Evaluation of a bioflavonoid product supplement as an alternative for synthetic vitamin A and E in broiler diets

by

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Declaration

I, An Marshall declare that the thesis, which I hereby submit for the degree MSc(Agric) Animal Nutrition at the University of Pretoria, is my own work and has not been submitted by me for a degree at this or any other tertiary institution.

Signature: ……

Date: ……

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Abstract

The supplementation of the bioflavonoid product, Biored®, as an alternative for synthetic vitamin A and E in broiler diets

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Biological systems are subject to oxidative stress and free radical attack. Free radicals and reactive oxygen species are formed as by-products of aerobic metabolism and redox reactions that occur naturally in living organisms. When there is an imbalance in their production and elimination they can cause extensive tissue damage. In order to protect itself from damage of ROS and free radicals the body has antioxidant defence systems in place which consists of enzymatic- and non –enzymatic systems. Vitamin A and Vitamin E are two antioxidants included in the diet that form part of these defence system and are well known for their ability to prevent lipid peroxidation in cell membranes of tissue.

Bioflavonoids are polyphenolic compounds found naturally in plants and have been shown to contain strong antioxidant properties and can be added to the diet to help protect the body from oxidative damage. Biored® is a commercial antioxidant product made from bioflavonoids that is added to animal feed to serve this purpose.

The main objective of the study was to determine if Biored® can partially replace the synthetic vitamins A and E included in broiler chicken diets. Broiler growth performance, liver vitamin A and E concentrations, blood oxidation levels, meat quality and carcass traits and shelf life of chicken thigh meat were measured. Replacing half of the vitamin A and E requirement of the chickens with Biored® could also be a cost saving practice as vitamin A and E are expensive to supplement in the diet. According to ADVIT Animal Nutrition Pty Ltd, vitamin E (500 IU) is sold for R82.55/kg and vitamin A (1000 000 IU) sold at R360.85/kg. Currently Biored® is sold at R35.00/kg to feed manufacturers.

Eight different dietary treatments were used that included different Biored® concentrations and either standard levels of synthetic vitamin A and E, only half these levels or no additional vitamin A and E. The dietary treatments were set up as follows, Treatment 1, 100% vitamin A and E, 0 g/ton Biored®; Treatment 2, 0% vitamin A and E, 0 g/ton Biored®; Treatment 3, 0% vitamin A and E, 150 g/ton Biored®; Treatment 4 100% vitamin A and E, 150 g/ton Biored®; Treatment 5, 50% vitamin A and E, 0 g/ton Biored®; Treatment 6, 50% vitamin A and E, 100 g/ton Biored®; Treatment 7, 50% vitamin A and E, 150 g/ton Biored®; Treatment 8, 50% vitamin A and E, 200 g/ton Biored®. In treatment 1 with the 100% vitamin A and E inclusion levels the actual cost of vitamin A in the grower diet for this trial amounts to R1.26 and for vitamin

E, R2.92. Treatment 3 that only contains 53g Biored® and no vitamim A and E the total cost of Biored® amounts to R1.86. In treatment 8 the diet contains 70.7g Biored® at a cost of R2.47, while half of the vitamin A (1.8g) and vitamin E (141.5g) amounted to R0.63 and R1.46 respectively. These calculations show that the inclusion of Biored® could potentially decrease the cost of the diet due to full or partial replacement of vitamin A and E.

The body weight of broilers on day 35 that received half the vitamin A and E specification with different incremental levels of Biored® did not differ significantly from the Positive Control group (100% vitamin A and E and no Biored®) and with increasing level of Biored® in the feed the weights of the groups seemed to increase as well, though the differences were not significant. The cumulative feed intake was improved when diets with 50% of vitamin A and E specification was supplemented with Biored® as well as the average daily gain over the whole 35 day trial period. Biored® did not have a vitamin A and E sparing effect in the liver.

Biored® helped improve oxidative stress in the blood of broilers at the end of the growth trial when added at the highest concentration of 200 g/ton. Carcass or portion weights in birds were not affected by the dietary treatments. Meat quality factors including drip loss, cooking loss and shear force in breast meat portions was also not affected by the inclusion of Biored®. The shelf life of thigh meat portions was also not affected by the addition of Biored® to the feed. The absence of vitamin A and E in the feed caused the most deterioration overall.

More research has to be done to determine the exact mode of action of the flavonoids present in the product and to what extent the compounds are metabolized and absorbed into the system of the birds.

Keywords: oxidation, flavonoids, antioxidant vitamins, chicken

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Chapter 1

Introduction

Oxidative stress in living cells is caused by oxidants which include reactive oxygen and reactive nitrogen species and free radicals (Jensen, 2003). Halliwel (1989) defined free radicals as 'any species capable of independent existence that contains one or more unpaired electrons'. Reactive oxygen species include oxygen centred radicals and non-radicals that are derived from oxygen (Middleton *et al*., 2000). Free radicals are highly reactive species and are small molecules or ions which can induce tissue damage in cells when allowed to accumulate (Jensen, 2003). Free radicals can be of endogenous origin, forming via normal cellular metabolism as by-products of redox reactions or it can come from exogenous sources such as drugs, pollutants, heavy metals, heat and exposure to radiation or to oxidizing substances (Machlin and Bendich, 1987; Kitts, 1997; Wang and Quin, 1999). Biological molecules such as proteins, DNA and lipids are especially susceptible to free radical attack (Machlin and Bendich, 1987; Middleton *et al*., 2000). Reactive oxygen species (ROS) are primarily produced by aerobic cellular metabolism, formed as by- products of reactions (Oliveros *et al*., 2000; Jensen, 2003). Free radicals considered important are hydroxyl ($OH^{\cdot}$), superoxide ($O_2^{\cdot}$), nitric oxide ($NO^{\cdot}$) and peroxyl ($RO_2^{\cdot}$) species. Molecules that are not considered free radicals but which can lead to free-radical reactions include peroxynitrite ($ONOO^-$), hypochlorous acid (HOCL), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and ozone (O_3). ROS include radicals ($OH^{\cdot}$, $O_2^{\cdot}$, $NO^{\cdot}$ and $RO_2^{\cdot}$) as well as non-radicals ($ONOO^-$, HOCL, H_2O_2, $^1O_2$ and O_3) species (Aruoma, 1998). The most reactive radical species is the OH molecule and Jensen (2003) mentioned that more than half of damage caused by free radicals is due to the OH radical. It is involved in reactions like lipid peroxidation, hydroxylation, oxidation of protein thiols and sugars and cleavage of strands of nucleic acids and damage to DNA (Avanzo *et al*., 2001; Jensen, 2003). The other oxidants show more selectivity with regard to the molecules reacted with (Jensen, 2003). ROS and free radical production is a normal part of cellular metabolism and is not a threat to biological molecules as long as they do not overwhelm the anti-oxidative capacity of the cell.

A living organism naturally has systems in place in order to protect itself against tissue damage caused by ROS and free radicals. Antioxidants remove or stabilise the harmful reactive molecules thereby protecting cells and ensuring their structural integrity (Chew, 1996). They can do this either through enzymatic or non-enzymatic pathways (Kitts, 1997; Middleton *et al*., 2000). Mitochondria, for example, are very susceptible to ROS and to protect themselves they have enzymatic (superoxide dismutase, catalase and glutathione peroxidases) as well as non-enzymatic (glutathione redox cycle, α-tocopherol) antioxidant protection (Avanzo *et al*., 2001). The enzymatic system in the body includes super-oxide dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase. Some enzymes target specifically ROS like SOD and catalase while other enzymes reduce thiols (Middleton *et al*., 2000). Non-enzymatic antioxidants consist of vitamins and micronutrients, which are less specific in their radical scavenging ability (Kitts, 1997;

Middleton *et al*., 2000). The hydrophilic non-enzymatic antioxidants include vitamin C (ascorbic acid) and urate while lipid soluble antioxidants include vitamin E (tocopherols), vitamin A (retinoids), carotenoids and ubiquinols (Middleton *et al*., 2000). Lipid peroxidation can be reduced by antioxidants through a mechanism where the antioxidant donates hydrogen to the free radical. Antioxidants can also act directly as free radical scavengers (Kitts, 1997). Vitamin E is one of the most important natural antioxidants and being fat soluble is involved in processes preventing lipid peroxidation. It is an essential nutrient that needs to be supplemented in the diet as broilers do not synthesise the vitamin in their bodies (Fellenberg and Speisky, 2005). The clinical signs of a vitamin E deficiency typically include retarded growth, exudative diathesis, encephalomalacia and several other diseases as well as a high morbidity rate (Avonzo *et al*., 2001; Yuming *et al*. 2001).

Bioflavonoids, polyphenolic compounds found in plants, have been shown, in numerous studies, to contain antioxidative properties. Heim *et al*. (2002) listed the antioxidative functions of flavonoids as follows: ROS scavenging, chain breaking antioxidants, metal chelators and reducing agents, quenchers of the formation of singlet oxygen and providing protection to vitamin C. They can also react with $OH^{\cdot}$ and can function as chain-breaking antioxidants (Heim *et al*., 2002). Flavonoids might be able to have an additive effect on the antioxidant capacity of the body (Nijveldt *et al*., 2001). Biored® (Biorem Biological Products, Oudtshoorn; V20924, Act 36 of 1947) is a feed additive and is made from bioflavonoids consisting of 7.8g/100g flavonoids and pro-anthocyanidins at 39.8 g/100g.

The main objective of the study was to determine if Biored® can be included in broiler diets to partially replace the more expensive synthetic vitamins A and E that are routinely included in these diets. Biored® was included in the broiler diets containing either standard levels (according to levels set by vitamin A and E manufacturers) of synthetic vitamin A and E, only half these levels or no additional vitamin A and E. The efficacy of Biored® as an antioxidant to replace vitamins A and E was evaluated by measuring the broiler growth performance, liver vitamin A and E levels, blood oxidation levels, meat quality and carcass traits and shelf life of the chicken meat.

Because of the similarity between the antioxidant functions of vitamins A and E and that of bioflavonoids found in Biored®, it was hypothesised that bioflavonoids can replace a proportion of vitamin A and E in the diet of broiler chickens. It was expected that the birds that received half of the standard addition of vitamin A and E in combination with Biored® would have performed similar in terms of above mentioned parameters than the birds that received standard levels of vitamin A and E.

This can possibly save on feed costs as vitamin A and E are expensive to supplement in the diet. According to ADVIT Animal Nutrition Pty Ltd, vitamin E (500 IU) is sold for R82.55/kg and vitamin A (1000 000 IU) sold at R360.85/kg. Currently Biored® is sold at R35.00/kg to feed manufacturers.

Chapter 2

Literature Review

2.1 Vitamin E

Vitamin E has been identified as the most important lipid soluble, chain breaking antioxidant present in the body and can be found in plasma, cells and red cells (Brigelius-Fohle and Traber, 1999; Ibrahim *et al*., 1999; Frank, 2005). Its major role according to Wang and Quinn (1999) is to protect cells and specifically unsaturated lipids from free radical damage through scavenging peroxyl radicals before they can damage their target lipids. Vitamin E is also involved in the expression of several genes that cause apoptosis, cell growth and metabolism (Alpsoy and Yalvac, 2011). Prevention of oxidation of lipids is a possible mechanism through which vitamin E can enhance the immune response (Chew, 1996). Oxidation of lipids can be responsible for an increased susceptibility to infectious diseases and also for impaired physiological function like growth and reproduction (Gladine *et al*., 2007). Due to its lipophilic nature vitamin E is partitioned into the lipid components of tissue or in molecular structures like lipoproteins (Wang and Quinn, 1999). It is mainly located in membranes like those of the mitochondria, the nucleus and microsomes where they prevent lipid peroxidation (Fukuzawa *et al*., 1980). α-Tocopherol is situated near the surface of cell membranes and as it goes deeper into the cell membrane it tends to lose its efficiency to scavenge radicals (Niki *et al*., 1995). Lipid peroxidation poses a real threat to cell membranes due to their high concentration of polyunsaturated fatty acids and their close proximity to oxygen, transition metals and peroxidases (Oliveros *et al*., 2000). This highlights the fact that the location of radical formation and the location of the antioxidants are important as well as its mobility (Niki *et al*., 1995). Figure 2.1 shows the reaction of lipids with free radicals and indicates the radical mediated chain reaction that occurs. The main role of vitamin E is to react with the lipid peroxyl radical before it attacks the lipid membrane (Wang and Quinn, 1999). Antioxidants can either act as hydrogen donors or as free-radical scavenging molecules in order to reduce the effect of lipid peroxidation by free-radicals (Kitts, 1997). Vitamin E is regarded as a scavenger of active oxygen radicals and singlet oxygen but it also has a secondary role of stabilising the cell membrane by binding to destabilising molecules inside the membrane (Oliveros *et al*., 2000). According to Kitts (1997), there is substantial evidence indicating that α-tocopherol is the best antioxidant for reducing lipid oxidation. It reacts with peroxyl radicals, thereby breaking the free-radical chain reaction, and forms harmless pheroxyl radicals and hydroperoxides. The tocopheroxyl radicals can be converted to tocopherol by reacting with ascorbate, reduced glutathione, urate and ubiquinol (Alpsoy and Yalvac, 2011).

Apart from its major role as an antioxidant, vitamin E also has other important functions. Wang and Quinn (1999) summarised a few possible functions based on studies done on the deficiency symptoms of vitamin E. These functions include a role in the regulation of haem synthesis, indirect involvement in regulation of haem-containing protein activity, a modulatory role in the immune response and in gene regulation, protection of selenium containing proteins and playing a part in the electron transport chain.

Rietjens *et al*. (2002) mentioned that vitamin E is also capable of having pro-oxidant effects where the accumulation of α-tocopherol radicals is able to induce oxidative stress. When the vitamin reacts with a free radical it becomes a radical itself. Other antioxidants, such as carotenoids, are needed to reduce this newly formed radical (Koivula and Eeva, 2010).

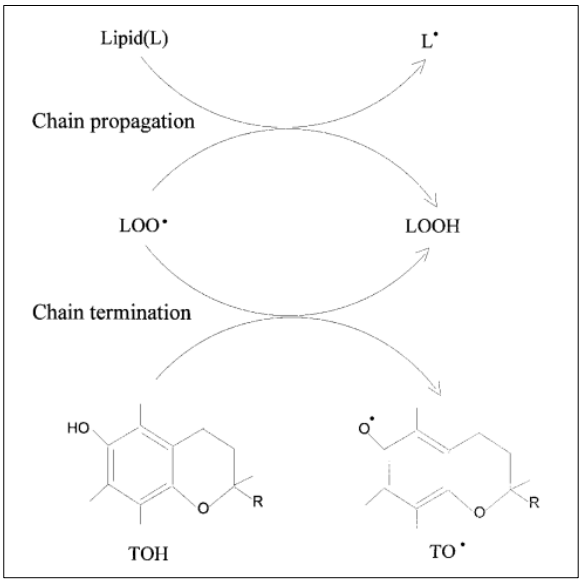


Figure 2.1 Reaction of a radical species with alpha tocopherol (TOH). $LOO^{\cdot}$ =lipid peroxyl radical, $L^{\cdot}$ = carbon-centered lipid radical. $LOO^{\cdot}$ can react with oxygen to produce other lipid peroxyl radicals, lipid hydroperoxide (LOOH), α-tocopheroxyloxyl radical ($TO^{\cdot}$**)** (Wang and Quinn, 1999)

2.1.1 Chemical structure

Vitamin E refers to a group of biological active compounds called the tocopherols and tocotrienols and their derivatives (Bast and Haenen, 2002). There are 8 isomeric molecules that consist of four tocopherols (α, β, γ, and δ) and four tocotrienols (α, β, γ, and δ) (Brigelius-Fohle and Traber, 1999; Wang and Quinn, 1999). R,R,R,-α-Tocopherol is the most abundant and most biologically active compound of them all (Figure 2.2, Brigelius-Fohle and Traber, 1999), followed by β -tocopherol, γ -tocopherol and δ –tocopherol (Bramley *et al*., 2000).

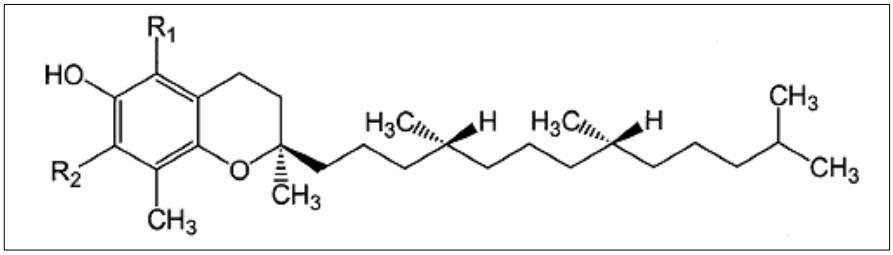


Figure 2.2 Chemical structure of R,R,R-tocopherols and in α-tocopherol. R1 and R2 are methyl groups (CH_3). (Bast and Haenen, 2002)

The vitamin has two distinctive parts which include a chroman head and a phytyl chain and an important characteristic of the molecule is the three asymmetrical carbon atoms on the phytyl tail. The chroman ring

with its OH- group is the structure that gives the vitamin its antioxidant activity as the hydrogen is donated to a free radical that can stabilise it (Bramley *et al*., 2000; Bast and Haenen, 2002). According to Traber and Atkinson (2007), α-tocopherol has superior antioxidative abilities due to its fully methylated chroman ring which prevents the formation of adducts. The ring faces the cytosol while the phytyl side chain is imbedded in between the fatty acids of the phospholipids in the cell membrane (Bast and Haenen, 2002).

2.1.2 Metabolism

Vitamin E is absorbed in the small intestine and transported to the liver (Frank, 2005). The absorption of vitamin E is dependent on the mechanisms used for lipid absorption. Vitamin E is transported in chylomicrons via the lymphatic circulation from the gastrointestinal tract after which it is secreted into the circulatory system. The chylomicrons in the circulatory system are then catabolised by lipoprotein lipase (LPL) to form chylomicron remnants. Some components that are excessive after catabolism of chylomicrons are transferred to high density lipoproteins (HDL). LPL can also function as a transfer protein that transports vitamin E to tissue in the body like skin, muscle and adipose tissue. LPL producing tissue like adipocytes might receive the vitamin E supply primarily by this route. Most of the tocopherol that is absorbed though is transported to the liver via the chylomicron remnants where the liver then controls the release of tocopherol into the circulation. The chylomicron remnants in the liver are then packaged into very low density lipoproteins (VLDL's) and again secreted into the circulatory system. VLDL has a very high concentration of α-tocopherol due to the α-tocopherol transfer protein (TTP) which shows preference for the binding of this homologue of vitamin E (Bramley *et al*., 2000; Frank, 2005). The VLDL in the circulatory system then gets catabolised by LPL, which produces VLDL remnants that are responsible for the transport of tocopherol to all the other lipoproteins (HDL, LDL or IDL). The lipoprotein that carries the most of the vitamin E is the LDL (Wang and Quinn, 1999). Vitamin E is transported to target tissue through various mechanisms including special receptors for LDL, HDL and IDL.

Vitamin E concentration varies from tissue to tissue. Mitochondria and microsomes have been found to contain high concentrations of α-tocopherol whereas the cytosol of cells has low concentrations, which reflects the importance for vitamin E as a radical scavenger during aerobic metabolism. The main route of excretion of tocopherols is the faeces with the urinary pathway being a minor route (Bramley *et al*., 2000).

2.2 Vitamin A

Vitamin A is a fat soluble vitamin and is found in three active forms in the body which is retinol (Figure 2.3), retinal and retinoic acid (Alpsoy and Yalvac, 2011; Mullin, 2011). These compounds have various important functions including the maintenance of epithelial tissue and its important role in the eye and vision as well as in reproduction, growth, the immune response, haematopoiesis and embryonic development (Oliveros *et al*., 2000; Alpsoy and Yalvac, 2011). It is also an important factor in the prevention of heart disease in humans (Palace *et al*., 1999; Oliveros *et al*., 2000). Many studies have demonstrated vitamin A's antioxidative properties, which are explained below. Vitamin A is not synthesised within the body and has to be obtained from the diet. Some carotenoids, especially β-carotene, are molecules present in plants which can be converted to vitamin A in the body (Alpsoy and Yalvac, 2011). Carotenoids are structurally similar to vitamin A and 50 carotenoids in a group of almost 600 compounds have pro-vitamin A activity (Palace *et al*., 1999). Food sources rich in vitamin A and carotenoids include animal products such as dairy products, organ meat, eggs and yellow and green vegetables and fruits (Palace *et al*., 1999; Alpsoy and Yalvac, 2011).

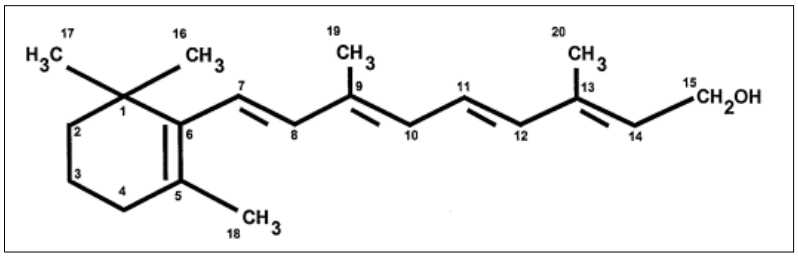


Figure 2.3 Structure of retinol (Palace *et al*., 1999)

2.2.1 Function as an antioxidant

The antioxidant properties of vitamin A can be ascribed to the polyene units (one or more sequences of alternating double and single carbon-carbon bonds) of the molecule. These units react with singlet oxygen, peroxyl radicals and thiyl radicals. The length of the polyene chain will determine the molecules' capacity to stabilise peroxyl radicals (Palace *et al*., 1999). Tesoriere *et al*. (1997) on the other hand, stated that retinol acts by a radical reacting with the cyclohexenyl ring. Vitamin A can function as a chain breaking antioxidant when it reacts with peroxyl radicals and thereby prevent lipid peroxidation.

Vitamin A can be found in the lipid membrane of cells and like vitamin E the cyclic carbon ring is positioned to the outer surface of the membrane in the more polar region and the polyene chain is imbedded in the inner, non-polar region. The antioxidative capacity of retinol and its ability to stabilise aqueous radicals are not as good as vitamin E due to the absence of a chroman ring and its related hydroxyl substituent but it is an effective scavenger of lipid peroxyl originating within the lipid bilayer of the cell membrane (Palace *et al*., 1999). Vitamin E has been shown to protect vitamin A from oxidation in the feed as well as in the body of poultry (Bieri, 1960). The antioxidant capacity of the different vitamin A molecules are as follows: retinol ≥ retinal >> retinyl palmitate > retinoic acid. An increase in lipid peroxidation can be expected in microsome membranes of organs like the liver, kidney, spleen and brain when there is a vitamin A deficiency as well as

a decline in essential fatty acids (Oliveros *et al*., 2000). Oliveros *et al*. (2000) also found that the enzymatic and non-enzymatic antioxidant defence mechanisms in the heart were altered when mice were fed a vitamin A deficient diet.

On the other hand, Tesoriere *et al.* (1995) demonstrated that all-*trans* retinol alone is not an effective antioxidant supressing lipid peroxidation in a unilamellar liposomal system from egg or soybeans. It was shown that all- *trans* retinol was consumed rapidly during peroxyl radical chain propagation and will only be effective when high concentrations of retinol is present in the system. The study did show that retinol together with α-tocopherol inhibited lipid peroxidation more effectively than any of the two vitamins separately suggesting a synergistic interaction.

2.2.2 Metabolism

As mentioned previously, β-carotene can serve as a pro-vitamin A molecule. When β-carotene is converted to retinol, two reactions are required which occurs in the intestinal mucosa of the small intestines. First of all, the enzyme β-carotene-15, 15'-dioxygenase converts β-carotene to retinal and then the enzyme retinal reductase converts retinal to retinol (Johannsen *et al*., 1998). The retinol is transported as retinyl esters in the chylomicrons (Sklan, 1983).

Some carotenoids can be absorbed by the brush border without being hydrolysed. These carotenoids form part of chylomicrons and are transported in the plasma as a part of lipoprotein particles. In cells outside the liver, the chylomicrons are then partially degraded by lipase enzymes that form retinyl esters. The retinyl esters are then transported to and taken up by the liver and cleaved by enzymes to produce vitamin A (Palace *et al*., 1999). The liver is the organ that stores the majority of vitamin A and is responsible for its regulation (Sklan, 1983). Retinyl esters found in the diet are hydrolysed to retinol in the gastrointestinal lumen and absorbed by the enterocytes. The retinol is also incorporated into chylomicrons. In the general circulation chylomicrons are broken down to chylomicron remnants which contain the absorbed retinol. These remnants are taken up by the liver but can also be absorbed by other cells including blood, bone marrow and adipose and spleen cells. In the liver retinol binds to retinol-binding protein and is stored as retinyl esters in lipid droplets in liver stellate cells (Norum and Blomhoff, 1992). The vitamin A can then be stored or secreted. Not all the β-carotene is converted to vitamin A and it has been reported that less than 50% of the activity of vitamin A is contributed by β-carotene. Vitamin A is transported in the circulatory system bound to a complex consisting of retinol binding protein (RBP) and transthyretin (TTR). An intra-cellular retinol binding protein (CRBP) is responsible for the uptake of retinol into the cell. Retinol can also form part of the cell membrane. The storage form of vitamin A in most cells is retinyl esters (Palace *et al*., 1999).

2.3 Bioflavonoids

Bioflavonoids, also known as flavonoids, are low molecular weight compounds found naturally in vascular plants. They can occur in most parts of the plant but especially in the photosynthetic cells (Yao *et al*., 2004). They have a wide variety of important functions acting as antioxidants, enzyme inhibitors, precursors of toxic substances, light screens against UV radiation and are involved in energy transfer (Russo *et al*., 2000; Yao *et al*., 2004). Other functions include giving colour, taste and texture to food, acting as a repellent for foraging animals as they are distasteful and they can attract or repel insects from plants (Formica and Regelson, 1995). More than 4000 of these compounds have been discovered in plants (Cao *et al*., 1997). The flavonoids are best known for their ability to act as antioxidants but they also have numerous other beneficial effects (Nijveldt *et al*., 2001). These include 1) anti-inflammatory effects through their ability to affect eicosanoid biosynthesis and their action on multiple pathways of the inflammation process 2) antithrombotic effects by preventing the aggregation of blood platelets 3) antihypertensive and antiarrhythmic effects by enhancing the relaxation of cardiovascular smooth muscles 4) antitumoral effects 5) hepatoprotective effects 6) antiviral effects (Formica and Regelson, 1995; Cao *et al*., 1997; Middleton *et al*., 2000; Majumdar and Srirangam, 2010). In their capacity to act as antioxidants they also can have a sparing effect on other antioxidants and can exert an effect in the digestive tract by protecting molecules during digestion from oxidative damage as well as protecting the intestinal epithelium (Goñi *et al*., 2007).

2.3.1 Chemical structure

Flavonoids can be described as polyphenols (Russo *et al*., 2000). They are benzo-γ-pyrone derivatives that are made up of phenolic and pyrane rings. All of the flavonoids have the same basic three ring nucleus structure as shown in Figure 2.4 (Middleton *et al*., 2000). It consists of two benzene rings, A and B, and a heterocyclic pyran/pyrone ring, C. The OH groups and sugars attached to the flavonoids give them hydrophilic properties while the methyl- and isopentyl groups give them lipophilic properties (Majumdar and Srirangam, 2010). The tocopherol molecule and flavonoids have a chroman ring in common. Flavonoids in plants are synthesised from the aromatic amino acids, phenylalamine and tyrosine, and acetate units (Middleton *et al*., 2000).

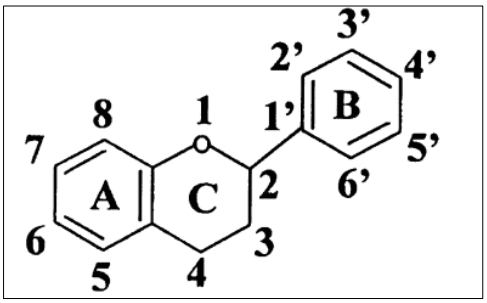
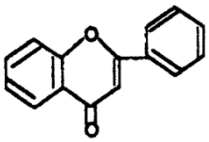
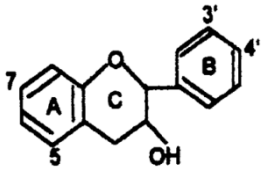
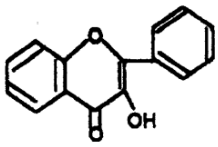
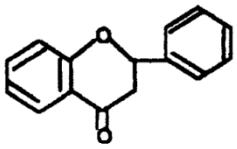
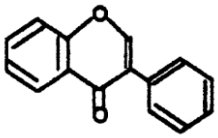
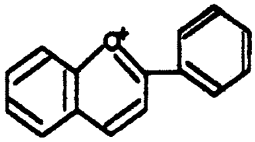


Figure 2.4: Nuclear structure of flavonoids (Heim *et al*., 2002)

There are a wide variety of flavonoids, differing in the arrangement of the hydroxyl (OH) group, their degree of hydroxylation, methoxy (O-CH3), and glycosidic (covalent bond that binds a carbohydrate molecule to another group) side groups and in the conjugation between rings A and B (Heim *et al*., 2002; Yao *et al*., 2004). Flavonoids are classed according to the substitutions present on the nuclear structure and can be classified into at least 10 groups. According to Yao *et al*. (2004), flavanones, flavones, iso-flavonoids, flavans (flavanols), anthocyanins, and flavonols are common in the diet of humans. Nijveldt *et al*. (2001) classified flavonoids into 4 main groups which include flavones, flavanones, catechins and anthocyanins, while Heim *et al*. (2002) divided the groups up into flavanol, flavone, flavonol, flavanone, isoflavone and anthocyanidin. Table 2.1 shows groups of flavonoids, their compounds, structure and the dietary sources in which it is found (Heim *et al*., 2002).

Table 2.1 Flavonoids and their general structure (Heim *et al*. 2002)

Main group of flavonoid	**General structure**	**Compound**
Flavones		Apigenin Luteolin Luteolin glycosides Rutin Chrysin
Flavanol		(+)-catechin (-)-epicatechin Epigallocatechin gallate
Flavonol		Kaempferol Quecertin Myricetin Tamarixetin
Flavanone (dihydroflavone)		Naringin Naringenin Taxifolin Eriodictyol Hesperidin
Isoflavone		Genistin Genistein Daidzin Daidzein
Anthocyanidin		Apigenidin Cyanidin

2.3.2 Antioxidant activity of bioflavonoids

The best known characteristic of almost every group of flavonoids is their antioxidant capacity (Nijveldt *et al*., 2001). Majumdar and Srirangam (2010) described the different mechanisms by which flavonoids exert their antioxidative effect as follows:

1) Flavonoids can directly scavenge free radicals due to the presence of hydroxyl groups on the nuclear structure. This group enables them to reduce oxidising free radicals like superoxide, peroxyl and hydroxyl species and make them less reactive by donating a hydrogen and electron to the radical (Heim *et al*., 2002).

2) Flavonoids can inhibit nitric oxide (NO) production. Endothelial cells and macrophages can produce NO which is important for the dilation of blood vessels. There is however an enzyme called inducible nitric oxide synthase (iNOS) that cause the production of higher concentrations of NO when oxidative damage occurs. The excess NO can react with free radical species to produce peroxynitrite that is highly reactive and can cause extensive tissue damage. Flavonoids can inhibit iNOS, decreasing the amount of NO produced and consequently peroxynitrite production.

3) Flavonoids can inhibit enzymes that are involved in the production of superoxide anions (xanthine oxidase and protein kinase C) or that are involved in ROS production (cyclooxygenase, lipoxygenase and NADH oxidase).

4) Flavonoids can chelate trace elements that can be involved in ROS generation like free iron and copper.

Majumdar and Srirangam (2010) mentioned some flavonoids that can potentially act as antioxidants and they include quercetin, apigenin, hesperidin, hesperetin, luteolin, epigallocatechin gallate, epicatechin gallate, rutin, cyanidin, naringenin, myricetin, chrysin, eriodictyol and kaempferol. Quercetin is the most studied as well as the most abundant flavonoid.

According to Rietjens *et al*. (2002) the effective antioxidant activity of flavonoids are due to the presence of a 3, 4-dihydroxy (catechol) part which strongly inhibits lipid peroxidation activity (Heim *et al*., 2002), the keto group on C4 (C=O), a 3-hydroxyl (OH) moiety and the double bond between C2 and C3 which describes the chemical structure of quercetin. This compound is also known for its iron-chelating ability (Nijveldt *et al*., 2001).

Russo *et al*. (2000) conducted an experiment to determine the free radical scavenging abilities of three flavonoids, rutin, catechin and naringin. The flavonoids showed a dose-dependent scavenging effect, they inhibited xanthine oxidase activity; they protected lipids from peroxidation as well as protection of DNA from cleavage by the OH free radicals. The antioxidative capacity of the molecules were attributed to 1) the catechol (3,4-dihydroxy) structure of ring B; 2) the C2 and C3 double bond and the 4-oxo function as well as 3) the presence of 5,7-hydroxyl groups. The flavonoid, rutin, has all of these structures and showed the highest scavenging ability.

Some flavonoids can prevent lipid peroxidation that occurs enzymatically or non-enzymatically. Lipid peroxidation occurs in three steps 1) initiation 2) propagation and 3) termination.

The three steps as shown by Middleton *et al*., (2000) occur as follows:

1) Initiation: $LH + {}^{\cdot}OH \rightarrow H_2O + L^{\cdot}$
2) Propagation: $L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$
   $LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot}$
3) Termination: $LOO^{\cdot} + LOO^{\cdot} \rightarrow$ Inert product
   $L^{\cdot} + L^{\cdot} \rightarrow$ Inert product
   $LOO^{\cdot} + L^{\cdot} \rightarrow$ Inert product

where LH is a lipid, $L^{\cdot}$ is a lipid radical, $LOO^{\cdot}$ is a lipid peroxyl radical.

The initiation step of lipid peroxidation can be prevented by free radical scavenging of the hydroxyl moiety and the chain reaction of step two can be prevented by peroxyl scavengers. The activity of flavonoids (FL) can be described by the following reaction:

$LOO^{\cdot} + FL\text{-}OH \rightarrow LOOH + FL\text{-}O^{\cdot}$

Flavonoids are proposed to react with the lipid peroxyl radicals and through this mechanism can prevent lipid peroxidation (Middleton *et al*., 2000). They either bind to the lipid membrane at the lipid-water interface or it penetrates the membrane to enter the hydrophobic core (Oteiza *et al*., 2005).

Raj Narayana *et al*. (2000) mentioned that stabilisation of meat lipids occurred with the addition of flavonoids (morin, myricetin, kaempferol and quercetin) at 200 mg/kg.

2.3.3 Metabolism

All the polyphenols are not absorbed and metabolised with the same efficiency and most of these compounds are metabolised by intestinal and hepatic enzymes and microflora in the gut before it reaches its target site of action (Manach *et al*., 2004). According to Nijveldt *et al*. (2001) the information on the absorption, the metabolism and excretion of bioflavonoids in humans are scarce as well as contradictory. In order to determine the biological activity of flavonoids it is important to know the intake, absorption, metabolism and excretion of these compounds and up to date this has been established for only a few compounds (Kay, 2010).

Several factors can influence the absorption of flavonoids like the dosage, form in which it is administered, diet, sex differences and the microbial population that is present in the colon (Heim *et al*., 2002). The size of flavonoid molecules as well as the hydrophobic properties of glucosides prevents them to be absorbed in the small intestine of the animal and the β-glucoside flavonoids can resist degradation of certain enzymes in the small intestine (Formica and Regelson, 1995). Micro-organisms in the gut play a role in the degradation of the polyphenolic substances. Flavonoids can undergo ring scission, demethylation and dehydroxylation, and bacteria can also produce glycosidase that can cleave sugar moieties on flavonoid glyxosides (Middleton *et al*., 2000). The form in which the flavonoid is present (glycosylated or aglycone form) affects the rate of absorption (Nijveldt *et al*., 2001). Flavonoids are also extensively metabolised in organs like the liver where they undergo conjugation reactions by adding sulphur or methyl groups that extend their retention within the

body and it can modify their activities (Nijveldt *et al*., 2001; Pavlica and Gebhart, 2010). Metabolites formed from flavonoids may contribute to the bioactivity and radical scavenging ability but are for the most part unknown (Kay, 2010). There is a lack of information regarding the exact absorption and metabolism of flavonoids and it depends on variables such as the type of flavonoid (Heim *et al*., 2002). Goñi *et al*. (2007) mentioned how polyphenolic extracts from grape and red wine can cause changes in intestinal microflora (decreasing the number of Propionibacteria, Bacteroides and Clostridia and increasing Lactobacilli and Bifidobacteria organisms) and proposed it as an alternative method to antimicrobial growth promoters which are banned in the EU.

Kay (2010) suggested areas of further research involving bioflavonoids and these include determining effects of processing, the bioavailability and metabolism of flavonoids, the development of physiologically applicable *in vitro* models and development of standard methods of analyses for flavonoids as well as appropriate clinical biomarkers.

2.3.4 Toxicity of flavonoids

It has been shown that flavonoids can have a pro-oxidant effect where they are responsible for the formation of free radicals. Flavonoids that contain a phenol ring on the B-ring like apogenin or naringenin can react with peroxidases and form phenoxyl radicals that are cytotoxic (Rietjens *et al*., 2002; Galati and O'Brien, 2004). The catechol group in flavonoids can form electrophilic quinone or quinone methide intermediates which can exert radical activity like binding to DNA (Galati and O'Brien, 2004). Cao *et al*. (1997) illustrated that the same flavonoid can act either as an antioxidant or a pro-oxidant depending on the flavonoid concentration and the radical interacting with it. Flavonoids can also act as pro-oxidants when reacting with redox active metal elements like Cu (Cao *et al*., 1997). According to Galati and O'Brien (2004) though these pro-oxidant effects was induced due to 'mega doses' of flavonoids each day and they suggested that further research needs to be conducted if flavonoids are to be used as therapeutic substances.

2.4 Vitamin A, vitamin E and bioflavonoids with regard to broiler production

Fat is normally included in broiler diets as a source of energy due to its high energy density. It is often supplemented in the form of vegetable oil which contains a high proportion of polyunsaturated fatty acids (PUFA) that are highly susceptible to oxidation (Tavárez *et al*., 2011). Animal diets are increasingly being supplemented with PUFA due to the desire to improve the fat quality of animal products (Gladine *et al*., 2007). Meat from poultry naturally contains higher levels PUFA compared to other meat types which makes it more susceptible to oxidative damage and quality deterioration during storage or cooking (Barroeta, 2007; Goñi *et al*., 2007; Aziza *et al*., 2010). Through the dietary manipulation of fats one can increase the PUFA levels in poultry muscle membrane (Goni *et al*., 2007). In the study done by Betti *et al*. (2009) it was showed that with prolonged feeding with flaxseed oil (containing high levels of n-3 PUFA) broiler breast and thigh meat became more susceptible to oxidative damage and meat quality factors were also affected.

Poultry meat contains low levels of natural antioxidants but also low levels of total fat compared to other animal species (Barroeta, 2007; Aziza *et al*., 2010). According to Barroeta (2007), the average fat content in poultry meat varies between edible portions ranging from 2.8 g/100g in breast muscle to 13 g/100g in thigh muscle with skin. In table 2.2 below the fatty acid content of chicken meat is shown.

Table 2.2 Major fatty acids present in chicken meat (g/100g total fatty acids) (Woods and Fearon, 2009)

Fatty acid	**16:0**	**18:0**	**Total trans**	**Total trans MUFA**	**Total trans PUFA**	**18:1 n-9**	**18:2 n-6**	**18:3 n-3**	**20:4 n-6**	**20:5 n-3**	**22:5 n-3**	**22:6 n-3**	**n-6: n-3**
Dark meat	20.4	6.0	0.8	0.8	Trace	42.7	16.6	2.6	0.4	ND	0.4	0.4	5.0
Light meat	18.9	6.0	0.9	0.9	Trace	36.1	13.7	1.7	0.8	Trace	0.8	0.8	4.4

Trace = Trace amounts which were undetected ND= not detected
MUFA = Mono unsaturated fatty acids
PUFA = Poly unsaturated fatty acids

Post-slaughter oxidation of lipids is affected by a number of factors including species, diet, phospholipid content and composition, anatomical location, sex and age. During processing it is further affected by factors such as composition of muscle, heating, chopping and deboning and exogenous compounds added like salt and nitrites (Kanner, 1994). Rancidity (oxidative off-flavour) of meat starts post-slaughter and continues in stored meat until the intensity becomes undesirable to consumers (Gray *et al*., 1996). The rancid flavour and off-odour is attributed to compounds that are the end-products of oxidation (Gobert *et al*., 2010). Compounds like aldehydes, ketones, alcohols and lactones which are responsible for undesirable odours and flavours in stored meat are formed from hydroperoxides which are formed during lipid peroxidation in meat (Gray *et al*., 1996; Ruiz *et al*., 2001). The muscle membrane phospholipids are more susceptible to oxidation due to their higher level of unsaturation compared to triacylglycerols which are termed the neutral lipids (Kanner *et al*., 1994; Kathirvel and Richards, 2009). Oxidation leads to a decrease in the nutritive value as well as deterioration of meat colour, texture and flavour (Mielnik *et al*., 2006). Lipid oxidation occurs spontaneously after slaughter and factors like lipid composition, iron, heating, disruption of membranes and storage over a long period of time can enhance this effect (Tang *et al*., 2001; Aziza *et al*., 2010).

Lipid oxidation can be prevented by making use of antioxidants and vacuum packaging which serves to limit oxygen exposure of meat products (Mielnik *et al*., 2006). Antioxidants can be added to meat during processing, cooking or packaging as an alternative to addition to the diet (Tang *et al*., 2001).

A study done by Tavárez *et al*. (2011) determined the effect of oil quality (fresh and oxidised soybean oil) and antioxidants in the diet on broiler meat quality, lipid oxidation and shelf life of meat. It was indicated that neither antioxidants nor oil quality had an effect on meat quality like drip loss, shear force or cooking loss in breast meat. These results are similar to other studies conducted. Antioxidant inclusion in the diet had

a sparing effect on vitamin A and E in the liver and serum but oxidised oil caused a reduction in the serum vitamin A and E levels. There was an interaction between antioxidant inclusion and oil quality and days of storage on the shelf life of meat. Antioxidants decreased lipid oxidation during storage even when oxidised oil was fed (Tavárez *et al*., 2011).

Oxidative damage post slaughter can also affect meat quality factors like meat colour. The colour of fresh meat is one of the most important criteria that will determine consumer acceptance of the product especially in beef. When the oxyhemoglobin (distinctive cherry red colour) in muscle is oxidised it produces methemoglobin which gives meat especially beef a brown colour (Kannar, 1994).

2.4.1 Vitamin E

Flachowsky *et al.* (2001) gave several reasons why supplementation of vitamin E to the diet of animals are beneficial and they include better performance of animals, improvement in health, improvement of antioxidant content of animal products, better quality animal products and higher concentrations of vitamin E in animal products is beneficial to human health. A vitamin E deficiency in chickens can manifest itself in a number of symptoms and diseases like exudative diathesis, nutritional muscle dystrophy, encephalomalacia, poor reproductive performance in broilers and depressed growth (Guo *et al*., 2001). It has been proven by numerous studies that vitamin E supplementation can stabilise lipid peroxidation as well as preserve colour in meat and prolong its shelf life (Lin *et al*., 1989; Kanner, 1994). It is a powerful antioxidant that prevents oxidation of phospholipids and cholesterol and dietary supplementation of vitamin E elevates its concentration in cell membranes, especially of the microsomes and mitochondria. Higher than normally recommended supplementation levels (supra-nutritional dietary supplementation) was proven to be advantageous for lipid stability in many experiments for beef, poultry, pigs and calves. It has been shown that the addition of 200 mg/kg α-tocopherol acetate to the diet of pigs reduced lipid oxidation and drip loss. Gray *et al*. (1996) reported that the dietary inclusion of 500 mg/kg of α-tocopherol acetate to pig diets also prevented PSE and improved water holding capacity of meat. Lauridsen *et al*. (1997) fed broiler chickens a more saturated fat (tallow) and an unsaturated fat (olive oil) and supplemented the diet with different levels of vitamin E (20 *vs*. 200 mg/kg feed). It was reported that with supplementation of vitamin E the concentration of α-tocopherol in mitochondria and microsomes increased. Broilers fed a diet high in olive oil and supplemented with 200 mg/kg vitamin E in feed showed a significant increase in the lipid stability of the membrane fractions when compared with a group where the diet was supplemented with 20 mg/kg vitamin E in feed. In conclusion it was found that muscle α-tocopherol levels are the more important factor with regard to oxidative stability in the membranes of muscles compared to the fatty acid composition (Lauridsen *et al*., 1997).

The requirement of vitamin E is determined by the quality, quantity and composition of fat that is present in the diet and is reported to linearly increase with PUFA inclusion, because an increase in PUFA content of muscle causes a decrease in α-tocopherol content (Rebolé *et al*., 2006; Barroeta, 2007). The α-tocopherol

content of muscle membrane phospholipids determine its oxidative stability and this can be manipulated via dietary supplementation of α-tocopherol acetate (Goni *et al*., 2007). Rebolé *et al*. (2006) has showed that dietary inclusion of vitamin E well above the recommended level (200 mg/kg instead of recommended 15-20 mg/kg) increased the body weight gain and feed efficiency of broilers.

Aziza *et al.* (2011) supplemented a broiler diet with camelina meal. Camelina meal is a good source of n-3 fatty acids, tocopherols and phenolic compounds like flavonoids. The authors reported that supplementation with camelina meal increased the γ-tocopherol concentration of thigh meat but not of the breast meat.

In the study done by Lin *et al*. (1989) the darker meat of poultry stored on average 50% more α-tocopherol than white meat. This can be attributed to the difference in physiology between the muscles, dark meat having higher lipid content and also a more developed vascular system. Rebolé *et al*. (2006) showed that the oxidative stability of dark meat in poultry was lower than white meat and attributed this to the higher absolute content of PUFA. Total fat content in dark meat is 3.5 times higher than in white meat and consequently makes it more susceptible to oxidative damage even though it has higher α-tocopherol content. The more developed muscular system in dark meat also could explain its higher susceptibility to oxidation due to higher number of pro-oxidative molecules coming from myoglobin and iron-containing proteins (Rebolé *et al*., 2006).

Voljc *et al*. (2011) aimed to measure the effectiveness of different α-tocopherol concentrations and different isomers in preventing oxidative stress in broiler meat with dietary induced stress. They determined *in vivo* lymphocyte DNA damage and it was shown that linseed oil induced higher levels of damage in lymphocyte DNA due to the higher levels of PUFA. Increased supplementation of all-*rac*-α-tocopherol has significantly increased the antioxidative protection of lymphocyte DNA. *In vivo* oxidative stress was also measured in plasma and liver of broilers by determining malondialdehyde (MDA) levels in tissue samples. MDA is the final product produced in the lipid peroxidation reaction. It was shown that the supplementation of vitamin E at 85 or 200 IU was insufficient to prevent all oxidative damage of the birds *in vivo*. Vitamin E supplementation did manage to significantly lower MDA levels in breast meat which was explained by the fact that less fat was deposited in breast muscle than liver tissue (Voljc *et al*., 2011).

Oxidative stress was induced in broilers by Gao *et al*. (2010) by administering a synthetic glucocorticoid (dexamethasone) to determine the effect that dietary supplementation of α-tocopherol acetate would have on certain parameters. These parameters include the post-mortem change in fatty acid profiles of muscles as well as the redox status of skeletal muscles. They showed that administration of the glucocorticoid significantly impaired growth of the broilers and increased the saturation level of muscle fatty acids. The supplementation of α-tocopherol acetate increased the storage of α-tocopherol in skeletal muscle and the dietary treatment of 200 mg significantly increased the lipid stability of the muscle (reflected in lower TBAR production) than 20 mg/kg supplemented feed. The fatty acid composition of muscle was not significantly altered by vitamin E supplementation. Overall it was shown that vitamin E did alleviate oxidative stress that was induced by dexamethasone in broilers (Gao *et al*. 2010).

Guo *et al*. (2001) performed 2 experiments. In the first experiment it was determined what the best level of α-tocopherol acetate level of supplementation is for broiler chicks aged between 0 and 3 weeks. A basal diet was supplied with 13 mg/kg of α-tocopherol acetate and treatments of 0, 5, 10, 50 and 100 mg/kg of α-tocopherol supplements. The treatments with high supplementation levels (50 and 100 mg/kg) of vitamin E increased body weight gain and improved feed utilisation of the broiler chicks during 0-3 weeks of age but they found no significant correlation between vitamin E supplementation and performance of the chicks. High levels of supplementation however, significantly increased α-tocopherol concentrations in the liver and significantly lowered the TBARS concentration which can be interpreted as a lower lipid peroxidation activity. Chicks supplemented with no extra vitamin E showed more severe levels of peroxidation in the liver as they aged from day 11. It was concluded that 23-27 mg/kg vitamin E supplementation in the diet was adequate to maintain a liver α-tocopherol concentration of 2-2.5 µg/g. In the second experiment (Guo *et al.* 2001), the effect of different supplemental levels of vitamin E on the performance and oxidative stability in thigh meat of broilers was evaluated. The grower diet contained 7 mg/kg and finisher diet contained 6 mg/kg α-tocopherol. Treatments included supplementation levels of 5, 10, 20, 50 and 100 mg/kg α-tocopherol acetate. No beneficial effect of high levels of vitamin E supplementation on growth throughout the growing period of the birds was found and it was concluded that the basal levels of 6 and 7 mg/kg were adequate for growth. There was a high positive correlation between supplementation of vitamin E and α-tocopherol levels in the liver on day 42 and it was concluded that the liver is a sensitive indicator of dietary vitamin E levels. It was found that there was a highly significant negative correlation between the level of vitamin E supplementation and the TBAR levels in the thigh meat when stored for four days at temperature under 4°C (Guo *et al*., 2001).

Li *et al*. (2009) conducted a study with Beijing-you chickens with the objective to determine the effect of vitamin E supplementation on the growth performance of the chickens, the susceptibility of tissue to oxidative damage and the meat quality and fatty acid profiles due to supplementation. The results indicated that with the supplementation of vitamin E in the diet, the α-tocopherol content of the breast and thigh muscle meat increased significantly and drip loss reduced while the tenderness of the meat improved. Vitamin E did not affect carcass yield, meat colour or the pH of the meat 24hr post-slaughter. Overall the lipid stability of meat was improved (for storage up to 7 days), fatty acid composition of the breast muscle changed to a greater proportion of polyunsaturated fatty acids (higher concentration of linoleic and linolenic acids) and meat quality was improved. The studies cited above confirmed the strong antioxidative properties of vitamin E and its essential role to prevent lipid peroxidation and increase meat shelf life.

2.4.2 Vitamin A

Vitamin A, also known as retinol, has a variety of functions in the body as mentioned previously, which include growth, reproduction, cell differentiation and vision (Sahin *et al*., 2002). This vitamin is very important in especially the first month of a chick's life as a deficiency of vitamin A causes higher mortality (Bieri, 1960). Vitamin A deficiency in birds can cause poor bone development and calcification. Excessive levels of vitamin A can also impart negative effects like increasing the incidence of tibial dyschondroplasia, decrease growth and decrease broiler carcass desirability due to changes in skin colour (Jiakui *et al*., 2008). There seems to be a relationship between vitamin A and E where vitamin E protects vitamin A from oxidation and it has been proposed that vitamin E might be involved in vitamin A absorption and transport (Sahin *et al*., 2002). Sklan (1983) showed that both the supplementation of vitamin A and E increased vitamin A levels in the liver. Sahin *et al*. (2002) reported that supplementation of vitamin A and E alleviated the effects of heat stress induced on broiler chickens. Malondialdehyde (MDA) levels in serum and liver samples were reduced with vitamin A and E supplementation, thereby indicated that a lower degree of oxidation occurred in the body (Guo *et al*., 2001).

Tesoriere *et al*. (1996) gave good evidence on the synergistic activity of vitamin A and vitamin E in liposomal membranes and showed that α-tocopherol prevented auto-oxidation of all-*trans*-retinol and in turn ensured better antioxidative function of the retinol and suggesting that these vitamins work together in cell membranes to scavenge lipoperoxyl radicals.

In rats Ciaccio *et al*. (1993) showed that those animals treated with vitamin A had an increased concentration in tissue membranes and that it served as a mechanism to protect cells from oxidative stress. Lipid peroxidation was induced by injecting the rats with doxorubicin. Oxidative damage was investigated in the rats that were treated with vitamin A and the controls. Results showed that brain and heart membranes had a delay in lipid peroxidation and produced less TBARS than rats in the control treatment, thus higher vitamin A levels resulted in resistance of lipid membranes to oxidation (Ciaccio *et al*., 1993). It has also been shown that a deficiency of vitamin A in rats resulted in a reduced antioxidant capacity and increased lipid oxidation (Wang *et al*., 2007).

Wang *et al*. (2007) demonstrated that TBARS level and drip loss decreased in the gluteus and longissimus muscle of cattle supplemented with vitamin A in their diet at 4400 IU/kg DM. It also reduced pigment oxidation of the meat. Overall, vitamin A does not play an important role in anti-oxidative effects, as retinol and retinal have weak abilities to scavenge radicals and they cannot react with singlet oxygen (Wang *et al*., 2007). Vitamin A can however have an effect on the levels of other antioxidants present in tissue (Wang *et al*., 2007).

2.4.3 Bioflavonoids

Due to the higher inclusion of PUFA in the diets of animals there is a growing demand for more bio-efficient ways to prevent lipid peroxidation of meat and meat products and to act upon consumer concerns of toxicity and safety on inclusion of synthetic antioxidants (Gladine *et al*., 2007).

Goñi *et al*. (2007) investigated the effect of grape pomace (that which is left of grapes after the extraction of juice) at different levels of inclusion in the diet of chicken. The skins and seeds of grapes are a good source of flavonoids including (+)-catechins, (-)-epicatechins, (-)- epicatechin-3-O-gallate and di-,tri- and tetrameric procyanidins. The grape pomace (GP) had no effect on growth performance of the chickens. GP did however, have a vitamin E sparing effect in the intestines which led to a higher level of vitamin E in the liver and they concluded that polyphenols used in the diet can improve vitamin E status of the chickens and reduce lipid peroxidation. GP also delayed lipid peroxidation in breast and thigh meat in storage and concluded that 15-30 g/kg can be effective to prevent lipid oxidation when compared to the inclusion of 200 mg/kg α-tocopheryl acetate (Goñi *et al*., 2007).

It has been reported that tea contains an effective amount of bioflavonoids. Tang *et al*. (2001) used catechins extracted from green tea containing the flavonoids epigallocatechin gallate, epigallocatechin, epicatechin gallate and epicatechin and added it to processed meat patties of different animal species. The oxidative stability of the cooked meat patties was then measured every 3 days. Lipid oxidation in the cooked patties of poultry was significantly reduced with the addition of 300 mg/kg^{-1} tea cathecin extract. It was proposed that it was due to the high affinity of tea catechins for the cell membrane of muscles and the ability of these compounds to scavenge radicals in these parts. A similar study conducted by Mielnik *et al*. (2005) showed that addition of grape seed extract to minced turkey prior to cooking and storage decreased lipid oxidation in a dose dependant way with doses ranging between 0.4-1.6 mg/kg. The study done by Gobert *et al*. (2010) also indicated that dietary supplementation of plant extracts rich in polyphenols together with vitamin E had an antioxidative effect on PUFA-rich meat as well as a vitamin E sparing effect in finishing cattle.

Erener *et al*. (2011) used green tea extract (GTE) in the diet of broilers and determined the performance, carcass traits, gastrointestinal tract traits, caecal coliform bacterial count, pH and colour of the breast muscle. Dietary inclusion of 0.1 and 0.2 g/kg GTE increased the body weight of the birds compared to the control which had no GTE inclusion. The group fed the 0.2 g/kg also showed an improvement in feed efficiency during the first 3 weeks of feeding. Several factors contributed toward the improved body weight and feed efficiency of the broilers including the composition of the basal diet, the origin of the green tea extract and its polyphenol content, time of harvest, the preparation method of the extract and environmental conditions. Erener *et al*. (2011) concluded that with regard to the improved body weight and feed efficiency, the trial was not performed under the ideal conditions. The authors argued that because highly digestible diets were fed throughout the study, the total coliform bacteria counts in the caecal intestine may have been low, and therefore the growth promoting effect of the additive may have been limited. It was further reported that the coliform count of the GTE supplemented birds were lower compared to the control fed group and that the

colour of the meat of supplemented birds differed from the control. The 0.1 g/kg supplemented group had a paler breast meat colour and both the GTE feeding levels produced more red and yellow meat than the control.

Leusink *et al*. (2010) determined the performance, meat quality and gut flora of broilers fed different levels of cranberry fruit extract (CFE) at levels of 0, 40, 80 and 160 mg/kg. Cranberries have been shown to contain antioxidant activity and contain polyphenolic compounds like anthocyanins, flavonols, flavan-3-ols, procyanidins and proanthocyanidins. CFE had no influence on performance parameters (body weight, feed intake, and feed efficiency) at any treatment level. Early mortality cases were decreased by 50% for the treatment group receiving 40 mg/kg compared to the control. There was no significant differences between treatment groups in carcass and meat quality characteristics like fat content, moisture content, pH, texture and colour, although the carcass weight and breast circumference was slightly higher for the 80 and 160 mg/kg treatments. Intestinal health was also not affected. On day 28 the birds receiving 80 mg/kg CFE had significantly lower *Enterococcus* spp. in caecal and cloacal samples and the group supplemented with 160 mg/kg had a significantly higher amount of these bacteria. These bacteria produced lactic acid which the authors speculated might have affected the growth of other micro-organisms in the gut. Overall there were no significant effects on bird performance with the different levels of CFE supplementations (Leusink *et al*., 2010).

Quercetin supplementation in the diet of pigs had a α-tocopherol sparing effect when α-tocopherol in the diet was low. An antioxidative effect on lipid oxidation with pigs fed a diet with fish oil and low vitamin E levels was observed and the authors suggested that it could be possible to partially replace α-tocopherol with flavonol supplements (Luehring *et al*., 2011).

In the study done by Mitsumoto *et al*. (2005) two different levels of tea cathecins (TC; 200 and 400 mg/kg meat) were added to raw beef and chicken patties. The addition of TC caused the discolouration of both beef and chicken patties after they were cooked. Lipid oxidation in the cooked beef patties were also reduced with TC addition. Tea cathecin treatment significantly lowered lipid oxidation in raw meat patties compared with vitamin C treatment of the patties. Mitsumoto *et al*. (2005) concluded that tea cathecins were a more potent antioxidant than vitamin C.

Kathirvel and Richards (2009) showed that quercetin was successful at inhibiting lipid peroxidation in mechanically separated turkey meat. They compared the activity of quercetin and its glucoside quercetin-3-D-glucoside and showed that quercetin had a higher affinity for membranes which made it an efficient radical scavenger. They also made the point that the locations of the flavonoids with regard to membranes are important for flavonoids to have an effect (Kathirvel and Richards, 2009).

2.5 Selenium as an important antioxidant mineral

Selenium (Se) is an important trace element that is included in animal diets and has a wide variety of functions including disease prevention, production and fertility (Cai *et al*., 2012). It is supplemented in

poultry diets to prevent exudative diathesis and pancreatic fibrosis and to maintain glutathione peroxidase activity (Payne and Southern, 2005). It forms part of the molecule glutathione and its enzyme glutathione peroxidase (GPX) where Se is transformed to selenocysteine and forms part of the enzyme's active centre (Mahmoud and Edens, 2003; Wang and Xu, 2008). This enzyme performs a very important role in the body's antioxidant defence system by protecting unsaturated bonds in cell membranes from free radical attack and lipid peroxidation (Mahmoud and Edens, 2003). The enzyme GPX is responsible for the decomposition of lipid hydroperoxides, ROS and reactive nitrogen species rendering them harmless and unable to cause any further damage (Mahmoud and Edens, 2003). Thioredoxin reductase, also a selenoprotein, together with GPX are the two most common selenium containing proteins, and together are responsible for the decomposition and elimination of hydrogen peroxide and lipid hydroperoxides (Zhang *et al*., 2010). Glutathione forms the largest part of an intracellular redox buffer and is a major non-enzymatic antioxidant in the cell (Cai *et al*., 2012). The selenium supply to the body will determine the activity level of GPX and glutathione (Wang and Xu, 2008; Cai *et al*., 2012). A deficiency in vitamin E and Se can lead to exudative diathesis in chicks due to ROS damage and it has been proven that a deficiency of either one of these nutrients or a combined deficiency can cause increased lipid peroxidation in cell membranes (Avanzo *et al*., 2001). Avanzo *et al*. (2001), in their study showed that vitamin E, rather than Se, had a more pronounced effect on lipid peroxidation in mitochondrial membranes of chicken breast meat. The birds fed a diet low in vitamin E and Se had the lowest concentration of glutathione and GPX activity, making tissue more susceptible to oxidative stress (Avanzo *et al*., 2001). The NRC (1994) set a minimum requirement for broilers for Se at 0.15 mg/kg and the maximum amount of Se allowed in the diet is 0.3 mg/kg (Yoon *et al*., 2007; Cai *et al*., 2012).

Se and α-tocopherol supplemented together can significantly decrease lipid oxidation in poultry meat as shown in the study of Kim *et al*. (2010). Vitamin E acts as the first line of defence in cellular membranes against lipid peroxidation and GPX is the second line of defence when vitamin E is not able to destroy all the peroxides. Vitamin E and Se (through GPX) can both be sparing mechanisms for each other (Puvača and Stanaćev, 2011). The absorption of vitamin E can be negatively affected when there is a severe Se deficiency (Skřivan *et al*., 2008). Skřivan *et al*. (2008) also showed that Se supplementation in the organic form can increase the α-tocopherol and Se content of broiler meat.

Vitamin E is a well-known antioxidant vitamin able to protect biological systems from attack from free radicals and reactive oxygen species and the fact that the animal cannot synthesise this vitamin makes the supplementation essential in the diet. Vitamin A has also been known to have antioxidant activity although it its potency as an antioxidant is still uncertain. Bioflavonoids, among several different functions in the body, have been shown to also have potent antioxidant activity with these molecules being widely available in plants and fruits. Broilers was used in a trial of 35 days in order to determine if a bioflavonoid based additive, Biored®, is able to partially replace vitamin A and E as an antioxidant.

It is hypothesized that Biored®, as a bioflavonoid based feed additive, is able to substitute a certain level of vitamin A and E in the diet of broiler chickens and effectively perform the antioxidative effects by either sustaining or improving performance parameters (body weight, feed intake, average daily gain and feed conversion) and sustain liver vitamin A and E stores when only half the vitamin A and E specification is used in the feed. Biored® is hypothesised to either improve or sustain meat quality factors (drip loss, cooking loss and shear force values), carcass traits (carcass weight, portion weights) and the shelf life of thigh meat when only half the vitamin A and E specification is used in the feed.

Chapter 3

Materials and Methods

The following trial attempted to determine if the bioflavonoid based product, Biored®, can partially replace the antioxidative function of vitamins A and E that are added to broiler diets. The ability of Biored® to replace vitamins A and E was evaluated by measuring the broiler growth performance, liver vitamin A and E levels, blood oxidation levels, meat quality and carcass traits and shelf life of the chicken meat.

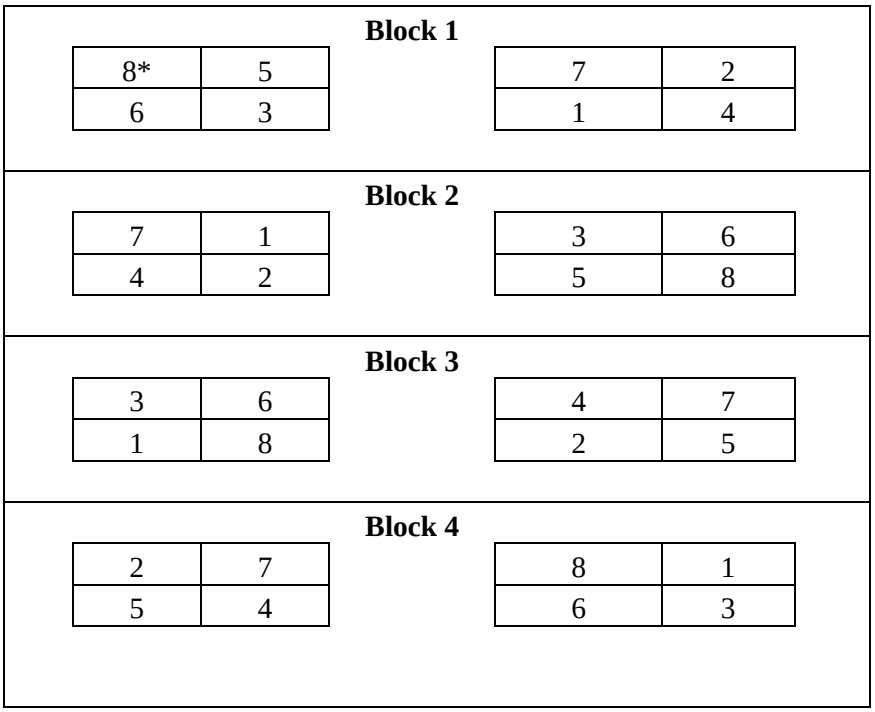
3.1 Experimental design and treatments

The trial had one grow-out cycle of 35 days. A total of 1 280 male Ross broiler chicken were randomly distributed among 64 pens, with a stocking density of 20 birds per pen. Eight different dietary treatments were used in the trial (Table 3.1) with each of the treatments replicated 8 times. The 64 pens were evenly distributed between 2 houses with each house containing 32 pens and 4 replications per treatment. Pen replicates were arranged according to a randomised complete block design with each block containing one replication of each treatment as demonstrated in Figure 3.1. Birds were randomly assigned to one of eight dietary treatments.

Table 3.1 Eight dietary treatments containing different levels of vitamin A and E as well as different inclusion levels of a bioflavonoid based product, Biored®, fed to broiler chickens

Treatment name	**Biored® inclusion level (g/ton)**	**Actual inclusion rate in diet (g)**		**Vitamin A and E inclusion level (%)***	**Actual inclusion rate in diet (g)**		
					Vitamin	A	E
Positive Control (1)	0	Starter	0.0	100	Starter	0.60	6.30
		Grower	0.0		Grower	3.50	35.40
		Finisher	0.0		Finisher	2.10	21.30
Negative Control (2)	0	Starter	0.0	0	Starter	0.0	0.0
		Grower	0.0		Grower	0.0	0.0
		Finisher	0.0		Finisher	0.0	0.0
3	150	Starter	7.9	0	Starter	0.0	0.0
		Grower	53.0		Grower	0.0	0.0
		Finisher	32.0		Finisher	0.0	0.0
4	150	Starter	7.9	100	Starter	0.60	6.30
		Grower	53.0		Grower	3.50	35.40
		Finisher	32.0		Finisher	2.10	21.30
5	0	Starter	0	50	Starter	0.30	3.20
		Grower	0		Grower	1.80	17.7
		Finisher	0		Finisher	1.10	10.70
6	100	Starter	5.3	50	Starter	0.30	3.20
		Grower	35.4		Grower	1.80	17.7
		Finisher	21.3		Finisher	1.10	10.70
7	150	Starter	7.9	50	Starter	0.30	3.20
		Grower	53.0		Grower	1.80	17.7
		Finisher	32.0		Finisher	1.10	10.70
8	200	Starter	10.5	50	Starter	0.30	3.20
		Grower	70.7		Grower	1.80	17.7
		Finisher	42.6		Finisher	1.10	10.70

* 100%, 50% or 0% inclusion of vitamin A and E relative to standard concentrations in commercial premixes.



* Numbers indicate the dietary treatment applied to the specific pen

Figure 3.1 Pen arrangements of treatments and replications for one of the two experimental houses, demonstrating the blocking of treatments

3.2 Husbandry and rearing of animals

The trail was conducted on the Experimental Farm of the University of Pretoria, Hatfield, Pretoria. The protocol for the project was approved by the University of Pretoria's Animal Ethics Committee (Project number: EC095-13) before commencement of the trial. Birds were housed in temperature controlled broiler houses. A total of 1600, vaccinated, day-old male broiler chicks (Ross 308) were purchased from a local commercial hatchery. Chicks were randomly divided between 64 pens with 20 chicks per pen at a stocking density of 8.89 birds/m^2 floor space. The individual pen sizes were 1.5 x 1.5 m giving a floor space of 2.25m^2 per pen. Three hundred and twenty day-old (320) chicks were randomly selected, removed and humanely euthanised for collection of blood samples to determine the baseline glutathione (GSH) and reduced glutathione (GSSG) concentrations in whole blood. A further 1 280 chicks were visually examined for health defects to ensure only healthy chicks were included in the trial. The concrete floor was covered with wood shavings of approximately 10 cm in depth. The broiler house was preheated for 48 hours prior to placement of day-old chicks to a floor temperature of 32°C. The temperature was then gradually decreased to reach 22°C at day 35. Heat was provided by electrical heaters installed in the broiler house. Ventilation throughout the house was provided by electrically controlled fans and air vents.

The lighting program consisted of 23 hour light and 1 hour darkness for the first week followed by 16 hours of light up to day 35. Each pen was equipped with 6 nipple drinkers and 1 tube feeder. Birds had *ad libitum*

access to feed and water throughout the trial. During the first week after chick placement one extra pan feeder and 1 fountain drinker were provided per pen in order to encourage and assist chicks to eat and drink.

3.3 Feeding and water supply

The birds received *ad libitum* feed and water according to the treatment they were assigned to. Feed consumption was monitored and the feeders refilled when needed on a daily basis. The feed was mixed by a local feed company specialising in producing small quantities for experimental purposes (Penville Pty Ltd., Pretoria) The same basal diet was mixed for all 8 treatments after which the premixes with the different concentrations of vitamin A, vitamin E and Biored® was added to the basal feed and remixed to form the 8 different dietary treatments. A three phase feeding regimen was followed consisting of a starter crumble, grower pellet and finisher pellet. The rations were mixed to specifications shown in Table 3.2.

The three phases were fed as follows:

- Starter: 1-10 days
- Grower: 11-28 days
- Finisher: 29-35 days

Biored® was incorporated into the premix at the levels mentioned in Table 3.1. The premixes added to all eight dietary treatments contained lower concentrations of selenium than normally added to limit the synergistic effect between selenium and vitamin E in the body. To prevent selenium deficiency in the chicks, sodium selenite was added to the premix to provide 150µg/kg feed. According to Wang and Xu (2007) the minimum amount of Se that needs to be supplemented in the diet of broilers in order to prevent severe deficiencies and still sustain growth is 100 µg/kg.

Table 3.2 Raw material inclusion (%) and calculated nutrient composition of the basal broiler diet for each of the three phases

Ingredients	Starter	Grower	Finisher
Yellow maize (fine)	59.09	62.53	66.74
Soya oilcake meal	26.50	18.98	14.39
Sunflower oilcake meal	3.00	3.00	3.00
Full fat soya	4.66	9.25	10.11
Gluten 60	3.00	3.00	3.00
Limestone ($CaCO_3$)	1.42	1.34	1.18
Sodium Bicarbonate ($NaHCO_3$)	0.073	0.038	0
Salt (Fine)	0.43	0.43	0.43
Mono calcium phosphate	0.949	0.560	0.313
Lysine HCL	0.314	0.299	0.286
DL Methionine	0.231	0.212	0.197
DL Threonine	0.063	0.051	0.038
Broiler Premix (excl. Vit A, E and Se)	0.3	0.25	0.25
Calculated nutrient values (g/kg):			
Dry matter	891.5	894.3	890.4
Moisture	108.5	105.7	109.6
Apparent metabolisable Energy	11.5	11.95	12.25
Crude protein	222.2	207.4	192.7
Crude fibre	37.3	38.6	38.3
Crude fat	35.6	44.3	46.6
Ash	47.9	44.4	40.4
Calcium	9.4	8.3	7.2
Total phosphorus	7.9	6.9	6.3

3.4 Measurements

3.4.1 Feed sampling and testing prior to start of trial

- *Proximate analysis and selenium*

Representative feed samples were taken from the eight different dietary treatments and for each phase (starter, grower and finisher). The feed samples (eight treatments and three phases) were analysed according to the proximate analysis system for their nutritional content at Nutrilab (Department of Animal and Wildlife

Science, University of Pretoria). Results of the analysis are presented in Table 3.3. This system determines seven fractions in food including dry matter, ash, crude protein, ether extract (lipid content), crude fibre, crude protein, calcium and total phosphorus. The twenty four feed samples were also analysed for selenium content. Dry matter of feed and ash were analysed according to AOAC's official method of analysis (AOAC, 2000, Official method of analysis 942.05). Moisture determination was done according to the method followed by AOAC's official method of analysis (AOAC, 2000, Official method of Analysis 943.01). Crude fibre was determined following the AOAC's method of analysis (AOAC, 2000, Official method of Analysis 962.09) as was crude fat (AOAC, 2000, Official method of Analysis 920.39). The Leco FP-428 (Leco Corporation, 3000 Lakeview Avenue, St. Joseph, MI 49085-2396) was used to analyse the nitrogen content of the feed and the method used was according to the AOAC's official method of analysis (AOAC, 2000, Official method of Analysis 988.05). The feed content for calcium, phosphorus and selenium were determined using the AOAC's official method of analysis, calcium (AOAC, 2000, Official method of Analysis 935.13), phosphorus (AOAC, 2000, Official method of Analysis 965.17) and selenium (AOAC, 2000, Official method of Analysis 996.16).

- *Vitamin A and E analysis*

Twenty four feed samples (eight treatments and three phases) were shipped to an independent laboratory, V and M Analysis (George, South Africa), for vitamin A and E analyses. All eight treatment diets for each of the three feeding phases (starter, grower and finisher) were analysed. There was a problem extracting the vitamin A and E out of the feed due to a coating around the vitamins that prevents oxidation in the feed. This resulted in the analysed vitamin concentration in the feed being much lower than was expected as can be seen in Table 3.4. Due to budget restrictions the analyses could not have been repeated at other laboratories due to the high costs of the analyses.

The level of vitamin A and E in the liver gives a good indication of the vitamin levels in the feed as the animal cannot synthesise the vitamins in the body and is totally depended on their supplementation in the feed as it has been shown in several studies (Jensen *et al*., 1998; Gao *et al*., 2010; Voljč *et* al., 2011). Both these vitamins are stored in the liver and mobilised from there to body tissues (Norum and Blomhoff, 1992; Wang and Quinn, 1999). As discussed in Section 3.4.3 below, vitamin A and E levels in the livers of broilers at day 35 of the trial were analysed and these values were used to verify the differences in vitamin A and E levels between treatments.

Table 3.3 Analysed nutrient values (on "as fed" basis) of the starter, grower and finisher feeds of the eight dietary treatments

Nutrient	Starter								Grower								Finisher							
	T1	T2	T3	T4	T5	T6	T7	T8	T1	T2	T3	T4	T5	T6	T7	T8	T1	T2	T3	T4	T5	T6	T7	T8
Dry Matter (%)	89.7	89.1	89.1	89.9	89.5	89.8	89.6	89.7	88.0	88.7	89.0	89.0	89.1	89.3	88.9	88.5	88.6	88.8	89.0	88.1	88.8	88.3	87.8	88.7
CrudeProtein (%)	21.5	20.4	20.8	21.2	22.6	22.2	21.0	21.5	20.2	20.2	20.1	20.5	20.2	20.8	20.7	19.7	18.8	18.8	19.4	19.1	18.8	19.0	18.6	18.7
Crude Fibre (%)	4.0	3.6	3.7	3.7	3.6	4.4	3.8	3.5	4.0	3.8	4.1	4.0	3.9	4.1	4.2	4.1	3.6	3.6	3.7	3.5	4.2	3.5	3.5	4.1
Crude Fat (%)	3.6	3.6	3.5	3.7	4.1	3.6	4.0	3.6	4.9	5.1	5.0	4.9	5.2	4.7	4.7	4.7	4.4	4.7	4.9	4.8	4.9	4.7	4.8	5.0
Ash (%)	6.1	5.5	5.6	6.0	5.0	5.3	5.7	5.8	4.5	5.2	4.2	4.1	4.7	4.4	4.8	4.2	4.5	4.3	4.4	4.0	3.9	4.2	3.9	3.9
Calcium (%)	0.81	0.82	0.79	0.84	0.86	0.85	0.92	0.79	0.78	0.70	0.65	0.72	0.70	0.68	0.74	0.65	0.63	0.59	0.58	0.62	0.59	0.58	0.58	0.61
Phosphorus (%)	0.57	0.56	0.58	0.58	0.59	0.60	0.62	0.55	0.51	0.49	0.48	0.49	0.49	0.50	0.49	0.47	0.42	0.42	0.43	0.43	0.43	0.43	0.42	0.43
Selenium (mg/kg)	0.17	0.25	0.17	0.21	0.18	0.26	0.26	0.21	0.18	0.20	0.32	0.0.37	0.32	0.31	0.37	0.37	0.21	0.18	0.27	0.23	0.24	0.28	0.25	0.24

*T1- 100% Vitamin A and E, 0 g/ton Biored®; T2- 0% Vitamin, 0 g/ton Biored®; T3-0% Vitamin A and E, 150 g/ton Biored®; T4- 100% Vitamin A and E, 150 g/ton Biored®; T5- 50% Vitamin A and E, 0 g/ton Biored®; T6- 50% Vitamin A and E, 100 g/ton Biored®; T7- 50% Vitamin A and E, 150 g/ton Biored®; T8- 50% Vitamin A and E, 200 g/ton Biored®.

Table 3.4 Expected and analysed vitamin A and E levels in the eight different dietary treatments

Phase	Treatment	Vitamin A		Vitamin E	
		Expected (mg/kg)	Analysed (mg/kg)	Expected (mg/kg)	Analysed (mg/kg)
Starter	1	11.15	2.03	117.10	29.90
	2	0.00	ND	0.00	< LOQ
	3	0.00	ND	0.00	ND
	4	11.15	2.16	117.10	27.32
	5	5.58	ND	59.48	ND
	6	5.58	1.57	59.48	14.81
	7	5.58	< LOQ	59.48	ND
	8	5.58	< LOQ	59.48	ND
Grower	1	9.65	1.27	97.63	3.82
	2	0.00	ND	0.00	ND
	3	0.00	ND	0.00	ND
	4	9.65	1.63	97.63	3.67
	5	4.96	1.02	48.81	2.17
	6	4.96	0.93	48.81	2.13
	7	4.96	0.84	48.81	< LOQ
	8	4.96	0.97	48.81	2.69
Finisher	1	9.61	2.59	97.44	91.25
	2	0.00	ND	0.00	< LOQ
	3	0.00	ND	0.00	< LOQ
	4	9.61	3.17	97.44	86.34
	5	5.03	1.76	48.95	46.98
	6	5.03	1.43	48.95	37.42
	7	5.03	2.10	48.95	38.41
	8	5.03	0.95	48.95	34.01

ND = Not Detected, LOQ = Limit of quantification

*T1- 100% Vitamin A and E, 0 g/ton Biored®; T2- 0% Vitamin, 0g/ton Biored®; T3-0% Vitamin A and E, 150 g/ton Biored®; T4- 100% Vitamin A and E, 150 g/ton Biored®; T5- 50% Vitamin A and E, 0g/ton Biored®; T6- 50% Vitamin A and E, 100 g/ton Biored®; T7- 50% Vitamin A and E, 150 g/ton Biored®; T8- 50% Vitamin A and E, 200 g/ton Biored®.

3.4.2 Performance parameters measured during rearing

1. *Body weight*

Body weight (BW) of broiler chickens was measured on a weekly basis. They were weighed as a group (20 per pen). They were weighed before the chicks were placed (day 0) and then on day 7, 14, 21, 28 and 35. The data was also used to determine the average daily gain of the birds.

2. *Feed intake*

Feed intake was measured weekly on the same day that the chicks were weighed on day 7, 14, 21, 28 and 35.

3. *Mortalities*

During twice daily inspections in the broiler house dead birds were collected and the weight of dead birds recorded.

4. *Feed Conversion Ratio (FCR)*

The weekly FCR was calculated as feed intake (g) / body weight (g). The FCR was corrected for BW of mortalities that occurred.

3.4.3 Parameters measured at the end of the trail on day 35

1. Vitamin A and E concentrations in the liver of birds

On day 35, five birds per replicate group were euthanised by way of cervical dislocation and the livers excised. The left lobe of the five livers were removed and placed together in marked plastic bags to form a pooled sample, placed in a black container (to protect samples from sunlight) and immediately frozen. Two days after slaughter the samples were moved to a -80°C freezer where the livers were stored till they were ready to be sent for analyses. The 64 pooled liver samples were shipped to an independent laboratory (VandM Analytical Toxicology Laboratory Services (Pty) Ltd., George) for analysis of vitamin A and E.

2. Glutathione/Reduced Glutathione (GSH/GSSG) assay in whole blood of birds

The GSH:GSSG ratio is a good indicator of oxidative stress occurring in the body (Avanzo *et al*., 2001). On day 1 and day 35, whole blood was collected from the birds to determine the GSH:GSSG ratio. At the start of the trial, 320 day-old chicks, were randomly selected and humanely euthanised by way of cervical dislocation after which blood was collected by way of cardiac puncture. On day 35, the blood of 5 birds per pen (amounting to 320 birds) was collected from the jugular vein. Blood was collected in tubes containing EDTA as an anti-coagulant.

For the GSH analyses 10 µL of the whole blood from each tube was added to a 1.5mL Eppendorf tube that ultimately gave a pooled sample of 50µL. The GSSG samples were prepared by adding 20µL whole blood from each EDTA blood tube to a 10µL M2VP solution. The M2VP (1-methyl-2-vinyl-pyridium

trifluoromethane sulfonate) solution was prepared by adding 0.008g of M2VP powder to 1mL of 0.1M HCl. The GSH and GSSG samples were immediately frozen at -20°C and after 2 days shipped to Cape Peninsula Universities' Oxidative Research Centre in Cape Town for analyses. All the samples for GSH and GSSG analysis were prepared in duplicate amounting to 256 samples in total.

3. *Meat quality*

Drip loss of breast meat

The right half of the breast (pectoralis major) from four birds per replicate pen was used to determine drip loss. The breast portion was weighed, placed in a marked bag and stored at 4°C for a period of 24 hours. The fridge was equipped with a light that was switched on permanently in order to simulate retail conditions. After 24 hours the breast sample was reweighed according to the procedure described by Tavárez *et al.* (2011). Drip loss was then recorded as a % using the following formula:

$$\text{Drip loss \%} = \frac{\text{Breast weight after slaughter} - \text{Breast weight after 24 hours}}{\text{Breast weight after slaughter}} \times 100$$

After the breast was reweighed, 24 hours after slaughter, it was frozen at -20°C until used for determining cooking loss and shear force.

Cooking loss

The right half of the breast portion (pectoralis major) was left to defrost at room temperature overnight. After the breast portion was thawed it was weighed and sealed in a plastic Ziploc bag. The bag with the breast was submerged in a water bath set at 80°C for 20 min as described by Liu *et al.* (2004). After 20 minutes in the water bath the bags were left to cool down to room temperature. The breasts were removed from the bag, the excess liquid on the meat dried off with paper towel and then reweighed. The cooking loss was determined as the loss of weight during the cooking as a percentage of the meat before cooking ([(weight of raw meat – weight of cooked meat)/ weight of raw meat] * 100) (Rossi *et al.*, 2013; Cao *et al.*, 2012). Breast portions of three birds per pen were used for determining cooking loss.

Shear force of breast meat

Shear force of cooked breast meat samples were measured according to the procedure described by Betti *et al.* (2009). Two 25 mm diameter cubes were removed from the thickest part of the breast meat portion and shear values were measured by placing meat fibres perpendicular to the blade of an Instron Universal Testing Machine. A 500 kg load cell was used with a crosshead speed of 500mm/min. Shear values were reported in Newton (N). Breast meat samples of three birds per pen were measured.

Relative portion yields

The eviscerated whole carcass, left half of the breast (pectoralis major) and left thighs of three birds per pen were weighed and the relative weights of the meat portions to the carcass weight were determined.

3. 5. Statistical analyses

Statistical analysis on data was done with the statistical software program SAS (Statistical Analysis System, 2014). The significance between treatments was determined by an analysis of variance with the general linear model (GLM). Means, standard error and significance of differences between means were determined by Fischers test (Sameuls, 1989) at the 95% confidence level. In all cases the level of statistical significance was $P<0.05$. Differences between treatments for mortalities were calculated with a Chi square.

Repeated Measures Analysis of Variance with the GLM model (SAS, 2014) were used for repeated period measures. Means and standard error of means for the different treatments were calculated and significant differences ($P<0.05$) between means were determined by Fischers test (Samuels, 1989).

The linear model used is described by the following equation:

$$Y_{ij} = \mu + T_i + H_j + TH_{ij} + e_{ij}$$

Where Y_{ij} = variable studied during the period

μ = overall mean of the population

T_i = effect of the i^{th} treatment

H_j = effect of the j^{th} house

TH_{ij} = effect of the ij^{th} interaction between treatment and house

e_{ij} = error associated with each Y

Chapter 4

Results

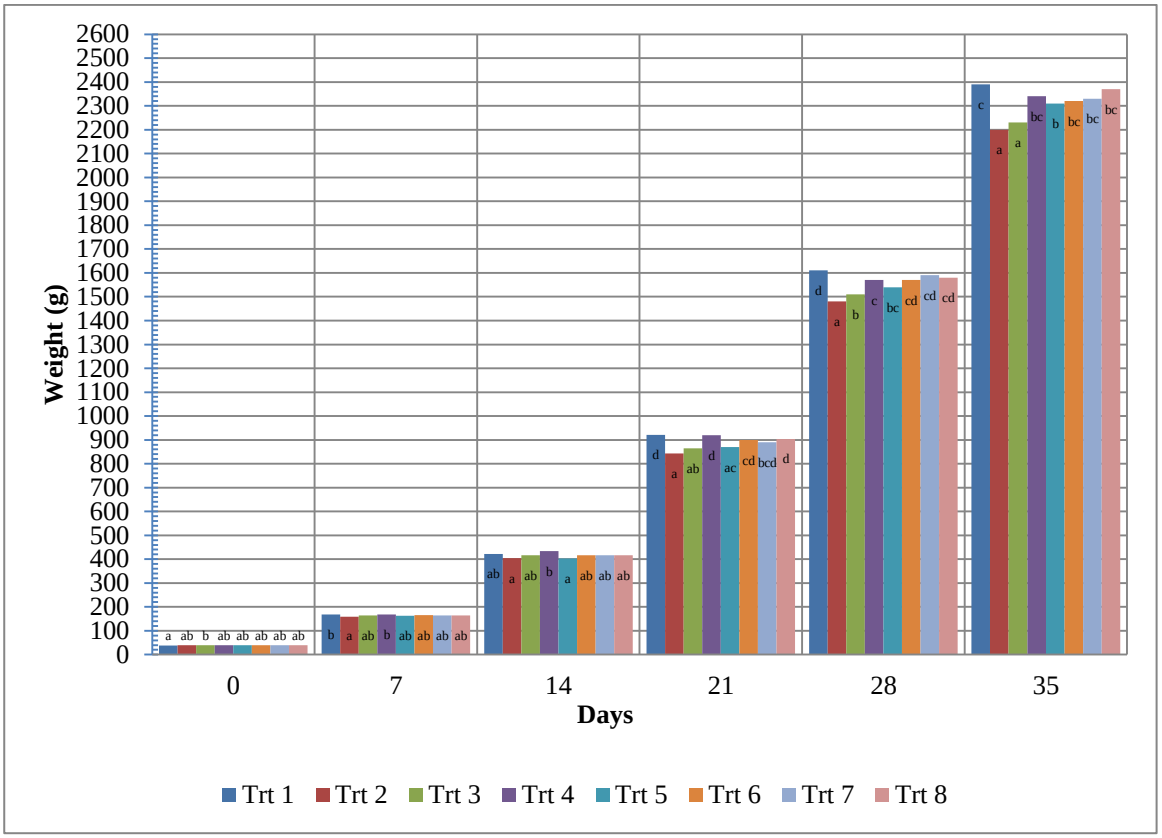
4.1 Performance data

4.1.1 Body weight

Table 4.1: Weekly body weight (g) of broiler chickens that received feed containing various concentrations of vitamin A and E and a bioflavonoid based product, Biored®

Treatment*	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
1	38.6^{a}	168^{b}	422^{ab}	921^{d}	1610^{d}	2390^{c}
2	39.1^{ab}	159^{a}	405^{a}	843^{a}	1480^{a}	2200^{a}
3	39.8^{b}	164^{ab}	417^{ab}	865^{ab}	1510^{ab}	2230^{a}
4	39.4^{ab}	168^{b}	434^{b}	919^{d}	1570^{c}	2340^{bc}
5	39.0^{ab}	163^{ab}	403^{a}	870^{ac}	1540^{cb}	2310^{b}
6	39.4^{ab}	165^{ab}	417^{ab}	899^{cd}	1570^{cd}	2320^{bc}
7	39.2^{ab}	164^{ab}	417^{ab}	890^{bcd}	1590^{cd}	2330^{bc}
8	39.3^{ab}	164^{ab}	417^{ab}	904^{d}	1580^{cd}	2370^{bc}
SE	0.0004	0.0024	0.0067	0.011	0.016	0.027
R^2	0.189	0.524	0.514	0.598	0.655	0.543

^{abcd} Column means with the same superscripts do not differ significantly from each other ($P < 0.05$)
*T1- 100% Vitamin A and E, 0 g/ton Biored®; T2- 0% Vitamin, 0 g/ton Biored®; T3-0% Vitamin A and E, 150 g/ton Biored®; T4- 100% Vitamin A and E, 150 g/ton Biored®; T5- 50% Vitamin A and E, 0 g/ton Biored®; T6- 50% Vitamin A and E, 100 g/ton Biored®; T7- 50% Vitamin A and E, 150 g/ton Biored®; T8- 50% Vitamin A and E, 200 g/ton Biored®



[abcd] Column means with the same letters do not differ significantly from each other ($P < 0.05$)
*T1- 100% Vitamin A and E, 0 g/ton Biored®; T2- 0% Vitamin, 0 g/ton Biored®; T3-0% Vitamin A and E, 150 g/ton Biored®; T4- 100% Vitamin A and E, 150 g/ton Biored®; T5- 50% Vitamin A and E, 0 g/ton Biored®; T6- 50% Vitamin A and E, 100 g/ton Biored®; T7- 50% Vitamin A and E, 150 g/ton Biored®; T8- 50% Vitamin A and E, 200 g/ton Biored®

Figure 4.1 Body weights (g) of broilers during a 35 day grow out period on 8 different dietary treatments with feed containing various concentrations of vitamin A and E and a bioflavonoid based product, Biored®

Table 4.1 and Figure 4.1 show the average weekly body weights of birds during the 35 day trial period.

Day 0

Day-old chicks in treatment 3 (0% vitamin A and E, 150 g/ton Biored®) were significantly heavier ($P \leq 0.05$) than chicks in the Positive Control (100% vitamin A and E, 0 g/ton Biored®). No other significant differences were found between birds in different treatments.

Day 7

The seven day BW of broilers in treatment 2 and birds in the Negative Control group (no vitamin A and E or Biored® inclusion), was significantly lower ($P \leq 0.05$) than that of birds in treatment 1 (Positive Control) and treatment 4 (150 g/ton Biored®) which contained the standard vitamin A and E concentration used commercially. However, birds in treatment 3 (0% vitamin A and E, 150 g/ton Biored®) that only received 150 g/ton of Biored® in the feed but no vitamin A and E, did not differ significantly ($P > 0.05$) from the birds in the Positive Control.

Day 14

Chicks from treatment 3 were heavier than the chicks in the Positive Control group on day 0 which might explain them being heavier on day 7 and 14 even though they did not receive vitamin A and E. On day 14, birds receiving treatment 4 (100% vitamin A and E inclusion and 150g/ton Biored®) were significantly heavier ($P \leq 0.05$) than the Negative control (0% vitamin A and E, 0 g/ton Biored®) birds and birds of treatment 5 that received only 50% of vitamin A and E specification and no Biored®. Birds receiving treatment 4 were the heaviest at 434g and birds in the Negative Control group were the lightest at 405g.

Day 21and 28

On day 21, the broilers of both the Negative Control (0% vitamin A and E, 0 g/ton Biored®) and 3 (0% vitamin A and E inclusion and 150g/ton Biored®) as well as birds in treatment 5 (50% vitamin A and E and 0 g/ton Biored®) had significantly lower BWs ($P \leq 0.05$) compared to birds in the treatments that received 100% vitamin A and E (Positive Control and treatment 4). Birds receiving treatment 5 (50% vitamin A and E and 0 g/ton Biored®) were heavier than the Negative Control birds and birds in treatment 3 (0% vitamin A and E inclusion and 150g/ton Biored®) on day 21, 28 and 35. However, the BW of birds from treatments 6, 7 and 8 that all received only 50% vitamin A and E, but with Biored® in the feed added in increasing levels, did not differ significantly ($P > 0.05$) from birds in the Positive Control group. These trends were also shown on day 28 and day 35 at the end of the study period.

Day 35

On day 35, at the end of the trial, the weights of birds in treatment groups 6, 7 and 8 (50% vitamin A and E with different incremental levels of Biored®) were not statistically ($P > 0.05$) different from the birds in the Positive Control (Treatment 1). Birds in treatment 5 (50% vitamin A and E, 0 g/ton) had significantly lower weights ($P \leq 0.05$) than birds in the Positive Control group. In treatment 6, 7 and 8 Biored® inclusion levels were increased with 50g/ton increments from 100g/ton (treatment 6) to 200g/ton (treatment 8) respectively. On day 35, as the level of Biored® increased the body weights of the birds also increased although the increase was not statistically significant ($P > 0.05$). The average BW difference between birds in treatment 6 and 7 was 10g and between birds in treatment 7 and 8, 40g.

In summary, broilers that received 0% of the standard vitamin A and E specification in their feeds, with or without Biored®, had significantly lower BWs on day 35. BW of birds that received 50% of vitamin A and E without Biored®, were significantly heavier than birds that received the 0% vitamin A and E, but significantly lighter than the 100% vitamin A and E treatment groups. When Biored® was added to the diets containing the 50% vitamin A and E levels; BW was improved and did not differ from the 100% vitamin levels, regardless of concentration of Biored® added.

4.1.2 Feed Intake:

Table 4.2: Average feed intake (kg/day) of broilers during a 5 week rearing period receiving 8 different dietary treatments containing different concentrations of vitamin A and E and different concentrations of a bioflavonoid based product, Biored®

Treatment	Day 0-7 (Week 1)	Day 7-14 (Week 2)	Day 14-21 (Week 3)	Day 21-28 (Week 4)	Day 28-35 (Week 5)	Day 0-35 (Cumulative)
1	0.164	0.401^{b}	0.684^{ab}	1.00^{c}	1.26^{c}	3.51^{e}
2	0.158	0.365^{ab}	0.651^{a}	0.948^{ab}	1.16^{a}	3.28^{ab}
3	0.160	0.370^{ab}	0.656^{ab}	0.930^{a}	1.19^{ab}	3.30^{bd}
4	0.169	0.389^{ab}	0.666^{ab}	0.974^{bc}	1.24^{bc}	3.44^{ce}
5	0.161	0.369^{ab}	0.646^{a}	0.953^{ab}	1.24^{bc}	3.37^{abc}
6	0.166	0.381^{ab}	0.673^{ab}	0.976^{bc}	1.24^{bc}	3.44^{ce}
7	0.155	0.373^{ab}	0.663^{ab}	0.978^{bc}	1.24^{bc}	3.41^{dce}
8	0.160	0.365^{b}	0.691^{b}	0.993^{c}	1.26^{c}	3.47^{ce}
SE	0.0075	0.012	0.014	0.014	0.021	0.041
R^2	0.191	0.278	0.283	0.426	0.375	0.473

^{abcde} Column means with the same superscripts do not differ significantly from each other ($P < 0.05$)
*T1- 100% Vitamin A and E, 0 g/ton Biored®; T2- 0% Vitamin, 0 g/ton Biored®; T3-0% Vitamin A and E, 150 g/ton Biored®; T4- 100% Vitamin A and E, 150 g/ton Biored®; T5- 50% Vitamin A and E, 0 g/ton Biored®; T6- 50% Vitamin A and E, 100 g/ton Biored®; T7- 50% Vitamin A and E, 150 g/ton Biored®; T8- 50% Vitamin A and E, 200 g/ton Biored®.

Feed intake of birds in the different treatment groups are presented in Table 4.2.

There were no significant differences ($P > 0.05$) between birds in different treatments for feed intake during the first week.

During week 2, broilers fed treatment 1 (Positive Control) consumed the highest amount of feed. Broilers fed treatment 2 (Negative Control) and 8 (50% vitamin A and E; 200 g/ton Biored®) consumed significantly less ($P \leq 0.05$) feed than birds in treatment 1 (Positive Control) but no other significant differences were noted and during week 3 (day 14-21) the broilers from treatment 8 ate on average the highest amount of feed which was significantly higher than both birds fed treatment 2 (Negative Control) and treatment 5 (50% vitamin A and E and no Biored®). During week 4 (21-28 weeks), birds in the two treatment groups (treatment 2 and 3) that received no additional vitamin A and E in their feed as well as birds from treatment 5, with only 50% vitamin A and E without Biored®, had significantly lower intakes than birds in the Positive control. During the last week of the study (day 28-35), the two groups of birds that did not receive additional vitamin A and E in their feed showed significantly lower intakes than the Positive control. Birds in treatment 3, which contained 150g/ton of Biored® in the diet and no vitamin A and E, had significantly lower intakes than birds of treatment 1 and treatment 8 (50% vitamin A and E and 200g/ton Biored®). The lowered intakes of birds in treatment 2 and 3 reflected in the day 35 body weights. The treatments containing Biored® did

not have higher feed intakes than the control diet. Birds in treatment 2 (Negative Control) and 3 (0% vitamin A and E, 150 g/ton Biored®) had significantly lower ($p<0.05$) feed intakes over the 35 day period than the birds in the Positive Control group (treatment 1). Birds in treatment 5 also had lower ($p<0.05$) intakes compared to birds in treatment 1. Birds in the Positive Control group had the highest feed intake of all the treatments and birds in the Negative Control the lowest.

4.1.3 Average Daily Gain (ADG)

Table 4.3 ADG (g/day) of chickens during a 5 week trial period receiving 8 different dietary treatments containing different concentrations of vitamin A and E and different concentrations of a bioflavonoid based product, Biored®

Treatment	Day 0-7	Day 7-14	Day 14-21	Day 21-28	Day 28-35	Day 0-35
1	18.51^{b}	37.09^{bc}	57.87^{c}	97.86^{b}	110.81^{bc}	67.15^{d}
2	17.14^{a}	35.13^{a}	50.04^{a}	90.88^{a}	102.14^{a}	61.60^{a}
3	17.77^{ab}	36.08^{ac}	51.25^{ab}	91.77^{a}	103.54^{ab}	62.61^{a}
4	18.35^{b}	38.00^{c}	56.83^{c}	92.25^{a}	111.50^{c}	65.84^{cd}
5	17.67^{ab}	34.41^{a}	54.05^{b}	96.36^{b}	109.36^{abc}	64.85^{bc}
6	17.91^{ab}	35.99^{ab}	56.35^{c}	96.51^{b}	108.43^{ac}	65.27^{cd}
7	17.79^{ab}	36.17^{ac}	55.02^{c}	98.59^{b}	106.68^{ac}	65.52^{cd}
8	17.81^{ab}	36.18^{ac}	56.78^{c}	96.83^{b}	107.45^{ac}	66.52^{cd}
SE	0.34	0.70	1.20	1.20	2.70	0.77
R^2	0.525	0.457	0.515	0.612	0.256	0.545

^{abcd} Column means with the same superscripts do not differ significantly from each other ($P < 0.05$)
*T1- 100% Vitamin A and E, 0 g/ton Biored®; T2- 0% Vitamin, 0 g/ton Biored®; T3-0% Vitamin A and E, 150 g/ton Biored®; T4- 100% Vitamin A and E, 150 g/ton Biored®; T5- 50% Vitamin A and E, 0 g/ton Biored®; T6- 50% Vitamin A and E, 100 g/ton Biored®; T7- 50% Vitamin A and E, 150 g/ton Biored®; T8- 50% Vitamin A and E, 200 g/ton Biored®.

ADG of birds in different treatment groups are presented in Table 4.3.

Birds in the Negative Control group (0% vitamin A and E, 0 g/ton Biored®) had the lowest growth rate throughout the trial period, which was consistently significantly lower ($P \leq 0.05$) than birds in the Positive Control (100% vitamin A and E, 0 g/ton Biored®). From Day 14 onwards the growth rate of birds in treatment 3 (0% vitamin A and E, 150 g/ton) was also significantly lower than birds in the Positive control group. Birds in treatment 4 (100% vitamin A and E, 150 g/ton Biored®) showed a lower body weight gain during week 4 which was significantly lower ($P \leq 0.05$) compared to birds in the Positive control (treatment 1), which is also apparent in the day 28 body weight of the group. Birds in all the other treatments performed similar to birds in the Positive Control. Over the whole 35 day period, birds in treatments receiving no vitamin supplementation (treatment 2 and 3) had a significantly ($p<0.05$) lower growth rate compared to

birds in all the other treatments. Birds of treatment 5 (50% vitamin A and E, 0 g/ton Biored®) also had a lower ($p<0.05$) growth rate compared to birds in treatment 1.

4.1.4 Feed Conversion Ratio

Feed Conversion Ratio (FCR) of broilers in different treatment groups are shown in Table 4.4.

The FCR values were calculated correcting for mortalities that occurred during the weeks.

No significant differences ($P > 0.05$) in FCR occurred between birds in the first two weeks of the study.

During week 3 (day 14-21), broilers from the Negative Control (treatment 2) had the highest FCR differing significantly ($P \leq 0.05$) from birds in treatment 4, 5, 6 and 7. Broilers in treatment 4 with 100% vitamin A and E and 150g/ton Biored® had the best FCR that significantly differed from broilers in treatment 2 and 3 that had no vitamin additions.

During week 5 (28-35 days), birds in treatment 8 (50% vitamin A and E, 200 g/ton Biored®) had the highest FCR and differed significantly form birds in treatment 1, 2, 5 and 6. The highest FCR over the whole 35 day growth period was reported for birds in treatment 2 (Negative Control) followed by birds of treatment 3 (0% vitamin A and E) and treatment 8 (50% vitamin A and E, 200 g/ton Biored®).

Table 4.4 Feed conversion ratio of chickens during a 5 week trial period receiving 8 different dietary treatments containing different concentrations of vitamin A and E and different concentrations of a bioflavonoid based product, Biored®

Treatment	Day 0-7 (Week 1)	Day 7-14 (Week 2)	Day 14-21 (Week 3)	Day 21-28 (Week 4)	Day 28-35 (Week 5)	Day 0-35 (Cumulative)
1	1.26	1.55	1.41^{cd}	1.46^{ab}	1.62^{b}	1.49^{ac}
2	1.33	1.49	1.49^{ac}	1.49^{a}	1.61^{b}	1.52^{a}
3	1.29	1.48	1.46^{bc}	1.45^{ab}	1.65^{ab}	1.51^{ab}
4	1.30	1.46	1.37^{d}	1.51^{ab}	1.65^{ab}	1.49^{ac}
5	1.28	1.53	1.40^{bd}	1.45^{ab}	1.60^{b}	1.48^{c}
6	1.32	1.52	1.40^{bd}	1.45^{ab}	1.64^{b}	1.49^{ac}
7	1.22	1.47	1.40^{bd}	1.42^{b}	1.66^{ab}	1.48^{bc}
8	1.33	1.48	1.42^{cd}	1.45^{ab}	1.71^{a}	1.51^{ac}
SE	0.063	0.046	0.031	0.024	0.022	0.011
R^2	0.110	0.166	0.256	0.390	0.336	0.297

^{abcd} Column means with the same superscripts do not differ significantly from each other ($P<0.05$)

*T1- 100% Vitamin A and E, 0 g/ton Biored®; T2- 0% Vitamin, 0 g/ton Biored®; T3-0% Vitamin A and E, 150 g/ton Biored®; T4- 100% Vitamin A and E, 150 g/ton Biored®; T5- 50% Vitamin A and E, 0 g/ton Biored®; T6- 50% Vitamin A and E, 100 g/ton Biored®; T7- 50% Vitamin A and E, 150 g/ton Biored®; T8- 50% Vitamin A and E, 200 g/ton Biored®.

4.1.5 Mortalities

No significant differences (P > 0.05) were found between treatments for mortalities.

Table 4.5 Cumulative mortalities of broilers for the 35 day grow out period receiving 8 different dietary treatments containing different concentrations of vitamin A and E and different concentrations of a bioflavonoid based product, Biored®

Treatment	Mortalities
1	14
2	9
3	10
4	15
5	14
6	15
7	9
8	13
Chi- Square	3.869
DF	7
Pr > ChiSq	0.7948

*T1- 100% Vitamin A and E, 0 g/ton Biored®; T2- 0% Vitamin, 0 g/ton Biored®; T3-0% Vitamin A and E, 150 g/ton Biored®; T4- 100% Vitamin A and E, 150 g/ton Biored®; T5- 50% Vitamin A and E, 0 g/ton Biored®; T6- 50% Vitamin A and E, 100 g/ton Biored®; T7- 50% Vitamin A and E, 150 g/ton Biored®; T8- 50% Vitamin A and E, 200 g/ton Biored®.

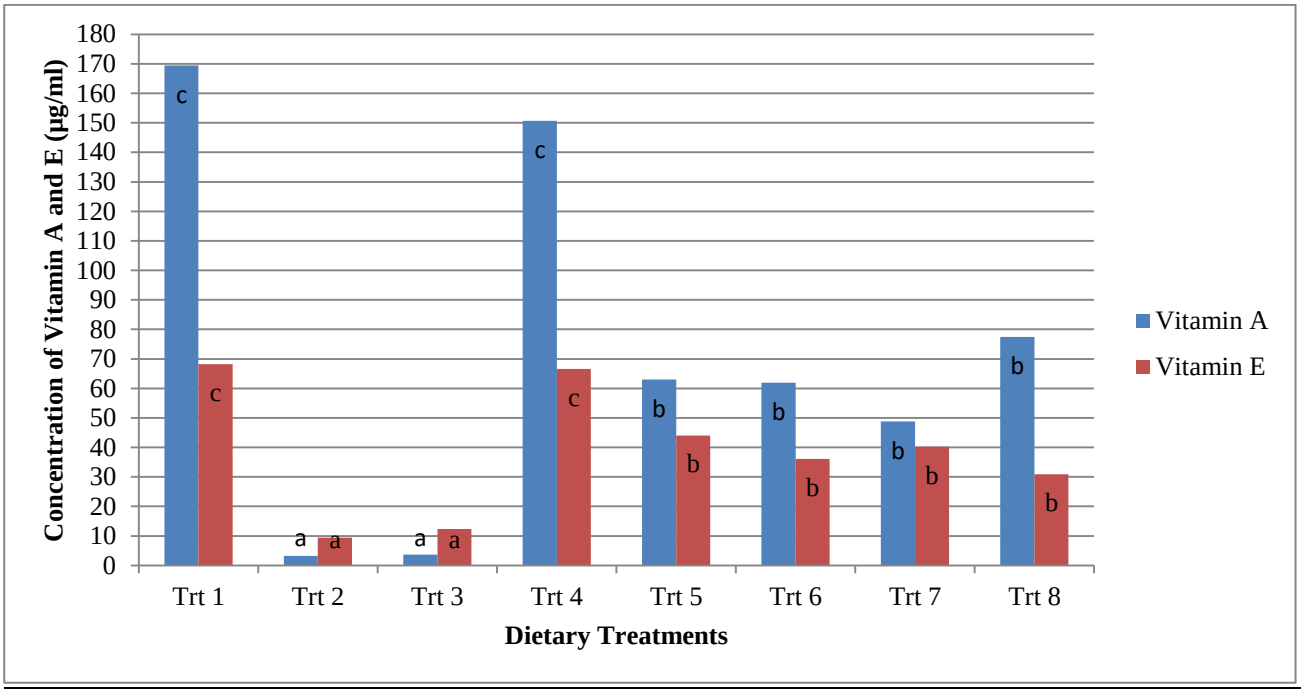
4.2 Vitamins in the liver

Concentrations of Vitamin A and E in the liver of broilers are shown in Table 4.6 and Figure 4.2. Birds of treatments 1 (Positive Control) and 4 (100% vitamin A and E, 150 g/ton Biored®) had the highest vitamin concentrations in the liver at slaughter. On the other hand birds of treatment 2 and 3 (no additional vitamin A and E in the diets with 0 and 150 g/ton Biored® respectively) had the lowest concentrations of vitamin A and E in the liver compared to all the other treatments. Vitamin concentrations in livers from broilers of treatments 5, 6, 7 and 8 were higher than the concentrations of birds that did not receive additional vitamin A and E in the feed but lower than those that received 100% of the standard vitamin A and E levels. Broilers in treatment 5 that had no Biored® and only 50% vitamin A and E in the diet showed no significant differences in the concentrations of these two vitamins in the liver than birds of treatment 6, 7 and 8 which contained incremental levels of Biored® at 100 g/ton, 150 g/ton and 200 g/ton, respectively.

Table 4.6 Vitamin A and E concentrations in the liver of Broilers receiving 8 different dietary treatments containing different concentrations of vitamin A and E and different concentrations of a bioflavonoid based product, Biored®

Treatment	Vitamin A (µg/ml)	Vitamin E (µg/ml)
1	169.45[c]	68.16[c]
2	3.20[a]	9.43[a]
3	3.70[a]	12.32[a]
4	150.66[c]	66.54[c]
5	62.97[b]	44.03[b]
6	61.88[b]	36.11[b]
7	48.82[b]	40.18[b]
8	77.48[b]	30.87[b]
SE	10.27	6.52
R^2	0.850	0.676

[abcd] Column means with the same superscripts do not differ significantly from each other ($P < 0.05$)
*T1- 100% Vitamin A and E, 0 g/ton Biored®; T2- 0% Vitamin, 0 g/ton Biored®; T3-0% Vitamin A and E, 150 g/ton Biored®; T4- 100% Vitamin A and E, 150 g/ton Biored®; T5- 50% Vitamin A and E, 0g/ton Biored®; T6- 50% Vitamin A and E, 100 g/ton Biored®; T7- 50% Vitamin A and E, 150 g/ton Biored®; T8- 50% Vitamin A and E, 200 g/ton Biored®.



[abcd] Column means with the same letters do not differ significantly from each other (P < 0.05)
*T1- 100% Vitamin A and E, 0 g/ton Biored®; T2- 0% Vitamin, 0 g/ton Biored®; T3-0% Vitamin A and E, 150 g/ton Biored®; T4- 100% Vitamin A and E, 150 g/ton Biored®; T5- 50% Vitamin A and E, 0 g/ton Biored®; T6- 50% Vitamin A and E, 100 g/ton Biored®; T7- 50% Vitamin A and E, 150 g/ton Biored®; T8- 50% Vitamin A and E, 200 g/ton Biored®

Figure 4.2 Vitamin A and E concentrations in the liver of broilers at the end of a 35 day grow out period receiving 8 different dietary treatments containing different concentrations of vitamin A and E and different concentrations of a bioflavonoid based product, Biored®

4.3 Glutathione/Reduced Glutathione ratio (GSH/GSSG) of whole blood

Values for GSH and GSSG in blood of broilers to indicate oxidative stress in the body is shown in Table 4.7. No significant differences (P > 0.05) were present in the GSSG levels in blood of chicken on day 0 or 35 and in GSH levels on day 0 of the trial. The GSH:GSSG ratio on day 35 of birds in treatment 8 where broilers received 200 g/ton Biored® and 50% of the standard levels of vitamin A and E in the feed, had a significant higher GSH:GSSG ratio than birds in the Positive Control group as well as birds in treatments 3, 4 and 5.

Table 4.7: Whole blood reduced glutathione (GSH), oxidised glutathione (GSSG) and GSH:GSSG as a marker of oxidative stress in whole blood of broilers receiving 8 different dietary treatments containing different levels of vitamin A and E and different concentrations of a bioflavonoid based product, Biored®

Treatment	GSH Day 0	GSSG Day 0	GSH:GSSG Day 0	GSH Day 35	GSSG Day 35	GSH:GSSG Day 35
1	1276.5	154.0	7.29	1091.8^{ab}	187.1	4.84^{a}
2	1125.3	151.5	6.80	1108.3^{ab}	185.9	4.97^{ab}
3	1363.6	152.0	8.10	1078.9^{a}	186.1	4.81^{a}
4	1295.1	162.1	7.00	1077.5^{a}	184.1	4.86^{a}
5	1173.8	159.6	6.33	1028.3^{a}	182.4	4.65^{a}
6	1220.9	154.8	7.77	1129.9^{ab}	185.3	5.11^{ab}
7	1233.0	155.8	7.01	1087.4^{ab}	183.0	4.95^{ab}
8	1205.3	159.1	6.73	1271.5^{b}	186.1	5.84^{b}
SE	140.9	0.34	1.10	66.62	0.34	0.325
R^2	0.457	0.525	0.515	0.457	0.525	0.515

^{abcd} Column means with the same superscripts do not differ significantly from each other (P > 0.05)
*T1- 100% Vitamin A and E, 0 g/ton Biored®; T2- 0% Vitamin, 0 g/ton Biored®; T3-0% Vitamin A and E, 150 g/ton Biored®; T4- 100% Vitamin A and E, 150 g/ton Biored®; T5- 50% Vitamin A and E, 0 g/ton Biored®; T6- 50% Vitamin A and E, 100 g/ton Biored®; T7- 50% Vitamin A and E, 150 g/ton Biored®; T8- 50% Vitamin A and E, 200 g/ton Biored®

4.4 Meat quality measurements

Table 4.8 shows the drip loss, cooking loss and shear force values for the left half of the breast (pectoralis major) of broiler chicken. There was no significant difference (P > 0.05) in drip loss between any of the birds in the treatments. Birds in the Negative Control (0% vitamin A and E, 0 g/ton Biored®) had the highest drip loss value whereas birds in treatment 8 (50% vitamin A and E, 200 g/ton Biored®) had the lowest drip loss. Significant differences (P > 0.05) were found between birds in treatment 8 (50% vitamin A and E, 200 g/ton Biored®) with a significantly lower (P ≤ 0.05) drip loss than the breast meat of the Negative Control (treatment 2) and also treatment 5 (no Biored®, 50% vitamin A and E) and birds in treatment 7 (150g/ton Biored®, 50% vitamin A and E). No statistically significant (P > 0.05) differences were found in loss of moisture during cooking of the breast meat between treatments. Breast meat of treatment 4 (150g/ton Biored® and 100% vitamin A and E) had the highest shear force measurements and differed significantly (P ≤ 0.05) from the breast meat from the Negative Control (treatment 2) and treatment 7 (150g/ton Biored® and 50% Vitamin A and E). The lowest shear force measurement was noted for the breast meat of birds in the Negative Control (P > 0.05).

Table 4.8: Drip loss (%), cooking loss(%) and shear force (N) measurements of the left half of the breast portions of broilers receiving 8 different dietary treatments containing different concentrations of vitamin A and E and different concentrations of a bioflavonoid based product, Biored®

Treatment	Drip loss	Cooking loss	Shear Force
1	2.11^{ab}	13.97	44.21^{ab}
2	2.43^{a}	14.49	40.74^{b}
3	2.21^{ab}	13.01	42.76^{ab}
4	2.15^{ab}	13.52	49.44^{a}
5	2.26^{ab}	13.81	42.65^{ab}
6	1.75^{ab}	13.01	43.85^{ab}
7	2.37^{a}	14.01	41.85^{b}
8	1.63^{b}	13.07	45.64^{ab}
SE	0.251	1.03	2.64
R^2	0.525	0.525	0.457

^{abc} Column means with the same superscripts do not differ significantly from each other (P > 0.05)
*T1- 100% Vitamin A and E, 0 g/ton Biored®; T2- 0% Vitamin, 0 g/ton Biored®; T3-0% Vitamin A and E, 150 g/ton Biored®; T4- 100% Vitamin A and E, 150 g/ton Biored®; T5- 50% Vitamin A and E, 0 g/ton Biored®; T6- 50% Vitamin A and E, 100 g/ton Biored®; T7- 50% Vitamin A and E, 150 g/ton Biored®; T8- 50% Vitamin A and E, 200 g/ton Biored®.

4.5 Carcass Traits

The birds of the Negative Control (treatment 2) and treatment 3 (0% supplementary vitamin A and E, 150 g/ton Biored®) had significantly lower ($p < 0.05$) carcass weights than the birds of the Positive Control group (treatment 1). The thigh portions of birds in treatment 2 (Negative Control) differed significantly ($p<0.05$) from birds in reatments 1 (Positive Control), 5 (50% vitamin A and E, 0 g/ton Biored®) and 8 (50% vitamin A and E, 200 g/ton Biored®). Birds of treatment 1 and 8 had the heaviest portions. The only significant difference in breast meat portions were between birds of treatment 1 (Positive Control) and 2 (Negative Control) with birds in the Positive Control that had the heaviest portion and birds in treatment 2 the lightest. Treatment 8 also had heavier meat portions than the rest of the birds in the other treatments but it was not significant ($P > 0.05$). There was no significant difference ($P > 0.05$) between the proportional weights of the thigh and breast meat samples to carcass weights.

Table 4.9: Weights of the carcass, right thigh, right half of the breast portion and proportional weights of broiler chicken on day 35 receiving 8 different dietary treatments containing different concentrations of vitamin A and E and different concentrations of a bioflavonoid based product, Biored®

Treatment	Carcass weight (kg)	Right thigh (g)	Right half of the breast (g)	Right thigh proportional to carcass	Right half of the breast proportional to carcass
1	1.81^{bc}	141.71^{b}	255.29^{b}	1.28	14.07
2	1.66^{ab}	129.54^{a}	236.42^{a}	1.28	14.23
3	1.73^{b}	135.88^{ab}	246.50^{ab}	1.27	14.27
4	1.79^{bc}	138.96^{ab}	249.21^{ab}	1.29	13.90
5	1.79^{bc}	140.83^{b}	246.00^{ab}	1.28	13.74
6	1.75^{bc}	133.13^{ab}	247.67^{ab}	1.32	14.18
7	1.79^{bc}	138.27^{ab}	244.83^{ab}	1.30	13.67
8	1.78^{bc}	141.33^{b}	253.33^{ab}	1.26	14.19
SE	0.025	3.34	6.38	0.026	0.249
R^2	0.469	0.345	0.212	0.204	0.285

^{abc} Column means with the same superscripts do not differ significantly from each other ($P > 0.05$)
*T1- 100% Vitamin A and E, 0 g/ton Biored®; T2- 0% Vitamin, 0 g/ton Biored®; T3-0% Vitamin A and E, 150 g/ton Biored®; T4- 100% Vitamin A and E, 150 g/ton Biored®; T5- 50% Vitamin A and E, 0 g/ton Biored®; T6- 50% Vitamin A and E, 100 g/ton Biored®; T7- 50% Vitamin A and E, 150 g/ton Biored®; T8- 50% Vitamin A and E, 200 g/ton Biored®.

4.6 Shelf life of thigh meat

Table 4.10 show the TBARS values of the thigh meat portions used in the shelf life study over a period of 6 days. Lipid peroxidation was measured by quantifying the TBARS content in thigh meat stored in retail simulated conditions. Three samples were collected over a six day period.

On day 0 of storage only meat portions of birds in treatment 3 (150 g/ton Biored® and 0% vitamin A and E) differed significantly from all the other treatments with a higher TBARS count than the other treatments. On both day 3 and day 6 of storage, the two groups that did not receive additional vitamin A and E in their feed (treatment 2 and treatment 3) showed significantly higher ($P \leq 0.05$) degrees of lipid peroxidation in the thigh meat portions than in birds of the Positive Control (treatment 1). No other differences were noted between thigh portions of different treatments.

Table 4.10 TBARS value (nmol/g) of thigh meat of broilers over a period of 6 days in broilers receiving 8 different dietary treatments containing different concentrations of vitamin A and E and different concentrations of a bioflavonoid based product, Biored®

Treatment	TBARS Day 0	TBARS Day 3	TBARS Day 6
1	10.68^{b}	10.46^{cd}	11.53^{b}
2	12.09^{b}	12.34^{ab}	16.78^{a}
3	17.90^{a}	12.98^{a}	16.18^{a}
4	11.49^{b}	9.41^{d}	10.66^{b}
5	11.74^{b}	10.41^{cd}	10.62^{b}
6	11.07^{b}	10.17^{cd}	10.99^{b}
7	12.26^{b}	10.55^{bcd}	11.13^{b}
8	10.51^{b}	11.30^{abc}	11.77^{b}
SE	1.98	0.656	1.30
R^2	0.525	0.525	0.457

^{abcde} Column means with the same superscripts do not differ significantly from each other (P > 0.05)
*T1- 100% Vitamin A and E, 0g/ton Biored®; T2- 0% Vitamin, 0g/ton Biored®; T3-0% Vitamin A and E, 150g/ton Biored®; T4- 100% Vitamin A and E, 150g/ton Biored®; T5- 50% Vitamin A and E, 0g/ton Biored®; T6- 50% Vitamin A and E, 100g/ton Biored®; T7- 50% Vitamin A and E, 150g/ton Biored®; T8- 50% Vitamin A and E, 200g/ton Biored®.

Chapter 5

Discussion

5.1 Performance data

Several studies have been done on flavonoid feed additives with variable results pertaining to broiler performance. In this study, the body weights (BW) of birds on day 7 and day 14 did not show extensive differences. This can be due to the fact that chicks are hatched with a store of fat-soluble as well as water soluble vitamins and other antioxidants and antioxidant enzymes originating from the maternal diet that is carried over to the embryo (Karadas *et al*., 2011). The newly hatched chick still contains a yolk sac originating from the egg, which provides it with sufficient nutrients until exogenous feed is consumed. This yolk sac makes up about 15-20% of the body weight of the chick at hatch and after the first 48hr of life, 90% is absorbed by the chick. After 10 days the whole yolk sac contents are absorbed (Esteban *et al*., 1990; Yadgary *et al*., 2010). On days 21, 28 and 35, broilers from both treatment 2 (negative control) and 3 (no vitamin A and E inclusion and 150g/ton Biored®) consistently had the lowest weight compared to birds of treatment 1 (100% vitamin A and E inclusion).

Vitamin E is one of the most important natural antioxidants and being fat soluble, is involved in processes preventing lipid peroxidation. It is an essential nutrient that needs to be supplemented in the diet as broilers do not synthesise the vitamin in their bodies (Fellenberg and Speisky, 2005). The clinical signs of a vitamin E deficiency typically include retarded growth, exudative diathesis, encephalomalacia and several other diseases as well as a high morbidity rate (Avonzo *et al*., 2001; Yuming *et al*., 2001). A deficiency of vitamin A can lead to poor growth performance as well as poor bone development (Li *et al*., 2008). These severe symptoms of the vitamin E deficiency were not seen in broilers of the negative control treatment. This might be due the fact that the basal diet contained these vitamins in the raw materials in sufficient quantities to prevent severe deficiencies. Commercial premixes are formulated to provide these vitamins in excess of what the animal needs to make up for any unforeseen losses during mixing or storage, or when birds might be subjected to stressors that increase their need for nutrients and sustain high rates of growth and production (Moravej *et al*., 2012).

Broilers receiving treatment 3 ate feed that only contained 150 g/ton of Biored® and no extra vitamin A and E. The birds in this treatment performed similar to birds in the negative control (treatment 2). Biored® (at 150 g/ton) was not able to replace vitamin A and E. Birds in treatment 5, having only 50% of the vitamin A and E specification (no Biored®), also had significantly lower BWs ($P \leq 0.05$) compared to birds receiving the positive control diet but had heavier weights than birds in the treatments with no vitamin inclusions. The amount of vitamins was included in a high enough concentration to have a growth promoting affect but not high enough compared to birds in the Positive Control treatment.

At the end of the trial, the body weights of Treatment groups 6, 7 and 8 that only received 50% vitamin A and E, with incremental levels of Biored®, did not differ significantly from the Positive Control (Treatment 1) while Treatment 5 (50% vitamin A and E, 0 g/ton Biored®) had significantly lower body weights than the Positive Control. This gives an indication that the bioflavonoid additive, Biored®, had a positive effect on body weight of broilers at low levels of dietary vitamin A and E. The body weights of Treatments 6, 7 and 8, however, were numerically, but not significantly, lower than the Positive Control (Treatment 1). Although not significant, there was a tendency that growth rate improved as Biored® was added to the feed (at 50% vitamin A and E) in increasing concentrations (0, 100, 150 and 200 g/kg). The birds in Treatment 5, that received no Biored® and only 50% vitamin A and E, had lower BWs than the treatment groups that contained Biored® (Treatment 6, 7 and 8) though the differences were not statistically significant ($P > 0.05$).

On day 35 the average difference in BW between Treatment 5 (50% vitamin A and E, 0 g/ton Biored®) and 6 (50% vitamin A and E, Biored® 100 g/ton Biored®) was 15 g, between Treatment 5 and 7 (50% vitamin A and E, 150 g/ton Biored®) was 20 g and between Treatment 5 and 8 (50% vitamin A and E, 200 g/ton Biored®), 60 g. Although these differences between the BWs were not significant it indicates that even higher inclusion levels of Biored® than used in this trial might have showed a more positive effect on BW gain. Biored® had no growth promoting effect when supplemented together with standard (100%) levels of vitamin A and E.

Feed intake was not affected by the bioflavonoid based feed additive, Biored®. Only the vitamin deficient diets (Treatment 2 and 3; 0% vitamin A and E) showed significant lower feed intakes compared to birds in the Positive Control group (treatment 1) during week 4 and 5 of the study. Goñi *et al*. (2007) did not see an effect in performance parameters like feed intake and FCR when he fed grape pomace (GP) at different concentrations to male Cobb broilers. Erener *et al*. (2011) on the other hand used Green Tea Extract (GTE) in their study with treatments containing 0, 0.1 and 0.2 g/kg in the feed. They found that the group consuming 0.1g/kg GTE consumed more feed than the control group did and explained it being due to the birds having a higher nutrient requirement to sustain higher growth rates and a larger body size.

The lower intakes of birds in treatment 2 and 3 reflected in the day 35 body weights. Birds in treatment 2 (Negative Control), 3 (0% vitamin A and E, 150 g/ton Biored®) and treatment 5 (50% vitamin A and E, 0 g/ton Biored®) had significantly lower feed intakes during the 35 day rearing period than birds in treatment 1 (Positive Control). Cumulative feed intake measured over the 35 day period showed that birds in treatment 5 (50% vitamin A and E, 0 g/ton Biored®) had a lower feed intake than birds in treatment 1 (Positive Control), while birds in treatment 6, 7 and 8, with incremental levels of Biored®, did not have a significantly different feed intake than birds of treatment 1. Biored® might have increased the feed intake levels of the chicken. Birds in treatment 8 (50% vitamin A and E, 200 g/ton) had the highest feed inclusion level of Biored® and had the highest feed intake level of all the treatments supplemented with the additive.

Throughout the trial birds in the Negative Control group (treatment 2) showed the lowest growth rate (measured as average daily gain) which was significantly lower ($P \leq 0.05$) than birds in the Positive Control

(treatment 1). From Day 14 onwards the growth rate of birds in treatment 3 was also significantly lower than the birds in the Positive control group. All other treatments had a growth rate not statistically significant ($P>0.05$) different to the birds of the Positive Control. In an unpublished study, Holstein steers receiving a diet with half the standard vitamin A and E specification and supplemented with Biored®, had a significantly higher average daily gain than steers that received the standard level of vitamin A and E in the diet without supplemented Biored® (unpublished data, personal communication: Mr. S. Slippers). Many factors influence the absorption of flavonoids including dosage, form that it is supplemented in, the diet of the animal, sex differences and the microbial population that is present in the colon (Heim *et al*., 2002).

FCR (feed conversion ratio) is a measure of performance level of an animal and measure the efficiency with which an animal converts its feed into body mass. Biored® had no effect on the FCR when compared to the Positive Control (100% vitamin A and E, 0g/ton Biored®) calculated during the 35 day period. According to Erener *et al*. (2011) the variation in feed efficiency and body weight between different studies can be due to several factors including the composition of the basal diet, the source and way in which the polyphenol feed additive was manufactured and in their case where Green Tea extract was used, the time of harvest of the leaves as well as environmental conditions.

5.2 Vitamins in the liver

Sahin *et al*. (2002) demonstrated that vitamin A and E act in a synergistic way to prevent oxidative stress caused by heat stress in poultry. Vitamin E cannot be synthesised inside the body by the animal itself so the amount present in the body is a reflection of the dietary intake. The order in which body tissue respond to intake of the vitamin is as follows: lungs/heart > liver > thigh meat > brain. The liver is a sensitive indicator for detection of dietary levels of α-tocopherol (Jensen *et al*., 1998; Guo *et al*., 2001; Voljč *et al*., 2011).

Almost 90% of the body's reserves of vitamin A are stored in the liver and there is an almost linear relationship between the vitamin concentrations in the liver and the feed (Johannsen *et al*., 1998). This can be seen in the results of the analysed liver samples of these vitamins in this study indicating that analysis of vitamin A and E in feed was probably flawed as stated previously because of the protective coating around the vitamins. Vitamin concentrations in livers from broilers of treatments 5 (50% vitamin A and E, 0 g/ton Biored®), 6 (50% vitamin A and E, 100g/ton Biored®), 7 (50% vitamin A and E, 150 g/ton) and 8 (50% vitamin A and E, 200 g/ton), that received half of the vitamin specification of the birds in the Positive Control, were higher than the concentrations of birds that did not receive additional vitamin A and E in the feed (treatment 2 and treatment 3) but lower than treatments that received 100% of the standard vitamin A and E concentrations. The addition of the additive Biored® did not seem to have a vitamin sparing effect on the vitamin A and E concentrations in the liver. Birds in treatment 1 (100% vitamin A and E, 0 g/ton Biored®) had the highest concentrations of both vitamin A and E in the liver, while birds in treatment 3 (0% vitamin A and E, 150g/ton Biored®) had similar concentrations of both vitamin A and E in the liver as birds in the Negative Control (0% vitamin A and E, 0 g/ton Biored®). Birds in treatment 5 (50% vitamin A and E,

0 g/ton Biored®) had similar vitamin A and E in the liver than birds of treatment 6, 7 and 8 that all had half the vitamin A and E specification and different incremental levels of Biored®. These results indicate that Biored® might not have a sparing effect on the antioxidative vitamins but have a different mode of action when it comes to performance as Biored® did have an effect on the body weight of broilers as discussed previously. Surai (2012) determined in their review on polyphenol compounds that the mode of action of antioxidant may not be the most important and only way in which they exert their function in the animal body. According to a review by Kamboh *et al*. (2015) flavonoids have shown to have numerous functions in *in vitro* and animal model studies that include anti-inflammatory, antibacterial and antiviral, hepatoprotective, antiallergic, antithrombotic, anticarcinogenic and immunomodulatory properties. It also can play a role in modulating oxidation reactions, detoxification of enzymes, apotosis and host immune system reactions.

5.3 Oxidative stress in whole blood

GSH and GSSG were measured in the whole blood of broilers to determine whether any of the treatment groups experienced oxidative stress due to either omission or lowering of vitamin A and E levels in the feed. Whole blood GSH and GSSG levels have been suggested to be an index of the oxidative status of the entire body (Giustarini *et al*., 2003). GSH is a potent antioxidant enzyme found in small concentrations in many tissues of the body including whole blood. GSH releases its H atom and donates it to a free radical or an oxidising substance in order to stabilise the molecule and in the process becomes the oxidised molecule GSSG (Wang *et al*., 1997; Fellenberg and Speisky, 2006). The decrease in the level of GSH, the increase in concentration of GSSG and the decrease in the GSH:GSSG has been suggested to be linked to contributing to certain diseases in humans like lung inflammation and Alzheimer disease (Giustarini *et al*., 2003).

At the end of the trial, on day 35, the GSH:GSSG ratio of birds in treatment 8 (50% vitamin A and E, 200g/ton Biored®) was significantly higher ($P < 0.05$) than birds in the Positive Control group (100% vitamin A and E, 0 g/ton Biored®) as well as birds in treatment 3 (0% vitamin A and E, 150 g/ton Biored®), 4 (100% vitamin A and E, 150 g/ton Biored®) and 5 (50% vitamin A and E, 0 g/ton Biored®) which indicates that this group experienced a lower level of oxidative stress than any of the other groups of broilers. Birds in treatment 5 (50% vitamin A and E, 0 g/ton Biored®) had the lowest ratio of GSH:GSSG indicating the highest level of oxidative stress although it was not significantly lower than the birds in the Positive Control (100% vitamin A and E, 0 g/ton Biored®). In broilers, Biored® at the highest inclusion rate of 200 g/ton, did have a lower level of oxidative stress compared to birds in the Control diet, treatment 3 (0% vitamin A and E, 150g/ton), treatment 4 (100% vitamin A and E, 150 g/ton Biored®) and treatment 5 (50% vitamin A and E, 0 g/ton Biored®) that indicates Biored® at a higher inclusion level of 200 g/ton might help suppress oxidative stress when subjected to lower levels of vitamin A and E.

5.4 Meat quality measurements

The muscle pH, drip loss (also known as water-holding capacity), shear force and meat colour are mainly used to determine meat quality. Drip loss and shear force are important meat quality factors for consumers and meat processors. Post slaughter drip loss is caused by the expulsion of water that is bound to myofibrils or that is present in extracellular protein. This loss of water is brought about by changes in pH, shrinkage of myofibrils and breakdown of protein in the costameres post slaughter (Allen *et al*., 1998). It influences the taste, succulence, colour, nutrient content and flavour of meat (Wei *et al*., 2014). A low water holding capacity of meat indicates drier and tougher meat due to loss of moisture and nutrients (Cao *et al*., 2012). Drip loss was measured on the right breast meat portion of the chickens. There were no significant differences ($P > 0.05$) in drip loss measurements in the breast meat of any of the dietary treatments compared to birds in the Positive Control (100% vitamin A and E, 0 g/ton Biored®). The breast meat of birds in the Negative Control (0% vitamin A and E, 0 g/ton Biored®) had the highest amount of drip loss which was significantly higher ($P < 0.05$) than birds of treatment 8 (50% vitamin A and E, 200 g/ton of Biored®) that had the lowest drip loss value. The high level of drip loss in meat of birds in treatment 2 could be due to the low vitamin E content of the diet. It is proposed that vitamin E prevents the oxidation of the phospholipids in the cell membranes in the meat helping to keep the cell membrane integrity and preventing the leaking out of the sarcoplasmic fluid through the cell membrane (Gray *et al*., 1996).

No statistically significant ($P > 0.05$) differences were found between treatments in loss of moisture during cooking of the breast meat.

Shear force gives an indication of meat tenderness and can provide information on the internal structure of the meat, muscle myofibril content, fat content, as well as the distribution and chemical structure of muscle connective tissue (Wei *et al*., 2014). Breast meat of birds in treatment 4 (150g/ton Biored® and 100% vitamin A and E) had the highest shear force measurements and differed significantly ($P \leq 0.05$) from the meat of birds in the Negative Control (Treatment 2) and treatment 7 (150g/ton Biored® and 50% Vitamin A and E). The lowest shear force measurement was noted for breast meat of birds in the Negative Control (0% vitamin A and E, 0 g/ton Biored®) group.

Other studies showed that flavonoids did have effects on meat quality. Cao *et al*. (2012) used fermented *Ginkgo biloba* leaves in his study to determine its effect on growth performance, meat quality and lipid metabolism in broilers. *Ginkgo bilboa* has high concentrations of flavonoids as well as polysaccharides. Their study indicated that diets containing the fermented form of the plant leave had significant effects on the 24 hour drip- and cooking loss percentage and shear force. Increasing levels of fermented *Ginkgo biloba* concentrations improved water holding capacity significantly compared to the normal diet. This was true for the cooking losses and shear force values as well. They contributed this improvement to enhanced antioxidative status due to elevated α-Tocopherol content in blood plasma. Peña *et al*. (2008) in their experiment put broilers under heat stress, fed them a mixture of ascorbic acid and Citric Flavonoids and then measured their performance and meat quality. The product used contained 10% ascorbic acid and 0.7%

flavonoids of which 50% consisted of quercetin and 50% rutin. Four treatments were tested containing 0, 250, 500 or 1000 g/ton of the product. No significant differences were found for shear force measurements but the product increased breast meat water loss at the age of 32 days of age. Genistein and hesperidin supplementation on the other hand improved water holding capacity for treatments containing 5 mg/kg genistein, 20 mg/kg hesperidin, a mixture of genistein and hesperidin at 5 mg/kg, 10 and 20 mg/kg. There was no difference in meat texture (including meat tenderness) between treatments which were judged by a sensory panel.

5.5 Carcass Traits

The carcass weights of birds in the Negative Control (0% vitamin A and E, 0 g/ton Biored®) and treatment 3 (0% vitamin A and E, 150 g/ton Biored®) were significantly lower ($P < 0.05$) than the birds in the Positive Control group (100% vitamin A and E, 0 g/ton Biored®). This is in agreement with their body weights on day 35, that was also significantly lower ($P < 0.05$) than the birds in the Positive Control (100% vitamin A and E, 0 g/ton Biored®). Birds in treatment 3 (0% vitamin A and E, 150 g/ton Biored®) had significantly lower carcass weights than birds in treatment 1 (100% vitamin A and E, 0 g/ton Biored®) suggesting that Biored® alone cannot compensate for the total absence of vitamin A and E in the diet. Birds in treatment 5 (50% vitamin A and E, 0 g/ton Biored®) did not have significantly lower carcass weights than birds in treatment 6, 7 and 8, with different levels of Biored®, which indicates that it did not have an effect on final carcass weights. Birds in treatment 5, with only half of the vitamin inclusion levels did not have a significantly lower carcass weight than birds in treatment 1 (100% vitamin A and E, 0 g/ton Biored®) which had the full vitamin A and E specification. This indicates that only the vitamin deficient diet of birds in treatment 2 (0% vitamin A and E, 0 g/ton Biored®) had a lighter carcass weight that might have been due to this groups lower cumulative feed intake during the 35 day growing period.

The thigh meat portion weights of birds in the Negative Control (0% vitamin A and E, 0 g/ton Biored®) were significantly lower ($P < 0.05$) than the birds in the Positive Control (100% vitamin A and E, 0 g/ton Biored®), treatment 5 (50% vitamin A and E, 0 g/ton Biored®) and treatment 8 (50% vitamin A and E, 200 g/ton Biored®). The only significant ($P < 0.05$) difference in breast meat portions were between the Positive Control (100% vitamin A and E, 0 g/ton Biored®) weighing 141.71g and the portions of the birds in the Negative Control (0% vitamin A and E, 0 g/ton Biored®) at 129.54g. Birds in treatment 3 (0% vitamin A and E, 150 g/ton Biored®) had no significantly ($P > 0.05$) different thigh and breast weight portions than birds in the Positive Control group (100% vitamin A and E, 0 g/ton) with only birds in the Negative Control (0 % vitamin A and E, 0 g/ton Biored®) having lower weights than the Positive Control. This could indicate that Biored® was able to prevent weight losses in the thigh and breast meat portions of birds when compared to the Negative Control but more research needs to be done. There were no significant differences ($P > 0.05$) between birds in any treatments with regard to portion weights proportional to the carcass weights.

5.6 Shelf life of thigh meat

Phospholipids in cell membranes are highly susceptible to oxidation and their risk of oxidation depends on the amount of polyunsaturated fatty acids in the lipid bilayer, the amount of reactive oxygen species produced and the amount of antioxidants present (Fellenberg and Speisky, 2006; Brenes *et al*., 2008). The highest level of lipid oxidation occurs when meat is handled, processed, stored and cooked. Compounds that are present in meat like haemoglobin, myoglobin, ferritin and hemosiderin release iron. The iron then forms chelates with molecules like amino acids, nucleotides and phosphates. These chelates catalyse lipid oxidation reactions in tissue (Voljč *et al.*, 2011). Lipid peroxidation in this study was measured by quantifying the thiobarbituric acid reactive substance (TBARS) content in thigh meat stored in retail simulated conditions. TBARS is a commonly used marker for detecting the development of rancid or off-flavours (Jensen *et al*., 1998). The thigh portion of the bird was chosen because this muscle group has a higher concentration of phospholipids and polyunsaturated fatty acids as it is a more active muscle group and consequently more susceptible to oxidation than the breast meat portion (Lauridsen *et al.*, 1997). On both days 3 and 6 of storage, thigh portions of birds in treatment 2 (0% vitamin A and E, 0 g/ton Biored®) and treatment 3 (0% vitamin A and E, 150 g/ton Biored®) showed significantly higher ($P \leq 0.05$) degrees of lipid peroxidation than birds in the Positive Control (100% vitamin A and E, 0 g/ton Biored®). It has been shown that there is a linear relationship between supplementation of vitamin E in the diet and its presence in meat of chicken. The higher level of vitamin E then provides a higher level of oxidative stability (Lanari *et al*., 2003; Voljč *et al*., 2011). Birds in treatment 2 and 3 had no additional vitamin A and E in the diet so the higher TBARS in the meat reflects the higher rate of oxidation in the meat which could be due to the lower levels of vitamins in the portions. Treatment 5 (50% vitamin A and E, 0 g/ton Biored®) with no Biored® inclusion and only half of the vitamin specification, was not significantly different ($P > 0.05$) from Treatment 6 (50% vitamin A and E, 100 g/ton Biored®), Treatment 7 (50% vitamin A and E, 150 g/ton Biored®) and Treatment 8 (50% vitamin A and E, 200 g/ton Biored®) that had different levels of the additive in the feed. On day 3 and 6, results showed that the diets supplied with only 50% of the vitamin requirement (Treatment 4 to 6) had no significantly different oxidation levels in the meat than the Positive Control (100% vitamin A and E, 0 g/ton Biored®) and Treatment 4 (100% vitamin A and E, 150 g/ton Biored®). The antioxidant additive Biored® seem to have an effect on oxidative stability in the thigh meat portions because on day 6 of the shelf life study of birds in treatment 6, 7 and 8 did not differ significantly ($P>0.05$) from the Positive Control (100% vitamin A and E, 0 g/ton Biored®) even though the birds in these treatments only received half of the vitamin A and E specification. The TBARS value of thigh meat portions of birds in treatment 6, 7 and 8 also differs significantly ($P<0.05$) from the TBARS value of the Negative Control and treatment 3(0% vitamin A and E, 150 g/ton Biored®). It might also be that only half the vitamin A and E specification in the diet is sufficient enough to supply the oxidative protection to the thigh meat portions as the TBARS value of the meat portions in treatment 1(Positive Control) does not differ significantly from meat portions in treatment 5

(50% vitamin A and E, 0 g/ton Biored®) that contained only half the vitamin A and E specification and no Biored®.

Simitzis *et al*. (2011) studied the effect that a flavonoid, hesperidin, had on broiler performance and chicken meat characteristics. Two diets were supplemented with either 1.5 g/kg or 3 g/kg of hesperidin and the fourth diet was supplemented with 0.02g/kg α-tocopheryl acetate. The extent of oxidation over a period of 9 days was measured in breast meat. They found that the supplementation of hesperidin delayed oxidation significantly from the control diet. The treatment group with the α-tocopherol acetate supplementation however had the lowest MDA levels, and thus the lowest oxidation level, at the end of the study. They also pointed to various studies using rosemary and sage extracts, tea cathechins, oregano essential oil, oregano herb, a combination of gallic acid and linoleic acid and a mixture of thymol and carvacrol, which all extended the shelf life of stored chicken meat.

Poultry meat contains high levels of polyunsaturated fatty acids and this makes the meat highly susceptible to oxidative deterioration. Goñi *et al*. (2007) used grape pomace as a feed additive which has been showed to have high concentrations of flavonoids. They showed that inclusion of grape pomace decreased oxidative deterioration of breast samples from day 4 and 7 and depressed MDA levels for thigh samples from day 7 of storage.

The antioxidant potential of flavonoids depends upon their lipophilic properties and their chemical structure (Simitzis, 2011).

Surai (2012) has written an insightful review on flavonoid research and how it has evolved up until today. A lot of attention has been given to the antioxidative capacity of flavonoids and its ability to scavenge free radicals. Many flavonoids though have been shown to be poorly absorbed from where it is digested in the gut and their concentrations in target tissues are often too low to exert a meaningful effect. Flavonoids have been implicated to prevent diseases like atherosclerosis, decrease the risk of heart attacks and improve endothelial function by mechanisms other than their antioxidative ability. They have also been shown to have a strong pro-oxidant effect. It is suggested that the relationship between polyphenols and vitamin E is not clear and that polyphenols might affect vitamin E redistribution between tissues and plasma. A lot more research is needed to determine exactly how flavonoids exert their function.

Chapter 6

Conclusion:

Antioxidants are essential in the body to protect cells and membranes from the harmful effects of free radicals and oxidative damage. Vitamin E in particular is considered as the most important lipid soluble antioxidant with the ability to prevent lipid peroxidation. Though studies have shown that vitamin A alone is not an effective antioxidant, vitamin A and E has been shown to work in synergy to help prevent and alleviate lipid peroxidation. Both these vitamins are stored in the liver and are supplemented in animal feeds. Bioflavonoids, compounds naturally occurring in vascular plants, are well known for their antioxidant abilities. They have proven to be effective free radical scavenging molecules with the ability to protect lipids from oxidation, a function homologous to vitamin A and E. Studies have reported the ability of flavonoids to have a vitamin E sparing effect in broiler chickens, improving vitamin E status and reducing lipid peroxidation and extending the shelf life of meat products. It was hypothesized that Biored®, as a bioflavonoid based feed additive, was able to substitute a certain level of vitamin A and E in the diet of broiler chickens and effectively perform the antioxidative effects by protecting cells from free radical damage and lipid peroxidation and also exerting a vitamin sparing affect. Performance parameters like final body weight at day 35, cumulative feed intake and average daily gain over the whole 35 day trial period were affected by Biored®. The final 35 day weight of the broiler chicken in treatments receiving only half the vitamin A and E specification with different incremental levels of Biored® did not differ significantly from the Positive Control group and with increasing level of Biored® in the feed the weights of the groups tended to increase as well though the differences were not significant. Cumulative feed intake was also improved when diets with 50% of vitamin A and E specification was supplemented with Biored® as well as the average daily gain over the whole 35 day trial period. Biored® did not have a vitamin A and E sparing effect in the liver. Treatments with the normal commercial specification for these vitamins had significantly higher levels in their liver then compared to the treatment groups receiving only half of the vitamins together with supplementation of Biored®. Biored® helped improve oxidative stress in the blood of broilers at the end of the growth trial when added at 200 g/ton. GSH levels and the GSH:GSSG ratio was significantly higher than the Positive Control diet indicating a lower level of oxidative stress in the blood and possibly the whole body. The inclusion of Biored® did not affect carcass or portion weights in birds. Meat quality factors including drip loss, cooking loss and shear force in breast meat portions was also not affected by the inclusion of Biored® in the diet. The shelf life of thigh meat portions was also not affected by the addition of Biored® to the feed. The absence of vitamin A and E caused the most deterioration overall. There are shortcomings in bioflavonoid research regarding the exact mechanism of absorption and metabolism that occurs within the body. In order to determine the biological activities of flavonoids one needs to understand the intake absorption, metabolism and excretion of these compounds and these parameters are only well established for a few compounds. Questions have also arisen whether the main mode of action of some

flavonoids is their antioxidative capacity. Flavonoids have been proven to exert anti-inflammatory, antibacterial and antiviral, hepatoprotective, anti-allergic, anti-thrombotic, anti-carcinogenic and immunomodulatory properties.

To accurately determine how Biored® influences broiler performance and production more research has to be done on the exact mode of action of the flavonoids present in the product and to what extent the compounds are metabolized and absorbed into the system.

Chapter 7

Recommendations:

1. Future experiments might want to test the product under environmentally challenged conditions that induces stress on the birds to see whether Biored® might support performance. The present study was performed in an environmentally controlled house. Stress can be induced by manipulating the stocking density and increasing it up to 21 birds/m^2 or increasing the environmental temperature. Growth performance parameters can be measured as well as GSH and GSSG levels that were measured in this trial in order to determine the oxidative state of the animals. The vitamin A and E status of the liver can also be determined as this is a sensitive indicator of the bodies' store of these anti-oxidative vitamins.

2. Determine whether Biored® has a different mode of action then the antioxidative function proposed by the study. Surai (2012) indicated that the antioxidative property is not the main mode of action of many flavonoids. A digestibility study could be done to determine the level of breakdown and absorption of flavonoids as many flavonoids have been shown to be poorly absorbed (Surai, 2012).

3. Make use of dose response trails with higher doses of Biored® in the diet. Make use of regression analyses to determine whether higher levels of Biored® will have an effect on performance parameters.

References:

Allen, C.D., Fletcher, D.L, Northcutt, J.K. and and Russell, S.M. 1998. The Relationship of Broiler Breast Colour to Meat Quality and Shelf-Life. Poultry Science 77, 361–366.

Alpsoy, L. and Yalvac, M.E., 2011. Key Roles of Vitamins A, C, and E in Aflatoxin B_1-Induced Oxidative Stress. Vitamins and Hormones 86, 286-305.

AOAC, 2000. Official method of analysis 920.39 (17th Edition) Volume I. Association of Official Analytical Chemists, Inc., Maryland, USA.

Aruoma, O.I., 1998. Free Radicals, Oxidative Stress, and Antioxidants in Human Health and Disease. Journal of the American Oil Chemists' Society 75, 199-212.

Avanzo, J.L., de Mendonca, C.X., Pugine, S.M.P. and Cesar, M. 2001. Effect of vitamin E and selenium on resistance to oxidative stress in chicken superficial pectoralis muscle. Comparative Biochemistry and Physiology Part C 129, 163-173.

Aziza, A.E., Quezada, N. and Cherian, G., 2010. Antioxidative effect of dietary Camelina meal in fresh, stored, or cooked broiler chicken meat. Poultry Science 89, 2711–2718.

Barroeta, A.C., 2007. Nutritive value of poultry meat: relationship between vitamin E and PUFA. World's Poultry Science Journal 63, 277-284.

Bast, A. and Haenen, G.R.M.M., 2002. The toxicity of antioxidants and their metabolites. Environmental Toxicology and Pharmacology 11, 251 – 258.

Betti, M., Schneider, B.L., Wismer, W.V., Carney, V.L., Zuidhof, M.J. and Renema, R.A., 2009. Omega-3-enriched broiler meat: 2. Functional properties, oxidative stability and consumer acceptance. Poultry Science 88, 1085–1095.

Bieri, J.G., 1960. Current Aspects of Vitamin A, Vitamin E, and Selenium in Poultry Nutrition. World's Poultry Science Journal 16, 245-258.

Bramley, P.M, Elfmadfa, I., Kafatos, A., Kelly, F.J., Manios, Y., Roxborough, H.E., Schuch, W., Sheehy, P.J.A. and Wagner, K.H., 2000. Review: Vitamin E. Journal of the Science of Food and Agriculture 80, 913-938.

Brenes, A., Viveros, A., Goñi, I., Centeno, C., Sáyago-Ayerdy, S.G., Arija, I. and Saura-Calixto, F. 2008. Effect of Grape Pomace Concentration and Vitamin E on Digestibility of Polyphenols and Antioxidant Activity in Chickens. Poultry Science 87, 307-316.

Brigelius-Flohe, R. and Traber, M.G., 1999. Vitamin E: function and metabolism. The Journal of the Federation of American Societies for Experimental Biology, 13: 1145–1155.

Cai, S.J., Wu, C.X., Gong, L.M., Song, T., Wu, H. and Zhang, L.Y., 2012. Effects of nano-selenium on performance, meat quality, immune function, oxidation resistance, and tissue selenium content in broilers. Poultry Science 91, 2532–2539.

Cao, G., Sofic, E. and Prior, R.L., 1996. Antioxidant and prooxidant behavior of flavonoids: Structure-Activity relationships. Free Radical Biology and Medicine 22, 749 – 760.

Cao, F.L., Zhang, X.H., Yu, W.W., Zhao, L.G. and Wang, T., 2012. Effect of feeding fermented *Ginkgo biloba* leaves on growth performance, meat quality, and lipid metabolism in broilers. Poultry Science 91, 1210-1221.

Chew, B.P., 1996. Importance of antioxidant vitamins in immunity and health in animals. Animal Feed Science Technology 59, 103-114.

Ciaccio, M., Valenza, M., Tesoriere, L., Bongiorno, A., Albiero, R. and Liverea, M.A., 1993. Vitamin A inhibits Doxorubicin-induced membrane lipid peroxidation in Rat tissue in Vivo. Archives of biochemistry and biophysics 302, 103-108.

Erener, G., Ocak, N., Altop, A., Cankaya, S., Aksoy, H.M. and Oztur, E., 2011. Growth Performance, Meat Quality and Caecal Coliform Bacteria Count of Broiler Chicks Fed Diet with Green Tea Extract. Asian-Australasian Journal of Animal Science 24, 1128-1135.

Esteban, S., Rayó, J. M., Moreno, M., Sastre, M., Rial, R. V. and Tur, J. A., 1991. A role played by the vitelline diverticulum in the yolk sac resorption in young post-hatched chickens. Journal of Comparative Physiology B. 160, 645-648.

Fellenberg, M. A., and Speisky, H., 2006. Antioxidants: Their effect on Broiler oxidative stress and its meat oxidative stability. World's Poultry Science Journal Vol 62, 54-70.

Ferket, P.R. and Gernat, A.B. 2006. Factors that affect Feed Intake of Meat Birds: A Review. International Journal of Poultry Science, 5(10), 905-911.

Flachowsky, G., Engelman, D., Su¨nder, A., Halle, I. and Sallmann, H.P., 2001. Eggs and poultry meat as tocopherol sources in dependence on tocopherol supplementation of poultry diets. Food Research International 35, 239–243.

Formica, J.V. and Regelson, W., 1995. Review of the Biology of Quercetin and Related Bioflavonoids. Food and Chemical Toxicology Journal 33, 1061-1080.

Frank, J., 2005. Beyond vitamin E supplementation: An alternative strategy to improve vitamin E status. Journal of Plant Physiology 162, 834 - 843.

Fukuzawa, K., Chida, H., Tokumura, A. and Tsukatani H., 1981. Antioxidative Effect of α-Tocopherol Incorporation into Lecithin Liposomes on Ascorbic Acid- Fe^{2+} -Induced Lipid Peroxidation. Archives of Biochemistry and Biophysics 206, 173-180.

Galati, G. and O'Brien, P.J., 2004. Potential toxicity of flavonoids and other dietary phenolics: Significance for their chemopreventive and anticancer properties. Free Radical Biology and Medicine 37, 287 – 303.

Gao, J., Lin, H., Wang, X.J, Song, Z.G. and , and Jiao, H.C., 2010. Vitamin E supplementation alleviates the oxidative stress induced by dexamethasone treatment and improves meat quality in broiler chickens. Poultry Science 89, 318–327.

Giustarini, D., Dalle-Donne, I., Colombo, R., Milzani, A. and Rossi, R. 2003. An improved HPLC measurement for GSH and GSSG in human blood. Free Radical Biology and Medicine 35, 1365-1372.

Gladine, C., Morand, C., Rock, E., Bauchart D. and Durand, D., 2007. Plant extracts rich in polyphenols (PERP) are efficient antioxidants to prevent lipoperoxidation in plasma lipids from animals fed n − 3 PUFA supplemented diets. Animal Feed Science and Technology 136, 281–296.

Gobert, M., Gruffat, D., Habeanu, M., Parafita, E., Bauchart, D. and Durand, D., 2010. Plant extracts combined with vitamin E in PUFA-rich diets of cull cows protect processed beef against lipid oxidation. Meat Science 85, 676–683.

Goñi, I., Brenes, A., Centeno, C., Viveros, A., Saura-Calixto, F., Rebole´, A., Arija, I. and Estevez, R., 2007. Effect of Dietary Grape Pomace and Vitamin E on Growth Performance, Nutrient Digestibility, and Susceptibility to Meat Lipid Oxidation in Chickens. Poultry Science 86, 508–516.

Gray, J.I., Gomaa, E.A. and Buckley, D.J., 1996. Oxidative Quality and Shelf Life of Meats. Meat Science 43, S111-S123.

Guo, Y., Tang, Q., Yuan, J. and Jiang, Z., 2001. Effects of supplementation with vitamin E on the performance and the tissue peroxidation of broiler chicks and the stability of thigh meat against oxidative deterioration. Animal Feed Science and Technology 89, 165-173.

Halliwell, B., 1989. Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis. British Journal of Experimental Pathology 70, 737-757.

Heim, K.E., Tagliaferro, A.R. and Bobilya, D.J., 2001. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. Journal of Nutritional Biochemistry 13, 572–584.

Hu, M. 2011. Dietary Polyphenols as Antioxidants and Anticancer Agents: More Questions than Answers. Chang Gung Medical Journal 34, 449-460.

Ibrahim, W., Lee, U., Szabo, J., Bruckner, G. and Chow, C.K., 1999. Oxidative stress and antioxidant status in mouse kidney: Effects of dietary lipid and vitamin E plus iron. Journal of Nutritional Biochemistry 10, 674 – 678.

Jensen, S.J.K., 2003. Oxidative stress and free radicals. Journal of Molecular Structure 666–667, 387–392.

Jensen, C., Lauridsen, C. and Bertelsen, G., 1998. Dietary vitamin E: Quality and storage stability of pork and poultry. Trends in Food Science and Technology 9, 62 – 72.

Jiakui, L., Dingren, B., Siyi, P., Yanhong, Z. and Donghai, Z., 2008. Effects of high dietary vitamin A supplementation on tibial dyschondroplasia, skin pigmentation and growth performance in avian broilers. Research in Veterinary Science 84, 409–412.

Johannsen, A. K. B., Jensen, S. K. and Jakobsen, K., 1998. A note on Vitamin A activity of ß-carotene in broilers. Acta Agriculturae Scandinavica, Section A, Animal Science 48, 260-263.

Kamboh, A. A., Arain, M. A., Mughal, M. J., Zaman, A., Arain, Z. M. and Soomro, A. H., 2015. Flavonoids: Health Promoting Phytochemicals for Animal Production – a Review. Journal of Animal Health and Production, 3(1): 6-13.

Kanner, J. 1994. Oxidative Processes in Meat and Meat Products: Quality Implications. Meat Science 36, 169-189.

Karadas, F., Surai, P.F. and Sparks, N.H.C., 2011. Changes in broiler chick tissue concentrations of lipid-soluble antioxidants immediately post-hatch. Comparative Biochemistry and Physiology, Part A 160, 68–71.

Kathirvel, P. and Richards, M.P., 2009. Mechanisms by which flavonol aglycones inhibit lipid oxidation better
than glycosylated flavonols in comminuted muscle tissue. Food Chemistry 117, 75–82.

Kay, C. D., 2010. The future of flavonoid research. British Journal of Nutrition 104, S91–S95.

Kim, Y.J., Park, W.Y. and Choi, I.H., 2010. Effects of dietary α-tocopherol, selenium, and their different combinations on growth performance and meat quality of broiler chickens. Poultry Science 89, 603–608.

Kitts, D. D., 1997. An evaluation of the multiple effects of the antioxidant vitamins. Trends in Food Science and Technology 8, 198-203.

Knak Jensen, S.J., 2003. Oxidative stress and free radicals. Journal of Molecular Structure (Theochem) 666–667, 387–392.

Koivula, M.J. and Eeva, T., 2010. Metal-related oxidative stress in birds. Environmental Pollution 158, 2359-2370.

Lanari, M. C., Hewavitharana, A. K., Becu, C. and de Jong, S., 2003. Effect of dietary tocopherols and tocotrienols on the antioxidant status and lipid stability of chicken. Meat Science 68, 155–162.

Lauridsen, C., Buckley, D.J. and Morrissey, P.A., 1997. Influence of Dietary Fat and Vitamin E Supplementation on α-Tocopherol Levels and Fatty Acid Profiles in Chicken Muscle Membranal Fractions and on Susceptibility to Lipid Peroxidation. Meat Science 46, 9-22.

Luehring, M., Blank, R. and Wolffram, S., 2011. Vitamin E-sparing and vitamin E-independent antioxidative effects of the flavonol quercetin in growing pigs. Animal Feed Science and Technology 169, 199–207.

Leusink, G., Rempel, H., Skura, B., Berkyto, M., White, W., Yang, Y., Rhee, J.Y., Xuan, S.Y., Chiu, S., Silversides, F., Fitzpatrick, S., and Diarra, M.S., 2010. Growth performance, meat quality, and gut microflora of broiler chickens fed with cranberry extract. Poultry Science 89, 1514–1523.

Li, J., Dingren B., Siyi, P., Yanhong, Z., and Donghai, Z. 2008. Effects of high dietary vitamin A supplementation on tibial dyschondroplasia, skin pigmentation and growth performance in avian broilers. Research in Veterinary Science 84, 409–412.

Li, W.J., Zhao, G.P, Chen, G.L, Zhengand, M.Q. and Wen, J., 2009. Influence of dietary vitamin E supplementation on meat quality traits and gene expression related to lipid metabolism in the Beijing-you chicken. British Poultry Science 50, 188— 198.

Liu, Y., Lyon, B.G., Windham, W.R., Lyon, C.E. and Savage, E.M., 2004. Principal Component Analysis of Physical, Color and Sensory Characteristics of Chicken Breasts Deboned at Two, Four, Six and Twenty-Four Hours Postmortem. Poultry Science 83, 101-108.

Lin, C.F., Gray, J.I., Asghar, A., Buckley, D.J., Booren, A.M. and Flegal, C.J., 1989. Effects of Dietary Oils and α-Tocopherol Supplementation on Lipid Composition and Stability of Broiler Meat. Journal of Food Science 54, 1457-1460.

Machlin, L.J. and Bendich, A., 1987. Free radical tissue damage: protective role of antioxidant nutrients. The Journal of the Federation of American Societies for Experimental Biology 1, 441-445.

Mahmoud, K.Z. and Edens, F.W., 2003. Influence of selenium sources on age-related and mild heat stress-related changes of blood and liver glutathione redox cycle in broiler chickens (Gallus domesticus). Comparative Biochemistry and Physiology Part B 136, 921–934.

Manach, C., Scalbert, A., Morand, C., Rémésy, C. and Jime´nez, L., 2004. Polyphenols: food sources and bioavailability. American Journal of Clinical Nutrition 79, 727– 747.

Majumdar, S. and Srirangam, R., 2010. Potential of the bioflavonoids in the prevention/treatment of ocular disorders. Journal of Pharmacy and Pharmacology 62, 951–965.

Middleton, E., Kandaswami, C. and Theoharides, T. C., 2000. The Effects of Plant Flavonoids on Mammalian Cells: Implications for Inflammation, Heart Disease, and Cancer. Pharmacological Reviews 52, 673–751.

Mielnik, M.B., Olsen, E., Vogt, E., Adeline, D. and Skrede G., 2006. Grape seed extract as antioxidant in cooked, cold stored turkey meat. LWT – Food Science and Technology 39, 191–198.

Mitsumoto, M., O'Grady, M.N, Kerry, J.P and Buckley, D.J., 2005. Addition of tea catechins and vitamin C on sensory evaluation, colour and lipid stability during chilled storage in cooked or raw beef and chicken patties. Meat Science 69, 773–779.

Moravej, H., Alahyari-Shahrasb, M., Baghani, M.R. and Shivazad, M. 2012. Withdrawal or reduction of the dietary vitamin premix on bone parameters of broiler chickens in two rearing systems. South African Journal of Animal Science 42, 169-177.

Mountzouris, K.C., Paraskevas, V., Tsirtsikos, P., Palamidi, I., Steiner, T., Schatzmayr, G. and Fegeros, K. 2011. Assessment of a phytogenic feed additive effect on broiler growth performance, nutrient digestibility and ceacal microflora composition. Animal Feed Science and Technology 168, 223-231.

Mullin, G.E., 2011. Vitamin A and Immunity. Nutrition in Clinical Practice 26, 495-496.

Muramoto, T., Nakanishi, N., Shibata, M. and Aikawa, K., 2003. Effect of dietary b -carotene supplementation on beef color stability during display of two muscles from Japanese Black steers. Meat Science 63, 39–42.

Nijveldt, R.J., van Nood, E., van Hoorn, DEC., Boelens, P.G., van Norren, K. and van Leeuwen, P.A.M., 2001. Flavonoids: a review of probable mechanisms of action and potential applications. American Journal of Clinical Nutrition 74, 418–425.

Niki, E., Noguchi, N., Tsuchihashi, H. and Gotoh, N., 1995. Interaction among vitamin C, vitamin E, and β-carotene. American Journal of Clinical Nutrition 62, 13225-65.

Norum, K.R., and Blomhoff, R., 1992. Vitamin A absorption, transport, cellular uptake, and storage. American Journal of Clinical Nutrition 56, 735-744.

Oliveros, L., Vega, V., Lic., Anzulovich, A.C., Ramirez, D. and Gimnez, M.S., 2000. Vitamin A deficiency modifies antioxidant defenses and essential element contents in rat hearts. Nutrition Research 20, 1139-1150.

Oteiza, A.I., Erlejman, A.G., Verstraeten, S.V., Keen, C.L. and Fraga, S.G., 2005. Flavonoid-membrane interactions: A protective role of flavonoids at the membrane surface?. Clinical and Developmental Immunology 12, 19–25.

Palace, V.P., Khaper, N., Qin, Q. and Singal, P., 1999. Antioxidant potentials of vitamin A and carotenoids and their relevance to heart disease. Free Radical Biology and Medicine 26, 746-761.

Pavlica S. and Gebhardt, R., 2010. Protective effects of flavonoids and two metabolites against oxidative stress in neuronal PC12 cells. Life Sciences 86, 79–86.

Payne, R.L. and Southern, L.L., 2005. Changes in Glutathione Peroxidase and Tissue Selenium Concentrations
of Broilers After Consuming a Diet Adequate in Selenium. Poultry Science 84, 1268–1276.

Peña, J. E. M., Vieira, S. L., López, J., Reis, R.N., Barros, R., Furtado, F.V.F. and Silva, P.X. 2008. Ascorbic Acid and Citric Flavonoids for Broilers Under Heat Stress: Effects on Performance and Meat Quality. Brazilian Journal of Poultry Science 10, 125-130.

Puvača, N. and Stanaćev, V., 2011. Selenium in poultry nutrition and its effect on meat quality. World's Poultry Science Journal 67, 479-484.

Raj Narayana, K., Sripal Reddy, M., Chaluvadi, M.R. and Krishna, D.R., 2000. Bioflavonoids classification, pharmacological, biochemical effects and therapeutic potential. Indian Journal of Pharmacology 33, 2-16.

Rebole, A., Rodrirguez, M.L., Ortiz, L.T., Alzueta, C., Centeno, C., Viveros, A., Brenes, A. and Arija, I., 2006. Effect of dietary high-oleic acid sunflower seed, palm oil and vitamin E supplementation on broiler performance, fatty acid composition and oxidation susceptibility of meat. British Poultry Science 47, 581— 591.

Rietjens, I.M.C.M., Boersma, M.G., de Haan, L., Spenkelink, B., Awad, H.M., Cnubben, N.H.P., van Zanden, J.J., van der Woude, H., Alink, G.M. and Koeman, J.H., 2002. The pro-oxidant chemistry of the natural antioxidants vitamin C, vitamin E, carotenoids and flavonoids. Environmental Toxicology and Pharmacology 11, 321 – 333.

Rossi, R., Pastorelli, G., Cannata, S., Tavaniello, S., Maiorano, G. and Corino, C., 2013. Effect of long term dietary supplementation with plant extract on carcass characteristics meat quality and oxidative stability in pork. Meat Science 95, 542-548.

Ruiz, J.A., Guerrero, L., Arnau, J., Guardia, M.D. and Esteve-Garcia, E., 2001. Descriptive Sensory Analysis of Meat from Broilers Fed Diets Containing Vitamin E or β-Carotene as Antioxidants and Different Supplemental Fats. Poultry Science 80, 976–98.

Russo, A., Acquaviva, R., Campisi, A., Sorrenti, V., Di Giacomo, C., Virgata, G., Barcellona, M.L. and Vanella, A., 2000. Bio£avonoids as anti-radicals, antioxidants and DNA cleavage protectors. Cell Biology and Toxicology 16, 91-98.

Sahin, K., Sahin, N., Sarı, M. and Gursu, M.F., 2002. Effects of vitamins E and A supplementation on lipid peroxidation and concentration of some mineral in broilers reared under heat stress (32°C). Nutrition Research 22, 723–731.

Samuals, M.L. 1989. Statistics for the life science. Collier MacMillan Publishers, Londen.

Simitzis, P.E., Symeon, G.K., Charismiadou, G.K., Ayoutanti, A.G. and Deligeorgis, S.G. The effects of dietary hesperidin supplementation on broiler performance and chicken meat characteristics. Canadian Journal of Animal Science 91: 275-282.

Sklan, D., 1983. Vitamin A absorption and metabolism in the chick: response to dietary intake, and to tocopherol. British Journal of Nutrition 50, 401–407.

Skřivan, M., Marounek, M., Dlouhá, G. and ŠevčÍková, S., 2008. Dietary selenium increases vitamin E contents of egg yolk and chicken meat. British Poultry Science 49:4, 482-486.

Statistical Analysis Systems, 2014. SAS User's Guide: Statistics Version 9.3. SAS Institute Inc. Cary, NC, USA.

Tang, S., Kerry, J.P., Sheeban, D., Buckley, D.J. and Morrissey, P.A., 2001. Antioxidative effect of added tea catechins on susceptibility of cooked red meat, poultry and fish patties to lipid oxidation. Food Research International 34, 651-657.

Tavárez, M.A., Boler, D.D., Bess, K.N., Zhao, J.,Yan, F., Dilger, A.C., Mckeith, F.K. and Killefer J., 2011. Effect of antioxidant inclusion and oil quality on broiler performance, meat quality, and lipid oxidation. Poultry Science 90, 922–930.

Tesoriere, L., Bongiorno, A., Pintaudi, A.M., D'Anna, R., D'Arpa, D. and Livrea, M.A., 1996. Synergistic Interactions between Vitamin A and Vitamin E against Lipid Peroxidation in Phosphatidylcholine Liposomes. Archives of biochemistry and biophysics 326, 57-63.

Tesoriere, L., D'Arpa, D., Re, R. and Livrea, M.A., 1997. Antioxidant Reactions of all-trans Retinol in Phospholipid Bilayers: Effect of Oxygen Partial Pressure, Radical Fluxes, and Retinol Concentration. Archives of Biochemistry and Biophysics 343, 13-18.

Traber, M.G. and Atkinson, J., 2007. Vitamin E, antioxidant and nothing more. Free Radical Biology and Medicine 43, 4 – 15.

Voljč, M., Frankič, T., Levart, A., Nemec, M. and Salobir, J., 2011. Evaluation of different vitamin E recommendations and bioactivity of α-tocopherol isomers in broiler nutrition by measuring oxidative stress in vivo and the oxidative stability of meat. Poultry Science 90, 1478–1488.

Wang, S., Bottje, W., Maynard, P., Dibner, J. and Shermer, W., 1997. Effect of Santoquin® and Oxidized Fat on Liver and Intestinal Glutathione in Broilers. Poultry Science 76, 961 – 967.

Wang, Y. and Xu, B., 2008. Effect of different selenium source (sodium selenite and selenium yeast) on broiler chickens. Animal Feed Science and Technology 144, 306–314.

Wang, W.J., Wang, S.P., Gong, Y.S.,Wang, J.Q. and Tan, Z.L., 2007. Effects of vitamin A supplementation on growth performance, carcass characteristics and meat quality in Limosin x Luxi crossbreed steers fed a wheat straw-based diet. Meat Science 77, 450–458.

Wang, X. and Quinn, P.J. 1999. Vitamin E and its function in membranes. Progress in Lipid Research 38, 309-336.

Wei, F.X., Hu, X.F., Sa, R.N., Liu, F.Z., Li, S.Y. and Sun, Q.Y. 2014. Antioxidant capacity and meat quality of broilers exposed to different ambient humidity and ammonia concentrations. Genetics and Molecular Research, 13(2): 3117 – 3127.

Woods, V.B. and Fearon, A.M., 2009. Dietary sources of unsaturated fatty acids for animals and their transfer into meat, milk and eggs: A review. Livestock Science 126, 1– 20.

Yadgary, L., Cahaner, A., Kedar, O. and Uni, Z. 2010. Yolk sac nutrient composition and fat uptake in late-term embryos in eggs from young and old broiler breeder hens. Poultry Science 89, 2441-2452.

Yao, L.H., Jiang, Y.M., Shi, J., Tomas- Barberan, F.A., Datta, N., Singanusong, R. and Chen, S.S., 2004. Flavonoids in Food and Their Health Benefits. Plant Foods for Human Nutrition 59, 113–122.

Yoon, I., Werner, T.M. and Butler, J.M., 2007. Effect of Source and Concentration of Selenium on Growth Performance and Selenium Retention in Broiler Chickens. Poultry Science 86, 727–730.

Yuming, G., Tang, Q., Yuan, J. and Jiang, Z. 2001. Effects of supplementation with vitamin E on the performance and the tissue peroxidation of broiler chicks and the stability of thigh meat against oxidative deterioration. Animal Feed Science and Technology 89, 165-173.

Zhang, W., Xiao, S., Samaraweera, H., Lee, E. and Ahn, D.U., 2010. Improving functional value of meat Products. Meat Science 86, 15– 31.