hormones and their roles in the reproductive performance of animals – a case study of leptin

Hormones are the chemical messengers that coordinates activities of different cells in multi-cellular organisms. Functionally, hormones are chemical messengers secreted by endocrine glands transmitted in the bloodstream to specific body target sites with receptors for the hormone where its actions are expressed. Unlike neuron messenger, the effects of hormones still linger on the target sites even long after reduction in its positive peak level. Following mechanisms of secretion, production and or transmission of hormones; hormones can be classified as neuroendocrine hormones, paracrine hormone, autocrine hormones, and pheromones. Neuroendocrine hormones are secreted by neuro tissues, for example, the hypothalamus hormones carried by blood portal to a specific site for receptors.

Paracrine are hormones secreted by neuro tissues from where they are carried to nearby associated cells rather than the blood portal system. Autocrine are hormones secreted at the cleft of synaptic neurons then feeds back on it for transmission to the target sites. Pheromones are another set of hormones that are secreted into the environment and then distributed to other organisms within the environment. Hormones irrespective of secretion or transmission mode are structurally categorized as steroid hormones, protein hormones, amino acid derivative hormones, and fatty acid derivative hormones. Glands from where hormones are secreted include pineal, hypothalamus, pituitary, thyroid, parathyroid, adrenal, kidney, pancreas and ovary glands (Squires, 2003).

Based on biological mechanisms of actions; hormones can express concerted (additives and non-additive), synergistic and or permissive mechanisms. In concerted hormonal mechanism, activities of two or more hormones on a biological response give the same effect if the sum total effect of the hormones gives a single equal sum, it is termed to be additive while if the sum is not equal; it is termed to be non-additive mechanisms. Synergistic mechanism effect is when different activities by two or more hormones enhance separate activities of each other while the permissive mechanism is where there is a need for action of a hormone in order to allow expression of effect of another hormone (Nelson and Cox, 2000; Scott and Pawson, 2000).

Hormones have a capacity of altering changes in cell metabolism but such changes are those ones a cell is capable of undergoing naturally. Hormones alter expression of genes through influence on the gene proteins synthesis. Hormones cause morphological changes, act as mitogens for cells differentiation, alter rates of catalytic enzymes activities, affect permeability of cell membranes for passage of ions and minerals. They can cause contraction of smooth muscles, for example in contraction of myoepithelium muscle for secretion of milk in mammals. Generally, hormones are chemical enhancers capable of alteration of bodily metabolic processes when secreted and moved only to the specific target sites with appropriate receptors. The receptors may be cell-surface receptors or intracellular receptors (Squires, 2003).

Receptors either as surface or intracellular receptors can be described as keys that open a way for the activity of a given hormone in a cell. At a given target site, there may be a single or many receptors for similar or different purposes in order to allow actions of the hormone on the target site. Receptors are protein present at a given target site purposely for aiding actions of a hormone transmitted to the site. If a receptor is located on the

surface of a given site, it may require another transmitter into the cell membrane while if it is located intra-cellularly it may not require any further transmitter into the cell (Falkenstein *et al*., 2000).

Reproduction has been identified as a huge cost for the survival of organisms because a large quantity of energy and some other important biological resources are used up for attaining puberty, development of reproductive activities such as oestrus cycle, ovulation, fertilization and development of the embryo, nurturing pregnancy, parturition and ensuring survival of new offspring. To accommodate increased responsibilities of reproduction, parents undergo damages which changes physiological activities in their bodily systems (Quinn *et al*., 2012). Studies on reproductive systems have implicated activities such as lactation, litter size, offspring growth and lots more on daily energy expenditure and risk of physiological loss. Reproduction can be regarded as sets of processes that survive on huge energy reserves in animals' adipose tissue.

Energy is a nutrient that is required in the animal body for fulfilling body metabolic needs and storage. The energy in feed is useful for the maintenance of body temperature, cardiac output, respiration, protein synthesis, and storage. It is measured in Kilocalories or Kilojoules (4.184 Kcal). It is a biologic source of fuel for the body system and it is described as the amount of heat required to raise the temperature by one-degree Celcius under normal atmospheric pressure. Energy is very important in animal metabolic activities as such its intake regulation and levels become a manipulative issue. Energy intake above energy expenditure results in positive energy balance and the excess is stored as fat in adipose tissue. Animals under different physiological states such as lactation, pregnancy or growing have different energy intake and expenditure. Lower energy intake to energy expenditure result in negative

energy balance and consequently lead to weight loss in animals. However, in some situations, animals maintain stable energy intake, expenditure, and storage levels. Energy intake and expenditure are affected by varied factors including age, environmental conditions, and physiological state of the animals, energy expenditure and sex of the animals (Speakman, 2007).

Apart from the stated preceding factors above, energy intake in animals is also under hormonal regulation. Leptin is the hormone in the body system that regulates energy intake and reserve depletion. Leptin is a hormone determining the level of energy reserve in animals. When there is a lot of adipose tissue, production of leptin increase to alert hypothalamus for reduction of feed intake and when there is a depleted level in adipose tissue reserve, there is reduced leptin which alerts higher feed intake. Leptin is multiple regulators in the body determining appetite, energy balance and body composition. If there is enough energy reserve, leptin informs increased luteinizing hormone production which consequently improves reproductive performances (Squires, 2003). Higher plasma serum leptin can be associated with potentially improved reproductive performance because it signals optimum energy reserves for supporting reproduction.

Leptin according to Cheung *et al*. (1997) was described as a permissive factor for attaining puberty while Masuzaki *et al*. (1997) also reported that leptin is secreted from the human placenta during pregnancy to the foetus which indicates the important role of leptin in pregnancy. From several researches and studies involving leptin, reproduction, energy and endocrine system, it can be generally deduced that leptin affects the attainment of puberty, pregnancy, and lactation through control of adiposity and feed

intake as demonstrated in studies involving rodents and primates (Ahima *et al*., 1997; Mounzih *et al*., 1998; Suter *et al*., 2000; Chehab, 2014).

Energy availability and storage reserve is a function of reproductive performance in animals because it affects activation of GnRH pulse generator (Chehab, 2014); Wattingney *et al*. (1999) and Kimm *et al*. (2001) also reported that increase in adipose tissue promotes attainment of sexual maturity. All these are pointers to the important roles of energy intake and storage levels as well as adipose tissue formation and maintenance in reproduction and given the central roles of leptin in these biological processes, it can more or less be agreed upon that leptin is a hormone of reproductive importance. Leptin deficiency has been associated with infertility (a widely accepted feature of reproductive dysfunction). This was reported in studies carried out by Chehab *et al*. (1996); Farooqi *et al*. (1999) and Gibson *et al*. (2004) in mice and humans.

The relationship between deficiency in leptin and infertility can be linked to ineffective roles of leptin in the stimulation of GnRH and its activity on LH as compared with its effectiveness of fertile mice or humans. Leptin roles in reproductive performance is very significant because Barkan *et al*. (2005) reported that leptin appears to exert independent effects of GnRH on reproductive system which induce ovulation in GnRH deficient mice and this was agreed upon by Nagatani *et al*. (1998), Reynoso *et al*. (2003) and Avelino-cruz *et al*. (2009) in their separate studies involving exploration of leptin roles in reproduction.

2.5.5. The hypothalamic-pituitary-ovarian axis in reproduction

The principal control of the reproductive endocrine system is the brain through its coordination of GnRH functions. The GnRH stimulate the anterior pituitary to release the gonadotropins (FSH and LH) which in-turn stimulate the gonads to release oestrogen and testosterone as the case may be for onward reproductive activities. The brain through its specialized cells found in a neuron-embedded network collectively referred to as Kisspeptin-Neurophin-and-Dynorphin (KNDY) controls this complex via the secretion of GnRH which in turn stimulate the gonads (ovaries and testis) to release oestrogen or testosterone, respectively. Positive feedback of this network to the brain increases the surge of the GnRH into the blood portal system and hence influence reproduction and reproductive performance; this network forms the bedrock of the hypothalamic-pituitary-ovarian axis in reproduction (de Roux *et al*., 2003).

In non-breeding animals, down regulation of the Kisspeptin is observed and is also involved in animals' weight changes as it is associated with reproduction; therefore, investigating nutritional manipulation associated with its upward and downward regulations can contribute to more understanding of the complex axis of hypothalamus-pituitary-ovarian linkage with the brain (George and Seminara, 2012). The axis is an important part of the reproductive system and it plays specific roles in the production of ovum and secretion of hormones. The steroid hormones Follicle-Stimulating Hormone (FSH) and Luteinizing Hormone (LH) enhances follicular growth and development as well as the enhancement of the follicle rupturing in preparation for fertilization. However, the reproductive importance of both hormones becomes expressed through oestrogen (Adashi, 1994). Oestrogen is a hormone associated with attainment of puberty and in rabbit the hormone contributes to sexual receptiveness (Cerventes, 2011).

Oestrogen is primarily produced by the ovary although smaller quantities are produced in other parts of the body including adrenal, breast, placenta and fat cells. It is a steroid hormone secreted by the ovary upon maturation of the follicle following secretion of the follicle-stimulating hormone by the hypothalamus-pituitary-ovary axis. Biologically, the secretion of oestrogen trigger by the development and release of follicles completes the reproductive cycle. Oestrogen is primarily involved in the growth and development of primary sex organs, development and growth of smooth muscles of reproductive tracts (uterus), stimulate the development of external genitalia and it also contributes to distribution of fat around reproductive organs.

2.6. Oxidative Stress: Sources, Mechanisms, and Effects on Animal Performances

Oxygen is the most valuable elements for the survival of living organisms (aerobic) because it is the main element for respiration. However, it is paradoxical that the same oxygen could be toxic to living organisms because of its derivatives known as pro-oxidants which include ozone oxygen, singlet oxygen, superoxide, hydrogen peroxide; and hydroxyl radicals. Formation of all these oxygen-related compounds made oxygen an element hitherto known to be beneficial to become hazardous and damaging to body systems. Although this does not occur under normal body functioning. In any situation where there is a shift in the balance of prooxidants and antioxidant defense either from exogenous or internal factors leading to overproduction of the prooxidants, it results into a biochemical phenomenon known as oxidative stress. Oxidative stress is a causal or at least ancillary factor reported in the pathology of many diseases (Sies, 1997).

Oxygen is available in abundance in the earth crust (approximately 54 % by weight) and it is 21 % by volume as well as 23 % by weight of dry air. It is a major component of water and is slightly soluble in water – a major reason why it supports aquatic lives. It is more soluble in organic solvents than in water. Biologically, oxygen is produced as a waste product from the process of photosynthesis in green plants after absorption of energy from sunlight by the chlorophyll for production of plant foods. This is the process that guarantees the availability of oxygen for respiration in humans and animals. Its comparable suitability for biological oxidative process depends on its appropriate physical state, satisfactory solubility in water and desirable combination of kinetic and thermodynamic properties. However, there is high cost associated with the use of oxygen in oxidative processes and this is its reduction to form prooxidants and other biological damaging species causing oxidative stress (Nelson and Cox, 2000).

Oxidative damage is associated with energy-demanding activities whereby there is the production of more Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) and because of their strong reactivity to pick up useful biomolecules in animals' system, they are capable of causing damages and diseases. Oxidative stress is closely linked with reproduction and causes more havoc as its mediator compared with other high energy-demanding activities such as thermoregulation which cause less oxidative damages compared with level of damages associated with the energy-demanding activities of reproduction. It was reported by some authors that oxidative damage in lactating animals was higher than oxidative damage for thermoregulation by non-breeding animals which are also an energy focus activity in animals. However, it is not economical and not even feasible to raise animals without them reproducing, therefore, overcoming challenges of oxidative damages associated with reproduction is highly

desirable (Micheal *et al.,* 2011; Berchieri-Ranchi *et al*., 2015; Zeweil and El-Gindy, 2016).

Oxidative stress has to do with a shift in redox balance leading to either negative or positive changes in the physiological processes of the body systems. It is a leading biochemical mechanism implicated in the development of high-profile diseases including infertility, endocrine dysfunctions, metabolic syndrome, overweight and obesity; type II diabetes mellitus, hyperlipidemia, coronary heart diseases, cardiovascular diseases, non-alcoholic fatty liver diseases, cognitive impairment, Parkinson's diseases and kidney-related diseases (Kochlik *et al*., 2017).

Oxygen derivative known as ozone (O_3) is one of the pro-oxidants capable of causing damage in living organisms. It is an unstable, toxic, pale blue diamagnetic gas with a distinctive odour. It has a melting point of -250° C and a boiling point of -112° C. It is only slightly more soluble in water than oxygen, but unlike oxygen, it can also react with water. Ozone is produced in the stratosphere by the action of sunlight on atmospheric oxygen during the Chapman cycle (Mustafa, 1990; Madronich, 1999). Ozone can cause respiratory problems (airway inflammation and decreased pulmonary function) and can damage the skin of humans and animals as well as the surface tissues of plants (Mustafa, 1990; Runeckles, 1994; Thiele *et al.*, 1997; Menzel and Meacher, 1999).

In studies for understanding biological damage and or significance of ozone; it was reported that inhaled ozone caused hyper-reactivity, neutrophil infiltration, increased epithelial macromolecular permeability, and the promotion of mucus secretion that eventually culminates in lung inflammation. Also, an investigation into the biological damage of ozone in plasma and skin models, there was a quick reaction between ozone

and uric acid, ascorbic acid and protein-thiol groups although it was a slow reaction in the plasma. In the skin, ozone depleted both ascorbic acid and α-tocopherol while increasing the concentration of malondialdehyde. Superoxide is another pro-oxidant of biological significant; it has both beneficial and detrimental effects on a living organism system because it contributes to body defense against invading pathogenic organisms. However, unwanted production of superoxide is a problem capable of causing inhibition enzyme activities, release of redox-active iron, and increasing oxidative stress (Acworth, 2003).

2.6.1. The physiological relevance of oxidative stress in reproduction

Production of reactive oxygen species triggers an imbalance between antioxidants and prooxidants which causes damage to reproduction and reproductive output from organism levels to minute cellular levels. Major problems of reproduction associated with oxidative stress include infertility, and reduced fertilizing capabilities of sperm; according to the extensive establishment of oxidative stress as pathophysiological complexities in the development of reproductive failures (Ashok, 2005). Damages associated with oxidative stress can be summarized as inhibition of production of primary and accessory reproductive cell functions, impairment of reproductive organ activities, poor conception, poor embryonic development, poor foetal growth and compromised offspring performance at early post-birth or long-term life performances. Unexplained infertility was linked with oxidative stress based because there were higher concentrations of oxidative stress products such as malondialdehyde concentrations, conjugated diene and lower total antioxidants capacity of peritoneal fluids in infertile subjects (Wang *et al.,* 1997; Polak *et al.,* 2001).

Reactive oxygen species cause oxidative stress highly relevant in reproductive inefficiencies in both humans and animals. In the male, it affects qualities of seminal plasma and spermatozoa functions while in female, it affects environmental suitability of the fallopian tube, vagina, and the uterus for oocytes maturation, spermatozoa movement and maturation, conception and development of embryo. These prooxidants increase in the body more than antioxidants shift the delicate balance to their favour – a situation leading to damages on vital biological components. This damage includes damage to DNA by destruction of purine and pyrimidine bases, destruction of protein and lipids biomolecules through fast reactions between the reactive species and the biomolecules such as proteins, carbohydrates, and lipids (Popko *et al.*, 2004).

Oxidative stress is also implicated as a mediator of aging and reproduction; because as reproduction takes place, oxidative stress increases leading to increase senescence. This is an indication that reproduction is a trade-off for aging. Oxidative stress also impairs animals' health either directly or indirectly; direct damaging effects include the peroxidative breakdown of lipids and macromolecules while indirect effects include residual effects on cellular membrane and cell components breakdown. When all these occur; they result in metabolic pathway disorders or mal-transformations disrupting the normal body's physiological activities. This phenomenon is a precursor of pathological conditions of animals and human diseases as reported by Adejuwon *et al*. (2014), Ashok *et al*. (2014) and Luddi *et al*. (2016).

Beyond physiological significance, oxidative stress effects in an animal are also economical because it results in economic loss. Losses from oxidative stress are economical because they reduced the productive capacities of animals, compromise product qualities and reduced viability of animal products for adequate processing.

According to Miller and Brzezinska (1993), more than half of cattle surveyed for oxidative stress-related ailment are associated with reduced productivity since it reduced milk yield and quality. This is a typical example of economic loss associated with oxidative stress in food-producing animals.

2.6.2. Environmental sources of oxidative stress in animals

Temperature increase, reduced humidity, and heat are environmental factors limiting livestock productivity in the Tropics. This continues at alarming rates in recent years. Oxidative stress is a direct consequence of these environmental extremes and whenever they occur there is always a reduction in animal performances and productivity. Temperature-Humidity-Index (THI) is one of the indicators for determining temperature comfort zone for animals, and it is an integrated condition because it also reflects the relative effects of temperature and humidity for optimum livestock performance and productivity. It is also associated with environmental temperature and heat generation as factors controlling energy metabolism and exchange hence avoidance of its extremes above and or below comfort zone can influence animal health and production as well as animal thermoregulatory status.

Application of THI has been used for determination of comfort zones for humans and different livestock species including dairy cattle, swine, turkeys, laying hens, broiler chickens and rabbits as a way of implementing management practices supporting optimum productivity (Ogunjimi *et al*., 2008; Behura *et al*., 2016). An evaluation of THI is a measure for identifying basal environmental conditions under which animals are protected against extreme effects of oxidative stress as biochemical consequence of elevated temperature and non-favourable environmental conditions.

Non-favourable environmental conditions apart from negatively affecting the physiological performance of animals; is also a leading factor contributing to high cost of production. In dairy production, environmentally induced stress contributes to high cost of milk production and reduced milk productivity especially for hot climates of Tropical countries where increased milk productivity depends on crossbreeding of local indigenous cattle with Temperate high producing cattle breeds. However, since productivity and heat tolerance are antagonistic; poor production of these cattle becomes glaring thereby leading to economic losses (Ravagnolo *et al*., 2000).

In poultry production, environmentally induced stress affects feed consumption and thus has direct implications on production performance. Under elevated temperature, feed intake reduces while under low-temperature feed intake increases and both conditions have physiological impacts influencing production levels (Sakomura *et al*., 2003). Climate data on temperature and relative humidity over a long period of years for a particular location, region and or country when obtained can be used for the determination of THI suitable for animal optimum performance for an area. An example of these formulae is the one involving evaluation of dry bulb and dew point temperatures for the determination of Temperature-Humidity-Index (THI) for a location as follows:

THI = dry bulb temperature ($_{o}$C) + (0.36 X dew point temperature) + 41.2 ……. i

(Source: Dairy Australia, 2016).

Kulkarni *et al.* (2017) when classifying THI suitable for optimum animal performance in a point-based index classified 70 as no stress, 70 and 75 as mild stress, 76 and 80 points as semi-moderate stress, 81 and 85 as moderate-high stress and 85 and 90 as severe stress. However, according to guidelines of heat stress management by Dairy Australia (2016), when the THI exceeds 72, cows are likely to begin experiencing heat stress which could affect their calving rates. When the THI exceeds 78, milk production is seriously affected and when the THI rises above 82, very significant losses in milk production are likely; cows will show signs of severe stress and may ultimately die.

In animal production, when the temperature and relative humidity are high, the tendency for high THI sets in and hence animal performance becomes negatively impacted. Vasna *et al*. (2011) confirmed this through a report that exposition of dairy cattle to high ambient temperatures and high relative humidity for extended periods decrease the ability of the lactating dairy cow due to decrease in feed intake, metabolism, reduced body weight and eventually reduction in milk yields as a way of alleviating the imbalance. Therefore, in the use of cross-bred cattle in Nigeria and other developing countries where cattle improvement for milk yield depends on crossbreeding with high-performance dairy cattle which are traditionally developed in the temperate region, special considerations for heat stress tolerance and understanding of the relationship between the THI and animal performance could be a valuable scientific endeavour. This is because most high performing dairy cattle including Holstein, Jersey, and Brown Swiss decrease daily milk yield in the amount of 3, 7 and 20 % of normal, respectively at 29° C temperature (Vasna *et al*., 2011). This calls for redirection of improving performance of indigenous cattle since it is hard to operate at environmental conditions that will promote optimum performance of the cross-bred animals.

In rabbit production, an elevated level of THI corresponds with higher heat and moisture production, especially in mature rabbits. Hence, to maintain optimum rabbit production a THI value of about 61.13 for rabbit using Livestock and Poultry Heat Stress Indices (LPHSI) formula which was modified for rabbit by Marai *et al*. (2002) is recommended. The value 61.13 arrived at is because it is below 70 (no stress condition) using the classifications mentioned earlier as well as following the recommendations of Ogunjimi *et al*. (2008). Physiological and productive condition of rabbits are both susceptible to heat stress because under critical high-temperature condition there is reduced feed intake and continuous use of metabolizable energy (ME) for non-productive activities such as panting and faster respiration rates for the purpose of survival; these reduce output and performance of the animals (El-Raffa, 2005; Pedersen, 2005; Ogunjimi, 2007).

In poultry production, broiler chicken exposed to elevated temperature and higher THI suffers from negative influences of oxidative stress damage because it promotes increasing generation of reactive oxygen species which are principal actors in the development of oxidative stress damage. It was also reported that high temperature and corresponding high relative humidity can lead to a decline in production of chicken because once these situations occur there will definitely be a rise in heat stress which is always directly proportional to oxidative stress (Chen *et al*., 2013; Rhoads *et al*., 2013). Under elevated temperature and relative humidity, there are always higher values for THI which is a situation capable of promoting an imbalance between the generation of ROS and body antioxidant systems (Kikusato and Toyomiza, 2013). These are confirmation that environmental conditions in Tropical countries require antioxidant nutrition for poultry and other animals to ensure adequate performance and productivity (Sikiru *et al*., 2018).

Direct consequences of elevated temperature are oxidative stress damages causing depressed mitochondria respiration which is reason why poor feed and energy intake is common affecting production performance in animals under elevated temperature condition. In addition to poor performance, there are also relationships between elevated temperature and excessive production of ROS and lipid peroxidation – a situation causing poor performance as results of diseases development (Yang *et al*., 2010). Apart from these physiological breakdowns, oxidative stress occasioned by higher THI can also result in reduced product qualities because oxidative damages have negative implications on muscle cell integrity and lipolysis as well as complete destruction of the cell membrane (Azad *et al*., 2010; Sunil-Kumar *et al*., 2011).

Oxidative stress damage as a result of heat stress also has negative implications on digestive tract functionalities. It was reported that in broiler chicken production, it decreases growth and also causes poor feed-to-meat conversion ratio. It is also a possible situation causing oxidative damage to the intestinal brush border in the intestinal lumen due to lipid peroxidation and hence reduce feed utilization efficiency (Lagana *et al*., 2007; Gu *et al*., 2012). Heat stress is a leading environmental hazard affecting animal production by limiting animal performance and productivity. Heat stress also affects animal nutritional health by increasing permeability to luminal pro-inflammatory molecules. When pro-inflammatory molecules get into the luminal tract; lipid peroxidation occurs, hence, hampering the absorptive capacity of the digestive tract (Fernandez, 2014). In the gut system, the negative impacts of heat stress start with damaging the small intestine (Kregel, 2002). Damages on small intestine lead to malfunctions of the digestive tract in the up-regulation of heat shock proteins (Flanagan *et al*., 1995).

Failure to up-regulate the expression of heat-tolerant genes makes animals susceptible to temperature elevation and subsequently oxidative stress damages. Heat stress also affects intestinal integrity through increased permeability of the intestine a situation leading to increase in the concentration of lipo-polysaccharide in the circulatory system compromising absorptive capacity of the intestine (Hall *et al*., 2001; Lambert *et al*., 2002; Pearce *et al*., 2013). Oxidative stress arising from elevated temperature effect is a prominent biochemical ailment affecting intestinal integrity due to heat stress because there is diversion of blood flows from the peripheral in an attempt to maximize heat dissipation and this usually led to hypoxia (Hall *et al*., 1999; Lambert *et al*., 2002).

In hypoxia conditions, enterocytes' broad borders become restricted and hence nutrients absorption is reduced. Reduction in nutrient absorption is the peak of the pathophysiology of heat stress in the gut because poor absorption will lead to poor digestibility, poor feed intake and lack of Adenosine Tri-Phosphate (ATP) for gene expressions and other related activities. In the temperate region, it was reported that during the summer period, generic cooling and hydration provide no remedies to heat stress susceptibility; that means improvement in pen construction and environment may not be a solution to heat stress management. It can be implied from this, that housing improvement option is not a feasible strategy for improving animal productivity under heat stress conditions. Hence, the discovery of pathophysiology of heat stress for the purpose of providing solution to the problem will contribute to increased livestock productivity especially in the Tropics where increasing temperature is a current challenge to livestock production output (Stowell *et al*., 2009; Leon and Helwig, 2010).

2.6.3. Genetic evidence of oxidative stress effects on reproductive performance

Under oxidative stress condition, increased activities of reactive oxygen species cause genomic instability through manipulation of deoxyribonucleic acid (DNA) coding. It also reduces the activities of protective antioxidants on cells, cellular membrane destruction and signal dysfunction damage and the leading mechanism of these compromise is increased generation of lipid peroxidation products including malondialdehyde, hydrogen peroxide and hydroxyl guanine (Zablocka and Janusz, 2008; Adejuwon *et al.*, 2014). Alvarez *et al.* (2002). Aziz *et al.* (2004) also reported that there are spermatozoa structural dysfunctions as results of overproduction of prooxidants from leukocytes originally meant for defense against infections during spermatogenesis. High levels of reactive oxygen species are also reported to induce oxidative damage of DNA in the sperm plasma membrane, mitochondrion, and nuclear genome; a complication on immediate reproductive performance of the patient and also is capable of causing childhood cancer and inherited infertility in the offspring (Aitken and Krausz, 2001). These are terrible effects of oxidative stress in reproduction occurring in both humans and animals.

Oxidative stress is also implicated in DNA fragmentation - a condition common in sub-fertile humans and animals displaying azoospermia. Fragmentation of spermatozoa causes mutation in offspring because it hardly repairs ahead of fertilization (Irvine *et al*., 2000). Furthermore, according to the study of Berchieri-Ronchi *et al*. (2015), oxidative stress affects reproductive performance through damage in different cellular components and biomolecules at all levels of reproduction from fertilization, to maintenance of pregnancy and lactation period in both humans and animals. Some of these biomolecules are cellular genetic materials useful for normal body functions and

non-adulteration of traits transfer from parent to their offspring. However, once compromised; they are wrongly transferred and hence performance is reduced and or other genetic complications developed.

It is also an established fact that oxidative stress triggers the balance between the prooxidants and antioxidants and it is a phenomenon promoting the destruction of vital bodily systems and structures and among them is cellular destruction leading to mutation and destruction of DNA. This is a clear genetic evidence linking oxidative stress with compromised reproductive performances. Prevention of this situation is required in order to sustain reproductive output of animals capable of fulfilling both productive and economics of livestock production and management especially in rabbit whose productivity and profitability depend solely on the number of kits produced by a rabbit doe in a year.

2.6.4. Antioxidant treatment effects on oxidative stress attenuation

In both animals and humans, there is an internal system of antioxidants defense system which put oxidative stress under check at all times. This is composed of internal antioxidant system and external antioxidant system. They are categorized broadly as enzymatic or non-enzymatic antioxidants. The antioxidant system in enzymatic antioxidants include catalase, superoxide dismutase, glutathione peroxidase or non-enzymatic antioxidants including glutathione reduced, tocopherol, astaxanthin, carotenoids, fucoxanthin, vitamins, and trace minerals. Antioxidants (either enzymatic or non-enzymatic), can be from natural or synthetic sources and can be applied to suppress activities of reactive oxygen species (Crowe *et al.*, 2001; Helena *et al.*, 2009; Tania and Cristian, 2015).

Prevention of oxidative stress damage through nutritional intervention focusing on exploiting antioxidants in food and or feeds currently stands as one of the most valid approaches to eliminating problems associated with oxidative stress. Kochlik *et al.* (2017) posited that nutrition – derived protection against oxidative stress can prevent several diseases and can stand as a measure for promoting quality life. The practice of exogenous antioxidant supplementation is thus a common practice for fertility treatment in men and for improved reproductive performance. Supplementation of vitamin E in infertile males was reported to improve post-thaw sperm motility and improvement of DNA integrity in cryopreservation of sperm (Taylor *et al*., 2009).

According to reports of Fanaei *et al*. (2014), exogenous supply of vitamin C in semen led to reduction in malondialdehyde level – an indication of reduction in sperm lipids peroxidation, reduction of DNA structural damage and improved sperm progressive motility as well as viability of sperm all resulting into improved fertility in human fertility patients. These researchers concluded that exogenous supplementation of antioxidants is a proven means of reducing oxidative stress associated with reproductive inefficiencies and suggested the need to discover different sources of antioxidants as supplements for improving reproductive performances.

Similarly, Luddi *et al*. (2016) in their study also reported on the potency of micronutrient supplementation for control of oxidative stress in female human subjects as a highly successful strategy for improving fertility. The study concluded that additional treatment with micronutrients, starting three months before *in vitro* fertilization cycles, protect follicular microenvironment from oxidative stress damage and thus increases oocytes' quality as well as conception rates. Micronutrients reported

for antioxidants propensity includes micro minerals such as selenium, manganese, and zinc.

Furthermore, Adejuwon *et al*. (2014) while exploring phytochemical sources for antioxidants capable of reducing oxidative stress reported that *Telfairia occidentalis* aqueous extract ameliorates oxidative stress in the brain of rats exposed to gamma rays. The study reported ($p < 0.05$) better activities of both enzymatic and non-enzymatic antioxidants in the brain of rats treated with the aqueous extracts of *Telfairia occidentalis* after different durations of exposure to gamma rays as a source of oxidative stress. Another reported source of phytochemical antioxidants for prevention of lipid peroxidation is turmeric. According to Mancini *et al.* (2016), turmeric powder used for preservation of rabbit meat prevents oxidative stress damages in the meat because the turmeric powder proved to be a strong antioxidant source. It prevented lipid peroxidation in rabbit meat even more than the synthetic antioxidant source ascorbic acid used in the preservation of rabbit meat. This work suggested turmeric as a natural source of antioxidants and recommend its utilization in the preservation of widely consume meat products such as meat burgers as an alternative to synthetic preservatives. Zeweil and El-Gindy (2016) also reported that supplementation of pomegranate peel in rabbit diet served as a source of natural antioxidant, improved reproductive performance ($p < 0.05$), and reduced lipid peroxidation. The study recommended using pomegranate peel for new-borns, which exhibit greater sensitivity to oxidative damage than adults, and for the development of the immune system in young animals as similarly reported by Debier *et al.* (2005).

2.6.5. Carotenoids crossing of the blood-brain barrier: potential roles of molecular receptors

The brain can be referred to as the most important organ in the body playing roles ranging from coordination to energy metabolism. Physiologically, the brain exerts central effects throughout the body functional systems. Apart from internal body responsibility of coordination, the brain is responsible for organism interactions with the environment, several neurons and nerves system associated with body systems from movement to reproduction are all depended on brain functionality. As a result; brain is central to all lives (von Bartheld *et al*., 2016). Primarily, the brain is responsible for neurotransmission and receptor activities, metabolism, perception and motor control, homeostasis, learning and memory management (Thomas *et al*., 2000; Balleine *et al*., 2009; Nieuwenhuys *et al*., 2014).

The blood-brain barrier is a semi-permeable border preventing direct interaction between internal and external metabolism between the brain and circulatory system; it allows independent metabolism within the brain different from the body blood circulatory system (Daneman and Prat, 2015). The barrier is a major protective part of the brain preventing crossing over effects of pathogens and unwanted materials into the brain. Reportedly under some disease's conditions such as epilepsy, liver diseases, brain trauma, oedema, neurodegenerative diseases and inflammation the blood-brain barrier becomes weak allowing entrance of unwanted materials including pathogenic organism (Tortora *et al*., 2004; Raza *et al*., 2005).

Youdim *et al*. (2003) however reported that in an *in-vitro* investigation, flavonoids demonstrated the capacity of crossing-over the blood-brain barrier. In further support of this investigation, the authors also reported that under *in-vivo* condition, metabolites of flavonoids are found crossing the blood-brain barrier. The metabolites were reported for playing a lipophilic role through the prevention of lipid peroxidation around the brain. Faria *et al*. (2010) also reported *in-vivo* transcellular transport of catechin, quercetin, and cyanidin-3-glucoside in blood-brain barrier of rat model. These compounds are antioxidants but according to Dragoni *et al*. (2006), these compounds have potential protective effects against cancer and cardiovascular diseases; which is the reason why the pursuit of their biological influence as a result of their capability to cross blood-brain barrier becomes necessary.

Evidence supporting antioxidant crossing of blood-brain barrier can be deduced from results of brain carotenoids evaluation found in varied proportions in human brains according to Vishwanathan *et al*. (2014), who reported that carotenoids including lutein, zeaxanthin, cryptoxanthin, and carotenes were found in brain of babies. Although significant levels of these carotenoids were reported in termed compared with preterm babies, that is babies born at the expected and before the expected parturition dates respectively). Lutein was reported as the most predominant carotenoid in the brain. These carotenoids presence in the brain were reported to play roles of cognitive development in babies as well as serve as antioxidant protection for the brain. Also, concentration of lutein and zeaxanthin in brain were reported as potential biomarkers for determining macula pigment concentration which is a primary brain antioxidant usually accumulated in the macula lutea of retina and it is responsible for sharp central vision (Vishwanathan *et al*., 2013).

Based on the evidence above, exogenous supplementation of algae and other bioresources rich in carotenoids can contribute to brain protection against oxidative damage via a crossing of the blood-brain barrier. Lutein and zeaxanthin which are reported to be associated with the brain are found in abundant quantities in microalgae such as *Chlorella vulgaris* and *Spirulina*; and the carotenoids were reported to influence concentration of macula pigment which is an important antioxidant protective compound (Krinsky, 1989; Ahmed *et al*., 2005). Internal synthesis of these carotenoids is not possible and since they have potential of crossing over the blood-brain barrier then they may have physiological importance beyond vision (Malinow *et al*., 1980). This is because apart from cognitive functions, these carotenoids play crucial roles in early lives which is a reason carotenoids diets are supplemented during pregnancy (Macais and Schweigert, 2001; Lietz *et al*., 2006). Therefore, foetal programming using carotenoid rich microalgae is worthy of exploitation as a way of producing offspring with improved capacity to survive neonatal stage of life.

2.6.6. Oxidative stress biomarkers: description of their roles and evaluations

Cells contain a large number of antioxidants that naturally prevent or repair the damage caused by reactive oxygen species and other prooxidants, as well as to regulate redox-sensitive signaling pathways. These antioxidant enzymes include Superoxide Dismutases (SOD), Catalase (CAT), Glutathione Peroxidase (GPX) and Glutathione Reductase (GR) (Weydert and Cullen, 2010). The shortfall of these internal body antioxidant systems is an indication of oxidative stress and this occurs from time-to-time in animals. Hence, feeding antioxidant-rich feedstuff such as algae can contribute to the improved internal antioxidant system in animals by contributing to capability of the enzymes to be available at optimum levels.

Reduced activities of these enzymes result in oxidative stress; superoxide dismutase work in complementation with another antioxidant enzyme called catalase. Superoxide dismutase converts superoxide radicals by dismutation into hydrogen peroxide while catalase converts hydrogen peroxide into water and molecular oxygen in order to become harmless to the body. Superoxide dismutase is a mineral dependent enzyme and it is defined by its associated metals Manganese Superoxide Dismutase (MnSOD) or Copper-Zinc Superoxide Dismutase (CuZnSOD). Manganese superoxide dismutase is located in the mitochondria while Copper-Zinc superoxide dismutase is located in the cytoplasm (Bailly, 1996).

Catalase is an antioxidant enzyme that convert hydrogen peroxide – a very strong oxidative damaging compound into water which is a harmless compound in the body and molecular oxygen used in cellular metabolism. Activities of catalase enzymes are largely located in subcellular organelles called peroxisome in the cell. Measuring of catalase can be done using a spectrophotometric procedure measuring peroxide removal (Cullen, 2003).

Glutathione peroxidase is an enzyme that catalyses the reaction which reduces peroxides such as hydrogen peroxides to protect the cell from oxidative damage. Higher concentration of this antioxidant can reduce oxidative stress effects in animals. These enzymatic antioxidants are useful because of their capability to limit the activities of prooxidants from causing oxidative stress damage. Increasing their availability in the body can then signal the level of oxidative stress development. Low activities of these antioxidants will allow oxidative stress damage while their strong activities will prevent oxidative stress damage. Hence, they are regarded as biological markers (biomarkers) indicating building-up or suppression of oxidative stress damage.

2.6.7. Future prospects of metabolites profiling in the study of oxidative stress

Metabolomics (metabolites profiling) is a technology that can contribute to understanding and providing solutions to problems related to oxidative stress damages because it has the potential for identification of novel biomolecules that have not been previously associated with oxidative stress through non-targeted global metabolites profiling. Researchers including Kaddurah-Daouk and Krishnan (2009), Theodoridis *et al*. (2012), Zhou *et al*. (2012) and Jenni *et al*. (2016) all pointed out this position in their reports on the application of metabolomics in the discovery of biomarkers associated with diseases of economic importance.

Metabolomics profiling is a systemic analysis of all endogenous metabolites associated with an organism including animals, plants, and microorganisms. The total aggregate of all the metabolites is referred to as metabolome and it is usually closely related to body physiological activities. During a period of any change to normal physiological body process, there is usually changes in metabolites production by the body as well and this is a major means of early disease detection and diagnosis techniques (Nicholas and Lindon, 2008). Metabolomics is a suitable tool for correlating changes in body functions in response to oxidative stress damage because the slightest change in biological system function can be determined using metabolites profile differences because at metabolites production levels, change in biological system can be more revealing.

The response of a biological system to environmental changes leads to the production of low molecular weight compounds usually between 150 and 1500 Da; these compounds are metabolites that can serve as significant biomarkers for understanding normal or abnormal condition of a biological systems under oxidative stress conditions (Royuaela *et al*., 2000). Identifying metabolites associated with these types of conditions could serve as novel approaches to oxidative stress damage evaluation in animals and will contribute to early detection of oxidative stress effects on productivity for better management of animals. Metabolite profiling will also contribute to the identification of biomarkers with both positive and or negative contributions in the development of oxidative stress damage as well as identification of metabolic pathways promoting and or inhibiting oxidative damages (Nyuk *et al*., 2013).

2.7. Algae as a Potential Functional Feed/Food for Animals and Man

Algae can be regarded as functional food/feed resources because of its use for promotion of nutritional values of foods and its uses as a natural source of feeds with health-promoting benefits in aquaculture and fisheries, specifically, as survival source of nutrients for newly hatched. In human nutrition, algae are functional foods because they contained essential oils extracted for use as additives in the formulation of infant foods; although it is an application just gaining ground. Man has been using algae as edible materials for more than a thousand years. In addition to algae use as food source in some parts of the world, algae have been used for waste water management and energy generations (Jensen *et al.*, 2001).

Algae attracted huge investments in different parts of the world because of the huge economic benefits associated with algae cultivation, processing, and uses. It was reported that as of more than two decades ago, there were up to 46 large-scale factories

cultivating and processing an average of 1000 Kg of algae daily in Asia alone. In other parts of the world, there is commercial production of algae such as Australia, USA as well as South America region (Spolaore *et al.*, 2006). Mass cultivation of algae remains a challenge inhibiting the development and use of algae in livestock nutritional system because of insufficient supply. However, there is feasible sustainable production of algae biomass for livestock consumption because algae are rapid growing plants with less negative impact on the environment when compare with other conventional feed resources such as soybean whose cultivation, processing and utilization usually involve destruction of the environment.

Algae, unlike other plant resources used as animal feeds resources, stand a bright chance of promoting intensive livestock management because waste generated in intensive livestock production can be put to use for the cultivation of algae which will, in turn, be used as livestock feed resources. From this perspective, algae use in livestock nutrition can then be regarded to have both positive economic and environmental potentials (Ahluwalia and Goyal, 2007).

Although several studies have identified and supported algae as a future livestock feed resource that can contribute to the production of meat, eggs, and milk for human consumption; mass cultivation of algae still remains largely un-established, in order to produce quantities that can support commercial livestock production activities. Leading factors necessary for excellent algae biomass performance include high temperature, abundant sunlight, adequate minerals, and nutrients supply as well as quality exchange of CO_2 and O_2; this is why effluents from livestock production can be explored for algae biomass cultivation.

Cultivation of algal biomass on livestock manure effluent stands as a potential strong contribution to environmental management. Algae grow well on organic waste effluents and waste waters because of mineral availability in these media; waste waters from agriculture such as livestock manure effluents are an example of good media suitable for algae cultivation and it will contribute to mitigation of negative environmental impacts of livestock production such as eutrophication. This is possible because there will be withdrawal of high concentration of nitrogen and phosphorus present in the waste water effluents by the algae for their own growth; in addition to these, cultivation of algae using agricultural waste waters can contribute to prevention of toxic chemicals from entering human food and consumption chain from waste water and organic effluents (Gonzalez *et al.*, 1997; Willkie and Mulbry, 2002; An, 2003).

Cultivation of algae under natural and less sophisticated environmental conditions is possible because natural daylight and a temperature range between 25 – 30° C can support high algae biomass cultivation in open ponds; this is capable of reducing the cost of cultivation. Variations in algae growth rates; temperature and photoperiods were reported to affect nutrients composition in algae. Rapidly growing algae are higher in protein and low in carbohydrate content (Sayegh and Montagnes, 2011). High temperature decreases protein and increases the accumulation of carbohydrates while in light and dark cycles, light favours the accumulation of carbohydrates and in the absence of light, cells obtain their energy by metabolizing carbohydrate which is used to synthesis protein (Sharma *et al.*, 2012). Based on these, mass cultivation of algal biomass under natural daylight and other necessary conditions is possible using waste effluents from livestock production as a way of producing cheap high-end animal feed resources.

2.7.1. Algae *Chlorella vulgaris*: its biology, cultivation, and applications

Chlorella vulgaris belongs to division *Chlorophyta* in the class *Chlorophyceae*; it is green algae because chloroplast is the most common pigment in the microalgae. The abundance of chloroplast in the algae makes it a strong photosynthetic plant. *Chlorella vulgaris* is a eukaryotic, unicellular, non-motile, fresh-water alga and it contains an appreciable quantity of hemicelluloses in addition to rich protein, vitamins, and minerals (Kay, 1991). It is a unicellular alga of round shape reported to be between 2 and 12 µm in diameter, is one of the most identified algae because of its survival and cultivation in freshwater, soil, and ease of cultivation (Kay, 1991).

Chlorella vulgaris is highly adaptive to varied environmental conditions ranging from frozen lands in the Scandinavian to the hot desert land in the Sahara. It was reported to be in existence over 2.7 billion years ago (Lee, 2008). However, the documentation for the discovery of the algae was in 1890 when it was discovered by a Dutch researcher named Martinus Willem Beijerink who name *Chlorella vulgaris* after the Greek words – *chloros* (Chloroplast) and – *ella* (green microscopic plant). It is a popular alga highly cultivated in different parts of the world because of its high nutritional value. *Chlorella vulgaris* reproduce rapidly and asexually by cell division (autospores). Within 24 hours, a *Chlorella* mother cell can complete a full cell division and multiply by autosporulation forming four (4) new daughter cells. In photobioreactors, *Chlorella* is cultivated by mitigation of limiting factors in open cultivation systems such as environmental variables including light intensity, temperature, carbon dioxides concentration, and pH; all these are provided in bioreactors in order to promote high *Chlorella* yield (Lee, 2008).

Approaches to the optimization of *Chlorella vulgaris* yield is referred to as the mixotrophic system of production whereby both autotrophic and heterotrophic systems are used for production. In this system, there is a supply of organic materials in addition to the natural growth conditions. The mixture of both autotrophic and heterotrophic conditions is reported to be excellent for productivity of algae *Chlorella vulgaris*; the yield is higher than when both autotrophic and heterotrophic are used independently. Mixotrophic condition was reported to have high dry biomass productivity (2–5 gL-1day-1). The main advantages of mixotrophic system is that it limits metabolic activities which usually results in algae biomass loss especially during dark periods of cultivation. Harvesting of *Chlorella vulgaris* involves a combination of two or more processes including centrifugation, flocculation, floatation, and filtration (Yeh and Chang, 2012).

Chlorella has been safely consumed as a food in Mexico and Central Africa for the past four centuries and currently, it is used as dietary supplements and a novel food ingredient, especially among Asians, including Asian-Americans. It is a popular functional food material for human approved to be generally safe for human consumption by the Food and Drugs Administration of the United States of America. However, in Africa apart from the unofficial local use in the Central Africa region, the use of *Chlorella* is scarcely documented for both animals and human uses. *Chlorella vulgaris* is referred to as a functional food because in addition to its regular nutritional composition of protein, carbohydrates, vitamins, and mineral; it was reported to have properties including hypoglycaemia, hypocholesterolemic, immune activation, antioxidant, anti-carcinogenic, and antitoxic effects when consumed (Kay, 1991; Kang *et al*., 2004).

In a bid to explore the potential toxicity of the microalgae, a research investigated the acute effects of *Chlorella vulgaris* in rats (up to 2,000 mg/kg BW/day) for a duration of 2 weeks. Forty male rats weighing between 150 – 220 g were divided into five different groups to receive the test compound at different dosage concentrations. There were no significant ($p > 0.05$) differences in mean body weights, food intakes, or organ weights among treatments and the control groups. The general behaviour of all the animals was normal. There was no abnormality in heart rate, respiratory rate, or muscle tone. Histopathological examinations showed no dose-related responses. Therefore, it was concluded in the study that there were no pathological changes in the organs studied that could be attributed to the use of *Chlorella vulgaris* in the experiment (Krishnaswamy, 2000a).

In a study by Janczyk *et al*. (2006) in which the effects of *Chlorella vulgaris* on reproductive performance and foetal development was investigated; consumption of 1.0 % *Chlorella vulgaris* (equivalent to approximately 1,560 mg/kg BW/day) was demonstrated in a three – generation reproduction study in mice. In the study, females from F0 generation groups of mice were fed control diet and *Chlorella*-supplemented food starting from day 21 of life after weaning. They were mated randomly on the 63rd day of life and all gave birth to pups. Litters were weighed, counted, and then standardized (4 males and 5 females per litter). Pups were weighed and counted on day 10 and day 21. After weaning, 2 females and 2 males (F1) from each litter were kept. Females were mated on day 63 of life. On the 18th day of pregnancy, 57 and 59 (control and algae group) were sacrificed; 51 and 53, respectively, gave birth to pups. Live, dead, absorbed foetuses, and corpora luteum were counted, and live foetuses were weighed. Born pups were counted, weighed and kept with dams without standardization, their number and weight recorded on days 10 and 21. Two females and

2 males (F2) were weaned and kept, and the procedure was repeated. Litters from the algae group were heavier at weaning. Females and males from the algae group also developed better than the ones from the control group.

Alcoholic extract of *Chlorella vulgaris* was examined for antioxidant potential in rat and it proved positive at 70 mg/kg bodyweight oral treatment. Rat whose antioxidant defense system decreased prior to the treatment became better off when treated with the algae. Both enzymatic (superoxide dismutase, catalase, and glutathione peroxidase) and non-enzymatic (glutathione reduced, ascorbic acid and alpha-tocopherol) antioxidant defense mechanisms increased after the algae treatment. It was concluded from the study that algae *Chlorella vulgaris* extract exerts chemo-preventive effects through modulation of antioxidants enzymes activities and inhibition of lipid peroxidation in rats exposed to naphthalene intoxication. Antioxidant compounds in the algae were reported to act as natural bioactive compounds offering protection against oxidative damages (Vijayavel *et al*., 2007; Dovi *et al*., 2012).

The microalgae are a natural source of antioxidants as reported by previous studies which identified the algae for its antioxidant composition and free radicals scavenging. *Chlorella vulgaris* extract increased antioxidant enzymes activities scavenged free radicals and increased total antioxidant concentrations as means of attenuating oxidative stress (Estevez *et al*., 2001; Miranda *et al*., 2001; Lee *et al*., 2003; Wu *et al*., 2005; Vijayavel *et al*., 2008; Bedirli *et al*., 2009; Azizzat *et al*., 2010; Wang *et al*., 2010). In a study by Rodriguez-Garcia and Guil-Guerrero (2008), it was also reported that the antioxidant activity of *Chlorella vulgaris* extract was higher compared with BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) which are the most

common synthetic antioxidants used for commercial application in foods and animal production.

Nutritional evaluations of the microalgae showed that it is rich in protein which is one of the most important nutrients for both humans and animals. The microalga contained between 42 and 58 % crude protein and protein quality standard stipulated by the World Health Organization (WHO) as well as the Food and Agricultural Organization (FAO) compared with egg and soybean as presented in Table 2.2.

Table 2.2: Amino acids profile of *Chlorella vulgaris* as compared with egg and soybean

Amino acids	*Chlorella Vulgaris*[1]	*Chlorella Vulgaris*[2]	*Chlorella Vulgaris*[3]	FAO/WHO Recommendation	Egg	Soya
Aspartic acid	9.30	10.94	9.80	N/A	11.00	1.30
Threonine	5.30	6.09	5.15	4.00	5.00	4.00
Serine	5.80	7.77	4.32	N/A	6.90	5.80
Glutamic acid	13.70	9.08	12.68	N/A	2.60	19.00
Glycine	6.30	8.60	6.07	N/A	4.20	4.50
Alanine	9.40	10.90	8.33	N/A	N/A	5.00
Cysteine	N/D	0.19	1.28	3.50	2.30	1.90
Valine	7.00	3.09	6.61	5.00	7.20	5.30
Methionine	1.30	0.65	1.24	N/A	3.20	1.30
Isoleucine	3.20	0.09	4.44	4.00	6.60	5.30
Leucine	9.50	7.49	9.38	7.00	7.00	7.70
Tyrosine	2.80	8.44	3.14	6.00	4.20	3.20
Phenylalanine	5.50	5.81	5.51	N/A	5.80	5.00
Histidine	2.00	1.25	1.97	N/A	2.40	2.60
Lysine	6.40	6.83	6.68	5.50	5.30	6.40
Arginine	6.90	7.38	6.22	N/A	6.20	7.40
Proline	5.00	2.97	4.90	N/A	4.20	5.30

Sources: Faheed and Abd. el-Fattah (2008); Naik *et al*. (2010); Safi *et al*. (2013); Carl *et al*. (2014).
N/A: Not Available
N/D: Not Determined

2.7.3. Microalgae as animal feed resources – application of *Chlorella vulgaris* in animal nutrition for improved performances

Factors including increasing cost, non-availability, price instability and the need for better and cheaper protein sources for animal production justifies the search for alternative protein feed resources for animal production. For example, soybean the leading animal protein source is feed resource whose alternative search has been a continuous scientific investigation due to the expensive nature of the ingredient and sometimes, it is not readily available for use in the feeding of animals. It is also under competition as food for man since it is used in formulation and production of infants' food and as milk replacers. Microalgae are identified as alternative unconventional feed resources with potential to replace soybean in animal production based on their nutritional values, fast growth and simplified production management. In addition to the nutritional valuable nature of microalgae, they contain bioactive compounds and metabolites which are of functional benefits to animals. These bioactive compounds are primary photosynthetic pigments and it includes chlorophyll, carotenoids, phycocyanin, allophycocyanin, and phycoerythrin. All these bioactive compounds are well documented as antioxidants of high values in animals (Adel *et al.,* 2017).

Microalgae including *Chlorella vulgaris*, *Dunaliella* and *Spirulina* have all been explored for the production of functional bioactive compounds such as vitamins, carotenoids, phycocyanin and other photosynthetic pigments useful for the promotion of animal health and productivity. They were all found capable of protecting against oxidative damages caused by reactive oxygen species (singlet oxygen, superoxide radical, hydroxyl radical and hydrogen peroxide) and other prooxidants. They are potential feed resources whose inclusion in animal feeds even at small quantities has

been shown to be of great benefits. Norambuena *et al.* (2015) stated that inclusion of microalgae at less than 10 % showed positive effects on growth performance and feed utilization efficiency because inclusion at 2.5 % and 10 % resulted in positive effects on growth performance by increasing weight gain, nutrient utilization, feed digestibility, carcass quality, physiological functions, and intestinal microbial environment.

According to Swiatkiewicz *et al*. (2015), it was concluded after evaluation of microalgae effects in animal nutrition that because of their chemical composition, microalgae can be efficiently used for the improvement of animal product quality and can as well partially replace conventional dietary protein sources such as soybean in animal diets. In their study, the researchers replace soybean at 0 %, 20 %, 40 %, 60 % and 80 % with *Chlorella vulgaris* for the production of diets fed to broilers chicken. The study proved that inclusion of the algae as replacement for soybean is feasible because it contains approximately 60 % crude protein with balanced amino acids as well as vitamins, minerals, and antioxidants.

Similarly, Kang *et al*. (2013) also explored *Chlorella vulgaris* as a source of non-chemical antibiotics as well as a growth promoter; the researchers reported that the alga is an alternative antibiotic source and growth promoter in chicken because it promotes production performance, immune system and intestinal microflora of the chicken. Furthermore, Kolrbacek *et al*. (1994) also reported that even at a very small quantity *Chlorella vulgaris* significantly influenced performance and improved physiological activities in chicken because it increases weight gain and phagocytic activity of white blood cells as well as lymphatic tissue development. Doucha *et al*. (2009) also explored the influence of selenium-enriched *Chlorella vulgaris* on performance of broiler chickens and concluded that there was positive effect of the preparations (0.3 mg of

Chlorella enriched selenium per Kg of diets) on body weight gain and activity of antioxidant enzyme glutathione peroxidase.

In a related study by Rezvani *et al*. (2012), it was also reported that inclusion of *Chlorella vulgaris* at 0.07 %, 0.14 % and 0.21 % in broiler chickens' diets from starter till 42 days, improved performance of the chicken through enhancement of feed conversion ratio and improved immune system. Also, *Chlorella vulgaris* inclusion at 1.25 % of laying bird diets positively affected oxidative stability of the egg yolk lipids and stored eggs according to Skrede *et al*. (2011) who evaluated replacement of microalgae with fishmeal. The study concluded that microalgae have potential replacement capability due to its nutritional compositions. *Chlorella vulgaris* in addition to its nutritional properties has also been widely investigated for its nutri-functional properties and a brief review of its uses with a specific focus on quantities of its supplementation and routes of administrations is summarized below in Table 2.3.

Table 2.3: Nutri-functional supplementation of *Chlorella vulgaris* and its derivatives in animals

	Authors & Years	Title	Remarks
1	Azizzat *et al*. (2010)	Modulation of oxidative stress by *Chlorella vulgaris* in streptozotocin (STZ) induced diabetic Sprague-Dawley rats.	150 mg/kg body weight for animals with bodyweight between 300 – 400 g for a period of 4 weeks.
2	Queiroz *et al*. (2016)	*Chlorella vulgaris* reduces the impact of stress on hypothalamic–pituitary–adrenal axis and brain *c-fos* expression.	Commercial diets and water were available *ad-libitum*, while *Chlorella vulgaris* supplementation was 50 and 200 mg/kg administered orally by gavages in a volume of 5 mL/kg
3	Kim *et al*. (2009)	Effect of *Chlorella vulgaris* intake on cadmium detoxification in rats fed cadmium.	5 or 10 % *Chlorella* of total kg diet for 4 weeks. 20.70 g/day feed intake; 1.035 – 2.07 g Chlorella vulgaris per day.
4	Mustafa (2015)	Potential alleviation of *Chlorella vulgaris* and *Zingiber officinale* on lead-induced testicular toxicity: an ultrastructural study.	50 mg/kg/rat of *Chlorella vulgaris* extract.
5	Zahran and Risha (2014)	Modulatory role of dietary *Chlorella vulgaris* powder against arsenic-induced immunotoxicity and oxidative stress in Nile tilapia (*Oreochromis niloticus*).	5 % and 10 % *Chlorella vulgaris* supplementation.
6	Furbeyre *et al*., (2018)	Effects of oral supplementation with *Spirulina* and *Chlorella* on growth and digestive health in piglets around weaning.	Orally administration of 385 mg/kg BW per day *Chlorella vulgaris* for 4 weeks.
7	Oh *et al*., (2015)	Effects of dietary fermented *Chlorella vulgaris* (CBT®) on growth performance, relative organ weights, cecal microflora, tibia bone characteristics, and meat qualities in Pekin ducks.	0, 1,000 or 2,000 mg/kg body weight of the ducks' administration of *Chlorella vulgaris* for 6 weeks.
8	Jeong *et al*. (2009)	Hypoglycaemic effect of *Chlorella vulgaris* intake in type 2 diabetic Goto-Kakizaki and normal Wistar rats.	Feeding of diets containing 0 %, 3 % or 5 % (w/w) *Chlorella* for 8 weeks; amounted to 44.19 g *Chlorella vulgaris* intake for the period.

9	Cheng *et al*. (2017)	Dietary *Chlorella vulgaris* ameliorates altered immunomodulatory functions in cyclophosphamide-induced immunosuppressive mice.	The *Chlorella vulgaris* (6 %), *Chlorella vulgaris* (12 %) and *Chlorella vulgaris* (24 %) were used for 6 weeks as a supplement in the diet; 0.7 g per day for 42 days.
10	Khalilnezhad *et al*. (2018)	Effects of *Chlorella vulgaris* on tumor growth in mammary tumor-bearing Balb/c mice: discussing the association of an immune-suppressed protumor microenvironment with serum IFN γ and IgG decrease and spleen IgG potentiation.	Supplementation of 0, 200, or 300 mg/kg for 42 days.
11	Tsiplakou *et al*. (2017)	The effect of dietary *Chlorella vulgaris* supplementation on micro-organism community, enzyme activities and fatty acid profile in the rumen liquid of goats.	Supplementation of 10 g lyophilized *Chlorella vulgaris*/kg concentrate (990 g control concentrate + 10 g lyophilized *Chlorella vulgaris*).
12	Morris *et al*. (2011)	Oral Administration of enzymatic protein hydrolysate from the green microalga *Chlorella vulgaris* enhances the nutritional recovery of malnourished mice.	Oral supplementation of 500 mg/kg *Chlorella vulgaris* for 8 days.
13	An *et al*. (2006)	Oral administration of hot water extracts of *Chlorella vulgaris* increases physical stamina in mice.	0.05 - 0.15 g/kg/day orally administered to mice for 7 days

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Experiment I - Influence of *Chlorella vulgaris* Supplemented Diets on Performances and Oxidative Stress Biomarkers of Prepubertal New Zealand White Rabbits

3.1.1. Location of the experimental studies

The was carried out at the Experimental Livestock Unit (ELU) of the National Institute of Animal Nutrition and Physiology (NIANP), Bangalore, India. The institute (NIANP) is a central government of India research establishment under the supervision of India Council for Agricultural Research (ICAR). The institute has a mandate for conducting and dissemination of research findings in all areas of animal nutrition and physiology. The institute is located at Adugodi area in Bangalore which is the capital city of Karnataka state, India. Agro ecologically, the area has Tropical Savannah vegetation and it is located on coordinates 12.97^{O} North (longitude) and 77.56^{O} East (latitude). The average monthly temperature is 23.90 ^{O}C while warmest temperature is 27.60 ^{O}C; average annual rainfall is 970 mm and usually spread clearly in two distinct seasons as dry and wet seasons (Sikiru *et al*., 2020). The average annual relative humidity of Bangalore is 65.2 % while average monthly relative humidity ranges from 45 % to 79 % (Santhosh *et al*., 2015).

3.1.2. Animals and their management

The rabbits were forty female weaned rabbits supplied by Biogen Biotechnology (Pvt) Ltd, India. Upon arrival at the ELU, they were acclimatized for a period of 14 days

before the commencement of the experimental trial. The animals were averagely eight weeks old with initial average weight of 1178.00 g, each. The animals were housed individually in battery cage in group for ease of identification of the animals. The animals were administered with a prophylactic dose of antibiotics during the acclimatization period. All the animals were fed with basal feed which is a commercial rabbits feed with proximate composition presented in Table 3.1. The protocol of the experiments was approved by the Institutional Animal Ethics Committee of the National Institute of Animal Nutrition and Physiology, Bangalore, India.

3.1.3. Experimental design

The experimental design was completely randomized design with 40 animals grouped into 5 categories as T0, T1, T2, T3, and T4 respectively. There were 4 replicates per each treatment whereby each replicate was assigned with 2 rabbits and 4 replicates were assigned to each treatment giving a total of 8 rabbits per each treatment group. Each treatment in addition to the basal feed and roughages, were supplemented with 0, 200, 300, 400 and 500 mg biomass of *Chlorella vulgaris* per kg bodyweight daily at 08:00 hours and by the afternoon 13:00 hours, 25 g wilted *Pennisetum purpureum* was served to each of the rabbit irrespective of their grouping differences (Table 3.3). The experimental model was simple linear model presented as follows:

$X_{ij} = \mu + e_{ij}$ .... (Equation i)

Where;

X_{ij} = the jth observation in the ith treatment

μ = the overall mean

X_i = effect of ith treatment

e_{ij} = Random error

3.1.4. Data collection

Data on performance parameters including feed intake, bodyweight changes, and feed conversion ratio were recorded on daily and weekly basis respectively. While oxidative stress biomarkers including malondialdehyde concentration, protein carbonyl content, total antioxidant capacity, and activities of antioxidant enzymes including superoxide dismutase, catalase, and glutathione were determined at the end of the experiment as described below:

3.1.4.1. Performance parameters

3.1.4.1.1. Feed intake: This was recorded as the quantity of feed consumed by each animal on a daily basis determined in grams. It was calculated through evaluation of differences between feed supplied and feed remnants on daily basis.

3.1.4.1.2. Bodyweight changes: This was recorded as the animal's weekly bodyweight changes determined from differences in body weight loss or gain at the beginning of the study and every week throughout the experimental period. It was determined by weighing the animals individually using a small digital scale of 0.20 g accuracy (Atom Scale, India).

3.1.4.1.3. Feed conversion ratio: This ratio was used in measuring the rate and efficiency with which the rabbits converted feed into weight gain. It was estimated as feed to gain per animals. Quantity of feed consumed per animal was recorded and expressed against weight gain by the animal within the period of the experiment.

Table 3.1: Chemical and nutritional composition of the basal feed fed to the rabbits

Parameters	Amount	Unit
Dry matter	90.89	% dry matter
Crude protein	18.55	% of dry matter
Total ash	7.90	% of dry matter
Metabolizable energy	2700	kcal/kg
Crude fibre	9.73	% of dry matter
Ether extract	2.99	% of dry matter

Source: Laboratory analysis by the researcher (2018).

Table 3.2: Chemical composition and nutritional values of *Pennisetum purpureum* supplied as roughage to the rabbits

Parameters	Amount	Unit
Dry matter	17.90	as fed
Crude protein	9.70	% dry matter
Crude fibre	36.10	% dry matter
Neutral detergent fibre	71.50	% dry matter
Acid detergent fibre	42.50	% dry matter
Lignin	5.70	% dry matter
Ether extract	2.00	% dry matter
Ash	13.80	% dry matter
Metabolizable energy	4158.70	kcal/kg

Source: Heuzé *et al*. (2016).

3.1.4.2. Serum oxidative stress and antioxidant enzymes activities

3.1.4.2.1. Blood collection, processing for serum separation and storage

In the last week of the experiment, 2 ml of blood was obtained from each animal through bleeding of mid-ear veins. The blood collected was subjected to the procedure of serum separation. The blood was obtained into vacutainers without anticoagulants, kept at room temperature for periods between 15 and 30 minutes allowing the blood to clot, the blood was then centrifuged at 2,000 x g for 10 minutes at 4 ^{O}C (Remi Centrifuge, India). Then serum samples were collected into test bottles and stored at -80 ^{O}C ahead of downstream biochemical analysis for oxidative stress biomarkers determination, which include malondialdehyde, protein carbonyl, total antioxidant capacity, superoxide dismutase, catalase, and glutathione.

3.1.3.2.2. Determination of serum lipid peroxidation concentrations

The lipid peroxidation was determined using the protocol described by Buege and Aust (1978) as reported by Ayala *et al*. (2014); Niki (2014); Gaschler and Stockwell (2017); Guvala *et al*. (2016) and Kagan (2018) with slight modifications. The procedures are described briefly as follows, a reaction mixture containing 500 µL of serum sample each, 200 µL of 8 % sodium-*n*-dodecyl-sulfate (SDS), 1000 µL of 20 % acetic acid and 1000 µL of 0.8 % 2-Thiobarbituric Acid (TBA). These reaction mixtures were individually boiled for 1 hour at 95 ^{O}C in a water bath (Brookfield, USA); and the reactions were terminated by cooling the mixtures on ice for 10 minutes. Then 1000 µL mixture of *n*-butanol and pyridine (15:1; v/v) was added to each reaction then vortex briefly and centrifuge at 4000 rpm for 10 minutes. Organic supernatant layers were obtained and set for absorbance reading with a microplate reader at 532 nM; molar

extinction coefficient of malondialdehyde was used for estimation of lipid peroxidation concentration in each sample expressed in nanomole (nmol) per mL.

3.1.4.2.3. Determination of serum protein carbonyl content

The procedure for protein carbonylation described by Colombo *et al.* (2016) was adopted for this study; the procedures involved evaluation of protein carbonyl derived from conjugation of the samples with 2,4-dinitrophenylhydrazine (DNPH) and quantified using spectrophotometric measurement. Briefly, 100 μL of serum samples were incubated with 100 μL of DNPH (20 mM prepared in 2N HCl) for 60 minutes in the darkroom. After the incubation, 1000 μL of 20 % Trichloroacetic Acid (TCA) was added then incubated on ice for 15 minutes and centrifuge at 10000 rpm for 5 minutes at 4 ^{O}C; supernatant from the centrifugation was discarded while protein pellets were washed twice in trichloroacetic acid and in ethyl acetate and ethanol mixture (1:1; v/v) then vortex mixed and followed by centrifuge at 3000 rpm for 15 minutes at 4 ^{O}C. This 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 a period of 30 minutes. After complete dissolution of the pellets in the guanidine solution, they were put up for absorbance reading at 366 nm in a Thermo Multiskan GO microplate reader (Thermo Fisher Scientific, Finland). Protein carbonyl concentration (μmol/mL) was calculated using molar absorption coefficient of 22,000 $M^{-1}cm^{-1}$.

3.1.4.2.4. Determination of serum total antioxidant capacities

There was an assessment of the overall antioxidant capacity of the serum using protocol described by Benzie and Strain (1999). This protocol was developed based on redox reactions of iron (III) to iron (II) at low pH. It is a chemical assay used for measuring ferric reducing antioxidant power of samples. In this study, a working assay was prepared and it contained 25 mL sodium acetate buffer (300 mM, pH 3.6), 2.5 mL of 10 mM 2,4,6-Tri-(2 pyridyl)-s-trizine prepared in 40 mM HCl and 2.5 mL ferric chloride (20 mM prepared in distilled water). The reaction mixture contained 50 µL of serum sample and 200 µL working solution incubated for 30 minutes at 37 ^{o}C (Labnet Inc., USA). Ascorbic acid was used as standard for calculating antioxidant power associated with colour changes and absorbance of reduced Fe^{3+} to Fe^{2+} at 493 nm. The total antioxidant concentration was expressed as µmol per mL of the serum samples.

3.1.4.2.5. Determination of serum superoxide dismutase activity

Superoxide dismutase activity was determined using chemical assay developed against autooxidation of pyrogallic acid (Marklund and Marklund, 1974) in the presence of DEPTA (Diethylenetriaminepentaacetic acid) at the pH 8.5 by 50 %; its modification by Guvvala *et al*. (2016) was adopted for this study. The superoxide dismutase enzyme activities assay used in this study comprised of 50 mM Tris cacodylate buffer prepared from Tris base and sodium cacodylate and with the addition of 1 mM DEPTA all dissolved into 200 ml distilled water. Twenty (20) Mm of 1,2,3-trihydroxybenzene (pyrogallic acid) solution was also prepared in 10 ml of 10 mM hydrochloric acid. This assay was used to prepare blank reaction mixture (Tris buffer 990 µL; pyrogallol 10 µL) and test reaction mixture (Serum 50 µL; Tris buffer 940 µL and pyrogallol 10 µL),

respectively. The reaction mixtures were individually set for absorbance reading against the blank at wavelength 420 nm by addition of Tris-DEPTA buffer (pH 8.5) at zero time and after 3 minutes of the addition of pyrogallol using UV-Vis spectrophotometer (Biochrom Libra S32, UK); change in absorbance were monitored at every 30 seconds, recorded, then used at the end of spectrometer absorbance readings for calculation of superoxide dismutase enzyme activity; percentage inhibition of pyrogallic autooxidation was used for estimating activity of the superoxide dismutase enzymes in each serum samples.

3.1.4.2.6. Determination of serum catalase enzyme activity

Serum obtained from separation protocol described above was used for assessment of catalase activities using the serum ability to decompose hydrogen peroxide (H_2O_2) into H_2O and O_2. The rate of the H_2O_2 decomposition was measured by decrease absorbance of the hydrogen peroxide at 240 nm over a period of 3 minutes. The assay used made up of a blank and reaction mixtures. The blank solution contained 900 µL of 50 mM potassium phosphate buffer (pH7.0), and 100 µL of 100 µM hydrogen peroxide while the reaction mixture contained 800 µL of potassium phosphate buffer, 100 µL hydrogen peroxide solution, and 100 µL serum sample. Spectrophotometer readings at 240 nm absorbance changes were noted at every 30 seconds interval for a period of 3 minutes reaction period which was used for calculating the catalase enzyme activities using the millimolar extinction coefficient of H_2O_2 at 240 nm.

3.1.4.2.7. Determination of serum glutathione reductase activity

The role of glutathione reductase enzyme in the prevention of oxidative stress is through oxidation of glutathione into reduced glutathione which serves as a primary antioxidant agent. In this study, an enzyme assay was prepared to contain 100 mM potassium phosphate buffer, 3.4 mM ethylene diamine tetraacetic acid (EDTA) pH 7.6 at 25°C, 30 mM Glutathione Substrate Solution (GSSG), and 0.8 mM ß-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form Solution (ß-NADPH). After the preparation of the reagents, reaction mixtures were prepared and put up for spectrophotometer reading at 340 nm for 5 minutes.

3.1.5. Data Analysis

All raw data obtained in the experiment I were subjected to one – way Analysis of Variance (ANOVA) using SPSS version 20.0 (IBM Corporations, USA). Significant means were determined at $P < 0.05$ level of probability and were subjected to homogeneity test using the Duncan test in the *Post hoc* tools. Furthermore, there was an analysis of nutri-functional relationships between the supplement diets prepared from the microalgae for the purpose of ranking them for their potential roles as antioxidant supplements using non-parametric analysis of variance tool known as Kruskal-Wallis test.

3.2. Experiment II – Influence of *Chlorella vulgaris* Supplemented Diets on Pre- and Post-partum Performances, and Oxidative Stress Biomarkers of New Zealand White Rabbits

3.2.1. Animals and their management

The animal management practices described in the section 3.1.2. was maintained for this experiment with gestational specific modifications. Briefly, the forty New Zealand White rabbits at the end of the experiment I were naturally serviced by active bucks not supplemented with the microalga in a mating ratio of 1:2 (buck:does), and kindling boxes were provided when the rabbits were approaching parturition.

3.2.2. Experimental design

As described in experimental I.

3.2.3. Experimental design

As described in experiment I.

3.2.4. Data collection

3.2.4.1. Performance parameters

Similar to data collection procedures described in experiment I; records of animal performance parameters and oxidative stress biomarkers were obtained as described below:

3.2.4.1.1. Feed intake: As described in experiment I.

3.2.4.1.2. Bodyweight changes: As described in experiment I.

3.2.4.1.3. Conception rates (%): This is the number of does that conceived from each group following pregnancy detection tests on days 16 and 21 days after mating.

$$\text{Conception rate (\%)} = \frac{\text{Number of pregnant does}}{\text{Number of does mated}} \times 100 \text{ .... (Equation ii)}$$

3.2.4.1.4. Gestation length: This is the number of days between mating and kindling. The mating day was recorded as day 0 of gestation while the kindling day was recorded as the last day of gestation; differences between day 0 of gestation and kindling days were taken as gestation length for each rabbit.

3.2.4.1.5. Litter size: This is a record of birth per rabbit doe; it was done through individual counting of the kits delivered per rabbit as described by Kalaba and Abdel-Khalek (2011).

3.2.4.1.6. Neonatal mortality: This is the number of kits which died during parturition per rabbit doe, it was subtracted from litter size after the counting within the first 12 hours of kindling which was used in the computation below:

$$\text{Neonatal mortality (\%)} = \frac{\text{Number of dead kitsx100}}{\text{Litter size}} \text{ .... (Equation iii)}$$

3.2.4.1.7. Viability rate: This is the number of rabbit kits which survived within the first 12 hours after birth per doe; the number of these kits per doe was used in the computation as follows:

$$\text{Viability rate (\%)} = \frac{\text{Number of live kits at birth}}{\text{Number of kits born}} \times 100 \text{ .... (Equation iv)}$$

3.2.4.1.8. Kindling rate: This is the computational percentage of rabbits does which kindled out of the rabbits does mated in each treatment using the formula:

$$\text{Kindling rate (\%)} = \frac{\text{Number of kindled does}}{\text{Number of mated does}} \times 100 \quad \text{......... (Equation v)}$$

3.2.4.1.9. Litter weight at birth: This is the total weight of kits kindled per rabbit doe; it was determined from the summation of individual rabbit kit weight at kindling by weighing using laboratory digital scale of 0.01 g accuracy (Atom Scale, India). The litter weight per rabbit was computed using the formula:

$$Y = X_1 + X_2 + X_3 \ldots X_n \quad \text{......... (Equation vi)}$$

(Where Y is the litter size in Kg and X_1, X_2, X_3 … Xn is the weight of each kit in the litter per rabbit doe).

3.2.4.1.10. Reproductive Index: This is the qualitative ranking of animal reproductive performance in a hierarchical order for comparison purposes. In rabbit production, determination of reproductive index makes use of litter size, kindling and viability rates values in cumulative evaluation which is a product of litter size, kindling rate and viability rate computed with the formula below:

Reproductive index (RI) = Litter size x Kindling rate x Viability rate … (Equation vii)

3.2.4.1.11. Gestation gain: Additional foetal weight from pregnancy is a factor leading to increasing of rabbits' weight during the gestation period. Apart from this, higher feed intake by the rabbits during gestation is another factor. Therefore, this

increase in weight due to pregnancy for each of the pregnant rabbits was monitored weekly throughout the gestation period by weighing the animals weekly and calculating differences in body weight changes.

3.2.4.2. Serum oxidative stress and antioxidant enzymes activities

3.2.2.2.1. Lipid peroxidation: As described in experiment I

3.2.4.2.2. Protein carbonylation: As described in experiment I.

3.2.4.2.3 Serum antioxidant enzymes: As described in experiment I.

3.2.5. Data Analysis

All data obtained were subjected to one – way Analysis of Variance (ANOVA) using SPSS version 20.0 (IBM Corporations, USA). Significant means were determined at P < 0.05 level of probability and were subjected to a homogeneity test using the Duncan test in the *Post hoc* tools.

3.3. Experiment III – Influence of *Chlorella vulgaris* Supplemented Diets on Performance and Oxidative Stress Biomarkers of Lactating New Zealand White Rabbits

3.3.1. Animals and their management

Thirty-five lactating New Zealand White rabbit randomly selected from the experiment II above standardized to have an equal number of kits (n = 5) were managed as described in the experiment I with modifications involving allowing the dams to suck their kits once daily. The lactation lasted for a period of forty-two days out of which the kits were made to depend exclusively on milk production from their dams for twenty-one days.

3.3.2. Experimental design

As described in experiment I.

3.3.3. Data collection

3.3.4.1. Performance parameters

Similar to data collection procedures described in the experiment I which include the records of animal performance parameters, oxidative stress biomarkers, and antioxidant enzymes activities were determined on daily, weekly basis, and at the end of the experimental study through animal observation and laboratory biochemical analysis.

3.3.4.1.1 **Feed intake**: As described in experiment I.

3.3.4.1.2. **Body weight changes**: As described in experiment I.

3.3.4.1.3. Body conditions of the rabbit does: This is animal body condition scorings done weekly throughout the lactating period; observed body conditions were described by Prebble *et al*. (2015) and presented in Table 3.3.

Table 3.3: Body condition score characteristics used for the assessment of the lactating rabbit does

Score	Description	Characteristics of the animals' body conditions
1	Very thin	Rabbits with more than 20 % body weight less than normal body weight of lactating and non-lactating rabbits. Sharp hip bones, ribs, and spine; curved rump area.
2	Thin	Rabbits with 10 - 20 % body weight less than normal body weight of lactating and non-lactating rabbits. Flat rump area; easily felt ribs, spine and hip bones.
3	Ideal	Rabbits with a normal body weight of lactating and non-lactating; these are rabbits which have flat rump without a sharp spine.
4	Overweight	Rabbits with 10 – 15 % above normal body weight of lactating and non-lactating rabbits; easily felt hip bones, ribs, and spine.
5	Obese	Rabbits with more than 15 % above normal body weight of lactating and non-lactating rabbits; very hard to feel spine and hips, ribs cannot be felt and rump area bulges out.

Source: Prebble *et al*. (2015)

3.3.4.1.4 Survivorship to weaning: This is the number of kits which survived from 12 hours after kindling to weaning as computed using the formula below:

$$\text{Survivorship to weaning (\%)} = \frac{\text{Number of kits at weaning X 100}}{\text{Number of kits at kindling}} \quad \text{… (Equation viii)}$$

3.3.4.1.5 Kits weight changes: Weekly body weight changes of each kit were recorded in gram. The difference in weight of a given week and the previous week was used to determine weight change for that week.

3.3.4.1.6 Litter size at weaning: This is the number of rabbit kits in each treatment at the time of weaning. This was done by counting of rabbit kits in each replicate, addition of which was used to estimate for the total number of rabbit kits at weaning for each treatment.

3.3.4.1.7. Milk yield capacity: Milk yield production was determined by estimation of the kit's growth with milk consumption using the formula below:

MY (g) = 1.69 x WGL+ 362 …. (Equation iii)

Where:

MY = Milk Yield in g for lactation period 0 – 21 days

WGL = Weight Gain of the Litter in g for lactation period 0 – 21 days (Fortun-Lamothe and Sabater (2003).

3.3.4.2. Serum oxidative stress and antioxidant enzymes activities

As described in experiment I.

3.3.5. Data Analysis

Data obtained were subjected to one – way Analysis of Variance (ANOVA) using SPSS version 20.0 (IBM Corporations, USA). Significant means were determined at $P < 0.05$ level of probability and were subjected to a homogeneity test using the Duncan test in the *Post hoc* tools of the software.

3.4. Experiment IV – Influence of Maternal Gestational Intakes of *Chlorella vulgaris* Supplemented Diets on Performances and Oxidative Stress Biomarkers of New Zealand White Rabbit Kits

3.4.1. Animals and their management

Fifteen rabbit kits were randomly selected from each group in experiment III on the 21st-day post-kindling. The animals were maintained in groups and management conditions as described in experiment I.

3.4.2. Experimental design

The design is as described in experiment I with slight modification in the number of animals per replicate. In this experiment, the number of animals per replicate was 5 rabbit kits and 3 replicates per treatment giving a total of 15 rabbit kits per each treatment group.

3.4.3. Data collection

3.4.3.1. Performance parameters

Performance parameters and growth of the rabbit kits observed in this experiment followed the description of performances data records described in experiment I:

3.4.3.1.1. Feed intake: As described in experiment I

3.4.3.1.2. Body weight changes: As described in experiment I

3.4.3.1.3. Feed conversion ratio: As described in experiment I

3.4.3.1.4. Serum oestrogen and testosterone levels: Concentrations of these reproductive hormones were determined using estradiol and testosterone ELISA kits according to the manufacturer protocols (Calibiotech, Inc., USA) while the serum preparation as described in experiment I was followed. For oestrogen concentration, 25 µL of each sample and standards were added with 100 µL working solution comprising anti-estradiol polyclonal antibody then incubated at 25 ^{O}C for 60 minutes in coated wells. After incubation, the wells were washed three times with wash buffer and blotted with absorbance paper. The washed wells were then incubated with 100 µL 3,3′,5,5′-Tetramethylbenzidine (TMB) reagents for 30 minutes at 25 ^{O}C then the reaction was stopped with 50 µL stop solution provided in the kit; absorbance readings were observed at 450 nm in a microplate reader and concentration of estradiol was determined in pg/mL using the standard plot curve.

For testosterone concentration, 50 µL of each sample and standards were added with 100 µL working solution containing conjugated testosterone enzymes and 100 µL biotin reagent then incubated at 25 ^{O}C for 60 minutes in coated wells. After incubation, the wells were washed three times with wash buffer and blotted with absorbance paper. The washed wells were then incubated with 100 µL 3,3′,5,5′-Tetramethylbenzidine (TMB) reagents for 30 minutes at 25 ^{O}C and the reaction was stopped with 50 µL stop solution provided in the kit; absorbance readings were observed at 450 nm in microplate reader and concentration of testosterone was determined in pg/mL using the standard plot curve.

3.4.4.2. Serum oxidative stress and antioxidant enzymes activities

As described in experiment I.

3.4.4.3. Serum biochemical profiling

Serum was processed from blood samples as described in experiment I and from the serum samples; biochemical parameters including Alanine Aminotransaminase (ALT), Aspartate Aminotransaminase (AST), creatinine, Alkaline Phosphatase (ALP), and bilirubin were determined. In addition, there was an evaluation of serum and blood urea concentration. These parameters were determined using commercial chemical kits measured using spectrophotometric absorbance. Kits for alanine amino transaminase, aspartate aminotransferase, alkaline phosphatase, and phosphorus were supplied by Proton Biologicals India (P) Ltd; while bilirubin, calcium and uric acid kits were procured from Sirus Biocare India (P) Ltd, Tara Clinical System, and Span Cogent India, respectively. The procedures of biochemical profiling were followed according to the instructions and protocol of their respective manufacturers while absorbance of kinetic reactions was obtained using spectrophotometer (Biochrom Libra S32, UK) and absorbance of endpoint reactions obtained using Multiskan plate reader (Thermofisher, Finland).

3.4.2.3. Gene expression analysis

For gene expression studies, sample preparations and procedures of cDNA synthesis, as well as quantification are described detailed in the appendix J. However, a set of genes investigated includes genes associated with a chemokine signaling pathway, interleukin, androgen receptor, nuclear factor kappa-light-chain-enhancer of activated B cells and cytochrome P450. The information about these genes and their respective oligo primers used is given in Table 3.4. The primers were designed by blasting of the nucleotides of

their respective genes from the database of National Centre for Biotechnology Information (NCBI).

3.4.4. Data Analysis

All raw data obtained in the experiment were analyzed using SPSS version 20.0. In the software, data were subjected to Analysis of Variance (ANOVA) and Duncan test was used for means separations while significant means were determined at $P < 0.05$ level of probability. Gene expression stability and fold changes analysis was carried and also subjected to Analysis of Variance (ANOVA) and Duncan test was used for means separations while significant means were determined at $P < 0.05$ level of probability.

Table 3.5: The genes and their respective oligo primers used for the quantitative gene expression studies in progenies of the rabbits

S/N	Gene Name	Primer type	Primer Sequence (5' to 3')	Primer Length	Product length	Accession No
1	Growth hormone receptor (GHR), mRNA	F	TTCACAGCCTTTACCCAGACA	21	200	NM_001082636.1
		R	CTGGACTACTTGGAGGGAAATAA	23		
2	Interleukin 6 (IL6), mRNA	F	GGAGGACTCCAACACCAAGG	20	230	NM_001082064.2
		R	AGGTCTCATTATTCACCGCCG	21		
3	Interleukin 1 alpha (IL1A), mRNA	F	GCACTTGAGTCGGCAAAGAAAT	22	228	NM_001101684.1
		R	GGAAGGTGAGGTTGGGTGAC	20		
4	Interleukin 2 (IL2), transcript variant 1, mRNA	F	TGCACTAACTCTTGCACTCCT	21	249	NM_001163180.1
		R	AGCATCCTGGAAAGTTTGGA	20		
5	Androgen receptor (AR), mRNA	F	GACTCTGTGCAGCCTATTGC	20	208	NM_001195724.1
		R	GTGCGGTGGAGTTAGGGAAA	20		
6	Nuclear receptor subfamily 3 C member 1 (NR3C1), mRNA	F	AAGGGCAGTGAAAGGACAGC	20	243	NM_001082147.1
		R	TGTGGTAATGCTGCAGGAACT	21		
7	Nuclear factor kappa B subunit 1 (NFKB1), mRNA	F	TCCACAAGGCAGCAGCTAGA	20	247	XM_017347386.1
		R	CCTTCCGGTGGGCAATACAG	20		
8	Cytochrome P450 2E1 (LOC100342572), mRNA	F	AAAGAGTACACACTCGCAAGA	21	248	XM_002718772.3
		R	AGTTTCTCTTCGATCTCGGGG	21		
9	Glutathione S-transferase pi 1 (GSTP1), mRNA	F	TGTCCCAGAACAAGGATGGC	20	248	XM_002724272.3
		R	AGGTCCCACAAACCCTCACT	20		
10	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA	F	GCTCCCGTTGCTGTCG	16	214	NM_001082253.1
		R	ATACTGGAACATGTAGACCATGTAG	25		

3.5. Experiment V – Influence of *Chlorella vulgaris* on Performance, Oxidative Stress Biomarkers and Genes Expression of Finishing New Zealand White Rabbits

3.5.1. Animals and their management

Forty weaned rabbits unrelated with the dams and kits of rabbits used in experiment I-III obtained at weaning and raised to finishing stage covering a period of 120 days after were used for this study. In addition to the animal management described in section 3.1.2. of experiment I, 3 rabbits per group were sacrificed and their respective liver, kidneys, uterus, and ovaries were collected for evaluation of tissues oxidative stress and genes expression studies. Details of animals' sacrifice, evisceration and excising of the organs were provided in the appendix L.

3.5.2. Experimental design

As described in experiment I.

3.5.3. Data collection

3.5.3.1. Performance parameters

Performance parameters, growth, feed conversion ratio, carcass traits, and meat yield recorded in this experiment followed the description of performances data records described in experiment I:

3.5.3.1.1. Feed intake: As described in experiment I.

3.5.3.1.2. Bodyweight changes: As described in experiment I.

3.5.3.1.3. Feed Conversion Ratio: As described in experiment I.

3.5.3.1.4. Haematopathological examination

Peripheral blood smear slides were prepared from blood drops obtained from the mid-ear veins of the animals were used for assessment of oxidative stress effects on the structure of the red blood cells. The slides were fixed in Giemsa stain for 72 hours (Nice Chemical (P) Ltd., India), and the stained slides were later examined under a fluorescence microscope (Nikon Eclipse 80i, USA).

3.5.3.1.5. Internal organs weight

Upon sacrificing the animals; internal organs including brain, liver, kidney, spleen, heart, uterus and ovaries were excised and weighed.

3.5.3.2 Serum oxidative stress and antioxidant enzymes activities

3.5.3.2.1. Sample collection and storage

Tissues including liver, ovaries, and uterus of the sacrificed animals were sampled approximately in 200 mg each in triplicate and stored cold PBS pH 7.4 and stored ahead of downstream analysis at -80 ^{O}C (Thermo Fisher Scientific, India).

3.5.3.2.2. Preparation of tissue homogenates

Homogenates of each the tissue were prepared as 10 % of the tissues in Tris HCl buffer (1 mM ethylene diamine tetraacetic acid, 1 mM dithiothreitol, and 50 mM Tris HCl) prepared in the laboratory as a homogenizer. One hundred (100) mg of the tissue sample each in triplicate were lysed under cold liquid nitrogen then homogenized with 1 mL of the homogenizer after which they were centrifuged at 3500 rpm for 15 minutes at 4 ^{O}C

(REMI Instrument, India); the supernatants were aliquot and stored at -22 ^{o}C for antioxidant enzymes activities.

3.5.3.2.3. Protein estimation

Concentration of protein in each tissue homogenate was determined using Bicinchoninic Acid (BCA) protein assay kit (G-Biosciences, USA). Following the manufacturer's protocol; there was the preparation of protein standards from Bovine Serum Albumin (BSA) through serial dilution of 300 µL of BSA from the stock into 150 µL Tris HCl buffer to produce final concentration of standards and a blank. In addition to the standard, a BCA working solution comprising the BCA reagent and copper solution was prepared, then 25 µL of each homogenate sample and 200 µL of the BCA working solution were sampled into microplate; and incubate at 37 ^{o}C in hybridization oven (Labnet Inc., USA). After the incubation; absorbance of the samples and the standards were determined at 562 nM using Thermo Multiskan GO microplate reader (Thermo Fisher Scientific, Finland).

3.5.3.2.4. Serum and tissues lipid peroxidation

As described in experiment I.

3.5.3.2.5. Serum and tissues protein carbonylation

As described in experiment I.

3.5.3.2.6. Total Antioxidant Capacity (TAC)

As described in experiment I.

3.5.3.3. Carcass characteristics and meat quality attribute

Upon sacrificing the animals, carcass traits including slaughter weight (g), dressing (%); commercial weight, meat yield, and weights of internal organs such as liver, heart, kidney, spleen, and lungs were taken. In addition to these physical characteristics; proximate chemical composition and fatty acid profile of the meat obtained from the rabbits were determined as attributes of the meat quality of the rabbits (Anjumoni *et al*., 2018).

3.5.3.4. The pattern of antioxidant genes expression in the rabbits

For gene expression, real-time quantitative polymerase chain reaction (RT-qPCR) protocol described by Penna *et al*. (2011) and Guvvala *et al*. (2019) were adopted with modification considering similarities and differences in the tissues selected for evaluation. The details of the gene expression protocol are given in appendix J. The investigated genes in the tissues samples include the oviductal glycoprotein 1 (OVGP1), uncoupling protein 1 (UCP1), oxidative stress-responsive 1 (OXSR1), brain and reproductive organ-expressed (TNFRSF1A modulator) (BRE), superoxide dismutase 1 (SOD1) and glutathione peroxidase 1 (GPX1) while glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The genes, their primers, and other associated information are presented in Table 3.5.

Table 3.5: The genes and their respective oligo primers used for quantitative gene expression studies of the ovaries and livers of the finisher rabbits

S/N	Gene Name	Primer type	Primer Sequence (5' to 3')	Primer Length	Product size	Accession No
1	Oviductal glycoprotein 1 (OVGP1)	F	TTCCTTGCCTGAGACCAGTT	20	121	NM_001105687.1
		R	CTCTCTCTCGGGGTGGTCATC	21		
2	Uncoupling protein 1 (UCP1)	F	CCTAACAACTGGAGGCGTGG	20	177	NM_001171077.1
		R	GGAGTTGTCCCTTTCCACAGA	21		
3	Oxidative stress responsive 1 (OXSR1)	F	GTTTGCGTAAAGGTGGGTGG	20	120	XM_008252717.2
		R	AGCAGGCACACACATCACTG	20		
4	Brain and reproductive organ-expressed (TNFRSF1A modulator) (BRE)	F	TCAGTCACTTTGGCACAGGT	20	120	XM_002709945.3
		R	GGAAAAACAGGGGCAGGTCA	20		
5	*Oryctolagus cuniculus* superoxide dismutase 1 (SOD1)	F	GGTGGTCAAGGGACGCATAA	20	205	NM_001082627.2
		R	CACATCAGCCACACCATTGC	20		
6	*Oryctolagus cuniculus* glutathione peroxidase 1 (GPX1)	F	TTTGGGCATCAGGAGAACGC	20	211	NM_001085444.1
		R	TGATGAACTTGGGGTCGGTC	20		
7	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	F	TCGGAGTGAACGGATTTGGC	20	181	NM_001082253.1
		R	TTCCCGTTCTCAGCCTTGAC	20		

3.5.3. Data Analysis

All raw data obtained were analyzed using SPSS version 20.0. In the software, data were subjected to Analysis of Variance (ANOVA) and Duncan test was used for means separations while significant means were determined at $P < 0.05$ level of probability. Gene expression stability and fold changes analysis was carried and also subjected to Analysis of Variance (ANOVA) and Duncan test was used for means separations while significant means were determined at $P < 0.05$ level of probability.

CHAPTER FOUR

4.0. RESULTS

4.1. *In-vitro* Antioxidant and Nutritional Properties of the *Chlorella vulgaris* Supplements

Chlorella vulgaris biomass and its derivatives were prepared and evaluated *in-vitro* for their antioxidants and nutritional properties which formed the basis for their selection for use in this study. They were used as supplements based on their antioxidant composition, ferric reducing antioxidant power, hydrogen peroxide scavenging potentials and total phenolic content. The mean total antioxidant concentration of the microalgae and its derivatives was 621.83±36.41 µmol/g ascorbic acid equivalent ($p < 0.001$) while minimum and maximum antioxidant concentrations components were 601.21 and 902.54 µmol/g ascorbic acid equivalent, respectively. Ferric reducing antioxidant power (FRAP) was 1.28±0.09 ($p < 0.001$) while minimum and maximum antioxidant FRAP were 1.20 and 2.48, respectively. Hydrogen peroxide scavenging (HPS) potential capacity was 34.04±3.18 % ($p < 0.001$) while minimum and maximum hydrogen peroxide scavenging capacities were 23.26 and 59.58 %, respectively. The total phenolic content of the diets was 30.54±0.05 mg/g ellagic acid equivalent ($p < 0.001$) while minimum and maximum total phenolic contents were 36.28 and 43.93 mg/g ellagic acid equivalent respectively; the supplements were iso-caloric and have functional properties as presented below (Table 4.1).

Table 4.1: Proximate composition and nutri-functional properties of the *Chlorella vulgaris* supplemented diets to the rabbits at different physiological stages

Parameters	T0	T1	T2	T3	T4	Mean	Units
Dry matter	91.15	93.95	94.38	93.54	93.33	93.80	% dry matter
Crude protein	3.00	30.46	44.36	53.86	61.15	47.45	% of dry matter
Total ash	2.60	2.23	3.59	4.30	5.28	3.85	% of dry matter
Metabolizable energy	3076.00	3033.00	3025.00	3083.00	3048.00	3053.80	kcal/Kg
Crude fibre	9.73	0.92	1.75	1.71	2.90	1.82	% of dry matter
Ether extract	2.99	1.22	1.13	1.18	0.80	1.08	% of dry matter
Chlorella vulgaris	0.00	200	300	400	500	350	mg/500 mg
Total phenolic content	0.22	39.38	36.28	41.16	43.93	30.54	mg/g ellagic acid equivalent
Total antioxidant concentration	4.41	670.30	601.21	760.00	902.54	621.83	µmol /g ascorbic equivalent
H_2O_2 Scavenging Power	0.24	47.44	23.26	59.58	41.14	34.04	%
FRAP	0.00	1.34	1.20	1.52	2.48	1.28	-n/a-

Source: Laboratory analysis by the researcher (2018).
FRAP - Ferric Reducing Antioxidant Power (FRAP).
T0 – Control group without any form of supplementation
T1 – group supplemented with 200 mg *Chlorella vulgaris* biomass
T2 – group supplemented with 300 mg *Chlorella vulgaris* biomass
T3 – group supplemented with 400 mg *Chlorella vulgaris* biomass
T4 – group supplemented with 500 mg *Chlorella vulgaris* biomass
-n/a - Not applicable.

4.2. Effects of *Chlorella vulgaris* Supplementation on Production Performance of the Pre-Pubertal Rabbits

There was no significant difference in initial body weights of the rabbits which was 1178.00±13.64 g ($p > 0.05$). However, supplementation of the microalgae led to a significant increase in the animal's final bodyweight. The overall mean final body weight was 2077.56±20.74 g ($p < 0.01$) while minimum and maximum final body weights of the rabbits were 1960.85±18.64 and 2143.72±36.34 g, respectively. Rabbits in the treatment groups had significantly higher final body weight compared with rabbits in the control group (Table 4.2).

Furthermore, there was no significant difference in feed intake but the control group has highest feed intake; the mean feed intake was 5902.84±116.66 g ($p < 0.56$) while minimum and maximum feed intakes were 5604.14±116.36 and 6084.36±118.64 g, respectively. However, there was a significant difference in weight gains of the rabbits, mean weight gain was 1445.69±27.75 g ($p < 0.003$) while minimum and maximum weight gains were 1003.35±20.52 and 1244.92±20.25 g, respectively. There was also a significant difference in feed conversion ratio ($p < 0.003$); mean feed conversion ratio was 5.19±0.23 ($p < 0.003$) while minimum and maximum feed conversion ratios were 4.52±0.24 and 6.12±0.12, respectively. Although there was no difference in feed intakes and despite the same levels of the feed intakes, rabbits in treatment groups had higher weight gains and better feed conversion ratios compared with the control group (Figure 4.1).

Table 4.2: Effects of *Chlorella vulgaris* supplemented diets on performances of the rabbits at the pre-pubertal stage

Parameters	T0	T1	T2	T3	T4	Mean	p – value
Initial weight (g)	957.50±24.13	920.77±31.34	925.76±25.65	953.88±24.15	954.25±48.58	1178.00±13.64	0.710
Final bodyweight (g)	1960.85±18.64[b]	2143.72±36.34[a]	2080.28±30.57[a]	2110.90±25.25[a]	2092.03±20.15[a]	2077.56±20.74	0.010
Weight gain (g/day/rabbit)	16.72±0.34[b]	20.38±0.42[a]	19.24±0.40[a]	19.28±0.34[a]	18.96±0.33[a]	18.92±0.46	0.003
Feed intakes (g/day)	101.40±1.98	95.14±2.00	101.15±1.91	100.80±2.08	93.40±1.94	98.38±1.98	0.560
Feed conversion ratio	6.12±0.12[a]	4.82±0.22[b]	5.27±0.18[b]	5.24±0.14[b]	4.52±0.24[b]	5.19±0.23	0.003

[abc]Means with different superscripts along the same rows are significantly different ($p < 0.05$) for each of the parameters measured. Control – no supplementation, T1 – group supplemented with 200 mg *Chlorella vulgaris* biomass, T2 – group supplemented with 300 mg *Chlorella vulgaris* biomass, T3 – group supplemented with 400 mg *Chlorella vulgaris* biomass and T4 – group supplemented with 500 mg *Chlorella vulgaris* biomass.

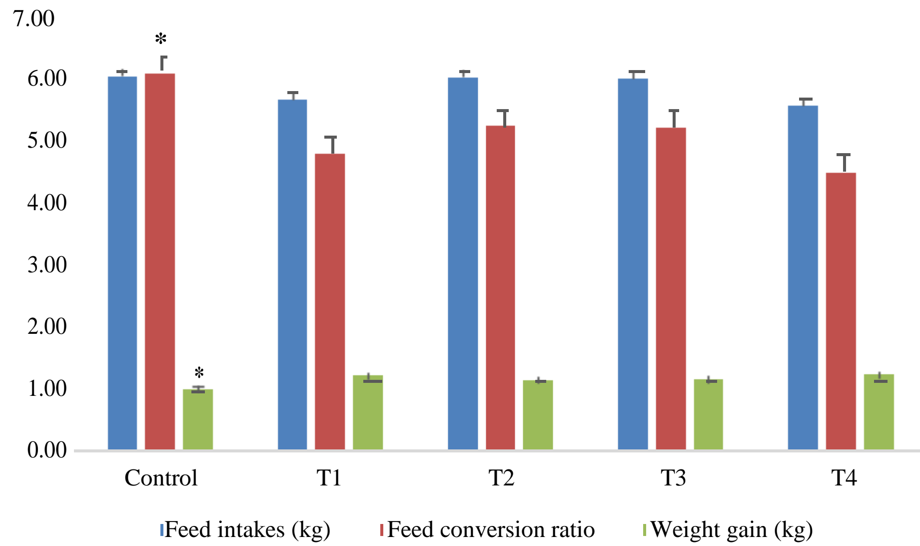

Fig. 4.1: Comparative effect of *Chlorella vulgaris* supplementations on the efficiency of feed utilization of the rabbits.

Control – no supplementation, T1 – group supplemented with 200 mg *Chlorella vulgaris* biomass, T2 – group supplemented with 300 mg *Chlorella vulgaris* biomass, T3 – group supplemented with 400 mg *Chlorella vulgaris* biomass and T4 – group supplemented with 500 mg *Chlorella vulgaris* biomass. Bars with different labels are significantly different ($p < 0.05$).

4.3. Effects of *Chlorella vulgaris* on Oxidative Stress in the Pre-Pubertal Rabbits

There was a significant difference in the oxidative stress status of the rabbits due to supplementation of the microalgae as revealed by concentrations of malondialdehyde and total antioxidant capacity of the serum. The overall mean malondialdehyde concentration was 11.62±2.03 nmol/mL ($p < 0.001$) while minimum and maximum concentrations were 6.02±0.40 and 27.95±3.60 nmol/mL, respectively. Similarly, there was a significant difference in total antioxidant capacities of the rabbit's serum. The overall mean total antioxidant capacity was 100.00±22.4 µmol/mL ($p < 0.003$) while minimum and maximum total antioxidant capacities were 120.04±26.10 and 363.80±14.41 µmol/mL, respectively. The results indicated that supplementation of *Chlorella vulgaris* reduced oxidative stress because there was a higher concentration of malondialdehyde and lower levels of total antioxidant capacity in the control group compared with the treatment groups (Figure 4.2).

In addition, there were significant differences in activities of antioxidant enzymes in the rabbit's serum; the overall mean superoxide dismutase enzyme activity was 79.42±5.47 U/mL ($p < 0.001$) while minimum and maximum activities were 33.66±3.14 and 98.31±8.52 U/mL, respectively for the control and T4. There was also a significant difference in activities of catalase, the overall mean activity of the enzyme was 28.88±3.38 U/mL ($p < 0.003$) while minimum and maximum were 11.05±1.02 and 48.50±1.15 U/mL, respectively for the control and T4. There was also a significant difference in glutathione reduced concentration. The overall mean concentration of glutathione reduced was 32.83±2.04 µmol/mL ($p < 0.001$) while minimum and maximum were 21.06±3.30 and 39.82±8.90 µmol/mL, respectively (Table 4.3).

Table 4.3: Effect of *Chlorella vulgaris* supplemented diets on oxidative stress of the rabbits at the pre-pubertal stage

Parameters	Control	T1	T2	T3	T4	Mean	p-value
Malondialdehyde (nmol/mL)	27.95±3.60[a]	7.39±1.98[b]	6.96±2.44[b]	9.78±0.66[b]	6.02±0.40[b]	11.62±2.03	0.001
Total antioxidant (µmol/mL)	120.03±26.10[a]	247.50±22.47[b]	266.40±27.70[b]	247.80±9.94[b]	363.80±14.41[c]	100.00±22.40	0.003
Catalase activity (U/mL)	11.05±1.02[a]	32.45±2.27[b]	27.01±2.36[b]	25.39±0.67[b]	48.50±1.15[c]	28.88±3.38	0.001
Superoxide dismutase (U/mL)	33.66±3.14[a]	84.21±5.88[bc]	77.28±1.73[b]	88.63±4.86[bc]	98.31±8.52[c]	79.42±5.47	0.001
Reduced glutathione (µmol/mL)	21.06±3.30[a]	39.82±8.90[b]	30.74±7.30[b]	36.48±4.70[b]	36.04±7.50[b]	32.83±2.04	0.01

[abc]Means with different superscripts along the rows are significantly different ($p < 0.05$) for each of the parameters measured. Control – no supplementation, T1 – group supplemented with 200 mg *Chlorella vulgaris* biomass, T2 – group supplemented with 300 mg *Chlorella vulgaris* biomass, T3 – group supplemented with 400 mg *Chlorella vulgaris* biomass and T4 – group supplemented with 500 mg *Chlorella vulgaris* biomass.

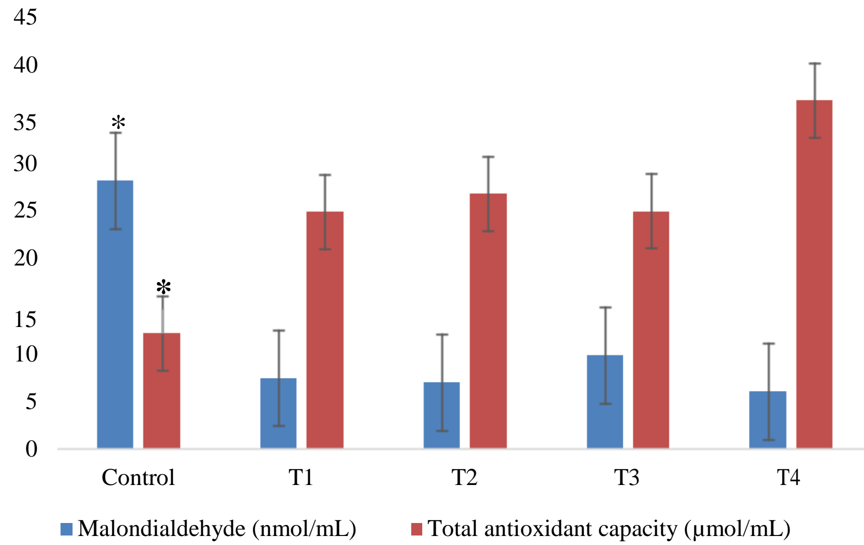

Fig. 4.2: **Comparative effect of *Chlorella vulgaris* on lipid peroxidation and total antioxidant capacity of the rabbits.**

Control – no supplementation, T1 – group supplemented with 200 mg *Chlorella vulgaris* biomass, T2 – group supplemented with 300 mg *Chlorella vulgaris* biomass, T3 – group supplemented with 400 mg *Chlorella vulgaris* biomass and T4 – group supplemented with 500 mg *Chlorella vulgaris* biomass. Bars with different labels are significantly different ($p < 0.05$).

4.4. Effects of *Chlorella vulgaris* on Performances of the Rabbits During Gestation Period

Supplementation of the microalgae led to a significant increase in the reproductive index of the rabbits ($p < 0.01$). The overall mean reproductive index was 5.08 while minimum and maximum reproductive indexes were 3.57 and 7.00, respectively. The supplementation also led significant increase sexual receptivity of the rabbits ($p < 0.02$). The overall mean sexual receptivity was 50.30 seconds, while minimum and maximum duration of sexual receptivity were 12.85 and 112.36 seconds, respectively upon introduction of female rabbits to the bucks.

There was no significant difference in the rabbit's gestation weight gain ($p < 0.21$), the overall mean gestation gain was 362.02±21.88 g while minimum and maximum gestation gains were 300.97 and 426.91 g, respectively. There was a significant difference in gestation length ($p < 0.03$), the overall mean gestation length was 31.61±0.27 days while minimum and maximum gestation lengths were 30.43 and 32.80 days, respectively. There was also a significant difference in the kindling rates as a result of the supplementation ($p < 0.01$). The overall mean kindling rate was 76.46 % while minimum and maximum kindling rates were 57.14 and 100 %, respectively (Table 4.4).

There was significant correlation between reproductive index and other parameters including gestation gain ($p <0.01$), litter size ($p <0.01$), number of live kits ($p <0.01$), gestation length ($p <0.01$) and viability rates ($p <0.01$). Similarly, there was significant correlations between kindling rate and other parameters including gestation gain ($p <0.01$), gestation length ($p <0.02$) and viability rates ($p <0.05$) as presented in Table 4.5.

These are indications that the supplementation significantly influenced reproductive performances of the rabbits because the supplementation of *Chlorella vulgaris* significantly increased both the reproductive index and kindling rates (Table 4.6). However, there was no significant difference in daily feed intakes of the rabbits ($p < 0.81$), the overall mean feed intake per day was 92.02±1.48 g while minimum and maximum feed intake were 88.95 and 94.99 g respectively.

Table 4.4: Effects of *Chlorella vulgaris* supplemented diets on pre- and post-partum performances of the New Zealand White rabbit does

Parameters	Control	T1	T2	T3	T4	Mean	SEM	*p*-value
Daily feed intake (g)	93.05	94.99	91.70	88.95	91.88	92.11	1.48	0.08
Sexual receptivity (sec)	112.36[a]	57.08[ab]	12.85[b]	25.57[b]	44.64[ab]	50.30	10.77	0.02
Gestation length (days)	31.29[a]	32.80[a]	32.14[a]	32.29[a]	30.43[ab]	31.61	0.31	0.05
Gestation gain (g)	420.66	335.93	300.97	321.89	426.91	362.02	21.83	0.20
Litter size at birth (No)	6.00	5.00	5.00	4.00	6.00	5.20	1.00	0.67
Viability rates (%)	80.95	63.89	64.62	55.10	100.00	72.91	7.14	0.20
Kindling rates (%)	85.71[b]	83.33[c]	57.14[d]	57.14[d]	100[a]	76.46	2.98	0.01
Reproductive index	5.36[b]	5.62[ab]	3.85[bc]	3.57[c]	7.00[a]	5.08	0.37	0.01

[a,b,c] Means with a 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 4.5: Correlations between reproductive index, kindling rates, and other reproductive parameters of the rabbits does fed *Chlorella vulgaris* supplemented diets

	Reproductive index		Kindling rate	
	r – value	*p* – value	*r* – value	*p* – value
Gestation gain	0.669	0.01**	0.565	0.01**
Litter size	0.899	0.01**	0.217	0.22
No of live kits	0.920	0.01**	0.247	0.16
Gestation length	0.692	0.01**	-0.376	0.03*
Viability rate	0.874	0.01**	0.340	0.05*

** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

4.5. Effects of *Chlorella vulgaris* on Oxidative Stress Status of Pregnant Rabbits

Analysis of serum lipid peroxidation, protein carbonylation and total antioxidant capacity of the rabbits during gestation period indicated that the supplementation of the microalgae significantly improved antioxidant protection of the rabbits. There was significant difference in malondialdehyde (MDA) concentration across the experimental groups ($p < 0.003$); mean concentration of the malondialdehyde was 15.36±1.23 nmol/mL while minimum and maximum MDA concentrations were 11.82 nmol/mL and 23.85 nmol/mL, respectively; although, there was no significant difference in protein carbonyl ($p < 0.66$), there was significant difference in total antioxidant capacity (TAC) of the serum; mean total antioxidant capacity was 61.09±10.06 µmol/mL ($p < 0.02$) while minimum and maximum TAC were 11.68 and 102.05 µmol/mL, respectively (Table 4.6).

Similarly, the supplementation improved activities of serum antioxidant enzymes because there were significantly higher antioxidant enzymes activities in the treatment groups compared with control; mean superoxide dismutase (SOD) activities was 9.06±0.34 U/mL ($p < 0.04$) while minimum and maximum SOD units per mL were 6.82 U/mL and 9.87 U/mL, respectively. Mean catalase (CAT) activities was 9.59±0.34 U/mL ($p < 0.005$) while minimum and maximum CAT activities were 7.61 U/mL and 10.38 U/mL, respectively. Glutathione reduced concentration (GSH) was 63.12±1.17 µmol/mL ($p < 0.001$) while minimum and maximum glutathione reduced were 51.65 and 71.62 µmol/mL, respectively (Table 4.7).

Table 4.6: Effects of *Chlorella vulgaris* supplemented diets on oxidative stress status of the rabbits during gestation

Parameters	Control	T1	T2	T3	T4	Mean	*p*-value
Malondialdehyde concentration (nmol/mL)	23.85±2.61[a]	13.54±1.32[b]	14.05±1.59[b]	11.82±1.68[b]	13.54±2.19[b]	15.36±1.23	0.003
Protein carbonyl concentration (µmol/mL)	30.32±4.76	9.01±11.96	7.53±14.13	8.30±21.81	5.62±14.40	12.15±13.41	0.66
Total antioxidant capacity (µmol/mL)	11.68±2.61[b]	102.05±22.47[a]	58.28±27.72[ab]	48.42±9.92[ab]	85.01±14.42[a]	61.09±10.06	0.02

[a,b,c] Means with a 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 4.7: Effects of *Chlorella vulgaris* supplemented diets on antioxidant enzymes activities in the rabbits during gestation

Parameters	Control	T1	T2	T3	T4	Mean	*p*-value
Superoxide dismutase activities (U/mL)	6.82±0.74[b]	9.59±1.13[a]	9.87±0.39[a]	9.62±0.28[a]	9.36±0.35[a]	9.06±0.34	0.02
Catalase activities (U/mL)	7.61±1.02[b]	9.46±2.27[a]	10.38±2.36[a]	9.37±0.67[a]	11.12±1.15[a]	9.59±1.49	0.005
Glutathione reduced activities (µmol/mL)	51.65±1.01[b]	64.51±4.00[a]	63.89±1.01[a]	71.62±2.00[a]	63.89±1.01[a]	63.12±1.17	0.001

[a,b,c] Means with a 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.

4.6. Effects of *Chlorella vulgaris* Supplementation on Lactation and Pre-Weaning Performances of the Rabbit Does and their Kits

Supplementation of *Chlorella vulgaris* led to a significant difference in body condition scores of the rabbits ($p < 0.009$); mean body condition score was 2.90±0.14 while minimum and maximum body condition scores were 1.50 and 2.75, respectively. There was also a significant difference in milk yield of the rabbit does ($p < 0.001$); mean milk yield was 1333.15±77.47 g, while minimum and maximum milk yields were 879.25 and 1570.00 g, respectively. There was a significant difference in litter weight gain on 21-days post kindling ($p < 0.009$) when the rabbit kits exclusively depended on their dams' milk for survival; mean litter weight gain was 574.65±45.86 g, while minimum and maximum litter weight gains were 305.75 and 715.00 g, respectively. There was no significant difference in feed intake of the rabbit does during the lactation period ($p < 0.477$), mean feed intake was 97.45±1.91 g while minimum and maximum feed intakes were 92.27 g and 102.87 g, respectively (Table 4.8).

Table 4.8: Effects of *Chlorella vulgaris* supplemented diets on performance of the rabbit does and their kits during the lactation period

Parameters	Control	T1	T2	T3	T4	Mean	SEM	*p*-value
Body conditions score	1.50[b]	2.00[ab]	2.75[a]	2.75[a]	2.50[a]	2.90	0.14	0.009
Milk yield (g)	1118.00[b]	1533.25[a]	1570.00[a]	1565.25[a]	879.25[b]	1333.15	77.47	0.001
Litter weight at 21 days (g)	447.50[b]	693.00[a]	715.00[a]	712.00[a]	305.75[b]	574.65	45.86	0.001
Daily feed intakes (g)	102.87	97.92	92.27	99.56	94.64	97.45	1.91	0.48

[a,b,c] Means with a 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.

4.6.1. Relationships between lactation performances of the does and pre-weaning performances of their kits

Relational analysis between the does and their kits' performances due to interactions between supplementation of *Chlorella vulgaris* and cumulative performance of the rabbit does from pre-pubertal to lactation stages indicated there were no significant effects of the supplementation on feed intake ($p > 0.05$); but there were significant effects of the supplementation on body condition scores ($p < 0.03$); milk yield ($p < 0.009$); and litter weight gain (0.009). The relational performance analysis of the rabbits and their kits ranked treatment groups as better performers compared with control (Figure 4.3).

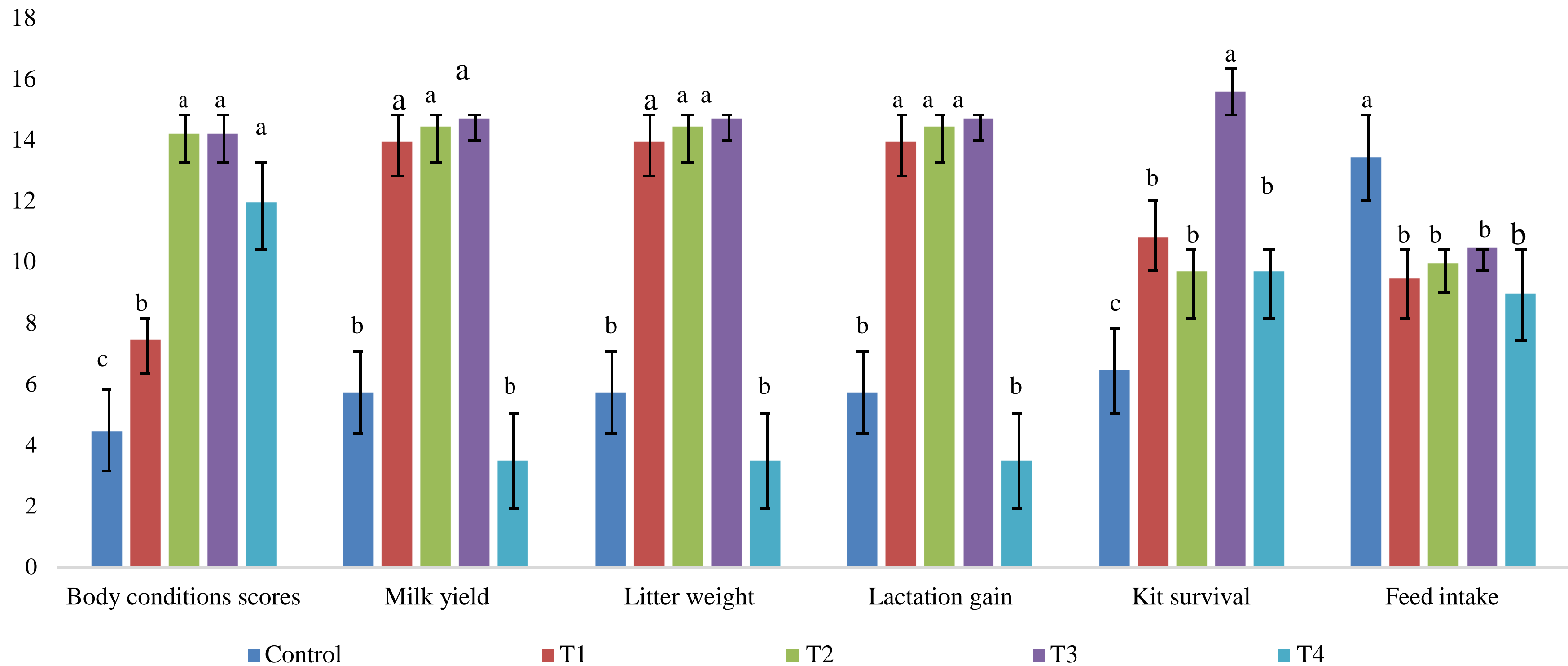

Fig. 4.2: Effects of interactions between *Chlorella vulgaris* supplemented diets and performance of the rabbit does and their kits during lactation period
Control – no supplementation, T1 – group supplemented with 200 mg *Chlorella vulgaris* biomass, T2 – group supplemented with 300 mg *Chlorella vulgaris* biomass, T3 – group supplemented with 400 mg *Chlorella vulgaris* biomass and T4 – group supplemented with 500 mg *Chlorella vulgaris* biomass. Bars with different labels are significantly different ($p < 0.05$).

4.7. Effects of *Chlorella vulgaris* on Oxidative Stress Status of Lactating Rabbits

There were significant differences in the distribution of all oxidative stress biomarkers of the rabbits determined during lactation. The mean concentration of malondialdehyde was 4.99±0.68 nmol/mL ($p < 0.001$), superoxide dismutase enzyme was 9.12±0.46 U/mL ($p < 0.001$), catalase enzyme was 10.25±0.90 U/mL ($p < 0.005$), glutathione reduced concentration was 6.27±0.86 µmol/mL ($p < 0.001$), protein carbonyl concentration was 2.72±0.61 µmol/mL ($p < 0.001$) while total antioxidant capacity was 34.68±0.67 µmol/mL ($p < 0.036$). Control group had significantly highest concentration of malondialdehyde and lowered antioxidant enzymes activities (Table 4.9).

There were significant inverse correlations between concentration of malondialdehyde and total antioxidant capacity ($p < 0.006$), superoxide dismutase enzyme activities ($p < 0.012$), glutathione reduced concentration ($p < 0.008$). Similarly, there was a significant inverse correlation between the concentration of protein carbonyl and superoxide dismutase enzyme activities ($p < 0.001$), glutathione reduced concentration ($p < 0.010$) (Table 4.10).

Table 4.9: Effects of *Chlorella vulgaris* supplemented diets on oxidative stress of the rabbit does during lactation

Parameters	Control	T1	T2	T3	T4	Mean	*p*-value
Malondialdehyde (nmol/mL)	9.79±1.16[a]	3.72±0.42[b]	3.35±1.42[b]	5.11±1.78[b]	2.99±0.29[b]	4.99±0.68	0.001
Superoxide dismutase (U/mL)	5.63±0.62[a]	9.84±0.88[b]	10.28±0.24[b]	10.38±0.20[b]	9.36±0.35[b]	9.12±0.46	0.001
Catalase (U/mL)	6.96±0.50[a]	9.46±1.74[a]	16.00±2.36[b]	8.87±0.67[a]	9.95±0.92[a]	10.25±0.90	0.005
Glutathione reduced (µmol/mL)	4.96±5.80[a]	6.45±1.00[b]	6.38±1.00[b]	7.16±2.00[b]	6.36±1.00[b]	6.27±0.86	0.001
Protein carbonyl (µmol/mL)	6.85±1.36[c]	2.90±0.38[b]	1.80±0.55[ab]	0.16±0.84[a]	2.23±0.19[ab]	2.72±0.61	0.001
Total antioxidant capacity (µmol/mL)	31.05±0.55[a]	34.69±1.53[ab]	34.52±0.98[ab]	36.05±1.96[b]	37.08±0.38[b]	34.68±0.67	0.036

[a,b,c] Means with a 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 4.10: Correlations between oxidative stress biomarkers and the rabbits' antioxidant activities during lactation period in rabbit does fed *Chlorella vulgaris* supplemented diets.

	Total antioxidant capacity		**Superoxide dismutase**		**Glutathione reduced**	
	r – value	*p* – value	*r* – value	*p* – value	*r* – value	*p* – value
Malondialdehyde	-0.595	0.006**	-0.649	0.01**	-0.573	0.008**
Protein carbonyl	-0.315	0.18	-0.0689	0.001**	-0.822	0.010**

**Correlation is significant at the 0.01 level (2-tailed).

4.8. Effects of Maternal *Chlorella vulgaris* Supplementation on Performances of Rabbit Kits

Maternal *Chlorella vulgaris* supplementation led to a significant increase in weight gain of the rabbit kits following outcome of performances assessment. The mean weight gain of the rabbit kit at the pre-pubertal stage was 1335.99±24.32 g ($p < 0.003$) while 1169.50 and 1426.26 g were minimum and maximum weight gains, respectively. There was also a significant difference in the animals' daily feed intakes; mean feed intake was 53.64±2.10 g ($p < 0.03$) while minimum and maximum feed intakes were 45.15 and 63.85 g, respectively. Similarly, there was significant difference in feed conversion ratio of the rabbits; mean feed conversion ratio was 1.93±0.11 ($p < 0.012$) while minimum and maximum feed conversion ratio were 1.63 and 2.60, respectively. There was also significant difference in the concentrations of reproductive hormones; mean oestrogen concentration was 2.32±0.002 pg/mL ($p < 0.001$) while minimum and maximum oestrogen concentrations were 2.30 and 2.32 pg/mL, respectively. Furthermore, there was significant difference in testosterone concentrations; mean testosterone concentration was 15.04±0.14 pg/mL ($p < 0.001$) while minimum and maximum testosterone concentrations were 14.17 and 15.70 pg/mL, respectively. In all the parameters recorded, treatment groups were significantly better compared with the control group (Table 4.11).

Table 4.11: Effects of *Chlorella vulgaris* supplemented diets on performance of the rabbit kits

Parameters	Control	T1	T2	T3	T4	Mean	SEM	*p*-value
Birthweights (g)	34.89[b]	40.22[a]	39.33[a]	41.73[a]	50.23[a]	41.28	1.69	0.040
Weight gain (g)	1169.50[b]	1387.74[a]	1310.15[a]	1386.32[a]	1426.26[a]	1335.99	24.32	0.003
Daily feed intake (g)	63.85[a]	57.24[ab]	45.15[b]	52.21[ab]	49.77[b]	53.64	2.10	0.030
Feed conversion ratio	2.60[b]	2.00[a]	1.66[a]	1.76[a]	1.63[a]	1.93	0.11	0.010
Oestrogen (pg/mL)	2.30[b]	2.32[a]	2.32[a]	2.32[a]	2.32[a]	2.31	0.02	0.001
Testosterone (pg/mL)	14.17[c]	15.20[b]	14.87[bc]	15.70[a]	15.26[b]	15.04	0.14	0.001

[a,b,c] Means with a 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.

4.9. Effects of *Chlorella vulgaris* on Serum Oxidative Stress Status of the Rabbit Kits

Maternal supplementation of *Chlorella vulgaris* significantly increased oxidative stress protection in the kits following outcome of the oxidative stress biomarkers assessment. Mean serum malondialdehyde (MDA) concentration was 18.35±2.04 nmol/mL ($p < 0.002$), protein carbonyl concentration 7.28±0.33 µmol/mL ($p < 0.001$), superoxide dismutase (SOD) activities was 14.89±3.47 U/mL ($p < 0.04$), catalase (CAT) activities was 4.31±1.06 U/mL ($p < 0.28$) and glutathione reduced concentration (GSH) was 11.24±0.70 µmol/mL ($p < 0.001$) respectively (Table 4.12).

Similarly, maternal supplementation of the microalgae significantly increased tissue-level oxidative stress protection via the reduction of lipid peroxidation product generation and enhanced antioxidant enzyme activities (Table 4.13). The mean tissue malondialdehyde concentration was 9.12±0.32 nmol/mg ($p < 0.001$) while minimum and maximum tissue malondialdehyde concentrations were 8.05 and 26 nmol/mg, respectively. The mean protein carbonyl content was 1.07±0.35 nmol/100mg ($p < 0.001$) while minimum and maximum protein carbonyl contents were 0.37 and 3.51 nmol/mg, respectively. Furthermore, the mean antioxidant capacity of the tissue evaluated in the F1 generation kits was 13.89±0.81 µmol/mg ($p < 0.14$) while minimum and maximum antioxidant capacities were 9.64 and 21.29 µmol/mg respectively. Superoxide dismutase enzyme activity was 4.30±0.18 U/mg ($p < 0.004$) while minimum and maximum SOD activities were 3.39 and 5.07 U/mg. However, there were no significant differences in activities of catalase and glutathione reduced concentration; mean catalase activity was 2.36±0.15 U/mg ($p < 0.40$) while glutathione reduced was 10.93±0.13 µmol/g ($p < 0.10$), respectively (Table 4.13.).

Table 4.12: Effects of *Chlorella vulgaris* supplemented diets on serum oxidative stress status of the F1 generation rabbit kits

Parameters	Control	T1	T2	T3	T4	Mean	p-value
Malondialdehyde (nmol/mL)	26.69±4.02[a]	22.01±1.15[b]	18.55±4.71[b]	19.51±2.24[b]	5.00±0.65[b]	18.35±2.04	0.002
Protein carbonyl (µmol/mL)	7.74±0.55[a]	5.88±0.22[b]	6.98±0.48[ab]	7.60±0.23[b]	6.23±0.29[b]	7.28±0.33	0.001
Total antioxidant capacity (µmol/mL)	72.92±16.07	75.08±2.05	72.41±2.92	85.27±9.20	101.31±5.15	81.40±7.07	0.14
Superoxide dismutase (U/mL)	13.29±0.95[ab]	17.83±2.29[b]	13.06±1.47[ab]	13.21±1.31[ab]	17.06±0.38[b]	14.89±3.47	0.04
Catalase (U/mL)	4.30±0.59	4.70±0.60	4.78±0.53	3.21±0.58	4.54±0.16	4.31±1.06	0.28
Reduced glutathione (µmol/mL)	6.22±0.61[a]	11.63±0.81[b]	13.73±0.24[c]	14.50±0.51[c]	10.13±0.17[b]	11.24±0.70	0.001

[a,b,c] Means with a 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 4.13: Effects of *Chlorella vulgaris* supplemented diets on tissue oxidative stress status of the F1 generation rabbit kits

Parameters	Control	T1	T2	T3	T4	Mean	p-value
Malondialdehyde (nmol/mg tissue)	11.26±0.22[a]	9.12±0.10[b]	8.05±0.17[b]	8.68±0.67[b]	8.51±0.28[b]	9.12±0.32	0.001
Protein carbonyl (nmol/mg protein)	3.51±0.72[a]	0.55±0.22[b]	0.37±0.18[b]	0.38±0.15[b]	0.54±0.14[b]	1.07±0.35	0.001
Total antioxidant capacity (µmol/mg)	11.50±1.30	14.25±3.56	14.81±1.57	13.42±1.31	12.95±1.00	13.89±0.81	0.80
Superoxide dismutase (U/mg tissue)	3.39±0.04[a]	3.89±0.02[ab]	4.59±0.25[bc]	4.57±0.30[bc]	5.07±0.32[c]	4.30±0.18	0.004
Catalase (U/mg tissue)	2.18±0.44	1.93±0.08	2.77±0.32	2.19±0.18	2.71±0.43	2.36±0.15	0.35
Reduced glutathione (µmol/g tissue)	10.59±0.15	11.38±0.24	10.81±0.38	10.56±0.16	11.33±0.18	10.93±0.13	0.10

[a,b,c] Means with a 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.

4.10. Effects of Maternal Gestational *Chlorella vulgaris* Supplementation on Functional Genes Expression in F1 Rabbits

Maternal gestational supplementation of *Chlorella vulgaris* significantly led to the up-regulation of functional genes in the rabbit kits. The evaluation of fold changes of the quantified target genes indicated that the microalgae enhanced expression of all the genes determined. Fold change values for glutathione-s-transferase-1 (*GSTP1*) gene was 2.00±0.95 ($p < 0.002$), for *cytochrome p-450* (*CYP1A1*) gene it was 6.15±1.39 ($p < 0.05$), for nuclear factor kappa beta unit 1 (*NFKB1*) gene it was 1.59±0.58 ($p < 0.21$), for Nuclear receptor subfamily 3 C member 1 (NR3C1) gene it was 3.75±1.08 ($p < 0.001$), for Androgen receptor (*AR*) gene it was 2.55±1.82 ($p < 0.001$), for interleukin-1 (*IL1A*) gene it was -2.84±1.11 ($p < 0.42$), for growth hormone receptor (*GHR*) gene it was 2.05±0.78 ($p < 0.001$), for interleukin-2 (*IL2*) gene it was 1.29±0.44 ($p < 0.001$) and for inteleukin-6 (*IL6*) gene it was 2.08±0.82 ($p < 0.02$). The regulations of these genes indicated that supplementation of the microalga *Chlorella vulgaris* significantly up-regulated these target genes differently across the treatment groups (Figures 4.4 – 4.12).

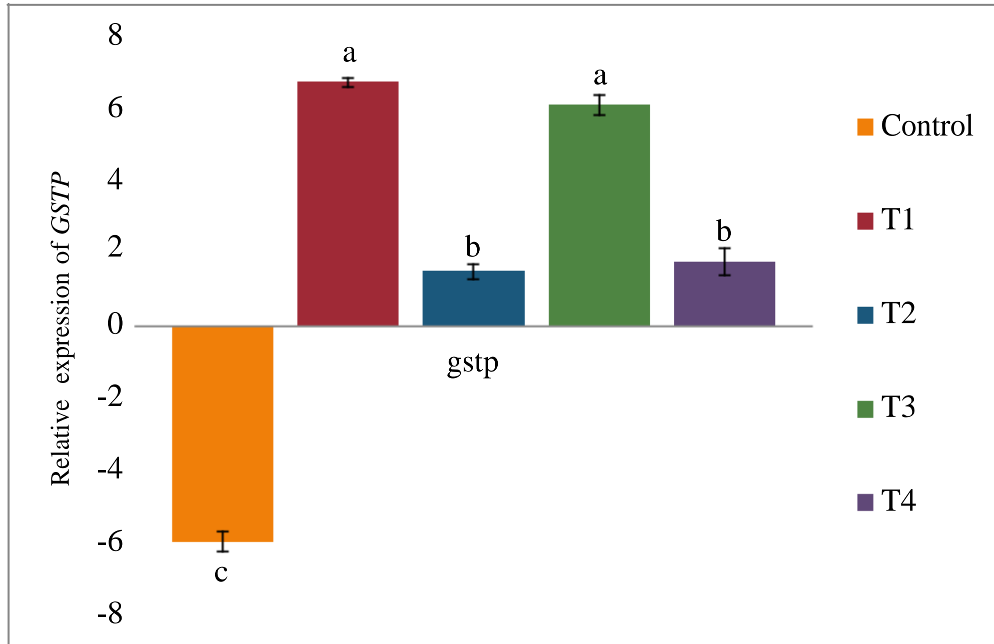

Fig. 4.3: Effects of *Chlorella vulgaris* on relative expression of GSTP1 gene in the rabbits' progenies

Control – no supplementation, T1 – group supplemented with 200 mg *Chlorella vulgaris* biomass, T2 – group supplemented with 300 mg *Chlorella vulgaris* biomass, T3 – group supplemented with 400 mg *Chlorella vulgaris* biomass and T4 – group supplemented with 500 mg *Chlorella vulgaris* biomass. Bars with different labels are significantly different ($p < 0.05$).

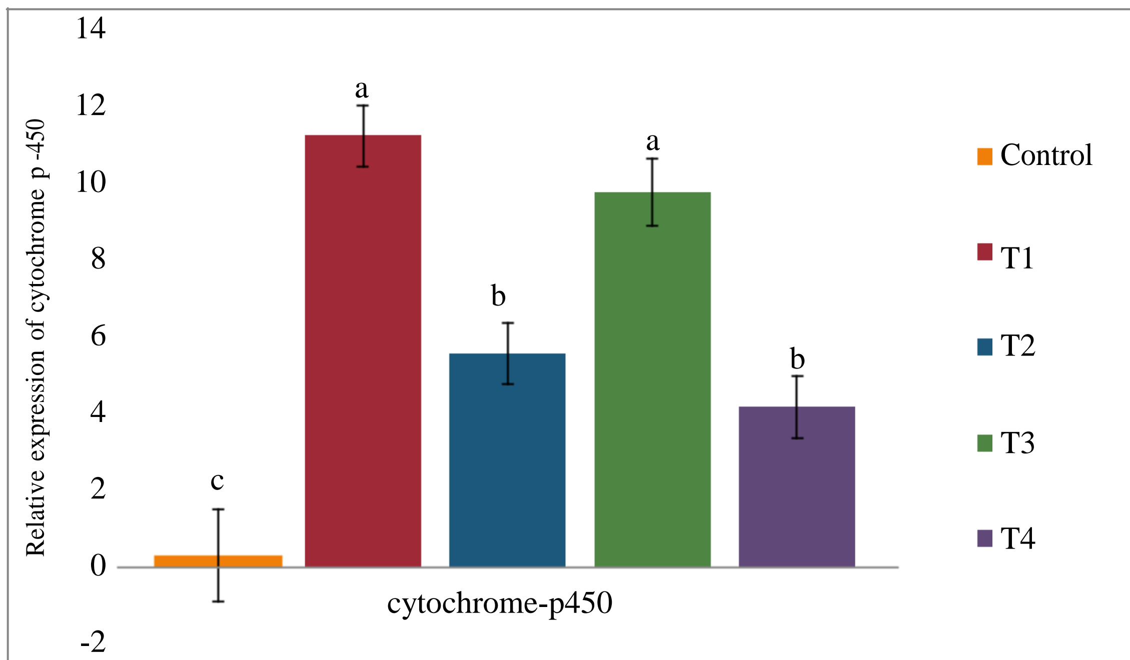

Fig. 4.4: Effects of *Chlorella vulgaris* on relative expression of cytochrome p450 gene in the rabbits' progenies

Control – no supplementation, T1 – group supplemented with 200 mg *Chlorella vulgaris* biomass, T2 – group supplemented with 300 mg *Chlorella vulgaris* biomass, T3 – group supplemented with 400 mg *Chlorella vulgaris* biomass and T4 – group supplemented with 500 mg *Chlorella vulgaris* biomass. Bars with different labels are significantly different ($p < 0.05$).

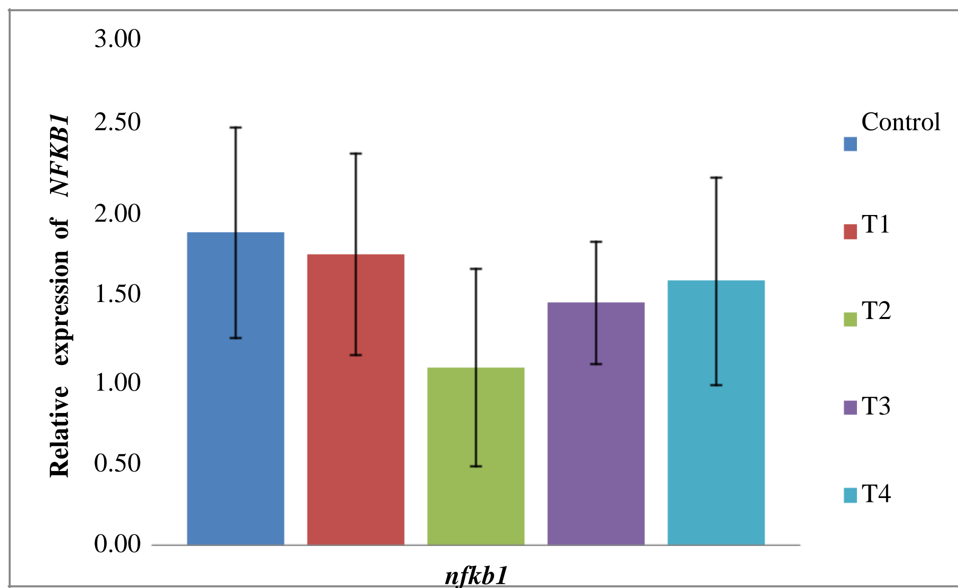

Fig. 4.5: Effects of *Chlorella vulgaris* on relative expression of *nfkb1* gene in the rabbits' progenies

Control – no supplementation, T1 – group supplemented with 200 mg *Chlorella vulgaris* biomass, T2 – group supplemented with 300 mg *Chlorella vulgaris* biomass, T3 – group supplemented with 400 mg *Chlorella vulgaris* biomass and T4 – group supplemented with 500 mg *Chlorella vulgaris* biomass. Bars with different labels are significantly different ($p < 0.05$).

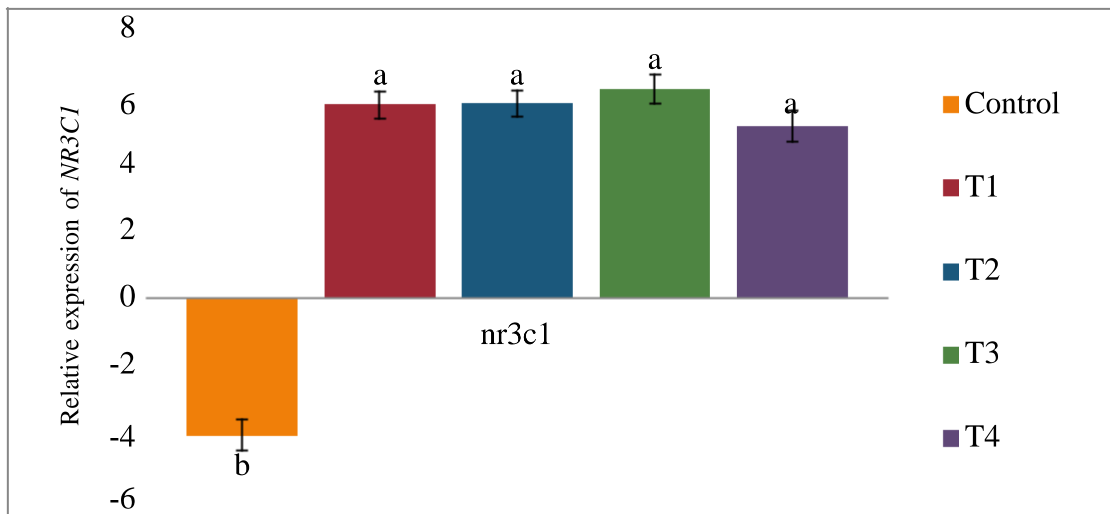

Fig. 4.6: Effects of *Chlorella vulgaris* on relative expression of *nr3c1* genes in the rabbits' progenies

Control – no supplementation, T1 – group supplemented with 200 mg *Chlorella vulgaris* biomass, T2 – group supplemented with 300 mg *Chlorella vulgaris* biomass, T3 – group supplemented with 400 mg *Chlorella vulgaris* biomass and T4 – group supplemented with 500 mg *Chlorella vulgaris* biomass. Bars with different labels are significantly different ($p < 0.05$).

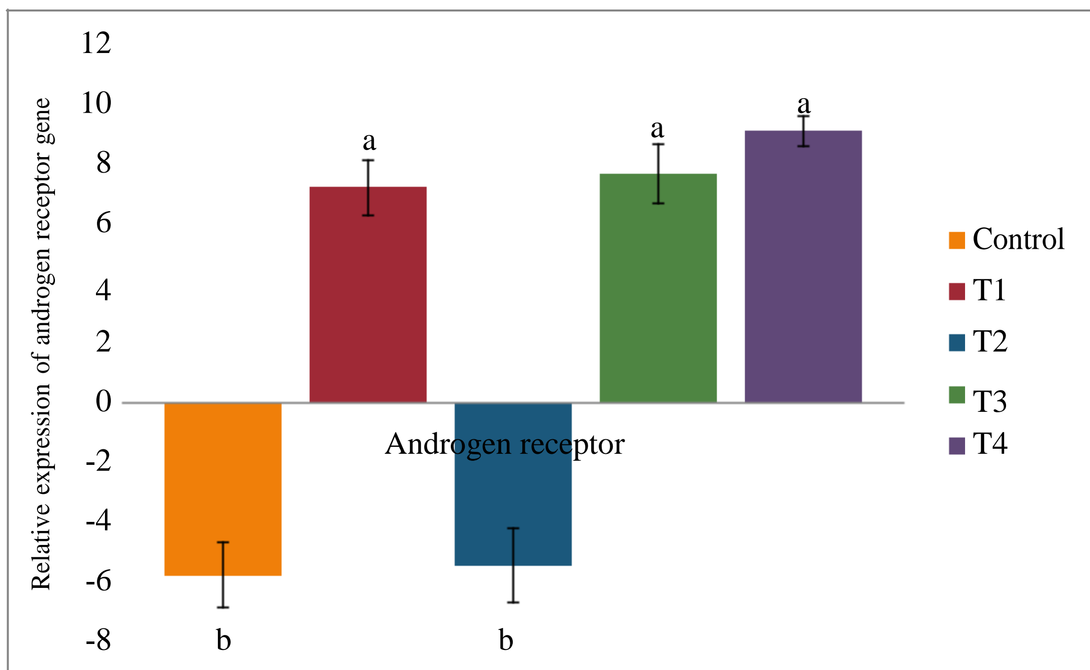

Fig. 4.7: Effects of *Chlorella vulgaris* on relative expression of androgen receptor gene in the rabbit progenies

Control – no supplementation, T1 – group supplemented with 200 mg *Chlorella vulgaris* biomass, T2 – group supplemented with 300 mg *Chlorella vulgaris* biomass, T3 – group supplemented with 400 mg *Chlorella vulgaris* biomass and T4 – group supplemented with 500 mg *Chlorella vulgaris* biomass.
Bars with different labels are significantly different ($p < 0.05$).

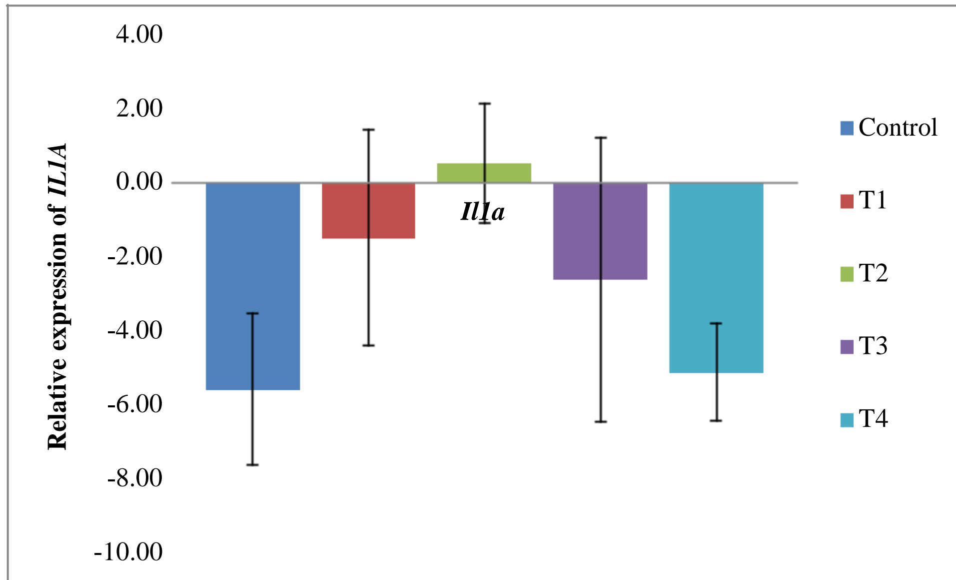

Fig. 4.8: Effects of *Chlorella vulgaris* supplements on relative expression of *Il1a* in the rabbits' progenies

Control – no supplementation, T1 – group supplemented with 200 mg *Chlorella vulgaris* biomass, T2 – group supplemented with 300 mg *Chlorella vulgaris* biomass, T3 – group supplemented with 400 mg *Chlorella vulgaris* biomass and T4 – group supplemented with 500 mg *Chlorella vulgaris* biomass. Bars with different labels are significantly different ($p < 0.05$).

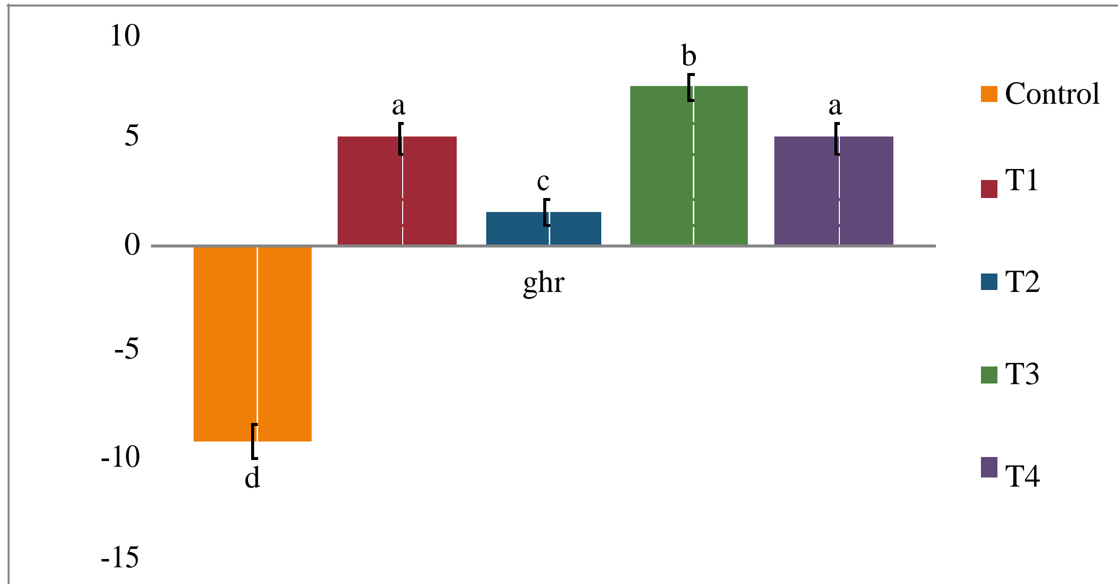

Fig. 4.9: Effects of *Chlorella vulgaris* on relative expression *ghr* genes in the rabbits' progenies
Control – no supplementation, T1 – group supplemented with 200 mg *Chlorella vulgaris* biomass, T2 – group supplemented with 300 mg *Chlorella vulgaris* biomass, T3 – group supplemented with 400 mg *Chlorella vulgaris* biomass and T4 – group supplemented with 500 mg *Chlorella vulgaris* biomass. Bars with different labels are significantly different ($p < 0.05$).

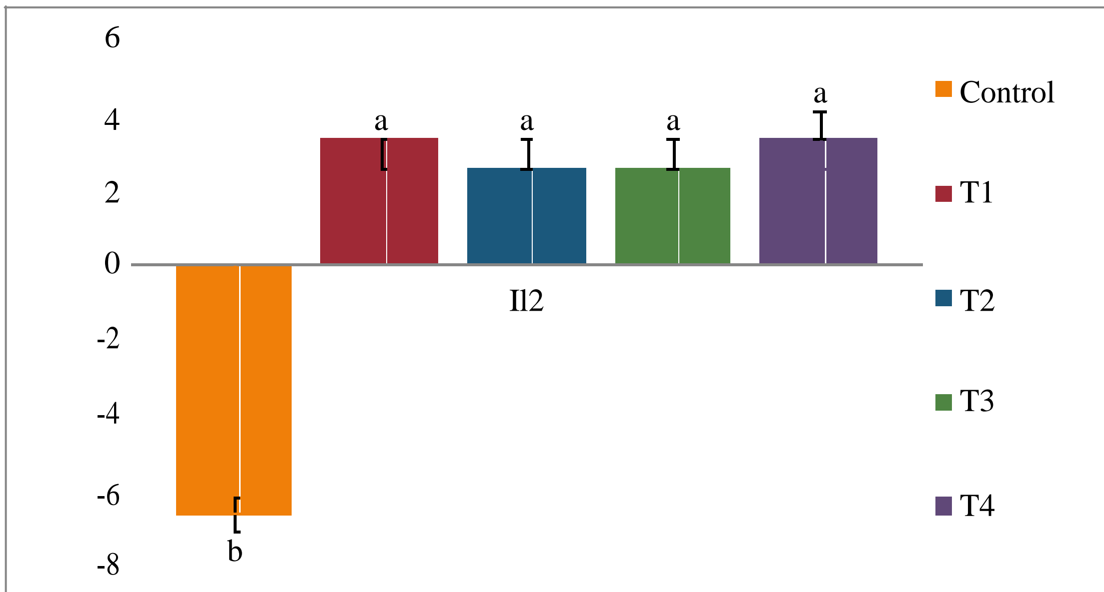

Fig. 4.10: Effects of *Chlorella vulgaris* on relative expression *IL2* in the rabbits' progenies Control – no supplementation, T1 – group supplemented with 200 mg *Chlorella vulgaris* biomass, T2 – group supplemented with 300 mg *Chlorella vulgaris* biomass, T3 – group supplemented with 400 mg *Chlorella vulgaris* biomass and T4 – group supplemented with 500 mg *Chlorella vulgaris* biomass. Bars with different labels are significantly different ($p < 0.05$).

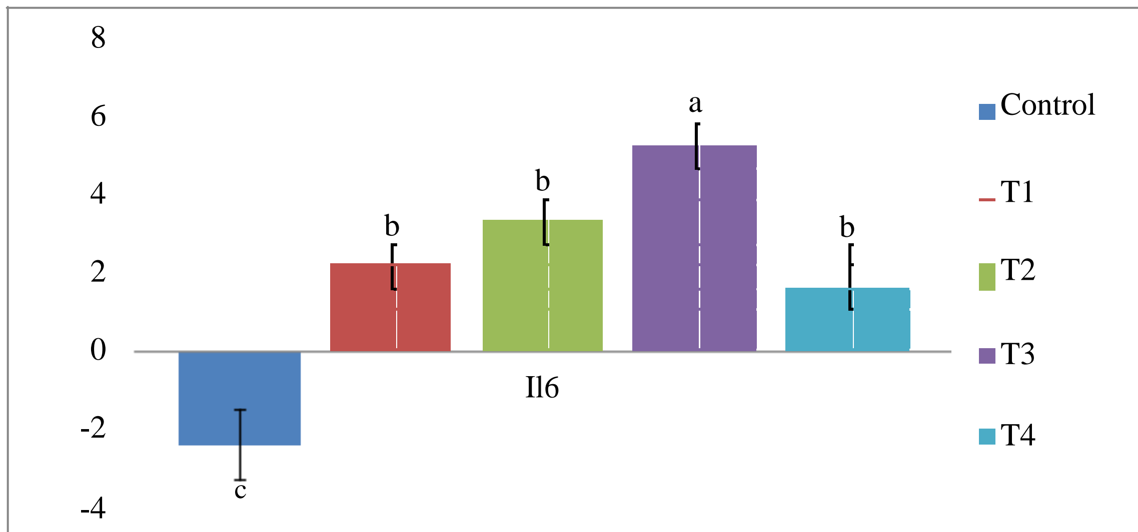

Fig. 4.11: Effects of *Chlorella vulgaris* on relative expression *IL6* in the rabbits' progenies Control – no supplementation, T1 – group supplemented with 200 mg *Chlorella vulgaris* biomass, T2 – group supplemented with 300 mg *Chlorella vulgaris* biomass, T3 – group supplemented with 400 mg *Chlorella vulgaris* biomass and T4 – group supplemented with 500 mg *Chlorella vulgaris* biomass. Bars with different labels are significantly different ($p < 0.05$).

4.11. Effects of Maternal Gestational *Chlorella vulgaris* Supplementation on Serum Biochemical Profile of the Rabbits' Progenies

There were no significant differences in serum biochemical profiles of the F1 rabbits foetal programmed with *Chlorella vulgaris* (Table 4.14). Mean serum alanine aminotransferase was 25.56±0.92 IU/L ($p < 0.40$) while minimum and maximum serum alanine aminotransferases were 20.07 and 29.14 IU/L, respectively. Mean serum aspartate aminotransferase was 15.19±0.97 IU/L ($p < 0.61$) while minimum and maximum were 12.91 and 18.14 IU/L, respectively. Mean serum alkaline phosphatase concentration was 55.26±2.25 IU/L ($p < 0.26$) while minimum and maximum were 45.87 and 61.07 IU/L, respectively. Similarly, serum urea and blood urea nitrogen were not significantly different. There was a significant difference in total bilirubin whereby the control group had a higher total bilirubin but still within the normal range of healthy rabbits (Figure 4.13).

Table 4.14: Effects of *Chlorella vulgaris* maternal supplemented diets on serum biochemical profile of F1 rabbit kits

Parameters	Control	T1	T2	T3	T4	Mean	SEM	p-value
ALT (IU/L)	29.14	24.07	25.06	28.28	26.23	26.56	0.92	0.40
AST (IU/L)	12.91	14.82	18.14	14.60	15.48	15.19	0.97	0.61
ALP (IU/L)	58.67	54.40	61.07	45.87	56.27	55.26	2.25	0.26
Serum Urea (mg/dl)	37.06	35.11	33.83	35.54	33.04	34.92	0.81	0.62
BUN (mg/dl)	17.31	16.40	15.80	16.60	15.43	16.31	0.37	0.63

[a,b,c] Means with a 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. ALT – Alanine Aminotransferase. AST – Aspartate Aminotransferase. ALP – Alkaline phosphatase. BUN - Blood Urea Nitrogen.

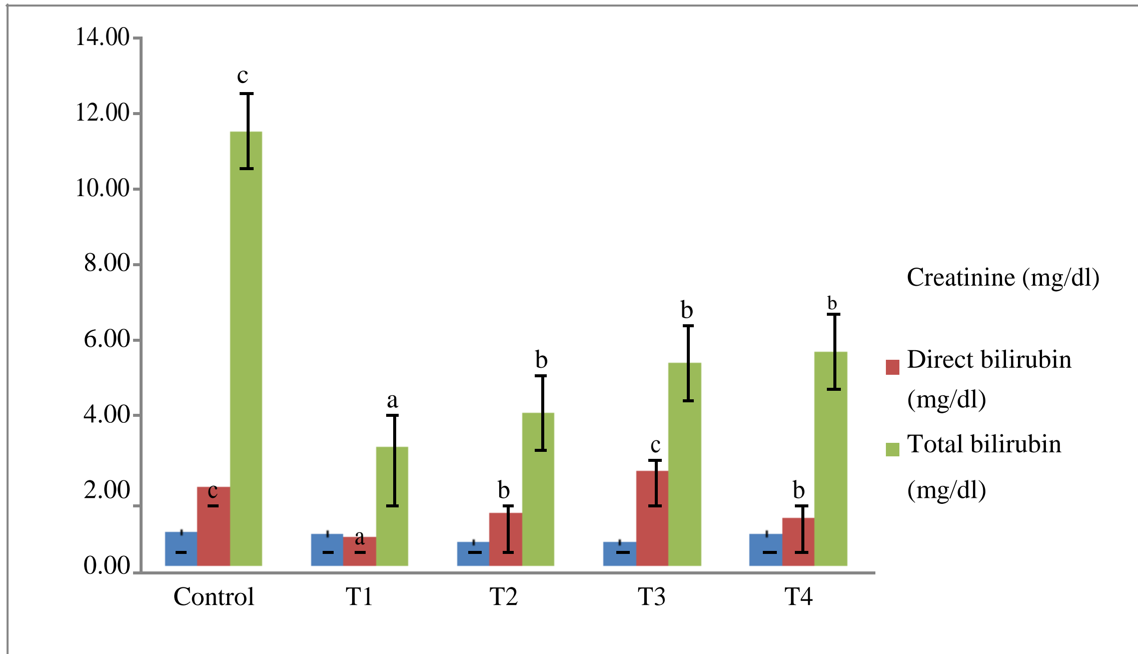

Fig. 4.12: Effects of *Chlorella vulgaris* on serum creatinine and bilirubin levels of the rabbit progenies
Control – no supplementation, T1 – group supplemented with 200 mg *Chlorella vulgaris* biomass, T2 – group supplemented with 300 mg *Chlorella vulgaris* biomass, T3 – group supplemented with 400 mg *Chlorella vulgaris* biomass and T4 – group supplemented with 500 mg *Chlorella vulgaris* biomass. Bars with different labels are significantly different ($p < 0.05$).

4.12. Effects of *Chlorella vulgaris* on Performances of Rabbits Supplemented to Finishing Stage

There was no significant difference in the initial weights of the rabbits; mean initial body weight was 1060±29.42 g ($p < 0.17$). However, there was significant difference in final body weights of the rabbits, the mean finishing weight was 2383±40.99 g ($p < 0.008$); there was also significant difference in feed intakes ($p < 0.01$); mean feed intake was 100.56±1.22 g while minimum and maximum feed intakes were 93.67 g and 105.92 g, respectively. Furthermore, there was a significant difference in commercial carcass weight ($p < 0.001$); similarly, a significant difference was also observed in the empty carcass weight ($p < 0.001$) as well as the rabbits' skin weight ($p < 0.001$). Weight after slaughtering was 2325±39.07 g ($p < 0.004$), blood weight was 58.94±5.54 g ($p < 0.006$), commercial carcass weight was 1419.72±34.36 g ($p < 0.001$), skin weight was 236.96±7.20 g ($p < 0.001$), and feed conversion ratio was 10.11±0.45 ($p > 0.05$), respectively (Table 4.15).

Supplementation of the microalgae also led to a significant increase in commercial primal cuts yield of the rabbits; similarly, the supplementation also led to a significant increase in the rabbits' meat yield from the carcasses. Mean commercial cuts percentage yield was 81.12±0.93 % ($p > 0.001$), while minimum and maximum commercial cut yields were 78.89 and 89.19 % respectively. Mean meat yield was 1613.03±41.95 g ($p < 0.001$) while minimum and maximum meat yield were 1429.66 and 1920.65 g, respectively; in both commercial yield and meat quantities, the treatment groups were better compared with the control group (Table 4.16).

Table 4.15: Effect of *Chlorella vulgaris* supplemented diets on performances of the finisher rabbits

Parameters	**Control**	**T1**	**T2**	**T3**	**T4**	**Mean**	**SEM**	***p*-value**
Feed intake (g)	100.32[a]	105.92[a]	101.81[a]	101.11[a]	93.67[b]	100.57	1.22	0.014
Initial body weight (g)	1031.50	987.55	1199.25	1065.25	1019.25	1060.56	29.42	0.170
Final body weight (g)	2152.30[b]	2322.70[ab]	2545.30[a]	2428.20[a]	2471.30[a]	2383.96	40.99	0.008
Weight after slaughter (g)	2103.10[b]	2268.30[ab]	2503.50[a]	2374.00[a]	2376.20[a]	2325.02	39.07	0.004
Blood weight (g)	49.20[b]	54.40[b]	41.80[b]	54.20[b]	95.10[a]	58.98	5.54	0.006
Commercial carcass weight (g)	1269.10[b]	1332.70[ab]	1666.10[a]	1428.50[a]	1402.20[a]	1419.72	34.36	0.001
Skin weight (g)	203.20[bc]	240.40[ab]	285.50[a]	234.20[ab]	221.50[b]	236.96	7.20	0.001
Feed conversion ratio	10.12	10.95	10.84	10.42	8.21	10.11	0.45	0.324

[a,b,c] Means with a 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 4.16: Effect of *Chlorella vulgaris* supplemented diets on meat yield and primal cuts of the finisher rabbits

	Control	T1	T2	T3	T4	Mean	SEM	*p*-value
Meat yield (g)	1429.66^{bc}	1523.93^{b}	1920.65^{a}	1613.76^{a}	1577.13^{a}	1613.03	41.95	0.001
Commercial cuts yield (%)	78.99^{b}	79.39^{ab}	89.19^{a}	79.12^{ab}	79.89^{ab}	81.12	0.93	0.001

^{a,b,c} Means with a 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.

The supplementation of *Chlorella vulgaris* also significantly affected the weights of ovaries and uterus of the rabbits. The rabbits in control group had ovaries with smaller weights compared with rabbits in the treatment groups ($p < 0.002$); mean ovaries weight was 0.44±0.03 g, minimum ovaries weight was 0.30 and maximum ovaries weight was 0.60 g respectively. Also, the weight of uterus of rabbits in the treatment groups were heavier than the uterus of the control group ($p < 0.001$); mean uterus weight was 7.78±0.30 g while minimum and maximum uterus weights were 6.70 and 9.10 g, respectively; there was also significant impacts of the supplementation on the rabbits gastro-intestinal weights; mean gastro intestinal tract (GIT) weight was 346±9.83 g ($p < 0.02$) while minimum and maximum weights of the GIT were 293.30 and 379.20 g, respectively (Table 4.17).

Table 4.17: Effect of *Chlorella vulgaris* supplemented diets on internal organs of the finisher rabbits

Parameters	Control	T1	T2	T3	T4	Mean	SEM	*p*-value
Liver (g)	56.40[c]	58.10[c]	67.10[b]	56.60[c]	74.30[a]	62.10	1.79	0.001
Heart (g)	4.60[b]	4.80[ab]	4.70[b]	5.10[ab]	5.30[a]	4.90	0.09	0.050
Kidney (g)	10.70[b]	13.30[a]	13.20[a]	12.00[ab]	12.70[a]	12.38	0.28	0.009
Lungs (g)	8.50[b]	11.20[a]	8.20[b]	11.00[a]	11.20[a]	10.02	0.32	0.001
GIT (g)	293.30[c]	351.20[ab]	349.50[ab]	334.10[b]	379.20[a]	341.46	7.99	0.002
Ovaries (g)	0.30[d]	0.40[c]	0.60[a]	0.50[b]	0.40[c]	0.44	0.02	0.001
Brain (g)	7.20	7.90	7.70	7.20	7.90	7.58	0.12	0.085
Uterus (g)	6.70[c]	9.00[a]	7.40[b]	9.10[a]	6.70[c]	7.78	0.25	0.001

[a,b,c] Means with a 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. GIT: Gastro Intestinal Tract (GIT).

4.13. Effects of *Chlorella vulgaris* on Serum, Liver, and Uterus Oxidative Stress Status of the Finisher Rabbits

There was a significant difference in serum oxidative stress status of the rabbits. The overall mean malondialdehyde concentration (MDA) was 18.40±0.91 nmol/mL ($p < 0.010$) while minimum and maximum concentrations were 15.37 and 23.90 nmol/mL, respectively. The overall mean total antioxidant capacity (TAC) was 71.38±8.60 µmol/mL ($p < 0.005$) while minimum and maximum total antioxidant concentration were 37.90 and 112.50 µmol/mL, respectively. The overall mean protein carbonyl contents (PCO) was 7.29±0.34 µmol/mL ($p < 0.001$) while minimum and maximum concentrations were 5.88 and 9.61 µmol/mL, respectively. Superoxide dismutase (SOD) activities was 172.48±7.80 U/mL ($p < 0.04$) while minimum and maximum activities were 146.20 and 203.80 U/mL, respectively. Catalase (CAT) enzyme activities were 4.06±0.26 U/mL ($p > 0.05$) while minimum and maximum activities were 3.33 and 5.13 U/mL, respectively. Glutathione reduced (GSH) concentration was 10.11±0.62 µmol/mL ($p < 0.017$) while minimum and maximum concentrations were 7.18 and 13.19 µmol/mL, respectively. Apart from catalase where there was no significant difference, all the oxidative stress biomarkers determined indicated that the treatment groups were better protected compared with the control group (Table 4.18).

Table 4.18: Effect of *Chlorella vulgaris* supplemented diets on serum oxidative stress markers in the finisher rabbits

Parameters	Control	T1	T2	T3	T4	Mean	*p* – values
MDA (nmol/mL)	23.90±1.66[a]	15.37±2.24[b]	16.88±0.94[b]	19.09±0.92[b]	16.78±1.32[b]	18.40±0.91	0.01
PCO (µmol/mL)	7.74±0.55[ab]	5.88±0.22[c]	6.99±0.49[ab]	9.61±0.23[a]	6.23±0.30[b]	7.28±0.34	0.001
TAC (µmol/mL)	37.9±4.50[c]	112.5±21.3[a]	62.80±20.10[bc]	63.4±20.05[bc]	80.30±5.75[ab]	71.38±8.60	0.005
SOD (U/mL)	158.5±9.50[b]	203.80±22.97[a]	146.2±14.70[c]	157.70±13.10[b]	196.20±3.80[ab]	172.48±7.80	0.04
CAT (U/mL)	3.80±0.35	4.64±0.57	5.13±0.53	3.33±0.58	3.41±0.50	4.06±0.26	0.10
GSH (µmol/mL)	7.18±0.54[c]	9.05±0.40[ab]	10.21±0.22[abc]	10.92±0.47[ab]	13.19±2.27[a]	10.11±0.62	0.02

[a,b,c] Means with a 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. MDA – Malondialdehyde. PCO – Protein Carbonyl. TAC – Total Antioxidant Composition. SOD – Superoxide Dismutase. CAT – Catalase. GSH - Glutathione.

Similarly, there was a significant difference in malondialdehyde (MDA) concentration in the liver, the overall mean malondialdehyde concentration was 6.61 ± 1.54 nmol/mg ($p < 0.001$) while minimum and maximum concentrations were 0.54 and 18.91 nmol/mg, respectively. The overall mean superoxide dismutase (SOD) enzyme activity in the liver was 169.74 ± 18.3 U/mg ($p < 0.002$) while minimum and maximum activities were 110.10 and 286.70 U/mg, respectively. The mean catalase (CAT) enzyme activity in the liver was 8.81 ± 0.30 U/mg ($p < 0.010$) while minimum and maximum activities were 8.00 and 10.35 U/mg, respectively. The overall mean glutathione reduced (GSH) concentration was 6.41 ± 0.37 μmol/g ($p < 0.001$) while minimum and maximum glutathione reduced were 5.00 and 8.85 μmol/g of the liver, respectively (Table 4.19).

Table 4.19: Effect of *Chlorella vulgaris* supplemented diets on liver oxidative stress biomarkers of the finisher rabbits

Parameters	Control	T1	T2	T3	T4	Mean	*p* – values
MDA (nmol/mg)	18.91±0.38[a]	0.54±0.15[c]	2.58±1.00[bc]	5.05±1.31[b]	5.99±1.89[b]	6.61±1.54	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	6.67±0.75	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	59.23±1.06	0.28
SOD (U/mg)	110.10±22.4[c]	190.40±18.10[b]	135.3±4.40[bc]	126.21±3.18[bc]	286.70±52.4[a]	169.74±20.09	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]	8.81±0.30	0.01
GSH (µmol/g)	6.30±0.10[ab]	6.58±0.38[ab]	5.30±0.35[b]	5.00±0.13[b]	8.85±1.12[a]	6.41±0.37	0.001

[a,b,c] Means with a 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.

Furthermore, the supplementation led to a significant difference in malondialdehyde (MDA) concentration in the uterus of the rabbits, the overall mean concentration was 1.70±0.46 nmol/mg ($p < 0.001$) while minimum and maximum concentrations were 0.08 and 5.25 nmol/mg of the uterus, respectively. The overall mean total antioxidant concentration (TAC) was 244.06±18.00 µmol/mg ($p < 0.01$) while minimum and maximum antioxidant capacity were 149.50 and 332.20 µmol/mg uterus, respectively. Superoxide dismutase (SOD) enzyme activity in the uterus was 144.48±18.40 U/mg ($p < 0.002$) while minimum and maximum activities were 84.80 and 261.50 U/mg, respectively. Mean catalase (CAT) enzyme activity in the uterus was 3.87±0.02 U/mg ($p < 0.001$) while minimum and maximum activities were 3.75 and 3.94 U/mg of the uterus, respectively. Mean glutathione reduced (GSH) concentration was 9.53±0.48 µmol/g ($p < 0.001$) while minimum and maximum glutathione reduced were 7.59 and 11.58 µmol/g, respectively (Table 4.20).

Table 4.20: Effect of *Chlorella vulgaris* supplemented diets on uterus oxidative stress biomarkers in the finisher rabbits

Parameters	Control	T1	T2	T3	T4	Mean	p-value
MDA (nmol/mg)	5.25±0.10[a]	2.57±0.24[b]	0.22±0.10[c]	0.38±0.20[c]	0.08±0.01[d]	1.70±0.46	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	22.11±3.73	0.40
TAC (µmol/mg)	149.50±6.00[b]	332.20±64.15[a]	255.50±13.14[a]	248.10±9.80[a]	235.00[a]	244.06±18.00	0.01
SOD (U/mg)	84.80±13.2[c]	165.10±10.5[b]	110.10±9.6[b]	100.90±5.8[b]	261.50±5.8[a]	144.48±18.40	0.002
CAT (U/mg)	3.85±0.01[bc]	3.89±0.02[b]	3.93±0.14[ab]	3.94±0.12[a]	3.75±0.01[a]	3.87±0.02	0.001
GSH (µmol/mg)	8.36±0.01[bc]	7.59±0.32[c]	10.94±0.39[ab]	11.58±1.02[a]	9.17±1.14[abc]	9.53±0.48	0.02

[a,b,c] Means with a 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.

4.14. Effects of *Chlorella vulgaris* on the Relative Expression of Antioxidant Genes in Finisher Rabbits Supplemented from Weaning

In the liver, there was a significant difference in the relative expression of primary antioxidant genes *sod1* ($p < 0.009$) and *gpx1* ($p < 0.004$). The overall mean relative expression of *sod1* was 2.02±0.35-fold changes while minimum and maximum expressions were 0.34- and 5.18-fold changes, respectively. The antioxidant gene *gpx1* was up-regulated in all the treatment groups with an overall mean fold change of 5.89±0.76-fold change while minimum and maximum fold changes were 0.22 and 9.88-fold changes, respectively. All treatment groups had a significantly higher relative expression of *gpx1* compared with control group (Fig. 4.14). Similar patterns of genes expression were found in the ovaries where *gpx1* was significantly up-regulated although there were no significant differences in the relative expression of *sod1* and *bre*. The overall 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-fold changes, respectively (Fig. 4.11). Comparative analysis of variance between relative expression of *sod1*, *gpx1* and *bre* using Mann-Whitney U statistics also indicated that there was significant difference in the level of *gpx1* expression between liver and ovaries ($p < 0.001$); but there were no significant differences in the expression of *sod1* ($p < 0.25$) and *bre* ($p > 0.05$). However, the relative expression of the two genes ranked higher in liver than ovaries (Fig. 4.15).

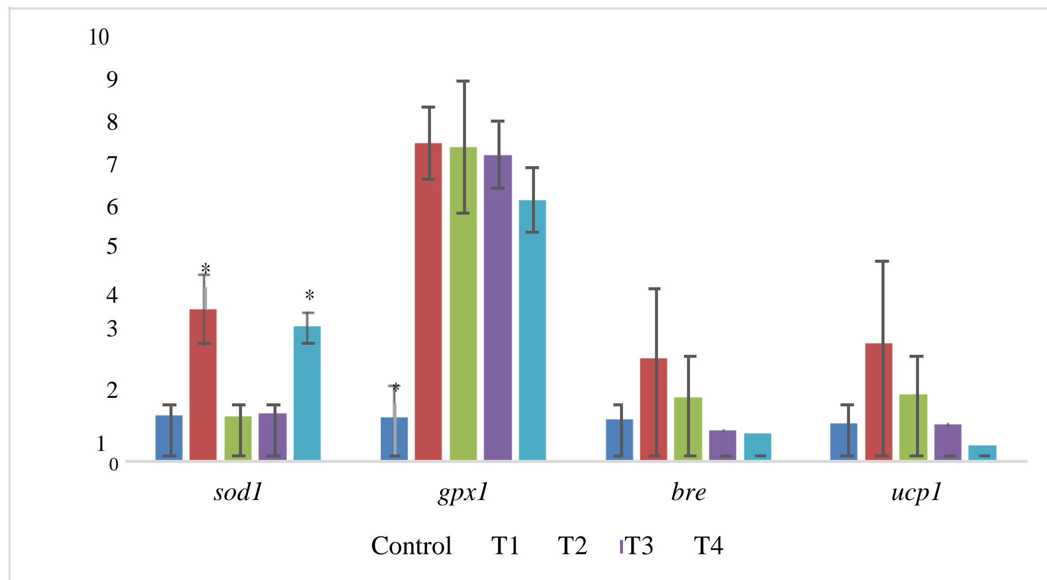

Fig. 4.13: Relative expression of primary antioxidant genes in the liver of the finisher rabbits
Control – no supplementation, T1 – group supplemented with 200 mg *Chlorella vulgaris* biomass, T2 – group supplemented with 300 mg *Chlorella vulgaris* biomass, T3 – group supplemented with 400 mg *Chlorella vulgaris* biomass and T4 – group supplemented with 500 mg *Chlorella vulgaris* biomass. Bars with different labels are significantly different ($p < 0.05$).

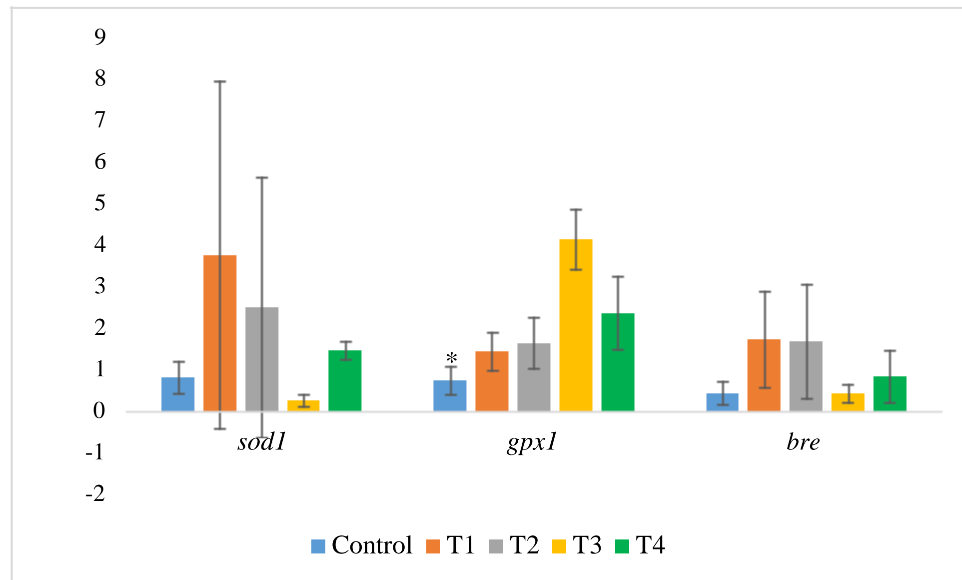

Fig. 4.14: Relative expression of primary antioxidant genes in ovaries of the finisher rabbits
Control – no supplementation, T1 – group supplemented with 200 mg *Chlorella vulgaris* biomass, T2 – group supplemented with 300 mg *Chlorella vulgaris* biomass, T3 – group supplemented with 400 mg *Chlorella vulgaris* biomass and T4 – group supplemented with 500 mg *Chlorella vulgaris* biomass. Bars with different labels are significantly different ($p < 0.05$).

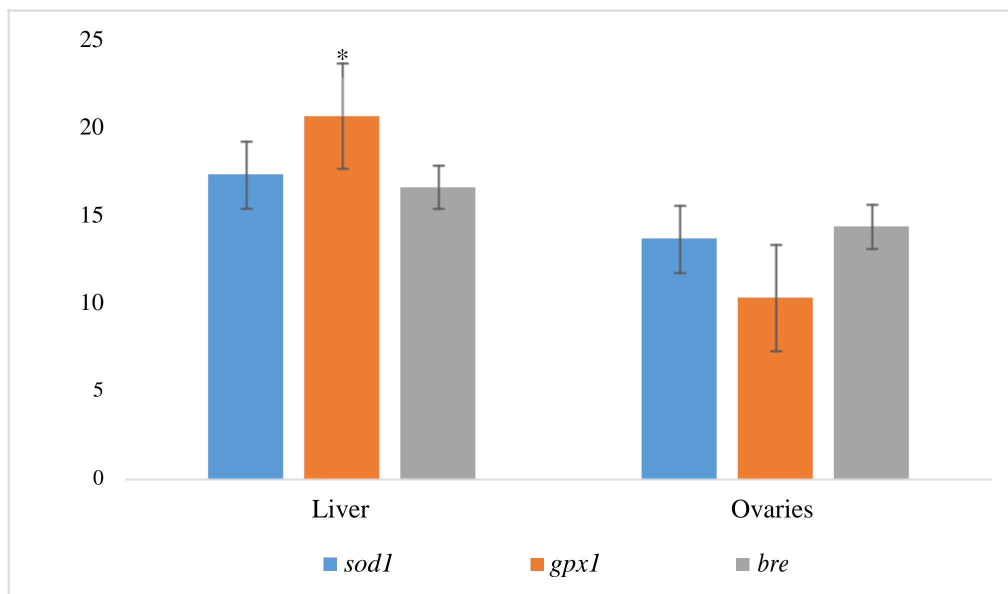

Fig. 4.15: Comparative expression of primary antioxidant genes in the liver and ovaries of the finisher rabbits Control – no supplementation, T1 – group supplemented with 200 mg *Chlorella vulgaris* biomass, T2 – group supplemented with 300 mg *Chlorella vulgaris* biomass, T3 – group supplemented with 400 mg *Chlorella vulgaris* biomass and T4 – group supplemented with 500 mg *Chlorella vulgaris* biomass. Bars with different labels are significantly different ($p < 0.05$).

4.15. Effects of *Chlorella vulgaris* on Haematopathological Status of the Rabbits

Examination of the blood cells indicated that there was no significant difference in the sizes of the red blood cells of the animals ($p > 0.05$). However, rabbits in the control group had lower red blood cell sizes compared with treatment groups. The overall mean red blood cell size was 6.88±0.13 µm ($p > 0.05$) while minimum and maximum red blood cell sizes were 6.22 and 8.13 µm, respectively (Figure 4.17).

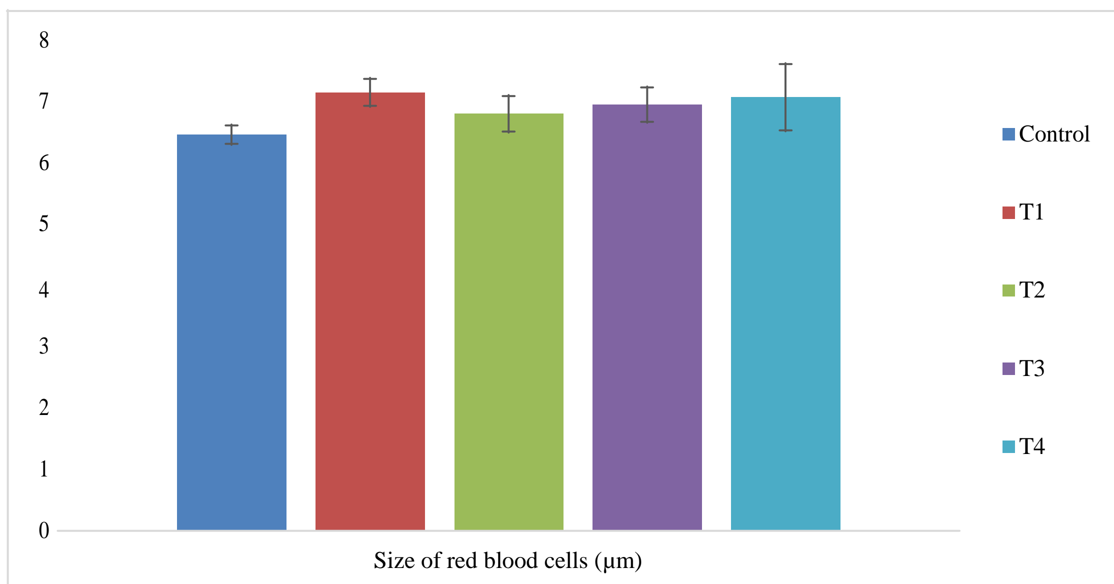

Fig. 4.16: Effects of *Chlorella vulgaris* supplementation on sizes of red blood cells of the finisher rabbits
Control – no supplementation, T1 – group supplemented with 200 mg *Chlorella vulgaris* biomass, T2 – group supplemented with 300 mg *Chlorella vulgaris* biomass, T3 – group supplemented with 400 mg *Chlorella vulgaris* biomass and T4 – group supplemented with 500 mg *Chlorella vulgaris* biomass. Bars with different labels are significantly different ($p < 0.05$).

CHAPTER FIVE

5.0. DISCUSSION

5.1. Nutri-functional Properties of the *Chlorella vulgaris* Supplement Diets

Chlorella vulgaris and its derivatives as revealed in the results highlighted is a suitable microalga with functional use in animal feeds and nutritional management taking advantage of bioactive components it contained. Specifically, the antioxidant components concentration, total phenolic content and ferric reducing antioxidant power of the diets indicated that they are suitable antioxidant supplements for improved productivity of animals. This is in agreement with submissions of Yan *et al*. (2012) who also investigated the *in-vitro* antioxidant capability of the microalga. The antioxidant compounds including astaxanthin, lutein, fucoxanthin, and carotenoids which are present in the microalga could be responsible for the recorded *in-vitro* capabilities of the supplements. These observations were also recorded by Boussiba and Vonshak (1991); Fan *et al.* (1994); Harker (1996); and Desai (2016) who have all previously investigated the *in-vitro* antioxidant capabilities of the microalga.

In agreement with these observations, *Chlorella vulgaris* was also described as a feed resource suitable for enhancement of physiological activities, growth promotion, antioxidant protection, immune modulation as well as protection against pathogenic microorganisms according to separate reports by Ishibashi (1972), Guzman *et al*. (2003) and Lee *et al*. (2010). Further to these assertions, the outcome of the *in-vitro* evaluation of the *Chlorella vulgaris* biomass used in this study suggests that the microalga in the forms evaluated in this study has suitable antioxidant properties.

Nutri-functional evaluation of the *Chlorella vulgaris* supplements used in this study indicated that the supplements have functional usefulness in addition to nutrients supply; these functional properties are potential features of the microalga making it a suitable supplement for the promotion of critical physiological activities such as immunity and physical strength in animals (An *et al*, 2006). Chemical and proximate composition of the microalga also indicated that the alga is rich in bioactive compounds sufficient as supplements for improving animal performance because these compounds are capable of reducing blood cholesterol, prevention of anaemia, serve as anti-viral agent, antioxidant against stress, probiotics as well as protection against chemical toxicants (Beheshtipour *et al*., 2013). This study have also shown that the microalga could be used as a novel animal supplement for improved productivity through oxidative stress attenuation (Roohinejad *et al*, 2017).

Furthermore, manipulation of this microalgal and other similar microalgae as done in this study is a prospective approach to development of animal nutraceutical taking advantage of abundant carotenoids, chlorophyll, sterol, vitamins, minerals and phycobilin abundant in the microalga; it could also serve as antimicrobial agents for diseases control and management (Pina-Pérez *et al*, 2017). Similarly, according to Graça *et al*. (2018), *Chlorella vulgaris* enriched with wheat flour was reported to lead to the production of healthier bread and other related products; it was also observed that small quantity of the microalgal can be used to enriched wheat flour.

5.2. *Chlorella vulgaris* Supplementation Effects on Performances of the Pre-pubertal New Zealand White Rabbits

Supplementation of the microalga at pre-pubertal stage in the rabbits indicated that *Chlorella vulgaris* improved weight gain of the rabbits. This corroborates previous reports that the microalgal is a growth promoter as reported by Yan *et al*. (2012) who supplemented the microalga *Chlorella vulgaris* to growing pigs. These researchers reported that the microalga positively increased the average daily gain of the animals. Bioactive compounds including S-nucleotide adenosyl peptide complex, polysaccharides and phenolic compounds present in abundant quantity in the microalga are responsible for these gains of growth promotion (Kay and Barton, 1991). Antioxidant protective capacity of the microalgae for improving immune functions is another factor that could be responsible for the growth promotion. This was linked with the ability of the *Chlorella vulgaris* to increase concentration of probiotics in the intestinal tracts of animals leading to optimal utilization of feed intake and promoted growth as complementary mechanism for improved immunity (Janczyk *et al*., 2006).

In addition to these nutrition-related performance mechanisms, the microalgal is also reported with the capacity for suppression of pathogenic bacteria in animals (Rania and Hala, 2008). Juxtaposing these with outcomes of this study as regards the reduction in feed intake without negative impacts on growth performance; it can be hypothesized that the supplementation improved efficiency of feed utilization due to growth-promoting principles in the microalga. Also, rather than exploration of the microalgae as a major source of dietary nutrients for rabbits; it supplementation at 200 and 500 mg per kg BW of rabbits at pre-pubertal stage can serve as a way of reducing intake of basal feed without

compromising growth – a strategy that can be employed for huge economic gains in commercial production systems.

Furthermore, in a study on exploring the supplementation effects of similar microalgae like *Chlorella vulgaris*, Heidarpour *et al*. (2011) also reported that supplementation of the microalgae enhanced growth in Holstein's calves. The authors pointed out that the microalgae just like *Chlorella vulgaris* contained bioactive compounds with exploitable functional properties as livestock supplement for improving productivity. Similarly, according to Mahmoud *et al*. (2017) suggested that the microalga *Chlorella vulgaris* can be explored as a potential substitute for soybean because it led to higher growth rates in rabbits. The improvement of performances due to *Chlorella vulgaris* supplementation, however, is not limited to rabbits because apart from rabbits, the microalgae supplementation in chicken, calves, sheep, and pigs were reported as natural growth promoters and potential substitute for expensive major sources of protein. However, this present research is contrary to a report which indicate that the microalgae *Chlorella vulgaris* supplementation only promote growth but not higher final body weight attainment (Grinstead *et al*., 2000). This present study is contrary with the report of inability of the microalga to increase final bodyweight attainment because both the growth rate and final weight of the rabbits were significantly influenced as results of its supplementation in this present study.

Growth promotion is a desirable and expected benefit of nutritional supplementation which this microalga has demonstrated, but besides that, it also increased final weight gain and this has practically showed that *Chlorella vulgaris* supplementation is a performances improvement supplement. In agreement with this, An *et al*. (2006) also reported that supplementation of dried *Chlorella vulgaris* powder significantly promotes growth and

final weights of broiler chickens. Therefore, this present study concluded on common ground that *Chlorella vulgaris* supplementation promotes both growth and final body weight in addition to performances improvements across different animal species and the microalgal was suggested as a suitable natural source of growth promotion, immune-boosting, tissues rebuilding and antioxidant protection (Guzmán and Calleja, 2001).

5.3. *Chlorella vulgaris* Supplementation Effects on Oxidative Stress Status of the Pre-pubertal New Zealand White Rabbits

This study showed that supplementation of *Chlorella vulgaris* significantly suppressed the generation of malondialdehyde in rabbits of the treatment groups with corresponding elevated antioxidant enzyme activities compared with control. Oxidative stress was higher in the control group compared with the treatment groups due to higher concentration of malondialdehyde. This is an indication that reproductive development involving maturation of secondary reproductive organs and the overall growth of the rabbits are potential sources of stress in pre-pubertal rabbits; it is evident because since there was no exogenous antioxidants supplementation in the control groups, oxidative stress became established and antioxidant enzyme activities were reduced. Thus, there was higher generation of malondialdehyde. This observation is in agreement with reports of increasing lipid peroxidation products in prepubertal females and decreased antioxidant enzymes activities because of fast growth and development of the reproductive system whose processes is closely associated with severe lipid peroxidation and a major indicator of oxidative stress damage at the prepubertal period (Kolesnikova *et al*., 2015).

Similarly, in a chemical-induced oxidative stress damages in pre-pubertal rat model, higher concentration of malondialdehyde with corresponding lower antioxidant enzymes

activities. Also, at the pre-pubertal stage, increasing of the lipid peroxidation marker malondialdehyde is a prominent impact of oxidative stress and can lead to reproductive dysfunction typically such as alterations in serum steroid hormone levels, disruptions in spermatogenesis, and loss of fertility (Cattani *et al.*, 2013). In pre-pubertal male rabbits, lipid peroxidation was also reported to correspond with reduced antioxidant enzymes activities in rabbits born during the warm season and has negative effects on spermatogenesis because at puberty the rabbits had lower testosterone concentration (García-Tomás *et al*., 2010).

However, the present study demonstrated that in an un-induced condition; there is the natural possibility of oxidative stress development because of active maturity at the prepubertal stage and free radical generation association with active metabolism and steroidal maturation of the reproductive tissues (Ishii *et al*., 2005). In addition to these, oxidative stress is almost inevitable and it is involved as part of mechanism of reproductive system complex used for elimination of xenobiotics from the reproductive tracts. Therefore, enhanced antioxidant capacity of animals is required via exogenous dietary antioxidant supplementation.

Malondialdehyde – a major product of lipid peroxidation is a terrible consequence of oxidative stress damage. It has both physical and biochemical impacts on the reproductive system. According to Puppel *et al*. (2015), it causes denaturation of cell membrane components and modifies synthesis of protein, DNA and RNA which are all cellular complications in reproductive performance. Therefore, inhibition of malondialdehyde formation as demonstrated by *Chlorella vulgaris* supplementation in this study is a contribution to cellular protection against oxidative stress. In agreement with this submission, *Chlorella vulgaris* supplementation was reported to improve the hepatic

imbalance of the prooxidant/antioxidant status of rats under deltamethrin intoxication and elimination of oxidative tissue injuries (Elsheikh *et al*., 2018).

In the female, there are available biological shreds of evidence linking stress with compromised reproductive performance; but because of its complexity, more questions are raised than available answers. Molecular propositions had it that under natural conditions, reproductive functions are directly and or indirectly affected by endocrine disturbance via hypothalamus-pituitary-gonadal axis; according to these propositions, stress affects majorly, expression of molecular signals associated with oestrogen and progesterone surge which are important mechanism behind sexual receptivity and ovulation behaviours (Pfaff *et al*., 2018). This is an indication that oxidative stress is a cellular reproductive compromise and the need for secondary feed-based antioxidant supplementation for its attenuation.

According to Miller and Brzezinska (1993), oxidative stress led to depletion of primary antioxidant defense in animals followed by a cascade of degeneration causing reduced productivity. It was suggested by these authors that dietary supplementation of secondary antioxidants can reverse the imbalance between the prooxidant and primary antioxidant defense enzymes. Outcomes of this present study thus confirmed the hypothesis that oxidative stress reduces animal performances and has also identified *Chlorella vulgaris* supplementation as suitable secondary source of antioxidants with potential chain-breaking capacity in rabbit since it reduced formation of lipid peroxidation products and increase antioxidant enzyme activities.

Apart from *Chlorella vulgaris* supplementation in food-producing animals for improved productivity, outcomes of antioxidant protection in this study due to reduction in MDA

formation has opened oppourtunities for further exploration of health and functional properties of the microalga in oxidative stress pathophysiology in cancer, psychiatric and cardiovascular-related ailments because high MDA concentration is a common clinical biomarker of these health challenges (Marrocco *et al*., 2017).

Attenuation of oxidative stress through reduction of MDA concentration then configured this study with nutri-functional ameliorative status of physiological importance for correcting oxidative stress-linked infertility because according to Agarwal *et al*. (2012), increasing oxidative stress is a major cause of reproductive challenges including endometriosis, polycystic ovary syndrome and unexplained infertility complexities while exogenous supplementation of antioxidants for oxidative stress attenuation was suggested as dietary therapy for management of the reproductive ailments.

Supplementation of the microalgal *Chlorella vulgaris* in this study fulfilled secondary antioxidant roles in oxidative stress attenuation via utilization of its bioactive compounds including fucoxanthin, zeaxanthin, astaxanthin and carotenes to scavenge free radicals in order to reverse the shift in oxidative stress balance against prooxidants and in favour of antioxidant enzymes which was the reason why the activities of the antioxidants enzymes were upregulated in the treatment groups compared with control (Sharma and Agarwal, 2004). It is important to protect against oxidative stress for ensuring optimum reproductive functions because the process of reproduction, especially in the female is naturally linked with the generation of reactive oxygen species (Ruder *et al*., 2014). For example, during ovulation, the surge in luteinizing hormone involves production of some pre-ovulatory precursors which are closely linked with generation of reactive oxygen species; meanwhile these precursors are vital to ovulation hence highly inevitable for successful ovulation (Shkolnik *et al*., 2011).

Although nutri-functional evaluation has been reported widely for *Chlorella vulgaris* before this present study, the uniqueness and novelty of this study is the attempt to explore the microalga beyond nutrition and also the establishment of supplemental levels of the alga for animals' benefit using rabbit as a model. Earlier studies specifically explored *Chlorella vulgaris* as source of protein for animals and most of the studies concluded with shortcomings of lack of significant differences in animals' weight gain, digestibility and high cost of feeding because of the expensiveness of the microalga.

Therefore, studies similar to this present one with focus on its functional properties for promotion of health and physiological functions ranging from its anti-diabetic properties are warranted and can introduce novel feed additives for promoting animal health and performances (Azizzat *et al*., 2010). In line with this justification, the microalgal was investigated for its ameliorative effects of stress associated with hypothalamus and brain (Queiroz *et al*., 2016). It was also investigated for its cadmium detoxification in rat model (Kim *et al*., 2009). The *Chlorella vulgaris* as anti-toxicant was investigated for its potency on testicular protection against lead toxicity in Nile tilapia (Zahran and Raha, 2014); and it was investigated for its improvement of digestive tract health in piglets (Furbeyre *et al*., 2018).

There was also assessment of its effects on performance, microbiome, enzymatic activities and fatty acids profile of goat milk (Tsiplakou, 2017). These studies confirmed excellent suitability of the microalgal *Chlorella vulgaris* as a feed additive and nutraceutical resource suitable for improved performances, antioxidant supplement, and supplement for improved products qualities rather than its use as source of major nutrient supply for animal consumption. This study also has contributed in the area of exploring microalgae for improving animal productivity and performance building on these health and

physiological benefits. However, the novelty in this study is that it filled one of the gaps left by previous studies which are in the area of determining lowest, medium and optimum supplementation levels of the microalga in rabbits for oxidative stress protection without negatively affecting growth and performances.

5.4. *Chlorella vulgaris* Supplementation Effects on Pre-gestation and Gestation Performances of the Pregnant New Zealand White Rabbits

This study also evidently showed that *Chlorella vulgaris* supplementation in pregnant rabbits improved reproductive output because even before the gestation period, it significantly increased receptivity for mating and after the mating, it shortened the gestation length, increased kindling rates and reproductive index; while the reproductive index and kindling rates were positively correlated with other reproductive parameters. The rabbits supplemented with the microalga also had higher values for all these evaluations and these indicated that the supplementation improved the reproductive performance of the rabbits.

Specifically, improvement of the sexual receptivity of the rabbits is an indication that the supplementation improved reproductive performances. This is because sexual receptivity is a determinant of successful reproduction and positive correlations exist between sexual receptivity and concentration of estradiol which could be responsible for successful conception recorded in the rabbits. Although, wide variations in sexual receptivity in rabbits exist, but lower sexual receptivity correlates with infertility (Rebollar *et al*., 2004; Boiti *et al*., 2006).

Therefore, the improvement of the sexual receptivity of the supplemented groups in this study demonstrated that the microalga enhanced sexual capability of the rabbits and it is

capable of being exploited for sexual enhancement to improve the efficiency of rabbits and other food-producing animals. In a large-scale trans-generational study exploring factors affecting sexual receptivity in rabbits, female rabbits were identified as major determinant of sexual receptivity (Theau-Clément *et al*., 2012). Therefore, dietary supplementation of *Chlorella vulgaris* which led to its improvement as demonstrated in this study can serve as a strategy for increasing rabbit reproductive output especially in nulliparous rabbit does (Blache *et al*., 2011).

Biological justification for this can be linked with interactions between phenolic compounds in the microalgae and sex hormones in the rabbits because these chemical interactions could be responsible for the up-regulation of receptor genes associated with sexual receptivity (Oberdörster and Cheek, 2001). Up-regulation of these receptor genes could lead to faster and higher acceptance of male by female rabbits hence, leading to a reduction in waiting time before female rabbits allowed bucks for mating. This observation is in agreement with an earlier submission that there are interactions between *Chlorella vulgaris* and sex hormones leading to up-regulation of oestrogen receptor genes according to reports of Amin (2009). This author stated that administration of the microalgae *Chlorella vulgaris* extract in mice model restored oestrogen receptor genes and this mechanism act on the anti-carcinogenic properties of the microalgae.

Furthermore, observations of this study linking *Chlorella vulgaris* supplementation with an increase in sexual receptivity in rabbits could be associated with higher oestrogen action in the rabbits supplemented. This is also in agreement with observations of Janczyk *et al*. (2006) which stated that microalgae *Chlorella vulgaris* increase sexual receptivity in mice models because of its capacity of increasing plasma oestrogen concentration and hence hypothesized that the algal is an oestrogenic source.

Supplementation of the microalgae also led to an improvement of the rabbits' reproductive index. This improvement can be linked with the biological roles of minerals, vitamins, phenolic compounds and carotenoids in the microalgae. These nutrients are minute functional requirements for normal reproductive performances. They are not usually produced *de novo* in animals and also not usually available in bulk feed resources but only from supplemental additives such as *Chlorella vulgaris,* the microalgae supplemented in this study.

In agreement with this observation, Attia *et al.* (2015) reported that supplementation of bee pollen because of its high profile of minerals, vitamins and carotenoids contributed to improved reproductive index of rabbits. It could also be deduced from the outcome of the reproductive index in this study that the microalgae *Chlorella vulgaris* supplementation contributed both quantitatively and qualitatively to the reproductive performances of the rabbits because the reproductive index is a product of conception, viability and kindling rates. Therefore, the higher reproductive index could be interpreted to be a result of increased conception and viability rates on one hand as qualitative improvement while higher litter size on the other side is quantitative improvement because of the microalgae supplementation.

Reproductive index is a product of litter size per parturition and immediate post-natal survivorship of the kit; considering its outcomes in the treatment groups where higher litter size, viability and kindling rates were recorded compared with control. Thus, it can be conveniently inferred that the supplementation of microalgal improved the rabbits' productivity. A similar submission was reported by Kapoor and Mehta (1993) who stated that supplementation of *Spirulina* in mice model led to higher litter size, litter weight, and post-natal survivor. However, it is important to note that in this study, in addition to the

stated reproductive improvement, supplementation of the *Chlorella vulgaris* led to early parturition which is an indication that the microalga promoted foetal growth and modulate activities of parturition related hormones.

This study identified antioxidant richness of the microalgae *Chlorella vulgaris* as the reasons for all the recorded improvement of reproductive performances because reproduction is always under threat from oxidative stress as a result of increased metabolic demand during gestation. Under oxidative stress condition, elevated levels of reactive oxygen species cause genomic instability through manipulation of deoxyribonucleic acid and it negative effects on reduced activities of protective antioxidants enzymes at the cells levels thereby resulting into cellular destruction and or damages (Adejuwon *et al.*, 2014); this mechanistic effect of oxidative stress was also reported to be strongly associated with poor reproductive performances according to Aitken and Krausz (2001).

Meanwhile, *Chlorella vulgaris* supplementation in this study demonstrated its capability of direct free radicals scavenging which was why oxidative stress was attenuated in the supplemented group as the reasons for improved reproductive indexes of the rabbits supplemented. In a similar submission to this observation; natural antioxidants in pomegranate were reported to improve reproductive performance in the rabbit does (Zeweil and El-Gindy, 2016).

5.5. *Chlorella vulgaris* Supplementation Effects on Gestational Oxidative Stress Status of the Pregnant New Zealand White Rabbits

This study also showed that gestation as a physiological state is associated with oxidative stress because there was significantly higher malondialdehyde (MDA) concentration and reduced total antioxidant capacities in rabbits of the control group compared with treatment

groups where malondialdehyde concentrations were lower and total antioxidant capacities were higher. These showed that antioxidant compounds in the microalga effectively attenuated oxidative stress in the supplemented rabbits. Oxidative stress is one of the most critical biological mechanisms compromising reproductive performances; hence, its attenuation via antioxidant nutritional supplements can lead to improving reproductive performance of animals (Miller and Brzezinska, 1993). This study outcome identified *Chlorella vulgaris* supplementation as a practical example of such nutritional strategy suitable for reducing concentrations of malondialdehyde and increasing total antioxidant capacity.

Oxidative stress is a biochemical phenomenon underlying poor reproductive output and whenever it occurs, there is always reduction in animal performance and productivity (Lee *et al.*, 2017); therefore, the ability of microalgae *Chlorella vulgaris* in this study countered oxidative stress by decreasing oxidative stress products concentration could be the reason for improved reproductive index recorded in the treatment groups where reproductive indexes were significantly higher ($p < 0.05$); because of lowered MDA and higher total antioxidant capacity linked with higher antioxidant enzyme activities.

Malondialdehyde increase during gestation is a physiological condition caused by high metabolic demand of pregnancy and elevated requirement of tissue oxygen; it causes oxidative stress due to electron escape from the electron transport chain, a situation causing cascades of oxidative stress compromise and reduced antioxidant defense (Olayaki *et al.*, 2008). This is a pathophysiological condition responsible for reproductive compromises occurring both in humans and animals as a result of increased free radical generation and decreased antioxidant capabilities (Ercan *et al.,* 2017). The supplementation of *Chlorella vulgaris* in this study demonstrated that carotenoids in the microalga such as xanthophylls are bioactive compounds with antioxidant power that protected the rabbits against gestation stress, and supported foetal growth and development through placental transportation channels where they served as modulators of better foetus development. These are what led to significant improvement of kindling rates, viability rates and eventually, higher reproductive index of the rabbits in the treatment group with the highest intake. This deduction agreed with Zieliński *et al.* (2017) who posited that carotenoids, including lutein and zeaxanthin (present in *Chlorella vulgaris*) play significant role in foetal nervous and optical developments and suggested empirical scientific pieces of evidence such as the ones demonstrated in this study as a way of re-emerging the role of carotenoids in the prevention of disorders affecting preterm neonates because all these disorders are caused by oxidative stress.

Efficacy of *Chlorella vulgaris* supplements in this study has shown that microalga is a useful supplement for the prevention and management of reproductive complications including preeclampsia and gestational diabetes because of its capacity to reduce malondialdehyde concentration, increased antioxidant enzyme activities and increased total antioxidant capacity. Preeclampsia and gestational diabetes are reported to occur during

gestation and positive associations exist between these complications and increased malondialdehyde production as well as reduced antioxidant protection. Therefore, supplementation of *Chlorella vulgaris* could aid their management during gestation (Glenville, 2006). In agreement with this proposition, reports of antioxidant supplementation in pregnant diabetic rat model indicated that there was improved foetal morphology and also prevention of associated birth defects; similarly, it was also reported that supplementation of antioxidant vitamins reduced incidence of pre-eclampsia in a population at high risk of the condition (Cederberg and Eriksson, 2005; Vadillo-Ortega *et al.*, 2011).

From the perspective of animal production, this study has shown that minimal supplementation of *Chlorella vulgaris* is capable of qualitative significant improvement of reproductive output since the reproductive index of the rabbits in the lowest and highest supplemented groups was significantly higher compared with the control group. In addition to higher reproductive index of the supplemented groups, significant increase in kindling rates and positive significant correlations of reproductive index and gestation gain, litter size, number of live kits, gestation length and viability rates have shown that the microalga could be exploited as animal supplement for improved productivity rather than feed resource for supplying of primary nutrients such as protein to animals considering cost of its incorporation as a source of major nutrient.

Furthermore, higher reproductive index and kindling rates due to the supplementation as well as their correlations with litter size indicated that the supplementation had strong economic implications because it positively influenced overall output per rabbit doe. Some of these reproductive traits are an important factor in profitable rabbit production; however, they are difficult to improve by selection and even intensive researches are still

not yielding significant improvement as reported by Cartuche *et al*. (2014). Therefore, supplementation of *Chlorella vulgaris* as demonstrated in this study is a simple alternative nutrition – reproduction strategy for increasing productivity.

5.6. *Chlorella vulgaris* Supplementation Effects on Performances of the Lactating New Zealand White Rabbits

This study indicated that supplementation of *Chlorella vulgaris* at different levels led to improved rabbits' production performances during the lactation period. Patterns of milk yield, litter weight gain, kits survival, and body conditions were similar; therefore, it can be deduced that the supplementation of *Chlorella vulgaris* led to improved productivity. However, lower milk yield was recorded in treatment group T4 which did not correspond with lower kits survival rate because, in T4 where milk yield was lower, the kit survival rates were higher compared with control group, this implies that the quality of milk was better than the control due to the supplementation.

This study has shown that body conditions of the rabbit dams, milk yield, and litter weight gain were influenced by dietary supplementation. The physiology behind this performance is that *Chlorella vulgaris* supplementation prevented stress and hence the rabbit had enough energy reserve to support milk production. These observations are in agreement with the submission of Szendro *et al*. (2002) which confirmed that survival of the kits is a determining factor in rabbit milk yield while the quality of the milk produced is a factor affecting the kits' performances such as promoting of weight gain and improved immunity.

This study also confirmed that the number of kits per doe is a major factor affecting milk yield since the rabbit does at the beginning of the lactation were standardized with an equal number of kits; it was however discovered later in the course of the study that does which

lost their kits had reduction in milk yield. This observation agreed with reports of previous authors who submitted that number of kits per rabbit doe is a major factor affecting milk yield; some of these authors including Partridge and Allan (1982), who reported 24.1 % increase in milk yield when the number of kits was doubled in rabbits does. Mohamed and Szendrö (1992) also reported a 3.3 % and 5.4 % increase in milk yield by an additional increase of kits per does from 6 and 10 kits respectively. Apart from the milk yield, kits number per doe was also reported as a factor affecting litter weight gain; this could be linked with outcomes of this study which showed that does with a smaller number of kits had lower litter weight gain while does with higher litter had higher weight gain.

The high significant milk yield, litter weight gains and body condition score of the rabbits recorded in this study due to *Chlorella vulgaris* supplementation can summarily be referred to as a nutritional manipulation of both biological and economic importance because milk production which has been reported to get little attention in the rabbits' selection programmes is key in kit survival and post-weaning performance. Therefore, nutritional supplementation for its increase as shown in this study with *Chlorella vulgaris* demonstrated a nutritional approach for increased rabbits' productivity during lactation (Maertens *et al*., 2006).

The supplementation of *Chlorella vulgaris* also led to an improvement of body condition score which is another production performance advantage; all the rabbit does in the treatment groups had better body condition scores compared with control despite lower feed intake. This observation is in agreement with the findings of Pascual *et al*. (2002) who observed that rabbits with higher body condition score produced more milk compared with rabbits with poor body conditions because the production of milk is negatively correlated with depletion of body fat reserves.

Body condition scoring is an indicator of well-being and optimum productivity as well as optimum animal reproductive performance; it is directly related to energy balance and it is an underlying factor affecting lactation, the animal capacity to return to breeding and long live animal existence (Sadiq *et al*., 2017). During lactation, energy from body reserves is mobilized into the mammary glands for milk production; this mobilization often leaves animals in either negative or positive energy balance. Since rabbit reproductive rhythm supports both gestation and lactation, concurrent gestation and lactation pose much burden on energy demand and hence rabbits are most liable to poor body condition score during lactation. However, this study has demonstrated that supplementation of *Chlorella vulgaris* is capable of improving body conditions of lactating rabbits because, despite higher milk production in the treatment groups, the body conditions score falls within the range of ideal body condition of matured rabbits (Pascual *et al*., 2003).

The antioxidant composition of the microalgae could be identified as the major biochemical mechanisms behind the better body condition in the treatment groups because the antioxidant composition provided additional protection complementing antioxidant enzymes activities of the rabbits against oxidative stress effects of energy partitioning between mammary glands for milk production and other body tissue during lactation (Jamali *et al.*, 2017). It could be concluded from the outcomes of lactation performances recorded in this study that antioxidant superiority of the microalgae promoted the animals' productivity during lactation through free radicals scavenging; this submission is in agreement with previous studies in lactating rabbits which reported that higher body condition scores reflected decrease in oxidative stress because body condition scores and oxidative stress are closely related to energy generation process at the cellular levels, and

that when its markers are reduced, it signifies less occurrence and usually lead to improved body conditions (Dobbelaar *et al.*, 2010; Jimoh and Ewuola, 2017).

5.7. Effects of *Chlorella vulgaris* Supplementation on Pre-pubertal Performances and Oxidative Stress Status of F1 Generation Kits of the New Zealand White Rabbits

Oxidative stress is a biochemical phenomenon associated with inefficient performances of an organism at early age and adulthood; it is positively correlated with increasing growth rates and reduced productivity. As an animal growth increases, oxidative stress also increases, and at some points when internal antioxidant defense becomes incapable of protection against oxidative stress, growth becomes reduced (Mangel and Munch, 2005; Christensen *et al.*, 2016). However, according to outcomes of this study, maternal supplementation of *Chlorella vulgaris* led to a reduction in oxidative stress through inhibition of malondialdehyde production which led to higher growth rates in the rabbit kits.

Similarly, maternal supplementation of the microalgae during gestation improved growth and cumulative antioxidant status of the F1 rabbits. Although the supplementation slightly affected catalase enzyme activities, there was a significant increase in the activities of superoxide dismutase, glutathione reduced and total antioxidant capacity. The relationships between performances of the rabbit kits, oxidative stress status and antioxidant protective activities indicated that *Chlorella vulgaris* biomass maternal supplementation is a suitable supplement for programming rabbit kits that can grow effectively fast and had a very high feed to gain conversion capabilities.

Rapid growth is closely related with oxidative stress caused by free radicals due to competitive concurrent allocation of metabolized energy and antioxidant resources for growth promotion and oxidative stress protection (Beckman and Ames, 1998); meanwhile, growth acceleration is a required performance for driving animal productivity, survival and reproductive advancement (Dmitriew, 2011). Therefore, in order to prevent growth-related complications by oxidative stress while implementing growth acceleration in animals for increase productivity, antioxidants supplementation is required for free radical scavenging, this was confirmed through this study because *Chlorella vulgaris* supplementation to the rabbits reduced oxidative stress and enhanced growth of the rabbits.

In agreement with the above proposition, Kumari *et al*. (1997) and Sastre *et al*. (1998) posited that antioxidants from plants and non-plant origins supplementation in senescence-accelerated mice models led to extended life span and enhanced performances via attenuation of oxidative stress and scavenging of reactive oxygen species. Although conflicting reports argued against these but such arguments could not be accepted as pieces of evidence that antioxidant supplementation is not a reliable approach to oxidative stress management since human and its models are complex organisms (De Block and Stoks, 2008). The protection against generation of oxidative stress products and increased antioxidant enzymes activities in the F1 generation kits in this study is also an indication that maternal *Chlorella vulgaris* supplementation can be used to enhance early growth rates without negative impacts on reproductive development because the foetal programmed kits had higher growth and higher levels of reproductive hormones compared with control group which is an indication of possible early pubertal attainment.

According to Bell *et al*. (2007), stress-related perturbation in animal compromise foetal Hypothalamus-Pituitary-Adrenal (HPA) axis functions; a condition implicated to be involved in entire lifetime reproductive impairments. Similarly, it was also reported that foeto-maternal supplementation of antioxidant related minerals such as selenium, improved reproductive performances through increased conception rates, lowering abortion and avoiding postnatal and prenatal foeto-maternal disorders. The underlying mechanisms are however, largely unknown (Palmieri and Szarek, 2011). Therefore, it can be deduced from outcome of this study that nutritional supplementation of *Chlorella vulgaris* is an innovative nutritional supplementation approach for preventing foetal related reproductive disorder at early peri-natal life because the animals' growth in the supplemented group were not inhibited while levels of their reproductive hormones were higher and was adequate in quantities compared with F1 of the control group.

Furthermore, apart from improved offspring performances; antioxidant supplementation is also capable of promoting reproductive success and well-being of the pregnant dams because antioxidant supplementation during gestation was reported to prevent oxidative stress – a condition associated with successful pregnancy carriage (Jenkins *et al.*, 2000). In a similar submission, Al-Gubory (2013) stated that maternal supplementation is needed to enhanced reproductive performance of both parents and their offspring because a variety of plant-derived antioxidant compounds such as vitamins, carotenoids, polyphenols, and trace elements can be used for oxidative stress inhibition. Therefore, it could be submitted that the supplementation of *Chlorella vulgaris* in this study because of its richness in vitamins, minerals, carotenoids, and polyphenols was responsible for better performances of the F1 rabbits' kits.

5.8. *Chlorella vulgaris* Supplementation Effects on Functional Genes Expression and Regulation in F1 Generation Kits of the New Zealand White Rabbits

Fetal programming of the rabbit progenies for protection against oxidative stress using the microalga *Chlorella vulgaris* biomass has led to the up-regulation of antioxidant genes including glutathione-s-transferase (gstp1) and cytochrome p450 (loc). This is a confirmation that the programming with the microalga has molecular impacts on the oxidative stress protection in the rabbit progenies. Glutathione-s-transferases are enzymes involve in a complex antioxidant protection network (Farina and Aschner, 2019). They are responsible for utilization of reduced glutathione which is a non-enzyme antioxidant because the enzymes (glutathione transferases) catalyzes the conjugation of free radicals especially hydrogen peroxide with reduced glutathione to decompose into molecular oxygen and water as a way of making it harmless in the body.

Up-regulation of the gene gstp1 indicated that there was active free radical detoxification in the rabbit progenies using reduced glutathione abundance. The gene is a protein-coding gene involved in oxidative stress protection; it negatively regulates expression of pro-inflammatory genes via translocation of p25 and p35 as oxidative stress mechanism (Cheung and Ip, 2012); the up-regulation of the gene (gstp1) as results of the maternal intake of *Chlorella vulgaris* biomass could also be link with non-enzymatic capacity of the microalga in protection of the animals against lipid peroxidation because reduced glutathione which is antioxidant catalyzed by these enzymes protects against oxidative stress through donation of its proton to membrane lipids for free radicals scavenging and inhibition of lipid peroxidation processes capable of complicating physiological functions (Birben *et al*., 2012). It could be concluded from the up-regulation of the gene glutathione-s-transferase that a molecular propelled mechanism of microalga oxidative stress

prevention is the promotion of glutathione transferase enzymes in decapacitation of free radicals.

Similarly, maternal supplementation of the microalga *Chlorella vulgaris* during gestation in the rabbits led to up-regulation of androgen receptor gene in their progenies; the implication of this observation is that there are increasing activities of androgens development in the fetal programmed progenies compared with their control counterparts because the androgen receptors in the fetal programmed progenies were more active. This outcome confirmed that fetal programming in protection against oxidative stress is a strategy for improving reproductive development in the rabbits and they have more effective masculinity imprinting due to activeness of their androgens (Neumann, 1983). The up-regulation of this gene is therefore advantageous especially during the pre-pubertal stage when the rabbits are being investigated; because this genetic apparatus promoting reproductive development is critical during these periods of their pre- and post-fetal lives since their fertility at adulthood is being programmed during these periods; and failure of the imprinting process cannot be made up for later in their lives (Neubert, 2002).

In this present study, there was up-regulation of interleukin genes; these are indications that supplementation of microalga *Chlorella vulgaris* promoted active immune functions and ignition of metabolic adaptation of the rabbit progenies to oxidative stress. This is because interleukin genes played essential roles in the activation and differentiation of immune cells, as well as their proliferation, maturation, migration, and adhesion (Angel and Ahmad, 2019). However, they also have pro-inflammatory and anti-inflammatory properties but since in the up-regulated rabbit progenies there was less concentration of oxidative stress markers as well as higher antioxidant enzyme activities; the up-regulations could be rather linked with improvement of immune functions and metabolic adaptations

by the rabbit progenies fetal programmed with the microalga, this agreed with submission of Wankhade *et al*. (2019) which submitted that up-regulation of interleukin genes is a function of active immunity and metabolic adaptation to reproductive related stress insults.

Interleukins are large group of proteins with related activities revolving round differentiation and activation of immune cells; they modulate active immunity through their effects on B or T cells; for example, IL-2 activate natural killer cells and T lymphocytes which makes it a suitable immune therapy approved for treatment of malignant cells (Angel and Ahmad, 2019). Therefore, enhanced activities of IL-2 genes due to supplementation of the microalga *Chlorella vulgaris* is a pointer that the microalga exploited immune modulation as mechanism of oxidative stress attenuation which implies that the rabbit progenies programmed with the microalga increased immune function to counter oxidative stress. These observations agreed with previous reports that microalga *Chlorella vulgaris* is an immune booster as reported by Gateau *et al*. (2017); the implication of this immune boosting potential of this microalga is that it is a suitable nutritional supplement for improving performance of young animals which are usually low or weak in immunity compared with their adult counterparts.

5.9. *Chlorella vulgaris* Supplementation Effects on Performances, Oxidative Stress Status and Genes Expression Patterns of the Finishing New Zealand White Rabbits

Chlorella vulgaris is a microalga commonly cultivated on a commercial scale in different parts of the world. Its applications cut-across food industries, pharmaceuticals and animal production. In animal production, it is being used as animal and fish feed additives because of its nutritional profile (Lum *et al*., 2013). In this present study, supplementation of the microalga to rabbits showed that it has potential for improving production performances

and efficiencies of feeds utilization because despite the reduction in feed intake as a result of the supplementation, final body weight of the rabbits in the treatment groups were higher compared with control group. The reduction in feed intake may not be because of non-palatability of the microalga or as results of toxicity but may be 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 similar compared with the treatment groups. All these are indications that supplementation of the microalga has significant economic benefits since it reduced feed intake and increased productivity.

Chlorella vulgaris supplementation in this study due to a 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 the submission of Halle *et al*. (2009) who observed that supplementation of *Chlorella vulgaris* in laying birds led to a reduction in feed intake without a 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 the control group because the supplemented groups had higher egg weight, higher laying percentages, higher hen-day production, and lower values for feed conversion ratio.

Similarly, supplementation of *Chlorella vulgaris* in fish was also reported to improve productivity, dietary lipid utilization and muscle pigmentation which were all identified as product quality improvement in fish (Gouveia *et al*., 1998). *Chlorella vulgaris* supplementation and the reduced feed intake observed in this study could be linked to improved biodiversity of intestinal microbes of the rabbits thereby contributing to better

gut health. Supplementation of the microalga since it is a green alga with enourmous amount of starch and cellulose, probably led to increased population of lactic acid bacteria and the ability of the gut microflora to degrade algal polysaccharides and other complex plant polymers in the basal feed and in the overall, led to better digestive efficiency of the animals which is the reason why despite the reduced feed intake, there was higher product yields.

This is possible in rabbits because they are hindgut fermenters capable of degrading complex biomolecules in their caecum as a result of the increased microflora population and diversity. Again, as a way of improving gut health, diversity is capable of reducing the population of non-beneficial microbes in the guts of the rabbit and hence contributed to improvement of the animals' health and productivity (Zheng *et al*., 2012). 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 attestations to improved nutritional efficiencies as a result of the supplementation. These observations are in agreement with previous studies on *Chlorella vulgaris* feeding in other animal species where it was regarded as microalga with great potential nutritive values capable of enhancing some biochemical and physiological functions for improved immunity and growth (Kang *et al*., 2013).

Oxidative stress product generation is a major pathophysiological mechanism of oxidative stress damages; malondialdehyde and protein carbonyls are leading among these products and their increasing concentration corresponds with poor performance (Agarwal and Allamaneni, 2004). In this study, supplementation of *Chlorella vulgaris* significantly reduced the generation of these products on the 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 supplementation is a strategy for promoting animal production performances (Fulia *et al*., 2001).

Chlorella vulgaris is a rich source of polyphenolic compounds; hence, the reduction observed in the oxidative stress product concentration in this study can be linked with antioxidant capabilities of the polyphenolic contents of the microalga (Li *et al*., 2007). 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 the possibility of maximum bioavailability of the polyphenols because both enzymatic and microbial degradation of bounded polyphenols takes place within the gastrointestinal tracts (Dangles *et al*., 2001). The higher bioavailability of polyphenols as a result of these multiple absorptions was probably responsible for the increased total antioxidant concentration in the supplemented animals in this study. The absorbed polyphenols from the microalga at cellular level, prevented the activation of NF-*k*B and hence inhibited oxidative stress and its associated compromise while several repeats of this process could lead to non-disturbance of cellular activities and therefore, reduce oxidative stress products generation (Vendrame and Klimis-Zacas, 2015).

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, oxidative stress can be attenuated using microalga *Chlorella vulgaris* because of the 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 the production of oxidative stress products including malondialdehyde (Mazo *et al*., 2004; Kim *et al*., 2010).

Lipid peroxidation is the chief oxidative stress effect and the most prevalent form of oxidative stress compromise at the cellular level. It is a toxic process to the cells causing cell loss of membrane integrity, cell death and activation of genetically programme cell death (Riss *et al*., 2004). Compromised reproductive inefficiencies in males such as spermatozoa dysfunctions, low epididymal sperm concentrations, poor sperm motility, and abnormal sperm morphology are also linked with increasing lipid peroxidation (Türk *et al*., 2016). In the 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 a mechanism of oxidative stress associated with the decline of oocyte quality. 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 (Janczyk *et al*., 2006; Higuera-Ciapara *et al*., 2006; Kouba *et al*., 2014).

This study outcome 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 of the significant up-regulation of the primary antioxidant genes *sod1* and *gpx1* indicating an immediate cellular defence stage. In specific, it could be translated that there are adequate protective actions of antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase in the suppression and detoxification of free radicals. This could be achieved via dismutation of superoxide radicals, decomposition of hydrogen peroxides and the overall prevention of free radicals' formation by inhibiting the mechanisms leading to their production (Ighodaro and Akinloye, 2018).

The up-regulation of these genes in the supplemented rabbits confirmed the molecular relevance of *Chlorella vulgaris* as a source of antioxidants capable of preventing metabolic and 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 the factors leading to up-regulation of these genes (Turrens, 2003).

Sod1 a protein-coding gene with the capacity of biding copper and zinc ions is one of the enzymes primarily responsible for protection against superoxide radical destruction. It is also active against superoxide radicals and hydrogen peroxide in both the cytoplasm and mitochondria. Therefore, its up-regulation as a result of *Chlorella vulgaris* supplementation indicated complete cellular protection. *Sod1* is also involved in the pharmacokinetics and reactive oxygen species detoxification pathways hence, the microalga is a drug candidate in the development of therapeutic solution for oxidative stress attenuation (Wang *et al*., 2018; Abbasi-Oshaghi *et al*., 2019). It is also evident from

the results of the study that as the rabbit matures, there was increasing metabolic activities due to growth and development of the reproductive organs leading to cellular generation of reactive oxygen species and superoxide radicals.

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 a higher adequate cyclical reaction for 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 (Peinado *et al*., 2013). The molecular basis of these protective mechanisms was the up-regulation of the antioxidant genes. Furthermore, the up-regulation of *sod1* 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. These genes along with *sod1* jointly contribute to cellular prooxidant detoxification and specifically detoxification of reactive oxygen species.

Glutathione peroxidase (*gpx1*) is another antioxidant gene up-regulated as a 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 the reduction of organic hydroperoxides and detoxification of hydrogen peroxide. The 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 (López-Lázaro, 2007; Burk and Hill, 2015). Molecular advantage of this is that the microalga *Chlorella vulgaris*

can be exploited as a feed additive and or drug agent with bioactive compounds that can contribute to favourable cellular transcriptions.

The gene was expressed in both the liver and ovaries of the rabbits indicating that metabolically and reproductively, there was adequate oxidative stress protections in the rabbits. This is the ultimate benefit of *Chlorella vulgaris* supplementation because of the up-regulation of *gpx1* as 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 substrates acts as electron donor then becomes oxidized, while the other acts as electron acceptor and becomes reduced (Ashburner *et al*., 2000). This cellular mechanism is responsible for the interactive relationship between *gpx1* and *sod1* for protection against oxidative stress.

The molecular function of *gpx1* expression is also related to the enhancement of glutathione-transferase activities – is a 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 signifies that the microalga supplementation contributed to total antioxidant protection (Forgione *et al.*, 2002). The specific benefit of this gene in the 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.

Deficiency of glutathione peroxidase expression is related to the occurrence of many diseases including haemolytic anaemia, diabetes mellitus, ischemia and retinal vascular diseases among others; what is common to all these diseases however are all associated with oxidative stress complexities. Therefore, its up-regulation in the treatment groups could explain the reason for reduced lipid peroxidation products, higher antioxidant potential, and enhanced antioxidant enzyme activities since its deficiency is related to higher oxidative stress damages (Sandstrom *et al.*, 1994). The 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 a source of antioxidants with a wide range of biological protective activities against oxidative stress.

In addition, this study also investigated impact of oxidative stress on the hematopathology of the rabbits via possible changes and perturbations on morphology of the red blood cells. The results showed no significant difference in the sizes of the red blood cells of the animals. However, there were pieces of evidence of oxidative stress perturbation in the red blood cells of rabbits in the control group compared with the treatment group which could be as results of oxidative stress damages.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1. Conclusions

The following conclusions were made after critical evaluation of the results obtained in line with the objectives stated at the beginning of this study:

i. Determination of proximate composition and *in-vitro* antioxidant capacities of the microalgae provided empirical data which led to conclusion that the microalga *Chlorella vulgaris* supplements used in this study are suitable antioxidants sources that can be used for management of oxidative stress and its ancillary biochemical complications in rabbits, because its total antioxidant capacities showed that they are rich in total antioxidant composition, and it contained high levels of phenolic compounds, and it equally demonstrated excellent ferric reducing antioxidant power.

ii. Supplementation of the microalga *Chlorella vulgaris* after comparative assessment of the serum and tissues concentrations of malondialdehyde, protein carbonyl, and total antioxidant capacities indicated that the supplemented groups were protected against oxidative stress compared with the control through reduction of malondialdehyde and protein carbonyl while increasing total antioxidant capacities of the animals. Therefore, it was concluded thereof, that dietary supplementation of the microalga *Chlorella vulgaris* prevented oxidative stress through reduction of serum and tissue malondialdehyde concentrations, protein carbonyl and increased total antioxidant capacities of the rabbits at all the physiological stages. Since the reduction in the

concentrations of malondialdehyde and protein carbonyl corresponded with increasing total antioxidant capacities of the rabbits supplemented; hence, it was concluded that the microalga can be used for both serum and tissue oxidative stress protections.

iii. *Chlorella vulgaris* and its derivatives supplemented in this study attenuated oxidative stress through modulations of antioxidant enzymes at the different physiological stages of the rabbits development; prepubertal stage, gestation stage, lactation stage as well as finishing non-reproductive stages; supplementation influenced activities of superoxide dismutase and reduced glutathione more than activities of catalase and from these it was concluded that the microalga is an excellent source of antioxidant since it has strong potential to serve as superoxide radical inhibitors and can as well serve as house-keeping antioxidant indicating that it functions at both the beginning and end of oxidative stress pathway.

iv. *Chlorella vulgaris* supplementation in this study contributed to reproductive performances of rabbits quantitatively and qualitatively at each stage of the study; at gestation stage, it led to higher reproductive index; during lactation stage, it improved body conditions of the rabbit does, it also contributed to higher milk yield and led to better survival and higher pre-weaning weight gains of the rabbit kits. In the finishing non-reproductive stage, the supplementation led to the rabbits finishing with higher commercial weights and thus, the microalga was concluded as an animal supplement suitable for improvement of reproductive and production performances of rabbits.

v. Supplementation of the microalga *Chlorella vulgaris* has no negative impacts on rabbit physiological performances and internal organs functions because all the serum biochemical parameters determined in the rabbits were within the normal range of healthy rabbits. Maternal supplementation of the microalga led to enhanced estrogen and testosterone levels, prevented oxidative stress in foetal programmed rabbits, increased activities of antioxidant enzymes as well as up-regulation of primary antioxidant genes including *sod1, gpx, cytochrome-p450* and *glutathione-s-transferase* in the rabbits.

vi. *Chlorella vulgaris* biomass between 200 mg and 500 mg per kg body weight are capable of preventing occurrence of oxidative stress, improved activities of primary endogenous antioxidant enzymes without negatively hampering productive performances of the rabbits at different physiological development stages. Supplementation of the microalgae led to a reduction in feed intake without decreasing weight gain of rabbit, therefore, its supplementation can contribute to nutritional optimization in rabbits.

vii. Supplementation of the microalgae in this study led to positive significant correlation ($p < 0.01$) relationships between gestation gain and litter weight at birth; and between reproductive index and litter size. This suggested that there are interactions between phenolic compounds in the microalgae and oestrogen; the interaction between phenolic compounds and oestrogen was also concluded to be responsible for increased sexual receptivity and higher litter size; conception, and kindling rates which were all identified as drivers behind the higher reproductive index observed due to the supplementation of the microalga.

viii. Supplementation of *Chlorella vulgaris* during gestation as a way of foetal programming rabbits in protection against oxidative stress *in-utero* as discovered in this present study resulted into production of rabbit progenies with higher birth attributes and better growth rates. Also, foetal programming of the rabbit progenies for protection against oxidative stress using the microalga *Chlorella vulgaris* biomass led to the up-regulation of antioxidant, growth and interleukin genes; this is confirmation that the programming with the microalga has molecular impacts on the oxidative stress protection in the rabbit progenies. It could also be concluded that the foetal programming of rabbit progenies using this microalga led to enhanced reproductive imprint and development of active immunity in the rabbit progenies and these could be linked with antioxidant capabilities of the bioactive compounds in the microalga *Chlorella vulgaris* biomass interactions with the fetus at the placental-fetus interface.

ix. Finally, this study demonstrated that oxidative stress is an inevitable biochemical process compromising animal performances and the implications transcend beyond physiological loss to economic loss since it manifested as a relevant mediator of reduced animal productivity in form of lower finishing weights as discovered in this study. Meanwhile, supplementation with microalga *Chlorella vulgaris* in this study ameliorated the production inefficiencies as well as enhanced the animals' physiological performances and also showed molecular oxidative stress protection through up-regulation of antioxidant genes through expression modification.

6.2. Recommendations

This study recommended the followings:

i. *Chlorella vulgaris* biomass supplementation at 200 mg and 500 mg per kg body weight of rabbits at pre-pubertal and gestation stages should be supplemented as additive for production enhancement and promotion of the rabbits' health via oxidative stress attenuation.

ii. *Chlorella vulgaris* biomass supplementation at 300 mg, 400 mg and 500 mg per kg body weight of rabbits at lactation and finishing stages be included as supplemental additive for enhancement of milk production, better carcass and meat yield traits and promotion of the rabbits' health via oxidative stress attenuation.

iii. The exploration of *Chlorella vulgaris* biomass supplementation at 200 mg and 500 mg per kg body weight in rabbits as models for development of nutraceutical for management and attenuation of oxidative stress in food producing animals and humans.

iv. The exploration of the microalga and other similar antioxidants supplement effects on expressions of functional genes in the intestines of rabbits and other food-producing animals in relation with gut microbiome and the reproductive performances of the animals.

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APPENDIX A

PUBLICATIONS AND AWARDS ASSOCIATED WITH THE STUDY

JOURNAL ARTICLES

1. **Sikiru, A.B**, Alemede, I.C., Arangasamy, A., Egena S.S.A., & Ijaiya, A.T, (2020). Temperature Elevation Effects on Livestock Production - A Study and Forecast of Bengaluru Climate Data. *Ecologia*, 10 (1): 1-8, 2020.
2. **Sikiru A.B**., Arangasamy A., Alemede I.C., Guvvala P.R., Egena S.S.A., Ippala J.R, & Bhatta R. (2019). *Chlorella vulgaris* supplementation effects on performances, oxidative stress and antioxidant genes expression in liver and ovaries of New Zealand White rabbits. *Heliyon* 5 (2019) e02470.
3. **Sikiru A. B.**, Arangasamy A., Alemede I. C., Egena S. S. A., & Bhatta, R. (2019). Dietary supplementation effects of *Chlorella vulgaris* on performances, oxidative stress status and antioxidant enzymes activities of prepubertal New Zealand White rabbits. *Bulletin of the National Research Centre*, 43(1), 162.
4. **Sikiru, A.**, Arangasamy, A., Ijaiya, A., Ippala, R., & Bhatta, R. (2019). Effects of *Chlorella vulgaris* supplementation on performances of lactating nulliparous New Zealand white rabbits does and their kits. *International Journal of Livestock Research*, 9 (9), 49 – 58.
5. **Sikiru A.B.,** Egena S.S.A., Alemede I.C., and Makinde O.J. (2018). Environmental source of stress in livestock productivity – a study of Minna climate data. *Biotechnology in Animal Husbandry*. 34 (2), p 159-170, 2018.
6. **Sikiru A.B.,** Alemede, I.C., Egena, S.S.A and Ijaiya, A.T. (2018). Oxidative stress and reproductive inefficiencies: the science, evidences, and solutions. *Agricultural Extension Journal*. 2(1):17-26.
7. **Sikiru A.B.,** Alemede, I.C., Egena, S.S.A and Ijaiya, A.T. (2017). Foetal Programming: Potential Tool for Improving Reproductive Performance of Rabbits – A Review. *Wayamba Journal of Animal Science*. P1610 - P1615.

CONFERENCE PAPERS AND POSTERS

8. **Sikiru A.B**., Arangasamy A., Alemede I.C., Egena S.S.A, & Ijaiya A.T (2019). Feta programming for oxidative stress protection: impacts of Chlorella vulgaris supplementation on birth attributes and prepubertal growth rates of rabbit progenies. *XXXV Annual Convention of ISSAR and International Symposium*, Namakkal, Tamil Nadu, India. 29 – 30.

9. **Sikiru A.B**., Arangasamy A., Alemede I.C., Egena S.S.A, Raghavendra B., & Ijaiya A.T (2019). Biochemical and molecular effects of *Chlorella vulgaris* antioxidants in rabbit model: a green-red biotechnology for promotion of food security and human well-being. *DBT – TWAS Conclave,* Chennai, India.

10. **Sikiru A.B.**, Arangasamy A., Alemede I.C., Selvaraju S., and Bhatta R. (2019). Molecular assessment of carotenoids crossing of brain - blood barrier: looking into antioxidant protection of brain for improving animal fertility. *Proceedings of 7th Pan Commonwealth Veterinary Conference* 2019 held at ICAR-National Institute of Animal Nutrition and Physiology, Bangalore, India. Pp 178.

11. **Sikiru A.B.**, Arangasamy A., Selvaraju S., and Bhatta R. (2018). *Chlorella vulgaris* and its derivatives are potential antioxidant supplements for improving livestock reproductive performance. *XXXIV Annual Convention of ISSAR and International Symposium*, Anand, Gujarat, India. 132 – 133.

12. **Sikiru A.B.,** Alemede I.C., Egena S.S.A. and Ijaiya A.T. (2018). Overview of Rabbit production as an option for fighting malnutrition in nigeria. *Proceedings of 43rd Annual Conference of the Nigerian Society for Animal Production,* Owerri 2018. 1424 – 1425.

13. **Sikiru A.B.,** Alemede I.C., and Egena S.S.A. (2017). Algae as functional food/feed – exploring functional potential of *chlorella vulgaris* against oxidative stress for improved reproductive performance. *Book of Abstract*, International Conference of Alexandra von Humboldt Foundation, Akure Kolleg 2017.

AWARDS

1. Busary Award by Alexandra von Humboldt Foundation, Germany; for attending the International Conference of Alexandra von Humboldt Foundation, Akure Kolleg, Federal University of Technology, Akure, Nigeria; 2017.

2. Doctoral Research Fellowship Award by The World Academy of Sciences (TWAS), Italy; and Department of Biotechnology (DBT), India; 2017 – 2019.

3. Best Young Scientist Award by Indian Society for Study of Animal Reproduction (ISSAR), at International Conference of ISSAR held at Veterinary College and Research Institute, Namakkal, India; 2019.

APPENDIX B

FERRIC REDUCING ANTIOXIDANT POWER ASSAY (FRAP)

1. Reagents and preparation

a. Preparation of Potassium ferricyanide solution (1%)

1 gram of potassium ferricyanide was dissolved into 100 ml volumetric flask with a small quantity of distill water first then filled up to the flask volume.

b. Preparation of Trichloroacetic acid solution (10%)

10 grams of trichloroacetic acid (HiMedia Laboratories Pvt. Ltd, India) was dissolved into 100 ml volumetric flask with small quantity of distill water first then filled up to the flask volume.

c. Preparation of Ferric chloride solution (0.1%)

0.1 gram of ferric chloride was dissolved into 100 ml volumetric flask with a small quantity of distill water first then filled up to the flask volume**.**

d. Preparation of Potassium phosphate buffer

1.212 g potassium hydrogen phosphate – K_2HPO_4 (S & D Fine Chemical Pvt. Ltd, India) and 0.414 g of potassium dihydrogen phosphate – KH_2PO_4 was dissolved into 100 ml volumetric flask with small quantity of distill water first then filled up to the flask volume.

e. Preparation of Ascorbic acid standard (0.1%)

1 mg of ascorbic acid was dissolved in 1 ml of distilled water.

2. Procedures

1 ml in triplicate from each *Chlorella vulgaris* concentrate solution (20%, 40%, 60%, and 80%) were sampled separately. 2.5 ml of phosphate buffer and 2.5 ml of potassium ferricyanide was added. This reaction mixture was incubated for 20 minutes at 50° C after which 2.5 ml of trichloroacetic acid was added. The mixtures were centrifuged at 3000 rpm for 10 minutes each where 2.5 ml of supernatant solution were obtained mix with 2.5 ml distilled water and 0.5 ml ferric chloride (0.1%) then put up for absorbance reading. Ascorbic acid was used as analytical standard while absorbance measurement at 700 nM for measurement and determination of reducing power of the algae.

APPENDIX C

PROXIMATE ANALYSIS PROTOCOL

Moisture and dry matter of the samples were determined by oven drying of the samples to constant weight at 80° C. The difference in weights before and after oven drying was used for estimation of the moisture and dry matter components. The loss in weight to the oven drying represented the moisture component while the weight after oven drying represented the dry matter component. The formula below was used for computation of the moisture level in percentage:

$$\text{Dry matter (\%)} = \frac{\text{After dry weight}}{\text{Initial Weight}} - \text{Empty Petri weight} \times 100$$

For the determination of minerals content of the samples, there were triplicates sampling of each feed separately ignited in muffle furnace for 4 hours at 550° C after which the ash was evaluated for mineral analysis using atomic absorption spectroscopy measurements (Ayoola *et al.*, 2011). The minerals content was determined for nutritional reasons in order to establish presence of minerals in the algae as well as determining presence of toxic or heavy minerals which may be harmful upon consumption by animals. Ash percentage was determined on dry matter basis using the formula:

$$\text{Percentage ash (\%)} = \frac{\text{Weight}_{\text{ash}}}{\text{Weight}_{\text{dried}}} \times 100$$

The ashes samples were dissolved in 15 ml 5N HCl then make up to 100 ml boiled on dry bath and filtered; the filtrates were again made up to 100 ml with distilled water to form an aqueous solution for minerals evaluations in Inductively Coupled Plasma-Optical Emission Spectrometer (PerkinElmer, Massachusetts, USA). It is a machine that works through transmission of radiations through the atomized samples and absorption of the radiation at wavelengths corresponding to different minerals for measuring each mineral element. Two sets of mineral solutions were prepared; the undiluted ash aqueous samples used for determination of macrominerals and diluted ash aqueous solution (aliquot 1ml sample and 10 ml ash solution) used for determination of micro minerals.

Protein, Kjeldahl procedure involving digestion, distillation, and titration of the samples were used for protein evaluation (Abdul-Mumeen *et al.*, 2013). For digestion, 0.5 g of each sample was measured in triplicates into digestion tubes added with 15 ml of concentrated tetra-oxo-sulfate (vi) acid H_2SO_4; 0.5 g of copper sulfate, 5 g sodium sulfate and a speck of selenium tablet were also added. The set-ups were placed into fume cupboard, heat from an electric source was applied and the digestion was allowed to run for a period of 45 minutes until the digesta became clear pale green.

Automatic Gerhardt Vapodest 45s apparatus (Gerhardt Analytical System, Germany) was used for the distillation, titration and the determination of the protein. 100 N H_2SO_4 and 40 % NaOH as well as 4 % boric acid indicator were automatically fed by the system for the titration; the volume of acid used was recorded after each run and was used to compute the crude protein values based on standardized formula for the apparatus below:

$$\text{Crude protein (\%)} = \frac{\text{Total volume - Blank}}{\text{Sample weight}} \times 0.8$$

Crude fibre determination was done using 10 g of the samples in triplicates; fat was extracted from the samples using petroleum ether. The de-fatted samples were individually added into a solution of pre-heated 1.2 % H_2SO_4 and then boiled gently for 30 minutes. Neutral residue from the boiled samples was put into 200 ml of pre-heated 1.25 % Na_2SO_4 then boiled for another 30 minutes. The boiled solutions were then washed in both ethanol and water filtered while the residues were dried at 65° C for 24 hours and weighed. The residues were put into porcelain crucible and ashed for 4 hours then cooled on silica desiccator and weighed. Crude fibre percentage was computed using the formula:

Crude Fibre (%) = Dry weight before ashing - Weight of ash x 100 The initial weight of the sample

Metabolizable energy which is the amount of energy available in a given sample of feed material for total heat production was determined by estimation using Atwater conversion (Livesey, 2001). Respective Atwater conversion factors for protein (4.0 Kcal/Kg), fat (9.0 Kcal/Kg) and carbohydrates (4.0 Kcal/Kg) were multiplied with the respective nutrients determined in the sample then added up and multiply by 10 in order to get final value for the samples metabolizable energy (FAO, 2003).

APPENDIX C

HYDROGEN PEROXIDESCAVENGING POTENTIAL

1. Reagents and preparation

a. Preparation of Phosphate buffer (pH 7.4)

3.48 g potassium hydrogen phosphate and 2.72 g of potassium dihydrogen phosphate were dissolved into 100 ml volumetric flask with small quantity of distilled water first then filled up to the flask volume.

b. Preparation of 40 mM H_2O_2 in PBS (pH 7.4)

Preparation of 40 mM solution, 4.09 ml of 30% H_2O_2 stocked solution (Sigma Aldrich) was added to 1 litre distilled water.

2. Procedures

1 ml in triplicate from each *Chlorella vulgaris* test samples were sampled separately. 1 mL of H_2O_2 solution (40 mM/L) was added to respective mixture of the test samples then incubated for 10 min at room temperature. The absorbance of H_2O_2 at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. Absorbance readings were taken for each of the solutions in triplicates. The scavenging capacity for each of the sample was calculated using the following formula:

$$H_2O_2 \text{ Scavenging capacity } (\%) = \frac{Abs_0 - Abs_1}{Abs_0} \times 100$$

Where Abs_0 is initial absorbance reading (230 nm) and Abs_1 is the final absorbance reading after 10 minutes for each of the test tubes against blank.

APPENDIX D

SUPEROXIDE DISMUTASE ASSAY

This assay capacity was developed on the background involving autooxidation of pyrogallol in the presence of DEPTA (Diethylenetriaminepentaacetic acid) at the pH 8.5 by 50 %; a principle established and verified by Marklund and Marklund (1974), modified by Guvala (2019). The assay is very efficient, easy and affordable because all the reagents can be produced in the laboratory as described below:

1. Reagents:

a. **Tris Cacodylate buffer (pH 8.5)**: 50mM Tris buffer (base) with molecular weight 121.14 was prepared by sampling 1211.4 mg; 2140 mg of Sodium cacodylate (molecular weight 214) and addition of 78.67 mg 1mM DEPTA (molecular weight 393.35) all into 200 ml distilled water.

b. **20mM Pyrogallol solution**: 25.22 mg of pyrogallol dissolved in 10 ml of 10 mM HCl.

c. **HCl (10 mM)**: 8 µl of concentrated HCl into 10 ml with distilled water.

2. Reaction mixture:

Ingredients	Sample	Blank
Serum	50 µL	0
Tris buffer	950 µL	950 µL
Pyrogallol	10 µL	10 µL

3. Absorbance reading: Absorbance reading will be taken at wavelength 420 nm by addition of Tris-DEPTA buffer (pH 8.5) at zero time and after 3 minutes of the addition of pyrogallol.

4. Calculations: At the end of spectrometer absorbance readings; percentage inhibition of pyrogallol autooxidation and activity of the superoxide dismutase enzymes in the samples calculated using the formulae below:

$$\% \text{ inhibition of pyrogallol autooxidation} = \frac{\text{Abs(blank)} - \text{Abs (test)}}{\text{Abs(blank)}} \times 100$$

$$\text{One unit of SOD activity (u/ml)} = \frac{\% \text{ inhibition of pyrogallolautooxidation}}{50\ \%}$$

Where;

$Abs_{(blank)}$ - Absorbance for blank; $Abs_{(test)}$ - Absorbance for treatment

One unit of SOD activity (u/ml) is the amount required for inhibiting pyrogallol autooxidation by 50% per min.

APPENDIX E

LIPID PEROXIDATION ASSAY

Chemicals and reagents preparation

Acetic acid (20 %); thiobarbituric acid (TBA; 0.8%); Sodium-*n*-dodecyl (SDS; 8.1 %); The TBA prepared in distilled water may be mildly heated to assist dissolution of TBA.

Samples homogenization

Homogenisation of tissue; after the animals were decapitated the organs were removed carefully, immediately weighed and homogenized with cold ice and 1.15% potassium chloride (KCl) to make 10% homogenate solution (i.e. the tissue is w/v for tissue and the potassium chloride solution as 1% tissue and 9% the solution). For serum samples; the serum was used directly without addition of KCl solution.

Procedures

200µL of the samples each combined with 0.2mL of 8.1 % SDS; 1.5 mL acetic acid and 1.5 mL TBA the solution made up to 4 mL with distilled water; then the solution is boiled for 60 minutes in a boiling water bath (95 ^{o}C); after cooling, the reaction product (TBA–MDA complex) was extracted by adding 1 mL of n-butanol-pyrimidine (15:1; v/v). The flocculent precipitate was removed by centrifugation at 3500 rpm for 15 mins; then supernatant is obtained and absorbance reading of the supernatant at 532 nm against a blank that contains the reagents minus the samples. The malondialdehyde concentration of the sample was calculated using the adduct extinction coefficient of 1.56 x 10^{5} $M^{-1}cm^{-1}$ for MDA.

Malondialdehyde concentration (M) = $\frac{Abs535}{155}$

MDA conc. (µM): M x 1000 = MDA conc. (µM/mL) of the sample

APPENDIX F

PROTEIN CARBONYLATION

Chemicals and reagents preparation:

1. 10 mM DNPH solution in 2 N HCl: Dissolution of 19.8 mg of 2,4,-dinitrophenylhydrazine (DNPH) (m.w. 198.1) in 10 ml 2 N HCl and stirred overnight in the dark to allow complete solubilization. The solution was used within 24 h because reactivity could decrease over time, and the DNPH solution was kept at room temperature protected from light because DNPH is light-sensitive.
2. Protein sample (1 mg/ml); for plasma samples, direct utilization of 100 µL
3. HCl (2N): 166 ml of 37% HCl (fuming hydrochloric acid) was diluted with 834 ml of distilled water.
4. 20% (v/v) trichloroacetic acid (TCA) solution, ice-cold: TCA stock solution (100%) was prepared by dissolving 100 g TCA with 45.4 ml of water. Dilute TCA stock solution 1:5 with water (10 ml of 100% TCA + 40 ml water) to obtain 20% (v/v) TCA. Store up to 6 months at room temperature.
5. 1:1 (v/v) ethanol:ethyl acetate
6. 6 M guanidine hydrochloride: Dissolve 57.32 g of guanidine hydrochloride (m.w. 95.53) in 100 ml of total water solution.
7. 0.2% (w/v) SDS in 20 mM Tris–HCl (pH 6.8)
8. The bicinchoninic acid (BCA) protein assay kit

Procedures:

1. 100 µl of the samples in triplicates obtained sampled into two parts in 1.5 ml tubes; where one serves as blank and the other one as a test sample. For plasma (sample without protein extraction but for tissue samples extracted protein included at 1 mg/ml).

2. Add 200 µl of 10 mM DNPH solution to the protein which will serve as the test sample, and prepare a blank sample adding 200 µl of 2 N HCl (without DNPH) to blank protein sample.

3. Vortex-mix samples and leave them in the dark at room temperature for 60 min, with vortex-mixing every 10–15 min.

4. Add 1.2 ml of 20 % TCA solution to the protein samples and incubate on ice for 15 minutes.

5. Centrifuge samples at 10,000 × g in a tabletop microcentrifuge for 5 min, at 4 ^{O}C.

6. Discard supernatants, wash protein pellets once with 1 ml of 20% TCA and vortex-mix.

7. Collect pellets centrifuging at 10,000 × g for 5 min, at 4 ^{O}C.

8. Discard supernatants, wash protein pellets with 1 ml of 1:1 (v/v) ethanol: ethyl acetate and mix by vortexing in order to remove any free DNPH.

9. Repeat Steps 6 and 7 at least twice until supernatants are completely transparent; Note: extend washes to completely remove any free DNPH, otherwise, residual DNPH in the protein pellet could lead to overestimation of sample PCO content.

10. Collect pellets centrifuging at 10,000 × g for 5 min, at 4 ^{O}C and discard supernatants.

11. Let the pellets vacuum dry for about 5 min to allow complete solvent evaporation; Note: constantly check pellets during vacuum drying to avoid over-drying, which could make pellet re-suspension harder to obtain and incomplete.

12. Re-suspend protein pellets in 1 ml of 6 M guanidine hydrochloride (dissolved in 50 mM phosphate buffer, pH 2.3) then incubate at 37 ^{O}C for 15–30 min with vortex mixing; Note: Protein pellets can alternatively be resuspended in 20 mM Tris–HCl, pH 6.8, containing 0.2% (w/v) SDS in case of protein quantification with the BCA protein assay and subsequent SDS-PAGE.

13. Once protein pellets are completely dissolved, carbonyl contents can be determined from the peak absorbance at 366 nm by using a molar absorption coefficient of 22,000 M-1 cm-1. The protein sample incubated with 2 N HCl without DNPH should be used as a blank in order to subtract intrinsic protein absorbance to the absorbance of specific DNP-adducts. Advice: scan samples in the wavelength range from 300 nm to 500 nm by a spectrophotometer in order to singularly check the profile quality of all sample spectra and choose the correct maximum peak.

Calculation:

14. PCO concentration in the cuvette (expressed as M) or sample carbonyl content (expressed as nmol PCO/mg protein), use the following formulas:

PCO Concentration (cuvette) = PCO (μm)

$$= 10^{6}\ (\text{Abs366nm} / 22{,}000\text{M}^{-1}\text{Cm}^{-1})$$

PCO Concentration (sample) = PCO (nm/mg protein) =

$$[10^{6}\ (\text{Abs366nm}/22{,}000\text{M}^{-1}\text{Cm}^{-1})]/\text{protein (mg/ml)}$$

APPENDIX G

CATALASE ACTIVITY

This assay is based on samples (enzyme) ability to decompose hydrogen peroxide (H_2O_2) into H_2O and O_2 and the rate of decomposition of H_2O_2 can be measured by the decrease of its absorbance at 240 nm over a period of time 3 min.

Reagents

- **Potassium phosphate buffer – 50 mM (pH-7.0)** di-Potassium hydrogen phosphate (M wt. 174.18) – 0.5034 g Potassium dihydrogen phosphate (M. Wt. 136.09) – 0.2871 g Makeup to 100 ml with double distilled water and check the pH
- **Hydrogen peroxide 100µM**

Take 56.5 µL H_2O_2 (30%) and makeup 5 ml with potassium phosphate buffer. Note; It has to be **prepared fresh** and avoid from light.

Procedure

Take the test tube, add the following ingredients:

Ingredients	Blank	Sample
50 mM Potassium Phosphate buffer	900 µL	890 µL or 880 µL
Serum Sample	-	10 µL or 20 µL
100µM H_2O_2	100 µL	100 µL

1. Mix properly and immediately the reaction mixture (prepare in a test tube) is transferred to the cuvette and the initial reading was recorded at 240 nm along with the successive reaction readings for every 30-sec intervals for 3 min.
2. The non-enzymatic reaction rate was correspondingly assessed by replacing the sample by 50 mM potassium phosphate buffer (pH-7.0)
3. The change in absorbance (ΔA 240) per minute so obtained for the blank was subtracted from each assay readings of the test sample.

4. Once the sample is added to the mixture the absorbance starts to decrease and the rate of change of absorbance is dependent on the amount of the Catalase present in the sample.

$$\text{Catalase activity (U/mL of sample)} = \frac{[\Delta A_{240/min}(\text{sample}) - \Delta A_{240/min}(\text{blank})] \times DF}{0.0436 \times \text{volume of the sample}}$$

$\Delta A_{240/min}$ =change in absorbance at 240 nm/min of sample or blank.

DF = Dilution factor of the original sample, if any, before adding to the reaction mixture

0.0436 = millimolar extinction coefficient of H_2O_2 at 240nm.

APPENDIX H

GLUTATHIONE PEROXIDASE ASSAY

1. Reagents

a. Phosphate buffer (100 mM, pH 7.6): Solution A: 1.78 g of disodium hydrogen phosphate will be dissolved in 100 ml of distilled water. Solution B: 1.38 g of sodium dihydrogen phosphate will be dissolved in 100 ml of distilled water. 13 ml of Solution A and 87 ml of Solution B will be mixed and made up to 200 ml with distilled water then the pH will be adjusted to 7.6.

b. EDTA (0.01M): 37.224 mg of EDTA will be dissolved in 10 ml of distilled water.

c. Sodium azide: 6.5 mg of sodium azide will be dissolved in 10 ml of distilled water.

d. Glutathione reductase: 31.25 mg of glutathione reductase will be dissolved in 5 ml of distilled water.

e. Glutathione reduced: 30.733 mg of glutathione (reduced) will be dissolved in 10 ml of distilled water.

f. NADPH (0.2 μM): 16.667 mg of NADPH was dissolved in 10 ml of distilled water

2. Procedures:

There will be the preparation of reaction cocktails, the first cocktail will be prepared by pipetting (in milliliters) sodium azide (9.20 ml), glutathione reductase (0.10 ml), and glutathione reduced (0.05 ml) into ß-NADPH. This mixture will be mixed by inversion and it will be adjusted to pH 7.0 at 25°C. From the cocktail and other reagents, there will be sampling (in ml) for the set-up below:

Ingredients	Blank	Sample
Cocktail	3.00 ml	3.00 ml
Sodium phosphate buffer	0.05 ml	0.00
NADPH	0.00	0.05 ml

Each of the test and blank mixtures will be mix by inversion and equilibrate to 25°C; the spectrophotometer will be monitored to absorbance of 340nm until constant after with there will be addition of 0.05 ml H_2O_2 into each of the test tube; then the decrease in absorbance at 340nm for approximately 5 minutes will be recorded to obtain change in absorbance for both the test and blank.

3. CALCULATIONS

$$\text{Glutathione activity (u/ml)} = \frac{(Abs_{340}(\text{sample}) - Abs_{340}(\text{blank}))\ (2)\ (3.1)\ (df)}{(6.22)\ (0.05)}$$

Where;

2 = 2 µmoles of GSH produced per µmole of ß-NADPH oxidized
3.1 = Total volume (in millilitres) of assay
df = Dilution factor
6.22 = Millimolar extinction coefficient of ß-NADPH at 340nm
0.05 = Volume (in millilitres) of NADPH used

APPENDIX I

GLUTATHIONE REDUCTASE ASSAY

The role of glutathione reductase in redox balance for the prevention of oxidative stress involves catalyzing of glutathione into oxidized glutathione which is further reduced into glutathione peroxidase that is a primary antioxidant enzyme; inhibition of glutathione reductase limit actions of glutathione peroxidase in hydrogen peroxide scavenging (Couto *et al*, 2016). This assay according to method of Carlberg and Mannervik (1985), a 3.00 ml reaction mix has a final concentration including 75 mM potassium phosphate, 2.6 mM ethylenediaminetetraacetic acid, 1 mM glutathione, 0.09 mM ß-nicotinamide adenine dinucleotide phosphate, reduced form, 0.13% (w/v) bovine serum albumin, and 0.03 - 0.06 unit of glutathione reductase.

1. REAGENTS:

a. 100 mM Potassium Phosphate Buffer with 3.4 mM Ethylenediaminetetraacetic Acid (EDTA), pH 7.6 at 25°C: Preparation of 200 ml in double distilled water involves using Potassium Phosphate, Monobasic, Anhydrous, and Ethylenediaminetetraacetic Acid, Dipotassium Salt, then follow by adjustment of the pH to 7.6 at 25°C with 1 M KOH.

b. 30 mM Glutathione Substrate Solution (GSSG): Preparation involve 5 ml in distilled water using Glutathione, Oxidized Form, Disodium Salt.

c. 0.8 mM ß-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form Solution (ß-NADPH): Prepare 5 ml in cold reagent A using ß-Nicotinamide Adenine Dinucleotide Phosphate, Tetrasodium Salt.

d. 1.0% (w/v) Bovine Serum Albumin (BSA): Prepare 100 ml in Reagent A using Albumin, Bovine. Note; it must be kept in cold.

e. Glutathione Reductase Enzyme Solution: Immediately before use, there will be the preparation of a solution containing 0.30 - 0.60 unit/ml of Glutathione Reductase in cold BSA.

PROCEDURE: After the preparation of the reagents, there will be pipetting of the reagents as given in the table below (in milliliters) into suitable cuvettes for spectrophotometer readings:

Reagents	**Test**	**Blank**
Double distilled water	0.65	0.65
Reagent A (buffer)	1.50	1.50
Reagent B (GSSG)	0.10	0.10
Reagent C (NADPH)	0.35	0.35
Reagent D (BSA)	0.30	0.40

The set-up above will be mixed by inversion and equilibrate to 25°C then monitor the absorbance until constant at 340nm. Then there will be an addition of 0.1 ml of reagent E to the test cuvette tube. This will be mixed immediately by inversion and record the decrease in the absorbance at 340nm every minute for approximately 5 minutes; to obtain change in absorbance from 340nm for both the Test and Blank.

3. CALCULATIONS

Glutathione reductase activity (u/ml)

Where;

3 = Total volume (in milliliters) of

assay df = Dilution factor

6.22 = Millimolar extinction coefficient of ß-NADPH at 340nm

0.1 = Volume (in milliliters) of enzyme used

APPENDIX J

GENE EXPRESSION STUDIES

SAMPLE COLLECTION AND STORAGE:

200 mg of each tissue sample obtained and stored in RNAlater at -80 ^{O}C; RNAlater is a chemical for maintenance of RNA integrity. 100 – 200 µL of RNAlater is required for each 200 mg sample of tissue, however; only 50 mg of each tissue sample is required for the gene expression studies.

Materials required for weighing and homogenization: beakers, scissors, grinding mortar and pestle, cold nitrogen, aluminum foil for weighing, RNAseZAP for wiping of hands, beakers in between and during weighing and homogenization process; and sensitive analytical balance.

Sample weighing: Wiping and cleaning of the beakers and other materials with RNAseZAP and working alcohol; avoidance of talking and eating, cutting of aluminum foil for weighing; washing of tissues in PBS (cold pH 7.2 – 7.4).

RNA ISOLATION:

Weighing of 30 – 50 mg tissue for RNA isolation using 1 ml Trizol solution; Trizol is Thermo fisher RNA isolation kit. After weighing, if the tissue is not immediately processed for other downward activities; the tissue can be stored between 4 and -20 ^{O}C before RNA isolation process.

After weighing the samples, it then follows crushing of the sample first then the addition of cold liquid nitrogen and lysed properly; 1 mL of Trizol can now be added for homogenization of the sample for further downstream molecular processes. After the homogenization, the samples will be aliquot into Eppendorf tubes then set in cell disruptor for mechanical separation of membranes from the cells.

After cell disruptor process, the sample will be incubated for 5 minutes at room temperature; followed by phase separations into RNA (first layer), Genomic DNA (second layer) and Proteins & other organic compounds (third layer). The separation is to be done by addition of 1 mL chloroform into each of the Trizol homogenized samples followed by vigorous mixing for 15 seconds each. Then each of the samples will be centrifuge at 12000 x g (rcf) for 15 minutes at 4 ^{O}C. After the centrifuge about 500 µL RNA clear aliquot will be obtained into 500 µL isopropanol.

The mixture of RNA and isopropanol will then gently mix for 15 seconds each and incubated for 10 minutes for RNA precipitation; then centrifuge again for 10 minutes at 12000 x g at 4 ^{O}C.After this centrifuge, the RNA pellets will be collected while the isopropanol will be discarded then the pellets will be individually washed with 1 mL ethanol and centrifuge for 5 minutes at 12000 x g at 4 ^{O}C; after the centrifuge, the pellets should be allowed to evaporate.

After evaporation, the RNA pellet will be dissolved by adding 50 µL nuclease-free water; upon addition, the pellets should be allowed to properly dissolved in the nuclease-free water completely then take the solution for RNA quality and quantity evaluation using nanodrop spectrophotometer. Specific attention is to be paid to Abs 260, 230, 280 and their ratios as well as ng/µL of the RNA in the solution.

DNASE TREATMENT

The need to eliminate excess contamination of DNA in the RNA is necessary and it is the reason for DNase treatment; after the successful RNA extraction and quantification; the quantity of RNA determined by the nanodrop is then evaluated to 10000 ng, the volume of the RNA supplying this much is then dissolved into Nuclease free water to give a total volume of 25 µL.

Preparation of master mix: The master mix per reaction of 25 μL above includes the following; RNase inhibitor 0.5 μL per reaction, DNAse buffer 10X = 3 μL per reaction, Turbo DNAse = 1 μL per reaction. The buffer and the Turbo can be calculated for the entire reactions then added for each reaction. After addition of the master mix, the reaction mixtures are gently mixed then incubated at 37 ^{O}C for 30 minutes shake in between 3 times. The incubation is followed by addition of inactivation buffer 3 μL per reaction added individually to each reaction mixture; then incubated for 5 minutes at 37 ^{O}C centrifuges at 10000 rcf at 4 ^{O}C for 1 minute. The supernatant is collected while the pellets are discarded then the treated solution is set for nanodrop evaluation again after which the reaction mixtures can be store at -80 ahead of other downstream processes. **NB**: the quantity ng/μL will reduce from thousands to hundreds while the 260/280 and 230/280 absorbance will be approximately 2.

cDNA SYNTHESIS

Thermo Fisher kit (Revertaid cDNA kit) is used for cDNA synthesis; maximum volume is 20 μL containing a maximum of 5000 ng DNase treated RNA. The processes of cDNA synthesis are as follows in a 0.2 ml Eppendorf tube:

RNA template: maximum volume of 11 μL containing 5000 ng of the DNase treated RNA; the balance volume to the 11 μL is then makeup with Nuclease Free Water (NFW).

Oligo DT: in the kit, 1 μL oligo DT is added to each RNA template prepared above and mix properly by brief centrifuge briefly and incubate at 65 ^{O}C for 5 minutes in PCR thermocycler then chilled on ice for 5 minutes.

Addition of master-mix: a master-mix is prepared and added to each of the RNA templates to make up to maximum of 20 μL. The composition of the master mix is as follows using the kit:

- 5x reaction buffer 4 μL
- RNA inhibition (ribolock) 1 μL
- 10 mm dNTP 2 μL
- Reverse transcriptase enzymes 1 μL

The master-mix can be prepared in bulk for all the reaction and sampled into the RNA template then incubate at 42 ^{o}C for 60 minutes and for 5 minutes at 70 ^{o}C; then the reaction mix can be stored at -20 oC for other downstream processes (qPCR) or sampled for northern blotting gel.

APPENDIX K

TISSUE OXIDATIVE STRESS PROTOCOL

i. Tris-HCl homogenization buffer

   a. 1mM Ethylenediaminetetraacetic acid (EDTA): 18.08mg EDTA (452.2g MW) into 40ml distilled water

   b. 1mM Dithiothreitol (DTT): 1.54mg DDT (154.3g MW) into 10ml distilled water

   c. 50mM Tris HCl: 394mg Tris HCl (157.6) into 50ml distilled water; then make up to 100ml with the EDTA and DDT solutions.

ii. 0.9% NaCl solution; ice-cold for sample collection and washing prior to homogenization.

200 mg of each organ crush under cold liquid nitrogen (~ 100 ml) in ceramic mortar and pestle the homogenized with 4ml of the Tris-HCl homogenization buffer. The homogenized solution then centrifuges at 4000 rpm for 15 minutes then the supernatants are collected; concentration of protein was determined using BCA assay.

Reaction mixture for lipid peroxidation including 100µl of the sample, 200µl of 8.1 % SDS, 1.5ml of 20% acetic acid solution (pH 3.5), 1.5ml of 0.8% TBA solution. The final solution was made to 4 ml with distilled water; then boiled at 95 ^{O}C for 1 hour in boiling water bath. After cooling with tap water, 1.0ml of distilled water and 5.0ml of the mixture of n-butanol and pyridine (15:1; v/v) added and shaken vigorously then centrifuge at 3500 rpm for 15 minutes. Then absorbance of the organic layer measured at 532nm against a blank containing other reagents without the samples.

Total antioxidant capacity of the tissues is determined using ferric reducing the antioxidant potential of the tissue homogenates. 100µl of the homogenate in triplicate from each sample separately; 250µl of phosphate buffer and 250µl of potassium ferricyanide was added. This reaction mixture was incubated for 20 minutes at 50° C after which 250µl of trichloroacetic acid was added. The mixtures were centrifuged at 3000 rpm for 10 minutes each where 250µl of the supernatant solution was obtained mix with 250µl distilled water and 500µl ferric chloride (0.1%) then put up for absorbance reading. Ascorbic acid was used as analytical standard while absorbance measurement at 700 nM for measurement and determination of total antioxidant capacity using FRAP score. Antioxidant enzymes activities including SOD, CAT, and Glutathione reductase were also determined.

APPENDIX L

CARCASS CHARACTERISTICS AND MEAT QUALITY PROTOCOL

The animals were fasted overnight but were provided with water; then randomly selected after which they were sacrificed. The process of slaughtering follows descriptions of Apata *et al* (2012) whereby there was cutting through both jugular vein and carotid arteries below the rabbits' jaws concurrently; after slaughtering the rabbits were bled and hot carcass weight was taken.

Physical characteristics of the meat

Carcass traits:

Traits including live weight bled weight, blood loss (g), dressed weight (%), Chilled carcass weight (%), chilling loss were determined.

Primal cuts

The slaughtered rabbits were cut into retail primal cuts including head, ear, neck, shoulder, forefoot, foreleg, loin, rib, hip, belly, rump, tail, hind leg, shank, and hindfoot; relative weight of these cuts were determined per the animal body weight (Martínez-Bas *et al*, 2018).

Drip loss:

100g meat samples in triplicates from each animal were sampled from the cold carcass and put into a cotton pouch and kept at 4 ^{O}C for 48 hours; the exudates from the meats were discarded while the meats were re-weighed from where the drip losses were calculated (Anjumoni *et al*, 2018).

Cooking loss:

100g meat samples in triplicates from each animal were sampled and roasted in the oven at 190 oC for 25 minutes then cooled to 25 ^{O}C; cooking loss was determined and expressed in percentages (Aaslyng *et al*, 2007).

Chemical and nutritional composition

Lipid peroxidation:

100g meat samples in triplicates from each animal were sampled homogenized then put up for lipid peroxidation assay and total antioxidant capacity.

Nutritional composition:

Proximate composition of the meat and poly-unsaturated fatty-acids profile of the meat was also determined.

APPENDIX M

PROTEIN EXTRACTION

a. **Reagents**

i. Lysis buffer: (7M urea, 2M thiourea, 4% CHAPS, 40Mm Tris, pH 8.5, 0.002% bromophenol blue, 65mM DTT, 1 mM EDTA and 1mM PMSF)

ii. Preparation of the lysis buffer (7M urea (60.06g mw), dissolve 4.204g in 10 mL distilled water; 2M thiourea (76.12g mw), dissolve 1.5224g in 10 mL distilled water; 4% CHAPS (614.88g mw), dissolve 400mg in 10mL distilled water; 40mM Tris (121.14g mw), dissolve 48.456mg in 10mL water and adjust the pH to 8.5; 65mM DTT (154.3g mw), dissolve 10.0295mg in 1mL of distilled water; 1 mM EDTA (452.2g mw), dissolve 4.522 in 10 mL distilled water and 1mM PMSF (174.2g mw), dissolve 1.742g in 10 mL; then add 0.002% bromophenol blue to all the solutions and mix well).

b. **Procedures**

i. Place frozen sample of the animal tissue (150 g) in a cold ceramic mortal with approximately 100 ml liquid nitrogen

ii. Crush the sample under liquid nitrogen to fine powder

iii. Transfer frozen sample into 1.5 ml tube with screw cap.

iv. Add 1.5 ml of lysis buffer (with 1X protease inhibitors); vortex

v. Set on ice for 10 minutes

vi. Centrifuged the homogenate at 16,000g for 20 min at 4 °C and discarded the pellet; then BCA protein quantification then precipitation of supernatant with chloroform/methanol.